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Optimization of the Extraction Process of Bioactive Compounds from *Zingiber officinale* Roscoe, Evaluation of Acetylcholinesterase Enzyme Inhibition and Cytotoxic Activity of the Free and Encapsulated Extract

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The present study optimized the extraction process of bioactive compounds present in ginger (*Zingiber officinale*) dried at 80 °C, using ethanol:water 70:30 (v/v) as solvent. The extracts were evaluated for antioxidant activity by the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical reduction methods and by the chelating activity of Fe^{II} ions. It was found that the extraction condition with a temperature of 60 °C and an extraction time of 12 h showed better responses to the tests. Then, the characterization of the compounds was carried out by mass spectrometry and thermal analysis (thermogravimetric (TG), differential thermal analysis (DTA), and differential scanning calorimetry (DSC)), identifying that the main compounds of ginger were gingerols and shogaols, being confirmed by the intensities and characteristics of the thermal graphs. The inhibition of the enzyme acetylcholinesterase (AChE) was evaluated using the Ellman test, which did not show an inhibitory action. Regarding cytotoxic activity, the free extract and encapsulated in liposomes were tested, showing antiproliferative effect at different concentrations for human kidney tumor cells (786-0), liver cells (HUH7.5), and *Macaca mullata* normal kidney cells (LLC-MK2). Given the results obtained, ginger presents itself as a renewable source of bioactive compounds and can be indicated for applications in the pharmaceutical industry.

Keywords: antioxidants, main component analysis, factorial planning, AChE, cancer

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Introduction

Ginger (*Zingiber officinale* Roscoe) is an herbaceous plant with a branched underground rhizome belonging to the Zingiberaceae family. This food has many bioactive compounds, such as phenolic compounds and terpenes, with a more significant proportion being gingerols, shogaols, and paradols.[1,2]

Bioactive compounds and antioxidants are widely evaluated and used in industries, due to the inhibition or delay of free radicals, being used in several pharmacological activities, including anti-inflammatory, anticancer, antimicrobial, antioxidant action and neuroprotective action.[3-7] These properties allow ginger to be used in the treatment of diabetes, nausea, gastric diseases, colds, obesity control, and others.[2,8-16]

Although synthetic drugs are efficient, they have several side effects and high costs. Thus, natural alternatives are sought, such as some vegetables, that provide the desired effectiveness, both in the prevention and treatment of degenerative diseases, and these searches are based on traditional medicine as a starting point.[17-19]

Bioactive compounds extracted from ginger were tested *in vitro* and *in vivo* to evaluate their potential to inhibit acetylcholinesterase (AChE). This study showed that the improvement in brain activities is related to ginger's antioxidant activity, which increases the level of neurotransmitters.[10]

It was also observed that the oral administration of ginger extract (GE) in rats increased memory capacity and cognition processes. The extract was used to affect the brain's cholinergic functions and to reduce neurodegradation caused by oxidative stress.[10]

When evaluating during 24 h the *in vitro* cytotoxic action of ginger on different cancer strains, Kumara *et al*.[20] confirmed its chemopreventive and chemotherapeutic action and observed a 13% reduction in fibrosarcoma cell viability, 25% in colon cancer cells, and 26% in macrophages from mice with leukemia.

Despite the numerous benefits of bioactive compounds, obstacles such as low solubility and short shelf-life influence their bioavailability when ingested. Furthermore, phytochemical compounds have low specificity, being easily absorbed by healthy cells as a nutrient source.[21,22]

Nanotechnology associated with conventional therapies has been increasingly implemented to overcome such factors. In particular, liposomes favor targeted delivery of the encapsulated material through endocytosis or by fusion mechanisms, as well as protecting against environmental and biological degradation and allowing controlled release.[22,23]

Based on the neuroprotective and cytotoxic actions related to the bioactive compounds present in ginger, the objectives of this work were to optimize the extraction of bioactive compounds from dried ginger by evaluating the antioxidant activity using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging methods and the iron(II) chelation capacity. For the best extract, the neurotoxic action related to AChE enzyme inhibition, encapsulation of the extract in liposomes, and evaluation of the cytotoxic activity of the encapsulated and free extract against monkey kidney tumor cells, liver cells, and normal kidney cells were evaluated.

Experimental

Preparation of sample

The ginger rhizomes were purchased locally at Apucarana City, Paraná, Brazil, in September 2019. Initially, samples were washed, peeled, sliced, and kept in an oven with air circulation (Solab model SL-102, Apucarana, Brazil) at 80 °C for 7 h.[3] Subsequently, the samples were ground, and the powder was packed in airtight bags and kept in a refrigerator until the chemical analysis was conducted.

Experimental design-optimization of extraction by conventional extraction

The experiments were developed based on a central composite rotatable design (CCRD) with two factors, including the central point with three replications. Independent variables were selected as temperature and extraction time, each in five coded levels (–1.41, –1, 0, +1, +1.41) for a total of eleven experiments (Table 1).

Table 1. Coded and real levels of independent variables

Independent variables	Coded and real levels of independent variables				
	–1.41	–1	0	+1	+ 1.41
Temperature / °C	20	25	40	55	60
time / h	0.5	4	12	20	24

Extractions were carried out as follows: 50.0 mL of ethanol:water (EtOH:H_2O) 70:30 (v/v) (Sigma-Aldrich, Barueri, Brazil) solvent system was added to 0.5 g of dehydrated ginger, under constant agitation at 140 rpm in a shaker (Marconi model MA-420, Apucarana, Brazil), as *per* experimental design time and temperature. The extracts were filtered, and the volumes adjusted with ethanol in a

50.0 mL volumetric flask (0.01 g mL^{-1}), then transferred to amber flasks and stored under refrigeration for subsequent analysis. The extracts were prepared in a random order, to avoid a systematic error. All experiments were performed in duplicate and with a random sequence.

DPPH radical scavenging activity of the ginger extract

The method involving the scavenging of the free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) was performed according to Brand-Williams *et al*.[24] with modifications by Bressiani *et al*.,[25] in which it is observed the decay of the DPPH• radical color (0.1192 mM) (Sigma-Aldrich, Barueri, Brazil) when in contact with the sample (0.01 g mL^{-1}). The antioxidant activity (AA) was expressed as a percentage of inhibition of the control. Analysis was performed in triplicate.

ABTS radical scavenging activity of the ginger extract

The methodology used to perform the radical $ABTS^{\bullet+}$ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) cation scavenging test was described by Rufino *et al*.,[26] with modifications, in which the decay of the $ABTS^{\bullet+}$ radical coloration (2.4214 mM) (Sigma-Aldrich, Barueri, Brazil) is observed when in contact with the sample (0.01 g mL^{-1}). After the reaction period (6 min), absorbance readings were taken in a spectrophotometer (Agilent Technologies Cary 60 UV-Vis model, Santa Clara, CA, United States) at 734 nm. The antioxidant activity was expressed as a percentage of inhibition of the control. Analysis was performed in triplicate.

Iron(II) chelating ability

To determine the iron(II) chelating ability, the methodology developed by Stookey[27] was applied, with modifications.[28,29] The readings were carried out in a spectrophotometer (Agilent Technologies Cary 60 UV-Vis model, Santa Clara, CA, United States) at 562 nm. The calculation of the percentage of iron(II) chelating ability (CA) of ginger extracts was obtained from equation 1, where Ab is the initial absorbance of the complex ion $[Fe^{2+}(ferrozine)_3]^{2+}$, A_0 is the absorbance of the sample and A_1 is the initial absorbance of the complex ion $[Fe^{2+}(ferrozine)_3]^{2+}$ with the sample. Analysis was performed in triplicate.

$$CA\,(\%) = \frac{\left[Ab - (A_1 - A_0)\right]}{Ab} \times 100 \quad (1)$$

Liposome production

The liposomes were produced as proposed by Zômpero *et al*.,[30] with modifications. First, the phospholipid (soy lecithin molecular weight (MW) 780 g mol^{-1}) was dispersed in ethanol at a concentration of 4.64 mM in an ultrasonic bath (ultrasonic bath Cristofoli, 42 KHz, Apucarana, Brazil) up to controlled temperature. The proportion of ginger extract was 10, 20, and 30% (m/m) of phospholipids. Then, the organic solution was dripped in water at room temperature, considering a ratio of 10% (v/v) of an organic solution in water at a flow rate of 10 mL min^{-1}, with a controlled stirring speed of 130 rpm. The characterization of the liposomes was performed by hydrodynamic mean diameter, polydispersity index, and zeta potential through the DLS Litesizer 500 equipment (Aton Paar, Apucarana, Brazil), at 25 °C.

Thermogravimetric analysis

Thermal gravimetry (TG) and differential thermal analysis (DTA)

TG and DTA of the optimized extract were obtained with the thermal analysis system TGA-50 (Shimadzu, Tokyo, Japan), under a synthetic air atmosphere with an argon flow of 50 mL min^{-1}. The samples were heated from room temperature (ca. 23 °C) to 800 ºC with a heating rate of 10 ºC min^{-1}. The mass of the sample used was 7.3 mg.

Differential scanning calorimetry (DSC)

DSC curves of the optimized extract were obtained using the thermal analysis system model DSC-60 Plus (Shimadzu, Tokyo, Japan), the curves were recorded in a synthetic air atmosphere with an argon flow of 50 mL min^{-1}, a heating rate of 10 °C min^{-1} to 350 °C, and samples weighing about 5.9 mg.

Mass spectrometry

Mass spectra of the optimized extract were acquired using a Premier XE quadrupole mass spectrometer (Waters, Milford, MA, USA), equipped with electrospray ionization (ESI), by the multiple reaction monitoring (MRM) method under two different conditions (Table 2), channels were created as reported in previous studies.[31-35] For the mobile phase, a solution of MeOH and NH_4OH (0.1%) was used (Sigma-Aldrich, Barueri, Brazil). Initially, the ginger extract was diluted in the mobile phase with an initial concentration of 1000 µg mL^{-1}, then the solution was filtered through Millex-HV 0.45 µm. For analysis, the filtered solution was diluted three times in the mobile

phase before injection into the equipment, with a final concentration of 1.0 μg mL^{-1}. Data were analyzed using MassLynx software version 4.4.

Table 2. Program for the mass spectrum for the MRM method

Component	Condition 1	Condition 2
Desolvation gas temperature / °C	200	250
Source gas temperature / °C	110	110
Ionization mode	MS^-	MS^-
Capillary voltage / kV	2.0	2.5
Cone voltage / V	20.0	40.0
Collision energy / V	15.0	30.0
Collision gas pressure / mbar	3.5×10^{-3}	3.5×10^{-3}

MS: mass spectrometry.

Cytotoxic activity

Cell culture

The human kidney tumor cells (786-0), liver cells (HUH7.5), and *Macaca mullata* normal kidney cells (LLC-MK2) were grown in 25 cm^2 culture flasks containing 10 mL of Dulbecco's Modified Eagle Medium (DMEM) culture medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and incubated in an incubator-type oven at 37 °C with 5% CO_2.

Cytotoxicity assay

To determine the cytotoxic activity, the MTT (3-(4,5-dimethilthiazol-2-il)-2,5-diphenyl tetrazolium bromide) assay was used, according to the protocol suggested by Mosmann,[29] with modifications. 96-well culture plates were used, and 1.0×10^4 786-0 or HUH cells or 1.0×10^5 LLC-MK2 cells were seeded in each well, except the cell-free control well (blank). After 24 h, the culture medium from each well was discarded and 100 μL of complete medium were added to the groups: negative control (CO^-) (culture medium), positive control (CO^+) with 500 μM of the cytotoxic agent methyl methanesulfonate (MMS) and treatments with different concentrations of the extract.

