Evaluation of immunomodulatory activities of essential oils by high content analysis

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Abstract

Nowadays, research concerning immunomodulatory products are of great interest, particularly in the treatment of inflammatory diseases or the prevention of infectious diseases. These activities are usually evaluated on cell cultures, by tracking different factors requiring dedicated manipulation. Evaluation of the immunomodulatory activities of essential oils and pure compounds using several technics adapted to high content analysis is described in this study. This approach allows a multiparametric evaluation on a single cell batch, in order to obtain an overall response. The developed method is based on the simultaneous evaluation of phagocytosis, production of iNOS and secretion of IL-6, induced by contact of RAW 264.7 cells with LPS. The results highlight the immunomodulatory activities of cinnamon and clove essential oils. They also provide information, particularly concerning the inhibitory activity of mint essential oil, which inhibits the LPS-induced phagocytosis of RAW 264.7 cells by 42%, at 100 µg/ml. This work presents for the first time the adaptation of high content analyses for the monitoring of immunomodulatory activities of essential oils. This protocol could be adjustable to other cell types and supplemented by the evaluation of additional parameters.

Keywords: high content analysis, essential oils, immunomodulation, RAW 264.7

1 Introduction

Inflammation is a natural response from the innate immune system (IS) in case of injury or microbial invasion (Beningo and Wang, 2002), that leads to the destruction of incoming pathogens. Phagocytic cells and especially macrophages are first line effectors of the IS, leading to the detection and elimination of pathogenic micro-organisms invading the hosts' body (Beningo and Wang, 2002; Underhill and Ozinsky, 2002). Activation of macrophages is associated to the recognition of pathogen-associated molecular patterns (PAMPs), like the lipopolysaccharides (LPS) associated to Gram negative bacteria or lipoproteins for Gram positive bacteria, by Toll Like Receptors (TLRs) expressed on cell surface of monocyte and macrophages (Chao et al., 2008). The pathogens thus recognized are absorbed by the cells and lysed, through the phagocytosis process. Binding of LPS to TLR-4, also activates the transduction of intracellular signals resulting in the production of tumor necrosis factor α (TNFα), interleukine 1 (IL-1) and 6 (IL-6) (Serafino et al., 2008; Underhill and Ozinsky, 2002). The pro-inflammatory cytokines of the IL-1 family are important in antimicrobial responses and are responsible for the acute phase response including fever, acute protein synthesis and somnolence (Bachiega et al., 2012). The regulatory cytokine IL-6 is involved in the regulation of cell proliferation, differentiation and survival and also in the management of the expression of inflammatory genes (Bachiega et al., 2012). The exposure of monocytes or macrophages to PAMPS also lead to the induction of inducible Nitric Oxide Synthase (iNOS) responsible for the production of Nitric Oxide (NO) (Tung et al., 2008). This endogenous free radical plays a key role as a toxic agent for pathogens, but also triggers cytotoxicity and tissue damage (Tung et al., 2008). Even if this is a normal and helpful process, NO has also been associated with oxidative stress (Miguel, 2010). Thus, the measurements of phagocytic activity, IL-6 production and iNOS activation allow the monitoring of immunomodulatory activity.

Besides their perfume and aroma, essential oils (EO) develop different biological properties such as antioxidant (Hussain et al., 2010; Miguel, 2010), anti-inflammatory (De Cássia Da Silveira E Sá et al., 2013; Miguel, 2010), antiviral (Astani et al., 2011; Pilau et al., 2011), antibacterial (Ballester-Costa et al., 2013; Du et al., 2015) and anticancer activities (Adorjan and Buchbauer, 2010; Periasamy et al., 2016), which have been widely reported. Nevertheless, very few studies were reported concerning immune-modulatory activities of essential oils. Cinnamon and clove EOs - or their main compounds cinnamaldehyde and eugenol - are among the most studied ones (Bachiega et al., 2012; De Cássia Da Silveira E Sá et al., 2013; Gunawardena et al., 2013; Kim et al., 2010). Cinnamon and its main compound cinnamaldehyde, are well known for their anti-inflammatory activities. The work of Tung et al. (2008) has demonstrated that *Cinnamomum osmophloeum* EO was able to strongly inhibit NO production of RAW 264.7 cells after an exposure to LPS, with IC_{50} ranging from 9.7 to 15.5 µg/ml. They also highlighted the involvement of cinnamaldehyde in this activity. The ability of cinnamaldehyde, used at a concentration of 2.64 µg/ml to reduce the synthesis of iNOS mRNA in RAW 264.7 cells has also been assessed by Kim et al. (2010). Clove essential oil has also been reported for its immunomodulatory activity. According to Bachiega et al. (2012), clove EO at a concentration of 100 µg/well leads to a significant inhibition of the production of interleukins by macrophages. The production of IL-6 was inhibited at the concentration of 100 µg/well when compared to control, while a concentration of 5 µg/well strongly enhanced its secretion by ten (Bachiega et al., 2012). Moreover, most of the studies consider the effect of the essential oils on the secretion of interleukins or the NO production, but do not deal with their ability to modulate the phagocytic activity.

High Content Analysis (HCA) is a powerful method that combines efficient high-throughput techniques to cellular imaging, in order to collect numerical data from biological systems (Zanella et al., 2010). In contrast to traditional cell-based screening methods, HCA acquires

functional and morphometric information from individual cells (Zanella et al., 2010). The algorithms used to analyze the pictures automatically extract diverse information like changes in cell morphology or cell movement, fluorescence intensity or distribution (Zanella et al., 2010). Thereby, multiple independent features of interest can be quantified simultaneously, for individual cells or cell populations, allowing the screening of active extracts (Zanella et al., 2010).

