Original Communication

Increased anti-inflammatory activity and enhanced phytochemical concentrations in superfine powders obtained by controlled differential sieving process from four medicinal plants

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Abstract: Anti-inflammatory effect of *Rosa canina*, *Salix alba*, *Scrophularia nodosa* and *Hedera helix* were studied in LPS-stimulated primary peripheral blood mononuclear cells (PBMCs) from mice (n=18) by comparing homogeneous powders of small microparticles (50–100 μm, 100–180 μm and 180–315 μm) obtained from plants via a controlled differential sieving process (CDSp) *versus* total plant materials obtained via hydroethanolic (HE) extraction. Further, phytochemical composition of the fine powders and HE extracts was determined by LC-PDA-ESI/MS analyses. Results showed that a one-hour pretreatment of PBMCs with fine powders, particularly those with superfine particle sizes (i.e. 50–100 μm and 100–180 μm), significantly inhibited TNFα, IL-1β, IL-6 and NO production in LPS-stimulated PBMCs, by at least ca. 20% more than HE extracts (all, $p<0.05$). For each of the plants studied, their superfine powdered fractions were more concentrated in phenolic contents than their HE extracts. Overall, our results further confirm CDSp, as an environmentally friendly method, for improving the concentration of bioactive compounds as well as their biological activities.

Keywords: Controlled differential sieving process, PBMCs, Anti-inflammatory effect, Plant powders, Phytochemical concentrations

Introduction

Controlled Differential Sieving process (CDSp) is a novel and patented green dry extraction method [1]. This solvent-free extraction method comprises three main stages, namely (i) drying the moist materials (herb, plant, or fruit) at moderate temperatures (20–35 °C), (ii) pulverizing to the point of obtaining micrometric particles, followed by (iii) controlled differential sieving, to discriminate powders of homogeneous particle sizes, ranging from 20 μm to 500 μm [1, 2, 3, 4].

Bioactive compounds in plants such as tannins, flavonoids, phenolic acids, carotenoids and terpenoids belong to various phytochemical classes and as such have different molecular weights. The CDSp is based on the concept of sequential separation of particles using sieves of decreasing mesh size. As a consequence, CDSp produces powders with differential biological activity commensurate with the concentration of bioactive compounds in powders which vary inversely with their size [2, 5, 6, 7, 8]. We previously demonstrated that combined grinding/sieving method is more efficient *versus* conventional solvent extraction methods [2, 3, 4], and it allows producing homogeneous granulometric fractions with good storage abilities [5]. As highlighted in our previous studies [5, 6], it is important to note that CDSp fractions (20–500 μm) with the highest content of active phytochemicals vary from one plant to another. Fractions larger than 315 μm, and more specifically those larger in size than 500 μm are expected to contain less bioactive compounds, since large particles are more likely to be derived from plant parts such as fibers, given their relative higher degree of difficulty to grind [5, 6, 7]. In our recently published studies, we compared the bioactivity of 3 different granulometric fractions (50–100 μm, 100–180 μm and

180–315 μm) from 4 medicinal plants in mouse primary spleen cells exposed to H_2O_2. Findings showed that higher antioxidant and greater preventive effects were observed in the cells treated with superfine (<180 μm) plant powders [3, 4]. In these studies, we have further demonstrated that the superfine powder gives better results in term of bioactivity compared to hydroethanolic extract obtained from total plant materials [3, 4]. This suggested that CDSp, which is a novel and environmentally friendly method, could be a good alternative to conventional solvent extraction techniques. For industrial purposes requiring high volume production, CDSp presents the advantage to be technically easily implemented. Compared to the existing conventional and non-conventional extraction methods, currently and commonly used to obtain bioactive fractions or even pure active phytochemicals, CDSp does not rely on the use of potentially toxic solvents. On the other hand, it has the potential of increasing yield (given its demonstrated ability to produce powder fractions of varying bioactive concentrations) if used as a preliminary step in the chemical method of isolation of bioactive compounds.

The inflammation is the cause of many chronic diseases. In the present study, we aimed to evaluate, for the first time, the effects of the fine powders obtained by CDSp on the anti-inflammatory activity of medicinal plants. Thus, we determined the phytochemical composition and anti-inflammatory activity of three fine (50–100 μm, 100–180 μm and 180–315 μm) powder fractions obtained by CDSp from 4 medicinal plants (the willow bark (*Salix alba* L.), the rose hip (*Rosa canina* L.), the common figwort (*Scrophularia nodosa* L.) and the common ivy (*Hedera helix* L.)), known for their anti-inflammatory properties. Phytochemical composition of the powders as well as that of the total extract of each plant material were determined using LC-PDA-ESI/MS analytical methods. Their anti-inflammatory preventive effects as well as that of the different total extract were studied *in vitro* by measuring lipopolysaccharide (LPS)-induced inflammatory responses in primary mouse peripheral blood mononuclear cells (PBMCs) using the levels of tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, IL-6, IL-10 and nitric oxide (NO) as biomarkers. Figure 1 shows the experimental design of the study.

Materials and methods

Plant material

Rosa canina L. (RC) hips, *salix alba* L. (SA) bark, *scrophularia nodosa* L. (SN) aerial plant parts and *hedera helix* L. (HH) leaves were provided dried, after natural air-drying at ambient temperature and 10% moisture content by Cailleau Herboristerie (Chemillé, France). SN and HH were manually harvested in March 2015 and June 2015. While, SA and RC were manually harvested in October 2015.

Controlled differential sieving process (CDSp)

As plant materials used in this study were already dried, CDSp was then performed using only two main steps: (i) grinding, using the Ultra Centrifugal Mill ZM 200 (Retsch, France) at 8000 × g and ambient temperature for 2 minutes, and (ii) controlled differential sieving of plant powders with vibratory sieve shaker Analysette 3 Spartan (Fritsch, Idar-Oberstein, Germany) at 0.5 mm vibration amplitude for 10 minutes to obtain the following powder fractions: 50–100 μm; 100–180 μm; 180–315 μm as described previously [1, 3, 4, 5, 6]. Powders were stored in a hermetically dry container.

Hydroethanolic (HE) extraction

Dried plant materials (20 g) were ground for 15 sec using an electric grinder WSG30 (Waring, USA) and macerated in the dark, under ambient air in 200 mL of a solution of water/ethanol (70/30, v/v), at room temperature under horizontal shaking for 16 hours, and then at 37 °C for 14 hours. The extract was filtered, and then concentrated using a Rotary Evaporator Laborota 4000 (Heildoph, Germany) at 40 °C and 250 rpm. The yield extracts were immediately frozen, and lyophilized using a laboratory freeze dryer alpha 1–2 (Christ, Germany).

