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**Supercritical $CO_2$ extraction of bioactive compounds from radish leaves: yield, antioxidant capacity and cytotoxicity**

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**Graphical abstract**

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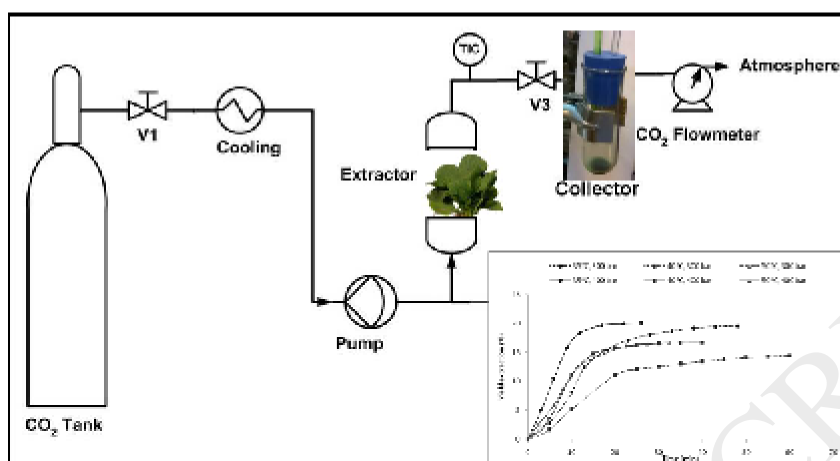


## Highlights

- Supercritical fluid extracts optimization were done from radish leaves.
- The best results were obtained at 35 ºC/400 bar and 40 ºC/400 bar.
- Maturation of dendritic cells was not promoted being these extracts promising and interesting sources of antioxidative and anti-inflammatory agents.


## Abstract

Supercritical Fluid Extraction by using $CO_2$ can be employed as an alternative to conventional methods for extracting functional compounds. Overall extractions curves


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for extractions of bioactive compounds from radish leaves were performed using $scCO_2$ adding ethanol as co-solvent. Yield, total phenolic compounds, total flavonoids and antioxidant capacity were determined for each extract. The best results were obtained at 35 ºC/400 bar and 40 ºC/400 bar, with values of total phenolics 1375 mg GAE/ 100 g d.m. and 1455 mg GAE/ 100 g d.m., respectively. Antioxidant capacity by DPPH values were 359 mg TE/ 100 g d.m. (35 ºC/ 400 bar) and 403 mg TE/ 100 g d.m. (40 ºC/ 400 bar). Maturation of dendritic cells was not promoted being these extracts promising and interesting sources of antioxidative and anti-inflammatory agents for future uses in the pharmaceutical and alimentary industries.

*Keywords:* bioactive compounds, integral use, supercritical carbon dioxide, *Raphanus sativus*, cytotoxicity, dendritic cell maturation

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## 1. Introduction

The amount of functional compounds recovered from raw plant materials depends on the used method for extraction [1]. The conventional techniques to obtain plant extracts, such as hydrodistillation and solvent extraction, have a number of disadvantages that are related with the use of high amounts of organic solvents and consequently the production of toxic solvent waste [2, 3]. These disadvantages promote the search of new alternatives leading to "solvent free" extracts, through more efficient techniques avoiding the thermal degradation of the active compounds. The use of supercritical carbon dioxide (sc$CO_2$) has been accepted in the last decades, because is chemically inert, non-polar, non-toxic, and non-flammable as well as an accepted food grade solvent. Also, sc$CO_2$ has shown an optimal behavior as extraction solvent for thermolabile compounds [1, 4]. Thus, Supercritical Fluid Extraction (SFE) by using $CO_2$ can be employed as an alternative to conventional methods for extracting and/or fractionating functional compounds [3, 4].

The extract antioxidant activity is related, mainly, with the phenolic compounds content of the starting vegetable. Considering the polar nature of most of the phenolic compounds and taking into account the non-polarity of $CO_2$, in many extractions result necessary to modify the solvent power of the fluid by the use of a co-solvent to improve the yield extraction [2]. Ethanol is generally recognized as safe (GRAS) solvent and environmental benign, and can therefore be used in food extraction processes [3, 5]. The efficiency of the SFE depends on several parameters such as pressure, temperature, solvent flow rate, particle size of the substrate and the use of a co-solvent.

