Research Article

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Optimization of polyphenol extraction, phenolic profile by LC-ESI-MS/MS, antioxidant, anti-enzymatic, and cytotoxic activities of *Physalis acutifolia*

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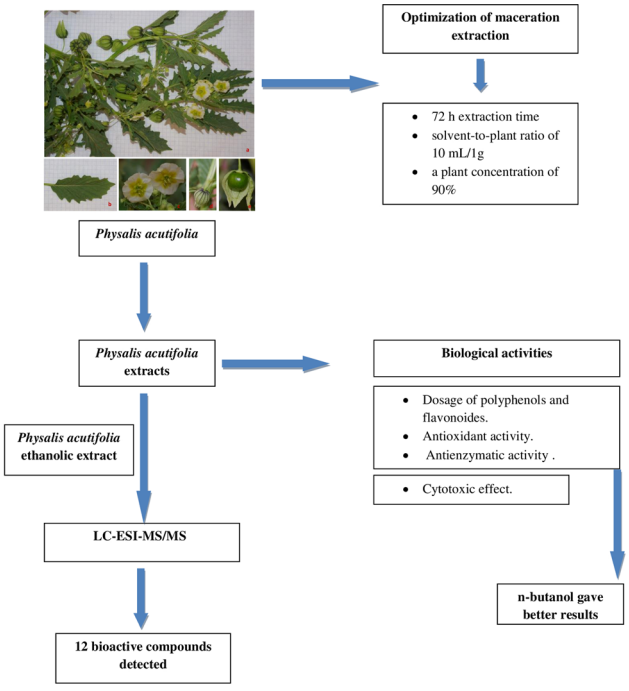
Abstract: During the current study, the phenolic and flavonoid contents were measured in *Physalis acutifolia* extracts with Folin–Ciocalteu and $AlCl_3$ methods, respectively. Various antioxidant assays, including 1,1-diphenyl-2-picrylhydrazyl, ABTS, reducing power, Fe^{2+}-phenanthroline reduction, and silver nanoparticle assays, were also conducted, along with anti-enzymatic assays. The cytotoxicity of the ethanolic



Graphical abstract

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extract was assessed on cancer cell lines (i.e., CAPAN-1 and dld-1) and a healthy cell line (i.e., L929). Optimal parameters for polyphenol extraction were determined: extraction time of 72 h, solvent-to-plant ratio of 10 mL/1 g, and plant concentration of 90%. The *n*-butanol extract showed the highest phenolic and flavonoid contents (i.e., 263.84 mg GAE/g dw and 72.03 QE/g dw, respectively), along with superior antioxidant and anti-enzymatic activities with IC_{50} values of 49.77 and 187.12 µg/mL with acetylcholinesterase and butyrylcholinesterase assays, respectively. LC-ESI-MS/MS analysis revealed 12 components, with hesperidin being the most abundant (i.e., 1829.0001 µg/g). The ethanolic extract exhibited cytotoxic effects on cancer cell lines, with an IC_{50} value of 0.959 mg/mL for dld-1, but with no effect on healthy cells. The bioavailability scores of the identified components support the *in vitro* findings and confirm the different assessed therapeutic effects.

Keywords: polyphenols, *Physalis acutifolia*, flavonoids, pharmacokinetics, biological activity, LC-ESI-MS/MS

1 Introduction

Plants have been used since ancient times as remedies for various diseases and comprise important research directions of modern medicine due to various bioactive constituents, sometimes with fewer side effects compared to synthetic drugs [1–3]. Nowadays, the use of new bioactive molecules is considered complementary to traditional medicine [4]. The polyphenols comprise a very diverse and well-known group of bioactive compounds produced by plants. In general, polyphenols are beneficial to human health due to their antioxidant, antibacterial, antithrombotic, antihypertensive, anti-atherogenic, anticancer, and anti-inflammatory activities [5–7]. Polyphenols decrease oxidative stress and protect human health by acting as radical scavengers, hydrogen donors, metal chelators, and reducing agents [8]. Furthermore, by controlling key metabolic enzymes, polyphenols also play an essential role in the treatment of Alzheimer's disease and diabetes mellitus [9]. Alzheimer's disease is characterized by the cholinergic deterioration of neurons and subsequent low levels of acetylcholine in the brain, causing cognitive impairment [10]. The enzymes acetylcholinesterase (AchE) and butyrylcholinesterase (BchE) play crucial roles in this process, with the former type dominant in the early stage, whereas the latter in the final stages of the disease [11]. Natural compounds are increasingly preferred to synthetic inhibitors because they are effective and induce fewer complications [12]. Moreover, the identification of plant bioactive compounds used in the control and treatment of diseases such as cancer is paramount to minimize the suffering in patients and reduce treatment costs [13].

Physalis sp. is a genus appurtenant to the Family Solanaceae and comprises approximately 100 species, mainly distributed in tropical and subtropical areas [14]. The plants of this genus gained great commercial interest, given that their derived products have a high nutritional and medicinal value [15]. The use of *Physalis* is well-known in folk medicine for the treatment of many human diseases, such as dermatitis, tracheitis, rheumatism, cancer, leukemia, and hepatitis [14,16,17]. Moreover, several species of the *Physalis* genus, such as *P. angulata*, *P. peruviana,* and *P. alkekengi*, have therapeutic potential due to their antioxidant, cytotoxic, anti-inflammatory, and antimicrobial activities [18–20]. *P. acutifolia* (Miers) Sandw is a plant native to the southwestern United States and northern Mexico, but it can also be found in central north Algeria [15]. The phytocomplex and the biological properties of this plant have not been fully investigated. Xu et al. [21] reported for the first time the isolation of physalins from the plant, which demonstrated a high cytotoxic effect against NCIH460 (non-small-cell lung cancer), SF-268 (glioma cancer), PC-3 (prostate adenocarcinoma), and MCF-7 (breast adenocarcinoma) [21]. Therefore, the current study considered, for the first time, to the best of our knowledge, the optimization of polyphenol extraction from *P. acutifolia*, their thorough characterization, and the assessment of their antioxidant and anti-enzymatic activities. In the current study, we have undertaken the optimization of extraction by maceration on a mixture of solvent (i.e., ethanol-distilled water) of polyphenols of *P. acutifolia*, followed by testing the therapeutic potential by measuring the antioxidant and anti-enzymatic activities of the ethanolic, chloroformic, ethyl acetate, and butanolic extracts, respectively, of *P. acutifolia* by different methods. Both bioavailability and pharmacokinetic attributes of the *P. acutifolia* identified compounds have been studied using the *in silico* approach. In the end, we have also assessed the cytotoxic effect of the ethanolic extract against two cancer cell lines (i.e., CAPAN-1 and dld-1) and a healthy cell line (i.e., L929). The composition of the ethanolic extract of *P. acutifolia* was determined by LC-ESI-MS/MS analysis, which revealed the specific molecules accountable for the observed effects.

2 Materials and methods

2.1 Chemicals and reagents

High-quality analytical-grade chemicals and solvents, comprising the reagents Folin–Ciocalteu (FCR), 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), butylated

hydroxytoluene (BHT), quercetin, and α-tocopherol, were purchased from Merk (St. Louis, United States).

2.2 Plant material

The flowering aerial parts of *P. acutifolia* were gathered in August 2021 from naturally occurring individuals in the Ouled Madi region of Msila province, northeast of Algeria (361 m a.s.l., 35°36′25″N, 04°30′16″E). The taxonomic identification of the plant material was performed by Prof. K. Rebbas from the University of Msila using accessible literature and the Algeria Flora [22]. A voucher specimen (No KR0044) was preserved in the herbarium of the same university. The collected samples were rinsed with Milli-Q water, dried at room temperature (25°C) away from sunlight for several days, then grounded with a blender to fine powder, and stored at 4°C before use.

2.3 Preparation of extracts

2.3.1 Optimization of extraction by maceration

To optimize the extraction process of polyphenols from *P. acutifolia* aerial parts, maceration was selected as the extractive technique, and a mixture of ethanol/water as the extractive solvent. The extractions were carried out at room temperature (25°C). For preliminary tests, a face-centered central composite design (CCDC) was used to generate surface responses. The optimized extraction parameters were as follows: the extraction time (X_1) ranged from 24 to 72 h, the liquid–solid ratio (X_2) ranged from 20 to 10 mL/g, and the ethanol concentration (X_3) ranged from 50 to 90% (Table 1). The total phenolic content (TPC) was the response evaluated for each extraction. The optimized parameters were then selected for the subsequent solid–liquid extraction.

