



# In Vitro Anti-inflammatory Activity of Three *Inula* Species Essential Oils in Lipopolysaccharide-Stimulated RAW 264.7 Macrophages

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## Abstract

In the face of the undesirable effects induced by anti-inflammatory drugs, there has been a return, nowadays, to the search for active ingredients based on plants. Herein, for the first time we study the anti-inflammatory activity of essential oils of three species of the genus *Inula*: *Inula viscosa*, *Inula graveolens* and *Inula crithmoides* in lipopolysaccharide (LPS)-activated macrophages. Essential oils have shown excellent preventive anti-inflammatory potential by causing inhibition of nitric oxide (NO) production in LPS-activated RAW264.7 macrophages with IC50s ranging between 15 and 35  $\mu\text{g mL}^{-1}$ . On the other hand, the major acidic compounds, more precisely  $\alpha$ - and  $\beta$ -costic acids, have been isolated from *Inula viscosa* and *Inula graveolens* essential oils and evaluated for their anti-inflammatory effect. These compounds appear to have a moderate preventive inhibitory effect on NO production relative to the significant effect generated by the neutral minority components present in the oils such as borneol, bornyl acetate, (*E*)-nerolidol, caryophyllene oxide, *T*-cadinol and eugenol. Therefore, we can deduce that the studied essential oils could be used as anti-inflammatory agents for the treatment of various inflammatory pathologies.

**Keywords** *Inula* species · Essential oils · Anti-inflammatory activity · Acidic compounds · Preventive effect

## Abbreviations

EOs	Essential oils
NO	Nitric oxide
LPS	Lipopolysaccharide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
IVEO	Essential oil of <i>Inula viscosa</i>
IGEO	Essential oil of <i>Inula graveolens</i>
ICEO	Essential oil of <i>Inula crithmoides</i>
N.F	Neutral fraction
A.F	Acidic fraction

## 1 Introduction

Inflammation is the immune system response against external harmful pathogens or endogenous stimuli such as damaged cells, resulting in either tissue healing or chronic pathologies if the acute inflammation became uncontrolled [1, 2].

The inflammation process involves a set of molecules called inflammatory mediators including cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IL-6, etc.), nitric oxide (NO), lipid mediators and also oxygenated free radicals. These mediators are secreted by immune cells including macrophages, and are known to be involved in a wide range of chronic diseases including multiple sclerosis and cancer [3]. Over the past years with the pandemic outbreak of COVID-19, scientific research has reported that cytokine storm is the most frequently mentioned pathological theory in COVID-19 [4–6]. As an example, it has been revealed that in COVID-19 patients [6], the IL-6 is the major upregulated inflammatory cytokine. IL-6 and TNF- $\alpha$  can cause superoxide generation in neutrophils, and hydrogen peroxide can stimulate IL-6 generation. It has been shown that inhibiting NO synthesis can reduce IL-6 production by more than 50% [7].

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The commonly used anti-inflammatory agents including aspirin, the world's most used therapeutic agent [8] and nonsteroidal anti-inflammatory drugs (NSAIDs) [9] could be associated with adverse effects such as gastrointestinal toxicities, cardiovascular risks, renal injuries, hepatotoxicity, hypertension as well as platelet dysfunction, [10, 11]. Therefore, the search for new anti-inflammatory drugs with reduced side effects is crucial.

Medicinal plants present a valuable source of new bioactive compounds. They have long been used as a folk remedy for inflammatory conditions such as fever, pain, migraine and arthritis. In recent years, the researchers managed to identify and highlight the potential anti-inflammatory effect of these products classified in various families namely: alkaloids [2], terpenoids [12], polyphenols [13] and sulfur-containing compounds [14].

Several studies have investigated the inhibitory action of essential oils obtained from various plants, such as *Eucalyptus camaldulensis* Dehnh, *Artemisia jordanica* Danin., *Cinnamomum camphora* L., *Thymus vulgaris* L., *Origanum vulgare* L., *Baccharis dracunculifolia* DC. against the production of different inflammatory mediators. The essential oils extracted from these species exhibited a potent anti-inflammatory effect, which was explained by the presence of monoterpenes such as 1,8-cineole, borneol, bornyl acetate, thymol, carvacrol, *p*-cymene, nerolidol, limonene [15].

