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Microwave- and Ultrasound-Assisted Extraction of *Cucurbita pepo* Seeds: a Comparison Study of Antioxidant Activity, Phenolic Profile, and In-Vitro Cells Effects

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Abstract: Nowadays there is a growing demand for nutraceuticals to prevent diseases related to redox imbalances, such as atherosclerosis and diabetes, being crucial to search for new matrixes rich in bioactive compounds. This work aims to characterize the value-added compounds extracted from *Curcubita pepo* seeds using green methodologies, namely microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE), employing water as an extracting solvent for two ratios (condition 1: 1 mg/20 mL; condition 2: 2.5 mg/20 mL). The extract with the best antioxidant/antiradical activity in FRAP (71.09 μmol FSE/g DW) and DPPH (5.08 mg TE/g DW) assays was MAE condition 1, while MAE condition 2 exhibited the highest activity in the ABTS assay (13.29 mg AAE/g DW) and TPC (16.89 mg GAE/g DW). A remarkable scavenging capacity was observed, particularly for HOCl, with IC50 values ranging from 1.88–13.50 μg/mL. A total of 21 phenolic compounds were identified, being catechin (4.567–7.354 mg/g DW), caffeine (1.147–2.401 mg/g DW) and gallic acid (0.945–1.337 mg/g DW) predominant. No adverse effects were observed on Caco-2 viability after exposure to MAE extracts, while the other conditions led to a slight viability decrease in NSC-34. These results highlighted that the extract from MAE condition 2 is the most promising as a potential nutraceutical ingredient.

Keywords: *Curcubita pepo* seeds; green extraction techniques; nutraceutical industry; valorization; sustainability

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1. Introduction

Nutraceuticals are bioactive compounds extracted from their original food matrix [1] that provide medical or health benefits by preventing or treating diseases [2]. In the past years, nutraceuticals received particular attention from consumers due to their potential to improve health, delay the aging process, increase life expectancy, prevent chronic diseases, and even support the structure or function of the body [3]. Different studies reported that nutraceuticals, such as ginseng, green tea, sumac, folic acid and cod liver oil, present promising results in different pathological complications, such as atherosclerosis [4,5], cardiovascular diseases [6,7], diabetes [8], cancer [9,10] and neurological disorders [11]. Furthermore, recently published reports demonstrated the positive effects of nutraceutical plants as *Zizyphus jujube* [12] or *Lavandula officinalis* [13] on the Alzheimer's disease, learning and memory. Extracts from coneflowers or herbs of other plants, namely *Echinacea angustfolia*, *E. pallida* and *E. purpurea*, revealed to improve immune function and lower susceptibility to some diseases [3]. The ability of

nutraceuticals to neutralize these conditions is often related with their capacity to restore redox balance [14–16].

The Cucurbitaceae family, also known as cucurbits, are a large group of almost 800 species that include squashes, pumpkins, melons and gourds [17]. *Cucurbita pepo*, also referred to as "summer squash" or "zucchini", originates from Central and South America, being currently cultivated worldwide in warm regions [18]. It is the most produced species of the Cucurbitaceae family, and one of the oldest known cultivated species, with Mexican archaeological evidence dating from 7000 BC [19,20]. *C. pepo* is an economically important crop in which immature fruits, leaves, peel, blossoms, and seeds are consumed due to their nutritional and medicinal benefits [21].

The seeds of the Cucurbitaceae family are globally used for the treatment of different diseases, particularly due to their antiviral, anti-inflammatory, anti-ulcerative, antidiabetic and antioxidant activities as well as analgesic for urinary disorders [17]. A few studies using *C. pepo* seed extracts were published in the past years, revealing wound healing [22], hair growth promotion [23] and anthelmintic [24] properties, besides their ability to inhibit the cell growth of hyperplastic and cancer cells [25]. Moreover, the treatment with *C. pepo* extract conducted to a substantial improvement of the lower urinary tract symptoms that are suggestive of benign prostatic hyperplasia [26].

According to different authors, *C. pepo* seeds are rich in several bioactive compounds, such as polyphenols [27], which have anti-aging and anticarcinogenic effects and may protect against vascular inflammation and cardiovascular and neurodegenerative diseases [28–31]. α- and γ-tocopherols [32] are also present, defending cells from oxidative stress and inflammation [33], while carotenoids (e.g., β-carotene and β-cryptoxanthin) may protect against different chronic diseases, including cancer [34] and cardiovascular diseases [35]. Besides that, carotenoids improve the cognitive and visual functions [36]. In addition, *C. pepo* seeds are rich in polyunsaturated fatty acids [37], zinc [38], and phytosterols such as β-sitosterol [32], which may reduce the blood cholesterol [39] and decrease the risk of certain types of cancer [40]. β-sitosterol is also the bioactive compound responsible for the successful use of *C. pepo* seeds in the treatment of benign prostatic hyperplasia [41–43]. Berberine and palmatine are also present in considerable amounts [24], conferring to nematocidal [24], antimalarial [44], antileishmaniasis [45], anti-schistosomiasis [46] and *Toxoplasma gondii* inhibitory properties [47].

