Anti-inflammatory Properties of an Aldehydes-enriched Fraction of Grapefruit Essential Oil

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ABSTRACT: Chronic inflammation is linked to the development of numerous diseases and is accompanied by increased cytokine secretion. Macrophages provide a first line of defense against pathogens which under inflammatory stimuli release pro-inflammatory cytokines. The essential oil (EO) fractions obtained from Citrus spp. rich in different compounds have gained the attention of both researchers and users during the last decades. In particular, Grapefruit (Citrus paradisi) peel is rich in phenolics and flavonoids with several health benefits, including anti-inflammatory actions. Additionally, its EO consists of a large number of compounds such as terpenes, sesquiterpenes, hydrocarbons, alcohols, aldehydes, esters, and oxides. Among the methods for encapsulating EOs, spray-drying is the main one. In the present study, we aimed to determine the in vitro anti-inflammatory activity of EO from C. paradisi (GEO) (whole and fractions) in a LPS-induced inflammation model. Results indicate that Fr-GEO and Fr-GEO_SD exert protective effects against LPS-induced inflammation by decreasing gene expression and levels of pro-inflammatory cytokines as IL-6 and TNF-α. Terpenes as the most common components, as well as aldehydes and sesquiterpene might be responsible for such effects, although a synergistic action is not excluded. Furthermore, a higher percent of aldehydes is linked to improved olfactory properties. Our findings support the anti-inflammatory effects of selected Fr-GEO with a great potential for the development of new nutraceuticals and/or functional food for the treatment of inflammatory-associated diseases.

Practical Application: The findings of this study support the anti-inflammatory effects of selected Fr-GEO with a great potential for the development of new nutraceuticals and/or functional food for the treatment of inflammatory-associated diseases.

Keywords: aldehydes; citral; Citrus paradisi; essential oil; inflammation; nutraceuticals; spray-dry.

1. Introduction

Inflammation represents a physiological response of the immune system to pathogens or tissue destruction however, under specific conditions, it may progress into a chronically pro-inflammatory state. Inflammation is an underlying and/or established risk factor for several diseases even without clinical symptoms, and, finally, it may lead to tissue damage. Such states are also characterized by deregulated cytokine secretion and macrophages are directly involved in these processes (Li et al., 2011). Under inflammatory stimuli, macrophages release pro-inflammatory cytokines and other inflammatory mediators, such as tumor necrosis factor α (TNF-α), interleukin (IL)-1, IL-6, and IL-1β (Arango Duque & Descoteaux, 2014). Briefly, TNF-α is a necessary and sufficient mediator of local and systemic inflammation (Liedtke & Trautwein, 2012). IL-6, known as a traditional marker of inflammation, promotes the induction of acute-phase proteins, but it may be also involved in the regulation of the transition from acute to chronic inflammation and stimulation of T and B lymphocytes (Dienz & Rincon, 2009). Both IL-1β and TNF-α play an important role in the onset of inflammatory processes that regulate the expression of other cytokines and chemokines (Arend et al., 2008).

Macrophage activation by lipopolysaccharide (LPS), a major component of Gram-negative bacteria's outer membranes, results in increased levels of pro-inflammatory cytokines, thus leading to the cytotoxic and pro-apoptotic mechanisms which belong to the innate response in several mammals (Bosca et al., 2005). On the other hand, such cytokine overproduction by activated macrophages is involved in the pathophysiology of various inflammatory diseases. Therefore, LPS-stimulated macrophages provide a useful model for studying inflammation as

well as the potential mechanisms of action of anti-inflammatory compounds (Oishi & Manabe, 2018).

In the Mediterranean diet *Citrus* fruits and their derivatives are highly consumed and very extensive studies over the last years have reported their numerous health properties, such as anti-inflammatory, neuroprotective, anti-aging, antimicrobial, and anticancer effects, highlighting their role in the prevention and management of chronic diseases (Gandhi et al., 2020; Klimek-Szczykutowicz et al., 2020). Grapefruit (*Citrus paradisi. L*) belongs to the *Citrus* genus, family *Rutaceae*, uniquely recognized among other *Citrus* species by its taste, odor, and utilities characterized by its content in organic acid, sugar, and phenolic compounds (Mohammed et al.). In recent years, numerous evidence have highlighted Grapefruit's nutritional and antioxidant properties, indicating several *Citrus* compounds to be responsible for anti-inflammatory, anticancer, antiviral, antiallergenic as well as analgesic activities (Cristobal-Luna et al., 2018; Mohammed et al.).

Essential oils (EOs) are natural volatile complex compounds characterized by a strong scent and produced by aromatic plants as secondary metabolites (Aziz et al., 2018). More than 3000 EOs have been identified, and approximately 10% of them are commercially important, particularly in the pharmaceutical, food, and cosmetic industries (Mitoshi et al., 2014). Over the past century their anti-inflammatory, antioxidant, antitumor, and anti-bacterial properties have been largely reported (Mancianti & Ebani, 2020). Specifically, an accumulating body of evidence has described the anti-inflammatory effect as well as the chemical composition of EO from *Citrus* (Pucci et al., 2020), and there is a continuous research interest in novel and safer natural products containing such EOs. Grapefruit EO (GEO) is called the "dieter's friend" because of its

anti-obesity effects, as it facilitates lipolysis, inhibits adipogenesis, promotes body cleansing and removal of toxins and excess fluids, but also it exerts strong antioxidant, antibacterial, antifungal, and anticancer activity (as reviewed in (Dosoky & Setzer, 2018; Gonzalez-Mas et al., 2019; Li et al., 2022)).

To have healthier, balanced, and more natural and efficacy food products and nutraceuticals, the use of unique drying processing technology as a method for preservation, pulverization, and microencapsulation is increasing and, in this year, it has reached the 150th anniversary of its invention (Assadpour & Jafari, 2019; Seid Mahdi & Katarzyna). Spray drying represents a capable method for the protection of functional oils, with more than 90% microencapsulation efficiency, high oxidative stability, longer shelf life, but also low operational cost, compatibility with labile materials and large-scale production (Aguiar et al., 2020; Jafari & Rashidinejad, 2021). The formulation of grapefruit powder by spray drying is of great interest with high potential as a nutraceutical food due to several bioactive compounds and its potential health effects (Gonzalez et al., 2019). Novel therapeutic strategies target both pro-inflammatory and pro-oxidant mediators and, consequently, an increasing number of studies have focused their attention on developing inhibitors from natural resources to prevent or mitigate chronic inflammatory conditions, and that can be used with minimal side effects (Boukhatem et al., 2020; Pucci et al., 2020; Qu et al., 2020; Song et al., 2021).