Table 1: Experimental design (CCDC) involved coded and real independent variables at different levels

Factor	Symbol	Factors levels		
		−1	0	1
Extraction time (h)	X_1	24	48	72
Liquid–solid ratio (mL/g)	X_2	10	15	20
Ethanol concentration (%)	X_3	50	70	90

2.3.2 Liquid–liquid extraction

P. acutifolia powder (40 g) was subjected to maceration at room temperature. The extract was filtered and evaporated under reduced pressure using a Büchi rotavapor R-215 (BüchiLabortechnik AG, Switzerland) at a temperature of 40°C. The dry extract was reconstituted by adding 100 mL of distilled water at a temperature of 100°C. Subsequently, liquid–liquid extraction was performed using a separating funnel with solvents of increasing polarity (POLA), namely chloroform, ethyl acetate, and *n*-butanol. Every extraction was performed twice.

2.4 LC-ESI-MS/MS analysis

The samples for LC-ESI-MS/MS analysis were prepared following the method of Griffith et al. [23], with minor modifications. A 50 mg of ethanolic extract was dissolved in a mixture of 1 mL of methanol and 1 mL of *n*-hexane in an Eppendorf tube of 2 mL, vortexed by a Bioprep-24 homogenizer for 2 min at 4°C, and centrifuged at 9,000 rpm for 10 min at 4°C by a Hettich Universal 320R (Germany). The methanol phase was then separated and diluted at a ratio of 1:9 in distilled water. Finally, the samples were filtered using a Captiva premium syringe filter with a polypropylene shield, a nylon membrane of 25 mm in diameter, a pore size of 0.45 µm, and an injection volume of 5.12 µL. The LC-ESI-MS/MS analysis was performed in an Agilent 1260 Infinity II LC System coupled with a tandem mass spectrometer. The flow rate of the method was 0.5 mL/min, the total run time was 30 min, and the oven temperature was set at 25°C. The chromatographic separation was carried out in a reversed-phase Agilent Poroshell120 EC-C18 analytical column (100 mm × 3.0 mm, 2.7 µm). Eluent A (consisting of water with 5 mM ammonium formate) and eluent B (acetonitrile with 0.1% formic acid) were employed as mobile phases in isocratic conditions of 75% A and 25% B. Mass spectrometry was carried out utilizing an Agilent 6460 Triple Quadrupole Mass Spectrometer System equipped with electrospray ionization (LC–ESI–MS/MS) to detect the compounds. The acquisition was performed in both positive and negative ionization modes. The data were analyzed using Agilent Mass Hunter Software. A multiple reaction monitoring approach was used to accurately identify and quantify the phytochemical compounds. The collision energies were chosen to guarantee ideal fragmentation and transmission of the targeted ions. The mass spectrometer operated with a nitrogen (N_2) drying gas flow of 15 mL/min, a nitrogen nebulizing gas flow of 11 mL/min, a capillary voltage

of 4,000 V, and a gas temperature of 350°C [24]. According to Yilmaz [25], validation parameters for the method, including limit of detection (LOD), limit of quantification (LOQ), and linearity range, were studied and calculated.

2.5 Total phenolic compounds

2.5.1 TPC

The TPC of *P. acutifolia* extracts was determined using the FCR method of Müller et al. [26] with slight modifications. Within a 96-well microplate, 20 mL of each extract at a concentration of 1 mg/mL was diluted with 75 mL of 7.5% sodium carbonate solution and 100 mL of FCR (1:9 ratio in distilled water). After the incubation of the solutions in the dark at room temperature for 2 h, the absorbance was measured at 765 nm. The total phenolic concentration was calculated using the linear regression equation ($y = 0.0034x + 0.1044$, $R^2 = 0.997$) calculated using the standard gallic acid calibration curve (concentrations ranging from 0 to 200 g/mL).

2.5.2 Total flavonoid content (TFC)

The TFC in the *P. acutifolia* extracts was estimated using the aluminum colorimetric method following the procedure of Topçu et al. [27], with slight modifications. A volume of 50 µL of each extract (at a concentration of 1 mg/mL), 10 µL of potassium acetate (1 M), 10 µL of aluminum nitrate (10%), and 130 µL of methanol were added to a 96-well microplate. The solutions were stored for 45 min at room temperature, and then the optical density was measured at 415 nm. The curve of quercetin was prepared under the same conditions using concentrations of 0 to 50 µg/mL, and the linear regression equation ($y = 0.004x$, $R^2 = 0.997$) was used to calculate the TFC.

2.6 Biological activities

All tests were done on a 96-well microplate, and the absorbance was measured using a PerkinElmer Multimode Plate reader EnSpire (Waltham, MA, USA). The IC_{50} and $A_{0.5}$ (µg/mL) values were calculated using a regression equation, and regression analysis was carried out using the best-fit approach. Three replicates' worth of findings are displayed on average.

2.6.1 Antioxidant activities

2.6.1.1 DPPH radical scavenging assay

The free radical scavenger potential of *P. acutifolia* extract was examined by the free radical DPPH method following that reported by Blois [28]. In a 96-well microplate, 160 µL of DPPH solution (1 mM in methanol) was added to 40 µL of various concentrations of the plant extracts solubilized in methanol. The solutions were maintained in the dark for 30 min at room temperature. The optical density was determined at 517 nm. The absorbance of the blank (methanol) was read to calculate the inhibition percentages for each concentration. The DPPH scavenging assay results were determined using the following equation:

$$\text{DPPH}^{\cdot} \text{ scavenging effect (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100, \quad (1)$$

where A_{control} is the optical density of the reference and A_{sample} is the optical density of the sample obtained from the microplate reader. The obtained inhibitions were plotted with the concentrations of the samples. These resulting plots were then utilized to determine the IC_{50} values, which represent the concentration of the samples necessary to reduce DPPH by 50%. BHT and BHA were used as positive controls.

2.6.1.2 ABTS cation radical scavenging assay

The evaluation of antioxidant activity was performed by the ABTS scavenging assay following the Re et al. [29] method, with minor modifications. The $ABTS^{+}$ solution was first prepared by mixing 7 mM ABTS in H_2O with 2.45 mM potassium persulfate. Then, the mixture was placed for 12 h obscured at room temperature to start the oxidation process of ABTS. In a 96-well microplate, various concentrations (0.0625–4 mg/mL) of the plant extracts prepared in methanol (40 µL) were mixed with 160 µL of the $ABTS^{+}$ solution. The optical density was measured after incubation in the dark for 10 min at 734 nm. The optical density of the blank (methanol) was read to calculate the inhibition percentages for each concentration. BHA and BHT were used as positive controls.

2.6.1.3 Reducing power

The reducing ability of *P. acutifolia* plant extracts was determined following the Oyaizu method [30] with slight modifications. Various concentrations of the plant extracts solubilized in methanol (10 µL) were mixed with 40 µL of buffer phosphate (pH 6.6) and 50 µL of 1% potassium ferric cyanide ($K_3Fe(CN)_6$). The mixture was maintained at 50°C for 20 min, and then 50 µL of 10% trichloro acetic acid was

added with 40 µL of purified water and 10 µL of 0.1% ferric chloride ($FeCl_3$). The optical density reading was obtained at 700 nm, utilizing ascorbic acid as the positive control.

2.6.1.4 Phenanthroline activity

Phenanthroline potential was evaluated following the Szydlowska–Czerniaka method [31] in a 96-well microplate reader. In detail, 10 µL of different concentrations (0.0625–4 mg/mL) of the plant extracts dissolved in methanol was blended with 50 µL of 0.2% $FeCl_3$, 30 µL of 0.5% phenanthroline, and 110 µL of methanol. Consequently, the mixture was incubated at 30°C for 20 min. The optical density was determined at 510 nm by a microplate reader. BHA was used as a control positive.

