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Improved Recovery of Antioxidant Compounds from Refined Pumpkin Peel Extract: A Mixture Design Method Approach

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Abstract: This study employed the mixture design method to determine optimal solvent combinations, aiming to obtain refined extracts from squash peels with enhanced antioxidant properties. We optimized extraction solvents, focusing on recovering the total phenolic compounds (TPC) and increased antioxidant properties using a second-order polynomial equation through the response surface methodology (RSM). Six solvents (MeOH, Hexane, DCM, EtOAc, BuOH, and water) were assessed for their effects on TPC and antioxidant activity in preliminary experiments. The refined extracts underwent a HPLC analysis for a phenolic composition determination and were further evaluated for their antibacterial activity and cytotoxicity. The results revealed a rich phenolic content in the refined extract from peels of Bejaoui landrace, primarily catechin (8.06 mg/g dry extract (DE)), followed by epicatechin and kaempferol (5 mg/g DE). Antibacterial tests against *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Staphylococcus aureus* showed significant antimicrobial activities, especially for Karkoubi and batati landraces, where the growth inhibitions were 99%, 96%, 97%, and 80% and 94%, 89%, 98%, and 96% for the respective bacteria. The peel extracts exhibited a negligible cytotoxicity on the RAW264.7 cell line, even at high concentrations. Our findings emphasize the potential antioxidant and antibacterial properties of peel extracts due to diverse phenolic compounds, suggesting the potential use of squash peels in the food and nutraceuticals industries as sources of natural antimicrobial agents.

Keywords: *Cucurbita maxima* Duchesne; phenolic compounds; antioxidant activity; antimicrobial properties; squash by-products; response surface methodology

1. Introduction

Currently, two pivotal strategies are employed within the environmental protection curricula, namely the circular economy and Zero Waste. The first refers to a non-linear economic system that recovers energy and raw materials as much as possible, aiming at the sustainable use of natural resources. The second strategy stresses the importance of the rational use of products and the reduction in the amount of waste produced [1]. The handling of crop waste is one of the most important problems that the agricultural and food sector has to address nowadays [2]. Processing fruits and vegetables into commercial products generates significant quantities of bulk waste, depending on the species (peels,

seeds, shells, skins, leaves, and roots, etc.), which may amount to up to 25–30% of total production [3,4]. For the modern food sector, the sustainable processing of food is a major issue, combined with the reduction in waste production. The processing of fruit and vegetable products needs intensive modernization to exploit the waste and by-products produced throughout the process, especially because they include a high content of health beneficial compounds. Regardless of the fact that these by-products are commonly transferred to other economically feasible but low-added-value non-food purposes such as composting, bio-energy production, or animal feeding, their rich content in phytochemicals highlights the importance of their transformation into high-added-value products [5]. Consequently, exploiting the waste of fruit and vegetable crops for bioactive components is an alternative and very promising waste management strategy in regard to sustainability, effectiveness, and global human health improvement [6]. Moreover, there is a high demand to consider food as a means of improving well-being and health, and not only as a nutrient source and for sensorial appeal [7]. With this respect, various studies have demonstrated that most antioxidants in fruits are located in the skin (peel) or outer soft shell, which indicates that significant amounts of valuable compounds are lost when fruits are processed (e.g., peeled, roasted, or blanched) in the food industry [8,9].

Pumpkin (*Cucurbita maxima* Duchesne) belongs to the *Cucurbitaceae* family, and the processing of fruit generates tons of seeds and peels as by-products. However, the fruit waste possesses beneficial nutrients and dry matter that can be utilized in many ways. It has been reported that pumpkin peels are rich in glucose, which can be converted through multiple steps in bioethanol for energy production, including pretreatment, enzymatic saccharification, and fermentation, which usually employs fungi to obtain fermentable sugar, followed by distillation [10]. In addition, pumpkin peels contain high amounts of β-carotene, which are rarely exploited to their full potential, and pectin that can be extracted through acid hydrolysis and could be used as gelling agent or thickener in the food industry [11,12]. In addition, pumpkin peel is an excellent source of minerals, protein, fibers, and isoforms of vitamin E. These valuable compounds are associated with the nutritional quality of food, as evidenced by Mala et al. [13], who showed that peels are rich in minerals, such as phosphorus and iron, as well as indietary fiber. Kubra Gungor et al. [2] used β-carotene from pumpkin peels (*C. moschata*) generated in the fruit processing industry using high-efficiency technology that produced zero waste and had no harmful effects on the environment. In fact, pumpkin peels contain several compounds that may act as potent antioxidants and antimicrobial agents, or they could be used for their peel powder or extracts for the isolation and characterization of bioactive compounds that could be implemented in the food, nutraceuticals, and pharmaceuticals industries.

It is noticeable that foodborne pathogens impose a considerable burden of infection and present an important public health problem in the world. Among the leading foodborne bacteria, *Salmonella typhimurium* and *Staphylococcus aureus* (Gram+) are the most common and frequent bacteria responsible for food poisoning and food-related infections [14,15]. *Enterococcus faecalis* (Gram+) is natural inhabitant of the gastrointestinal tract of humans, and it is frequently found in many food products, including those of meat, dairy, and vegetable origin [16,17]. On the other hand, the Gram-negative bacteria *Pseudomonas aeruginosa* is a common foodborne pathogen, mainly found in meat products and drinking water [18]. Therefore, the use of novel antimicrobial agents, especially those of natural origin, is considered to be of major importance for fulfilling the needs of the food industry sector and consumers who are seeking healthy foods without synthetic additives [19].

Optimized extraction methods ensure the maximum recovery of bioactive compounds, enhancing their potential for use in functional foods, nutraceuticals, and natural preservation systems. The utilization of the Response Surface Methodology (RSM) holds significant importance for obtaining refined preserving compounds from pumpkin by-products [20]. The Response Surface Methodology is a statistical technique used to optimize processes and understand complex relationships between multiple factors. It involves creating mathematical models to predict how a response variable changes based on the levels of different input