Although the modulation of the inflammatory system seems to be an asset in the fight against pathogens, it remains a complex process that might be modulated by natural extracts. In this study, the immunomodulatory effect of five EOs and two main compounds is evaluated on RAW 264.7 murine macrophages. We also propose herein a multiparametric analysis method including HCA, allowing the observation of the overall effects of essential oils using non-invasive methods.

2 Materials and methods

The experimental design, involving different measurements on the same cell batch by a high-content screening approach, can be observed in the Fig. 1. RAW 264.7 cells were first pre-incubated during 24 h with essential oils or pure chemical compounds, at non-cytotoxic concentrations. We performed then consecutively three different assays: (1) the evaluation of the phagocytic capacity (§2.5), using pH dependant fluorescent bioparticles (2) the measurement of the expression levels of iNOS (§2.6), by immunofluorescence on the fixed cells (3) the determination of the concentration of IL-6 (§2.8) secreted in the supernatant recovered after step 1.

2.1 Plant extracts and pure components

Commercial essential oils (EOs) of cinnamon (*Cinnamomum cassia* J. Presl, 1825), clove (*Syzygium aromaticum* (L.) Merr. & L. M. Perry, 1939), lemon (*Citrus ×limon* (L.) Burm. f., 1768), peppermint (*Mentha ×piperita* L., 1753) and oregano (*Origanum vulgare* L., 1753) were purchased from BioArmor S.A. (Plaintel, France). Cinnamon, clove, peppermint and oregano EOs were extracted by steam distillation of specific organs: leaves and branches for cinnamon, leaves for clove and peppermint and the whole plant for oregano. Lemon EO was obtained by expression of fruit pericarps. EOs were stored in the dark at room temperature. Provided by the supplier, the major compounds of each EO are presented in table 1. *Trans*-cinnamaldehyde and carvacrol, the respective main constituent of cinnamon and oregano EOs were purchased from Sigma Aldrich (St Louis, MO, USA).

2.2 Cell culture

Murine macrophages from cell line RAW 264.7 (ATCC, Manassas, VA, USA) were grown in plastic culture flasks (Greiner Bio-One GmbH, Kremsmünster, Austria) using MEM / F12 supplemented with 2mM L-glutamine, 10% FBS (Foetal Bovine Serum) and 1% penicillin / streptomycin solution (Dominique Dutcher, Brumath, France). Cells were incubated under 5% CO_2 at 37 °C. When 80% confluence was reached, cells were removed from the culture flask by scraping and were then centrifuged for 5 min at 1500 rpm. The medium was then removed and the cells were resuspended with fresh media. Cell counts and viability were performed using a standard trypan blue cell counting method. The cell concentration was adjusted to 5 x 10^5 cells/ml or 1 x 10^5 cells/ml in the same medium.

2.3 Cytotoxicity assay

Cytotoxicity was evaluated using Hoechst® 33342 and propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA) to reveal the total and the dead cells respectively. Toxicity was evaluated during 24 h on $5x10^4$ cells/well. EOs and main compounds were diluted in culture medium in order to achieve final concentrations ranging from 0.98 to 1000 µg/ml for cinnamon, clove, mint and oregano EO and the major compound cinnamaldehyde and carvacrol, and 1.95 to 2000 µg/ml for lemon EO. Dimethyl sulfoxide (DMSO, Sigma Aldrich, MO, USA) at the maximal final concentration of 0.3% was used to disperse EOs in the culture medium. Cells treated with DMSO at the maximal concentration of 0.3% were used for the negative control. The assay for each concentration of samples was performed in triplicates. The 96 well culture plates were kept at 37 °C with 5% (v/v) CO_2. After 24 h of incubation, 100 µl of medium was removed from each well. Subsequently, 100 µL of staining solution (16.2 µM Hoechst® 33342 and 0.66 µM PI diluted in MEM / F12) were added and plates were incubated for a further 30 min. The supernatant was then removed and cells were washed twice using phosphate buffered saline (PBS, 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4, Dominique Dutcher, France) before to perform microscopic analysis as described in §2.7.

2.4 Cell pre-treatment conditions

In order to determine the effects of essential oils on phagocytosis, iNOS expression and IL-6 secretion, a 24 h pre-treatment was performed. For this, a final concentration of $1x10^4$ cells per well was used. Cell suspension obtained from a confluent culture was first spread in the wells of a 96 well culture plates and incubated for 6 h. Non-adherent cells were then discarded, and the supernatant was replaced by solutions of EOs at 100 µg/ml for clove, lemon and peppermint EOs, or at 10 µg/ml for cinnamon and oregano EOs, cinnamaldehyde and carvacrol. The pre-treatment was realised in presence or absence of lipopolysaccharide

(LPS, from *E. coli* 0111:B4, Sigma Aldrich, MO, USA) at a concentration of 0.1 µg/ml. Again, DMSO was used to disperse EOs, at a maximal concentration of 0.5%. Negative and positive controls consisted in medium containing respectively DMSO at the maximal calculated concentration (0.5%) or LPS at 0.1 µg/ml.