The present study aimed to investigate the anti-inflammatory activity of aldehydes-enriched fractions of Grapefruit Oil (Fr-GEO), which due to their improved aroma profile may represent food additives of commercial importance, and also when added to food products, may provide additional health benefits. In this regard, spry-dried (SD) GEO was obtained by cold-pressed

114 extraction from the fresh fruit, and the Fr-GEO was isolated by chromatographic technique.
115 LPS-stimulated murine and human macrophages were used to investigate the anti-
116 inflammatory effects of whole and Fr-GEO, while Fr-GEO_SD were tested only in human
117 macrophages.
118

2. Materials and Methods

2.1 Purification of Enriched Fractions from Grapefruit Essential Oil

Grapefruit essential oil (GEO) was obtained by cold extraction from fresh fruit at the company Agrumaria Corleone S.P.A.(AgruCo) (Palermo, Italy). Fractions enriched in aldehydes were obtained by a new column chromatography method according to the previously described protocol that is currently covered by trade secret (Pucci et al., 2020).

2.2 Qualitative and quantitative Analysis of Essential Oils: Gas Chromatography (GC-MS and GC-FID) Analyses

The volatile portion of whole grapefruit oil (GEO) and each extracted fractions were diluted in hexane at a ratio of 1:10 and then was analyzed on the Agilent 6890 N Network gas chromatograph (GC) coupled with an Agilent 5973 mass spectrometer (Agilent Technologies Inc., Santa Clara, CA, USA) with a DB-5MS fused silica column (30 m × 0.25 mm ID, 0.25 µm film thickness, Agilent Technologies Inc., Santa Clara, CA, USA). The oven program and all other technical details have been reported before (Pucci et al., 2020). The identification of each compound in each sample was determined by comparing the mass spectrum with a library of NIST MS Search and Wiley 138 mass spectra, as well as with literature data. A GC FID analysis, which allowed us to quantify the compounds present in grapefruit essential oil, was performed with the Agilent 7890 A GC (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a DB-5 nonpolar capillary column (Agilent Technologies Inc., Santa Clara, CA, USA) (length: 20 m; inner diameter 100 µm; film thickness 0.1µm). The oven temperature program and all other technical details have already been reported in our previous paper (Pucci et al., 2020). GC-FID analysis of the sample furnished the relative percentage amount of the individual components contained in the analyzed

141 samples. The value of each analyte is expressed as the percent area of the peak relative to the total
142 composition of the EO obtained from the GC-FID analysis.
143
144 **2.3 Preparation of grapefruit oil emulsion and spray dryed**
145 To produce Fr-GEO in spray dryed form (Fr-GEO_SD), an aqueous emulsion was prepared containing the
146 aldehyde-enriched fraction of pink grapefruit essential oil (which will form the core material),
147 maltodextrin (Nutriose FM06, Roquette) and gum arabic (which will form the wall material) according to
148 the following proportion: Oil:Maltodextrin:Gum arabic = 1:2:1. The percentage of GEO in the solution
149 was 5%.
150 An amount of 500 g of grapefruit emulsion was spray-dried using a small-scale laboratory spray drier
151 (Bu¨chi Mini Spray Dryer B-290) with a yield of 60%. The drier operates co-currently and has the dual
152 fluid nozzle with an orifice having a diameter of 0.7 mm. The conditions of spray drying were: Inlet
153 temperature: 150-165°C, outlet temperature: 90C, peristaltic pump: 20-25%, Aspirator: 100%, Flow air:
154 65mm (800L/h) (this value was adjusted regularly to keep the outlet drying temperature constant). Once
155 the water is evaporated during the process, the final product will contain 25% of GEO. In our
156 experiments, a blank powder containing the above indicated proportions of Maltodextrin:Gum Arabic
157 (2:1), without Fr-GEO, was used as control .
158
159 **2.4 Cell culture and treatment**
160 The murine macrophage RAW264.7 and the human monocyte THP-1 cell lines, (ATCC, Manassas, VA,
161 USA) were respectively cultured in DMEM (supplemented with 10% FBS, 2mM L-glutamine, 100U/mL
162 penicillin, and 100 µg/mL streptomycin) and in RPMI-1640 medium (supplemented with 10% FBS, 2mM
163 L-glutamine, 100U/mL penicillin, and 100 µg/mL streptomycin (Euroclone, UK), and 0.05 mM 2-
164 mercaptoethanol). To obtain differentiated M0 macrophages (THP-1 M0), THP-1 human monocytes

were seeded at 1 x 10^5 cells/mL and incubated in the presence of Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Saint Luis, MO, USA, 50 ng/mL) at 37 °C with 5% CO2 for 48 hours (Shiratori et al., 2017). Afterward, the medium containing PMA was replaced with a fresh medium for 3 days for cell recovery.

GEO and Fr-GEO were diluted in a solution consisting of 95% FBS and 5% DMSO with a final concentration of 0.005%, 0.01%, and 0.02% used to treat RAW264.7 and THP-1 M0. The final DMSO concentration in the cell cultures were no greater than 0.25%; this concentration did not affect cell viability. DMSO treated cells were used as a control (CN) in cell viability assay.

Spry-dried (SD) formulation of Fr-GEO (Fr-GEO_SD) containing 25% of Fr-GEO and 75% of biopolymers (maltodextrin and Arabic gum) was diluted in properly supplemented RPMI-1640 medium to get the final Fr-GEO concentrations of 0.005%, 0.01%, and 0.02%. Also, blank SD (containing only biopolymers) was diluted in properly supplemented RPMI-1640 medium with a final concentration of biopolymers equivalent to that used in the working diluitions of Fr-GEO (0.005%, 0.01%, and 0.02%).

For evaluating the anti-inflammatory activity of both Fr-GEO and Fr-GEO_SD, cells were pre-treated for 2 hours with Fr-GEO and Fr-GEO_SD, and then exposed to LPS (500 ng/mL for RAW264.7 cells and 1 µg/mL for THP-1 M0 cells) for further 6 hours (total 8 hours). The cells treated only with LPS were used as a positive control of inflammation induction.

2.5 Cell viability assays

RealTime-Glo™ Cell Viability Assay (Promega Italia S.r.l, Milan, Italy) was used to determine RAW264.7 and THP1 M0 cell viability after 24, 48 and 72h of treatment with GEO and Fr-GEO (0.005%, 0.01%, 0.02% and 0.05%), following the manufacturer's instructions. The fluorescence, proportional to the percentage of viable cells, was measured by Glomax (Promega).