variables. By conducting experiments with various combinations of these variables, the RSM helps identify the optimal conditions for achieving the desired outcome. This method is valuable for finding the best settings for processes in fields such as engineering, chemistry, and agriculture. By employing the RSM, it is possible to optimize the combination of several extraction parameters, namely the extraction temperature, extraction time, and the concentration of solvents, as well as to improve the recovery of preserving compounds from pumpkin waste [2]. Moreover, the RSM facilitates the construction of response surface models that provide valuable insights into selective extraction process. These models allow for the prediction of the response variables within the experimental design space, enabling a deeper understanding of the optimal conditions required for obtaining refined preserving compounds. Furthermore, the RSM aids in reducing the number of experiments needed for optimization by employing statistical techniques such as factorial designs, central composite designs, or Box–Behnken designs. This approach not only saves time and resources, but also provides a more efficient and systematic strategy for optimizing the extraction procedure. In the current study, we employed the RSM in obtaining refined preserving compounds from pumpkin peels, aiming to enhance the efficiency, reproducibility, and scalability of the extraction process. This work presented a comprehensive analysis of the most suitable extraction conditions for obtaining refined preserving compounds from pumpkin peels and also evaluated their potential antioxidant activity. The potential cytotoxicity and antimicrobial and phytochemical profiles (HPLC) of squash peel-refined extracts were also assessed. Overall, the use of the response surface methodology was proven as a useful tool that allowed for identifying those conditions which ensured the best extraction efficiency of polyphenols and also improved the antioxidant potential and antimicrobial properties of the obtained extracts. Considering the increasing environmental pressure from the inefficient management of crop by-products, this study highlights the importance of valorizing pumpkin fruit waste for the recovery of bioactive compounds and further increasing the added value of the crop.

2. Materials and Methods

2.1. Chemicals and Reagents

Chemicals and reagents were purchased from Fluka (99.8 and 98% purity Buchs, Switzerland). HPLC pure standards were purchased from Sigma (St. Louis, MO, USA). DPPH (2,2-diphenyl-1-picrylhydrazyl) and Folin–Ciocalteu and sodium carbonate reagents were purchased from Sigma-Aldrich (São Paulo, Brazil). Ultrapure water was prepared with the Millipore system (Billerica, MA, USA). Solvents were obtained from Loba Chemie Pvt. (97% purity). Mueller–Hinton broth and Sabouraud broth were purchased from BIOKAR Diagnostics (Allonne, France).

2.2. Plant Sampling and Peel Extraction

In this study, three landraces of *Cucurbita maxima* peels (Batati (NGBTUN 746), Karkoubi (NGBTUN748), and Bejaoui (NGBTUN751)) were evaluated. Fruits were collected at the same stage of ripeness [21]. For all the analyses, samples from six fruits were prepared after separating the peels from the pulp based on the methodology previously described by our team [20].

2.3. Preliminary Fractionation

Peel extract fractionation involved the separation of different compounds present in the extract using various solvents. In this process, n-butanol, dichloromethane, ethyl acetate, n-hexane, methanol, and water were used successively as extraction solvents [20]. The extract was initially mixed with one of the solvents, and the mixture was vigorously shaken to allow the compounds to partition between the solvent and the aqueous phase. A separation funnel was then used to separate the two distinct phases based on their density difference. Water, being the polar solvent, helped in extracting hydrophilic compounds, such as phenolic compounds and water-soluble vitamins. n-Butanol was chosen to ex-

tract lipophilic compounds, including terpenoids. Methanol is a versatile solvent that can extract a wide range of compounds, including polar and non-polar ones, making it useful for comprehensive extraction. Ethyl acetate was employed to extract non-polar compounds, such as flavonoids and alkaloids. Dichloromethane is effective in extracting moderately polar compounds, while hexane is predominantly used to extract non-polar lipids and hydrocarbons.

2.4. Determination of Total Phenolic Compounds Content

The total phenolic compounds (TPC) content was evaluated with the Folin–Ciocalteu reagent using the adapted protocol of Mansour et al. [20]. The TPC was expressed as milligrams of Gallic acid equivalent per gram of extract (mg GAE/g E). All the samples were analyzed in triplicate.

2.5. DPPH Radical Scavenging Activity Assay

The antiradical activity against 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical was measured as previously described by Hatano et al. [22]. The inhibition percentage (IP %) of DPPH radical was calculated using the following equation described by Mansour et al. [20]:

$$IP (\%) = [(A_0 - A_1)/A_0] \times 100$$

where A_0 and A_1 are the absorbance of the control and the sample, respectively, at 514 nm, using a microplate spectrophotometer (EZ Read 2000, Biochrom, Cambridge, UK). The results are presented as the inhibition percentage (IP) at a concentration of 0.01 g/mL. All the analyses were performed in triplicate.

2.6. Mixture Design and Optimization of the Extraction Process Using Response Surface Methodology (RSM)

In the current study, we implemented a plan of centered mixtures (simplex-centroid designs). The factors X1, X2, and X3 represent the solvents MeOH, ethyl-acetate, and water based on the results of the preliminary fractionation (see Section 2.2), which ranged from 0 to 1 without constraints, according to formula described by Zeouk et al. [23]:

This analysis was carried out using the NEMRODW software package (Mathieu, Nony and Phan-Tan-Luu, version 2000, Marseille, France). The experimental design included 13 experiments performed in a standard order (Table 1).

Table 1. Three components axial screen matrix (X1: methanol, X2: ethyl acetate, and X3: water) and the values of the experimental responses for total phenolic contents (Y1, expressed in mg GAE/g extract) and antiradical activity (Y2, expressed in inhibition percentage (%)) for pumpkin landraces Batati (NGBTUN 746), Karkoubi (NGBTUN748), and Bejaoui (NGBTUN751).

	Experimental Factors			Landraces					
Experiment N°	X1	X2	X3	Batati		Bejaoui		Karkoubi	
				Y1	Y2	Y1	Y2	Y1	Y2
1	1.0000	0.0000	0.0000	14.44	53.32	14.59	56.17	15.42	48.82
2	0.0000	1.0000	0.0000	17.08	29.57	12.31	22.46	27.50	71.32
3	0.0000	0.0000	1.0000	12.64	37.98	10.00	34.47	10.35	28.44
4	0.6667	0.3333	0.0000	15.92	66.09	15.97	72.08	12.92	32.54
5	0.3333	0.6667	0.0000	16.10	55.23	14.00	50.96	19.12	63.06
6	0.6667	0.0000	0.3333	12.53	27.89	9.62	30.56	18.96	58.97
7	0.3333	0.3333	0.3333	15.85	49.62	9.23	51.97	16.85	59.60
8	0.0000	0.6667	0.3333	15.30	44.93	8.47	48.07	16.30	51.52
9	0.3333	0.0000	0.6667	11.02	31.59	6.04	36.93	10.67	30.25