Radish (*Raphanus sativus* L.) is a root vegetable of the Cruciferaceae family and it is an important vegetable crop worldwide [6]. In particular, radish leaves constitute an underutilized leafy vegetable, and scarce information is available on the chemical

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content of such raw material. Goyeneche, et al. [7] reported that leaves have shown excellent antioxidant capacity, and the extraction of their bioactive compounds should be optimized [8].

Moreover, it has been reported the presence of antigenic component(s) from mature leaves of cruciferous plants such as the radish, turnip, and rape [9, 10]. Therefore, possible oral administration of these vegetal extracts much be tested against the presence of potential antigens, preventing food hypersensitivity reactions such as food allergy. Dendritic cells (DCs), a subset of innate immune cells, are critical sentinels that perform multiple roles in immune responses. Immature DCs reside in peripheral tissues where they can capture a broad spectrum of antigens through a variety of mechanisms, before their migration to the secondary lymphoid organs. In the gut, DCs meet exogenous, non-harmful food antigens as well as vast commensal microbes and its function is to maintain the intestinal immune homeostasis. The internalized antigens are processed into peptides and loaded onto Major Histocompatibility Complex Class I and Class II (MHC I and MHC II) for presentation to T cells. In absence antigen components, DCs become tolerogenic. Conversely, when DCs are activated by microbial or “antigen” signals, they become “immunogenic” cells essential for triggering adaptive immunity [11].

The aim of the present work was to obtain extracts rich in bioactive compounds from red radish leaves by using supercritical $CO_2$ extraction with ethanol as co-solvent. The effect of $CO_2$ pressure and temperature on the performance of $scCO_2$ extraction processes was studied. The overall extraction curves (OEC), yield, total phenolic compounds, total flavonoids and antioxidant capacity were determined for each extract at different conditions. These extracts were also exposed to DCs to check their potential

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antigenic properties and to determine if they are able to induce DCs maturation or on the contrary, are innocuous to activate the immune system.

## 2. Materials and methods

### *2.1. Plant material and sample preparation*

Radishes (*Raphanus sativus* L.) were purchased from a local market in Mar del Plata, Argentina. Radish roots were separated from leaves, and the roots were discarded. Leaves were washed with tap water, dried with absorbent paper, frozen with liquid nitrogen and lyophilized (Vitris, Advantage Plus, Gardiner, NY, EEUU) for 72 h. Finally, dried material was powdered with a mortar and five grams was weighted and stored on polyethylene sealed bags.

### *2.2. Supercritical fluid extraction*

Sc$CO_2$ extraction was performed on a supercritical fluid extractor (HPE500, Eurotechnica, Germany) with an extractor volume of 500 mL. The schematic diagram of sc$CO_2$ extractor unit is shown in Fig. 1.

In each experiment, 5 g of lyophilized and milled radish leaves were mixed with 20 mL of ethanol and 60 g of glass spheres, to improve mass transfer. Co-solvent (ethanol) was added to improve polyphenols extraction. Previous assays with pure $CO_2$ resulted on very low percentage of extraction and polyphenol content (data not shown). Moreover, the distribution particle diameter was calculated from the fractions retained on five sieves, according to the ASAE [12] procedures. The prepared mixture was introduced into the extractor vessel of a High Pressure Extraction Unit trapped by filter paper to prevent any carryover of particles. The sc$CO_2$ flow rate used in the SFE experiments was 0.6 kg/h. The obtained extract mass was registered as a function of the $CO_2$ passage

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time. The extraction curves were obtained at P = 300 bar and 400 bar; and at T = 35, 40 and 50 °C.

The crude extraction yield of bioactive compounds was then measured gravimetrically and reported as follows:

$$Y = \frac{m_{extract}}{m_{leaves}} * 100$$

where Y is the crude extraction yield (%), $m_{extract}$ is the crude extract mass (g) and $m_{leaves}$ is the extracted leaves mass (g).

*2.3. Total phenolic content*

Total phenolic content (TPC) was determined colorimetrically by the Folin–Ciocalteu method according to Ayala- Zavala, et al. [13] with slight modifications. 15 µL aliquot of the extract solution was transferred to a hole with 75 µL of Folin–Ciocalteu reagent. After 3 min, 60 µL of 7.5 % $Na_2CO_3$ solution were added. After 120 min of incubation at room temperature, the absorbance was measured in a microplate spectrophotometer (ELx800, Biotek, USA) at 765 nm and compared to a gallic acid equivalent (GAE) calibration curve. Results were expressed as mg GAE/100 g of dry matter (d.m.). All reagents were purchased from Merck (Merck KGaA, Darmstadt, Germany). All measurements were done in triplicate.