Nevertheless, most of the authors employed organic solvents in the extractive step or, at least, used cold pressing methods aiming to obtain the lipidic fraction. Therefore, the use of water as an alternative solvent led to a greener extraction process and, simultaneously, avoided the extraction of a significant amount of lipids that are naturally present in *C. pepo* seeds. Allied to the selection of less-polluting solvents, it is imperative to select eco-friendly extraction methods. Therefore, technologies such as microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) arise as an alternative to the traditional ones [48]. MAE is a conventional, automated green extraction technique that allows the extraction of active components from different matrices [49,50]. By using microwave energy, MAE heats the solvents in contact with samples, disrupts the cell membrane, and releases the intracellular components into the solvents [50,51]. This is opposite to the conventional methods, such as Soxhlet extraction, that often requires 12–24 h of extraction periods and hundreds of milliliters of organic solvents [50]. When compared to such methods, MAE requires shorter extraction times, decreasing the degradation of the extracted components as well as the costs and the volume of the solvents used, and therefore improving the purity of the final extracts [52–56].

On the other hand, UAE is also an environmentally friendly, simple, effective, and inexpensive technique, with applications in pharmaceutical, cosmetic, and alimentary fields that have become more popular since 2007 [57–59]. This sustainable technique uses ultrasounds waves, which induce cavitation and thermal and mechanical effects in the extraction medium, and disrupt the cell walls of the matrix [58]. This phenomenon leads to the release of intracellular components into the solvents, without producing

considerable modifications in the structure and properties of the compounds [60]. Some advantages of this technique include the use of small amounts of matrixes and solvents, short extraction times and samples throughput increment [61]. UAE also avoids the thermal decomposition of heat sensitive compounds, since it is a non-thermal process [62]. Furthermore, UAE achieves higher extraction yields in comparison with maceration and Soxhlet extractions [63]. Overall, these methodologies are less time-consuming, easy to execute, low-cost and usually lead to higher extraction yields, being reported by different authors as successfully in the recovery of high-added value compounds from plants [64–66].

Considering these points, the main goal of this study was the extraction of value-added compounds from *C. pepo* seeds using two green extraction methods, namely MAE and UAE, and water as solvent. Besides that, aiming to evaluate the influence of the ratio on the bioactive composition of the extracts, two different amounts of sample were used, namely 1 g/20 mL (condition 1) and 2.5 g/20 mL (condition 2). Afterwards, the extracts were characterized regarding antioxidant activity, radical scavenging capacity, phenolic profile, and in vitro cellular effects, aiming to determine their potential use as ingredients for nutraceutical purposes. To the best of our knowledge, this is the first study that uses these techniques and solvent to valorize *C. pepo* seeds. The possibility of it being used as a nutraceutical ingredient was comprehensively evaluated by analyzing its antioxidant activity, radical scavenging capacity, phenolic profile, and in vitro cellular effects.

2. Materials and Methods

2.1. Chemicals

Trolox and 2,2-diphenyl-1-picryl-hydrazyl (DPPH) were obtained from Sigma-Aldrich (Steinheim, Germany). Folin–Ciocalteu's reagent and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Hank's Balanced Salt Solution (HBSS), non-essential amino acids, penicillin, streptomycin, and trypsin–EDTA were obtained from Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain). Dimethyl sulfoxide (DMSO) was supplied by AppliChem (Darmstadt, Germany).

HPLC solvents were provided by Sigma-Aldrich (Milan, Italy). Phenolic compounds' individual standards used for the identification or quantification in extracts were purchased from Sigma-Aldrich (Steinheim, Germany) and their purity was at least above 95%.

The Caco-2 cell line was obtained from American Type Culture Collection (ATCC, USA). Mouse Motor Neuron-Like Hybrid cells (NSC-34 cell line) were obtained from Cedarlane (Hornby, ON, Canada).

2.2. Samples

C. pepo seeds were obtained from local producers in September 2021, Braga, Portugal. The seeds were dehydrated (Excalibur Food Dehydrator, Sacramento, CA, USA) at 41 °C for 24 h, grinded in a miller (Moulinex A320) and stored at 4 C °in the dark until further extraction.

2.3. Preparation of C. pepo Extracts

The MAE was performed on a MARS-X 1500 W (Microwave Accelerated Reaction System for Extraction and Digestion, CEM, Mathews, NC, USA), using closed Teflon extraction vessels. Samples of grinded *C. pepo* seeds were extracted with 20 mL of deionized water. Two different quantities of samples (1 mg/20 mL and 2.5 mg/20 mL) were used in order to determine the best ratio. Microwave power was fixed at 300 W and the extraction was performed at 25 °C for 30 min, with constant medium stirring, according to the procedure described by Silva et al. [67].

The UAE was accomplished using a Sonic Vibracell (model VC 750, Newtown, CT, USA), at room temperature for 30 min. Sonication was performed with a probe of 13 mm diameter with 40% of amplitude. The power was fixed at 750 W and the frequency at 20 kHz, as reported by Lameirão et al. [68].

After extraction, the solutions were filtrated through a Whatman No. 1 filter paper and frozen at −80 °C for subsequent lyophilization (Telstar, model Cryodos−80, Spain). After lyophilization, extracts were stocked at room temperature (20 °C) and kept in the dark. For the further experiments, the final residue was dissolved in deionized water, except for the DPPH assay, in which the extracts were dissolved in a hydroalcoholic (1:10, *v*/*v*) solution.

In the following sections, the nomenclature used will be MAE condition 1 (1 mg/20 mL), MAE condition 2 (2.5 mg/20 mL), UAE condition 1 (1 mg/20 mL) and UAE condition 2 (2.5 mg/20 mL). These ratios were based on preliminary studies performed by the research team (data not shown).