Mouse PBMCs isolation

Nine-week-old Swiss albino male mice (CD1, Janvier Labs, France), at the time of reception, were housed in groups (3 per cage) with a 12-h light: 12-h dark schedule (lights on at 8:00 a.m.) with *ad libitum* access to water and food (SDS Dietex-France) under constant temperature (21±2 °C) and a relative humidity of 55±10%. Mice (n=18) were anesthetized with isoflurane, sacrificed following the relevant European Union regulations (Directive 2010/63/EU) and their blood samples collected in heparinized tubes and diluted with Dulbecco's Phosphate-Buffered Saline (PBS) (ratio 1: 1). The diluted samples were subsequently subjected to density gradient separation on Hitopaque-1077 medium (ratio 1:1) and centrifuging at 500g for 30 min. Afterward, the PBMCs layer was collected and washed with Hank's Balanced Salt Solution (HBSS). PBMCs were recovered and resuspended in a complete RPMI-1640 medium, i.e. supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine (200 mM), 1% mixture of penicillin (100 IU/ml) and streptomycin (100 μg/mL) (Figure 1).

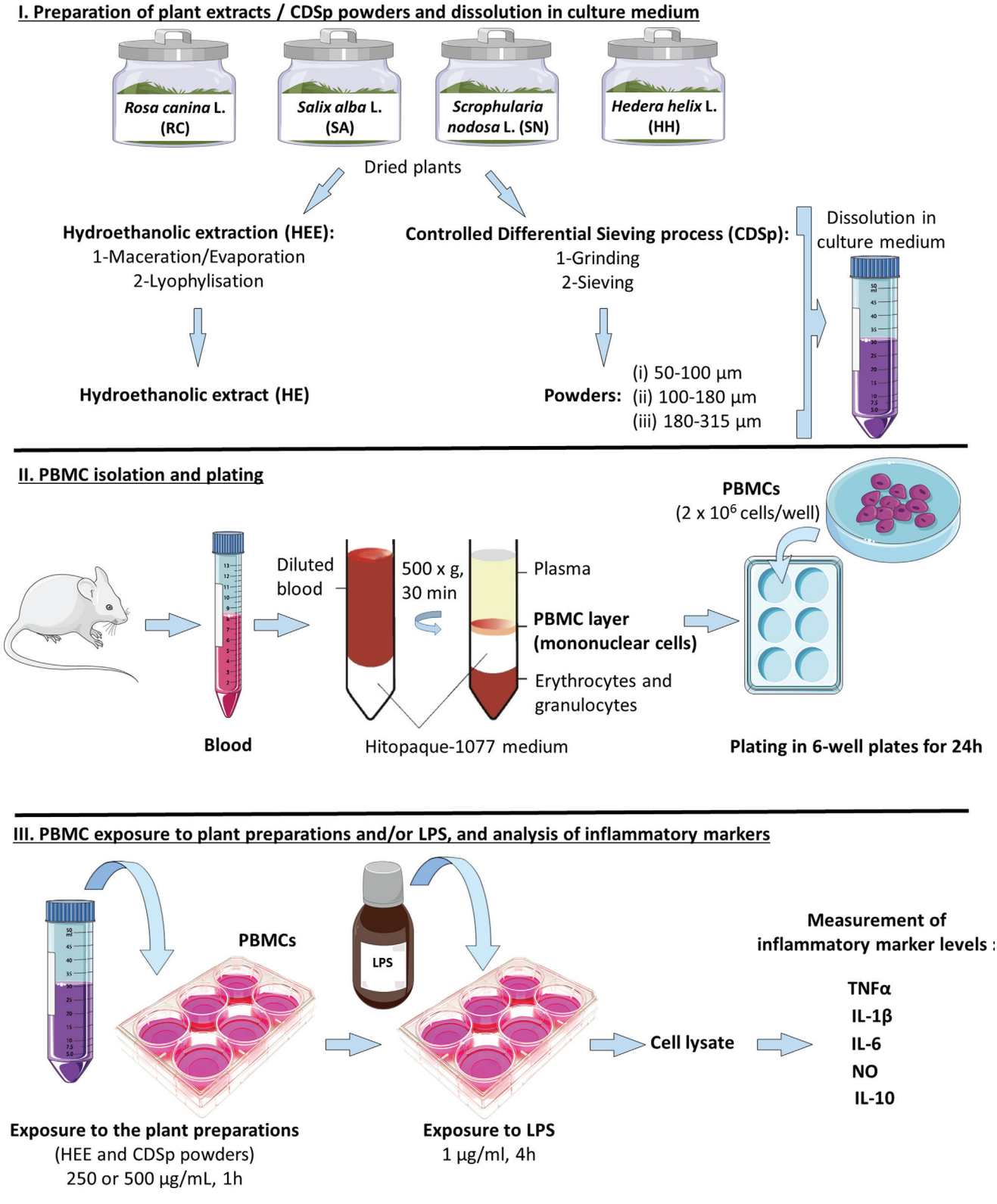

Figure 1. Experimental design of the study of anti-inflammatory effects of *Rosa canina* L. (RC), *Salix alba* L. (SA), *Scrophularia nodosa* L. (SN) and *Hedera helix* L. (HH) against LPS-induced inflammatory responses in primary mouse peripheral blood mononuclear cells (PBMCs). PBMC layer is constituted by mononuclear cells including lymphocytes and monocytes. Created with some illustrations from Servier Medical Art with the Creative Commons Attribution 3.0 Unported License. CDSp: controlled differential sieving process; HEE: hydroethanolic extraction; HE extract: hydroethanolic extracts; IL: interleukin; LPS: lipopolysaccharide; NO: nitric oxide; TNFα: tumor necrosis factor alpha.

Cell treatment and *in vitro* anti-inflammatory activity

The anti-inflammatory preventive effects of plant fractions and HE extracts were assessed using peripheral blood mononuclear cell (PBMCs) assay (Figure 1). PBMCs were incubated at a density of 2×10^6 cells/well in 6-well plates for 24 h at 37 °C and 5% CO_2. CDSp powders and HE extracts were dissolved directly in the cellular buffer followed by a filtration step, using a sterile cell strainer with a filter size of 70 µm. HE extracts and CDSp fractions were then added in plated cells to reach final doses of 250 and 500 µg/mL. Plates were incubated for one hour. After that, lipopolysaccharide (LPS, 1 µg/mL) was added to PBMCs and further incubated for 4 hours to induce inflammatory responses. After pretreatment with different plant preparations, LPS-exposed PBMCs were collected, homogenized and stored at −80 °C until analysis. Anti-inflammatory activity of plant fractions and HE extracts was evaluated in pretreated PBMCs *vs*. negative control (PBMCs exposed only to LPS) by assessing several cytokines and NO production (Figure 1).

Cytokine measurements

Four mouse ELISA kits were used to assess the level of interleukin (IL)-1β, IL-6, IL-10 and tumor necrosis factor alpha (TNFα). The assessment was carried out following the manufacturer's protocol and using the standard curve provided in each kit (R&D Systems, Minneapolis, MN, USA) and a microplate reader MR96 A (Mindray, China) at 450 nm.