*2.4. Flavonoids content*

Total flavonoid content (TFC) of extracts was quantified following the methodology described by Zhishen, et al. [14] with some modifications. An aliquot of ethanolic extract (100 µL) was added to 430 µL of an aqueous $NaNO_2$ solution (0.35% w/v), and the mixture was incubated for 5 min at room temperature. 30 µL of an $AlCl_3$ solution

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(10% w/v) were added, the mixture was incubated for 1 minute, and 440 μL of NaOH 0.454 M were added. 150 μL of mixture were transferred to a microwell plate, and the absorbance was read at 496 nm (ELx800, Biotek, USA). TFC was calculated from a calibration curve using quercetin as a standard, and the results were expressed as mg quercetin/100 g d.m. All measurements were done in triplicate.

*2.5. Antioxidant capacity*

The antioxidant capacity was determined by the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, according to the procedure described by Ayala- Zavala, et al. [13] with some modifications. An aliquot of 140 μL of 0.15 mmol/L DPPH radical in ethanol was added to a hole with 10 μL of the extract. The reaction mixture left to stand at room temperature in the dark for 60 min. The absorbance was measured at 515 nm, using a microplates spectrophotometer (ELx800, Biotek, USA). 80% (v/v) ethanol was used as blank solution. Calibration curve was made using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). The results were expressed as mg TE (Trolox equivalent)/100 g d.m. All measurements were done in triplicate.

*2.6. Cell culture*

Bone Marrow-derived Dendritic Cells (BMDCs) were generated from mouse bone marrow cultures as described previously with minor modifications [15]. Briefly, bone marrow was flushed from femurs and tibias and depleted of erythrocytes with RBC lysing buffer (BD Bioscience, San Jose, CA). Cells were subsequently plated on 6-well tissue culture plates at $1 \times 10^6$/mL in 3 ml of complete RPMI 1640 medium containing 5 % heat-inactivated fetal calf serum (Gibco; Invitrogen), 100 U/mL

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penicillin/streptomycin, 10 µg/mL gentamicin and 2 mM L-glutamine, (all from Life Technologies, Grand Island, NY). To induce DC-differentiation, cells were supplemented with 100 ng/mL Flt3-L (R&DSystems) and cultured at 37 °C in 5 % $CO_2$ for 6 days. On day 7, to characterize our DC-population, cell surface molecules from DC-cultures were analyzed by flow cytometry using fluorescence-conjugated monoclonal antibodies (mAbs) directed against CD11b (M1/70), CD11c (HL3), CD3 (145-2C11), and CD45R/B220 (RA3-6B2) (BD Biosciences Pharmingen, San Diego, CA). Approximately 70–90 % of the cells were $CD11c^+$ (data not shown).

*2.7. Quantification of cytotoxicity and maturation measurement of BMDCs by flow cytometry*

Viability and cell cytotoxicity were analyzed after cultivate BMDCs in absence (control) or presence of radish leaf extracts for 18 h. An apoptosis detection kit was used according to manufacturing methods (Immunotech, Marseille, France). Briefly, the cells were labeled with propidium iodide (PI) immediately prior to evaluation of fluorescence by flow cytometry. PI positive cells were defined as dead cells. Additionally, to evaluate maturation, differenced BMDCs were stimulated with 100 ng/ml Lipopolysaccharide (LPS, Sigma-Aldrich Co, positive control) or with 200 µL of radish leaf ethanolic extracts (30 % v/v ethanol) for 18 h at 37 °C. After incubation, cells were harvested, washed with PBS, resuspended in 100 µL of fluorescence in isothiocyanate (FITC) and phycoerythrin (PE) conjugated mAbs directed to MHCII and CD86, respectively (eBioscience, San Diego, CA, dilution 1:100) and incubated for 30 min at 4 °C. In all cases, isotype matched control antibodies were used, and a gate (R1) was defined in the analysis to exclude nonviable cells and debris, based on size and PI staining. Analysis was performed using a FACS flow cytometer and a CellQuest

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software (BD Bioscience, San Jose, CA). The results were expressed as the mean fluorescence intensity or as the percentage of positive cells.

*2.8. Statistical analysis*

Data were analyzed using the software package SAS (version 9.0, SAS Institute Inc., Cary, U.S.A., 2002). All experiments were performed in triplicate. Results were presented as a value ± standard deviation (SD). Significant levels were defined at $P < 0.05$.