2.5 Evaluation of phagocytic capacity

After pretreatment, the culture media was removed and cells were washed twice using PBS to avoid traces of EOs or LPS. Cells were incubated with pHrodo™ Red *S. aureus* Bioparticles™ diluted at a final concentration of 100 µg/ml and mixed with Hoechst at 1:1000 (Invitrogen®, Thermo Fisher Scientific, Waltham, MA, USA). The pHrodo™ Red *S. aureus* Bioparticles™ (Invitrogen, Carlsbad, CA, USA) consist of *Staphylococcus aureus* cell particles coupled with pH dependent rhodamine, becoming fluorescent when pH reaches 5, *i.e.* after phagocytosis. Fluorescence intensity was measured at t=0 h, 1 h and 2 h using automated epifluorescence microscopy as described in §2.7. The fluorescence intensity at t = 2 h is used to express the percentages of phagocytosis in the cells.

2.6 Measurement of iNOS expression

Once the phagocytosis step over, the cell supernatants were recovered to determine the effect of EOs on IL-6 secretion (see §2.8). Wells were washed twice with PBS to remove non phagocyted pHrodo™ probes. Remaining cells were then fixed using a formaldehyde solution (Dominique Dutcher, Brumath, France) during 15 minutes and washed twice again. Quantification of the inducible nitroxyde synthase (iNOS) expressed in cells was then performed using immunostaining. Briefly, 100 µl of murine macrophage iNOS specific antibody (ABR iNOS, Invitrogen®, Thermo Fisher Scientific) were added on the cells at a dilution of 1:250 (v:v) in PBS. Recognition was processed during 3h at 4 °C before washing

and addition of the secondary antibody (Anti-Rabbit F(ab')2 Alexa Fluor 647 4414S (Ozyme, Montigny-le-Bretonneux, France)). After 1 h of incubation, a last washing step took place before fluorescence quantification using epifluorescence microscopy as described in §2.7.

2.7 Microscopy analysis and calculation

Cells were observed with an automated epifluorescence microscope (ImageXpress Micro XLS, Molecular Devices, San Jose, CA, USA) using appropriate excitation/emission wavelength: 350/461 nm for Hoechst; 533/617 nm for propidium iodide; 560 / 585 nm for pHrodo *S. aureus* Bioparticles; 650/665 nm for Anti-Rabbit F(ab')2 Alexa Fluor 647 4414S. Four fields of view were taken of each well at a magnification of 20x. The obtained images were processed using the associated MetaXpress® software, to obtain quantitative data.

Cytotoxicity was expressed as CC_{50} which is the concentration leading to 50% of PI positive cells in comparison to cells treated with DMSO at 0.3% (negative control). Phagocytic activity was expressed as a percentage of fluorescence intensity, relative to the cells that did receive DMSO alone as a pre-treatment (value set to 100%). The rate of iNOS expression was calculated using fluorescence intensity, relative to the cells that did receive DMSO alone as a pre-treatment (value set to 1).

2.8 Determination of IL-6 secretion

Production of IL-6 by phagocyting macrophages was determined on cell supernatant using a specific commercial Elisa Kit (Termo Fisher Scientific). The test was performed according to the supplier's recommendation. Concentrations in the samples were calculated by linear regression from the values obtained with the calibration range of IL-6 (0 - 2000 pg/ml). This experiment was performed at 24 °C and samples were assayed in duplicates.

2.9 Statistical analysis

Cytotoxic concentrations, phagocytic activity and iNOS expression levels were determined using triplicates whereas IL-6 concentrations were calculated from duplicates. Conventional statistical methods were used to calculate means and standard deviations. Analysis of variance (ANOVA) was performed to determine differences ($p < 0.05$). Means were then compared pairwise using Tukey's test. The statistical analyses were realised using XLSTAT for windows.

3 Results and discussion

3.1 EOs composition

The chemical compositions of the essential oils (EOs) are presented in table 1. Cinnamon EO obtained from the leaves, contains 80.0% of cinnamaldehyde. Lemon and peppermint are rich in terpenes, containing respectively 64.5% of d-limonene and 32.5% of menthol. Clove and oregano EOs are rich in phenolic compounds, containing respectively 80.5% of eugenol and 70.7% of carvacrol as main compounds. The chemical profiles of the essential oils are in agreement with the previously published data (Ooi et al., 2006; Viuda-Martos et al., 2010; Yang et al., 2010).

3.2 Assessment of cytotoxic concentrations

In order to establish working concentrations, the cytotoxicity of EOs and main compounds has been established on RAW 264.7 cells. Integration of propidium iodide (PI) by cell nuclei has been used as a marker of the cell mortality and results are shown in supplementary Fig. S1. Example of images taken during the cytotoxicity assay are also shown in Fig. S2, present in the supplementary material. The EOs of clove and peppermint did not develop a CC_{50} when tested at concentrations ranging from 1 to 1000 µg/ml. The same observation is made for

lemon EO, tested at concentrations ranging from 2 to 2000 µg/ml. The cytotoxicity of lemon and mint essential oils on RAW 264.7 cells has not been reported in the literature yet. However, these two essential oils are rich in terpenic compounds, namely limonene and menthol, which are known for their low cytotoxicity on eukaryotic or prokaryotic cells (Bakkali et al., 2008). The absence of cytotoxicity of clove EO on murine macrophages, had also been noticed by Bachiega et al. (2012) in concentrations ranging from 5 to 100 µg/well (Bachiega et al., 2012). When tested in concentrations ranging from 1 to 1000 µg/ml, oregano EO developed a CC_{50} of 244.63 µg/ml. In comparison, its main compound carvacrol (70.7 %) showed a similar CC_{50} of 287.24 µg/ml. The presence of 70.7% carvacrol in Oregano essential oil, as well as the similarity of the CC_{50} measured here, suggest that carvacrol is mainly responsible for the cytotoxic activity of oregano essential oil. Cinnamon EO and its main compound cinnamaldehyde triggered the highest cytotoxicity with respective CC_{50} of 37.87 µg/ml and 40.59 µg/ml. This also suggests that cytotoxicity of cinnamon EO is supported by cinnamaldehyde. Tung et al. (2010) observed no cytotoxic effect on RAW 264.7 cells after a treatment with *Cinnamomum osmophleum* EOs (also rich in cinnamaldehyde) when using concentrations up to 100 µg/ml, suggesting that the cytotoxicity of essential oils can be variable and is related to their global chemical composition. Based on these results, it was possible to determine the maximal non-cytotoxic concentrations of EO used for the pre-treatment, namely 10 µg/ml for the essential oils of cinnamon and oregano, as well as for their major constituents cinnamaldehyde and carvacrol and 100 µg/ml for essential oils of lemon, clove and mint.