2.6.1.5 Silver nanoparticle (SNP) assay

The antioxidant power of *P. acutifolia* plant extracts was estimated by the silver ion (Ag^+) reduction method, according to Ozyurek et al. [32]. About 130 mL of SNP solution (1 mM $AgNO_3$, 1% citrate solution) and 50 mL of distilled water were added to 20 µL of various doses (0.0625–4 mg/mL) of the plant extracts dissolved in methanol. The mixture's optical density was obtained at 423 nm after incubating for 30 min at 25°C. Ascorbic acid and Trolox were used as control positives.

2.6.2 Anti-enzymatic activities

2.6.2.1 Anti-AchE activity

AchE and BChE inhibitory activities were determined according to Ellman et al. [33]. A total of 10 µL of various doses of the plant extracts prepared in ethanol was added to 150 µL of 100 mM sodium phosphate buffer (pH 8.0). Then, 20 µL of AChE (5.32×10^{-3} U) or BChE (6.85×10^{-3} U) solution was added, and the solution was incubated for 15 min at 25°C. After that, 10 µL of 5,5′-dithio-bis-2-nitrobenzoic acid (0.5 mM) and 10 µL of acetylthiocholine iodide (0.71 mM) or butyrylthiocholine chloride (0.2 mM) was added. The optical density of the mixture was determined at 412 nm: one reading at 0 min, two readings after 5 min, three readings after 10 min, and finally, four readings after 15 min. The effectiveness of the extracts was evaluated by the following percentage of inhibition:

$$I\% = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}, \quad (2)$$

where A_{control} is the optical density of the blank, and A_{sample} is the optical density of the test sample. Galantamine hydrobromide was used as a reference.

2.6.2.2 α-Amylase inhibition activity

The inhibitory activity of α-amylase was assessed following the Zengin et al. [34] method. A total of25 µL of various concentrations of plant extracts solubilized in methanol was incubated for 10 min at 37°C and 50 µL of α-amylase solution was added. The solution was incubated again for 10 min at 37°C after the addition of 50 µL of the starch solution (0.1%). The reaction was stopped by the addition of 25 µL of HCl (1 M). Then, 100 µL of iodine–potassium solution was added. The optical density was determined at a wavelength of 630 nm. The blank used for this assay did not contain the enzyme. The α-amylase inhibitory activity was determined using formula (2). Acarbose was used as a positive control.

2.6.2.3 Urease inhibition activity

The urease inhibitory activity was measured according to Taha et al. [35]. By using a 96-well microplate, 10 µL of various concentrations of the plant extracts solubilized in methanol were added to 25 µL of urease preparation and 50 µL of urea solution (17 mM). The mixture was incubated for 15 min at 30°C. Then, 45 µL of phenol reagent (0.1% w/v sodium nitroprusside and 8% w/v phenol) was added to each well with 70 µL of alkaline reagent (4.7% NaOCl active chloride and 2.85% NaOH). The optical density of the mixture was measured after 50 min incubation. Urease inhibitory activity was determined using formula (2). Thiourea was applied as the positive control.

2.6.3 Anticancer activity

To assess the anticancer potential, the L929 fibroblast cell line, CAPAN-1 pancreatic cancer cell line, and dld-1 colorectal adenocarcinoma cell line were used; they were procured from the laboratory of Prof. Dr. Mustafa Türk at Kırıkkale University (Turkey). All cell culture experiments were conducted in culture plates and multi-well plates (Corning, USA). The frozen cells were quickly defrosted at 37°C. The cells were shifted to a 15 mL cell culture tube inside a sterile laminar flow hood. The cell culture tube was rotated at 250 G for 5 min. Then, 3 mL of the corresponding medium (10% fetal bovine serum, 1% antibiotic) was added to the Falcon tube and made homogeneous before being seeded into 25 cm^2 flasks. The flask was incubated in a 37°C incubator with 5% CO_2 [36]. To prepare the samples, the extract was mixed with a nutrient medium to create concentrations of 1, 0.5, 0.25, and 0.125 mg/mL, which were thoroughly mixed using a vortex mixer. Cell application was carried out without any delay. It was ensured that each well had 10×10^3 cells based on the live cell count. In

detail, 100 µL of the cell suspension in complete medium was added to each well and incubated for 24 h. The adhesion of cells to the surface of the well plate was checked after 24 h. The media in the wells were removed, and the test materials were added to the wells at concentrations of 1, 0.5, 0.25, and 0.125 mg/mL. The negative control group received only the complete medium, while the positive control group received 10% DMSO. After 24 h of incubation, the media were removed, and MTT (tetrazolium salt) solution (1 mg/mL) was included in each well. The plate was incubated at 37°C for 2–2.5 h. Subsequently, the MTT solution was treated with 100 mL of MTT solvent (isopropanol). The optical density of the 96-well plate was determined at 570 nm using an ELISA plate reader to determine cell viability. The cell viability percentage for each group was determined using the following formula, with the control cell viability assumed to be 100%:

$$\text{Cell viability}(\%) = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100,$$

where A_{sample} is the optical density of the sample and A_{control} is the optical density of the reference.

Cells were added to a 48-well plate with 15×10^3 cells per well and allowed to stand for 1 day in a controlled environment. The medium was removed, and 200 µL of samples with a density of 0.5 mg/mL was added to each well, except for the negative control well which only received cells. The plate was then incubated for an additional 24 h. Afterward, 70 µL of double staining preparation was included in each well, covered, and stored in the dark for 15 min [37].

2.7 Bioavailability and pharmacokinetic properties

The bioavailability, which depends on the physicochemical properties, such as molecular size, lipophilicity, POLA, and insolubility, of the compounds was studied by *in silico* analyses, as described by Bédoui et al. [38] and Mhadhbi et al. [39]. Druggability and several pharmacokinetic attributes of the *P. acutifolia* identified phytochemicals were also studied based on their absorption, distribution, metabolism, excretion, and toxicity (ADMET) attributes as previously reported [40–42].

2.8 Statistical analysis

Multiple regression analyses were carried out in Minitab Release 19 (Minitab Inc., State College, Pennsylvania, USA), and Statistica v.10 (Stat Soft, France) was used to assess the response surfaces using the models. To compare the impact of variables on the responses, ANOVA was used. The appropriateness of models was then assessed by dividing the residual sum of squares into pure error and lack-of-fit, and the coefficient of determination (R^2) was calculated. Additionally, optimization was assessed using Minitab Release 19. An analysis of the variation in means among treatments was performed using Tukey's HSD multiple-range tests ($p < 0.05$).

3 Results and discussion

3.1 Optimization of extraction

The effects of specific extraction parameters (i.e., extraction time, solvent concentration, and liquid–solid ratio) of polyphenol recovery from *P. acutifolia* aerial part material were carried out. For the optimization study, a surface response methodology (RSM) was adopted. Each response was measured in terms of TPC as part of the experimental design. According to Box and Wilson [43], RSM is suitable for the optimization of the extraction of bioactive compounds and is a useful statistical tool for optimizing, processing, and reducing the number of experimental trials. RMS is widely employed when the extraction methods

Table 2: CCDC matrix and response values of the *P. acutifolia* extraction

Entry	X_1 (h)[a]	X_2 (%)[b]	X_3 (mL/g)[c]	TPC (mg GAE[d]/g dw[e])
1	48	70.0	15.0	46.1
2	24	90.0	10.0	26.3
3	48	70.0	15.0	43.4
4	24	50.0	10.0	75.4
5	48	50.0	15.0	48.2
6	48	70.0	15.0	43.4
7	72	50.0	20.0	65.1
8	24	70.0	15.0	48.3
9	24	50.0	20.0	75.6
10	72	90.0	20.0	77.5
11	48	70.0	15.0	43.4
12	72	70.0	15.0	80.7
13	72	50.0	10.0	83.5
14	48	70.0	15.0	43.4
15	48	70.0	20.0	49.9
16	24	90.0	20.0	37.2
17	48	90.0	15.0	34.7
18	48	70.0	10.0	61.1
19	48	70.0	15.0	46.0
20	72	90.0	10.0	77.2
R^2	= 0.975			

[a]X_1, extraction time; [b]X_2, solvent concentration; [c]X_3, liquid/solid ratio; [d]GAE, gallic acid equivalents; [e]dw, dry weight. CCDC: face-centered central composite design.

involve several factors and interactions and whenever the independent parameters have combined effects [44]. Table 2 lists the response values of 20 tests that were performed.