The cells were incubated for 24, 48, and 72 h, and, after that time, the culture medium was replaced by 100 μL of non-supplemented culture medium, plus MTT (0.167 mg mL^{-1}). The plate was incubated again for another 4 h and then the medium containing MTT was discarded and 100 μL of dimethyl sulfoxide (DMSO) were added to the wells to solubilize the formazan crystals. The absorbance reading was performed in a microplate reader (Thermo Plate, Belo Horizonte, Brazil) at 560 nm.

The results were presented as the mean and standard deviation of absorbances and submitted to analysis of variance (one-way ANOVA), followed by Dunnet's test, using the Action Stat program.[36] Differences were statistically significant when the *p*-value was less than 0.05.

The percentage values of cell viability (CV) were estimated using equation 2.

$$CV(\%) = \left\{ \frac{ABS_T}{ABS_{CO^-}} \right\} \times 100 \quad (2)$$

where CV is the cell viability (%), ABS_T is the treatment absorbance, and ABS_{CO-} is the absorbance of the negative control.

AChE enzyme activity

Dissection of brain structures

Mice were euthanized and the brains were stored in test tubes with ice-cold 10 mM Tris HCl buffer, pH 7.2, (1:10 (m:v)). Then, they were homogenized in a glass Potter and the structures were centrifuged at 1000 g (2500 rpm) for 15 min. The supernatant was separated into micro tubes and frozen at −20 °C for further use. The protein content of the samples was determined according to the method described by Bradford,[37] using bovine serum albumin as a standard.

Quantification of AChE enzyme activity

The homogenate supernatants were used for the enzymatic assay of the AChE activity, which was determined spectrophotometrically by the method of Ellman with modifications.[38]

For this analysis, 96-well plates were used and the procedures were developed as follows: 150 μL of 100 mM potassium phosphate buffer (pH = 7.0), 80 μL of distilled water, 20 μL of homogenate and 20 μL of Iso-OMPA (inhibitor of BChEi) were added to pre-incubating for 30 min. After that, 30 μL of acetylthiocholine (10 mM) were added to start the reaction, which was conducted for 10 min at 37 °C.

The reaction was stopped by adding 20 μL of 51 mM neostigmine bromide (AChEi inhibitor) and for development, 20 μL of 5,5-dithiol-bis-(2-nitrobenzoic acid) (DTNB), 8.5 mM. AChE-catalyzed hydrolysis of acetylthiocholine iodide forms the anion 5-thio-2-nitrobenzoate (TNB). This anion is formed by the reaction of DTNB with the thiocholine resulting from hydrolysis. At the end of the reaction, the concentration of this anion can be determined at λ_{max} = 412 nm, using a spectrophotometer at room temperature. AChE activity was expressed in μmol of hydrolyzed acetylthiocholine *per* hour *per* milligram of protein.

Ethical aspects

The work was approved by the Animal Ethics Committee (AEC) of UFMS registered under No. 757/2016. The handling and care of the animals were carried out by Law No. 11.794, of 2008 and the Brazilian guideline on Practice for the Care and Use of Animals for Scientific and Didactic Purposes.[39]

Statistical analysis

The results obtained were evaluated by analysis of variance ANOVA (n = 6), Tukey's test ($p < 0.05$), and central composite rotatable design (CCRD) 2^2 using R software[40] and principal component analysis (PCA) using Past 4.04 software.[41] The results obtained through the *in vitro* biological assays were analyzed by GraphPad Prism[42] through one-way analysis of variance (one-way ANOVA), followed by Dunnett's Test.

Results and Discussion

Optimization of the extraction of compounds from ginger

The results of antioxidant activity by DPPH and ABTS free radical scavenging methods and iron(II) chelating ability (CA) for ginger extracts are shown in Table 3.

Regarding the DPPH radical reduction test, all extracts showed a response above 81.77% with a maximum of 88.49%, that is, despite the change in extraction conditions (time and temperature), the difference between them was not expressive. From the data analysis, it is possible to observe that a time of 20 h and a temperature of 25 ºC were the conditions that provided the highest percentage of DPPH radical scavenging.

When comparing the extracts produced in 4 h with temperatures of 25 and 55 °C, the responses were significantly the same, 82.20 and 81.77%, respectively. About the times of 12 and 20 h, it was observed that the increase in temperature resulted in a decrease in AA, from 85.17 to 83.58% and from 88.49 to 82.52% for the times of 12 and 20 h, respectively. Tanweer *et al.*[12] performed the extraction with ethanol:water 50:50 (v/v) in a rotary flask and obtained 51.10% inhibition of the DPPH radical, demonstrating the influence of the solvent and type of extraction of the antioxidant compounds in ginger.[2,43,44]

The behavior of the $ABTS^{\bullet+}$ radical reduction activity and the chelating activity of Fe^{II} (CA) were similar to that described for the DPPH test. The variations in the percentage of activity were from 21.05 to 29.87% for the $ABTS^{\bullet+}$ radical and from 87.15 to 99.90% for the CA. The conditions that provided the highest percentage for ABTS radical scavenging activity were 12 h and 60 °C, and to Fe^{II} ion chelating ability, the conditions that provided the highest percentage were 20 h and 55 °C and 12 h and 60 °C showing no significant difference. The extract stood out from the other samples at 60 °C for 12 h, which was used for further analysis.

In the work developed by Tanweer *et al.*,[12] when evaluating the extract of different parts of ginger using ethanol:water (50:50 v/v) as a solvent, the obtained values for the CA test were below the found in the present work, being 32.02, 18.70 and 15.62% for leaves, flower, and rhizome, respectively. However, the results obtained in this work for the ABTS and DPPH radical reduction tests were lower than those obtained by Camargo *et al.*,[3] who evaluated different hydroalcoholic extracts of ginger.

Table 3. Results of percentages of antioxidant activity (AA) obtained by the central composite rotatable design (CCRD) model for the extracts of ginger

Run	Temperature / °C	time / h	ABTS / %	DPPH / %	CA / %
1	25	4	21.05 ± 0.21^{g}	$82.20 \pm 0.13^{f,g}$	$92.63 \pm 0.15^{b,c}$
2	55	20	27.29 ± 0.15^{b}	$82.52 \pm 0.08^{e,f}$	99.90 ± 0.02^{a}
3	40	0.5	$23.36 \pm 0.17^{d,e}$	86.34 ± 0.15^{b}	88.61 ± 0.15^{f}
4	25	20	27.60 ± 0.27^{b}	88.49 ± 0.13^{a}	90.35 ± 0.06^{e}
5	40	12	22.39 ± 0.22^{f}	$82.46 \pm 0.17^{e,f}$	91.60 ± 0.09^{d}
6	60	12	29.87 ± 0.21^{a}	83.58 ± 0.14^{d}	99.78 ± 0.06^{a}
7	40	12	$22.58 \pm 0.09^{e,f}$	82.78 ± 0.15^{e}	91.28 ± 0.18^{d}
8	55	4	23.52 ± 0.07^{d}	81.77 ± 0.05^{g}	92.28 ± 0.13^{c}
9	20	12	24.70 ± 0.11^{c}	85.17 ± 0.05^{c}	87.15 ± 0.22^{g}
10	40	12	$23.14 \pm 0.04^{d,e,f}$	$82.49 \pm 0.08^{e,f}$	91.27 ± 0.21^{d}
11	40	24	23.74 ± 0.17^{d}	85.66 ± 0.11^{c}	93.22 ± 0.11^{b}

Results expressed as mean (n = 6). Equal letters in the same column indicate that there are no significant differences ($p < 0.05$) by the Tukey's test. ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; DPPH: 2,2-diphenyl-1-picrylhydrazyl; CA: chelating ability.

Different authors[15,45-47] have shown that factors such as the sample mass:solvent volume ratio, the concentration of the extract used for the analysis, type of solvent (alkaline or acid), moisture, and the part of ginger studied (leaf, rhizome, or stem), type of agitation and/or extraction procedure, cultivation conditions (planting and harvesting at different times) and even soil conditions can influence responses related to the antioxidant activity.

From the results of the experimental design presented in Table 3, theoretical models were made to gain a clear view of the effects of time and temperature factors on the antioxidant activity of the ginger extract.

The effect of the experimental factors on the independent variables of the extraction process, time (t) and temperature (T), the statistical significance, and the linear, quadratic, and factor interaction coefficients are shown in Table 4. These results show a significant variation in the responses of the antioxidant tests by varying the T and t during the extractions and the model was significantly adjusted ($p < 0.05$) with an F value of the model of 45.49 for the ABTS, 24.89 for the DPPH and 138.50 for the chelating ability. The coefficient of determination (R^2) was 0.76, 0.63, and 0.90 for ABTS, DPPH, and CA, respectively. The antioxidant activity of the extracts produced was significantly ($p < 0.001$) affected by the linear effect of temperature and time.

Note that the quadratic factor of time did not present a significant response for the ABTS and CA tests. As for the quadratic factor of temperature, the effect was the opposite, being significant for both ABTS and CA and not significant for DPPH. The other linear terms and the interaction of factors, all showed a significant difference. After determining the terms, the polynomial equations for the antioxidant tests were generated, and they were established from the removal of non-significant terms (equations 3 to 5):

$$AA_{ABTS}\ (\%) = 22.97 + 1.18T + 1.36t - 0.69Tt + 2.08T^2 \quad (3)$$

$$AA_{DPPH}\ (\%) = 83.37 - 1.08T + 0.76t - 1.39Tt + 1.00t^2 \quad (4)$$

$$CA\ (\%) = 91.53 + 3.39T + 1.49t + 2.47Tt + 1.40t^2 \quad (5)$$

To verify the normal distribution of residuals, the Shapiro-Wilk test was performed, considering the null hypothesis as being true when the p-value is greater than 0.05. In all cases evaluated in this study, the p-value was higher than that considered in the null hypothesis, being 0.29, 0.24 and 0.12 for the ABTS, DPPH and CA, respectively.

As shown in Table 4, the regression coefficients of the models showed that the combined effect of the temperature and time variables have a significant influence ($p < 0.05$) on the antioxidant action of ginger extracts. The responses of the CCRD planning for the antioxidant tests of ginger extracts against the combined action of experimental variables are illustrated in Figure 1.

Table 4. Analysis of variance for central composite rotatable design (CCRD) for antioxidant activity by ABTS, DPPH, and CA methods

	Source of variation	Df	SS	MS	F value	Pr (> F)
ABTS	T	1	60.03	67.03	37.71	7.15×10^{-8} [a]
	T	1	88.61	88.61	49.85	1.97×10^{-9} [a]
	T^2	1	158.92	158.92	89.40	1.71×10^{-13} [a]
	t^2	1	2.67	2.67	1.50	0.22
	T × t	1	11.55	11.55	6.50	0.01[b]
	residuals	60	106.66	1.78		
DPPH	T	1	56.08	56.08	33.68	2.61×10^{-7} [a]
	T	1	27.75	27.75	16.67	1.34×10^{-4} [a]
	T^2	1	0.11	0.11	0.06	0.80
	t^2	1	39.08	39.08	23.47	9.31×10^{-6} [a]
	T × t	1	46.06	46.06	27.66	2.03×10^{-6} [a]
	residuals	60	99.90	1.67		
CA	T	1	548.66	548.66	345.80	2.20×10^{-16} [a]
	T	1	105.75	105.75	66.65	2.57×10^{-11} [a]
	T^2	1	72.18	72.18	45.49	6.80×10^{-9} [a]
	t^2	1	0.97	0.97	0.61	0.44
	T × t	1	146.83	146.83	92.54	9.08×10^{-14} [a]
	residuals	60	95.20	1.59		

Significance level and $p < 0.001$[a] and $p < 0.05$[b]. ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; DPPH: 2,2-diphenyl-1-picrylhydrazyl; CA: chelating ability; Df: degree of freedom; SS: sum of squares; MS: mean square; T: temperature; t: time.