3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was used to test cell viability after treatments with Fr-GEO_SD as previously described (Raimondo et al., 2015). Briefly, THP-1 M0 were plated in 48-well plates in triplicate at 2 x 10^4 cells/well and exposed to escalating doses (0.005%, 0.01%, 0.02%) of GEO_SD and Fr-GEO_SD for 24 and 48 hours. The absorbance was measured at 540 nm (Microplate Reader, BioTek, Winooski, VT, USA).

For both assays, means and standard deviations are generated from three independent experiments and values are expressed as a percentage of cell growth versus control (untreated) cells.

2.6 RNA Isolation

RAW264.7 cells were cultured at 5 x 10^4 cells/well, while THP-1 M0 at 1 x 10^5 cells/well in 12-well plates. After 24 h both cell lines were treated with Fr-GEO as described above in detail, while only THP-1 M0 were treated with Fr-GEO_SD. Commercially available Illustra RNA spin Mini Isolation Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was used to isolate total RNA, following the manufacturer's instructions. The total RNA concentration was detected using a Nanodrop spectrophotometer (Thermo Fisher®, USA). Then, total RNA from both cell cells was reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA).

2.7 Real-Time PCR

Gene expression analysis of IL-6 and TNFα was performed using Real-time polymerase chain reaction (RT-PCR). For quantitative SYBR Green Real-time PCR, the reaction was carried out in a total volume of 20 µL containing 2X SYBR Green I Master Mix (Applied Biosystems), 2 µL of cDNA, and 300 nM forward and reverse primers. The sequences of oligonucleotides used are listed in Table 1.

RT-PCR was performed in 48-well plates using the Step-One Real-Time PCR System (Applied Biosystems). Relative changes in gene expression between control and treated samples were determined using the

2(-Delta Delta C(T)) method. Levels of the target transcript were normalized to a GAPDH (or beta-actin) endogenous control. Final values were expressed as fold change.

2.8 Enzyme-Linked ImmunoSorbent Assay (ELISA) Assays

THP-1 M0 cells were seeded at a density of 1 x 10^5 cells/well in 12-well plates and after 24 hours were pre-treated with different doses of Fr-GEO (0.005%, 0.01%, and 0.02%) and Fr-GEO_SD (0.005% and 0.01%) for 2 hours and, then, exposed to LPS (1 μg/mL) and incubated without medium change for a further 6 hours (total 8 hours). After that, the cell-conditioned medium was collected and centrifuged to remove floating cells and cellular debris. The amounts of pro-inflammatory cytokines, IL-6 and TNF-α in the conditioned medium were determined using the IL-6- and TNF-α- specific ELISA kits (Thermo Fisher Scientific, Waltham, MA USA) according to the manufacturer's instructions.

2.9 Statistical Analysis

Data were obtained from at least three separate experiments and are represented as means ± SD. Statistical analysis was done using a Student's t-test and the differences were considered statistically significant when $p < 0.05$.

3. Results

3.1 Analysis of the volatile compounds of isolated fraction from GEO

Aldehydes and aldehyde-derived compounds are good candidates for the development of anti-inflammatory compounds useful not only for scientific research but also for the agro-food industry. In this work, our focus was on two fractions of grapefruit essential oil enriched with 68% aldehyde-derived compounds (Fr-11 + Fr-12) compared to the whole oil. As shown in table 2, the two fractions have a comparable chemical profile, so we chose to combine them in a single solution (Fr-GEO) to treat two different cell lines and test their biological properties. From the combination of GC-MS and GC-FID analysis (Table 2), we selected and quantified 42 compounds that contribute mostly to the aroma of grapefruit essential oil.

These compounds were divided into 6 chemical classes (Monoterpenes, Total aldehydes, Citral, Sesquiterpenes, Esters, and Alcohols) and were expressed as a percentage of the total area of each peak (Table 3). The results show, comparing the whole GEO with the fractions, an increase of 68% in total aldehydes and about 50% in Citral and 10% in Sesquiterpenes observed in each of the two selected fractions; no differences were found for monoterpenes, esters and alcohols.

To ensure the reproducibility of the process, for each sample, three analyses were carried out. In the three technical replicates of the fractionation process, we found that 63% of the compounds showed a coefficient of variation (CV%) ≤10 and 27% of the compounds showed a CV% ≤5 in fraction 11. In fraction 12, the percentage of compounds showing a CV% ≤10 is 46%, while 51% of the compounds have a CV% ≤ 5. CV% higher than 10% was not found for any

compound. Collectively, these results show that the fractionation process is highly reproducible (Figure 1).

Internal olfactory panel tests showed that fractions 11 and 12 had improved their aromatic properties compared to the whole GEO (data not shown), thus indicating that the increase in aldehydes results in better olfactory characteristics.

3.2 Viability of the murine and human macrophage cells after treatment with GEO and Fr-GEO

The viability of RAW264.7 and THP1 M0 cells exposed to increased doses of GEO anf Fr-GEO (0.005%, 0.01%, 0.02% and 0.05%) was evaluated for 24, 48 and 72H with RealTime-Glo™ Cell Viability Assay. As reported in the Figure 2A, we found that the tested doses of Fr-GEO did not exerted cytotoxicity, defined as a cell viability below 80%, in both cell types as compared to untreated control cells (dashed lines in the histograms). Conversely, the higher dose of GEO (0.05%) showed to have a significant cytotoxic effect on RAW264.7 at all observed time, and on THP1 M0 all doses already after 24h induced a reduction below 80% of cell viability (Figure 2B). Based on the obtained data, the doses 0.005%, 0.01% and 0.02% of Fr-GEO were used for the subsequent analyses to evaluate the anti-inflammatory activity.

3.3 Anti-inflammatory properties of Fr-GEO

To assess the anti-inflammatory effects of the aldehyde enriched fraction of GEO (Fr-GEO), RAW264.7 and THP1 M0 cells were pre-treated for 2 hours with 0.005%, 0.01%, 0.02% of Fr-GEO, and then with LPS for 6h (the dose 500 ng/ml RAW264.7 and 1000 ng/ml THP1 M0, respectively). The cells treated only with LPS were used as a positive control.

As reported in the histograms in figure 3A, we found that compared with the treatment with only LPS, the pre-treatment with Fr-GEO (specially the dose of 0.02%) induced in both RAW264.7 and THP1 M0 cells a significant change of IL-6 and TNF-α mRNA levels.