NO production

Nitric oxide (NO) production was determined using the Griess reaction assay previously described by Green et al. [9] and Dirsch et al. [10]. Briefly, NO was measured by adding 100 µl Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid) to 100 µL of PBMCs homogenate. The resulting color was measured by a DU 720 spectrophotometer (Beckman Coulter, USA) at 540 nm. The absorbance values were compared to a standard sodium nitrite curve and the absorbance values were converted to corresponding nitrite concentrations (µM).

LC-PDA-ESI/MS analyses

Phytochemical composition of RC powders and HE extract was determined using the LC-PDA-ESI/MS method as earlier described [2, 7]. As for SA, SN and HH, the phytochemical composition was reported from our precedent works [2, 7]. The LC-MS 2020 system (Shimadzu, Tokyo, Japan) consisted of a photodiode array detector (PDA) and a mass spectrometer detector (MS) associated with an electrospray ionization source (ESI). The ESI ion source operated in negative mode with 1.5 L/min nebulization gas flow, 12 L/min drying gas flow, 300 °C heat block temperature, 250 °C desolvation line temperature, and −4 kV probe voltage. MS data acquisition was performed in the selected ion monitoring (SIM). UV detector range was set at 200 nm and 800 nm. Chromatographic separation of compounds was achieved on a Gemini 3 µm C18 130 A reversed phase column (Phenomenex, Torrance, CA, USA) of 150 mm length and 4.6 mm using acidified water (0.5% formic acid) as solvent A and acetonitrile as solvent B. Elution started with a linear increase of the organic modifier percentage from 10% to 65% in 15 min; then in 0.5 min the component B was increased to 100% and maintained at this percentage for 5 another min; in 0.5 min component B was decreased to 10% and finally held for 4 min (re-equilibration step). The flow rate of the mobile phase was 600 µL/min; the injection volume was 20 µL and the oven temperature of the column was fixed at 30 °C.

Statistical analyses

All data were reported from quadruplicates as mean±SD. Equality of variances was verified by box plots. The normality of distribution was verified using plots, and was measured by conducting Shapiro-Wilk tests. Pretreatment of LPS-exposed PBMCs with powders and HE extracts was considered as fixed factor, and anti-inflammatory effects as observed factors. Following significant F-values, Bonferroni post-hoc tests were conducted for individual comparisons. A p-value below 0.05 (2-sided) was considered significant. All statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL).

Results

Effects of pretreatment with plant powders on TNFα production in LPS-stimulated PBMCs

Significant production of TNFα, a pro-inflammatory cytokine, was seen in LPS-activated PBMCs compared to untreated cells ($p<0.001$) (Figures 2A, 3A, 4A, 5A). In LPS-stimulated PBMCs, pretreatment with any of the three fine powders derived from the 4 studied medicinal plants, at both concentrations (250 and 500 µg/mL), significantly reduced TNFα level ($p<0.05$). However, only pretreatment of PMBCs with the highest concentration of hydroethanolic (HE) extracts (i.e. 500 µg/mL) from plants significantly decreased TNFα level ($p<0.01$), except for RC hips, in which HE extract did not inhibit TNFα production ($p>0.05$) in LPS-stimulated PBMCs. Comparing between