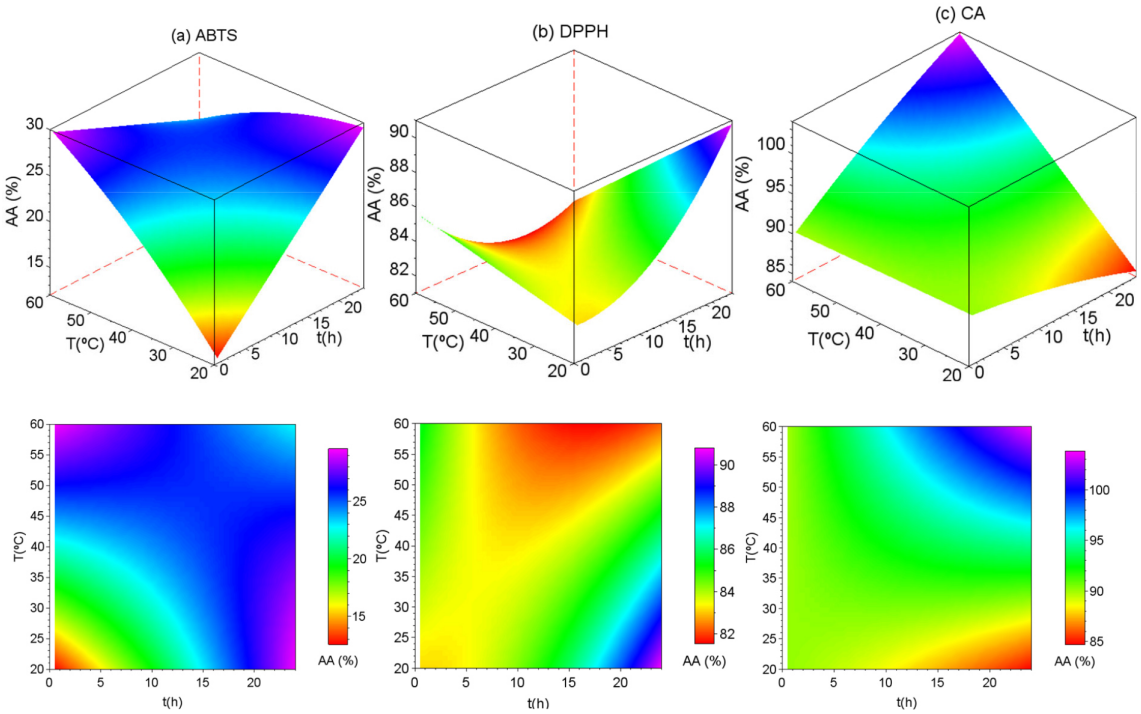

Figure 1. Response surface graph (3D and 2D) showing the combined effect of temperature and extraction time on antioxidant activity (a) ABTS; (b) DPPH and (c) CA.

According to the graphs generated, it is possible to observe that, for the ABTS test (Figure 1a), there are two regions of maximum percentage. The first is for extracts obtained at high temperatures (between 55 and 60 °C) for a short period (between 1 and 4 h). The second region, on the other hand, presents a greater range of temperature variation (20 to 40 °C) considering a smaller time variation (20 and 24 h), and the AA varies from 25 to 30% in both described situations.

Regarding the tests related to DPPH and CA, each one can present a single optimal range of AA. DPPH (Figure 1b) had a better response for extracts produced at low temperatures (between 20 and 30 °C) and long periods (20 and 24 h), with AA above 88%. The CA test (Figure 1c), with times above 10 h and temperature of 60 °C, showed a response above 95%.

It is known that the increase in temperature provides better diffusivity between the solvent and the plant matrix, providing better solubility with bioactive compounds present there.[46] A similar effect was reported by Darvishzadeh and Orsat,[43] which assessed different polyphenol extraction methods and the influence of temperature on such processes. The importance of assessing the effect of temperature in the extraction process is to be able to identify the moment of maximum response, because, above this detected limit, there may be a balance between the release of antioxidant compounds and the degradation of some others.[44]

Another way to evaluate the influence that the variables of temperature and extraction time exert on the antioxidant action of ginger is the principal component analysis (PCA), a method based on the correlation between the data obtained.[48] Table 5 describes the loadings and the percentage of variation explained for each axis for the terms evaluated in each antioxidant test applied.

Table 5 shows the participation of each variable for PC1 and PC2, that is, how much each term influenced the responses obtained by the CCRD planning. It is noticed that, in all tests, the weight of the variables T and t were

Table 5. Loadings of T and t variables for PC1 and PC2

	ABTS		DPPH		CA	
	PC 1	PC 2	PC 1	PC 2	PC 1	PC 2
T	0.9997	0.0213	–0.4407	0.8976	0.9237	–0.3832
t	–0.0213	0.9997	0.8976	0.4407	0.3832	0.9237
Variance / %	64.03	35.97	74.92	25.08	93.77	6.23

T: temperature, t: time; ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; DPPH: 2,2-diphenyl-1-picrylhydrazyl; CA: chelating ability.

identical, and each one had relevance for each component. For the ABTS test, the PC1 component justified 64.03% of the data, with temperature having a greater relevance for PC1 whereas time has a greater importance for PC2. Such behavior of the studied variables is repeated for the CA test, where PC1 explains 93.77% of the data variance. As for the DPPH test, the opposite is observed, with temperature having a greater weight in PC2 and time in PC1, with PC1 having the greatest influence on the data, with 74.92%.

The distribution of points by PCA analysis can be seen in Figure 2. The green arrows indicate the variables (T and t) and in which position the planning points are. For the ABTS (Figure 2a), as seen in Table 5, the T is positioned on the positive axis of PC1, a factor that receives greater relevance, indicating that point 1.41 of the planning was the one that most influenced the results. The t influenced only the PC2 axis, indicating that it had a greater intervention in point 1 of the planning. In Figure 2c, the same behavior trend is observed for the CA test, which indicates the planning points 1.41 and 1 for the variables T and t, respectively.

The model obtained with the PCA analysis is used to complement the statistical analyses discussed above, showing the relevance of each variable considered. This study observed that both temperature and time had similar weights for each antioxidant test, each with its significance.

After determining the CCRD planning points with the greatest influence on the results of antioxidant activity, the actual values applied were identified in Table 1, concluding that the point related to T 1.41 is equivalent to 60 °C and the point related to t 1 (20 h) would be the best extraction conditions. However, when the PCA was compared with the response surface, it is clear that times above 12 h should be avoided, since the results of antioxidant activity presented were significant. Thus, considering the reduction of the extraction cost, the conditions chosen to continue the studies were 60 °C for 12 h.

With the development of this work, it was observed that the time and temperature factors directly influence the extraction efficiency compared to the tests performed (ABTS, DPPH, and CA). With the increase in temperature, the process does not need to be subjected to long periods, to reduce the viscosity and surface tension of the solvent used, thus facilitating the penetration of the solvent into the matrix, favoring the mass transfer process.[49] In addition, there may be a weakening of interactions between antioxidant compounds and proteins or polysaccharides, in addition to cell rupture and opening of the cell matrix, increasing the rate of diffusion and availability of these compounds.[50]

Hsieh *et al.*[46] report that, at low temperatures, the extraction is not efficient because it hinders the solubilization of the compounds in the solvent, and at temperatures above 60 °C, they cause deterioration of the extracted compounds. Even with the different antioxidant activity responses between the studies presented and the one developed in this

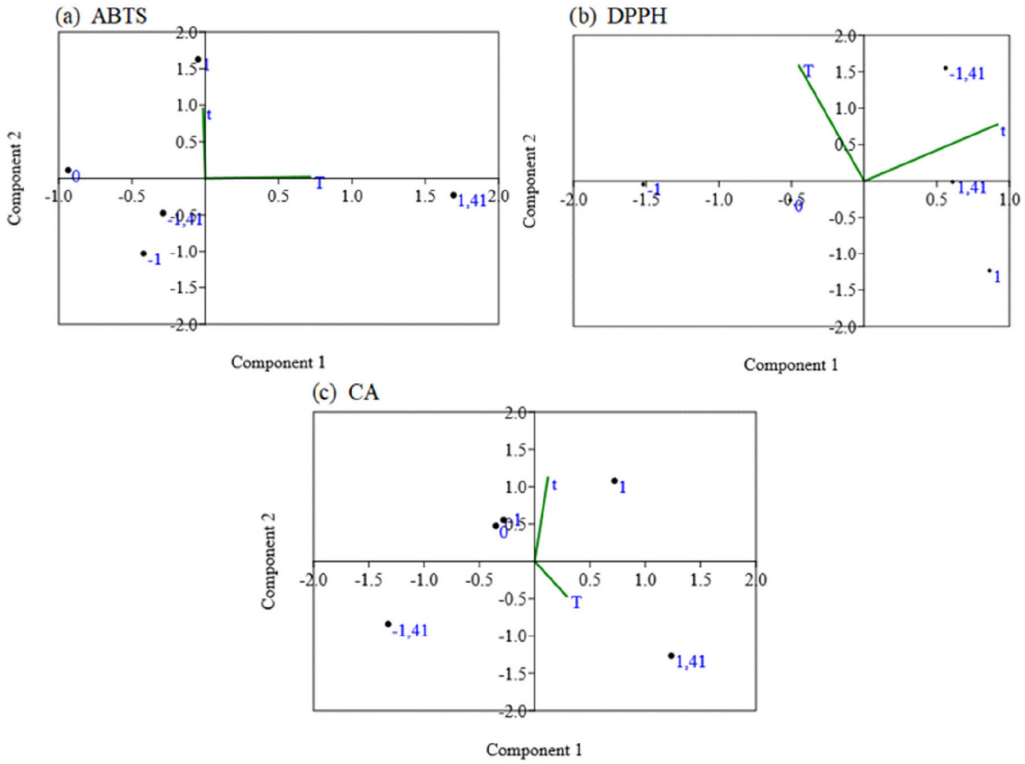

Figure 2. PCA analysis graph for the PC1 × PC2 temperature and time variables corresponding to the tests applied to the ginger extracts.

work, it is possible to prove the effective antioxidant activity of the ginger extract, and the differences in responses may be associated with factors such as maturation point and conditions of ginger cultivation, extraction solvent, drying conditions, and sample storage time.[12]

Thermal analysis (TG, DTA, and DSC) of the ginger extract optimized

The thermal properties of optimized ginger extract, evaluated by TG-DTA (Figure 3), provide the necessary data to predict the thermal stability of the compounds present in the samples.[51] On the other hand, DSC analysis associates the effects of heat with changes in physical state, such as phase transition, and chemical changes in samples, such as dehydration, and dissociation, among others.[52]

The graph generated by the TG-DTA analysis (Figure 3a) shows that the gradual increase in temperature caused four degradation bands for the ginger extract, both with endothermic characteristics. The first range is associated with the dehydration of the sample. In contrast, the second, third, and fourth ranges may be related to the decomposition, volatilization, and oxidation of organic material, respectively, and such actions populate energy absorption generating endothermic peaks. The fourth band is related to the completion of the decomposition of the sample, with the formation of ash as an indication.[53]

Regarding mass loss, the first degradation range occurred between 25 and 125 °C, from which it obtained about a 6.84% reduction. The second range, between 130 and 190 °C, showed a mass loss of 9.42%, indicating the beginning of the degradation of the organic matter present in the sample. The third range, with a reduction of 39.53%, appears between 200 and 355 °C and is related to the degradation of bioactive compounds present in ginger.[51] The last range, 600 to 720 °C, with the sharpest peak, presents a mass loss of approximately 48.20%, characterizing the consumption of the remaining organic matter.

