PulpIng – Development of Pumpkin Pulp Formulation Using a Sustainable Integrated Strategy

PRIMA-Section 2 project (2019)

Deliverable Number	3.2
Deliverable name	Report about the potential toxicity of refined extracts/individual compounds
Contributing WP	WP3- Refinement and stabilization of the identified preserving compounds
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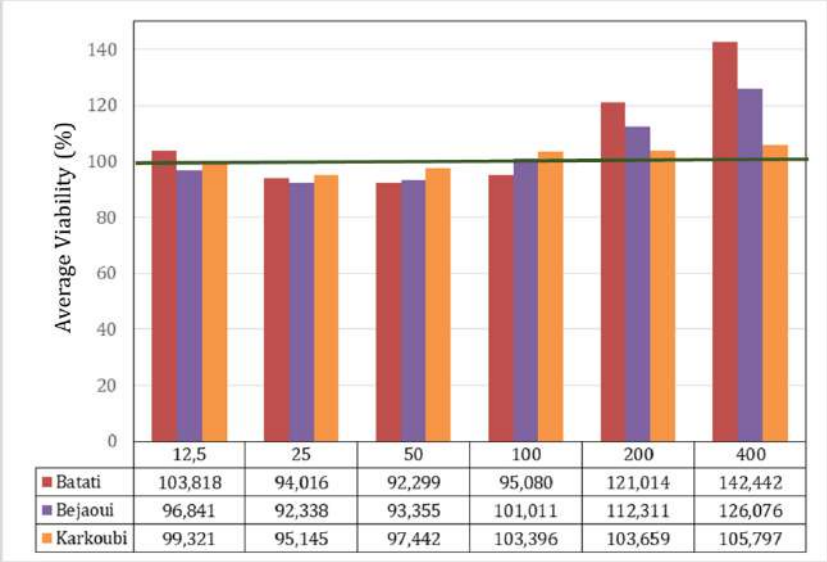
Responsible partner	CBBC
Reviewers	All partners
Version	1

Executive Summary

Background	Cytotoxicity is one of the most important indicators for biological evaluation *in vitro* studies. In order to determine the cell death caused by the tested samples, there is a need for cheap, reliable and short-term reproducible cytotoxicity and cell viability assays. *In vitro* cell viability and cytotoxicity assays using cultured cells have become employed for testing cytotoxicity effects of chemicals and plant extracts. These assays have gained significant interest in recent years due their rapidness cost-effectiveness, and animal free nature. Furthermore, they are useful for testing large number of samples. A wide range of cytotoxicity assays are currently used in the fields of toxicology and pharmacology. One of the most used techniques is the fluorometric assays. Fluorometric assays of cell viability and cytotoxicity are easy to perform with the use of a fluorometer, fluorescence microplate reader or flow cytometer, and offer many advantages over traditional dye exclusion and colorimetric assays. Fluorometric assays are also applicable for adherent or suspended cell lines and are easy to use. These assays are more sensitive than colorimetric assays and adapted to test plant extracts. At this point of the project, it is crucial to thoroughly evaluate the safety profile of these refined extracts to determine their suitability for human consumption. To conduct a comprehensive analysis, *in vitro* studies involving cell cultures are employed to assess the cytotoxicity of the refined extracts optimized in D3.1 and the extraction fractions from the Greek sample.
Objectives	The objective of this deliverable is to provide a comprehensive assessment of the potential cytotoxicity of refined extract derived from pumpkin peels, as obtained in the study reported in the Deliverable 3.1 and also in the extract fraction obtained from the Greek peel samples.
Methodology	***Cell viability.*** The cytotoxicity effect of the refined extracts optimized in D3.1 and the extract fractions were evaluated on the murine macrophage RAW 264.7 cell line (American Type Culture Collection, ATCC). Cells were grown in 24-well plates at a concentration of $2x10^5$ cells/ml during 24 h. The resazurin test was performed to verify the cytotoxicity of samples in the cells. The absorbance was measured at 540 nm. All samples were analyzed at least in six replicates. ***Cell culture.*** The murine macrophage cell line RAW 264.7 (American Type Culture Collection), was grown in flasks in RPMI 1640 medium (Dominique Dutscher; w/L-Glutamine), to which was added 10% fetal bovine serum (FBS) (Dominique Dutscher; Origin South America) and antibiotics (penicillin 100 U/mL and streptomycin 100 µg/mL). Cells were grown at 37 °C in a humid atmosphere containing 5% CO_2. Prior to each assay, RAWs (in exponential growth phase) were seeded in 24-well plates at a density of $2x10^5$ cells/well. They were then incubated for 24 h to allow them to adhere to the flask. ***Evaluation of extract cytotoxicity.*** The cytotoxicity of the refined extracts/extract fractions was assessed using the rezasurin assay.