Table 1. *Cont.*

| Experiment N° | Experimental Factors | | | Landraces | | | | | |
| | X1 | X2 | X3 | Batati | | Bejaoui | | Karkoubi | |
				Y1	Y2	Y1	Y2	Y1	Y2
10	0.0000	0.3333	0.6667	14.50	47.61	7.49	48.07	23.41	65.52
11	0.6667	0.1667	0.1667	15.20	58.95	11.13	55.73	15.48	55.50
12	0.1667	0.6667	0.1667	16.20	50.88	10.50	52.70	15.92	54.10
13	0.1667	0.1667	0.6667	14.64	35.40	10.00	53.71	16.16	46.97

2.7. HPLC Analysis

The characterization of the phenolic compounds was performed by the means of a high-performance liquid chromatography (HPLC) analytical instrument (Agilent Technologies 1260; Waldbronn, Germany) equipped with a reverse-phase C18 column (4.6 × 100 mm and 3.5 mm particle size) Zorbax Eclipse XD B C18 (Agilent Technologies 1260; Waldbronn, Germany), as previously described [24], and a diode array detector (DAD) detector. The analysis conditions were described in detail by Ben Mansour et al. [20]. The identification of the phenolic compounds was conducted by comparing the retention time and UV spectra of the identified compounds with pure standards.

2.8. Cell Viability

The cytotoxic effects of the refined extracts were evaluated on the murine macrophage RAW 264.7 cell line (American Type Culture Collection, ATCC) based on the protocol previously described by Pereira et al. [25]. All the samples were analyzed in six repetitions. For the evaluation of cytotoxicity, the following equation was used [20]:

$$\% \text{ viability} = [\text{Fluorescence (sample)} \times 100]/\text{Fluorescence (control)}$$

2.9. Antimicrobial Activity Evaluation

The antimicrobial properties of the extracts obtained after mathematical optimization were tested against food borne bacteria, e.g., *Salmonella typhimurium* (ATCC 14028); *Pseudomonas aeruginosa* (ATCC 8166), *E. faecalis* (ATCC 29212), and *Staphylococcus aureus* (ATCC 6538), according to previously described protocols [26].

The percentage of growth inhibition was determined using the following equation:

$$\text{Growth inhibition (\%)} = 100 - [((A_{\text{Sample}} - A_{\text{sc}})/A_{\text{GC}} - A_{\text{sc}}) \times 100]$$

where A_{GC}: the absorbance of the growth control (positive control); A_{SC}: the absorbance of the sterility control (negative control); and A_{Sample}: the absorbance of the samples [20].

2.10. Mixture Design Statistical Study

The F ratio (R/r) (ratio of the mean square regression and the mean square residual) was implemented to validate the statistical significance of the obtained model at a significance level of 95% [27]. Moreover, the Student's *t*-test was applied at a significance level of 95% to confirm or refuse the significance of the factors.

2.11. Statistical Analysis

For all the above-mentioned tests, from three to six replicates were used. The means were compared according to Duncan's Multiple Range test at a level of $p < 0.5$ when differences were significant after using the statistical package SAS 9.1 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Preliminary Fractionation

The first step for the extraction refinement was the development of a linear gradient solvent system in order to obtain compounds with a broad polarity range for the screening and separation of bioactive molecules from the pumpkin peels. To find a suitable gradient elution solvent system that allowed for the separation of compounds with a wide polarity range, various solvents were tested. In detail, common solvents used for liquid–liquid extraction (n-hexane, ethyl acetate, n-butanol, and water) were fixed, while solvents that modify polarity (methanol, ethanol, and dichloromethane) were also added. According to the results obtained in this preliminary fractionation (Supplementary Figure S1), an important variability was observed when comparing the different solvent extracts obtained from the peels of the tested landraces. Considering the extraction efficiency of the tested solvents, the ethyl acetate and methanolic fraction seemed to be the best-performing ones in terms of the total phenolic compounds recovery, with total polyphenol contents reaching 7.5 and 6.3 mg GAE/g extract for the Batati peels. The aqueous fraction also contained important TPC estimated at 5.5 mg GAE/g R extract, followed by hexane, dichloromethane, and butanol fractions, with TPC contents ranging from 4.1 to 4.2 mg GAE/g extract. When comparing the three landraces, it seems obvious that the Batati peels contained the highest total phenolic contents, ranging between 4.1 and 7.4 mg GAE/g extract for all the tested solvents, except for dichloromethane, where the extracts of the Karkoubi peels recorded the highest amounts of TPC (4.55 mg GAE/g extract). Moreover, the TPC content in the extracts of the Karkoubi peels ranged between 1.1 and 5.6 mg GAE/g extract, whereas the extracts of the Bejaoui peels contained the least amounts of TPC for all the tested solvents (TPC content ranged between 0.7 and 4.5 mg GAE/g extract).

As for the phenolic profile, results concerning the fractions' antiradical activities highlighted an important variability between the different solvent extracts and the three landraces (Supplementary Figure S2). Considering the solvent recovery efficiency and in accordance with the results of the total phenolic compounds content, the best antiradical activity was detected in the methanolic fraction with an IC_{50} value equal to 175 μg/mL, closely followed by the ethyl acetate fraction with an IC_{50} value of 190 μg/mL. The IC_{50} values of the other fractions were significantly higher and even reached 1140 μg/mL in the case of the dichloromethane fraction. Considering the variability in the tested landraces, the Batati peels, which recorded the highest total phenolic compounds content, also exhibited the best antiradical activity, expressed by the lowest IC_{50} values, which ranged between 175 and 320 μg/mL. For the Karkoubi extracts, the IC_{50} ranged from 200 to 640 μg/mL, while the best IC_{50} value for the Bejaoui extracts was 250 μg/mL and the least efficient one was 1140 μg/mL. Consequently, the three most active fractions (MeOH, ethylacetate, and water) were combined and further analyzed by implementing the mixture design method, aimed at suggesting the best ratio (see Table 1).