## 3. Results and discussion

*3.1. Extraction yield*

Fig. 2 shows the particle size distribution. Most particles was retained by 710 µm. Similar results were obtained for *Baccharis dracunculifolia,* with mean diameter 703 µm [16]; *Ocimum basilicum* L. [17], 660 µm and *Artemisa annua* L. leaves, 838 µm [18].

Fig. 3 shows data from extraction yield for all operational conditions. Extraction curves are useful for scale-up of processes and the calculation of manufacturing costs. One of the difficulties of using the supercritical technology is the high initial investment cost. However despite the high initial cost, several studies have demonstrated the economic viability of sc$CO_2$ extraction [18].

As can be seen, there were a high extraction rate at the beginning of the processes, and a rapid reduction in the extraction rate in the subsequent minutes. The extraction curves showed that by increasing the pressure, the yield is improved, obtaining more extract in a shorter time. As for temperature, the opposite effect was observed: as the extraction temperature increases, yield decreases. Similar results were reported by Elgndi, et al.

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[19] for essential oils and extracts of *Satureja montana* L., *Coriandrum sativum* L. and *Ocimum basilicum* L. obtained by supercritical fluid extraction. The increase of pressure (from 100 to 300 bar at constant temperature) enhanced the kinetics and greatly reduced the extraction time and significantly improve the extraction efficiency. This behavior is due the increase of the supercritical solvent density, which enlarges the solubility of $CO_2$ and thus enhances the extraction rate. According to Ghasemi, et al. [4], the extraction yields of *Myrtus communis* L. leaves were increased with a raise in the pressure. Similar results reported Zermane, et al. [20]: for all the temperature conditions tested, an increase in pressure leads to a maximize oil recovery from *Algerian Myrtus communis* L. leaves. Pressure and temperature affected both mass transfer and thermodynamic properties. In this experience, whatever the pressure, the highest temperature leads to smaller values of yield. This fact is related to the influence of both pressure and temperature on solubility. At low pressure, lower temperatures lead to higher solubility (that is convenient to avoid thermal degradation of the extracted species) whereas at very higher pressures an inverse behavior can be found. The solubility results from the volatility of the solute and the solvent power of the solvent. The former increases with temperature while the latter decreases due to decreasing density [21]. Moreover, the high selectivity is one of the most important properties of supercritical $scCO_2$ extraction. The selectivity of this extraction process can be adjusted by tuning the process pressure and temperature. Based on results, the extraction pressure is the most dominant parameter which affects the $scCO_2$ selectivity. Thus, radish leaves SFE curves could be divided in few extraction periods controlled by mass transfer mechanisms by convection, solubility as well as internal diffusion.

*3.3. Bioactive compounds and antioxidant capacity*

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Table 1 shows TPC and antioxidant capacity by DPPH for radish leaves extracts obtained at different pressure and temperature conditions.

Respect to Folin–Ciocalteu's assay, this is commonly applied to assess the TPC of natural products, however, various compounds, e.g. reducing sugars, are known to reduce the Folin–Ciocalteu's reagent as well, thus this assay in general measures reducing capacity of the whole sample [22]. As can be seen on Table 1 and Fig. 3, better results are obtained at higher pressure and lower temperature (35 ºC/400 bar and 40 ºC/400 bar), with values of 1375 mg GAE/ 100 g d.m. and 1455 mg GAE/ 100 g d.m., respectively. It should be noted that these values are higher than those reported for extracts by $scCO_2$ from broccoli leaves (461-794 mg GAE/ 100 g d.m.) [23], but lower than *Artemisia annua* L. leaves (4000-9000 mg GAE/ 100 g d.m.) [18] and *Prunus persica* leaves (2600-3700 mg GAE/ 100 g d.m.) [24]. On the other hand, Goyeneche, et al. [7] reported for radish leaves, using traditional methods of extraction, TPC values of 695.07 ± 36.67 GAE/ 100 g d.m. According to this, the use of supercritical extraction is effective to obtain extracts rich in phenolic compounds.

Antioxidants have been largely studied in the food and agriculture fields, and the DPPH assay has been widely used since it is a simple and highly sensitive method [25]. As can be seen on Table 1, when the polyphenol content is higher, better antioxidant capacity was obtained, with the following values: 359 mg TE/ 100 g d.m. (35 ºC/ 400 bar) and 403 mg TE/ 100 g d.m. (40 ºC/ 400 bar). Comparable results were obtained by Arnáiz, et al. [23] for extracts by $scCO_2$ from broccoli leaves (0.590-0.876 mg TE/g d.m.).