The anti-inflammatory effect of Fr-GEO was confirmed by evaluating the modulation of both inflammatory cytokines at protein level. The results obtained by the ELISA assay showed that the pre-treatment with 0.02% of Fr-GEO decreased the release of both IL-6 and TNF-α proteins (Figure 3B).

3.4 Treatment of human Macrophage cells with a spray dryed form Fr-GEO

In order to obtain a more stable and soluble formulation of the Fr-GEO, we have powdered it by spray drying (Fr-GEO_SD). After evaluating by the MTT Assay the non-toxicity of the used Fr-GEO_SD doses (Figura 4A), we assesed if the SD formulation of Fr-GEO kept the anti-inflammatory effects. The qRT-PCR and ELISA assay (Figure 4B and 4C respectively) confirmed that the pre-treatment of THP1 M0 cells with Fr-GEO_SD significantly counteracted, at both mRNA and protein levels, the LPS-induced expression of IL-6 and TNF-α. The lack of effect of the blank powder clearly indicated that anti-inflammatory activity was spefically due to the presence of the Fr-GEO in the spray-dried. It was interesting to note that in comparison to the liquid formulation the SD enhanced the anti-inflammatory effects of GEO. Indeed, we found that in human macrophages the dose of 0.02% was more effective compared to the liquid form; interestingly we found a strong reduction of IL-6 mRNA expression levels already with the dose 0.005%, and of TNF-α with the dose of 0.01% (Figure 4B). To further validate this observation, the levels of IL-6 and TNF-α protein in the conditioned medium of cells treated with 0.005% and

0.01% doses of Fr-GEO_SD were measured by ELISA. According to the qRT-PCR data, also at protein level the SD formulation showed a stronger activity than the liquid form. As shown in Figure 4C, when treated with the Fr-GEO_SD, the human macrophages showed a reduction of 61% for IL-6 and of 72.4% for TNF-α with the dose of 0.005%, against the ineffectiveness of the corresponding dose of the liquid Fr-GEO (Figure 3B), and of 91.4% for IL-6 and 46% for TNF-α with the dose 0.01 against the reduction of 42% for IL-6 induced by the same dose of the liquid Fr-GEO (Figure 3B). Overall the data reported encourage the use of the Fr-GEO spray dryed form.

4. Discussion

The composition of EOs is characterized by predominant volatile compounds of terpenes (the most common are limonen, myrcene, citral and terpin), but also hydrocarbons, alcohols, aldehydes, esters and oxides, with well-known healthy properties such as anti-inflammatory, antioxidant and antiinfective (Gonzalez-Mas et al., 2019; Lombardo et al., 2020; Mitropoulou et al., 2017; Quintans et al., 2019). Compared to other *Citrus*, GEOs are rich in monoterpenes with the predominance of d-limonene (from about 65% to about 95.9%) (Dosoky & Setzer, 2018), and some sesquiterpenes, which are responsible for their typical tart flavor. In addition to its nutritional supplements, grapefruit waste (leaf and peel) has been suggested to be an essential part of the diet due to its several actions against different illnesses (Miya et al., 2021). In our study, the anti-inflammatory effects of the whole GEO, Fr-GEO and Fr-GEO_SD were evaluated in a suitable *in vitro* experimental model of an inflammatory state. Our data show a significant reduction of IL-6 and TNF-α expression by Fr-GEO and Fr-GEO_SD, which was also confirmed by ELISA carried out for quantitative detection of the two pro-inflammatory cytokines. Monoterpenes, among which limonene, as is the major chemical constituent in GEO (more than 90%) [33-36], linalool, linalyl acetate, and α-terpineol, are responsible for such efficiency (de Cassia da Silveira e Sa et al., 2013; Huang et al., 2019; Wojtunik-Kulesza et al., 2019). Limonene has been recognized as a potent anti-inflammatory compound according to *in vivo* animal and human studies (d'Alessio et al., 2013; Rehman et al., 2014). Some authors reported that the main ingredients of *C. paradisi* oil (with the highest percent of limonene, then β-myrcene, and α-pinene) were consistent with those of different sorts of cold-pressed citrus peel oils (also with the predominance of limonene, then γ-terpinene, α-pinene, and myrcene) (Ou et al., 2015). The

GEO used in our study were obtained from grapefruit peel and limonene was also the major component among monoterpenes content. Interestingly, in an *in vitro* study where the cells were treated with volatile compounds of orange juice (Held et al., 2007), the authors found that α-terpineol effectively inhibited IL-6, while limonene showed a stimulating effects on IL-6 production. Further, it has been reported that phellandrene was the dominating compound in the leaf oils of three varieties of *C. paradisi* from South Africa ("Rose Pink", "Ruby Red" and "White Marsh") (Miya et al., 2021). The same authors also observed the anti-inflammatory potential of the oils, among which the "Rose Pink" peel oils had the highest and fastest efficacy (reducing inflammation within the first two hours compared to those after the 3rd and 4th hours with other color variants "White Marsh" and "Ruby Red", respectively) (Miya et al., 2021). These findings are somewhat similar to ours, given that Fr-GEO contained a higher percent of phellandrene compared to the whole GEO, but also of other monoterpenes such as geranyl acetate as well as the citral which content was increased up to 50%; all of them well known by their anti-inflammatory effects (Goncalves et al., 2020; Nadia et al., 2021). Hence, the anti-inflammatory activity of Fr-GEO seen in the present study, at least in part, could be attributable to the monoterpenes. However, other dominant compounds in Fr-GEO (aldehydes and sesquiterpene) should not be underestimated. Aldehydes and fatty alcohol are extensively found in different fruits and plants and are responsible for the aroma of plant EO, a better smeller, but also the anti-inflammatory activity (Foudah et al., 2021; Heidari et al., 2016). Fr-GEO used in the present study was enriched in aldehydes such as octanal, nonanal, citronellal, undecanal noted by their anti-inflammatory effects (Choi et al., 2020; Foudah et al., 2021; Uchikawa et al., 2020; Xiao et al., 2017). Nootkatone (NKT), a widely described sesquiterpenoid

compound isolated from grapefruit, inhibits the expression of pro-inflammatory and increased the expression of the anti-inflammatory cytokines (Choi et al., 2014; Park et al., 2021). NKT was present in the whole GEO, while its percent was lower in Fr-GEO compared to the whole GEO. However, Fr-GEO contained a higher percentage of another sesquiterpene, such as β-caryophyllene, which protective effects in chronic inflammation are well recognized (Scandiffio et al., 2020). NF-κB and PKA/CREB signaling pathways have been suggested to mediate such anti-inflammatory mechanisms. Similar mechanisms were suggested by studies *in vivo* in a mouse model (Nemmar et al., 2018; Tsoyi et al., 2011; Wang et al., 2018). In addition, the results from one *in vivo* study indicate that EOs from four *Citrus* species (*C. limon, C. latifolia, C. aurantifolia or C. limonia*) act similarly to immunomodulators in reducing cell migration and inflammatory mediator production (Amorim et al., 2016).