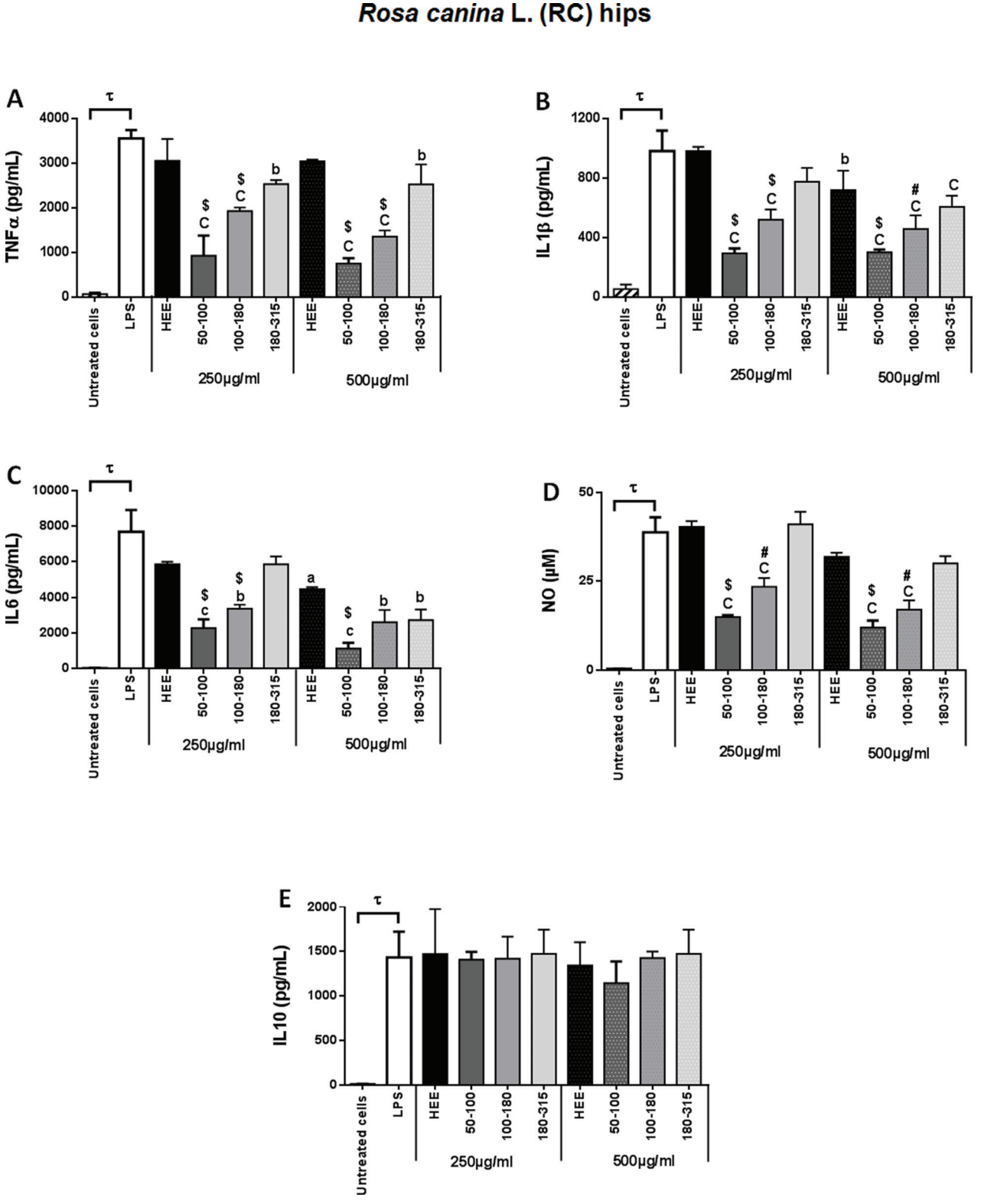

Figure 2. Effects of *Rosa canina* L. (RC) hip preparations derived from Controlled Differential Sieving process (CDSp) and hydroethanolic (HE) extraction on lipopolysaccharide (LPS)-induced inflammatory responses in PBMCs. (A) Tumor necrosis factor alpha (TNFα) production expressed in pg/mL. (B) Interleukin (IL)-1β levels expressed in pg/mL. (C) Interleukin (IL)-6 levels expressed in pg/mL. (D) Nitric oxide (NO) production expressed in µM. (E) Interleukin (IL)-10 levels expressed in pg/mL. All assays were performed in quadruplicate (n=4). The data are reported as the mean±SD. $^{\tau}p<0.001$: significantly different between control PBMCs and untreated PBMCs; $^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$ significantly different from the control cells (PBMCs exposed only to LPS); $^{\#}p<0.01$ and $^{\$}p<0.001$ significantly different from PBMCs pretreated with hydroethanolic (HE) extracts.

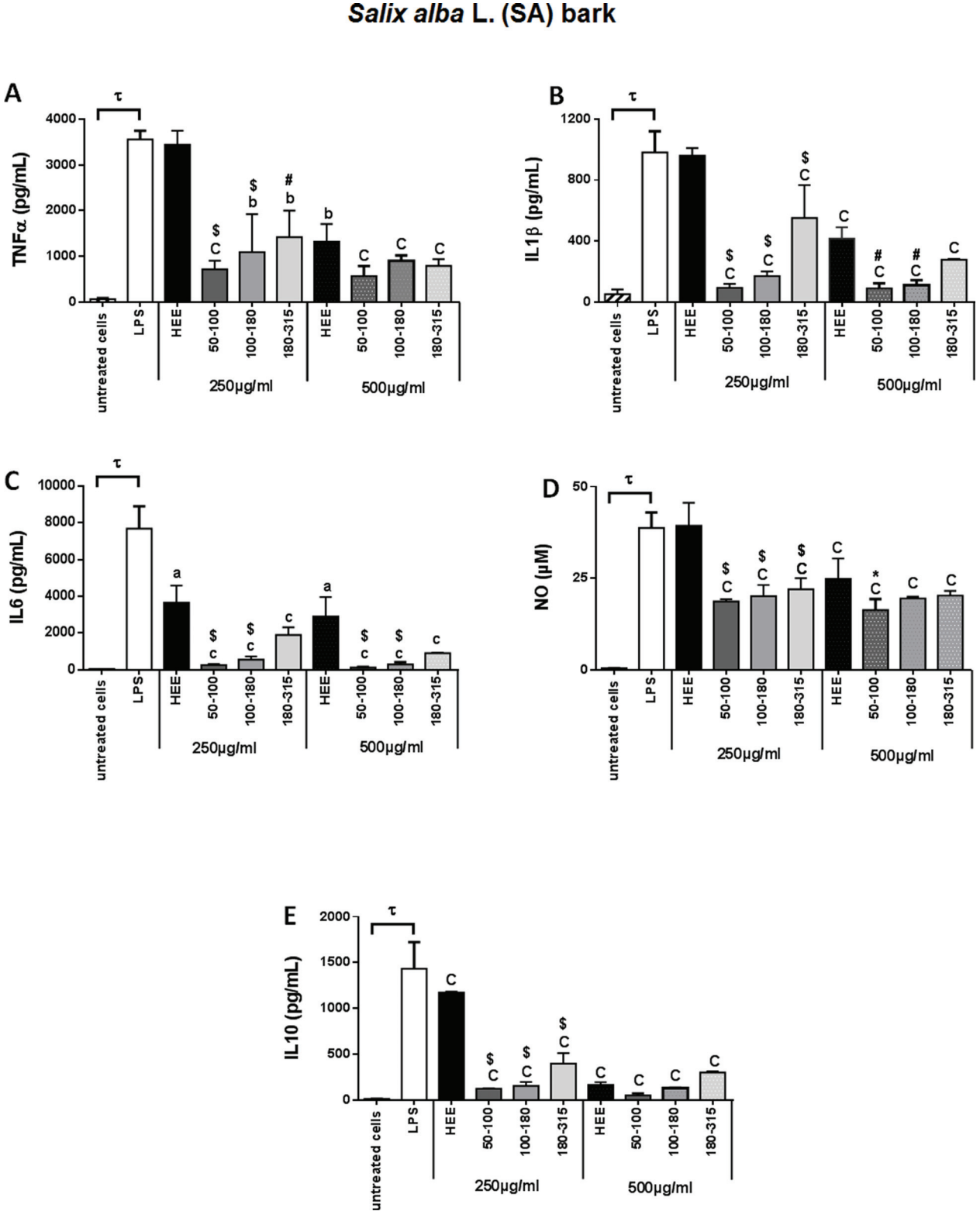

Figure 3. Effects of *Salix alba* L. (SA) bark preparations derived from Controlled Differential Sieving process (CDSp) and hydroethanolic (HE) extraction on lipopolysaccharide (LPS)-induced inflammatory responses in PBMCs. (A) Tumor necrosis factor alpha (TNFα) production expressed in pg/mL. (B) Interleukin (IL)-1β levels expressed in pg/mL. (C) Interleukin (IL)-6 levels expressed in pg/mL. (D) Nitric oxide (NO) production expressed in µM. (E) Interleukin (IL)-10 levels expressed in pg/mL. All assays were performed in quadruplicate (n=4). The data are reported as the mean±SD. $^{\tau}p<0.001$: significantly different between control PBMCs and untreated PBMCs; $^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$ significantly different from the control cells (PBMCs exposed only to LPS); $^{*}p<0.05$, $^{\#}p<0.01$ and $^{\$}p<0.001$ significantly different from PBMCs pretreated with hydroethanolic (HE) extracts.