3.2. Mixture Optimization

According to the results obtained using the mixture design matrix for the selected solvents (MeOH, ethyl-acetate, and water), the values of the TPC ranged between 11 and 17 mg GAE/g for the peel-refined extracts of Batati, between 8 and 16 mg GAE/g for the peel-refined extracts of Bejaoui, and between 10 and 27 mg GAE/g for the peel-refined extracts of the Karkoubi landrace (Figure 1).
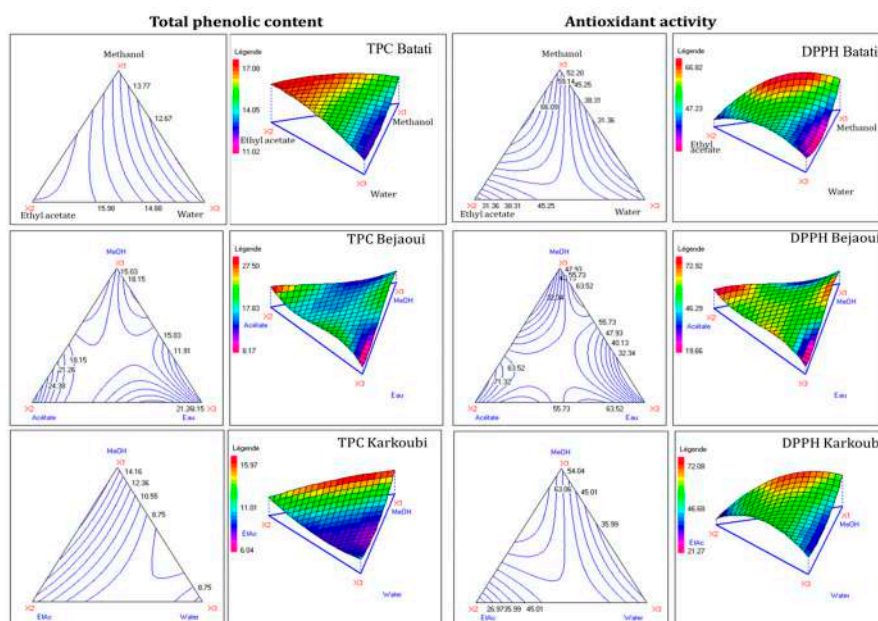
Figure 1. Isoresponses and mixture plot for the extracts obtained from the peels of Batati, Karkoubi, and Bejaoui landraces.

These results are interesting, since these values were higher than those obtained during the preliminary fractionation. For the DPPH assay, all the samples were tested at the same concentration of 1 mg/mL and the inhibition percentage ranged between 27 and 66% for Batati, between 20% and 73% for Bejaoui, and between 21% and 72% for the Karkoubi peel-refined extracts (Figure 1). For the purpose of generating the optimal zone of the solvents combination to fulfill the expectations of all the responses, an optimizer of response was used following the next formulas:

$$Y_{TPC(Batati)} = 14.47 \times X1 + 16.48 \times X2 + 12.52 \times X3$$

$$Y_{DPPH(Batati)} = 53.91 \times X1 + 28.97 \times X2 + 37.40 \times X3 + 95.07 \times (X1 \times X2) - 68.16 \times (X1 \times X3) + 57.22 \times (X2 \times X3)$$

$$Y_{TPC(Bejaoui)} = 14.78 \times X1 + 11.94 \times X2 + 10.14 \times X3 - 19.53 \times (X1 \times X3) - 12.31 \times (X2 \times X3)$$

$$Y_{DPPH(Bejaoui)} = 54.77 \times X1 + 21.27 \times X2 + 37.11 \times X3 + 107.64 \times (X1 \times X2) + 93.58 \times (X2 \times X3)$$

$$Y_{TPC(Karkoubi)} = 15.35 \times X1 + 27.41 \times X2 + 10.27 \times X3 - 24.63 \times (X1 \times X2)$$

$$Y_{DPPH(Karkoubi)} = 48.40 \times X1 + 71.30 \times X2 + 28.32 \times X3 + b12 \times (X1 \times X2) + b13 \times (X1 \times X3) + b23 \times (X2 \times X3)$$

These equations were transposed into isoprenic curves, as illustrated in Figure 1. The validity of the used model was justified through the Fisher test, as described in Table 2. According to the same table, the corresponding *p*-value indicates that independent factors significantly affected the fixed response. Actually, the effects of the solvent mixtures on the polyphenols extraction showed that the individual solvent coefficients were highly significant parameters, with *p*-values lower than 0.01.

Table 2. ANOVA results and mixture compound effects. Coefficient significance concerning extraction variables and two responses (DPPH activity and total phenolic content (TPC)) of the peel extracts obtained three squash landraces (Batati, Bejoui, and Karkoubi). Significance: $p < 0.1$ (*); $p < 0.01$ (**); $p < 0.001$ (***) mean very significant and strongly significant, respectively.

	Batati Peel					Bejaoui Peel					Karkoubi Peel				
	Coefficient	F.Inflation	Ecart-Type	t.exp.	Signif. %	Coefficient	F.Inflation	Ecart-Type	t.exp.	Signif. %	Coefficient	F.Inflation	Ecart-Type	t.exp.	Signif. %
Total Polyphenols Content															
b1	14.473	2.30	0.799	18.12	***	15.35	2.71	0.842	18.23	***	14.78	2.30	1.013	14.6	***
b2	16.485	2.30	0.799	20.64	***	27.41	2.71	0.842	32.57	***	11.94	2.30	1.013	11.79	***
b3	12.526	2.30	0.799	15.69	***	10.27	2.71	0.842	12.21	***	10.14	2.30	1.013	10.02	***
b12	4.369	2.25	3.528	1.24	25.5%	−24.63	2.72	3.763	−6.55	**	6.44	2.25	4.475	1.44	19.2%
b13	−4.783	2.25	3.528	−1.36	21.6%	8.39	2.72	3.763	2.23	11.1%	−19.53	2.25	4.475	−4.37	**
b23	4.120	2.25	3.528	1.17	28.1%	4.11	2.72	3.763	1.09	35.6%	−12.31	2.25	4.475	−2.75	*
Antiradical activity															
b1	53.91	2.30	4.022	13.4	***	48.409	2.71	4.185	11.57	***	54.77	2.30	5.39	10.15	***
b2	28.97	2.30	4.022	7.2	***	71.30	2.71	4.185	17.04	***	21.27	2.30	5.39	3.94	**
b3	37.4	2.30	4.022	9.3	***	28.32	2.71	4.185	6.77	**	37.14	2.30	5.39	6.88	***
b12	95.07	2.25	17.770	5.35	**	−54.62	2.72	18.706	−2.92	6.0%	107.64	2.25	23.84	4.51	**
b13	−68.16	2.25	17.770	−3.84	**	29.09	2.72	18.706	1.56	21.7%	−47.89	2.25	23.84	−2.01	8.3%
b23	57.22	2.25	17.770	3.22	*	34.86	2.72	18.706	1.86	15.9%	93.58	2.25	23.84	3.92	**

The used software concluded that the targeted limit could be achieved with a 99% desirability for the Batati landrace using a solvent mixture consisting of 53.4% Methanol+ 45.8% Ethyl acetate + 0.8% water. In the case of the Karkoubi peels, the refined extract could be reached with a 99% desirability using a solvent mixture consisting of 64.4% Methanol+ 34.4% Ethyl acetate + 1.1% water. Finally, the desired refined extract for the Bejaoui landrace could be achieved with an 87.4% desirability via a solvent mixture containing 87.3% Methanol+ 6.5% Ethyl acetate + 6.2% water. The experimental validation of these formulas is detailed in Table 3. Once the experimental validation confirmed the mathematical model, all the obtained refined extracts were assessed for their phenolic composition (using HPLC equipment), along with an assessment of their biological activities, such as antibacterial activity and cytotoxic effects.