2.4. Total Phenolic Content (TPC)

The determination of the total phenolic content (TPC) was performed following the Folin–Ciocalteu procedure designed by Singleton and Rossi [69], with slight modifications. Gallic acid was used as standard for the calibration curve (linearity range = 5–100 μg/mL; $R^2 > 0.997$). The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of extract on dry weight (DW) (mg GAE/g DW).

2.5. In Vitro Antioxidant and Antiradical Activities

2.5.1. $ABTS^{\bullet+}$ Radical Scavenging Activity Assay

The extracts $ABTS^{\bullet+}$ scavenging capacity was directly assessed in a 96-well microplate as described by Re et al. [70], with minor modifications. Ascorbic acid was prepared as standard for the calibration curve (linearity range: 5–50 μg/mL; $R^2 > 0.981$). The results were presented as mg ascorbic acid equivalents (AAE) per gram of extract on DW (mg AAE/g DW).

2.5.2. $DPPH^{\bullet}$ Radical Scavenging Activity Assay

The antiradical activity by scavenging of $DPPH^{\bullet}$ radicals was evaluated following the procedure previously described by Pinto et al. [71]. Trolox was the standard used for the calibration curve (linearity range: 5–75 μg/mL; $R^2 > 0.990$). Results were expressed as mg of Trolox equivalents (TE) per gram of extract on DW (mg TE/g DW).

2.5.3. Ferric Reducing Antioxidant Power Assay

Ferric reducing antioxidant power (FRAP) was determined based on the reduction of a ferric complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ) by antioxidants, according to Benzie and Strain [72], with minor modifications. The reaction mixture was incubated at 37 °C for 30 min and the absorbance was measured at 595 nm. The calibration curve was prepared with a solution of ferrous sulphate 1mM as standard (linearity range: 25–500 μM; $R^2 > 0.999$). The results were expressed in μmol of ferrous sulphate equivalents (FSE) per gram of extract on DW (μmol FSE/g DW).

2.5.4. Reactive Oxygen Species Scavenging Capacity Assays

Hypochlorous Acid Scavenging Assay (HOCl)

The hypochlorous acid (HOCl) quenching capacity of *C. pepo* extracts and the positive controls (catechin and gallic acid) was determined according to the protocol described by Gomes et al. [73]. The HOCl solution was prepared using NaOCl 1% (*w*/*v*) and the pH was adjusted to 6.2 using H_2SO_4. The fluorescence signal was monitored for 5 min at 37 °C.

Results were expressed as the inhibition, in IC_{50} (μg/mL), of HOCl-induced oxidation of DHR to rhodamine.

Superoxide Anion Radical Scavenging Assay ($O_2^{\bullet-}$)

The superoxide anion radical ($O_2^{\bullet-}$) scavenging assay was performed following the procedure described by Gomes et al. [73]. $O_2^{\bullet-}$ was produced using a non-enzymatic system (NADH/PMS/O_2) that induces the reduction of NBT into a purple-colored diformazan. The absorbance was measured at 560 nm for 5 min. The results were presented as the inhibition, in IC_{50} (μg/mL), of the NBT reduction to diformazan.

Peroxyl radical scavenging assay (ORAC)

The peroxyl radical scavenging assay was performed to determine the capacity of *C. pepo* extracts to quench this reactive oxygen species (ROS), following the procedure described by Gomes et al. [73]. The positive controls used were catechin and gallic acid. GraphPad Prism 9.2.0 software (La Jolla, CA, USA) was used to plot the curves of inhibition percentage *versus* extract concentration and calculate the IC_{50} values. Results were expressed as mg TE/g DW.

2.6. Identification and Quantification of the Polyphenols Profile

The polyphenol identification and quantification were performed by HPLC with photodiode array (PDA) detection, as described in detail by Moreira et al. [64]. A Gemini C_{18} column (250 mm × 4.6 mm, 5 μm, Phenomenex, Alcobendas, Spain) was used for the separation at 25 °C. The results were expressed as mg of each phenolic compound per gram of extract on DW (mg/g DW).

2.7. Cell Viability Assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate the effect of the extracts on intestinal and neuronal cell lines aiming to screen the absence of toxicity for future use as a nutraceutical ingredient. Briefly, cells were incubated during 24 h with fresh medium in the absence or in the presence of the extracts (0.1, 1, 10, 100 and 1000 μg/mL) dissolved in cell culture medium. Two cell lines were employed: Caco-2 (clone type C2BBe1) and NCS-34. Passage 69–70 and 29–30 of Caco-2 and NCS-34 were, respectively, used for the MTT assay. Cells were grown according to the methodology described by Pinto et al. [74].

2.8. Statistical Analysis

Data were presented as mean ± standard deviation of at least three independent experiments. IBM SPSS Statistics 28.0.1.0 software (SPSS Inc., Chicago, IL, USA) was used to investigate statistical differences among results. After the evaluation of the normality of the data, one-way ANOVA was applied to determine the differences between samples and *post hoc* comparisons of the means were carried out using Tukey's HSD test. A meaningful significance was accepted for $p < 0.05$.