The three species of *Asteraceae* family *Inula viscosa* (L.) Aiton, *Inula graveolens* (L.) Desf. and *Inula crithmoides* (L.) are well known in folk medicine for treating various pathologies [16]. These plants contain different substances such as phenolic compounds, flavonoids and terpenoids, which provide a great assortment of biological properties such as antioxidant [17–20], cytotoxic [13, 20] antibacterial [17, 19–21], anti-cancer [22, 23], antiproliferative [24] and anti-inflammatory activities [17].

The majority of studies made on these three species have been carried out on aqueous and organic (ethanol, methanol and dichloromethane) extracts. Essential oils (EOs) are also part of the secondary metabolites of these species. The chemical characterization of the essential oils of these plants has been the subject of several studies, including our previous published study, which also looked at the antioxidant and cytotoxic effects of these oils [25]. Other researchers have focused on other biological activities that can be exerted by these oils such as antibacterial [26, 27], antimicrobial [28, 29], insecticidal (only for *Inula viscosa* essential oil) [28], as well as anti-cholinesterase, anti-tyrosinase and anti-tumor which have been proven only for *Inula graveolens* essential oil [29]. Nevertheless, the anti-inflammatory activity of these EOs has never been studied.

Therefore, the present study focuses, for the first time, on the evaluation of the anti-inflammatory potential of three *Inula* species essential oils: *Inula viscosa* (IVEO), *Inula*

*graveolens* (IGEO) and *Inula crithmoides* (ICEO) through the inhibition of NO production in LPS-activated RAW264.7 macrophages. Further, a study of the anti-inflammatory activities of the acidic fractions of IVEO and IGEO that contain the main components is included, in order to determine if they are responsible for the anti-inflammatory activity exerted by the EOs or not.

## 2 Materials and Methods

### 2.1 Essential Oils Extraction

*Inula viscosa*, *Inula graveolens* and *Inula crithmoides* were collected in October in the Monastir region (Tunisia). Voucher specimens (*Dv1-5*; *Dgrbis1-5*; *Lcr1-5*) identified by the botanist Pr. Fethia Harzallah-Skhiri, were deposited in the herbarium of the laboratory of Botany, High Institute of Biotechnology of Monastir. The EOs of these three species were extracted from the dried aerial parts by hydrodistillation for 3 h using a Clevenger-type apparatus. The distilled essential oil was dried over anhydrous sodium sulfate, filtered and stored at 4 °C for further analyses.

### 2.2 Cell Line

Anti-inflammatory activity was assessed in cultured murine macrophage cells (RAW264.7). The cell line was purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) and incubated at 37 °C in 5% CO<sub>2</sub>.

### 2.3 MTT Assay for Cell Viability

The cytotoxicity test was evaluated according to the method described by Seisenbaeva et al. [30] on macrophage cell line, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay. This method is based on the ability of living cells to reduce the yellow soluble tetrazolium salt into insoluble purple coloured formazan crystals. Cells were seeded in 96-well plate at a density of 2000 cells per well in their respective culture medium, followed by incubation for 24 h. Then, cells were incubated with EOs for 24 h at concentrations range between 0 and 200 µg mL<sup>-1</sup>. Cells treated with the vehicle were considered as a control. After the end of the incubation period, MTT was added to each well to reach a final concentration of 0.5 mg mL<sup>-1</sup>. After incubation for 3 h, culture medium was aspirated and a mixture of ethanol/DMSO solution (1:1, v/v) was added in order to dissolve the crystals, which are proportional to the number of living cells. After 20 min of shaking, the absorbance was

read with a microplate reader Multiskan type (Thermo Scientific, Courtaboeuf, France) at 540 nm. The cell viability percentage was calculated according to the following equation: (absorbance test/ absorbance control\*100). The experiment was repeated three times. The mean lethal concentration (LC<sub>50</sub>) was determined at 50% of cell death from the dose-response curves using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA).

This experiment will help us to determine the non-toxic EOs concentrations to be used in the following experiments.

## 2.4 Anti-inflammatory Activity Assessment

Inflammation was induced by the addition of lipopolysaccharide (LPS) from *Escherichia coli*, purchased from Sigma-Aldrich, to the cell supernatant. The LPS induces the nitric oxide (NO) production. Therefore, this test is based on measuring the ability of EOs to inhibit NO production in LPS-activated RAW264.7 macrophages.

The anti-inflammatory activity of the EOs was tested in three conditions in order to demonstrate their preventive or curative effect as previously described in references [31] and [32]: (i) addition of EOs 2 h before the stimulation of cells by LPS, (ii) addition of EOs at the same time of the LPS addition and (iii) addition of EOs 2 h after the stimulation of cells by LPS.