3.3 Effect of essential oils on phagocytic activity

The percentage of phagocytic activity, measured after pre-treatment with 100 µg/ml of clove, lemon and mint EOs or 10 µg/ml of cinnamon and oregano EOs, as for their main compounds

cinnamaldehyde and carvacrol; was determined at the endpoint of the experiment. Results obtained in presence or absence of LPS as an inducer are presented in Fig. 2. No significant difference of phagocytic activity was observed between negative (100.00 ± 12.33%) and positive control (132.54 ± 11.10%), namely cells without treatment and cells treated with 0.1 µg/ml LPS. The absence of difference has already been observed by Suzuki & Umezawa (2006) when *E. coli* bioparticles were used to evaluate the phagocytic activity of RAW 264.7 cells. These results suggest that the activation of macrophages is a fast mechanism and the PAMPs present at the surface of the bioparticles are sufficient to activate the RAW 264.7 cells in the same way than the LPS (Suzuki and Umezawa, 2006). In comparison to untreated cells (control), the addition of EOs did not result in a significant change in phagocytosis levels ranging from 124.31 ± 31.93% for clove EO to 62.99 ± 7.79% for cinnamon EO. When LPS was added at 0.1 µg/ml, the pre-treatments consisting in 100 µg/ml of mint EO or 10 µg/ml of cinnamon or oregano EOs led to a significant decrease in phagocytosis (77.93 ± 14.37%, 72.06 ± 12.47% and 81.83 ± 7.73% respectively). These three EOs also triggered the most significant decrease in phagocytic activity when comparing the EOs to each other, in presence or absence of LPS during the pre-treatment. Interestingly, cinnamaldehyde and carvacrol, the respective major components of cinnamon and oregano EO, both reduced phagocytosis to 85.41 ± 8.88% and 78.70 ± 12.44%. This shows that these two compounds are involved in the activity of essential oils, even if other compounds may act synergistically.

The anti-inflammatory activity of cinnamon essential oil (Tung et al., 2010), has already been shown before , but the modulation of phagocytosis was not often a monitored parameter. Pérez-Rosés et al. (2015) assessed that clove EO at a concentration of 50.40 µg/ml inhibited phagocytosis of human neutrophils by 36%. Contrariwise, Serafino et al. (2008) identified eucalyptus EO as an enhancer of phagocytic activity of human monocyte derived macrophages (MDMs). Phagocytosis was evaluated using fluorescent beads, and a total of 17

fluorescent beads/cell were observed after treatment with 160 µg/ml of eucalyptus versus 3 beads/cells when LPS was used alone. Nevertheless, the results demonstrated in these studies are hardly comparable to ours, because of the cell type used. Indeed, the work of Merly and Smith (2017) displayed that the responses can be different between RAW 264.7 and stimulated human primary cells. The ability of essential oils to limit phagocytosis induced by LPS treatment suggests that they act as TLR4 inhibitors, in a similar way than DHMEP, a synthetic product tested by Suzuki & Umezawa (2006). TLR-4 is a receptor involved in the recognition of LPS in RAW 264.7 cells inducing the activation of NF-κB. The activation of this transcription factor is involved among others in a positive feedback on phagocytosis, in the production of NO and in the cytokine secretion (Suzuki and Umezawa, 2006). EOs and their main compounds are already known for their ability to inhibit the activation of NF-κB (De Cássia Da Silveira E Sá et al., 2013; Miguel, 2010). To our knowledge, this is the first time that the inhibitory activity of mint essential oil on phagocytosis is demonstrated using this method, on RAW 264.7 cells. Moreover, the mechanism of anti-inflammatory action of mint EO or his main compound menthol, on RAW 264.7 cells, has not been elucidated yet. It can however approach those of other terpenes, implicating the down regulation of TLR-4 or the inactivation of NF-κB (De Cássia Da Silveira E Sá et al., 2013; Miguel, 2010).

3.4 Modulation of iNOS expression in pre-treated cells

The activation of the inducible Nitric Oxide Synthase (iNOS) is mediated by the recognition of pathogenic specific patterns like LPS and results in the production of Nitric Oxide (NO). The iNOS protein was quantified by immunostaining in cells that had phagocytosis and expression rates are presented in Fig. 3. No significant difference was observed between control cells and cells treated with 0.1 µg/ml LPS (LPS control), with respective expression level of 1.00 ± 0.23 and 1.72 ± 0.47. In absence of LPS as an inducer during pre-treatment,