	Briefly, RAW 264.7 macrophages previously adhered to 24-well plates were treated for 24 h with different concentrations of each refined extract. After removing the supernatant, 1 ml of a 2% rezasurin solution in PBS (Dulbecco's Phosphate Buffered Saline, Dominique Dutscher) was added to each well. Fluorescence was measured after 60 min incubation, and cell viability was calculated against a control of untreated cells using the following equation: % viability = [Fluorescence (sample) x 100] /Fluorescence (control) The extracts are considered safe/non-toxic at the concentration studied for a viability percentage superior to 80%.
Results and implications	The Figure 1 below provides a detailed overview of the results regarding the cytotoxicity of the refined extracts from pumpkin peel obtained in D3.1. It is important to consider that viability percentages above 100% indicate potential cell growth stimulation, while percentages below 100% may suggest varying levels of cytotoxicity or decreased cell survival. The obtained results provide insights into the supposed cytotoxic properties and potential effects of the peel refined extracts at different concentrations. First, and regardless of pumpkin landraces, the cell viability for all the tested extracts was over 92%, indicating no significant toxicity of the studied samples even at the highest concentrations tested. Interstingly, at the maximum concentrations of 200 and 400 µg/ml, the average viability increased significantly surpassing 100% of the control cell growth, meaning a higher cell growth when compared to the control. This particular result may suggest a potential stimulatory effect of the refined extratcts on cell growth.  |	12,5	25	50	100	200	400
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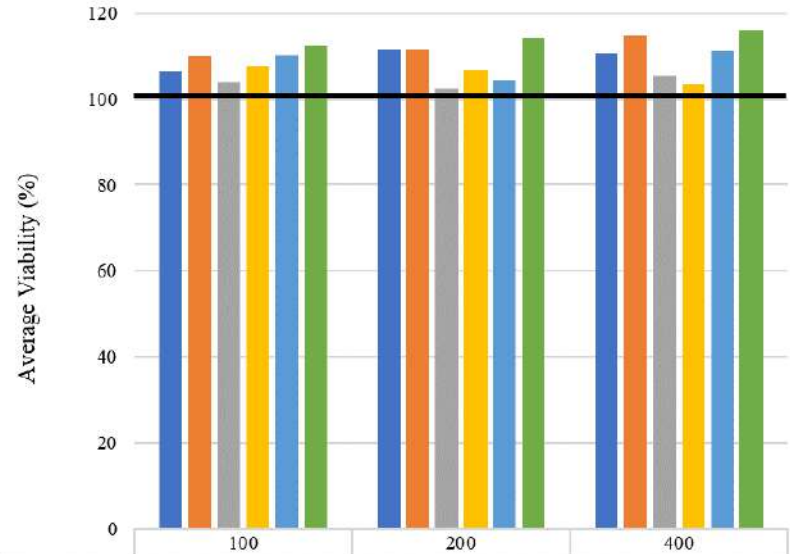
Batati	103,818	94,016	92,299	95,080	121,014	142,442
Bejaoui	96,841	92,338	93,355	101,011	112,311	126,076
Karkoubi	99,321	95,145	97,442	103,396	103,659	105,797	 **Figure 1.** The cytotoxicity of the refined extracts from pumpkin peel in terms of cell viability (%) in different extract concentrations (µg/ml). In Figure 2 is presented the cytotoxicity assessment for the extract fractions obtained from the peel samples of the pumpkin genotype 'Leuka Melitis' (round). Considering that viability percentages above 100% indicate potential cell growth stimulation and that all the extract showed

results higher than 100%, it's possible to affirm that none of the extracts have toxic effect in the cell culture tested, from 100 to 400 µg/ml.



	100	200	400
BnOH	106,49	111,47	110,61
Ethyl acetate	109,97	111,41	114,73
MeOH	103,79	102,38	105,31
Hexane	107,67	106,64	103,27
Water	110,05	104,30	111,07
DCM	112,45	114,15	115,94

Figure 2. The cytotoxicity of the extract fractions from the Greek pumpkin peel, in terms of cell viability (%) in different extract concentrations (µg/ml).

These findings highlight the overall safety profile of the extracts.