Flavonoids are phenolic substances isolated from a wide range of vascular plants, with over 8000 individual compounds known. They act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants, and for light screening. Many studies have suggested that flavonoids exhibit biological activities,

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including antiallergenic, antiviral, anti-inflammatory, and vasodilating actions. However, most interest has been devoted to the antioxidant activity of flavonoids, which is due to their ability to reduce free radical formation and to scavenge free radicals [26].

Table 1 shows TFC for radish leaves extracts obtained at different pressure and temperature conditions. As well as for polyphenols and antioxidant capacity measured according to DPPH, the best results for flavonoids were obtained at higher pressure and lower temperatures.

Goyeneche, et al. [7] reported similar results for radish leaves using traditional methods of extraction, with values of 1042.73 ± 49.95 mg quercetin/100 g d.m.

### *3.4. Cytotoxicity and maturation analysis of BMDCs*

Due to the best results of SFE were obtained at higher pressure and lower temperature, the biological assays were carried out employing the 35 °C, 400 bar and 40 °C, 400 bar radish leaf extracts. Dendritic cells are antigen presenting cells that play a central role in orchestrating immune responses to self and foreign antigens. Contact with inflammatory stimuli, can induce the maturation of DCs accompanied by functional and phenotypic changes. In their mature state, DCs are the unique cell type able to prime and activate naive T cells [27]. Different volume (100 to 1000 µL, equivalent at 30 to 300 mg d.m.) of radish leaf extracts were evaluated for cytotoxicity on BMDCs by flow cytometry using PI. Viability decreased as a function of extract concentration as shown in Fig. 4. Both extracts presented no cytotoxicity at least up to 200 µl after 18 h of incubation. The viability of BMDCs incubated in control conditions (negative control) was relativized to 100 % whereas the viability of BMDCs cultured in presence of 100 µL ethanol 70 % (toxicity control) decreased to 15 %. These results indicated that the

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extracts are innocuous at the concentration assayed for the bioactive and antioxidant capacity studies. Additionally, further studies were done to determine if radish leaf extracts were able to induce BMDC maturation. As can be observed in Fig. 5, the expression of the MHCII and the costimulatory molecule CD86 on DCs remained similar to control values when BMDCs were incubated with 200 µL of radish leaf extracts. In turn, BMDCs incubated in presence of LPS (positive control) upregulated both molecules expression indicating phenotypic maturation of DCs. These results allow us to suggest the absence of carbohydrates and proteins with potential antigenic properties in these extracts [9, 10]. Nevertheless, further studies are needed to determinate the tolerogenic function of DCs exposed to radish leaf extracts.

## 4. Conclusions

Overall extractions curves for extractions of bioactive compounds from radish leaves were performed using $scCO_2$. Yield, total phenolic compounds, total flavonoids and antioxidant capacity were determined for each extract. According to the results, it is recommended to extract bioactive compounds from radish leaves at a pressure of 400 bar and temperature of 35 or 40 ºC. With those conditions, we obtained maximum yield, and maximum bioactive compounds extraction, free of antigenic components. As we had expected, maturation of DCs was not promoted being these extracts promising and interesting sources of antioxidative and anti-inflammatory agents for future uses in the pharmaceutical and alimentary industries. Further studies will be carried out in our laboratory to isolate and identificate their active constituents.