only clove increased significantly the expression level of iNOS to 2.26 ± 0.40. The expression levels for the other EOs ranged from 0.83 ± 0.13 for lemon EO to 1.83 ± 0.32 for cinnamon EO. The combination of LPS to clove and cinnamon EOs led to a significant increase in expression levels (2.36 ± 0.92 and 3.02 ± 0.59 respectively). As compared with phagocytosis, cinnamaldehyde induced an identical response to that of cinnamon EO, by promoting the expression of iNOS (expression rate of 2.86 ± 0.26). That result suggests that the activity of cinnamon EO is partially supported by cinnamaldehyde. The inhibitory activity of eugenol, the main compound of clove EO, on iNOS expression and NO production was reported by Li et al (2006). Eugenol, at a concentration of 8.2 µg/ml reduced both the expression of iNOS, evaluated by western blotting, and NO secretion measured by the Griess reagent in LPS stimulated cells. In another study, carried out by Kim et al. (2010), the treatment of RAW 267.4 cells with 2.6 µg/ml cinnamaldehyde and 1 µg/ml LPS showed a 10 time inhibition on the synthesis of iNOS mRNA and also inhibited NO production. Due to the use of various methods, it is difficult to compare these data with the values obtained in our study. Moreover, in the study conducted by Li et al (2006), cells were pre-treated with eugenol only for one hour, while the pre-treatment time is 24h in our study. In the study realised by Kim et al. (2010), iNOS is quantified indirectly by evaluation of the mRNA, after 6 hours of incubation with cinnamaldehyde and LPS. An effect of the incubation time with cinnamon, cinnamaldehyde or clove essential oil on the regulation of iNOS can be suggested. The quantification of NO in the supernatant by the Griess reagent, in the presence of essential oils, may also include a bias, particularly related to the anti-radical and antioxidant activities of essential oils. Furthermore, it is important to consider that the macrophages studied here sustained the phagocytosis step prior to the quantification of iNOS. Both essential oils of clove and cinnamon could improve the macrophage efficiency by enhancing the production of toxic NO.

3.5 Inhibition of IL-6 production.

Interleukins are mediators of inflammation, produced by macrophages when pathogens are detected. They lead to the recruitment of immune cells and they promote the transition from innate to adaptative immunity (Meager and Wadhwa, 2001). The activity of EOs on IL-6 production by macrophages has been evaluated here on supernatants using an ELISA assay. Concentrations of IL-6 detected after two hours of phagocytosis are presented in Fig. 4. When LPS was absent from pre-treatment, concentrations of IL-6 were above the limit of detection of 31.25 pg/ml. Concentration of secreted IL-6 increased when LPS was added into the pre-treatment and reached 1444.75 ± 270 pg/ml when 0.1 µg/ml of LPS was added. EO of lemon, mint, cinnamon and oregano and the main compound carvacrol had no significant effect on the IL-6 production induced by LPS. Clove EO and cinnamaldehyde significantly inhibited by six and three times respectively the secretion of IL-6 by the cells. Clove EO was the most active, leading to a concentration of secreted IL-6 of 231.33 ± 79.20 pg/ml. This result is in agreement with the data published by Bachiega et al. (2012) concerning the efficiency of clove and eugenol on IL-6 inhibition (Bachiega et al., 2012). The addition of 100 µg/well of clove EO led to the reduction by 1.6 times of the IL-6 secretion when compared to LPS used alone (Bachiega et al., 2012). The addition of 10 µg/ml of cinnamaldehyde also led to a decreased concentration of IL-6 (489.58 ± 177.60 pg/ml) when compared to LPS treated cells. This result is also in agreement with previously published data. Chao et al. (2008) showed that cinnamaldehyde at a concentration of 3.18 µg/ml added on LPS treated cells, led to a significant decrease in IL-6 production, from 15 ng/ml to 6 ng/ml (Chao et al., 2008). EOs and their main compounds are known for their ability to inhibit the activation of NF-κB, a nuclear factor involved in the secretion of cytokines. Inhibition of IL-6 secretion and the tendency of EO to inhibit phagocytosis is consistent with inhibition of this transcription

factor. Clove EO and cinnamaldehyde are able to inhibit intracellular signalisation induced by PAMPs recognition in RAW 264.7 cells. This inhibition could limit the propagation of the inflammatory process. Moreover, EOs develop a long-lasting effect on phagocytic activity, even after their withdrawal.

4 Conclusion

The immunomodulatory activities of natural compounds are highly sought today, on the one hand to try to limit the use of antibiotics, but also to offer new possibilities for treating inflammatory diseases. A method applying the HCA to the study of the immunomodulatory activities of essential oils is proposed herein. RAW 264.7 cells are used in this analyse, allowing in a single and overall manipulation the monitoring of several parameters, namely the quantification of phagocytosis, of the intracellular expression of iNOS and of the secretion of IL-6 in the media. All these parameters are evaluated on the same batch of cells. The pre-treatment consisting in 100 µg/ml of Mint EO or in 10 µg/ml of cinnamon or oregano EOs led to an inhibition of the LPS induced phagocytosis. The activity of cinnamon and oregano EOs can be related to their main compounds, namely cinnamaldehyde and carvacrol, representing more than 70 % of the compounds. These two aromatic molecules have been widely reported for their biological activities, especially concerning anti-inflammatory properties. The *in vitro* inflammatory activities of menthol or menthone, which are the major components in mint EO have not been established yet. To our knowledge, this is also the first time that a modulating activity of phagocytosis is reported for Mint EO through an *in vitro* study. The expression of iNOS, also induced by LPS, is enhanced by the pre-treatments involving 100 µg/ml of clove or 10 µg/ml of cinnamon EOs. Clove EO is moreover able to induce this expression increase on its own. Concerning the secretion of IL-6, clove essential oil is again put forward for its ability to significantly inhibit the secretion of this cytokine. Cinnamaldehyde, the main

compound of cinnamon HE, provides an equivalent result. This study highlights the immunomodulatory activities of clove and cinnamon EOs, by the use of a rapid method. The prospect of this work is the addition of new parameters that will confirm the immune-modulatory nature of the essential oils. First, it will be necessary to confirm the overexpression of iNOS by cinnamon and clove EOs, in particular by monitoring the secretion of NO in the medium. It would also be interesting to follow the effect of the HE on TLR receptors, especially the TLR4 receptor, involved in the detection of LPS. Finally, it will also be important to understand the interaction of essential oils with the main regulators of the inflammatory response, including NF-κB. The analysis of these different parameters in the same experimental design is currently under study.