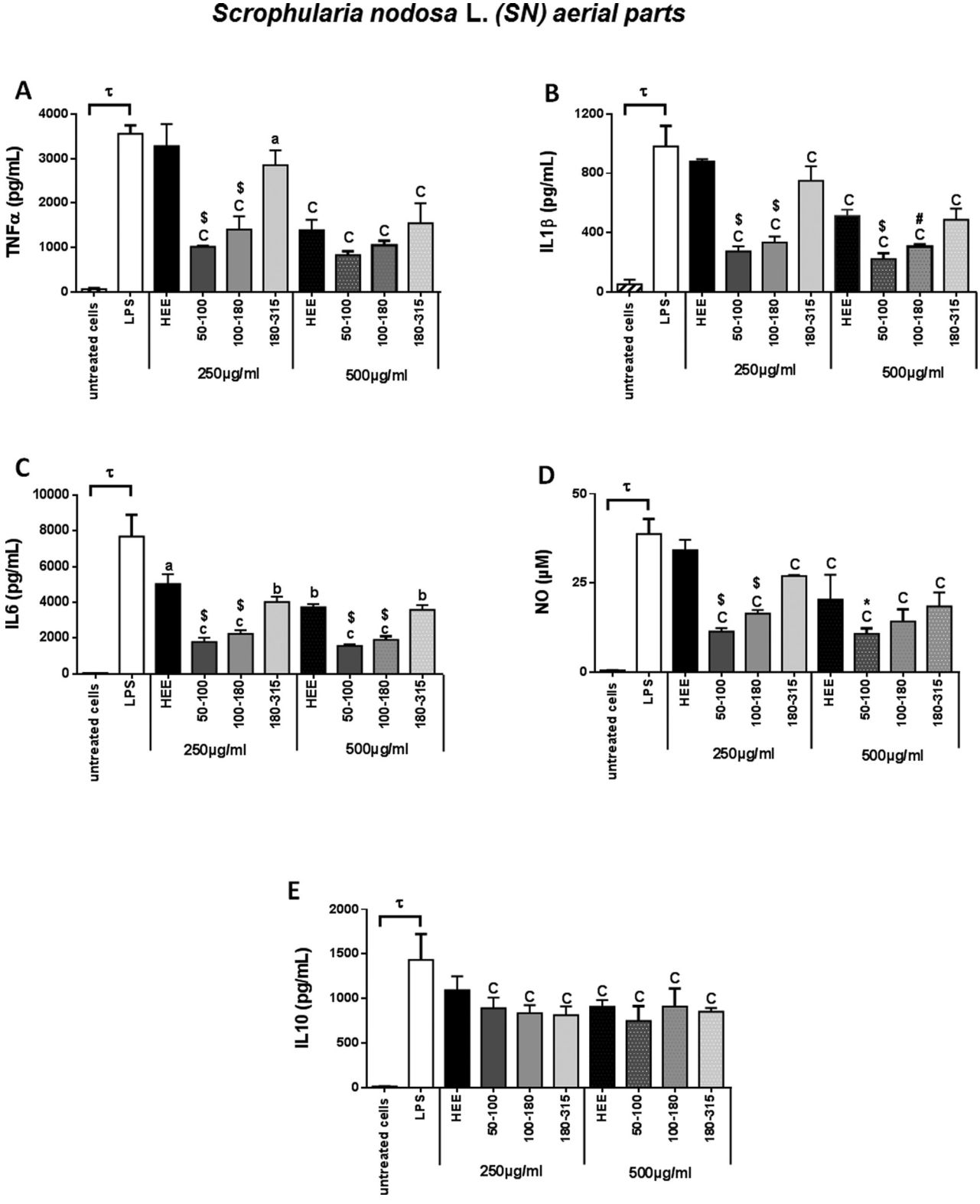

Figure 4. Effects of *Scrophularia Nodosa* L. (SN) preparations derived from Controlled Differential Sieving process (CDSp) and hydroethanolic (HE) extraction on lipopolysaccharide (LPS)-induced inflammatory responses in PBMCs. (A) Tumor necrosis factor alpha (TNFα) production expressed in pg/mL. (B) Interleukin (IL)-1β levels expressed in pg/mL. (C) Interleukin (IL)-6 levels expressed in pg/mL. (D) Nitric oxide (NO) production expressed in µM. (E) Interleukin (IL)-10 levels expressed in pg/mL. All assays were performed in quadruplicate (n=4). The data are reported as the mean±SD. $^{\tau}p<0.001$: significantly different between control PBMCs and untreated PBMCs; $^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$ significantly different from the control cells (PBMCs exposed only to LPS); $^{*}p<0.05$, $^{\#}p<0.01$ and $^{\$}p<0.001$ significantly different from PBMCs pretreated with hydroethanolic (HE) extracts.