Table 3. Total phenolic compounds content (TPC, expressed as mg GAE/g E) and antiradical activity (DPPH test, expressed as inhibition concentration at 50% or IC_{50}) of peels extracts.

	Batati		**Bejaoui**		**Karkoubi**	
	TPC	DPPH Test	TPC	DPPH Test	TPC	DPPH Test
Predicted values	16.44 ± 1.5	65.54 ± 7.2	16.06 ± 0.3	49.88 ± 10.7	15 ± 1.2	66.94 ± 2.3
Experimental values	15.60 ± 1.3	64.14 ± 2.1	16.17 ± 0.9	50.47 ± 3.2	16.17 ± 0.5	62.24 ± 2.2

3.3. HPLC Analysis of Peel-Refined Extracts

Figure 2 illustrates the chromatograms obtained for the three optimum extracts of the tested landraces, while the detailed phenolic compounds composition is presented in Table 4. The chromatographic profiles were rich in phenolic compounds, especially in flavonoids. Indeed, the analysis showed that the identified phenolic compounds and their concentrations varied among the landraces as well. Catechin was the most abundant flavonoid in the Bejaoui peel with a concentration of 8.06 mg/g extract, followed by epicatechin, kaempferol, and vanillin (5.00, 5.01, and 2.0 mg/gE, respectively). In addition, the profile of phenolic acids showed quantitative and qualitative differences between the extracts. The Bejaoui peel extract contained a high content of phenolic acids (gallic, caffeic, chlorogenic, vanillic, ellagic, and sinapic acid). The most abundant compound was chlorogenic acid with a content 2.17 mg/g extract, while the rest of the compounds were detected in low amounts. Moreover, the total amount of the individual compounds was

recorded for the extracts of the Bejaoui peels (24.9 mg/g extract), whereas the Batati and Karkoubi extracts contained 2.39 and 1.13 mg/g extract, respectively.
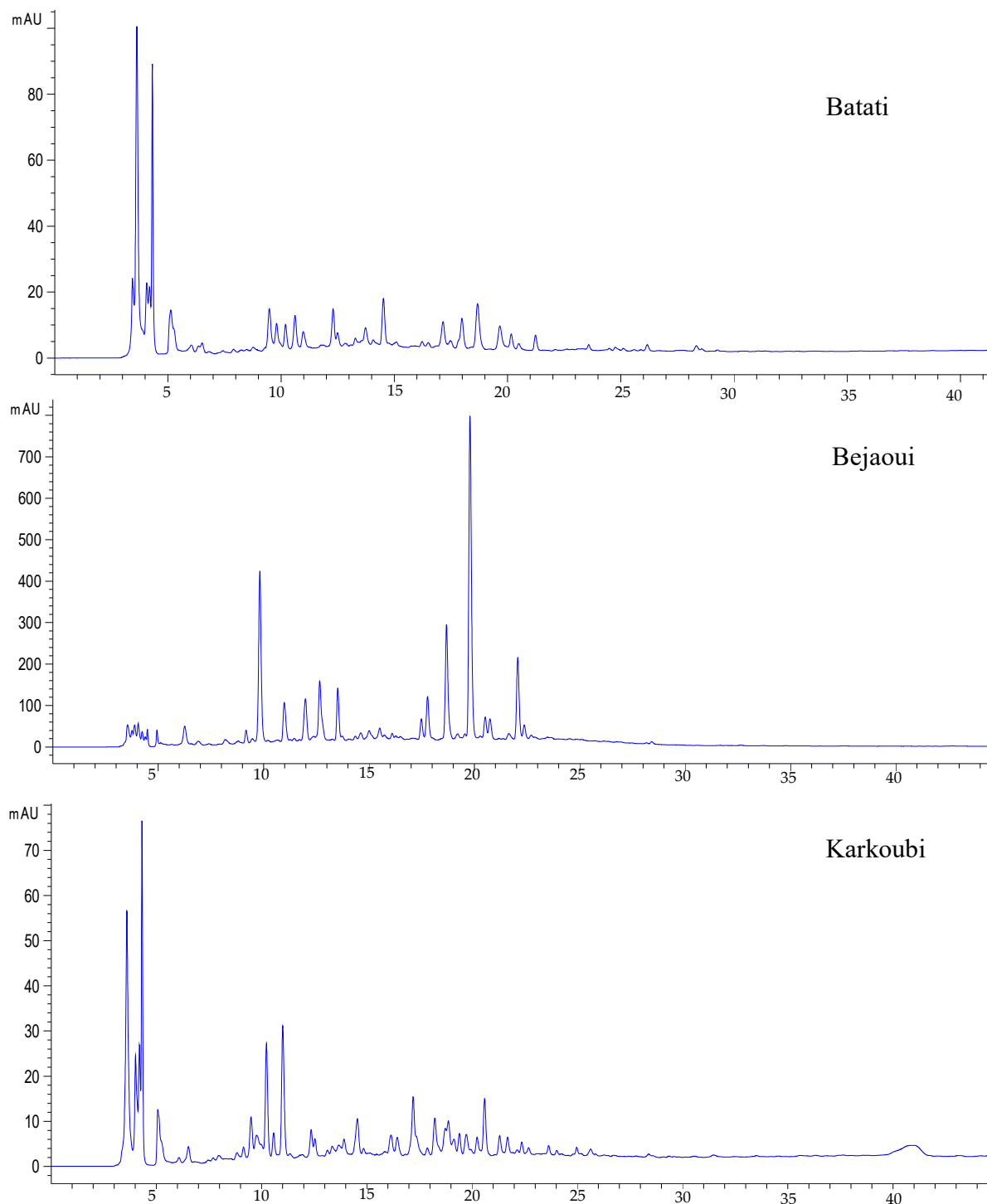

Figure 2. Chromatographs of three optimum peel extracts for the tested landraces, obtained after mathematical optimization (RSM).

Table 4. Identification and quantification of phenolic compounds from squash peel extracts obtained after mathematical optimization (Response surface methodology; RSM). Values are presented as milligrams per gram of extract (mg/g E; (means ± SD). Means followed by different letters within the same row are significantly different based on the Duncan's multiple range test (DMRT) at *p* < 0.05.