3. Results and Discussion

3.1. Extraction Yield of C. pepo Seed Extracts

Extraction yield is one of the principal factors for the selection of an extractive technique, depending not only on the matrix and solvents used, but also on the technique and respective extraction conditions (e.g., ratio, temperature, volume, among others) [64,75]. Therefore, to optimize the extraction efficiency, different extraction conditions were used, namely the solid–liquid ratios (1 g/20 mL and 2.5 g/20 mL) and the extraction techniques (MAE and UAE). As observed in Table 1, the extraction yields of *C. pepo* seed extracts varied between 16.30% (MAE condition 2) and 28.41% (UAE condition 2). UAE led to higher yields (25.19% and 28.41%, respectively, for condition 1 and 2) when

compared to MAE (23.62% and 16.30%, respectively, for condition 1 and 2). These differences may be due to a higher amount of compounds extracted by the UAE technique.

Table 1. Extraction yield, total phenolic content (TPC), in vitro antioxidant/antiradical activities (evaluated by ABTS, FRAP and DPPH assays) of *C. pepo* extracts prepared by MAE and UAE. Values are expressed as mean ± standard deviation (n = 3).

Extraction Techniques	Ratio (g/mL)	Extraction Yield (%)	TPC (mg GAE/g DW)	FRAP (μmol FSE/g DW)	ABTS (mg AAE/g DW)	DPPH (mg TE/g DW)
MAE	1/20 (condition 1)	23.62 ± 0.02	12.63 ± 1.01 ^{b}	71.09 ± 1.07 ^{a}	13.29 ± 0.69	4.93 ± 0.93
	2.5/20 (condition 2)	16.30 ± 0.07	16.89 ± 1.06 ^{a}	59.75 ± 1.09 ^{b}	12.42 ± 0.85	5.08 ± 0.84
UAE	1/20 (condition 1)	25.19 ± 0.04	12.17 ± 0.72 ^{b}	52.32 ± 1.95 ^{c}	12.87 ± 1.76	4.35 ± 0.73
	2.5/20 (condition 2)	28.41 ± 0.10	12.40 ± 0.91 ^{b}	45.80 ± 1.37 ^{d}	11.38 ± 3.54	4.54 ± 1.40

Different letters (a, b, c, d) in the same column indicate significant differences between extracts ($p < 0.05$). TPC: total phenolic content; FRAP: ferric reducing antioxidant power; FSE: ferrous sulphate equivalents; AAE: ascorbic acid equivalents; TE: trolox equivalents; DW: dry weight.

3.2. Antioxidant/Antiradical Activity

The extraction conditions and the experimental values of TPC, ABTS, DPPH and FRAP assays are presented in Table 1. The TPC varied from 12.17 mg GAE/g DW (UAE condition 1) to 16.89 mg GAE/g DW (MAE condition 2). These results are in line with the results obtained by HPLC–PDA analysis (Section 3.4), which confirmed that the MAE condition 2 extract has the highest content in phenolic compounds, while the UAE condition 1 extract achieved one of the lowest. The TPC values are considerably higher than the ones obtained for *C. pepo* seeds extracted using acetone as a solvent in an Ultra Turax mixer (8.37 mg GAE/g DW) [76]. The TPC of MAE condition 2 extract is in line with the value reported by Mondal et al. [77] for an ethanolic extract of *C. pepo* leaves and stems (17.49 mg GAE/g DW), using the cold extraction method. Oppositely, conventional extracts of *C. pepo* fresh seeds dried at 65 °C and prepared with ultrapure water displayed a substantially lower content (2.39 mg GAE/g DW) [78]. The TPC values are also considerably higher than the ones reported for *C. maxima* seed oil extracted using chloroform/methanol (54.41 mg GAE/Kg) as solvent [79]. Moreover, Peiretti et al. obtained a TPC of 9.82 mg of catechin equivalents (CAE)/g of *C. pepo* seed extract using a mixture of 80:20 methanol/water (*v*/*v*) as solvent, and a solid material to solvent ratio of 1:10 (*w*/*v*) in an ultrasonic water bath [27]. Nevertheless, the units used by the authors are not the same, making it not possible to completely compare.

Regarding the DPPH assay, the results ranged from 4.35 mg TE/g DW (UAE condition 1) to 5.08 mg TE/g DW (MAE condition 2), with no significant differences between the extracts. These values are lower than the ones reported using conventional extracts of *C. pepo* fresh seeds dried at 65 °C and employing water as a solvent (118.19 μmol of TE/g) [78]. As far as we know, no more studies have been performed regarding the scavenging capacity of this radical.

In regards to the antioxidant activity evaluated by the FRAP assay, the MAE condition 1 extract achieved the best result (71.09 μmol FSE/g DW), while the UAE condition 2 extract achieved the worst (45.80 μmol FSE/g DW). These values are in line with the ones reported by Peirerri et al. (54 μmol FSE/g DW) for *C. pepo* seeds using 80:20 methanol/water (*v*/*v*) as a solvent [27]. Nevertheless, it should be highlighted that these authors used organic solvents, while in the present study, water was the only solvent employed.

Concerning the ABTS assay, the results varied between 11.38 mg AAE/g DW (UAE condition 2) and 13.29 mg AAE/g DW (MAE condition 1), with no significant differences between extracts. These results are not in accordance with the TPC ones, which could be justified by the ABTS mechanism of assay. The ABTS assay measures the relative ability

of the antiradical compounds present in the extracts to scavenge the $ABTS^{\bullet+}$ cation generated in vitro. Nevertheless, some matrixes may have interferents, which do not scavenge the cation and lead to worse results. Therefore, the antiradical assays should always combine different radical assays (such as ABTS or DPPH).