The anti-inflammatory activity was evaluated according to the method previously described [31]:

For the condition (i): macrophage (RAW264.7) cells were seeded in 96-well plate at a density of  $2 \times 10^5$  cells/well. After 24 h after cell seeding, cells were treated with different concentrations of EOs, 2 h after incubation, cells were treated with LPS solution to reach a final concentration of  $100 \text{ ng mL}^{-1}$ .

For the condition (ii): 24 h after cell seeding, cells were treated simultaneously with different concentration of EOs and LPS (final concentration  $100 \text{ ng mL}^{-1}$ ).

For the condition (iii): 24 h after cell seeding, cells were treated for 2 h with LPS solution (final concentration  $100 \text{ ng mL}^{-1}$ ) followed by treatment with different concentration of EOs.

Untreated cells were considered as a negative control, while cells treated with LPS were considered as a positive control. Further cells were treated with EOs only at the concentrations used in the experiment in order to verify that EOs do not induce any NO by themselves.

## 2.5 Nitrite Determination

The nitrite assay test was performed 24 h after the three treatment conditions previously described. This test measures the production of nitrite ions ( $\text{NO}_2^-$ ) based on the Griess reaction [31]. A nitrite concentration range was

prepared from 0 to  $100 \text{ }\mu\text{M}$  to plot the calibration curve absorbance (at 540 nm) = f (nitrite concentrations).

A volume of  $100 \text{ }\mu\text{L}$  of the supernatant was taken and placed in another 96-well plate, followed by the addition of  $100 \text{ }\mu\text{L}$  of freshly prepared Griess's reagent, which is consisting of 0.1% N-1-naphthylethylenediamine dihydrochloride in  $\text{H}_2\text{O}$  (A) and 1% sulfanilamide in 5%  $\text{H}_3\text{PO}_4$  at 1:1 (v/v). After 15 min of incubation in the dark, the absorbance was measured with a Multiskan type microplate reader (Thermo Scientific, Courtaboeuf, France) at 540 nm. The amount of nitrite produced was determined by referring to the calibration curve.

In order to eliminate any interference due to cell death, a cytotoxicity test was performed as previously described.

## 2.6 Isolation of the Acidic Compounds from Essential Oil (Acid-Basic Partition of IVEO and IGEO)

In order to estimate the bioactive compounds in our EOs, we realized the separation of the acidic and neutral compounds from the IVEO and IGEO by an acid-base treatment. Briefly, the EO was dissolved in dichloromethane, then alkalized with a saturated solution of sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) in a separating funnel. After decantation, the neutral molecules that remained in the organic phase were recovered after evaporation, while the acid molecules remained in the ionized form in the aqueous phase. A volume of dichloromethane was added and the ionized molecules were acidified with sulfuric acid (1 N). The acids were moved into the organic phase, which was subsequently recovered and evaporated.

## 2.7 Ultra-Performance Liquid Chromatography (UPLC) Analyses Coupled with Mass Spectroscopy (MS)

Both acidic and neutral fractions were analysed by UPLC Class H (WATTERS) coupled to a mass spectrometer SQD2 and UV diode array. The analyses were carried out under the following conditions: a volume of  $2 \text{ }\mu\text{L}$  of the sample was injected to pass through a column C18 ( $1.7 \text{ }\mu\text{m}$ ,  $2.1 \times 50 \text{ mm}$ ). The sample was conveyed at a flow rate of  $0.6 \text{ mL min}^{-1}$  by a mobile phase consisting of two solvents: solvent A was water with 0.1% acetic acid ( $\text{C H}_3\text{COOH}$ ) and solvent B was acetonitrile with 0.1%  $\text{CH}_3\text{COOH}$ . The elution was carried out by gradient going from 100% of A to 100% of B. The analyses were carried out in negative mode with a mass range between 100 and 1000 Da, a source temperature of  $149 \text{ }^\circ\text{C}$ , a desolvation temperature of  $599 \text{ }^\circ\text{C}$ , a cone gas flow rate of  $50 \text{ L h}^{-1}$  and a desolvation gas flow rate of  $998 \text{ L h}^{-1}$ .

## 2.8 Statistical Analysis

The results are reported as the mean values  $\pm$  SEM of three independent experiments. The statistical analysis was performed using Graph Pad Prism 5.0 software. The comparison between groups was analyzed with Student's t-test. Differences between groups were considered to be significant at  $p < 0.05$  [17].