Compounds	Retention Time (min)	Content (mg/g E)		
		Batati	**Bejaoui**	**Karkoubi**
Vanillic acid	5.12	0.1 ± 0.01 [a]	0.04 ± 0.002 [b]	0.14 ± 0.02 [a]
Gallic acid	6.1	0.01 ± 0.001 [b]	0.43 ± 0.002 [a]	0.03 ± 0.001 [b]
Catechin gallate	7.34	-	0.15 ± 0.02 [a]	-
Hydroxytyrosol	9.15	0.25 ± 0.03 [b]	0.42 ± 0.04 [a]	-
Epigallocatechin	10.68	0.48 ± 0.02 [a]	0.24 ± 0.01 [b]	0.18 ± 0.02 [c]
Resorcinol	11.55	-	0.08 ± 0.002 [a]	-
Chlorogenic acid	11.6	-	2.17 ± 0.6 [a]	-
Catechin	12.18	0.50 ± 0.02 [b]	8.06 ± 0.5 [a]	0.26 ± 0.02 [b]
Catechol	13.4	-	0.07 ± 0.001 [a]	-
Epicatechin	13.82	0.36 ± 0.08 [b]	5.00 ± 0.32 [a]	-
Caffeic acid	14.25	-	0.05 ± 0.002 [a]	0.07 ± 0.001 [a]
Sinapic acid	14.47	0.12 ± 0.03 [a]	0.12 ± 0.03 [a]	-
Myrecitin 3-O-galactoside	15.37	-	0.08 ± 0.001 [a]	-
Rutin	16.44	0.04 ± 0.002 [b]	0.16 ± 0.02 [a]	0.08 ± 0.002 [b]
Ellagic acid	17.42	0.01 ± 0.001 [c]	0.35 ± 0.03 [a]	0.14 ± 0.01 [b]
Vanillin	17.79	-	2.00 ± 0.04 [a]	-
p-Coumaric acid	17.9	0.08 ± 0.001 [a]	-	-
Kaempferol	18.28	0.31 ± 0.02 [b]	5.01 ± 0.12 [a]	0.16 ± 0.01 [b]
Ferulic acid	18.87	-	-	0.07 ± 0.01 [a]
Myrecitin	22.54	-	0.08 ± 0.001 [a]	-
resveratrol	24.5	0.03 ± 0.002 [b]	0.07 ± 0.001 [a]	-
Quercetin	26.16	0.04 ± 0.001 [a]	0.03 ± 0.001 [a]	-
Apigenin	28.31	-	0.29 ± 0.02 [a]	-
Total		2.39 [b]	24.9 [a]	1.13 [c]

3.4. Antibacterial Activity of Peel-Refined Extracts

The results of the antibacterial activity of the peel extracts against different bacterial strains (*P. aeruginosa*, *S. typhimurium*, *E. faecalis*, and *S. aureus*) are presented in Figure 3. It is important to emphasize that the Batati and Karkoubi peels exhibited interesting antimicrobial properties. The two extracts inhibited the pathogen growth for all the tested bacterial strains with percentages up to 80%. The Karkoubi and Batati peel-refined extracts exhibited the best inhibitory effects (growth inhibitions of 99%, 96%, 97%, and 80% for Karkoubi and 94%, 89%, 98%, and 96% for Batati against *E. feacalis*, *P. aeruginosa*, *S. typhimurium*, and *S. aureus*, respectively). In contrast, the Bejaoui peel extract showed moderate efficacy against the *P. aeruginosa* and *S. aureus* pathogens (44% and 17% growth inhibition, respectively).

3.5. Cell Viability of Peel-Refined Extracts

The results concerning the presumed cytotoxicity of the peel-refined extracts are described in Figure 4. It is important to note that viability percentages above 100% indicate potential cell growth stimulation, while percentages below 100% suggest varying degrees of cytotoxicity or decreased cell survival. The obtained results provide insights into the cytotoxic effects of the refined peel extracts at different concentrations. First, regardless of the pumpkin landrace, the cell viability for all the tested extracts was over 92%, indicating no significant toxicity for the studied samples, even at the highest concentrations tested. Interestingly, at the maximum concentrations of 200 and 400 μg/mL, the average viability increased significantly, surpassing 100% of the control cell growth, indicating a higher cell growth compared to the control. This particular result may suggest a potential stimulatory effect of the refined extracts on cell growth.
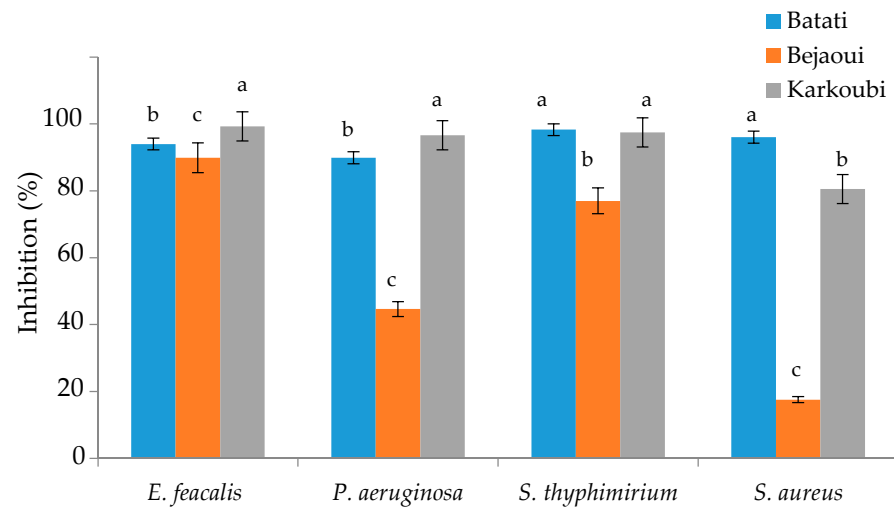
Figure 3. Microbial growth inhibition (expressed in percentage (%)) against four microbial strains of the optimum extracts of peels of the tested landraces obtained after mathematical optimization (RSM). Values are the means of six replicates and standard deviation. Values with different letters (a–c) within the same strain are significantly different at $p < 0.05$, according to Duncan's multiple range test (DMRT).
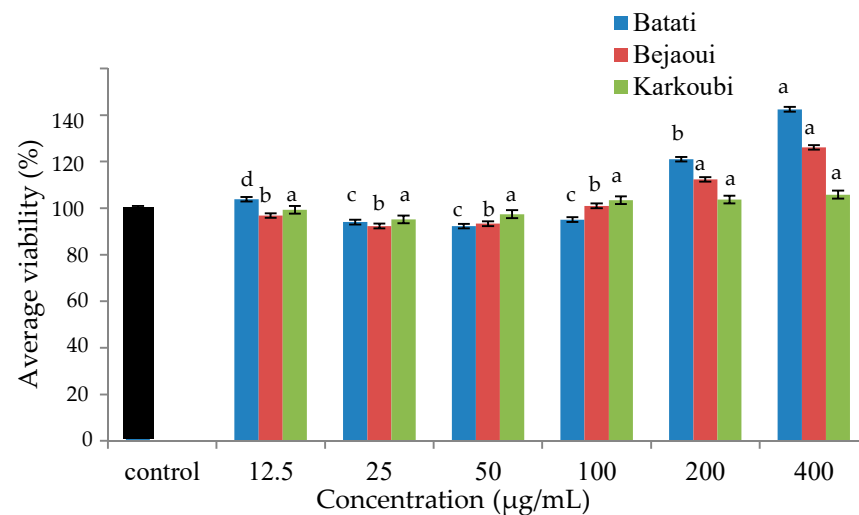