The TPC values for *P. acutifolia* extracts ranged from 26.3 to 83.5 mg GAE/g dw. The experimental data were regressively analyzed, and the importance of model coefficients in the extraction of phenolic compounds was assessed. According to Table 2, the R^2 value was 0.975, indicating a correlation between experimental results and the model for the selected parameters. The relationship between the extraction time, the concentration of the dried plant material, and the concentration of ethanol for the extraction of phenolic compounds is

$$\text{TPC} = 45.459 + 12.115X_1 - 9.493X_2 + 1.818X_3 + 17.237X_1^2 - 5.763X_2^2 + 8.232X_3^2 + 11.694X_1X_2 + 3.646X_1X_3 - 3.661X_2X_3.$$

The outcome of multiple regression analysis is consistent with the surface plot analysis results, as shown in Figure 1. The effects of extraction duration and ethanol concentration on the TPC are shown in Figure 1a. The extraction time is seen as a more significant quadratic variable in addition to its positive linear influence. Based on these results, TPC levels peaked between 70 and 90% ethanol in the range of 60–70 h. On the contrary, the lowest phenolic concentration was recorded at less than 50 h, with an ethanol concentration greater than 65%. Normally, the combination of water with organic solvents leads to a moderately polar medium that increases the interactions between the plant matrix and the extracting agent. This usually enhances the polyphenol extraction efficiency [45]. Moreover, the ethanol/water mixture is considered to be an eco-friendly solvent to be employed for natural compound extraction [46].

Figure 1b shows the results of the impact of extraction time and liquid/solid ratio on the TPC. The results show that the concentration of dry plant matter is an important factor in increasing the yield of polyphenols extraction. The TPC peaked after 65 h, while the impact of the liquid/solid ratio was more significant with low values (<1 g/12 mL).

The impact of the ethanol/distilled water ratio and the liquid/solid ratio on the polyphenol extraction (Figure 1c) had a slight effect on the quantity of polyphenols extracted. The maximum polyphenol extraction was obtained with an ethanol/distilled water ratio under 70% and a solid/liquid ratio of less than 1 g/16 mL. Based on these findings, the ideal conditions and anticipated values were determined by using a desirability function in the range of 0.95–1, with 1 denoting the most desirable result. To verify the experiment, three replicates were carried out using the ideal parameters obtained via RSM. The results are summarized in Table 3.

Table 3: Estimated optimal conditions and predicted and experimental values of the investigated responses

Optimum extraction parameters		
X_1 (h)[a]	X_2 (%)[b]	X_3 (mL/g)[c]
71.96	50.00	10.13
Response variables TPC (mg GAE[d]/g dw)[e]		
Predicted	**Experimental**	
83.47	83.50 ± 0.72	

[a]X_1, extraction time; [b]X_2, solvent concentration; [c]X_3, liquid/solid ratio; [d]GAE, gallic acid equivalents; [e]dw, dry weight; The values represent the means ± standard deviation (SD) obtained from three independent replicates.

3.2 LC-ESI-MS/MS results

To deepen the understanding of the chemical composition of plant extracts, the current study used LC-ESI-MS/MS analysis, a powerful analytical tool [47]. LC-MS-based approaches are

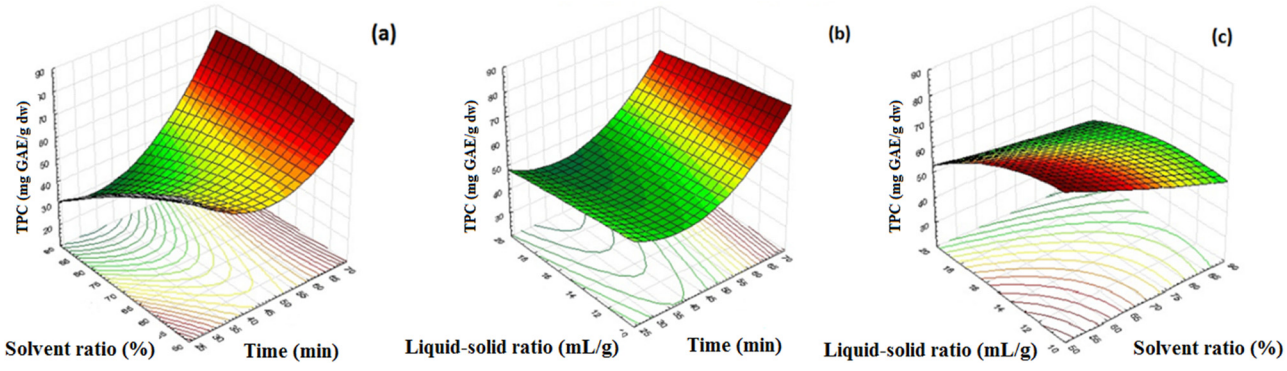

Figure 1: Response surface plots indicating combined effects of maceration parameters on TPC: (a) time and solvent ratio, (b) time and liquid–solid ratio, and (c) solvent ratio and liquid–solid ratio.

effective in understanding the biochemical diversity of such plant metabolites, which includes numerous semi-polar compounds with main groups of secondary metabolites [48]. However, this technique has its limitations due to the associated matrix effects, which are characterized by the co-elution phenomenon of residual matrix constituents impacting the ionization process of the target analytic and altering the ionization of analytes in LC-MS, affecting their response and compromising the accuracy of subsequent measurements [49,50]. This subsequently limits the applicability of this analysis for quantitative analysis due to sensitivity variations, reference alterations, imprecise results, retention time deviations, and distortion of chromatographic peaks [49]. Figure 2 depicts the chemical composition of the ethanolic extract of *P. acutifolia* by LC-ESI-MS/MS analysis. The choice of the ethanolic extract for LC-ESI-MS/MS analysis was related to the average POLA of the ethanol solvent, which helps in the efficient extraction of phenolic compounds in plants [51]. Twelve compounds were successfully identified in the ethanolic extract of *P. acutifolia* by utilizing their MS fragmentation patterns, high-resolution mass, and retention time (Table 4). Furthermore, the identified compounds were quantified.

The component present in significant quantities was mainly hesperidin, with a concentration of 1829.0 µg/g, followed by chlorogenic acid, with a concentration of 312.431 µg/g. However, fisetin was also present, albeit in lower concentrations (i.e., 1.907 µg/g). Indeed, hesperidin, the most abundant compound, is a natural phenol compound known for its antioxidant and anticancer properties [52], and has an inhibitory effect in neurodegenerative diseases [53]. The considerable concentration of this component in the ethanolic extract of *P. acutifolia* may account for the observed therapeutic potential of this plant, as evidenced by previous tests indicating its efficacy in reducing free radicals and inhibiting the proliferation of cancer cells. Moreover, compounds including protocatechuic acid, hydroxybenzaldeyde, caffeic acid, vanillin, *o*-coumaric acid, salicylic acid, isoquercitrin, kaempferol-3-glucoside, and naringenin were detected in this plant, which has shown therapeutic effects in previous studies [54–56]. The protocatechuic acid and the naringenin have notable antioxidant and antiproliferative properties [54,57]. Fisetin, a bioactive compound found in various vegetables and fruits, exhibits anticancer properties [55]. Vanillin, a safe-for-use food flavoring agent, has anti-metastatic potential and decreases the invasiveness of breast cancer cells, with great potential for use as cancer treatment [58]. Furthermore, salicylic acid improves the growth rate, photosynthesis, and antioxidant activity under moderate salt stress [59]. Additionally, chlorogenic acid, a prominent dietary polyphenol naturally occurring in green coffee, displays antioxidant activity [56]. The isolation and purification of these bioactive molecules and the assessment of their therapeutic potential open new scientific perspectives for future studies.