## 3 Results and Discussions

### 3.1 Cytotoxic Effect of Essential Oils on Macrophage Cells

The cytotoxic effect of the EOs on macrophage cells was studied after 24 h of cell treatment with a concentration range from 0 to 200  $\mu\text{g mL}^{-1}$ . Results in Figure S1 showed that the *I. viscosa* essential oil appeared to be the less toxic on macrophage cells with a  $\text{LC}_{50}$  equal to 112.2  $\mu\text{g mL}^{-1}$ , followed by the essential oils extracted from *I. crithmoides* and *I. graveolens*, which revealed  $\text{LC}_{50}$  equal to 70.7 and 56.2  $\mu\text{g mL}^{-1}$ , respectively. These results allowed us to set the concentration range of 10 to 60  $\mu\text{g mL}^{-1}$  for each oil in

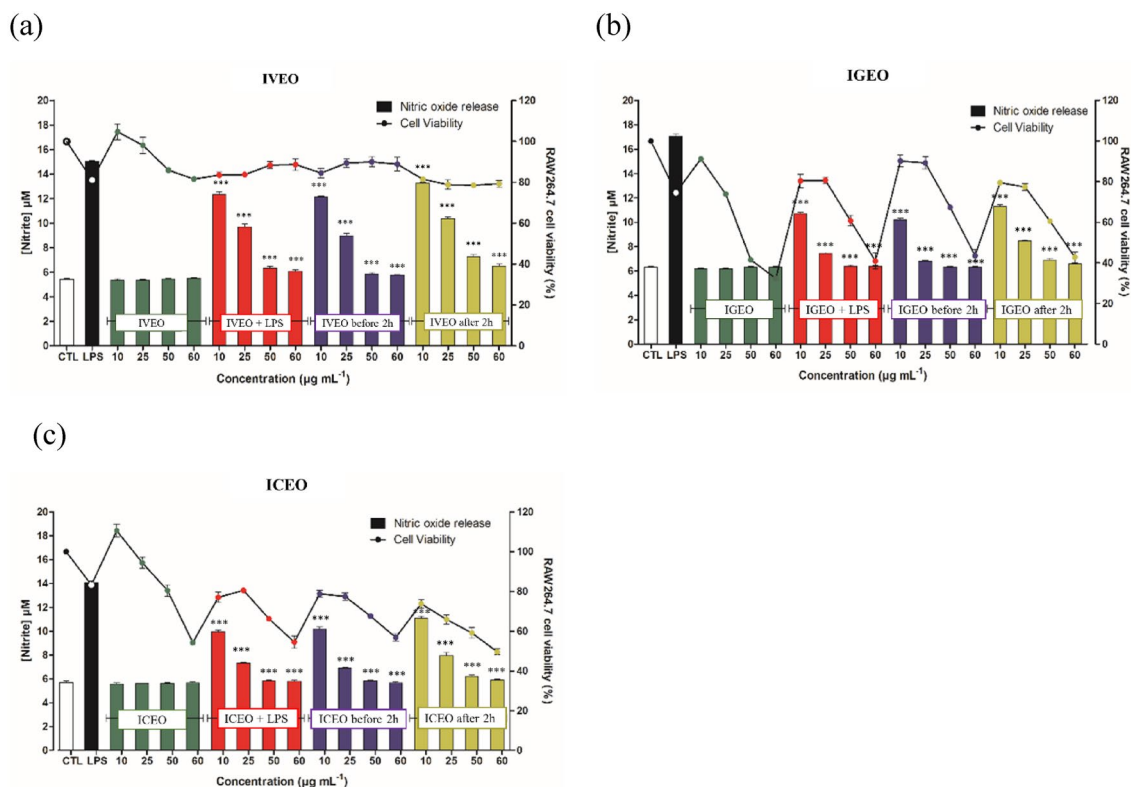
order to assess their anti-inflammatory activity to avoid an impact on cell viability.

### 3.2 Effect of Essential Oils on NO Production

The assessment of anti-inflammatory activity is based on the ability of our EOs to inhibit the production of nitrites induced by LPS. After the Griess reaction, the nitrite concentration was determined by extrapolation on the calibration curves (supplementary Figure S2). Figure 1 shows both the amount of nitrites produced by RAW264.7 macrophage cells and the curves of cell viability as a function of the concentration of IVEO (a), IGEO (b) and ICEO (c).

As a first analysis of the results presented in Fig. 1, it should be noted that our EOs tested alone (bar graph in green color) did not cause any inflammation effect. Comparing with the untreated control cell (bar graph in white color), the amount of nitrites remained almost the same for the three EOs.