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Tables

Table 1: Chemical compositions of commercial essential oils (%) determined by gas chromatography. The compounds are classified according to their concentration in all the tested EOs. These data are provided by the supplier.

	Cinnamon	**Clove**	**Lemon**	**Peppermint**	**Oregano**
Eugenol		**80.5**			
Trans-cinnamaldehyde	**80.0**				
Carvacrol					**70.7**
d-Limonene			**64.5**	3.2	1.7
Menthol				**32.5**	
(DL)-Menthone				26.3	
β-Caryophyllene		15.2		3.8	3.6
β-Pinene			13.5	1.6	1.2
γ-Terpinene			9.8		5.0
o-Methoxycinnamaldehyde	8.0				
Menthyl acetate				5.0	
p-Cymene			0.6		3.7
(D/L)-Isomenthone				4.1	
α-Pinene			2.3	1.0	
(D)-Neomenthol				3.2	
Linalool					3.0
Citral			2.6		
Cinnamyl acetate	2.5				
Menthofuran				2.4	
Thymol					2.1
Eucalyptol					1.4
α-Terpineol			0.2		0.8

Figure captions

Figure 1: Workflow of high content immunomodulation assay. The methodology used for this essay is diagrammed, in order to represent all the successive analysis steps undergone by the cells from the pre-treatment. Image was obtained and modified from https://www.thermofisher.com/order/catalog/product/P35361 (date of last access : 2019.03.15).

Figure 2: Phagocytic capacity of RAW 264.7 cells after 24 h of pre-treatment with EO, cinnamaldehyde and carvacrol, with (+) or without (-) LPS. Clove, lemon and mint EOs were added for pre-treatment at a concentration of 100 µg/ml when cinnamon and oregano EOs, cinnamaldehyde and carvacrol were added at 10 µg/ml. Fluorescence intensity was measured after two hours of incubation with pHrodo™ particles. Values are means ±SD (n=3). Samples with different letters are significantly different ($P<0.05$).

Figure 3: iNOS expression in RAW 264.7 cells after 24 h pre-treatment with EO, cinnamaldehyde and carvacrol, with (+) or without (-) LPS. Clove, lemon and mint EOs were added for pre-treatment at a concentration of 100 µg/m as cinnamon and oregano EOs, cinnamaldehyde and carvacrol were added at 10 µg/ml. Values are means ±SD (n=3). (*) values are significantly different from cells treated with LPS at 0.1 µg/ml ($P<0.05$).

Figure 4: Concentrations of IL-6 (pg/ml) secreted by RAW 264.7 cells after 24 h pre-treatment with EO, cinnamaldehyde and carvacrol, with (+) or without (-) LPS. Clove, lemon and mint EOs were added for pre-treatment at a concentration of 100 µg/m as cinnamon and oregano EOs, cinnamaldehyde and carvacrol were added at 10 µg/ml. Values are means ±SD (n=3). (*) values are significantly different from cells treated with LPS at 0.1 µg/ml ($P<0.05$).

Supplementary material

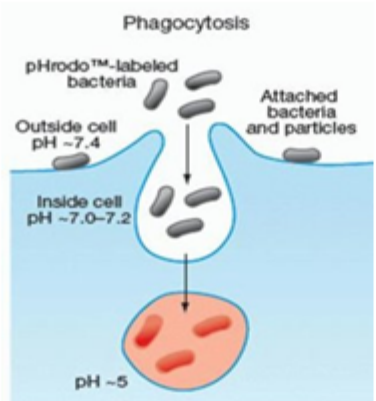
Figure S1: Cytotoxicity of essential oils and pure compounds evaluated on RAW 264.7 cells after 24 h treatment. Cinnamon, clove, mint and oregano EOs, as cinnamaldehyde and carvacrol, were tested in concentrations ranging from 0 to 1000 µg/ml. Lemon EO was tested in concentrations ranging from 0 to 2000 µg/ml. Cytotoxicity was established from the intensity of PI integrated by the cells. Values are means ±SD (n=3).

Figure S2: Comparison of the cytotoxic effect of lemon EO and cinnamaldehyde, using the integration of propidium iodide (PI) on RAW 264.7 cells. Cells were treated during 24 h with lemon EO or cinnamaldehyde at 1 µg/ml (Fig S2 C / E) or 200 µg/ml (Fig S2 D / F) respectively. DMSO at 0.3% (Fig S2 A) and methyl methanesulfonate (MMS) at 5 µM (Fig S2 B) were used as negative and positive control respectively. Total cell nuclei were identified using the nuclear fluorescent probe Hoechst (in blue on the image). Propidium iodide was added in a second step to evaluate the cytotoxicity of the products. Heavily deteriorated cells show high fluorescence levels in the nucleus, marked in red on the pictures. Fluorescence images were merged with transmitted light images taken with a 20X Super Plan Fluor lens.