Kulczyński et al. obtained an ABTS value of 79.30 mg Trolox/100 g DW for *C. maxima* seed extracts using an ultrasonic water bath for 1h at 30 °C, employing water as an extraction solvent [80]. Using ethanol and methanol as an extraction solvent, Nawirska–Olszańska et al. obtained ABTS values of 7.92 and 1.95 μM Trolox/g fresh weight (FW), respectively, using *C. pepo* (Miranda cultivar) seed extracts and the UAE technique [81]. Nevertheless, the results obtained in the present study could not be compared with these ones since the units are different.

3.3. Reactive Oxygen Species Scavenging Capacity Assays

Reactive species are the principal cause of oxidative stress, being divided in reactive oxygen and nitrogen species (ROS and RNS, respectively). These molecules, produced during the body aerobic metabolism, may cause oxidative damage of amino acids, DNA, lipids, and proteins [82–84]. The radical scavenging activity results are summarized in Table 2.

Table 2. Reactive oxygen species scavenging capacity (evaluated by $O2^{\bullet-}$, HOCl and ORAC assays) of *C. pepo* extracts prepared by microwave-assisted extraction and ultrasound-assisted extraction (MAE and UAE, respectively). Values are expressed as mean ± standard deviation (n = 3).

Extraction Technique	Conditions	Reactive Oxygen Species		
		$O_2^{\bullet-}$ IC_{50} (μg/mL)	HOCl IC_{50} (μg/mL)	ORAC μg TE/mg DW
MAE	Condition 1	-	2.29 ± 0.11 ^{c}	0.28 ± 0.025
	Condition 2	134.59 ± 29.31 ^{b,c}	6.32 ± 0.10 ^{b}	0.04 ± 0.01
UAE	Condition 1	221.88 ± 0.00 ^{a}	1.88 ± 0.23 ^{c}	1.13 ± 0.28
	Condition 2	178.68 ± 42.60 ^{a,b}	13.50 ± 0.75 ^{a}	0.67 ± 0.06
Positive controls				
Catechin		84.40 ± 10.33 ^{c,d}	0.31 ± 0.05 ^{d}	6.60 ± 9.29
Gallic acid		24.55 ± 3.49 ^{d}	3.27 ± 0.29 ^{c}	7.31 ± 2.63

Different letters (a, b, c, d) in the same column indicate significant differences between extracts (p < 0.05). IC_{50} = In vitro concentration required to decrease in 50% the reactivity of the studied reactive species in the tested media. ORAC: Peroxyl radical scavenging assay; TE: trolox equivalents; DW: dry weight.

Among ROS, superoxide radical ($O_2^{\bullet-}$) has particular importance as it is one of the most aggressive oxygen species in the human organism, being enrolled in the development of aging and chronic diseases, such as atherosclerosis, ischemic heart disease, diabetes mellitus, cancer, neurodegenerative diseases, immunosuppression and others [85–88]. Phenolic compounds have demonstrated a strong scavenging capacity of superoxide anion [89]. Concerning this species quenching assay (Table 2), gallic acid and catechin were the best scavengers, with IC_{50} values of 24.55 μg/mL and 84.40 μg/mL, respectively, followed by MAE condition 2 (IC_{50} = 134.59 μg/mL), UAE condition 2 (IC_{50} = 178.68 μg/mL) and UAE condition 1 (IC_{50} = 221.88 μg/mL). It is important to emphasize that there are no significant differences (p > 0.05) between MAE condition 2 extract and catechin used as positive control.

Among the ROS studied, the best results were achieved by hypochlorous acid (HOCl). For this species, the UAE condition 1 (IC_{50} = 1.88 μg/mL) and MAE condition 1 (IC_{50} = 2.29 μg/mL) extracts achieved the best results, being not significantly different from gallic acid (IC_{50} = 3.27 μg/mL) used as positive control. These results are followed by MAE condition 2 (IC_{50} = 6.32 μg/mL) and UAE condition 2 (IC_{50} = 13.50 μg/mL) extracts.

Regarding the oxygen radical absorbance capacity, the values of *C. pepo* extracts varied between 0.04 μg TE/mg DW (MAE condition 2) and 1.13 μg TE/mg DW (UAE condition 1), with no significant difference between the extracts and the positive controls employed.

To the best of our knowledge, this is the first study that screened the radical scavenging activity of *C. pepo* extracts. Nevertheless, comparing with other seed matrixes, the results are very interesting. For example, using tea seed oil extracted by cold pressing, Liu et al. obtained a IC_{50} = 1.73 mg/mL for the $O_2^{\bullet-}$ radical [90], which indicates that this extract has a lower radical scavenging capacity than the *C. pepo* extracts tested. Annatto seed extracts, obtained by a conventional extraction employing distilled water as the solvent, displayed an IC_{50} = 1.0 μg/mL for the HOCl and an IC_{50} = 0.11 μmol TE/mL for the ORAC assay. However, no $O_2^{\bullet-}$ radical scavenging activity was detected within the assayed concentration range (15–25 μg/mL) [91].

3.4. Phenolic Compounds of C. pepo Seed Extract

Phenolic compounds are a group of non-enzymatic antioxidants that have the ability to combat free radicals, which, when in excess, may lead to increased oxidative stress and consequently to cell aging. The phenolic composition of the *C. pepo* extracts is summarized in Table 3.

Table 3. Identification and quantification (mg /g DW) of the phenolic compounds present in *C. pepo* seed extracts obtained by UAE and MAE at different extraction conditions through HPLC–PDA analysis. Results are expressed as mean ± standard deviations (n = 3).