The curves obtained for the DSC (Figure 3b) indicated two endothermic peaks between 50 and 125 °C, which can be attributed to the dehydration of the sample, given the need to absorb heat so that water can be released, and thus be portrayed.[54,55] the need to absorb heat so that water can be released, and thus be portrayed.54,55 From that point on, two exothermic peaks are observed, justified by the possible ruptures in the compounds present, releasing the energy previously absorbed as soon as the degradation products begin to form.

The last peak formed, which appears in the range of 250 to 300 °C, may be related to the degradation of the products formed during the thermal decomposition of the compounds present in the ginger extract.[56]

Mass spectrometry (MS)

The identification of the main compounds present in the optimized ginger extract was performed by the MRM method, a selective method with greater sensitivity. The identified phenolic compounds are listed in Table 6.

For the MRM analysis, the software was programmed to identify a total of 24 compounds based on the references cited (Table 6).[32-35,37] In addition, two different conditions were evaluated of which capillary voltage, cone voltage, and collision energy were varied. However, only 9 compounds showed reproductive signals. 6-Gingerol and 8-shogaol, two of the main compounds in ginger, were identified for both conditions evaluated.

In addition to the compounds already mentioned, for the method of analysis of condition 2, ferulic acid was also detected. As for condition 1, another 6 compounds were detected, malic acid, gallic acid, 8-gingerol, 10-shogaol, 10-gingerol, and quercetin-3-*O*-hexoside. The phenolic compound with the greatest predominance in the ginger extract was 6-gingerol, reinforcing what was presented by Akamine *et al.*,[31] Asamenew *et al.*,[57] and Zhong *et al.*[58]

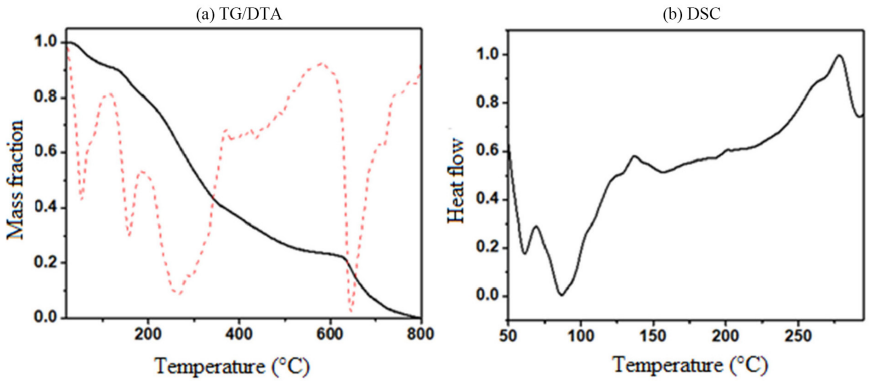

Figure 3. Curves (a) TG (solid line), DTA (dashed line), and (b) DSC.

Table 6. Identification of the main compounds present in the optimized ginger extract

Analyte	$[M - H]^-$	MRM transition (*m/z*)	Condition 1 (S/N:RMS)	Condition 2 (S/N:RMS)	Reference
Malic acid	133	133 > 115	98	–	33
Galic acid	169	169 > 125	65	–	33
Ferrulic acid	193	193 > 134	–	97	35
6-Gingerol	293	293 > 99; 193	820; 289	997	32
8-Shogaol	303	303 > 167	125	193	37
8-Gingerol	321	321 > 127	160	–	32
10-Shogaol	331	331 > 195	205	–	37
10-Gingerol	349	349 > 155; 193	323; 110	–	32
Quercetina-3-*O*-hexosideo	463	463 > 179	63	–	34

MRM: multiple reaction monitoring; S/N: signal to noise; RMS: root-mean-square.

Other studies[59,60] have reported the presence of different compounds, such as naringin, coumaric acid, caffeic acid, catechol, ascorbic acid, and gallic acid. In addition to these, other phenolic compounds derived from gingerol, shogaol, and paradol have been identified from the ginger extract.[57,61,62]

Inhibition of the enzyme acetylcholinesterase (AChE)

The AChE enzyme inactivation test was performed for the ginger extract that showed the best responses for the tested antioxidant activities (extract 6, 60 °C for 12 h). The *in vitro* analyzes were carried out with different concentrations of the extract (1, 10, 100, 500, and 1000 μg mL^{-1}), and the control was carried out under the same conditions, without the presence of ginger extract. The results are shown in Figure 4.

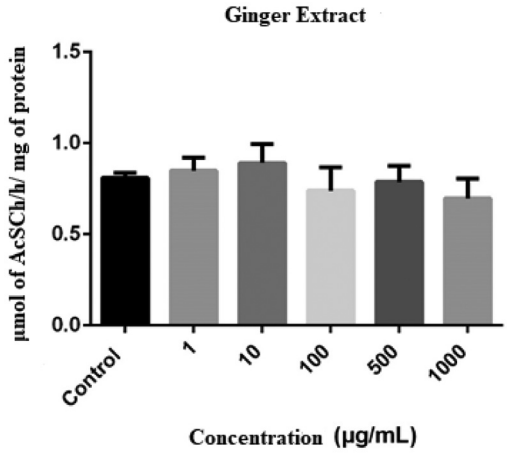

Figure 4. Inhibition of AChE activity by ginger extract. All data were expressed as mean ± S.E.M (standard error of the mean). Significant difference compared to control with H_2O (one-way ANOVA n = 5, followed by Dunnett's test, $p < 0.001$).

Figure 4 shows that the ginger extract did not present an inhibitory effect on the AChE enzyme; however, its regular development can be noted, indicating that the extract does not have neurotoxic action. The non-inhibition of AChE is also a biomolecular indicator of the absence of neurotoxicity and neurological side effects associated with the cholinergic system.[63] With this result, its application in other biological tests becomes viable, such as its anti-cancer action.

Fathy *et al.*[64] tested the AChE inhibiting activity of the methanol extract of ginger and obtained a similar result. Akinyemi *et al.*[65] evaluated the effect of a diet composed of ginger in mice with hypertension. They observed the preservation of AChE activity and improved cognitive system, such as memory, learning, and modulation of blood flow, indicating a possible increase in AChE production. Studies[63] have shown that ginger oil containing gingerols and shogaols was able to reduce the AChE inhibiting activity caused by an overdose of topiramate, a drug used to treat epilepsy and migraine, reducing its neurotoxic effect. These effects confirm the use of extracts from *Z. officinale* as an antioxidant activity and neuroprotective effects agent.[66]

Characterization of liposomes

A series of liposomes incorporated with different proportions of the optimized ginger extract was prepared by the ethanol injection method, with the concentration of soy lecithin, a source of phospholipids, maintained at 4.64 mM. The average hydrodynamic sizes, polydispersity, and zeta potential are presented in Table 7.

With the results obtained, it was observed that even after increasing the proportion of the ginger extract, the hydrodynamic diameter of the particles produced did not change significantly, ranging from 168.04 to 184.73 nm. A recent study[67] agrees with the results obtained in this research, in which, when producing liposomes by the extrusion method, liposomes smaller than 200 nm were obtained.

Table 7. Hydrodynamic diameter, polydispersity, zeta potential, and encapsulation efficiency of liposomes

	Ginger extract / %			
	0	10	20	30
D_h / nm	184.73 ± 0.52[a]	174.45 ± 0.70[a]	168.04 ± 5.02[a]	168.06 ± 5.14[a]
Polydispersity / %	26.17 ± 1.14[a]	25.33 ± 0.50[a]	25.93 ± 0.29[a]	26.23 ± 0.74[a]
ζ / mV	–34.80 ± 0.40[a]	31.80 ± 0.93[a]	30.80 ± 1.12[a]	31.47 ± 1.13[a]

D_h: hydrodynamic diameter; ζ: zeta potential.

Regarding polydispersity, the liposomes presented values between 25 and 26%, which means that the particles formed are homogeneous. Physical characteristics such as size, morphology, and surface charges are related to the application of the formed particle, the mode of insertion into the cells, and, consequently, the efficiency of the treatment. Previous studies[67,68] have shown that liposomes with sizes ranging from 100 to 300 nm and polydispersity less than 70% facilitate the localization of tumor tissue.

This characteristic is an important factor when the objective is the delivery of a drug, because the low diameter provides a larger surface area, consequently, its functions are potentiated. The negative surface charge of the liposomes (zeta potential) ranged from –30 to –34 mV. The negative charge of liposomes is caused by the presence of the hydrophilic groups present in phospholipids, which have a negative charge.[69,70]

Generally, the higher the absolute value of the zeta potential, the more stable the colloidal solutions become, as an energy barrier is formed that prevents the particles from aggregating.[71] Therefore, the liposomes charged with the ginger extract have a negative charge present itself as the most attractive carrier for biological application.

The encapsulation of bioactive compounds enables preservation and their bioavailability in the system, preventing their immediate degradation.[67] By the statistical analysis applied to the produced liposomes, no significant differences were obtained for the produced formulations, therefore, both formulations were submitted to the cytotoxic test, as will be presented in the next sections.

Cytotoxic/antiproliferative activity

The cytotoxic/antiproliferative activity of the ginger extract optimized and liposomes prepared by incorporation of this extract was evaluated by the MTT method, against human kidney tumor cells (786-0), liver cells (HUH7.5) and *Macaca mullata* normal kidney cells (LLC-MK2).

The cell viability data (Table 8) show that the concentration of 200 μg mL^{-1}, in the three evaluation times, presented a dose-dependent cytotoxic/antiproliferative effect, with the concentration of 1000 μg mL^{-1} reaching the lowest cell viability values of 52.25% (24 h), 38.03% (48 h) and 25.38% (72 h). From a concentration of 300 μg mL^{-1}, an effect dependent on the exposure time can also be observed,

Table 8. Viability percentage (VC) of the human tumor cells (786-0), (HUH7.5) and normal monkey kidney cells (LLC-MK2) in different concentrations of ginger extract optimized

Group	VC / %								
	Human kidney tumor cells (786-0)			Human liver tumor cells (HUH7.5)			Monkey kidney normal cell		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
CO^-	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
CO^+	65.24	45.09	20.74	39.76	12.94	27.31	6.32	35.52	29.63
5 **μg** mL^{-1}	115.77	122.77	100.00	98.06	112.23	97.33	106.31	94.13	117.98
10 **μg** mL^{-1}	99.89	121.97	122.00	101.67	115.91	105.51	95.49	106.83	101.39
50 **μg** mL^{-1}	121.57	145.20	94.39	105.55	106.04	87.84	98.72	102.05	101.33
100 **μg** mL^{-1}	98.93	136.07	111.21	85.15	93.06	89.63	101.83	106.63	90.84
200 **μg** mL^{-1}	136.48	127.86	89.75	98.33	77.05	85.49	108.06	84.90	93.62
300 **μg** mL^{-1}	132.08	138.15	71.67	95.63	58.07	97.81	115.57	74.68	94.56
400 **μg** mL^{-1}	113.52	108.55	56.06	75.92	56.42	66.37	78.76	64,.94	92.67
500 **μg** mL^{-1}	106.44	93.87	48.70	62.04	49.01	37.03	115.87	131.63	78.80
1000 **μg** mL^{-1}	52.25	38.03	25.38	99.93	27.01	31.28	121.55	110.86	63.76

CO^-: control negative; CO^+: control positive; VC: viability percentage.

in which the longer the exposure time, the lower the cell viability.