## Acknowledgments

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# Figures

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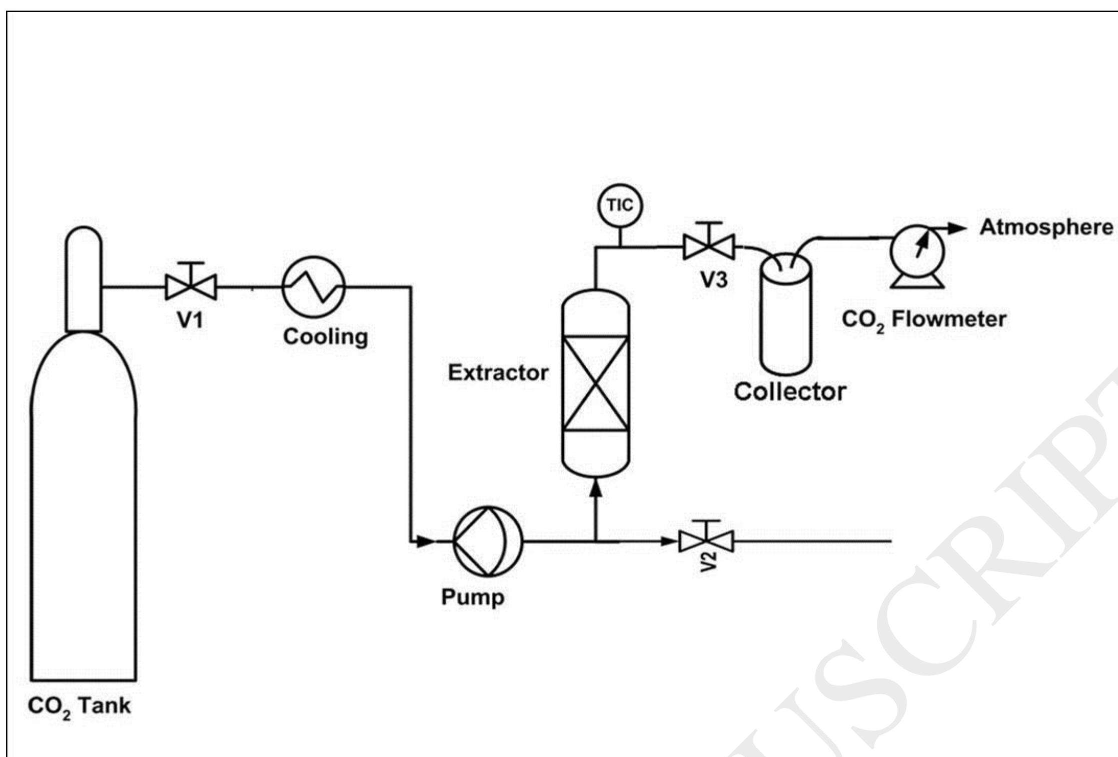


**Fig. 1.** Schematic diagram of supercritical fluid extractor.

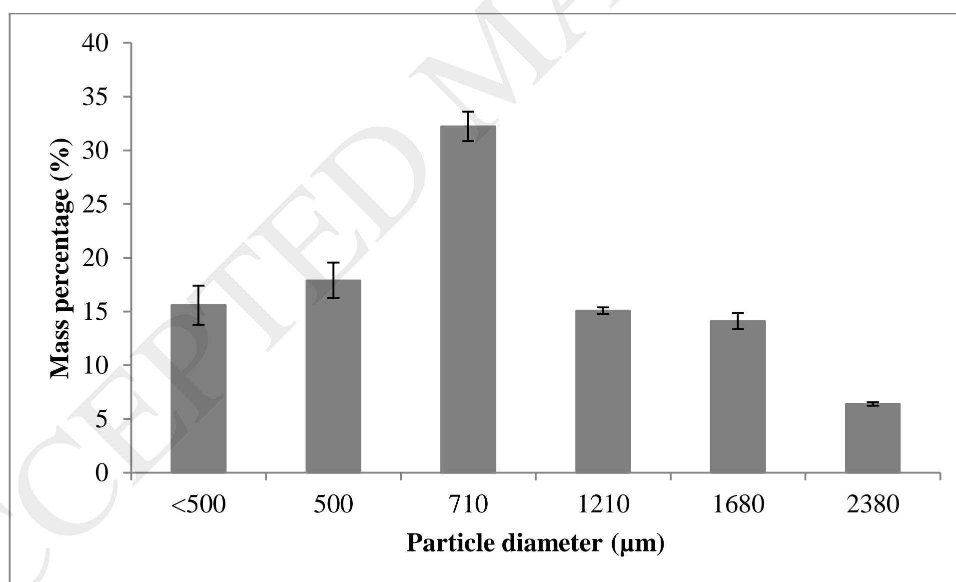


Fig. 2. Particle size distribution.