Compounds	UAE (mg/g DW)		MAE (mg/g DW)	
	Condition 1	**Condition 2**	**Condition 1**	**Condition 2**
Alkaloids				
Caffeine	2.401 ± 0.120	1.147 ± 0.057	1.483 ± 0.074	2.373 ± 0.119
Chalconoids				
Phloridzin	ND	ND	1.502 ± 0.075	2.062 ± 0.103
Flavanols				
Catechin	5.634 ± 0.282	4.567 ± 0.228	5.753 ± 0.288	7.354 ± 0.368
Epicatechin	0.278 ± 0.014	<LOD	0.549 ± 0.027	0.654 ± 0.033
Flavanones				
Naringin	0.016 ± 0.001	0.072 ± 0.004	0.102 ± 0.005	0.116 ± 0.006
Flavonols				
Rutin	ND	ND	0.045 ± 0.002	0.054 ± 0.003
Myricetin	0.299 ± 0.015	ND	0.392 ± 0.020	<LOQ
Phenolic acids				
Gallic acid	1.337 ± 0.067	0.945 ± 0.047	1.061 ± 0.053	1.236 ± 0.062
Protocatechuic acid	0.378 ± 0.019	0.712 ± 0.036	1.989 ± 0.099	1.970 ± 0.098
Neochlorogenic acid	0.224 ± 0.011	<LOD	0.106 ± 0.005	<LOQ
Caftaric acid	0.174 ± 0.009	0.176 ± 0.009	0.090 ± 0.005	0.081 ± 0.004
Chlorogenic acid	0.079 ± 0.004	0.111 ± 0.006	1.490 ± 0.074	1.419 ± 0.071
4-*O*-caffeyolquinic acid	0.396 ± 0.018	0.369 ± 0.018	0.399 ± 0.020	0.533 ± 0.0267
Vanillic acid	0.224 ± 0.011	0.269 ± 0.013	0.531 ± 0.027	0.835 ± 0.042
Caffeic acid	0.050 ± 0.002	0.032 ± 0.002	0.152 ± 0.008	0.184 ± 0.009
Syringic acid	0.047 ± 0.002	0.083 ± 0.004	0.183 ± 0.009	0.197 ± 0.010
p-Coumaric acid	0.123 ± 0.006	0.142 ± 0.007	0.257 ± 0.013	0.303 ± 0.015
trans-Ferulic acid	0.061 ± 0.003	0.044 ± 0.002	0.080 ± 0.004	0.132 ± 0.007
Sinapic acid	0.155 ± 0.080	0.093 ± 0.005	0.062 ± 0.003	ND
4,5-di-*O*-caffeoylquinic acid	0.555 ± 0.028	0.336 ± 0.017	0.693 ± 0.035	0.840 ± 0.042

Stilbenoids				
trans-polydatin	0.072 ± 0.004	0.055 ± 0.003	0.090 ± 0.004	<LOQ
Total	12.511	9.154	17.006	20.340

ND: not detected; LOD: limit of detection; LOQ: limit of quantitation; DW: dry weight.

MAE condition 2 achieved the highest concentration of phenolic compounds (20.34 mg/g DW), followed by MAE condition 1 (17.01 mg/g DW), UAE condition 1 (12.51 mg/g DW) and UAE condition 2 (9.15 mg/g DW). The lower concentration of phenolic compounds in the UAE extracts may be explained by the partial degradation of phenolic acids by ultrasonic waves and the creation of highly reactive hydroxyl radicals during the UAE process, which has been reported by several authors [92–94].

In all extracts, the phenolic compound present in the highest quantity was catechin. Quantitatively, in addition to catechin, the compounds identified in higher amounts were caffeine and gallic acid in the UAE condition 1 and UAE condition 2 extracts, and caffeine, phloridzin, gallic acid, protocatechuic acid, and chlorogenic acid in the MAE condition 1 and MAE condition 2 extracts. Moreover, caffeine, catechin, naringin, gallic acid, protocatechuic acid, caftaric acid, chlorogenic acid, 4-*O*-caffeyolquinic acid, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid and 4,5-di-*O*-caffeoylquinic acid are present in all samples. Phloridzin and rutin were only detected in the UAE extracts. The phenolic compounds 3,5-di-caffeoylquinic acid, quercetin-3-*O*-galactoside, resveratrol, quercetin-3-*O*-glucopyranoside, ellagic acid, cinnamic acid, quercitrin, kaempferol-3-*O*-glucoside, isorhamnetin-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside, naringenin, trans-epsilon viniferin, quercetin, phloretin, tiliroside, kaempferol, apigenin and chrysin were not detected in any of the *C. pepo* extracts.

To the best of our knowledge, this is the first work that identified and quantified the phenolic compounds present in *C. pepo* seed extracts. Nevertheless, it is possible to compare the results with other Cucurbita seed extracts. The results obtained by Ennebs et al. shown that *C. moschata* seed extract contains less phenolic compounds than *C. pepo*, as only six phenolic acids (quinic acid, protocatechuic acid, caffeic acid, syringic acid, trans-ferulic acid and 4,5-Di-O-caffeoylquinic acid) were identified in *C. moschata* seed extracts using LC-ESI-MS, employing hexane, chloroform, ethyl acetate and methanol as the extraction solvent [95].