The data in Figure 5 indicate the mean absorbance values and their respective standard deviations obtained using human kidney tumor cells (786-0) treated with different concentrations of ginger extract at 24, 48, and 72 h. According to the viability results, it can be verified that concentrations above 300 μg mL^{-1}, in the longest evaluation time (72 h), were cytotoxic for this tumor lineage, and the concentration of 1000 μg mL^{-1} showed this effect from the time of 24 h. However, some concentrations in the time of 24 h (200 and 300 μg mL^{-1}) and 48 h (50, 100, 200, and 300 μg mL^{-1}) showed mean absorbances higher than that of the negative control.

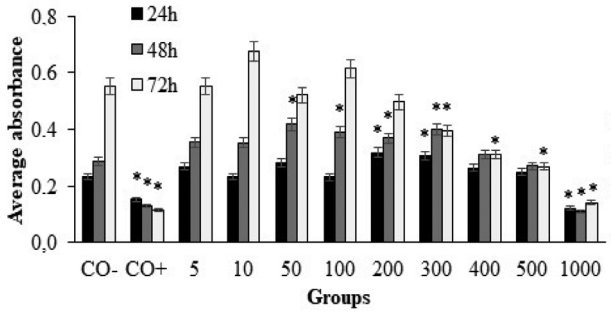

Figure 5. Mean absorbance and standard deviation of human kidney tumor cells (786-0) treated for 24, 48, and 72 h with concentrations (μg mL^{-1}) of ginger extract optimized. The result is statistically different from the negative control (Dunnet's test, $p < 0.05$).

Figure 6 presents the results of the average absorbances of treatments of human liver tumor cells (HUH7.5) with pure ginger extract. Statistical analysis shows that, as for kidney tumor cells, the extract showed cytotoxic/antitumor/

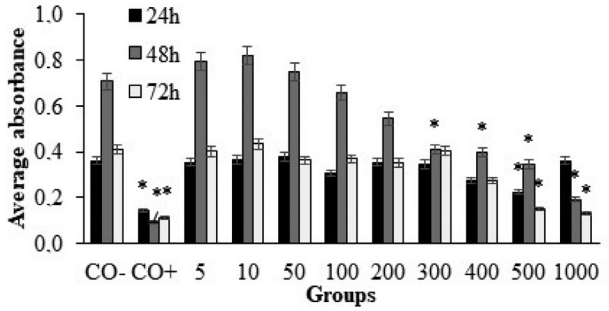

Figure 6. Mean absorbance and standard deviation of human liver tumor cells (HUH7.5) treated for 24, 48, and 72 h with concentrations (μg mL^{-1}) of ginger extract optimized. The result is statistically different from the negative control (Dunnet's test, $p < 0.05$).

antiproliferative activity. In this case, in the time of 24 h the concentration of 500 μg mL^{-1}, in the time of 48 h the concentrations above 300 μg mL^{-1} and in the time of 72 h the concentrations of 500 and 1000 μg mL^{-1} showed statistically lower mean absorbances than the negative control. Furthermore, cell viability (Table 9) reached minimum values of 27.01% (1000 μg mL^{-1}).

The data in Figure 7 indicate the mean absorbance values and their respective standard deviations obtained using normal monkey kidney cells (LCC-MK2) treated with different concentrations of ginger extract at 24, 48, and 72 h. It was possible to observe that only the highest concentration (1000 μg L^{-1}) of the ginger extract, in the time 72 h was cytotoxic. It can be noted that, in normal cells, cell proliferation occurred at all times, reaching a cell viability (Table 9) of 121.55% (1000 μg L^{-1}) in 24 h, 131.63% (500 μg L^{-1}) in 48 h and 117.98% (5 μg L^{-1}) in 72 h.

According to Radhakrishnan *et al.*,[72] 6-gingerol, identified in the ginger extract of the present study, played

Table 9. Percentage of viability of human kidney tumor cells treated in different concentrations of liposome without extract, encapsulated with 10, 20, and 30% of ginger extract

Group	VC human kidney tumor (786-0)											
	0%			10%			20%			30%		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
CO^-	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
CO^+	104.79	118.24	98.42	93.29	81.75	103.05	65.82	67.96	67.01	55.35	75.93	80.92
5 μg mL^{-1}	100.96	159.04	106.98	86.84	100.16	72.58	80.03	63.74	65.65	84.38	93.05	67.84
10 μg mL^{-1}	95.40	91.84	85.51	89.56	90.37	65.61	83.89	97.55	65.60	101.03	67.95	72.51
50 μg mL^{-1}	106.13	119.20	114.36	135.33	89.74	85.24	86.20	83.69	71.74	119.75	77.84	72.95
100 μg mL^{-1}	80.46	103.36	111.46	96.22	86.53	90.05	75.92	85.55	70.22	76.29	65.60	110.67
200 μg mL^{-1}	137.36	108.48	66.53	106.35	81.75	97.16	99.37	95.91	56.85	85.25	69.56	81.87
300 μg mL^{-1}	97.32	142.40	82.21	127.16	79.56	84.97	102.79	96.87	56.97	91.67	71.10	80.85
400 μg mL^{-1}	95.79	93.92	94.99	87.52	84.10	94.43	88.74	86.64	59.11	69.63	74.88	72.51
500 μg mL^{-1}	162.64	97.60	95.52	94.30	84.34	66.22	91.70	66.36	74.45	96.35	56.76	83.92
1000 μg mL^{-1}	139.85	146.72	76.42	83.21	60.22	71.29	102.55	59.51	51.72	56.15	60.22	148.25

CO^-: control negative; CO^+: control positive; VC: viability of human kidney tumor cells.

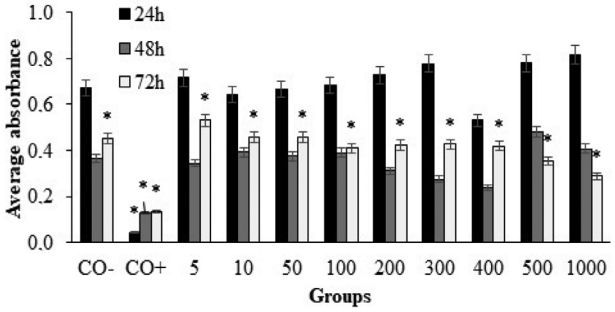

Figure 7. Mean absorbance and standard deviation of normal monkey kidney cells (LCC-MK2) treated for 24, 48, and 72 h with the concentrations (μg mL^{-1}) of the extract. The result is statistically different from the negative control (Dunnet's test, $p < 0.05$).

cytotoxic and antiproliferative effects on human colon cancer cells, while normal colon cells were not affected, corroborating with our results.

The cytotoxic/antiproliferative activity of ginger and its metabolites have been reported by other authors.[73-75] Among the studies already reported, ginger showed cytotoxic activity on tumor cells of rat lung (L929) and in prostate cancer cells *in vitro* and *in vivo*, thus corroborating with the present study.[76,77]

Interest in the benefits of ginger for human health has increased due to the presence of several active compounds.[78] Phytochemical studies have shown that ginger is rich in 6-gingerol and shogaol. These compounds have several biological activities, such as antioxidant and anti-inflammatory properties.[3,5] In addition, they exert antitumor activities against gastrointestinal cancer. This chemopreventive role is due to the ability to modulate signaling molecules such as NF-κB (nuclear factor kappa B), TNF-α (tumor necrosis factor α), COX-2 (cyclooxygenase-2), AP-1 (activator protein 1), Bcl-2 (B-cell lymphoma 2), caspases, and other cell growth regulatory proteins.[1,79]

Of the ginger-specific compounds, 6-shogaol has been shown to have the ability to introduce the G2/M cycle that will induce cancer cell arrest and apoptosis. Regarding gingerol, its antioxidant activity was attested by the inhibition of phospholipid peroxidation driven by the $FeCl_3$-ascorbate system and by the activity of xanthine oxidase.[80] Studies[81] show that 6-gingerol can reduce the levels of H_2O_2, NO, MPO (myeloperoxidase), MDA (malondialdehyde), TNF-α (tumor necrosis factor α) and caspase-3 and increase the concentration of antioxidant enzymes such as catalase, GPx (glutathione peroxidase), SOD (superoxide dismutase), GSH (glutathione), CAT (chloramphenicol acetyltransferase), Taq (Taq polymerase), and GST (glutathione S-transferas). It can also reduce NOx concentrations, proteins, and carbonyl content, significantly reducing oxidative stress.

To increase the synergistic action of the ginger extract as well as to evaluate the controlled release, the extract was encapsulated in liposomes and, subsequently, its cytotoxic action was evaluated. The data in Figure 8a present the results of the average absorbances of the treatments of the

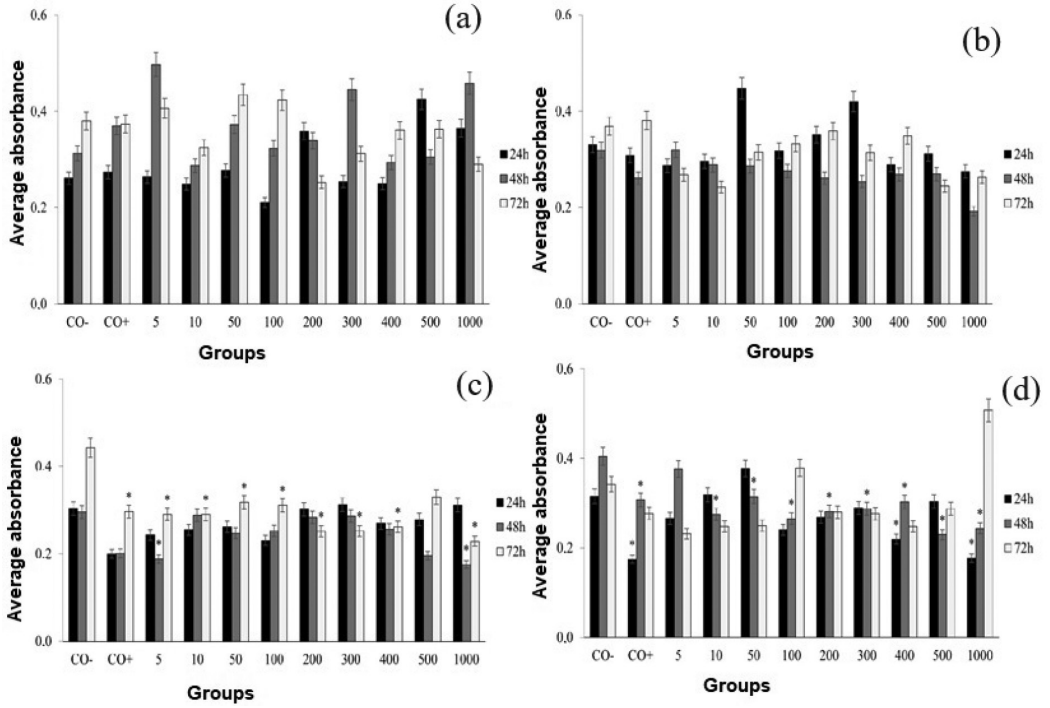

Figure 8. Mean absorbance and standard deviation of human kidney tumor cells treated for 24, 48, and 72 h with concentrations (μg mL^{-1}) of liposomes encapsulated with 0% (a), 10% (b), 20% (c), and 30% ginger extract (d). The result is statistically different from the negative control (Dunnet's test, $p < 0.05$).

human kidney tumor cells with the liposomes without the ginger extract, by the cytotoxicity/antiproliferative activity test. Statistical analysis shows that there was no significant cytotoxic activity and cell viability (Table 9) remained high. A similar result was obtained with a liposome with 10% ginger (Figure 8b), in which the statistical analysis showed that no concentration, at all evaluated times, showed cytotoxicity for this cell line. In addition, the minimum cell viabilities achieved were 83.21, 60.22 and 65.61% for 24 (1000 μg mL^{-1}), 48 (1000 μg mL^{-1}), and 72 h (10 μg mL^{-1}), respectively.