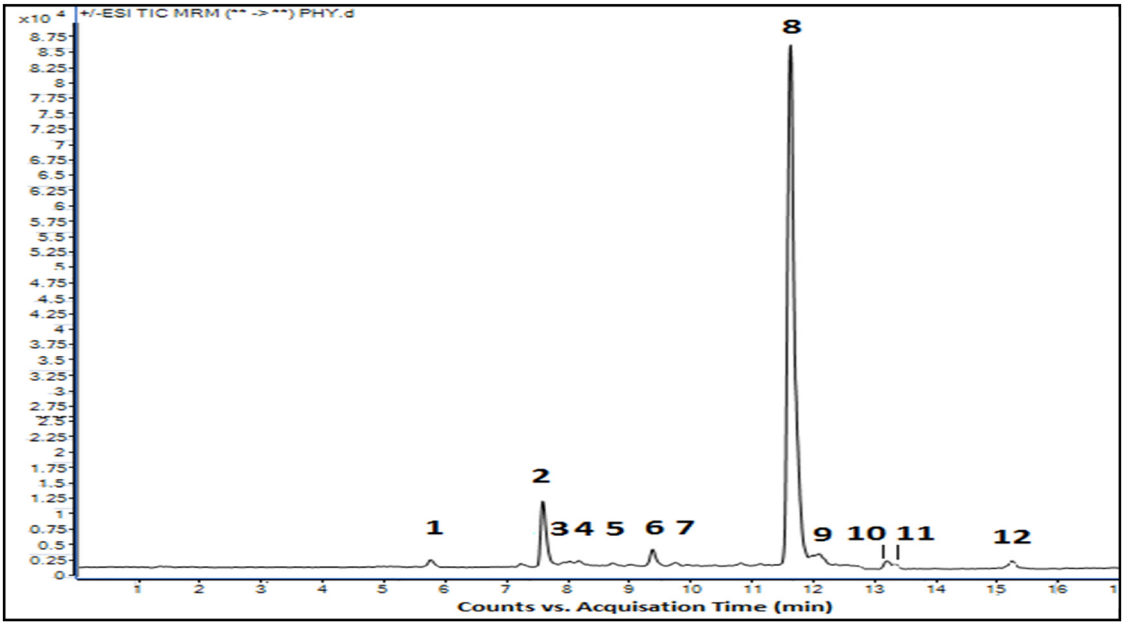

Figure 2: Chemical composition of *P. acutifolia* ethanolic extract.

Table 4: Phenolic compounds identified and quantified by LC-ESI-MS/MS analysis of *P. acutifolia* ethanolic extract

No	Retention time (min)	Compound	Concentration (µg/g)	Ionsource	Ion transitions	Ion mode	R^2	LOQ (µg/L)	LOD (µg/L)	Linearity range (µg/L)
1	5.897	Protocatechuic acid	20.993	ESI	153.0 → 109.0	Negative	0.9969	13.173	3.156	15.625–250
2	7.584	Chlorogenic acid	312.431	ESI	353.0 → 191.0	Negative	0.9981	25.902	11.589	31.25–500
3	7.9236	Hydroxybenzaldeyde	4.657	ESI	121.0 → 92.0	Negative	0.9993	12.865	4.9742	15.625–250
4	8.0313	Caffeic acid	10.686	ESI	178.9 → 135.1	Negative	0.9994	24.162	6.920	31.25–500
5	8.716	Vanillin	5.533	ESI	153.0 → 125.0	Positive	0.9949	40.541	14.588	62.5–1000
6	9.377	*o*-Coumaric acid	14.919	ESI	163.0 → 119.1	Negative	0.9996	7.9973	4.016	15.625–500
7	9.753	Salicylic acid	23.836	ESI	137.0 → 93.1	Negative	0.9981	82.964	47.669	112.5–1800
8	11.616	Hesperidin	1829.0001	ESI	611.0 → 302.9	Positive	0.9957	17.675	4.139	31.25–500
9	11.656	Isoquercitrin	52.565	ESI	464.9 → 302.8	Positive	0.9982	11.268	9.938	18.75–300
10	13.198	Kaempferol-3-glucoside	4.931	ESI	448.8 → 286.9	Positive	0.9997	4.5238	1.161	7.8125–125
11	13.217	Fisetin	1.907	ESI	287.0 → 137.0	Positive	0.9954	44.366	10.896	15.625–250
12	15.100	Naringenin	10.313	ESI	270.9 → 119.1	Negative	0.996	0.4575	1.369	31.25–500
Total quantification			**2291.7711 µg/g**							

Table 5: Phenolic and flavonoid contents of *P. acutifolia* extracts

Extract	TPC[a] ± SD[b] (mg GAE[c]/g dw[d])	TFC[e] ± SD (mg QE[f]/g dw)
Ethanol	83.50 ± 0.72[a]	54.29 ± 0.55[a]
Chloroform	71.96 ± 0.42[b]	6.20 ± 0.73[b]
Ethyl acetate	124.72 ± 0.85[c]	44.62 ± 1.05[c]
n-Butanol	263.84 ± 1.14[d]	72.03 ± 0.27[d]

[a]TPC, total phenolic content; [b]SD, standard deviation; [c]GAE, gallic acid equivalents; [d]dw, dry weight; [e]TFC, total flavonoid content, [f]QE, quercetin equivalents. The values reported are from three independent analyses. The unrelated characters (a, b, c, or d) denote significant variations between the values ($p < 0.05$).

3.3 TPC and TFC

Table 5 shows the TPC and TFC of *P. acutifolia* extracts. The results show that the *n*-butanol fraction is richest in polyphenols and flavonoids (i.e., 263.84 ± 1.14 mg GAE/g dw and 72.03 ± 0.27 mg QE/g, respectively). The lowest amounts of TPC and TFC were detected in the chloroform fraction (i.e., 71.96 ± 0.42 mg GAE/g dw and 6.20 ± 0.73 mg QE/g, respectively). The results confirm that the increasing POLA of the solvent enhanced the recovery of polyphenols and flavonoids in the extract [60]. The extraction efficiency and quality of end-products are strongly influenced by several co-occurring factors, encompassing the nature of solvents, ambient temperature, and treatment duration, along with the compositional and physicochemical properties of the samples *per se* [61]. However, the POLA of each solvent exerted a distinct influence on the dissolution rate of active compounds during the extraction process. Consequently, this variability significantly impacted the yield of end-product metabolites [62]. The solvents with the highest POLA, such as *n*-butanol and ethyl acetate, are also the most suitable for polyphenol extraction due to their increased affinity to these compounds compared to non-polar solvents [63]. Therefore, the lower ability to extract phenolics and flavonoids by chloroform can be explained by the low solubility of these compounds in low-polar solvents [64]. To the best of our knowledge, studies with a main focus on *Physalis* sp. using various solvent extracts are lacking. Banothu et al. [65] reported for the first time the abundance of flavonoids in the polar solvents of *P. minima* (i.e., ethyl acetate) compared to non-polar solvents (i.e., chloroform), which is in agreement with our findings.

3.4 Biological activities

3.4.1 Antioxidant activity

Table 6 lists the antioxidant activities of *P. acutifolia* extracts. Given that the polyphenols comprise the main

class of antioxidant phytochemicals [66,67], *in vitro* antioxidant assays were also performed (Table 6). The identification of phenolic compounds is usually followed by a thorough investigation of the antioxidant activity of extracts [68]. In the current study, the antioxidant activity of *P. acutifolia* extracts (i.e., ethanol, chloroform, ethyl acetate, and *n*-butanol) was evaluated by various methods, such as DPPH, ABTS, FRAP, phenanthroline, and SNP, respectively. The results of the antioxidant potential, measured in terms of IC_{50} or $A_{0.5}$, were compared with different reference standards (Table 6).