Figure 4. Cell viability of three peel-refined extracts obtained after mathematical optimization (RSM). Values are the means of six replicates and standard deviation. Values with different letters (a–d) within the same concentration are significantly different at $p < 0.05$, according to Duncan's multiple range test (DMRT).

4. Discussion

An efficient extraction technique uses the optimal solvent or mixture of solvents that facilitates the recovery efficiency of targeted compounds [28]. The preliminary experiment of our study allowed for the selection of methanol, ethyl acetate, and water as the main solvents that were the most efficient in extracting antioxidant molecules. Based on these results, an experimental design (mixture design type) was elaborated to refine the obtained extracts and fine-tune the extraction conditions. On the other hand, for antiradical activity, the individual solvents, as well as their combination, exhibited a significant synergistic effect. The TPC ranged between 11.02 and 17.08 mg GAE/g extract for the Batati peels, between 6.04 and 15.97 mg GAE/g extract for the Bejaoui peels, and between 10.35 and 27.5 mg GAE/g extract for the Karkoubi peels, which recorded the highest TPC content (Table 1). This finding was validated through data derived from the special cubic model

(R^2 = 0.992), which indicated that ethylacetate (X2) had a high effect on the TPC when implemented either purely or combined with methanol (X1 × X2). The linear effect of ethylacetate (X2) was positive, whereas water (X3) was less effective (TPC = 28.44 mg GAE/g extract). The use of water in the ternary mixture (X1 × X2 × X3) increased the extraction efficiency in comparison to the pure solvents, e.g., methanol (X1) and ethyl acetate (X2), as shown by the positive value of coefficient and the TPC value of 58.97 mg GAE/g extract. The binary interaction (X1 × X3) was also very significant ($p \leq 0.05$). These results point out that the extraction was dependent on the polarity of the solvents [20]. In addition, the positive effect of water and methanol could be explained by the presence of polar substances, such as phenolic glycosides, in the tested extracts, which resulted in an increased extraction efficiency.

Previous studies have pointed out that pure water does not facilitate the efficient extraction of phenolic compounds, which are more soluble in organic solvents less polar compared to water [29]. This is probably the reason that the TPC in combinations 3 and 9 for the Karkoubi peels had the lowest content. Numerous reports have shown that the efficiency of extraction protocols for phenolic compounds is improved when a mixture of water and organic solvents such as methanol and ethanol is used, whereas the extraction yield is reduced when pure solvents are used [30,31]. This is in agreement with our results, since the addition of methanol or ethyl acetate in water increased the TPC content (Table 1). Moreover, the extraction with pure ethyl acetate also gave considerable results for the tested peel-refined extracts, probably due to the particularities in the chemical profile of the pumpkin peels.

Regarding the impact of solvent type on the composition in terms of the phenolic compounds, a mixture of solvents was more effective than individual ones. Similar findings have been obtained in other studies on fruit or vegetable by-products, such as spent coffee grounds [32], artichoke waste [33], and mango by-products [34]. Generally, the high extraction efficiency of solvent mixtures can be attributed to phenolic compounds, which exhibit a broad range of solubility and are usually more soluble in solvents with a lower polarity than water [35]. Thus, the recorded differences in the extraction efficiency of the tested solvents could be attributed to differences in the affinity of the phenolic compounds present in the pumpkin peels. In addition, the extraction of natural substances from plant matrices is a complex procedure including many steps, such as the penetration of solvents in the plant matrix, the breakage of solute–matrix interactions, and the solubilization of the solute. Therefore, apart from affinity, the factors involved in the rest of the extraction steps should be considered, especially when taking into account that phenolic compounds can be found either in free or bound [35].

Our results also showed that the TPC contents recorded in this work were quite promising, particularly when compared to those found in other fruit or vegetable wastes [35–37]. It is important to know that the high availability of squash by-products at no cost make them a promising plant matrix for obtaining phenolic compounds. It should also be taken into account that the amounts of these compounds may vary depending on the pumpkin genotype and growth conditions, and could be increased through tailor-made agronomic practices [20].

4.1. HPLC Analysis of Peel-Refined Extracts

According to the HPLC data of each peel extract, the chromatograms revealed a phenolic profile principally consisting of flavonoids such as catechin, epicatechin, epigallocatechin, catechin gallate, myrecitin, myrecitin 3-O-galactoside, quercetin, kaempferol, and apigenin, followed by phenolic acids (gallic, caffeic, chlorogenic, sinapic, ferulic, and ellagic acids) and other compounds such as resveratrol, resorcinol, hydroxytyrosol, and catechol. It could be suggested that the analysis of the studied peel extracts resulted in different contents and compositions of phenolic compounds, thus indicating the significant impact of genotype on the phenolic compounds profile. Furthermore, within the flavonoid compounds, those belonging to the flavan-3-ol group (epigallocatechin, cate-

chin, and epicatechin 3-O-gallate) were the most abundant in all the optimized extracts of peels. Catechin was the most abundant compound in the Bejaoui peels with an amount of 8.06 mg/g extract and probably was responsible for the strong antiradical activity, which reached 50.47% of inhibition (Table 3). This compound was present in all the optimized extracts and is well-known for its powerful antioxidant activity, due to the O-dihydroxy and O-hydroxyketo groups that are present in its structure [38]. Additionally, epicatechin 3-O- gallate is also a potent bioactive compound which can act as an antioxidant agent [39]. Several works have reported epicatechin to be the major constituent in many varieties of Cucurbitaceae species, such as *C. maxima* [40], *C. moschata* Duchesne [41], and *Momordica caranthia* (bitter melon) [42]. Among the flavonoids found in the present study, kaempferol was present in appreciable amounts, especially in the refined extract of the Bejaoui peels (5.01 mg/g extract). Kaempferol has broad biological properties [43,44] and it has been shown to be a good antioxidant and efficient agent against various enveloped viruses [45]. Moreover, it has been suggested to have various antitumor activities, including breast, ovarian, bladder, cervical, liver, colon, lung, prostate cancer, and leukemia, etc. [46]. From a broad perspective, flavonoids have important antioxidant abilities, essentially due to their hydroxyls, which donate an electron (H+) to radicals, thus scavenging them and alleviating oxidative stress [47].