On the other hand, it is known that grapefruit peel is rich in flavonoids (such as flavanones, flavanone glycosides, and polymethoxylated flavones) with known anti-inflammatory and anti-oxidant properties that mainly have been attributed to naringin, naringenin, limonin, and quercetin (Heidary Moghaddam et al., 2020; Jain & Parmar, 2011; Tahaghoghi-Hajghorbani et al., 2019), indicating their benefits for the treatment of obesity, diabetes, hypertension, and metabolic syndrome, although the therapeutic uses of these flavonoids are limited by the lack of adequate clinical evidence (Alam et al., 2014). Interestingly, naringenin is structurally similar to the extensively studied polyphenol resveratrol, and also has hypolipidemic properties (Saenz et al., 2018); it is responsible for the grapefruit's bitter taste, and together with hesperidin, has been recognized as a major bioactive constituent responsible for the anticancer effects of grapefruit (Hung et al., 2017). The anti-inflammation activity of Fr-GEO and Fr-GEO_SD seen in

the present study may be a result of a complex interaction among their various chemical compounds, which might produce synergistic or additive effects, even for those present at low concentrations. Also, we do not exclude the possibility that such effects may be linked to the known antioxidant potential of grapefruit (Miya et al., 2021), which has not been tested in the present study. In addition, the anti-inflammatory activities of EOs are often related to their analgesic properties (de Cassia da Silveira e Sa et al., 2013), which further support their use in health care, but also potential clinical significance of our findings. Further, given that the consumption of grapefruit has decreased due to the interference of some compounds with drug absorption, there is a high need for obtaining grapefruit-like citrus varieties without such unfavorable effects (Garcia-Lor et al., 2021). Moreover, playing an important role in the onset and constancy of the inflammatory process, some pro-inflammatory cytokines, such as IL-6 and TNF-α (Kany et al., 2019) are important targets for developing new anti-inflammatory agents. Our results show the capacity of Fr-GEO and, particularly Fr-GEO_SD to regulate such pro-inflammatory cytokines.

It should be mentioned that the chemical composition of *Citrus* oils is different and depends on origin, season, ripening stage, climate, method of extraction, genetic background, age, etc. (Dosoky & Setzer, 2018; Mohammed et al.). In addition, it has been shown that environmental conditions and maturation may influence the amount of the compounds which interfere with drugs, and also there are differences in its mount between harvest times and seasons, that, further, all together make evaluation and selection of new varieties more difficult in practice (Garcia-Lor et al., 2021). The novelty of the present study is that GEOs were obtained from grapefruit peel using a particular type of stationary phase and Fr-GEO with high content in

aldehydes, thus influencing the product quality, its biological effects and making the combination of volatile compound unique compared to what has been already reported in the literature.

It is known that spray drying allows a one-step micronization and encapsulation of phytocomplexes within a vehicle increasing the bioavailability, the absorption, and the efficacy of nutraceuticals and it is attracting the interest of not only the food industry but also pharmaceutical and agrochemical industries (Rajam & Anandharamakrishnan, 2015). Of importance, this method is used in the design of micro delivery systems with increased the on-target and preserved off-target delivery (Almansour et al., 2022). Furthermore, a juice of extremely high sensorial quality can be obtained by rehydration of powdered fruit obtained by spray-drying; the rehydration of spray-dried grapefruit exerts the properties significantly different compared to commercial and natural juices (Martinez-Navarrete et al., 2019). Such evidence together with our data regarding SD formulation of GEO supports commercial production of fruit powder with healthy properties that further can be rehydrated and used to obtain a good-quality juice. The majority of the currently available publications of SD formulation are on vegetables and some fruits (Verma & Singh, 2015), while data on grapefruit (and citrus overall) are very limited and mostly focused on processes of spray-drying itself. Interestingly, it has been shown that pulverized grapefruit (*Citrus paradisi Macf.*) mesocarp at different doses (0.4, 0.8, 1.2, 1.6, and 2.0%) significantly influenced the composition of the encapsulated lemon juice, except the protein content (Alcantara Marte et al., 2018). In addition, the majority of the available data indicates high content in phenols and flavonoids as a source of oxidative protection, while, to the best of our knowledge, this is the first report

showing anti-inflammatory effects of GEO_SD. Yet, to date, little data exist regarding EOs and a spray-drying process, mainly on orange EO (Aguiar et al., 2020), that further highlights the novelty of our study and a need for future investigation *in vitro* and *in vivo*, including the development of a new nutraceutical and its use in clinical settings. Our group previously reported that a natural product in a SD formulation containing plant-derived extracellular vesicles modified two important risk factors in healthy volunteers (Raimondo et al., 2021).

Finally, the evidence from the present study increases the availability of data on GEO's benefits and its important role in the development of nutraceutical products, including its SD form. The combination of the selected Fr could not only improve the health but also might decrease the interaction with the drug interference, which remains to be tested in the future. Our data further strengthen the therapeutic potential of this natural product and the base for future investigation in the direction of personalized medicine, given that grapefruit-derived nanovectors, if coated by inflammatory-related receptors, could be directed to inflammatory or tumor sites, and may exhibit some potential in drug delivery (Wang et al., 2015).

5. Conclusion

The data obtained in the present study indicates that the fraction of pink grapefruit (Fr-GEO) shows a higher ability to decrease the levels of pro-inflammatory cytokines (IL-6, TNFα) after stimulation with LPS in both cell lines used. Also, Fr-GEO_SD significantly reduced IL-6 at all doses, while TNF-α significantly decreased at the two higher doses (0.01% and 0.02%). Such effects can be attributed to a high concentration of aldehydes, but also their possible synergistic action with other compounds that can be useful in the prevention and/or alleviating of chronic inflammatory disorders. We wish to highlight that more data, including metabolic effects in vivo, are needed to introduce such supplements in clinical use. Our results can pave the way for prospective clinical trials to evaluate the potential anti-inflammatory effects of Fr-GEO/Fr-GEO_SD, but also strengthen the idea to create a new nutraceutical product, either as a water-soluble liquid, or a SD powder.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data Availability

Data supporting the results reported in this study are available upon reasonable request.