The liposome with 20% ginger extract (Figure 8c) resulted in statistically lower and cytotoxic mean absorbances for human kidney tumor cells at concentrations of 5 and 1000 μg mL^{-1} at the time of evaluation of 48 h and all concentrations in 72 h (except 500 μg mL^{-1}). Furthermore, cell viabilities (Table 9) reached minimum values of 75.92, 59.51, and 51.72% for 24 (100 μg mL^{-1}), 48 (1000 μg mL^{-1}), and 72 h (1000 μg mL^{-1}), respectively. In this case, for concentrations of 50, 200, 300, 400, and 1000 μg mL^{-1}, an effect dependent on the exposure time can be observed, in which the longer the time, the lower the cell viability. This confirms the gradual release of the compound by the liposomes, caused by the instability when it is in an aqueous medium.[82]

About liposomes containing 30% of extract (Figure 8d), the results show that concentrations of 400 and 1000 μg mL^{-1} at 24 h, and all concentrations at 48 h (except for 5 μg mL^{-1}) showed a cytotoxic effect, with minimum viabilities of 56.15% (24 h, 1000 μg mL^{-1}) and 56.76 (48 h, 500 μg mL^{-1}).

The results demonstrate that the liposomes incorporated with 20% of the ginger extract showed greater antiproliferative/cytotoxic activity against kidney tumor cells when compared to the other liposomes. However, a decrease in cell viability is observed when compared to the free extract, mainly at a concentration of 1000 μg mL^{-1} at 24, 48, and 72 h, demonstrating that the free extract has greater antiproliferative/cytotoxic activity against this strain. For concentrations of 200 and 300 μg mL^{-1}, at times of 72 h, the liposome incorporated with 20% of the extract showed lower values of viability percentage (VC), resulting in a greater antitumor potential.

The liposome without ginger extract (Figure 9a) did not show cytotoxicity to human liver tumor cells at all evaluated times. In addition, in the evaluation time of 48 h, the concentrations of 50, 100, and 300 μg mL^{-1} showed cell proliferation, with cell viability (Table 10) of 141.40, 154.00, and 139.65% for the concentrations of 50, 100, and 300 μg mL^{-1}, respectively.

Liposomes containing 10% ginger extract (Figure 9b), as well as the one containing 0%, did not show cytotoxicity. In addition, it showed cell proliferation within 48 h at a concentration of 400 μg mL^{-1}, with cell viability of 135.42% (Table 10). A similar result was obtained with liposomes containing 20% of the extract, which also did not show cytotoxicity to liver tumor cells and induced cell proliferation at concentrations of 50 μg mL^{-1} (72 h), 100 μg mL^{-1} (48 and 72 h), 200 μg mL^{-1} (48 and 72 h), 400 μg mL^{-1}, 500 μg mL^{-1} and 1000 μg mL^{-1} (72 h), with cell

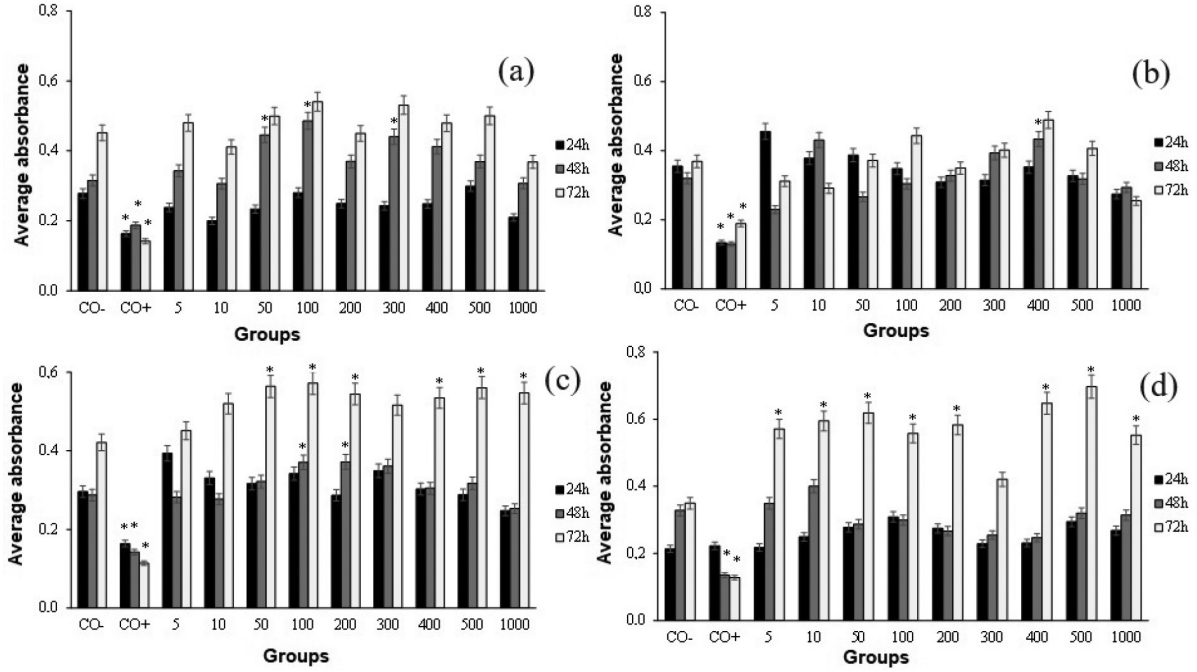

Figure 9. Mean absorbance and standard deviation of human liver tumor cells treated for 24, 48, and 72 h with concentrations (μg mL^{-1}) encapsulated with 0% (a), 10% (b), 20% (c), and 30% (d) ginger extract. The result is statistically different from the negative control (Dunnet's test, $p < 0.05$).

Table 10. Percentage of viability of human hepatoma cells treated in different concentrations of liposome without extract, encapsulated with 10, 20, and 30% of ginger extract

Group	VC human hepatoma											
	0%			10%			20%			30%		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
CO^-	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
CO^+	58.72	59.40	31.43	37.71	40.63	51.36	55.41	49.42	27.05	104.33	41.16	36.67
5 μg mL^{-1}	85.61	108.80	106.25	128.24	71.67	84.63	133.02	98.14	107.06	102.11	106.48	163.26
10 μg mL^{-1}	71.85	97.07	90.92	106.57	134.58	79.02	111.82	96.52	123.37	116.86	122.03	170.03
50 μg mL^{-1}	83.99	141.40	110.57	108.91	83.23	100.81	106.93	111.95	133.75	130.09	87.58	176.91
100 μg mL^{-1}	100.90	154.00	119.54	97.94	95.00	120.16	115.54	129.00	135.77	144.73	91.23	159.40
200 μg mL^{-1}	89.57	117.21	99.61	86.87	102.40	94.76	96.88	129.47	129.18	128.57	81.33	166.62
300 μg mL^{-1}	87.41	139.65	117.49	88.56	122.92	108.86	117.99	125.64	122.48	107.26	77.64	120.28
400 μg mL^{-1}	89.30	130.77	106.03	99.16	135.42	132.64	102.20	106.03	126.81	108.08	75.23	185.08
500 μg mL^{-1}	107.55	117.05	110.74	92.12	99.48	110.31	97.30	110.44	132.98	137.94	97.56	199.21
1000 μg mL^{-1}	75.36	97.46	81.52	77.20	91.77	69.17	83.61	88.05	129.77	125.64	95.73	157.83

CO^-: control negative; CO^+: control positive; VC: percentage of viability of human hepatoma cells.

viabilities (Table 10) greater than 126%. This proliferative effect was also observed with the liposome containing 30% of ginger extract because at the time of 72 h, all concentrations except 300 μg mL^{-1}, showed absorbances greater than that of the negative control with viability greater than 157% (Table 10). For a concentration of 1000 μg mL^{-1}, at times of 72 h, the liposome incorporated with 10% of the extract showed lower values of VC, resulting in a greater antitumor potential.

The results obtained with the hepatic lineage, different from the cytotoxic/antiproliferative activity of liposomes containing ginger (20 and 30%) for renal tumor cells can be explained by the type of cell lineage. In addition, it is possible that the ginger extract was not released in sufficient quantity from the liposomes in the liver cells and, therefore, although the pure extract was cytotoxic to the HUH cells when evaluated free, it did not present toxicity to the samples encapsulated in liposomes for not having been able to reach the concentration sufficient to act in the pathways of inducing the death of tumor cells.

On the other hand, liposomes with or without the extract generally induced the proliferation of normal monkey kidney cells (Figure 10 and Table 11). The 0% ginger extract liposome showed viabilities above 82, 119,

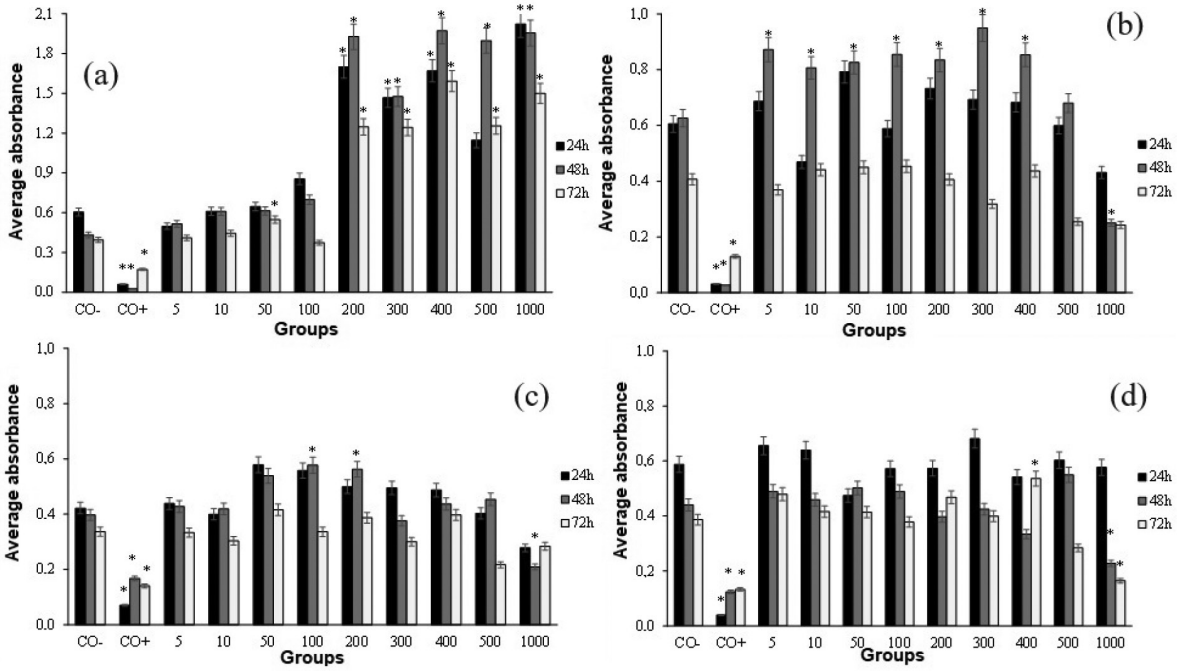

Figure 10. Mean absorbance and standard deviation of normal macaque kidney cells treated for 24, 48, and 72 h with concentrations (μg mL^{-1}) encapsulated with 0% (a), 10% (b), 20% (c), and 30% (d) ginger extract. The result is statistically different from the negative control (Dunnet's test, $p < 0.05$).