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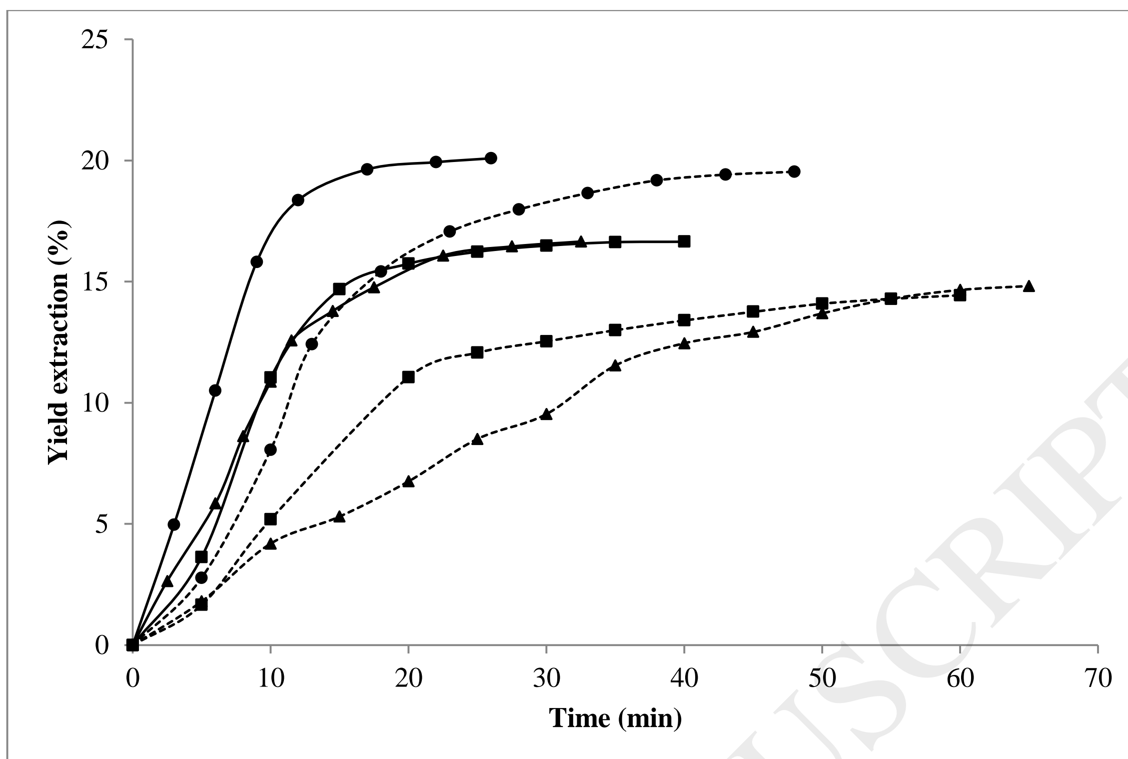


**Fig. 3**. Extraction curves at different temperatures and pressures from radish leaves, $CO_2$ flow rate = 0.6 kg/h ( 35 ºC, 400 bar; 40 ºC, 400 bar; 50 ºC, 400 bar; 35 ºC, 300 bar; 40 ºC, 300 bar; 50 ºC, 300 bar)

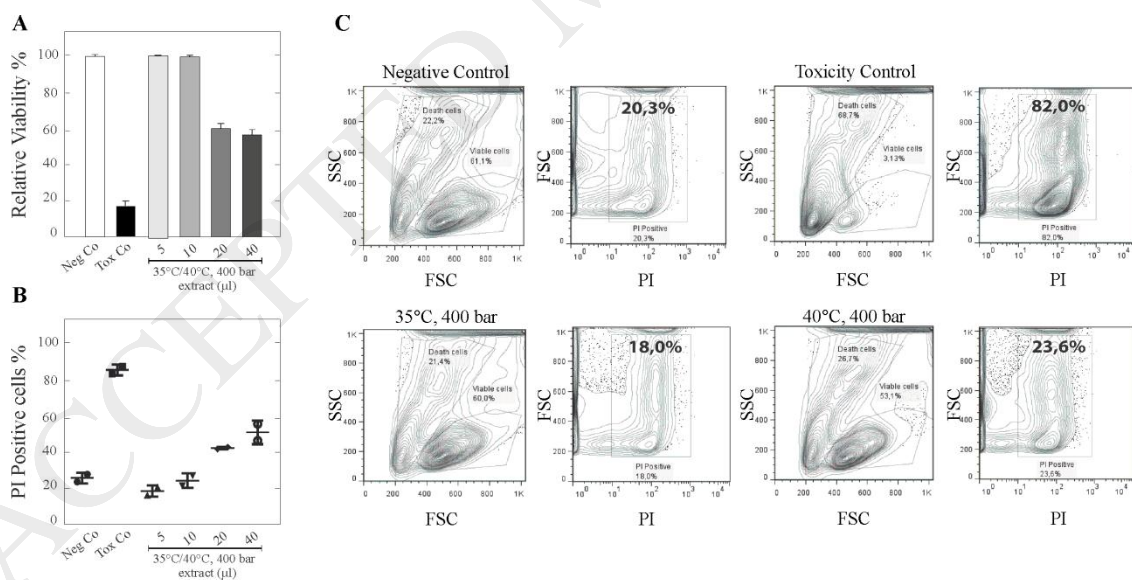


**Fig. 4**. Cytotoxic effect of radish leaf extracts on BMDCs**.**

**A.** Representative graph bar showing BMDC relative viability in presence of 30% ethanolic extracts obtained at 35°C, 400 bar. **B.** Representative scatter plot showing total percentages of positive PI cells viability in presence of 30% ethanolic extracts obtained at 40°C, 400 bar. **C.**