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686 # Tables

687 **Table 1** – Sequences of oligonucleotides used in RT-PCR

Gene	Forward	Reverse
Murine:		
GAPDH	CCCAGAAGACTGTGGATGG	CAGATTGGGGGTAGGAACAC
IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
TNFα	CACGTCGTAGCAAACCACCAAGTGGA	TGGGAGTAGACAAGGTACAACCC
Human:		
GAPDH	ATGGGGAAGGTGAAGGTCG	GGGTCATTGATGGCAACAATAT
β-actin	AGCACAGAGCCTCGCCTT	CATCATCCATGGTGAGCTGG
IL-6	GGTACATCCTCGACGGCATCT	GTGCCTCTTTGCTGCTTTCAC
TNFα	CCAGGCAGTCAGATCATCTTCTC	AGCTGGTTATCTCTCAGCTCCAC

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690 **Table 2** – List of the volatile compounds of isolated fraction from GEO reported in order of
691 elution.

No	VOLATILE COMPOUND	WHOLE LEO		Fr11		Fr12	
		Rt[a]	Area (%)[b]	Rt	Area (%)	Rt	Area (%)
1	α-Thujene	2.555	0.00450	2.762	0.005	2.763	0.0048
2	α-Pinene	2.661	0.46470	2.881	0.4594	2.882	0.4576
3	Camphene	2.847	0.00320	3.139	0.0028	3.139	0.0026
4	Sabinene	3.339	0.39120	3.613	0.3858	3.614	0.3855
5	β-Pinene	3.398	0.03920	3.674	0.0389	3.675	0.0389
6	Myrcene	3.696	1.86370	3.976	1.8546	3.977	1.8509
7	Octanal	3.898	0.43010	4.177	0.7271	4.178	0.7293
8	Phellandrene	4.358	0.02870	4.222	0.0341	4.221	0.0335
9	Limonene	4.583	93.89970	4.866	93.6864	4.865	93.5006
10	(Z)- β-Ocimene	4.659	0.01030	4.937	0.0129	4.937	0.0103
11	(E)- β-Ocimene	4.836	0.28760	5.115	0.2606	5.115	0.2592
12	γ-Terpinene	5.012	0.04860	5.292	0.0404	5.293	0.04
13	Cis-Sabinene hydrate	5.315	0.00280	5.544	0.0033	5.543	0.0031
14	Octanol	5.407	0.00770	5.590	0.0033	5.590	0.0033
15	Terpinolene	5.556	0.01100	5.832	0.009	5.832	0.0089
16	Trans-Sabinene hydrate	5.660	0.01420	-	-	-	-
17	Linalool	5.783	0.07890	6.053	0.0592	6.053	0.1386
18	Nonanal	5.851	0.03960	6.118	0.0681	6.119	0.0679
19	Citronellal	6.704	0.03060	6.963	0.0509	6.964	0.05
20	Terpinen-4-ol	7.083	0.00500	7.357	0.0039	7.356	0.0085

21	α-Terpineol	7.316	0.03640	7.662	0.0022	7.715	0.0018
22	Decanal	7.529	0.26170	7.828	0.4575	7.828	0.4414
23	Nerol+Citronellol	7.949	0.00260	-	-	-	-
24	Neral	8.127	0.05050	8.356	0.0752	8.356	0.0816
25	Geraniol	8.240	0.00270	8.612	0.0024	8.613	0.0023
26	Geranial	8.555	0.09100	8.800	0.1248	8.801	0.1546
27	Undecanal	9.293	0.00630	9.341	0.0138	9.342	0.0136
28	Alpha-Terpinyl acetate	9.879	0.00630	9.999	0.0099	10.000	0.0129
29	Citronellyl acetate	10.086	0.00870	10.137	0.0154	10.138	0.0157
30	Alpha-copaene	10.161	0.10870	10.316	0.1094	10.316	0.1097
31	Geranyl acetate	10.261	0.05780	10.393	0.103	10.394	0.1074
32	β-Caryophyllene	10.640	0.36890	10.889	0.3664	10.890	0.367
33	Valencene	11.560	0.00250	11.698	0.0024	11.697	0.0027
34	Bicyclogermacrene	11.756	0.02600	11.772	0.0268	11.772	0.0284
35	Epi-Alpha-Selinene	11.829	0.01120	11.803	0.0114	11.803	0.0113
36	Alpha Farnesene	11.989	0.00930	-	-	11.859	0.0085
37	Delta Cadinene	12.073	0.12340	12.005	0.1266	12.005	0.1266
38	(z)-Nerolidol	12.150	0.05400	12.302	0.0207	12.302	0.0201
39	Tetradecanal	12.488	0.00220	12.465	0.0041	12.525	0.002
40	Beta Sinensal	12.951	0.01450	13.112	0.0202	13.113	0.0224
41	Alpha Sinensal	13.215	0.00680	13.383	0.0026	13.328	0.0096
42	Nootkatone	13.547	0.13900	13.650	0.0192	13.651	0.0142

692 [a]Retention Time; [b]Percentage of peak's areas of compounds in whole and fraction essential oil.

693

694 **Table 3 -** Classification and percentage quantification of main chemical classes of whole and
695 fraction essential oil.
696

Chemical classes	Whole GEO (%)	Fr 11 (%)	Fr 12 (%)
Monoterpenes	97.06	96.80	96.60
Total Aldehydes	0.97	1.60	1.62
Citral	0.14	0.20	0.24
Sesquiterpenes	0.75	0.83	0.85
Esters	0.08	0.13	0.14
Total Alcohols	0.32	0.23	0.31