Pumpkin peels are reported to contain high contents of phenolic compounds with a powerful antioxidant capacity [20]. Previous works have reported high phenolic amounts for Cucurbitaceae varieties and revealed the group of flavonoids as the richest class of phenolic compounds in terms of the number and quantity of compounds detected [20,40,42]. Regarding the phenolic compounds profile, the observed differences in the literature reports could be probably associated with the variability in the genotypes studied and the environmental and agronomic conditions, or the extraction methods and the analytical instruments used [20]. Meanwhile, comparable results in terms of the phenolic compound contents in pumpkin peels was recently reported by Leichtweis et al. [40] for *C. maxima* fruit grown in Portugal, which showed that peel extract has a diversified phenolic profile and also high concentrations of total phenolic compounds (9.4 mg/g extract).

4.2. Antibacterial Activity of Peel-Refined Extracts

The peel-refined extracts obtained from the tested landraces exhibited significant growth inhibitions of 94, 89, and 99% against *E. faecalis*, and growth inhibitions of 98%, 77%, and 97% against *S. typhi* for the Batati, Bejaoui, and Karkoubi landraces, respectively. These results are very interesting, since phytochemicals such as flavonoids being present in parts of pumpkin fruit could be instrumental in the antibacterial properties of the extracts [48]. Many works have focused on the study of the antibacterial effects of peels of several fruits, including squash. In this context, Hussain et al. (2021) showed that pumpkin (*C. maxima*) peel methanolic extract (80%) exhibited inhibition zones of 6.60, 4.34, 7.03, and 13.41 mm against four bacterial strains (*S. aureus*, *B. subtilis*, *E. coli*, and *S. typhi*, respectively). In the same line, Asif et al. [49], who conducted antibacterial assays with squash peel extract, reported a good potential in inhibiting the growth and division of pathogenic bacteria, while Alabassi et al. [50] reported that pumpkin fruit is rich in potent antibacterial and antioxidant compounds. Dissanayake et al. [51] tested three bacterial strains, namely *S. aureus*, *B. subtilis*, and *E. coli*, to assess the antibacterial activity of extracts of pumpkin by-products (skin, seeds, and leaves) by using three types of solvents (e.g., acetone, methanol, and ethyl acetate). The authors suggested that the pumpkin peels extracted with methanol gave zones of inhibition of 7.6 mm against *S. aureus* and 4.8 mm against *B. subtilis*. Moreover, a study on the peel extract of *C. pepo* showed significant antibacterial activity that could be associated with the presence of steroids, flavonoids, tannins, alkaloids, and saponins, which were reported to possess antimicrobial properties [48]. In the same study, the recorded inhibition zones were in the range of 7–10 mm for both extracts of pumpkin peels obtained with ethanol and methanol against *S. aureus* and between 6 and 12 mm against *S. typhi* [48].

4.3. Cell Viability of Peel-Refined Extracts

The lack of toxicity of the peel-refined extracts obtained from the mixture design was corroborated by evaluating their toxicity in a culture of macrophage cells (RAW264.7). Our results indicated there was no cytotoxic effect up to the highest concentration of 400 mg/mL tested. Many studies have highlighted the possibility of using peel extracts as a source of phytochemicals with a protective effect against many types of cancer [52,53]. The presumed cytotoxic activity of pumpkin peel extracts has been studied previously, using liver [54] and human prostate [55] cancer cell lines. Extracts from C. pepo "Lungo Fiorentino" (zucchini) have been previously reported to have toxic effects against HaCaT keratinocytes at a concentration superior to 200 μg/mL [56]. Asif et al. [49] also reported that the inhibitory effect on the growth of the MDBK cancer cell line using the MTT assay indicated methanol extracts of squash peel and puree as the most efficient for inhibiting the growth (≈35%) and cell division of cancer cells. Gaweł-B et al. [57] evaluated the bioactive properties of 15 extracts obtained from the peels of five cultivars of *Cucurbita maxima* and *C. moschata* and reported that the studied peel extracts were not toxic against human keratinocytes up to a concentration of 1000 μg/mL, and therefore they could be considered as non-irritants for the skin, confirming the potential use of peel extracts in cosmetic products. These findings highlight the overall safety profiles of the refined extracts and indicate their potential to promote cell growth. However, further investigation is needed to fully comprehend the implications of these results and reveal the mechanisms of action for the observed properties.

5. Conclusions

This study employed a mixture design approach to identify the optimal combination of solvents that facilitated the extraction of refined compounds from squash peels with enhanced bioactivity. Our results revealed that the Bejaoui peel-refined extract exhibited a notable phenolic compounds content, prominently catechin (8.06 mg/g dry extract (DE), epicatechin (5 mg/g DE), and kaempferol (5 mg/g DE). Moreover, the peel-refined extracts demonstrated potent antioxidant and antibacterial properties against *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Staphylococcus aureus* (especially for the Karkoubi and batati landraces, where the growth inhibitions were 99%, 96%, 97%, and 80% and 94%, 89%, 98%, and 96% for the respective bacteria), while a negligible cyto-toxicity against macrophage cells (RAW264.7) was recorded. In conclusion, our findings indicate the potential health benefits of squash peel associated with its rich phytochemical composition and remarkable antioxidant and antibacterial effects, and highlight the importance of valorizing this by-product in the food value chain within the circular economy strategy. Moreover, our findings show the great potential of using peel extracts as novel and natural antimicrobial agents that could substitute synthetic compounds in the food and nutraceutical industries. Considering that several compounds may contribute to the antioxidant activity and bioactive properties of natural matrices, further studies are needed to optimize the extraction protocols to obtain other targeted compounds (e.g., carotenoids) from pumpkin by-products that also could be used as novel antimicrobial agents.

Conflicts of Interest: The authors declare no conflict of interest.

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