Table 11. Percentage of viability of normal macaque kidney cells treated in different concentrations of liposome without extract, encapsulated with 10, 20, and 30% of ginger extract

Group	VC normal monkey kidney											
	0%			10%			20%			30%		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
CO^-	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
CO^+	10.05	6.08	43.83	5.25	4.47	32.00	16.70	42.16	41.65	6.76	28.09	34.48
5 μg mL^{-1}	82.46	119.69	103.74	113.47	139.22	90.72	103.85	107.74	99.11	111.51	111.12	124.00
10 μg mL^{-1}	100.99	141.17	112.67	77.49	128.75	108.35	94.67	105.48	90.13	108.73	104.20	107.63
50 μg mL^{-1}	106.99	142.21	138.76	130.81	131.91	110.50	137.06	135.56	123.39	80.70	113.90	107.18
100 μg mL^{-1}	141.38	161.90	94.68	97.23	136.42	111.36	132.15	145.31	99.85	97.24	111.07	97.87
200 μg mL^{-1}	281.22	446.27	316.02	120.98	133.27	99.75	118.35	141.54	115.00	97.49	90.24	121.15
300 μg mL^{-1}	242.56	341.86	314.63	114.46	151.65	78.26	117.29	94.59	89.24	115.82	96.42	103.30
400 μg mL^{-1}	276.21	456.75	403.29	112.72	136.26	107.25	115.39	109.94	117.97	92.09	75.77	138.75
500 μg mL^{-1}	189.50	439.30	317.92	98.97	108.63	62.59	95.56	114.22	64.51	102.49	124.80	73.42
1000 μg mL^{-1}	334.53	452.75	379.90	71.21	40.02	59.77	65.90	52.70	84.43	98.13	51.70	42.63

CO^-: control negative; CO^+: control positive; VC: viability of normal macaque kidney cells.

and 94% for 24, 48, and 72 h. The concentrations below 400 μg mL^{-1} (48 h) of the liposome with 10% ginger extract showed a proliferative effect with viabilities above 128%. The concentrations of 100 and 200 μg mL^{-1} (48 h) of the liposome with 20% ginger extract showed cell viability of 145.31 and 141.54%, respectively. The 400 μg mL^{-1} liposome concentration with 30% ginger extract (72 h) showed 138.75% cell viability.

Only the concentration of 1000 μg mL^{-1} of the liposome with ginger extract at 10 and 20% for 48 h and 30% for 48 and 72 h showed a cytotoxic effect on normal monkey kidney cells, similar to that observed with the pure extract of ginger for this cell line.

The confrontation between the cytotoxic action of free and encapsulated ginger revealed that in its free form, the extract was toxic from 200 μg mL^{-1}. However, the extract encapsulated in liposomes showed a toxic effect at even lower concentrations, showing the effectiveness of the encapsulation process.

Even with the positive results, new studies must be carried out to evaluate new concentrations of raw material, as well as the possibility of comparing the effect of the compounds in isolation.

Conclusions

The optimization of the extraction of bioactive compounds from ginger, using ethanol:water 70:30 (v/v) as a solvent, showed that the time and temperature factors can directly influence the quality and action of the compounds obtained, as can be observed in the antioxidant tests (ABTS, DPPH, and CA), whose best extraction condition was determined at 60 °C for 12 h. Different phenolic compounds were identified, including gingerols and shogaols, the main phenolic compounds in ginger. In addition to the significant antioxidant activity, the studied extract showed cytotoxic action both in the free form and in its liposome-encapsulated form.

It was verified with this study that the ginger extract did not present an inhibitory effect on the AChE enzyme; however, its regular development can be noted, indicating that the extract does not have neurotoxic action. The non-inhibition of AChE is also a biomolecular indicator of the absence of neurotoxicity and neurological side effects associated with the cholinergic system. With this result, its application in other biological tests becomes viable, such as its anti-cancer action.

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Author Contributions

Danielli A. Nardino was responsible for original draft, data analysis, figures (preparation and editing) formal analysis, methodology, and writing (review and editing); Ana C. R. Aranha for figures (editing, formal analysis), and writing (review and editing); Lilian T. D. Tonin for formal analysis, review, and editing; Rafael O. Defendi for formal analysis, review, and editing; Sabrina Ishikawa for investigation

cytotoxic analysis; Patricia A. Bressiani for investigation cytotoxic analysis; Ana B. S. Santana for investigation cytotoxic analysis; Elisângela Dusman for investigation, supervision cytotoxic analysis, formal analysis, review; Murilo K. A. Yonekawa for investigation AChE enzyme inhibition; Jeandre A. S. Jaques for resources; Edson A. Santos for investigation supervision AChE enzyme inhibition, formal analysis, review; Angélica P. P. Tonin for investigation mass spectrometry data processing; Eduardo C. Meurer for supervision mass spectrometry; Caroline C. Sipoli for review and supervision; Rúbia M. Suzuki for review and supervision. All authors discussed the results and contributed to the final manuscript.

References

1. Prasad, S.; Tyagi, A. K.; *Gastroenterol. Res. Pract.* **2015**, *2015*, ID 142979. [Crossref]
2. de Souza, J. P.; Sarturi, L.; de Abreu, A. R.; de Sousa, T. A.; Geron, V. L. M. G.; *FAEMA* **2019**, *10*, 44. [Crossref]
3. Tonin, L. T. D.; Camargo, J. N. A.; Bertan, A. S.; Almeida, I. V. D.; Vicentini, V. E. P.; Dusman, E.; *Acta Sci. Technol.* **2020**, *42*, e45724. [Crossref]
4. Jacob, J.; Peter, G.; Thomas, S.; Haponiuk, J. T.; Gopi, S.; *Int. J. Biol. Macromol.* **2019**, *129,* 370. [Crossref]
5. Mustafa, I.; Chin, N. L.; Fakurazi, S.; Palanisamy, A.; *Foods* **2019**, *8*, 456. [Crossref]
6. Pan, D.; Zeng, C.; Zhang, W.; Li, T.; Qin, Z.; Yao, X.; Dai, Y.; Yao, Z.; Yu, Y.; Yao, X.; *Food Funct*. **2019**, *10*, 1203. [Crossref]
7. Talebi, M.; İlgün, S.; Ebrahimi, V.; Talebi, M.; Farkhondeh, T.; Ebrahimi, H.; Samarghandian, S.; *Biomed. Pharmacother.* **2021**, *133*, 111088. [Crossref]
8. Hassan, N. A.; Karunakaran, R.; Uma, S.; Aye, K. M.; *Int. J. Pharmacogn. Phytochem. Res.* **2016**, *13*, 3577. [Crossref]
9. Srinivasan, K.; *PharmaNutrition* **2017**, *5*, 18. [Crossref]
10. Sutalangka, C.; Wattanathorn, J.; *BMC Complement. Altern. Med.* **2017**, *17*, 135. [Crossref]
11. Belwal, T.; Ezzat, S. M.; Rastrelli, L.; Bhatt, I. D.; Daglia, M.; Baldi, A.; Devkota, H. P.; Orhan, I. E.; Patra, J. K.; Das, G.; Anandharamakrishnan, C.; Gomez-Gomez, L.; Nabavi, S. F.; Nabavi, S. M.; Atanasov, A. G.; *TrAC, Trends Anal. Chem.* **2018**, *100*, 82. [Crossref]
12. Tanweer, S.; Mehmood, T.; Zainab, S.; Ahmad, Z.; Shehzad, A.; *Clin. Phytosci.* **2020**, *6*, 12. [Crossref]
13. Fernandes, J. S. G.; de Andrade, M. S.; *Psicol. Saúde Doenças* **2017**, *18*, 131. [Crossref]
14. Fan, L.; Mao, C.; Hu, X.; Zhang, S.; Yang, Z.; Hu, Z.; Sun, H.; Fan, Y.; Dong, Y.; Yang, J.; Shi, C.; Xu, Y.; *Front. Neurol.* **2020**, *10*, 1312. [Crossref]
15. Czernicka, L.; Ludwiczuk, A.; Rój, E.; Marzec, Z.; Jarzab, A.; Kukula-Koch, W.; *Molecules* **2020**, *25*, 1643. [Crossref]
16. David, B.; Schneider, P.; Schäfer, P.; Pietruszka, J.; Gohlke, H.; *J. Enzyme Inhib. Med. Chem.* **2021**, *36*, 491. [Crossref]
17. Kukula-Koch, W.; Koch, W.; Czernicka, L.; Głowniak, K.; Asakawa, Y.; Umeyama, A.; Marzec, Z.; Kuzuhara, T.; *Molecules* **2018**, *23*, 1301. [Crossref]
18. Rungsaeng, P.; Sangvanich, P.; Karnchanatat, A.; *Appl. Biochem. Biotechnol.* **2013**, *170*, 934. [Crossref]
19. Sun, Z. K.; Yang, H. Q.; Chen, S. D.; *Transl. Neurodegener.* **2013**, *2*, 6. [Crossref]
20. Kumara, M.; Shylajab, M.; Nazeemc, P.; Babu, T.; *J. Dev. Drugs* **2017**, *6*, 1000167. [Crossref]
21. Kulkarni, A. D.; Belgamwar, V. S.; *Drying Technol.* **2019**, *37*, 1143. [Crossref]
22. More, M. P.; Pardeshi, S. R.; Pardeshi, C. V.; Sonawane, G. A.; Shinde, M. N.; Deshmukh, P. K.; Naik, B. N.; Kulkarni, A. D.; *Med. Drug Discovery* **2021**, *10*, 100082. [Crossref]
23. Senapati, S.; Mahanta, A. K.; Kumar, S.; Maiti, P.; *Signal Transduction Targeted Ther.* **2018**, *3*, 7. [Link] accessed in November 2023
24. Brand-Williams, W.; Cuvelier, M. E.; Berset, C.; *LWT* **1995**, *28*, 25. [Crossref]
25. Bressiani, P. A.; de Lima, G. R. F.; Düsman, E.; Tonin, L. T. D.; *J. Food Meas. Charact.* **2021**, *15*, 2743. [Link] accessed in November 2023
26. Rufino, M. S. M.; Alves, R. E.; Brito, E. S.; Morais, S. M.; Sampaio, C. G.; Pérez-Jiménez, J.; *Metodologia Científica: Determinação da Atividade Antioxidante Total em Frutas pela Captura do Radical Livre DPPH*; Embrapa: Fortaleza, 2007. [Link] accessed in November 2023
27. Stookey, L. L.; *Anal. Chem.* **1970**, *42*, 779. [Crossref]
28. Boroski, M.; Visentainer, J. V.; Cottica, S. M.; Morais, D. R.; *Antioxidantes: Princípios e M*étodos *Analíticos*, 1st ed.; Appris: Brazil, 2015.
29. Mosmann, T.; *J. Immunol. Methods.* **1983**, *65*, 55. [Crossref]
30. Zômpero, R. H. F.; López-Rubio, A.; Pinho, S. C.; Lagaron, J. M.; de la Torre, L. G.; *Colloids Surf., B* **2015**, *134*, 475. [Crossref]
31. Akamine, L. A.; Medina, D. A. V.; Lanças, F. M.; *Talanta* **2021**, *222*, 121683. [Crossref]
32. Ghareeb, M. A.; Sobeh, M.; Rezq, S.; El-Shazly, A. M.; Mahmoud, M. F.; Wink, M.; *Molecules* **2018**, *23*, 3238. [Crossref]
33. Pavlović, A. V.; Dabić, D. Č.; Momirović, N. M.; Dojčinović, B. P.; Milojković-Opsenica, D. M.; Tešić, Ž. L.; Natić, M. M.; *J. Agric. Food Chem.* **2013**, *61*, 4188. [Crossref]
34. Sinosaki, N. B. M.; Tonin, A. P. P.; Ribeiro, M. A. S.; Poliseli, C. B.; Roberto, S. B.; da Silveira, R.; Visentainer, J. V.; Santos, O. O.; Meurer, E. C.; *J. Braz. Chem. Soc.* **2020**, *31*, 402. [Crossref]
35. Tao, Y.; Li, W.; Liang, W.; Van Breemen, R. B.; *J. Agric. Food Chem.* **2009**, *57*, 10014. [Crossref]
36. *Action Stat*, version 3.7, Estatcamp, São Carlos, Brazil, 2021.
37. Bradford, M. M.; *Anal. Biochem.* **1976**, *72*, 248. [Crossref]