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Figures

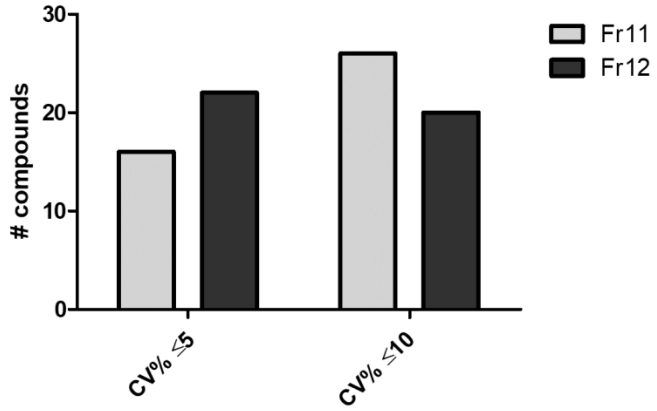
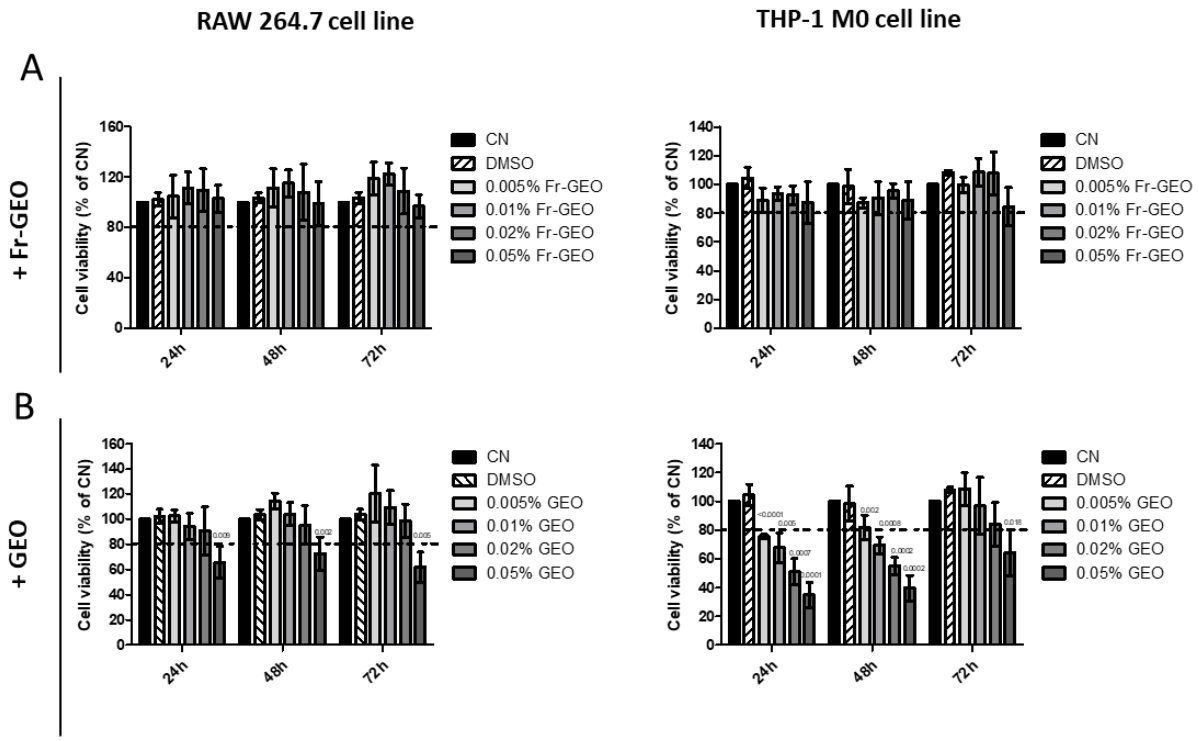


Figure 1: Analysis of the coefficient of variation of the compounds identified in the essential oil in the three replications of the fractionation process.



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708 **Figure 2:** Cell viability of RAW264.7 and THP1 M0. Cell viability (% of control) was measured using the
709 RealTime-Glo™ Cell Viability Assay after exposure of the RAW264.7 and THP1 M0 cells to 0.005%, 0.01%
710 0.02% 0.05% of Fr-GEO (A) and GEO (B), for 24, 48 or 72 h. Dashed lines represent threshold for
711 cytotoxicity (80% in comparison to control cells). The results are expressed as the mean ± standard
712 deviation of three independent experiments. Statistical significance reported in each histogram was
713 calculated vs untreated cells (CN).

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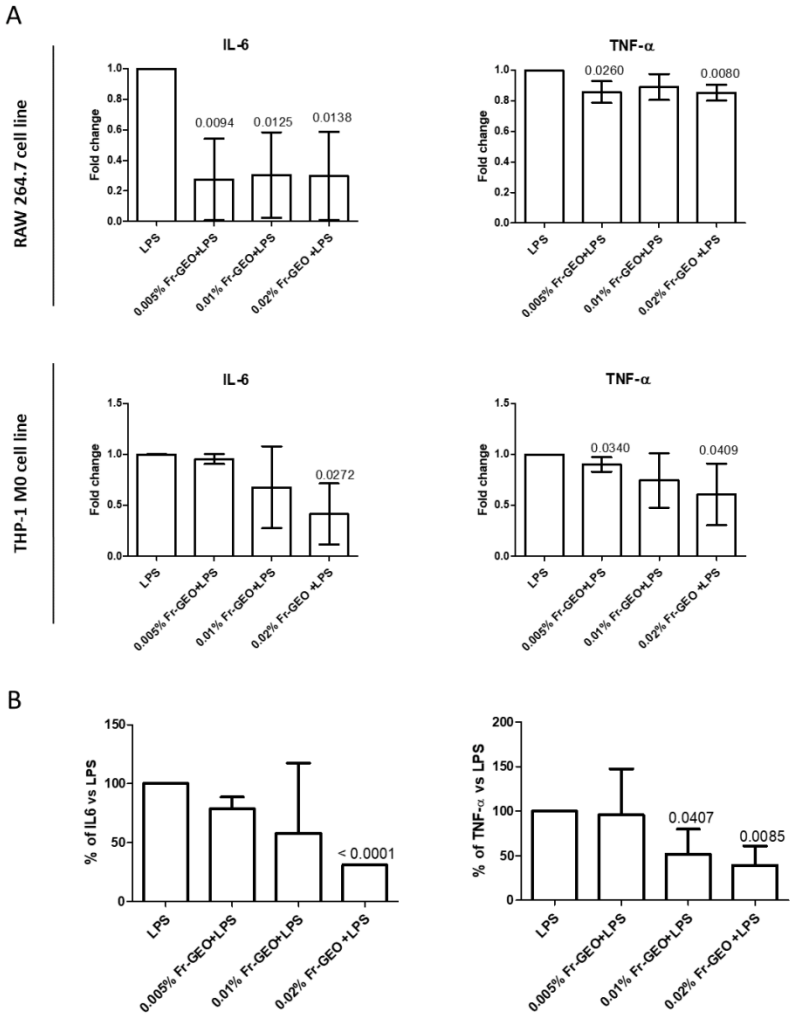