Employing ultrasounds as the extraction method and methanol/H_2O 80:20 (*v*/*v*) as the solvent, Iswaldi et al. identified 10 phenolic acids (*p*-coumaric acid, ferulic acid, caftaric acid, caffeic acid, 3-*O*-caffeoylquinic acid, caffeic acid, 2-*O*-caffeoylmalic acid, dicaffeoyltartaric acid, dicaffeic acid and sinapic acid) and 16 flavonoids (including luteolin *O*-glucoside, quercetin 3-*O*-rhamnosyl-rhamnosyl-glucoside, quercetin rutinoside, glucoside, isorhamnetin 3-rutinoside-7-rhamnoside and different kaempferol glycosides) in *C. pepo* whole fruit extracts, using LC-DAD-Q-TOF MS [96].

3.5. Cytotoxic Effects of C. pepo Seed Extracts towards Intestinal and Neuronal Cells

Caco-2, an intestinal cell line, was used to evaluate the cytotoxicity of the *C. pepo* extracts obtained by UAE and MAE. This cell line allows the study the compound absorption across the intestinal epithelium, being commonly employed to evaluate the extracts safety [97]. According to Figure 1, the exposure of this cell line to increasing concentrations (0.1–1000 μg/mL) of MAE *C. pepo* extracts showed a viability around 100%, without significant differences ($p > 0.05$). On the other hand, only the concentration of 0.1 μg/mL of the UAE condition 1 extract maintained a viability of 101.36%, being significantly different from the other concentrations ($p < 0.05$). At higher concentrations, the viability ranged between 70.20% (1000 μg/mL) and 77.82% (1 μg/mL). The UAE condition 2 displayed viabilities above 88.78%, being not significantly different ($p > 0.05$). The lower viability displayed by Caco-2 cells incubated with UAE *C. pepo* extracts may be explained by the higher content on neochlorogenic acid and caftaric acid present in the

extracts. These compounds have antiproliferative activity and may effectively inhibit the growth of colorectal cancer cells [98,99].

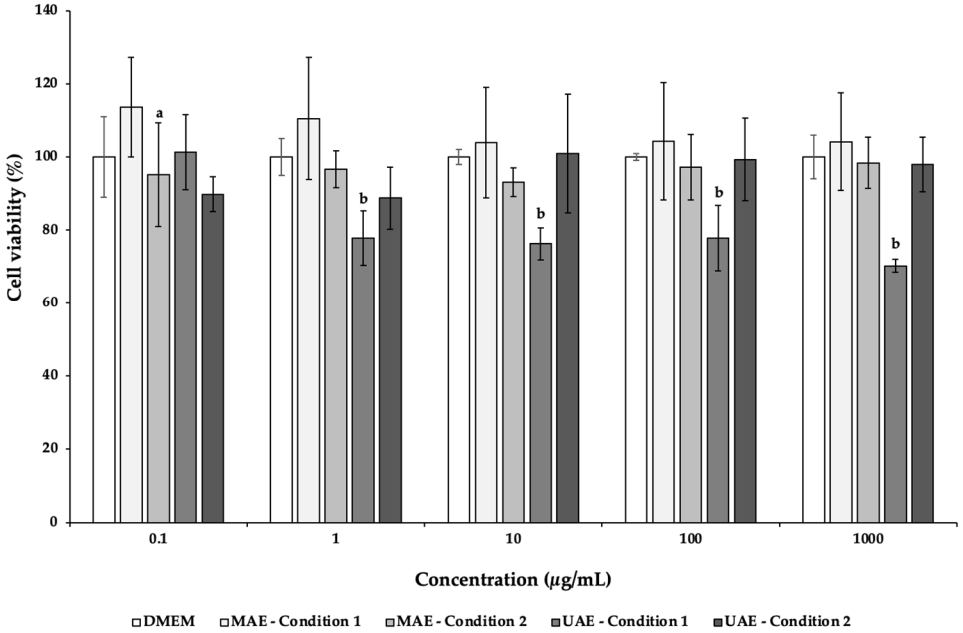

Figure 1. Effects of *C. pepo* extracts obtained by UAE and MAE exposure on the viability of Caco-2 cells at different concentrations (0.1–1000 μg/mL), measured by MTT assay. Values are expressed as mean ± standard deviation ($n = 3$). Different letters represent significant differences between concentrations of the same sample ($p < 0.05$), according to Tukey's HSD test.

In the present study, NSC-34 cell line was used to evaluate the potential neurotoxicity of *C. pepo* extracts obtained by UAE and MAE (Figure 2). NSC-34 is a hybrid cell line that has similar morphological and physiological properties to motor neurons [100]. This cell line in culture mimics the synthesis and storage of acetylcholine (ACh) and the expression of neurofilament proteins [100]. After 24 h of incubation in the presence of different concentrations (0.1–1000 μg/mL) of *C. pepo* extracts, it was possible to observe a decrease of the NSC-34 cell viability, mainly in the MAE and UAE condition 1 extracts (Figure 2). Indeed, at the highest concentration tested (1000 μg/mL), the cells' viability was 9.92% and 19.58%, for MAE condition 1 and UAE condition 1, respectively, with significant differences ($p < 0.05$). In these cases, it was possible to determine the IC_{50} values of 253.68 (MAE condition 1) and 95.32 μg/mL (UAE condition 1).

Oppositely, MAE and UAE condition 2 showed an increasing trend with increased concentrations. For MAE condition 2, the results varied from 54.17% (0.1 μg/mL) to 82.97% (1000 μg/mL). In the case of UAE condition 2 extracts, the highest cell viability (98.21%) was found for the highest concentration tested (1000 μg/mL), following in descending order 100, 1, 10 and 0.1 μg/mL.