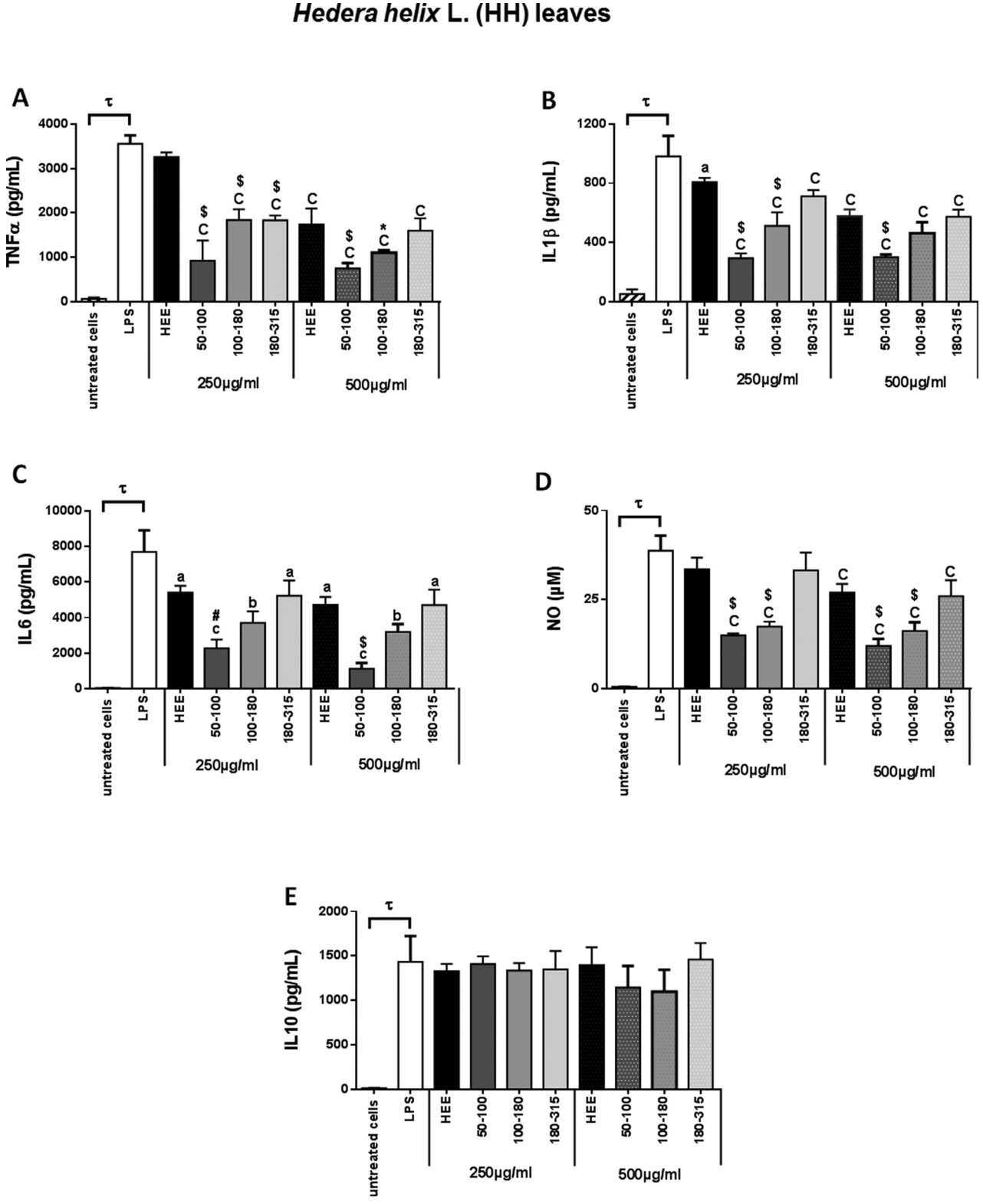

Figure 5. Effects of *Hedera Helix* L. (HH) preparations derived from Controlled Differential Sieving process (CDSp) and hydroethanolic (HE) extraction on lipopolysaccharide (LPS)-induced inflammatory responses in PBMCs. (A) Tumor necrosis factor alpha (TNFα) production expressed in pg/mL. (B) Interleukin (IL)-1β levels expressed in pg/mL. (C) Interleukin (IL)-6 levels expressed in pg/mL. (D) Nitric oxide (NO) production expressed in μM. (E) Interleukin (IL)-10 levels expressed in pg/mL. All assays were performed in quadruplicate (n=4). The data are reported as the mean±SD. $^{\tau}p<0.001$: significantly different between control PBMCs and untreated PBMCs; $^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$ significantly different from the control cells (PBMCs exposed only to LPS); $^{*}p<0.05$, $^{\#}p<0.01$ and $^{\$}p<0.001$ significantly different from PBMCs pretreated with hydroethanolic (HE) extracts.

the preventive effects of powders and HE extracts, at the lowest concentration (250 μg/mL), the reduction effects of superfine plant powders (i.e. 50–100 μm and 100–180 μm) derived from the 4 studied medicinal plants on LPS-induced TNFα production were significantly higher than those of HE extracts in PBMCs ($p<0.001$) (Figures 2A, 3A, 4A, 5AA). As for 180–315 μm fine powder, at the lowest concentration, markedly reduction was demonstrated only with SA bark ($p<0.01$) and HH leaves ($p<0.001$), as compared to HE extracts. However, at the highest concentration, there were significant differences between the superfine powders (50–100 μm and 100–180 μm) and HE extract of RC hips ($p<0.001$) and HH leaves ($p<0.05$) (Figures 2A, 3A, 4A, 5A).

Effects of pretreatment with plant powders on IL-1β production in LPS-stimulated PBMCs

Significant production of IL-1β, a pro-inflammatory cytokine, was seen in LPS-activated PBMCs compared to untreated cells ($p<0.001$) (Figures 2B, 3B, 4B, 5B). Except for 180–315 μm fine powder of RC hips, pretreatment of LPS-activated PBMCs with any of the fine powders derived from the 4 studied medicinal plants (at concentration level of 250 and 500 μg/mL) were observed to significantly ($p<0.01$) inhibit the production of IL-1β. On the other hand, similar pretreatment of LPS-stimulated PBMCs using HE extracts of the different plants was observed only for extracts with the highest concentration of 500 μg/mL. An exception was observed in the case of HE extracts from HH leaves where the lowest concentration (250 μg/mL) was also observed to significantly ($p<0.05$) inhibit the production of IL-1β. Irrespective of the plant source of fine powders (50–100 μm and 100–180 μm) their inhibiting effect at concentration levels of 250 μg/mL on the production of IL-1β was systematically observed to be significantly ($p<0.001$) different from that induced by the use of HE extracts. In addition, the fine powder (180–315 μm) from SA bark at concentration of 250 μg/mL was equally found to significantly ($p<0.001$) inhibit IL-1β production as compared to HE extracts in PMBCs stimulated with LPS. Furthermore, at 500 μg/mL, all 50–100 μm superfine powders at the highest concentration of 500 μg/mL were also found to significantly ($p<0.01$) reduced IL-1β production compared to HE. Except for 100–180 μm intermediate fine powder from HH leaves, noteworthy also was the observation that all intermediate fine powders at this highest concentration equally and significantly ($p<0.01$) inhibited the production of LPS-induced IL-1β production when compared to the effect of HE extract in PBMCs (Figures 2B, 3B, 4B, 5B).

Effects of pretreatment with plant powders on IL-6 production in LPS-stimulated PBMCs

The production of IL-6, a pro-inflammatory cytokine, was observed to be significantly enhanced in LPS-activated PBMCs compared to untreated cells ($p<0.001$) (Figures 2C, 3C, 4C, 5C). Except for 180–315 μm fine powder of RC hips, pretreatment with other fine powders derived from the 4 studied medicinal plants, at both concentrations (250 and 500 μg/mL), significantly reduced LPS-induced IL-6 production in LPS-treated PBMCs ($p<0.05$). On the other hand, HE extracts at both concentrations, except RC hips extracts at 250 μg/mL ($p>0.05$), significantly reduced IL-6 production in LPS-stimulated PBMCs ($p<0.05$). Comparing between the preventive effects of powders and HE extracts, except for 100–180 μm fine powder from RC hips, at the highest concentration, and that from HH leaves, at the two concentrations, the superfine particle size (50–100 μm and 100–180 μm) from all plants, at both concentrations, significantly diminished the production of IL-6 induced by LPS in PBMCs as compared to HE extract ($p<0.01$) (Figures 2C, 3C, 4C, 5C).

Effects of pretreatment with plant powders on NO production in LPS-stimulated PBMCs

A significant production of NO, a pro-inflammatory molecule, was seen in LPS-activated PBMCs compared to untreated cells ($p<0.001$) (Figures 2D, 3D, 4D, 5D). Pretreatment with fine powders from the studied plants significantly decreased the NO production induced by LPS in PBMCs ($p<0.001$), except in the case of 180–315 μm fine powder from RC hips, at both concentrations, and HH leaves, at the lowest concentration, which did not significantly ($p>0.05$) prevent LPS-induced NO production in PBMCs. Furthermore, pretreatment with HE extracts significantly decreased NO production only at the highest concentration for SA bark, SN aerial parts and HH leaves in LPS-activated PBMCs ($p<0.001$). Comparing between the preventive effects of powders and HE extract, at 250 μg/mL, superfine particle size at 50–100 μm and 100–180 μm from all plants significantly decreased NO production in LPS-stimulated PBMCs compared to HE extracts ($p<0.01$). In addition, at the lowest concentration, 180–315 μm fine powder from SA bark significantly reduced NO production in LPS-stimulated PBMCs as compared to HE extracts ($p<0.001$). While for the highest concentration, both superfine powders from RC hips and HH leaves (powders <180 μm) significantly decreased NO production in LPS-activated PBMCs more than HE extracts ($p<0.01$).