715

716 **Figure 3:** (A) Evaluation of IL-6 and TNF-α transcription levels by qRT-PCR analysis in RAW and THP1M0
717 cells. Cells were pre-treated with increasing doses of Fr-GEO (0.005%, 0.01% 0.02% 0.,05%) for 2h and
718 and were exposed to LPS (500 ng/ml RAW264.7 and 1000 ng/ml THP1 M0) for 6h. Data were normalized
719 to GAPDH as endogenous control. Values are reported as Fold change versus cells treated with LPS and
720 are the means of three biological replicates ± SD. Statistical significance reported in each histogram was
721 calculated vs LPS treated cells (LPS). (B) IL-6 and TNF-α protein levels were determined by ELISA assay in
722 the conditioned medium of THP1 M0 treated with Fr-GEO (0.005%, 0.01% 0.02%) for 2h and then
723 exposed to LPS for 6h. The values are the mean of two or three biological replicates ± SD. Statistical
724 significance reported in each histogram was calculated vs LPS treated cells (LPS).

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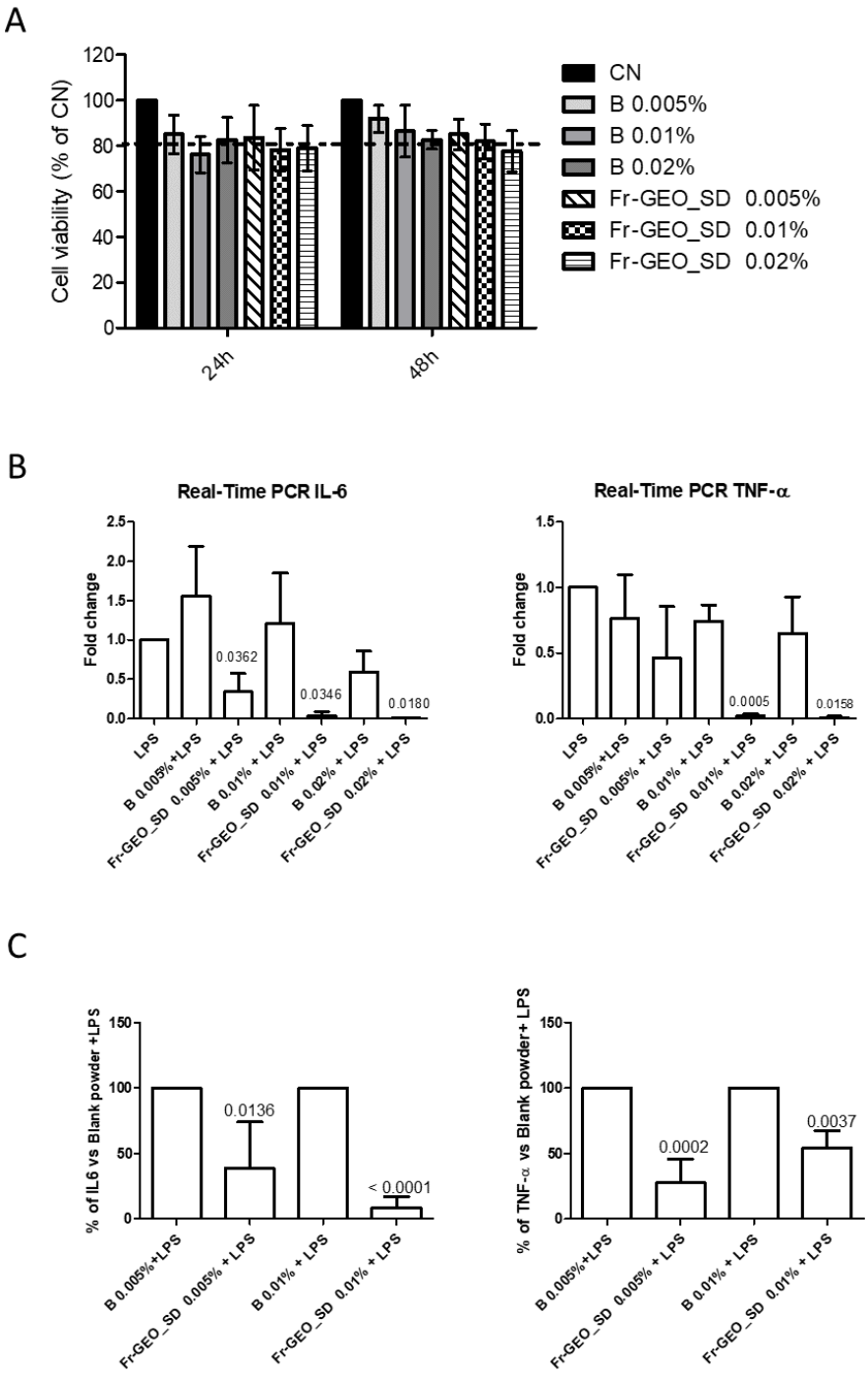

Figure 4: Treatment of human macrophage cells THP1 M0 with Fr-GEO_SD. (A) Evaluation of THP1 M0 cell viability measured by MTT assay after treatment with Fr-GEO_SD (0.005%, 0.01%, 0.02%) for 24h and 48h. The values are the mean of three biological replicates ± SD. CN: untreated control cells; B: blank powder (see Materials and Methods section). Dashed line represents threshold for cytotoxicity

(80% in comparison to control cells). (B) qRT-PCR analysis of IL-6 and TNF-α transcript levels, was performed to assess the anti-inflammatory effects of the different concentrations of Fr-GEO_SD (0.005%, 0.01%, 0.02%). THP1 M0 were pre-treated for 2h with the increased doses of FR-GEO_SD or blank powder (B) and then exposed to LPS for 6h. Data were normalized to GAPDH as endogenous control. Values are reported as Fold change versus cells treated with LPS and are the means of three biological replicates ± SD. Statistical significance reported in each histogram was calculated vs LPS treated cells (LPS). (C) ELISA assay was performed to determine the IL-6 and TNF-α protein levels in the conditioned medium of THP1 M0 cells treated with Fr-GEO_SD and with the blank powder (B) for 2h at different concentrations (0.005%, 0.01%) followed by exposure to LPS for 6h. The values are the mean of three or four biological replicates ± SD. Statistical significance reported in each histogram was calculated vs LPS treated cells (LPS).