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Representative dot-plots of 200µL of 35°C 400 bar and 200 µl of 40°C 400 bar radish leaf extracts.

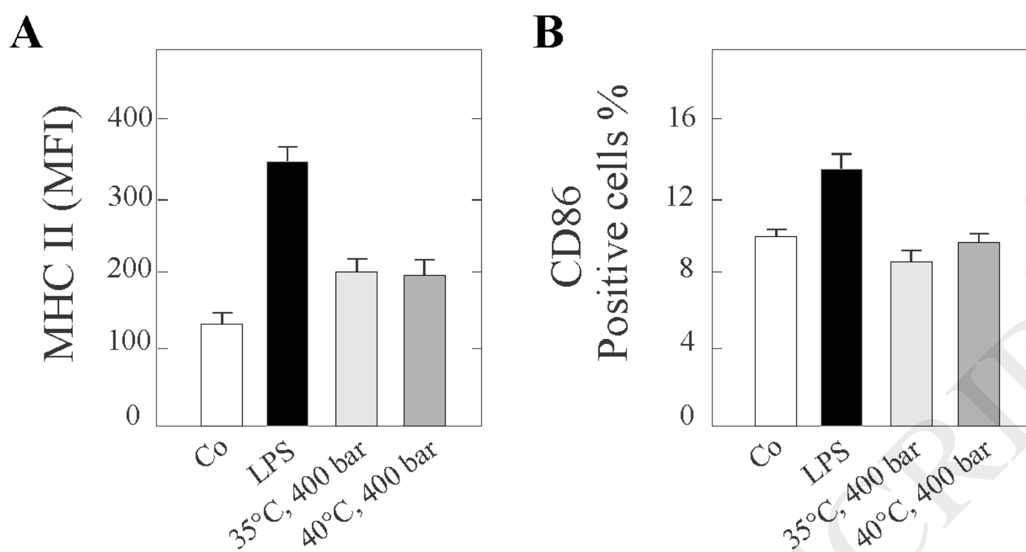


**Fig. 5**. Maturation analysis of BMDCs.

**A.** Representative graph bar showing the mean fluorescence intensity (MFI) of the MCH II in BMDCs (n=3). **B.** Representative graph bar showing the expression of the costimulatory molecule CD86 in BMDCs (n=3). LPS was used as positive control of BMDC maturation.

**Table 1.** Total phenolic content (TPC), antioxidant capacity (DPPH) and total flavonoids content (TFC) for extracted radish leaves at different pressure and temperature conditions.

| Temperature (ºC) | Pressure (bar) | TPC (mg EAG/g d.m.) | DPPH (mg TE/g d.m.) | TFC (mg quercetin/g d.m.) |
|---|---|---|---|---|
| 35 | 300 | 13.61$^{b}$ ± 0.157 | 0.290$^{c}$ ± 0.000 | 14.6$^{c}$ ± 1.3 |
| 35 | 400 | 13.75$^{b}$ ± 0.151 | 0.359$^{b}$ ± 0.118 | 13.9$^{c}$ ± 2.2 |
| 40 | 300 | 10.55$^{c}$ ± 0.075 | 0.274$^{d}$ ± 0.127 | 8.5$^{d}$ ± 0.5 |
| 40 | 400 | 14.55$^{a}$ ± 0.049 | 0.403$^{a}$ ± 0.003 | 21.8$^{a}$ ± 0.9 |
| 50 | 300 | 9.97$^{c}$ ± 0.012 | 0.310$^{c}$ ± 0.034 | 18.2$^{b}$ ± 2.4 |
| 50 | 400 | - * | - * | - * |

*The extract obtained at 50 ° C and 400 bar contained solid waste and could not be measured correctly.

Values are mean ± s.d., n=3. Values with the same lowercase letter in the same column are not significantly different (P<0.05)