As for SA bark and SN aerial parts, only the 50–100 μm superfine powder at 500 μg/mL significantly decreased NO production compared to HE extracts in PBMCs activated with LPS ($p<0.05$) (Figures 2D, 3D, 4D, 5D).

Effects of pretreatment with plant powders on IL-10 production in LPS-stimulated PBMCs

A significant production of IL-10, an anti-inflammatory cytokine, was seen in LPS-activated PBMCs compared to untreated cells ($p<0.001$) (Figures 1E, 2E, 3E, 4E). Pretreatment with powders as well as HE extracts from RC hips and HH leaves, at both concentrations, did not significantly modify the level of IL-10 production induced by LPS in PBMCs ($p>0.05$). However, at both concentrations, all powders from SA bark and SN aerial parts significantly inhibited LPS-induced IL-10 production in PBMCs ($p<0.001$). Only HE extracts derived from SA bark, at the two concentrations, and that derived from SN aerial parts, at the highest concentration significantly diminished the production of IL-10 in LPS-stimulated PBMCs ($p<0.001$). Moreover, at the lowest concentration, all fine powders from SA bark significantly decreased the production of IL-10 in LPS-activated PBMCs compared to HE extracts ($p<0.001$) (Figures 1E, 2E, 3E, 4E).

Phytochemical composition

RC fine powders (100–180 μm and 180–315 μm) contained significantly higher amounts of catechin, procyanidin B1, procyanidin B2, gallic acid and hyperoside than hydroethanolic (HE) extract obtained from total plant materials (Table 1). However, miquelianin was only significantly more abundant in the 180–315 μm fraction. Overall, Table 1 shows that the characterized compounds were significantly more concentrated in the fine powders derived from 4 medicinal plants, which obtained by CDSp compared to HE extracts.

Discussion

In this study, anti-inflammatory effects of 3 fine powders (50–100 μm, 100–180 μm and 180–315 μm) derived from 4 medicinal plants, obtained by CDSp, as well as those of HE extracts, obtained from total plant materials, on LPS-induced inflammatory responses in primary mouse PBMCs were studied, by monitoring the production of inflammatory markers such as TNFα, IL-1β, IL-6, IL-10 and NO. Results revealed significant preventive effects of all powders in PBMCs treated cells, as highlighted by a significant decrease of LPS-induced inflammatory response. Our results also demonstrate for the first time that CDSp powders, particularly the superfine fractions (50–100 μm and 100–180 μm) possess a greater anti-inflammatory inhibiting activity than that of HE extracts. Equally demonstrated was the potential of CDSp to produce powders with higher concentration yield of phytochemicals from the 4 medicinal plants compared to those in the HE extracts of the same plants. These findings further confirm the concept behind CDSp, which is a novel green solvent-free process, that can be considered as a good alternative method to chemical extraction process which make use of organic solvents.

Production of inflammatory mediators such as TNFα, interleukins and NO have been demonstrated in several inflamed tissues [11, 12, 13]. Inflammation involves the release of other pro-inflammatory molecules including prostaglandins (cyclooxygenase products) and leukotrienes (lipoxygenase products) [14, 15, 16]. LPS, an inflammatory stimulus, has been widely used to induce inflammatory responses in immune cells [17, 18, 19]. We have found that exposure of PBMCs to LPS resulted in a significant production of pro-inflammatory mediators including TNFα, IL-1β, IL-6 and NO (Figures 2, 3, 4, 5). Our results showed that pretreatment of the PBMCs with different concentrations of CDSp fine powders, and in particular with the superfine powders, significantly inhibited the production of such pro-inflammatory compounds (Figures 2, 3, 4, 5). Suppression of LPS-induced TNFα, IL-1β, IL-6 and NO production in immune cells is used as a useful marker in determining the anti-inflammatory effect of drugs and plant extracts [19, 20, 21, 22, 23]. CDSp superfine powders have further exhibited greater anti-inflammatory effects in LPS-stimulated PBMCs than HE extracts (Figures 2, 3, 4, 5). These findings thus demonstrate the potential of the CDSp technique to obtain fractions of the 4 plants with improved anti-inflammatory activity. In this study, exposure of PBMCs to LPS resulted in a significant production of IL-10 (Figures 2, 3, 4, 5), an anti-inflammatory agent that with other cytokines such as IL-4 and IL-13 play together a role in antagonizing pro-inflammatory response [11, 24]. However, in our study, all HE extracts and fine powders from RC and HH did not significantly alter IL-10 levels (Figures 2, 3, 4, 5). Powders from SN and SA have rather reduced IL-10 levels (Figures 2, 3, 4, 5). These results suggest that anti-inflammatory activity mechanisms of the 4 medicinal plants did not involve the activation of anti-inflammatory IL-10 production. Pro-inflammatory cytokines production including TNFα, IL-1β and IL-6 involves the activation of signal transduction pathways such as nuclear factor-kB (NF-kB) and mitogen-activated protein kinases (MAPKs) [11, 22, 25]. High production of NO, a pro-inflammatory mediator, involves the induction of the inducible nitric

Table 1. Concentration (mg/g of dry mater) of major compounds in powder fractions and unground plant part extracts of *S. nodosa*, *H. helix*, *S. alba* and *R. canina*