The *n*-butanol extract had high antioxidant potential compared to other *P. acutifolia* extracts, with IC_{50} values equal to 72.81 ± 0.44 and 49.77 ± 0.43 µg/mL by DPPH and ABTS assays, respectively, and $A_{0.5}$ values equal to 49.77 ± 0.72 and 7.33 ± 0.33 µg/mL by FRAP and phenanthroline assays, respectively. The activity of the *n*-butanol extract was followed by that of the ethyl acetate extract, whereas the chloroform extract had the lowest antioxidant activity. In summary, DPPH, FRAP, and phenanthroline assays showed that the effectiveness of the different extracts in terms of antioxidant activity was as follows: chloroform extract < ethanol extract < ethyl acetate extract < *n*-butanol extract. Regarding the ABTS assays, the resulting efficacy was as follows: chloroform extract < ethyl acetate extract < ethanol extract < *n*-butanol extract. Even if *P. acutifolia* extracts were all less effective in terms of antioxidant activity than the reference standards, they can be employed in several application fields. The *n*-butanol extract can then be considered to be a potent antioxidant depending on its IC_{50} values with DPPH and ABTS assays (72.81 ± 0.44 and 49.77 ± 0.43 µg/mL, respectively), and its $A_{0.5}$ values with FRAP and phenanthroline assays (49.77 ± 0.72 and 7.33 ± 0.33 µg/mL, respectively) [69]. The abundance of phenolic molecules and components with antioxidant properties identified by LC-ESI-MS/MS as hesperidin, protocatechuic acid, naringenin, and chlorogenic acid, respectively, can justify the overall antioxidant potential of plant extracts from *P. acutifolia* [52,54,56,57]. Naringin and hesperidin possess hydroxyl groups at positions 4 and 3, respectively, which very likely increased the antioxidative efficacy of flavonoids [70]. Moreover, caffeic acid was also considered an important candidate with antioxidant properties, as revealed by increased demonstrable efficacy in *in vitro* assays, mostly against ABTS and DPPH radicals, which were benchmarked against established standards, such as BHA and butylated hydroxytoluene [71]. The observed difference in the antioxidant activity can be explained by the variable POLA of the solvents used in the current study. This POLA induced the selective extraction of certain antioxidant compounds according to their chemical structures, polarities, and solubility, thus modulating the overall antioxidant capacity of the resulting extracts [72]. The information on the antioxidant activity of *P. acutifolia* and those comparing the antioxidant potential of polar and non-polar extracts of *Physalis* genus was not further explored in the current study. However, polar solvents such as ethyl acetate were reported for the first time in the current study to have the highest antioxidant activity in *P. minima* extracts compared to non-polar solvents such as hexane and chloroform, which showed the lowest activity [65].

3.4.2 Anti-enzymatic activities

The results of *in vitro* enzymatic activity of *P. acutifolia* extracts are presented in Table 7.

The chloroform extract of *P. acutifolia* showed an anti-BChE with an IC_{50} of 187.12 ± 1.36 µg/mL. However, the IC_{50} of the positive control galantamine was 34.75 ± 1.99 µg/mL. The other extracts were almost inactive against BChE with IC_{50} values higher than 200 µg/mL. Moreover, all the extracts tested were also nearly inactive against both AChE (IC_{50} >

Table 6: *In vitro* antioxidant activity of *P. acutifolia* extracts

Extracts and standards	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	FRAP $A_{0.5}$ (µg/mL)	Phenanthroline $A_{0.5}$ (µg/mL)	SNP $A_{0.5}$ (µg/mL)
Ethanol	331.74 ± 2.22^{a}	54.17 ± 0.37^{a}	>200	90.50 ± 0.50^{a}	>400
Chloroform	657.49 ± 7.7^{b}	222.84 ± 0.55^{b}	193.94 ± 1.42^{a}	170.66 ± 0.57^{b}	>400
Ethylacetate	138.46 ± 1.46^{c}	136.53 ± 0.73^{c}	141.05 ± 1.33^{b}	41.50 ± 0.33^{c}	>400
n-Butanol	72.81 ± 0.44^{d}	49.77 ± 0.43^{d}	49.77 ± 0.72^{c}	7.33 ± 0.33^{d}	>400
BHA	6.35 ± 0.13^{e}	12.11 ± 0.10^{e}	NT	1.04 ± 0.14^{e}	NT
BHT	12.59 ± 0.34^{e}	1.28 ± 0.05^{f}	NT	2.24 ± 0.06^{f}	NT
α-Tocopherol	NT	NT	34.50 ± 0.50^{d}	NT	NT
Ascorbic acid	NT	NT	6.50 ± 0.50^{e}	NT	7.14 ± 0.12^{a}
Trolox	NT	NT	NT	NT	34.17 ± 1.03^{b}

BHT, butylhydroxyltoluene; BHA, butylhydroxyanisole; NT, not tested. The values within the columns, denoted by unrelated characters (a, b, c, d, e, or f), exhibit significant differences ($p < 0.05$).

Table 7: *In vitro* enzymatic activity of *P. acutifolia* extracts

Extracts and standards	AChE IC_{50} (µg/mL)	BChE IC_{50} (µg/mL)	α-Amylase IC_{50} (µg/mL)	Urease IC_{50} (µg/mL)
Ethanol	>200	>200	>400	NA
Chloroform	>200	187.12 ± 1.36[a]	>400	NA
Ethylacetate	>200	>200	>400	NA
n-Butanol	>200	>200	>400	NA
Galantamine	6.27 ± 0.36	34.75 ± 1.99[b]	NT	NT
Acarbose	NT	NT	365.09 ± 2.07	NT
Thiourea	NT	NT	NT	11.57 ± 1.13

AChE, acetylcholinesterase; BChE, butyrylcholinesterase; NT, not tested; NA, not active; the values within the columns, denoted by different superscripts (a or b), demonstrate substantial variances ($p < 0.05$).

200 µg/mL) and α-amylase (IC_{50} > 400 µg/mL). These results are in contrast with the data reported in the literature, which reported the activity of plants from the *Physalis* genus against the α-amylase enzyme and the enzymes involved in Alzheimer's disease [73]. The high abundance of hesperidin, with its potent inhibitory properties against enzymes implicated in neurodegenerative diseases, emphasizes the pivotal role played in impeding the pathological processes associated with Alzheimer's [53]. Andrade et al. [74] reported the important role played by caffeic acid in preventing and treating Alzheimer's disease by the inhibition of aggregation and disruption of Aβ fibrils. Moreover, isoquercetin is a monoglycoside of quercetin, which was detected by LC-ESI-MS-MS in the ethanolic extract of *P. acutifolia* and is an effective inhibitor of alpha-amylase in a competitive mode [75]. In addition, *P. acutifolia* extracts were inactive against urease.

3.4.3 Anticancer activity

The cytotoxicity of the ethanolic extract of *P. acutifolia* against three cell lines, namely L929 (fibroblast cell line), CAPAN-1 (pancreatic cancer cell line), and dld-1 (colorectal adenocarcinoma cell line), was assessed using the MTT assay. The results are presented in Table 8.

The obtained results show a significant cytotoxic effect of *P. acutifolia* ethanolic extract against both dld-1 and CAPAN-1 cell lines at different concentrations with an IC_{50} value equal to 0.959 ± 0.02 mg/mL for dld-1 and more than 1 mg/mL for CAPAN-1 and L929 cell lines, concluding that the mean effectiveness of *P. acutifolia* ethanolic extract against dld-1 cell line was comparable with the other two. The cytotoxic effect, validated using simple linear regression analysis, showed a highly significant negative correlation between the concentration (*x*-axis) and cell viability against dld-1 (R^2 = 0.993; $p < 0.001$***); CAPAN-1 (R^2 = 0.943; $p < 0.001$***), and L929 (R^2 = 0.842; $p < 0.001$***) (Figure 3). The extract concentration of 1 mg/mL had the greatest impact, leading to a cell line viability of 46.42 and 57.74% for dld-1 and CAPAN-1, respectively. The cytotoxic effect of this extract was not significant at concentrations of 0.5, 0.25, and 0.125 mg/mL. Conversely, the ethanolic extract showed low toxicity against L929 cell lines, which are the reference healthy cell lines since cell viability was 93.25% for an extract concentration of 1 mg/mL. The observed anticancer potential of *P. acutifolia* may be enhanced by the presence of various bioactive molecules, such as hesperidin, which was

Table 8: Cytotoxic effect of *P. acutifolia* ethanolic extract on cancer cell lines

Concentration (mg/mL)	Cell viability (%)		
	dld-1	CAPAN-1	L929
1.0	46.42 ± 1.99[a]	57.74 ± 1.12[a]	93.25 ± 1.47[a]
0.5	110.59 ± 1.78[b]	147.60 ± 0.98[b]	304.97 ± 2.65[b]
0.25	148.48 ± 1.31[c]	152.02 ± 1.03[b]	302.33 ± 2.32[b]
0.125	172.37 ± 2.13[c]	178.98 ± 2.36[b]	307.67 ± 2.36[b]
IC_{50} (mg/mL)	0.959 ± 0.02	>1.0	>1.0
Control absorbance	0.2397 ± 0.001	0.2422 ± 0.002	0.1223 ± 0.001

Values within the columns, denoted by different superscripts (a, b, or c), demonstrate substantial variances ($p < 0.05$).