The inferior cells' viability observed after exposure to MAE condition 1 and UAE condition 1 extracts may be due to the presence in higher amounts of the antiproliferative compounds neochlorogenic acid and caftaric acid, which is not observed in MAE condition 2 and UAE condition 2 extracts. Besides that, MAE condition 2 and UAE condition 2 extracts present other compounds (e.g., catechin, gallic acid or protocatechuic acid) with benefic cell effects in higher quantities, justifying a possible protective effect. Further studies are needed to justify these differences.

To the best of our knowledge, this is the first study that screens the in vitro effects of *C. pepo* extracts on the viability of cell lines. Li et al. [101] evaluated the cytotoxicity effect of *C. moschata* polysaccharides on Caco-2 through the CCK-8 assay, reporting a viability

above 90% for all tested concentrations (0, 250, 500, 1000, 1500 μg/mL) [101]. These results are in line with the ones obtained in the present study for Caco-2.

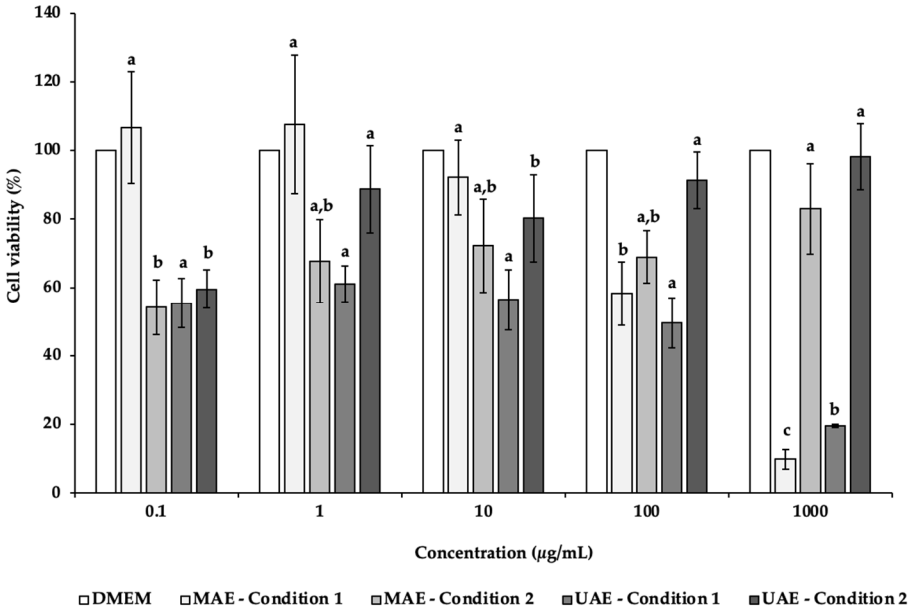

Figure 2. Effects of *C. pepo* extracts obtained by UAE and MAE exposure on the viability of NSC-34 cells at different concentrations (0.1–1000 μg/mL), as measured by the MTT assay. Values are expressed as mean ± standard deviation ($n = 3$). Different letters indicate significant differences between concentrations of the same sample ($p < 0.05$), according to Tukey's HSD test.

4. Conclusions

In this work, value-added compounds from *C. pepo* seeds were extracted using green techniques—UAE and MAE—and an eco-friendly solvent—water, aiming a potential use as nutraceutical ingredient. Two different ratio conditions were employed.

FRAP scavenging activity ranged from 45.80 to 71.09 μmol FSE/g DW (UAE condition 2 and MAE condition 1, respectively), while DPPH values varied between 4.35 mg TE/g DW (UAE condition 1) and 5.08 mg TE/g DW (MAE condition 2). The extract with the best performance at ABTS assay was MAE condition 1 (13.29 mg AAE/g DW), while UAE condition 2 was the worst (11.38 mg AAE/g DW). The extract with the highest TPC value was MAE condition 2 (16.89 mg GAE/g DW), and UAE condition 1 had the lowest (12.17 mg GAE/g DW). All extracts displayed a remarkable scavenging capacity for HOCl, with IC_{50} values ranging from 1.88 (UAE condition 1) to 13.50 μg/mL (UAE condition 2).

HPLC–PDA analysis revealed the presence of alkaloids, chalconoids, flavanols, flavanones, flavonols, phenolic acids and stilbenoids. A total of 21 phenolic compounds were identified, with catechin, caffeine and gallic acid being present in higher quantities. No adverse effects were detected in Caco-2 viability to MAE extracts up to 1000 μg/mL, while all the other conditions tested led to a slight decrease of cell viability.

The results shown that a higher sample-to-solvent ratio led to an increase in the amount of phenolic compounds present in both MAE and UAE extracts, which led to better results in ORAC, $O_2^{\bullet -}$, TPC and DPPH assays. Overall, these results highlighted that *C. pepo* seeds are a potential source of antioxidant/antiradical compounds and that the extract MAE condition 2 is rich in bioactive compounds with potential uses in the nutraceutical field.

Moreover, the results obtained in this study show that higher sample-to-solvent ratios using MAE technology can lead to the extraction of a greater amount of phenolic compounds. Therefore, further studies are needed to screen the effects on neuronal

enzymes (such as acetylcholinesterase or butyrylcholinesterase) as well as 3D intestinal permeation studies to screen the amounts that are absorbed at intestinal level.

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