Step 1 : RAW 264.7 cell pre-treatment

Incubation for 24h at 37°C and 5% CO_2

Step 2 : Evaluation of phagocytic capacity using pHrodo™ Red *S. aureus* Bioparticles™



Addition of Bioparticles ™

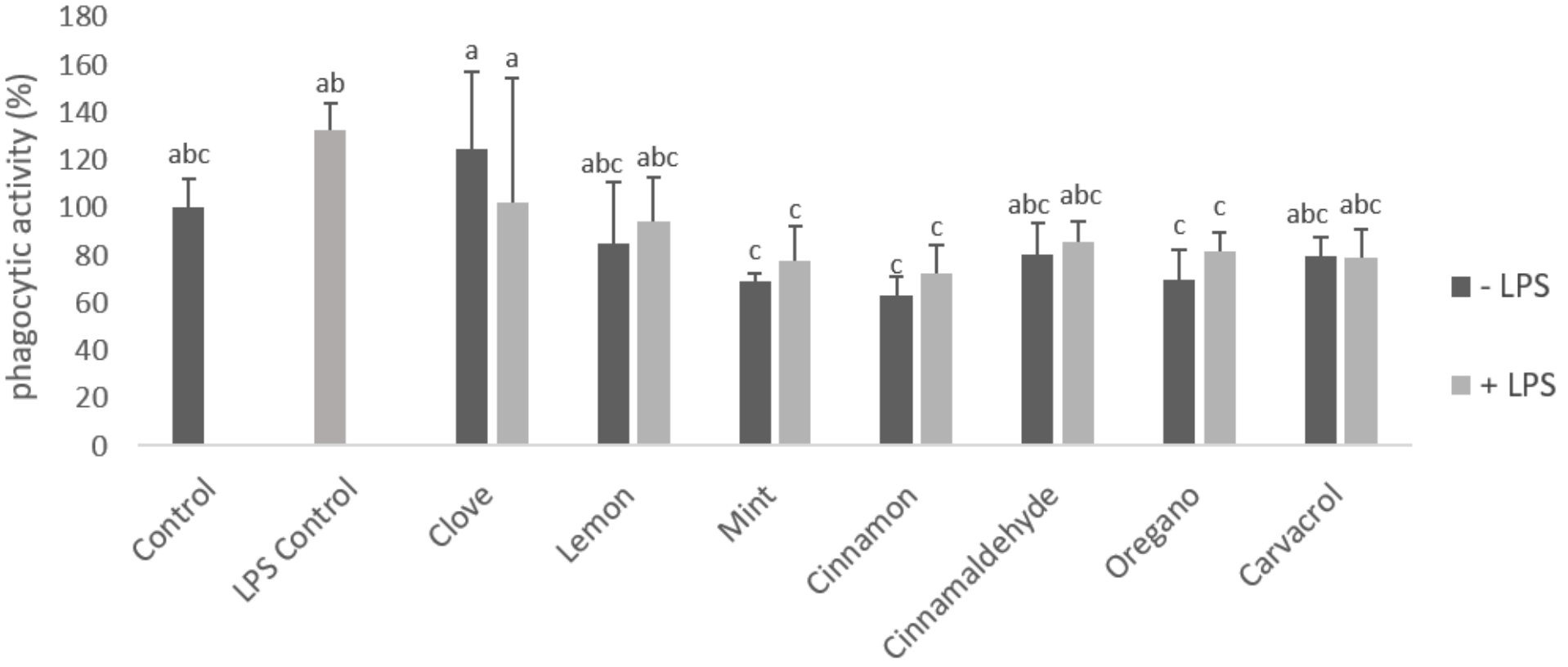
Fluorescence monitored after 2 hours

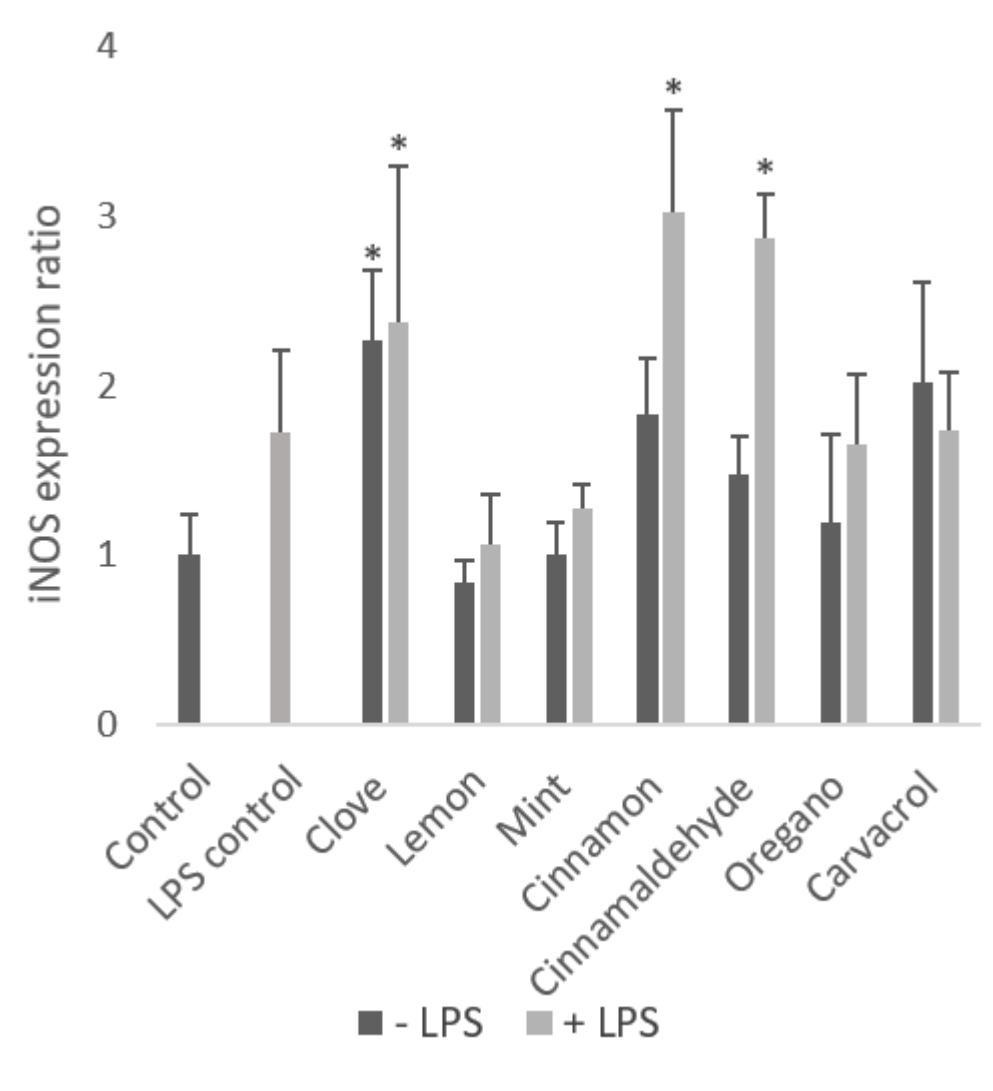
Fixation of remaining cells

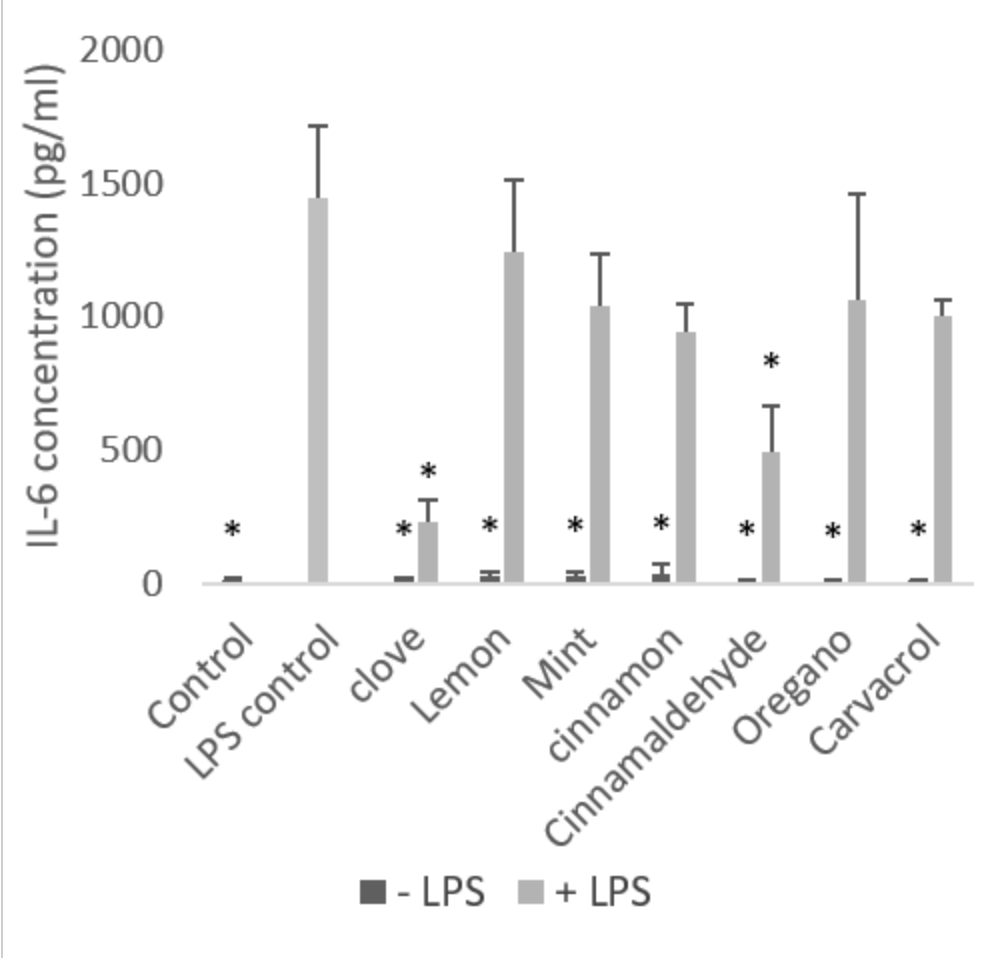
Recovery of the cellular supernatent

Step 3 : Measurement of iNOS expression
Quantification of iNOS expressed in the cells using immunofluorescence

Step 4 : Determination of IL-6 secretion
in cell supernatent using an Elisa assay


phagocytic activity (%)
180
160
140
120
100
80
60
40
20
0
abc
ab
a
a
abc
abc
c
c
c
c
abc
abc
c
c
abc
abc
Control
LPS Control
Clove
Lemon
Mint
Cinnamon
Cinnamaldehyde
Oregano
Carvacrol
- LPS
+ LPS


iNOS expression ratio
4
3
2
1
0
*
*
*
*
Control
LPS control
Clove
Lemon
Mint
Cinnamon
Cinnamaldehyde
Oregano
Carvacrol
- LPS
+ LPS


IL-6 concentration (pg/ml)
2000
1500
1000
500
0
Control
LPS control
clove
Lemon
Mint
cinnamon
Cinnamaldehyde
Oregano
Carvacrol
- LPS
+ LPS