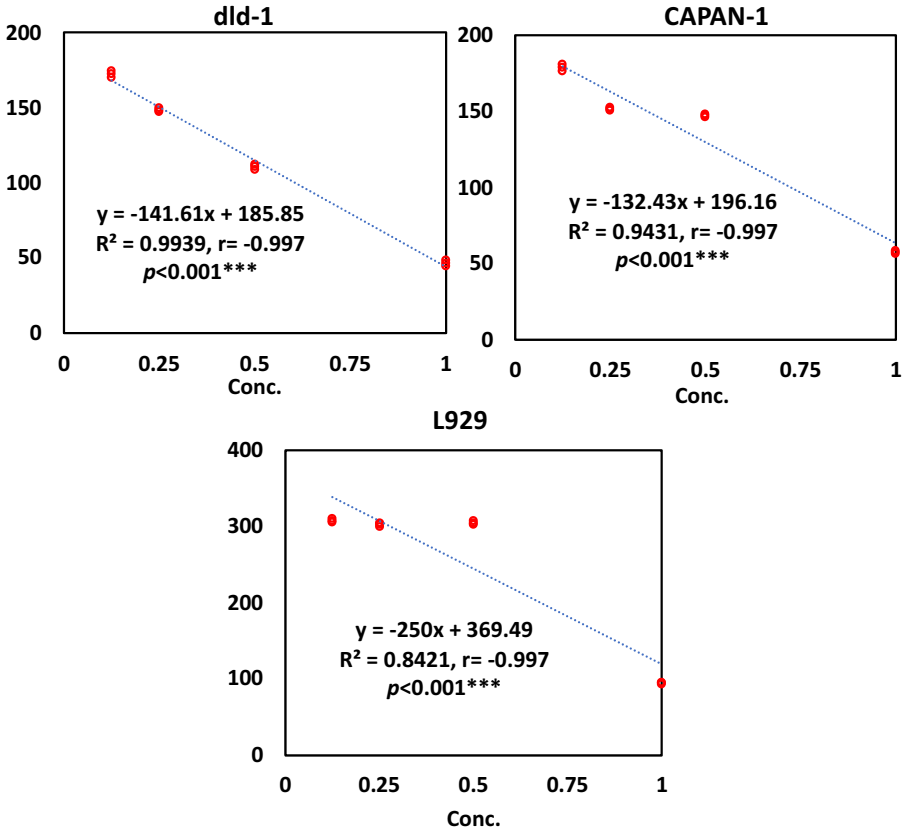

Figure 3: Regression trendline presenting the relationship between concentration and cell viability (%).

Table 9: ADMET properties of the *P. acutifolia* identified compounds

Entry	*P. acutifolia* identified compounds										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**9**	**10**	**11**	**12**
Physicochemical properties/lipophilicity											
Molecular weight	154.12	354.31	122.12	180.16	152.15	164.16	138.12	464.38	448.38	286.24	272.25
No. heavy atoms	11	25	9	13	11	12	10	33	32	21	20
No. arom. Heavy atoms	6	6	6	6	6	6	6	16	16	16	12
Fraction Csp3	0	0.38	0	0	0.12	0	0	0.29	0.29	0	0.13
No. rotatable bonds	1	5	1	2	2	2	1	4	4	1	1
No. H-bond acceptors	4	9	2	4	3	3	3	12	11	6	5
No. H-bond donors	3	6	1	3	1	2	2	8	7	4	3
Molar refractivity	37.45	83.5	33.85	47.16	40.34	45.13	35.42	110.16	108.13	76.01	71.57
TPSA (Å²)	77.76	164.75	37.3	77.76	46.53	57.53	57.53	210.51	190.28	111.13	86.99
Consensus Log $P_{o/w}$	0.65	−0.39	1.17	0.93	1.2	1.4	1.24	−0.48	−0.09	1.55	1.84
Druglikeness/bioavailability/pharmacokinetics											
Lipinski's rule	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes
Bioavailability score	0.56	0.11	0.55	0.56	0.55	0.85	0.85	0.17	0.17	0.55	0.55
GI absorption	High	Low	High	High	High	High	High	Low	Low	High	High
BBB permeant	No	No	Yes	No	Yes	Yes	Yes	No	No	No	No
P-gp substrate	No	No	No	No	No	No	No	No	No	No	Yes
CYP1A2 inhibitor	No	No	No	No	No	No	No	No	No	Yes	Yes
CYP2C19 inhibitor	No	No	No	No	No	No	No	No	No	No	No
CYP2C9 inhibitor	No	No	No	No	No	No	No	No	No	No	No
CYP2D6 inhibitor	No	No	No	No	No	No	No	No	No	Yes	No
CYP3A4 inhibitor	Yes	No	No	No	No	No	No	No	No	Yes	Yes
Log *K*p (cm/s)	−6.42	−8.76	−6.13	−6.58	−6.37	−5.86	−5.54	−8.88	−8.52	−6.65	−6.17
Synthetic accessibility	1.07	4.16	1	1.81	1.15	1.85	1	5.32	5.29	3.16	3.01

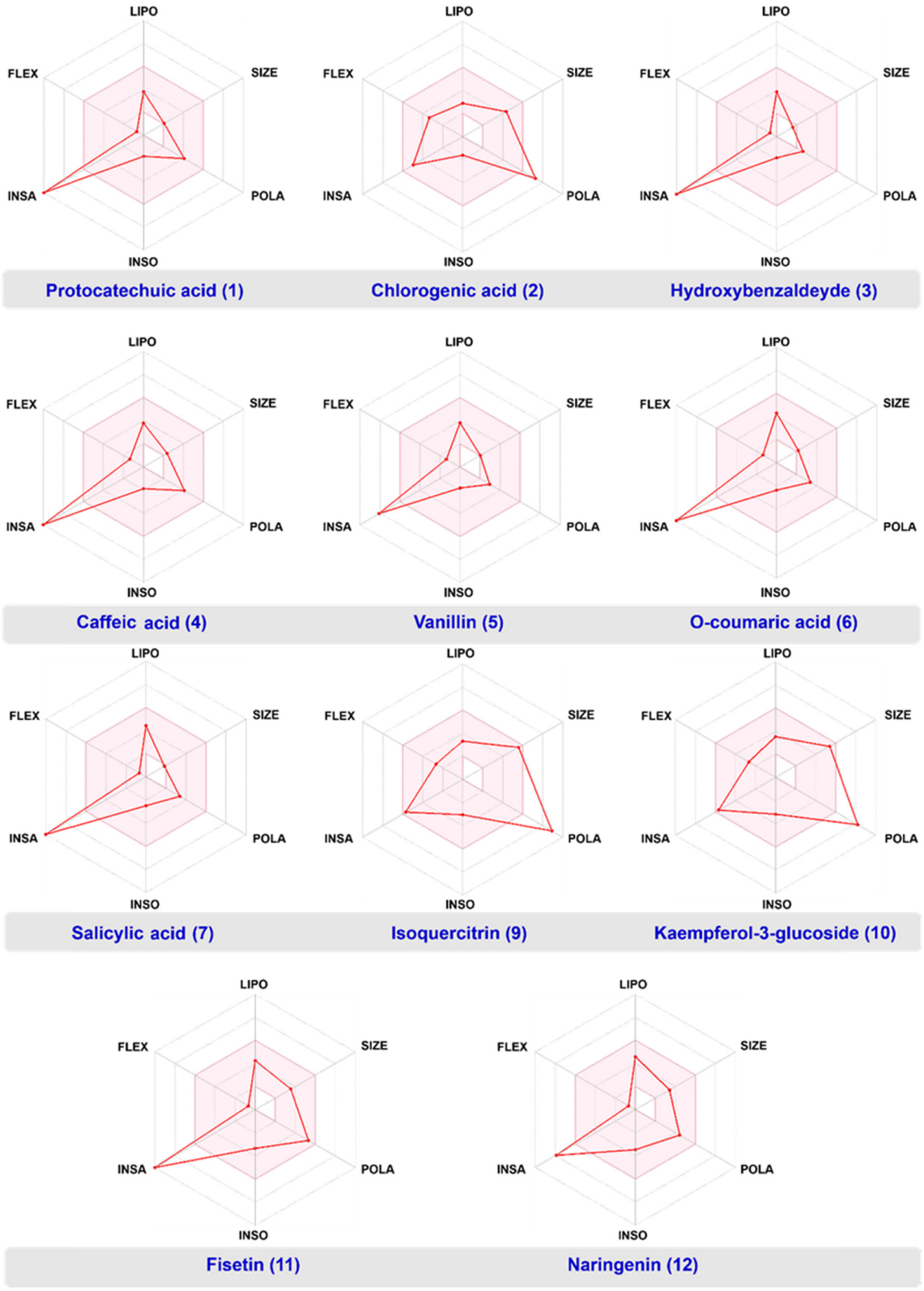

Figure 4: Bioavailability hexagons of the major *P. acutifolia* identified compounds as assessed by LC-ESI-MS/MS analysis of the ethanolic extract: (LIPO) lipophilicity, (SIZE) molecular size, (POLA) polarity, (INSO) insolubility, (INSA) unsaturation, and (FLEX) flexibility.