		Scrophularia nodosa[#]				*Hedera helix*[#]			
Compound	*m/z*	50–100 μm	100–180 μm	180–315 μm	Total extract	50–100 μm	100–180 μm	180–315 μm	Total extract
Caffeic acid	179	0.037±0.003[a]	0.044±0.002[b]	0.041±0.003[b]	0.037±0.005[a]	/	/	/	/
Quercitrin	447	0.041±0.002[a]	0.052±0.003[b]	0.049±0.002[b]	0.026±0.002[c]	/	/	/	/
Isoquercetin	463	0.21±0.02[a]	0.26±0.03[b]	0.26±0.02[b]	0.14±0.05[c]	/	/	/	/
Harpagoside*	493	29.32±0.52[a]	28.56±0.47[a]	25.17±0.50[b]	25.6±0.03[b]	/	/	/	/
Rutine	609	0.61±0.03[a]	0.63±0.02[a]	0.63±0.03[a]	0.39±0.07[b]	0.82±0.04[a]	0.86±0.04[a]	0.79±0.03[a]	0.63±0.03[b]
Verbascoside*	623	4.36±0.15[a]	5.05±0.20[b]	4.47±0.17[a]	3.30±0.08[c]	/	/	/	/
Chlorogenic acid	353	/	/	/	/	1.87±0.08[a]	1.86±0.07[a]	1.73±0.05[b]	1.57±0.05[c]
3,5-Dicaffeoylquinic acid	515	/	/	/	/	2.55±0.9[a]	2.54±0.11[a]	2.31±0.09[b]	1.82±0.07[c]
Kaempferol-rutinoside	593	/	/	/	/	0.22±0.01[a]	0.22±0.01[a]	0.20±0.02[a]	0.14±0.01[b]
α-Hederin*	795	/	/	/	/	1.04±0.04[b]	1.15±0.06[a]	1.10±0.03[b]	0.49±0.02[c]
Hederacoside C*	1265	/	/	/	/	3.99±0.12[a]	4.11±0.14[a]	3.45±0.12[b]	1.59±0.09[c]
		Salix alba[##]				*Rosa canina*			
Compound	*m/z*	50–100 μm	100–180 μm	180–315 μm	Total extract	50–100 μm	100–180 μm	180–315 μm	Total extract
Catechin	289	1.75±0.07[a]	1.60±0.07[b]	1.46±0.05[c]	1.35±0.04[d]	NR	0.13±0.02[a]	0.13±0.01[a]	0.025±0.007[b]
Chlorogenic acid	353	0.072±0.004[a]	0.076±0.006[a]	0.073±0.005[a]	0.054±0.003[b]	NR	/	/	/
Salicin	331	1.68±0.05[a]	1.52±0.05[b]	1.47±0.03[b]	1.45±0.05[b]	NR	/	/	/
Gallocatechin	305	3.6±0.08a	3.14±0.09[b]	2.25±0.06[c]	1.74±0.05[d]	NR	/	/	/
Procyanidin B1	577	5.87±0.11[a]	4.93±0.12[b]	3.79±0.10[c]	3.48±0.10[d]	NR	1.13±0.04[a]	1.09±0.06[a]	0.080±0.009[b]
Procyanidin B2	577	3.42±0.10[a]	3.24±0.07[b]	2.83±0.07[c]	2.29±0.09[d]	NR	0.20±0.02[a]	0.21±0.02[a]	0.025±0.01[c]
Procyanidin C1	865	1.81±0.05[a]	1.43±0.03[b]	1.20±0.03[c]	1.07±0.04[d]	NR	/	/	/
Gallic acid	169	/	/	/	/	NR	0.10±0.008[a]	0.11±0.009[a]	0.042±0.005[b]
Hyperoside	463	/	/	/	/	NR	0.15±0.01[b]	0.21±0.02[a]	0.12±0.009[c]
Miquelianin	477	/	/	/	/	NR	0.082±0.008[b]	0.12±0.01[a]	0.091±0.007[b]

Data are expressed as mean±standard deviation of triplicate measures. Values in one row not sharing the same letters are significantly different ($p<0.05$); the symbol "/" indicates that the compound was absent in the concerned plant; *m/z* indicated the ratio of mass *versus* charge. *Detected under the form of formic acid adduct; NR means not realized measures because the amount of this class powder was insufficient; Total extract was obtained via hydroethanolic extraction of ground and unsieved plant parts. [#] and [##] Sources from Zaiter et al. [3, 4], respectively.

oxide synthase (iNOS) [22, 26]. Thus, it is more plausible that anti-inflammatory activity of the 4 medicinal plants, used in this study, may result rather from inhibition (or attenuation) of iNOS, NF-kB and MAPK activation. In this context, several studies have highlighted that plant extracts and plant ingredients have the possibility to attenuate LPS-induced inflammatory response, by interfering with iNOS, NF-kB and MAPK pathways [22, 25, 26]. In addition, greater anti-inflammatory effects of the superfine powders as compared to HE extract and 180–315 μm powder fraction could be also explained by their higher antioxidant effects, as emphasized in our previous studies [3, 4]. In this respect, several studies have demonstrated a close relationship between anti-inflammatory effects of plant extracts as well as drugs and their antioxidant effects *in vivo* [14, 27, 28, 29].

In our previous studies, *in vitro* differential bio-effects were seen among the 3 CDSp fine powders derived from these 4 studied medicinal plants, as the superfine powders (i.e. 50–100 μm and 100–180 μm) exhibited better results in term of antioxidant activity in a cellular assay [3, 4]. The two concentrations of 250 μg/mL and 500 μg/mL have been chosen in this study, as they were the best dosages that permitted to differentiate between CDSp fine powders and HE extracts in term of cellular antioxidant activity [3, 4]. Indeed, compared to HE extracts, greater antioxidant and cytoprotective effects were found with the superfine powders against H_2O_2-induced oxidative stress in mouse primary spleen cells, highlighted by higher ROS elimination, increased GPx activity and lower MDA production [3, 4]. Overall, our precedent works have shown that grinding and sieving permit to obtain better results in terms of activity as well as in terms of bioactive compound concentrations compared either to unground plant materials [6, 7, 8] or unsieved powders [2, 3, 4, 5]. In this respect, total phenolic content in SA bark powders (20–500 μm), was significantly higher in the 20–50 μm and 50–100 μm fractions, followed by 100–180 μm and 180–315 μm fractions [7]. However, these two last fractions exhibited, in HH leaves, the highest total phenolics and total flavonoids compared to the other fractions (i.e. 20–50 μm, 50–100 μm, 315–500 μm and >500 μm) [2]. Nevertheless, SN aerial plant parts showed

no significant difference in total phenolics among the 3 studied fractions (50–100 μm, 100–180 μm and 180–315 μm) [2]. Regarding the concentration of phytochemicals, Table 1 shows that CDSp provided significantly enhanced yields than the conventional hydroethanolic method. In fact, except for caffeic acid in 50–100 μm fine powder from SN aerial plant parts, salicin in the 2 fractions with the highest particle size (i.e. >100 μm) from SA bark and miquelianin in 100–180 μm fine powder from RC hips, the concentration of all phytochemicals detected in the 4 medicinal plants by LC-PDA-ESI/MS analyses were significantly higher in the fine powders compared to HE extracts that obtained from total plant materials. These findings supported that CDSp is a favorable method that in addition to its potential to increase bioactivity, it also enhances phytochemical concentrations.

Conclusion

In summary, our results demonstrated that CDSp increased anti-inflammatory activity and enhanced phytochemical concentrations in the superfine powders obtained from medicinal plants compared to a conventional hydroalcoholic extraction. Thus, CDSp, which is a novel green solvent-free process, could be a good alternative method to chemical process employing organic solvents. Although this study was realized on primary cells, which have the advantageous to be physiologically normal, retaining *in vitro* many if not all native cellular functions, and thus may provide unaltered even "natural" experimental results [3, 4]; the limitations of single-cell-based models justify *in vivo* research on animal models to investigate in depth the bioactivity of fractions from CDSp.

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