38. Yonekawa, M. K.; Penteado, B. D. B.; Rodrigues, A. D. O.; Lourenço, E. M.; Barbosa, E. G.; Neves, S. C.; Oliveira, R. J.; Marques, M. R.; Silva, D. B.; Lima, D.; Beatriz, A.; Oses, J. P.; Jaques, J. A. S.; Santos, E. A.; *Bioorganic. Med. Chem. Lett.* **2021**, *47*, 128206. [Crossref]
39. Lei No. 11.794, de 8 de outubro de 2008, Regulamenta o inciso VII do 1o do art. 225 da Constituição Federal, estabelecendo *Procedimentos para o Uso Científico de Animais*; revoga a Lei no 6.638, de 8 de maio de 1979; e dá outras providências, Diário Oficial da União (DOU), Brasília, 2008. [Link] accessed in November 2023
40. *R: A Language and Environment for Statistical Computing*, R Core Team, Vienna, 2016.
41. Hammer, O.; *PAST - Paleontological Statistics*; Natural History Museum, University of Oslo, Oslo, 2018.
42. *GraphPad Prism*, version 9; GraphStats Technologies, New York, 2004.
43. Darvishzadeh, P.; Orsat, V.; *J. Appl. Res. Med. Aromat. Plants* **2022**, *27*, 100368. [Crossref]
44. Radojković, M.; Moreira, M. M.; Soares, C.; Barroso, M. F.; Cvetanović, A.; Švarc-Gajić, J.; Morais, S.; Delerue-Matos, C.; *J. Chem. Technol. Biotechnol.* **2018**, *93*, 1684. [Crossref]
45. Chen, C.-Y.; Cheng, K.-C.; Chang, A.-Y.; Lin, Y.-T.; Hseu, Y.-C.; Wang, H.-M.; *Int. J. Mol. Sci.* **2012**, *13*, 1762. [Crossref]
46. Hsieh, Y. H.; Li, Y.; Pan, Z.; Chen, Z.; Lu, J.; Yuan, J.; Zhu, Z.; Zhang, J.; *Ultrason. Sonochem.* **2020**, *63*, 104915. [Crossref]
47. Zhang, B.; Liu, Y.; Wang, H.; Liu, W.; Cheong, K.; Teng, B.; *Food Control.* **2021**, *130*, 108216. [Crossref]
48. Amadian-Kouchaksaraie, Z.; Niazmand, R.; Najaf Najafi, M.; *Innovative Food Sci. Emerging Technol.* **2016**, *36*, 234. [Crossref]
49. Si, Y.-Y.; Sun, S.-W.; Liu, K.; Liu, Y.; Shi, H. L.; Zhao, K.; Wang, J.; Wang, W.; *Processes* **2019**, *7*, 171. [Crossref]
50. Böhmer-Maas, B. W.; Otero, D. M.; Zambiazi, R. C.; Aranha, B. C. *Rev. Ceres* **2020**, *67*, 181. [Crossref]
51. Matos S. P.; *Técnicas de Análise Química: Métodos Clássicos e Instrumentais*, 1st ed.; Érica: Brazil, 2015.
52. Mitić, Ž.; Stolić, A.; Stojanović, S.; Najman, S.; Ignjatović, N.; Nikolić, G.; Trajanović, M.; *Mater. Sci. Eng.: C* **2017**, *79*, 930. [Crossref]
53. de Oliveira, C. S.; Andrade, M. M. P.; Colman, T. A. D.; Costa, F. J. O. G.; Schnitzler, E.; *J. Therm. Anal. Calorim.* **2014**, *115*, 13. [Crossref]
54. da Silva, J. A.; Sampaio, P. A.; Dulcey, L. J. L.; Cominetti, M. R.; Rabello, M. M.; Rolim, L. A.; *J. Drug. Delivery Sci. Technol.* **2021**, *61*, 102103. [Crossref]
55. Garavand, F.; Rouhi, M.; Razavi, S. H.; Cacciotti, I.; Mohammadi, R.; *Int. J. Biol. Macromol.* **2017**, *104*, 687. [Crossref]
56. Phinichka, N.; Kaenthong, S.; *J. Mater. Res. Technol.* **2018**, *7*, 55. [Crossref]
57. Asamenew, G.; Kim, H. W.; Lee, M. K.; Lee, S. H.; Kim, Y. J.; Cha, Y. S.; Yoo, S. M.; Kim, J. B.; *Food Res. Technol.* **2019**, *245*, 653. [Crossref]
58. Zhong, W.; Zhu, J.; Yi, J.; Zhao, C.; Shi, Y.; Kang, Q.; Huang, J.; Hao, L.; Lu, J.; *J. Ethnopharmacol.* **2022**, *290*, 115077. [Crossref]
59. Tohma, H.; Gülçin, İ.; Bursal, E.; Gören, A. C.; Alwasel, S. H.; Köksal, E.; *J. Food Meas. Charact.* **2017**, *11*, 556. [Crossref]
60. Yang, D.; Dunshea, F. R.; Suleria, H. A. R.; *J. Food Process. Preserv.* **2020**, *44*, e14497. [Crossref]
61. Jiang, Y.; Bu, C.; Fan, L.; Cao, G.; Sun, L.; Ho, R. J. Y.; Wu, D.; Gui, S.; Han, Y.; Hong, Y.; *Arabian J. Chem.* **2022**, *15*, 104151. [Crossref]
62. Li, M. Q.; Hu, X. Y.; Wang, Y. Z.; Zhang, X. J.; Li, J. P.; Song, Z. M.; Liu, Y. F.; Feng, W. S.; *Rapid Commun. Mass. Spectrom.* **2021**, *35*, e9029. [Crossref]
63. Mabrouk, D. M.; El Makawy, A. I.; Ahmed, K. A.; Ramadan, M. F.; Ibrahim, F. M.; *Environ. Sci. Pollut. Res.* **2022**, *29*, 87184. [Crossref]
64. Fathy, M. M.; Eid, H.; Hussein, M. A.; Ahmed, H. H.; Hussein, A.; *Res. J. Pharm. Biol. Chem. Sci.* **2015**, *6*, 735. [Link] accessed in November 2023
65. Akinyemi, A. J.; Thomé, G. R.; Morsch, V. M.; Bottari, N. B.; Baldissarelli, J.; Oliveira, L. S.; Goularte, J. F.; Belló-Klein, A.; Duarte, T.; Duarte, M.; Boligon, A. A.; Athayde, M. L.; Akindahunsi, A. A.; Oboh, G.; Schetinger, M. R. C.; *Planta Med.* **2016**, *82*, 612. [Crossref]
66. Rashid, S.; Wali, A. F.; Rashid, S. M.; Alsaffar, R. M.; Ahmad, A.; Jan, B. L.; Paray, B. A.; Alqahtani, S. M. A.; Arafah, A.; Rehman, M. U.; *Pharmaceuticals* **2021**, *14*, 146. [Crossref]
67. Thangavelu, P.; Sundaram, V.; Gunasekaran, K.; Mujyambere, B.; Raju, S.; Kannan, A.; Arasu, A.; Krishna, K.; Ramamoorthi, J.; Ramasamy, S.; Velusamy, T.; Samiappan, S.; *Chem. Phys. Lipids* **2022**, *245*, 105206. [Crossref]
68. Grace, V. M.; Sandeep, V.; Viswanathan, S.; Sathyabama, B. E.; *Indian J. Pharm. Sci.* **2018**, *80*, 1039. [Crossref]
69. Sepúlveda, C. T.; Alemán, A.; Zapata, J. E.; Montero, M. P.; Gómez-Guillén, M. C.; *Innovative Food Sci. Emerging Technol.* **2021**, *71*, 102708. [Crossref]
70. Taladrid, D.; Marín, D.; Alemán, A.; Álvarez-Acero, I.; Montero, P.; Gómez-Guillén, M. C.; *Food Res. Int.* **2017**, *100*, 541. [Crossref]
71. Wang, Y.; Wang, J.; Liu, Z.; Huang, X.; Fang, F.; Guo, J.; Yan, P.; *Sci. Total. Environ.* **2021**, *795*, 148682. [Crossref]
72. Radhakrishnan, E. K.; Bava, S. V.; Narayanan, S. S.; Nath, L. R.; Thulasidasan, A. K. T.; Soniya, E. V.; Anto, R. J.; *PloS One* **2014**, *9*, e104401. [Crossref]
73. Liao, D.; Cheng, C.; Liu, J.; Zhao, L.; Huang, D. C.; Chen, G.; *Int. J. Biol. Macromol.* **2020**, *151*, 894. [Crossref]
74. Venkatadri, B.; Shanparvish, E.; Rameshkumar, M. R.; Arasu, M. V.; Al-Dhabi, N. A.; Ponnusamy, V. K.; Agastian, P.; *Saudi J. Biol. Sci.* **2020**, *27*, 2980. [Crossref]

75. Yılmaz, N.; Seven, B.; Timur, H.; Yorgancı, A.; İnal, H. Á.; Kalem, M. N.; Kalem, Z.; Han, Ö.; Bilezikçi, B.; *J. Chin. Med. Assoc.* **2018**, *81*, 905. [Crossref]
76. Karna, P.; Chagani, S.; Gundala, S. R.; Rida, P. C. G.; Asif, G.; Sharma, V.; Gupta, M. V.; Aneja, R.; *Br. J. Nutr.* **2012**, *107*, 473. [Crossref]
77. Kottarapat, J.; Liju, V.; Kuttan, R.; *Int. J. Pharm. Pharm. Sci.* **2015**, *7*, 341. [Link] accessed in November 2023
78. Joshi, D.; Srivastav, S. K.; Belemkar, S.; Dixit, V. A.; *Biomed. Pharmacother.* **2017**, *91*, 645. [Crossref]
79. Masuda, Y.; Kikuzaki, H.; Hisamoto, M.; Nakatani, N.; *BioFactors* **2004**, *21*, 293. [Crossref]
80. Surh, Y.-J.; *Food Chem. Toxicol.* **2002**, *40*, 1091. [Crossref]
81. Promdam, N.; Panichayupakaranant, P.; *Food Chem. Adv.* **2022**, *1*, 100043. [Crossref]
82. Chen, M.; Li, R.; Lu, X.; Dai, Y.; Chen, T.; Xing, Y.; Xue, L.; Duan, Z.; Zhou, W.; Li, J.; *Food Chem.* **2023**, *398*, 133953. [Crossref]

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