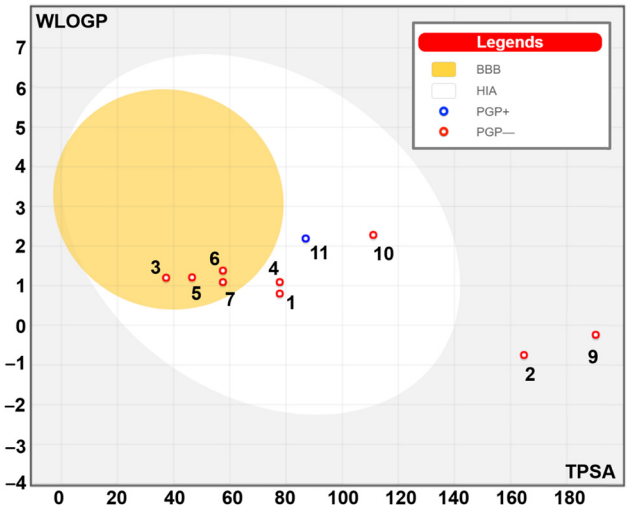

Figure 5: Boiled-egg model of *P. acutifolia* identified compounds as assessed by LC-ESI-MS/MS analysis of the ethanolic extract: (1) protocatechuic acid, (2) chlorogenic acid, (3) hydroxybenzaldeyde, (4) caffeic acid, (5) vanillin, (6) *o*-coumaric acid, 7) salicylic acid, (8) hesperidin; (9) isoquercitrin, (10) kaempferol-3-glucoside, (11) fisetin, and (12) naringenin.

the dominant compound in the current study and is known for its anticancer properties [52]. Moreover, hesperidin was reported to influence several types of cancer, such as gastric, colon, and breast, by inducing apoptotic death in the cancer cells via intrinsic and extrinsic pathways [76]. Furthermore, compounds such as protocatechuic acid, caffeic acid, and naringenin also exhibit significant antiproliferative properties [54,57]. Additionally, fisetin, a bioactive compound commonly found in vegetables and fruits, is also known for its efficient anticancer properties [55]. The assessment of the anticancer activity of some *Physalis* plants has already shown satisfactory results [14]. Among *Physalis* plants that have shown cytotoxic effects, *P. neomexicana* has demonstrated a notable cytotoxic effect on two human breast cancer cell lines, namely MDA-MB-231 and MCF-7, indicating its potential as a therapeutic agent for breast cancer treatment with IC_{50} values of 1.7 and 6.3 µM, respectively [77]. Similarly, withanolides from *P. peruviana* L. exhibited selective cytotoxic activity against two prostate cancer cell lines, LNCaP and 22Rv1, with IC_{50} values of 0.94 and 0.99 µM, respectively [78]. Additionally, withanolides extracted from *P. angulate* L. demonstrated cytotoxic activity against three different cell lines, A549, p388, and HeLa, with IC_{50} values of 11.36, 8.03, and 21.75 µM, respectively [79].

Withanolides extracted from the methanolic extract of *P. acutifolia* exhibited cytotoxic activity against a panel of human cancer cell lines, namely NCIH460 (non-small-cell lung), SF-268 (CNS glioma), PC-3 (prostate adenocarcinoma), and MCF-7 (breast adenocarcinoma), as well as normal human lung fibroblast cells (WI-38) with IC_{50} values of 6.5, 7.2, 2.3, 1.7, and 3.9 µM, respectively [21]. These findings indicate that the current study validates the cytotoxic potential of *P. acutifolia* against additional cell lines. Moreover, the presence of bioactive molecules suggests that further testing on different types of cell lines and cancer cell lines could be conducted to explore its potential for cancer treatment. *In vivo* tests are also recommended considering their crucial role in confirming the anticancer potential observed *in vitro* by offering essential insights into effectiveness, safety, and drug behavior in living organisms, and thus unraveling intricate biological processes. By evaluating the compounds in dynamic tumor environments, these studies validate initial observations and propel promising candidates toward clinical applications [80]. In addition, the use of other colorectal adenocarcinoma cell lines is advised. In fact, colorectal carcinomas present a disability by coherent chromosomal gains and losses, despite intratumoral heterogeneity. This highlights stable genomic alterations in the cancer cell population [81].

3.5 Bioavailability and pharmacokinetics

Both bioavailability and pharmacokinetic analyses are commonly explored for computer-aided drug design and to avoid drug failure at advanced stages [38,39,82]. Table 9 exhibits the bioavailability and pharmacokinetic properties of the compounds identified in *P. acutifolia* ethanolic extract. Our data showed that the majority of the compounds (10 out of 12) met the Lipinski rule and possessed acceptable bioavailability scores (BAS). BAS values varied between 0.11 and 0.85. The skin permeability of *P. acutifolia* identified compounds were predicted to be low to moderate, as log *K*p ranged between –5.54 and –8.88. Similar findings, particularly for the BAS results, supported that *P. acutifolia* compounds have biological activities without eventual violations and/or toxic outcomes. This was further supported by the bioavailability hexagons (Figure 4), which depend on the physicochemical characteristics of the compounds. While the majority of the components were associated with high gastrointestinal (GI) absorption, only four of them were blood–brain barrier permeants. These compounds are hydroxybenzaldeyde, vanillin, *o*-coumaric acid, and salicylic acid, which correspond to phytochemicals 3, 5–7, respectively. The boiled-egg mapping (Figure 5) supported these calculations. Interestingly, regardless of compound 12, all the others are not predicted to be substrates for P-glycoprotein (P-gp). Hence, it could be deduced that *P. acutifolia* phytochemicals induced no disruption of drug distribution and elimination [82–84]. The content of the studied extract is also safe for the

transportation and distribution of the drugs as most of the phytochemicals did not inhibit the majority of the cytochrome P450 (CYP) isoforms: CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4 [39,40]. Furthermore, eight compounds (2–10) did not inhibit the five studied CYPs. The synthetic accessibility of *P. acutifolia* phytochemicals ranged between 1.0 and 5.32, which indicates that they are easy to synthesize and suitable drug molecules [38,84,85]. The beneficial effects of *P. acutifolia* phytochemicals have already been reported in the current study itself through *in vitro* approaches. Altogether, our findings support the promising biological activities and health promotion potentialities of natural-derived compounds, phytotherapy, and medicinal plants including *P. acutifolia* [42,82,85].

4 Conclusions

The current study emphasized the polyphenol content extracted from *P. acutifolia,* a process optimized by maceration using the CCDC design. The concentration of polyphenols and flavonoids as well as the antioxidant and anti-enzymatic activities were assessed for the ethanolic extract and its fractions (i.e., chloroform, ethyl acetate, and *n*-butanol, respectively). The *n*-butanol fraction had the highest TPC and TFC, as well as superior antioxidant and anti-enzymatic activities. Moreover, the current study proved the cytotoxic effect of the ethanolic extracts of *P. acutifolia* against two cancer cell lines (i.e., CAPAN-1 and dld-1). However, the anticancer effect was almost negligible on the healthy cell line L929. The LC-ESI-MS/MS analysis identified 12 components in the ethanolic extract of the plant. Out of these, hesperidin, which has powerful antioxidant and anticancer properties, was measured in high concentrations. Both the bioavailability and pharmacokinetic properties of *P. acutifolia* might explain the antioxidant, anti-enzymatic, and anticancer effects, which can certainly be the consequence of the phytochemical constituents. These findings open further research opportunities to develop more efficient and effective food preservatives in terms of therapeutic agents and will be further improved in support of folk medicine and "*in vivo*" biological activities.

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