On the other hand, this same figure shows clearly that all three oils contributed significantly to the inhibition of nitrite production in a dose-dependent manner as the inhibitory effect increases with increasing EOs concentration.



**Fig. 1** Effect of essential oils on LPS-induced inflammatory mediators in RAW 264.7 macrophage-like cells; data presented as the mean  $\pm$  SEM of three independent experiments. \*Statistically significant

difference from the LPS group.  $P < 0.05$ , \*\* $P < 0.001$ , \*\*\* $P < 0.0001$ , nothing: not significant

As shown in Fig. 1, each oil exhibited significant inhibitory activity on nitrite release using the condition of extract addition 2 h before LPS stimulation. RAW264.7 cells produced  $15.04 \pm 0.09$ ,  $17.06 \pm 0.25$  and  $14.07 \pm 0.25$   $\mu\text{M}$  of nitrite after LPS stimulation, these amounts were reduced, respectively, to  $5.77 \pm 0.05$   $\mu\text{M}$  by IVEO (Fig. 1a, violet bars),  $6.33 \pm 0.06$   $\mu\text{M}$  by IGEO (Fig. 1b, violet bars) and  $5.69 \pm 0.05$   $\mu\text{M}$  by ICEO (Fig. 1c, violet bars) at  $60 \mu\text{g mL}^{-1}$  concentration.

Similarly, ICEO was the most potent in reducing nitrite production ( $5.91 \pm 0.09$   $\mu\text{M}$  at  $60 \mu\text{g mL}^{-1}$ ) in the third condition (addition of extract 2 h after LPS stimulation) (Fig. 1c, yellow bars). Similarly, in the second condition (simultaneous addition of extract and LPS), ICEO reduced the maximum level of nitrite to  $5.82 \pm 0.1$   $\mu\text{M}$  at  $60 \mu\text{g mL}^{-1}$  (Fig. 1c, red bars), followed by IVEO ( $6.12 \pm 0.14$   $\mu\text{M}$ ) (Fig. 1a, red bars) and IGEO ( $6.40 \pm 0.06$   $\mu\text{M}$ ) (Fig. 1b, red bars) at the same concentration.

According to the cytotoxicity results obtained in this test, the cell viability remained stable for the different concentrations of IVEO (Fig. 1a). Beyond  $25 \mu\text{g mL}^{-1}$ , a decrease in viability was observed during treatment with IGEO (Fig. 1b) and ICEO (Fig. 1c). At  $25 \mu\text{g mL}^{-1}$ , the quantity of nitrites inhibited by these two essential oils is interesting and reveals more than 50% inhibition in the case where the treatment with the sample is carried out 2 h before LPS addition. As for IVEO, it only achieves 60% inhibition at a concentration of  $25 \mu\text{g mL}^{-1}$  but it reaches 90% inhibition at  $60 \mu\text{g mL}^{-1}$  without damaging the cells of RAW264.7 macrophages.

These results suggest that the EO of this plant could be a good anti-inflammatory agent.

### 3.3 Chromatographic Analysis of the Acid and Neutral Fractions of IVEO and IGEO

The GC-FID and GC-MS analyses of IVEO and IGEO performed in our previously published study [25] showed that  $\alpha$ - and  $\beta$ -costic acids are the major components in *I. viscosa* and *I. graveolens* EOs. Indeed,  $\alpha$ - and  $\beta$ -costic acids represented 14.4% of the total composition of IGEO while  $\alpha$ -costic acid was the main compound in IVEO (65.4%). These compounds have been previously isolated from these plants and showed bactericidal [33] and acaricidal [34, 35] effects as well as activity against dermatophyte species [33]. However, no study has investigated their anti-inflammatory activity. Therefore, it was interesting to check the contribution of these compounds in the anti-inflammatory activity through their separation by acid-base treatment.

Following the separation of the acidic and neutral fractions, a chromatographic analysis was carried out in order to verify their chemical composition. Figures 2 and 3 show the chromatograms (LC-MS) of the total EO, the neutral fraction and the acidic fraction of IVEO and IGEO.

LC-MS analyzes of IVEO (Fig. 2) showed that the main peak, which appeared at 4.12 min in the total essential oil, corresponded to  $\alpha$ -costic acid according to its mass spectrum which gave a parent peak at  $m/z$ : 234 (supplementary Figure S3). Contrary to the other peaks appearing at 4.84,

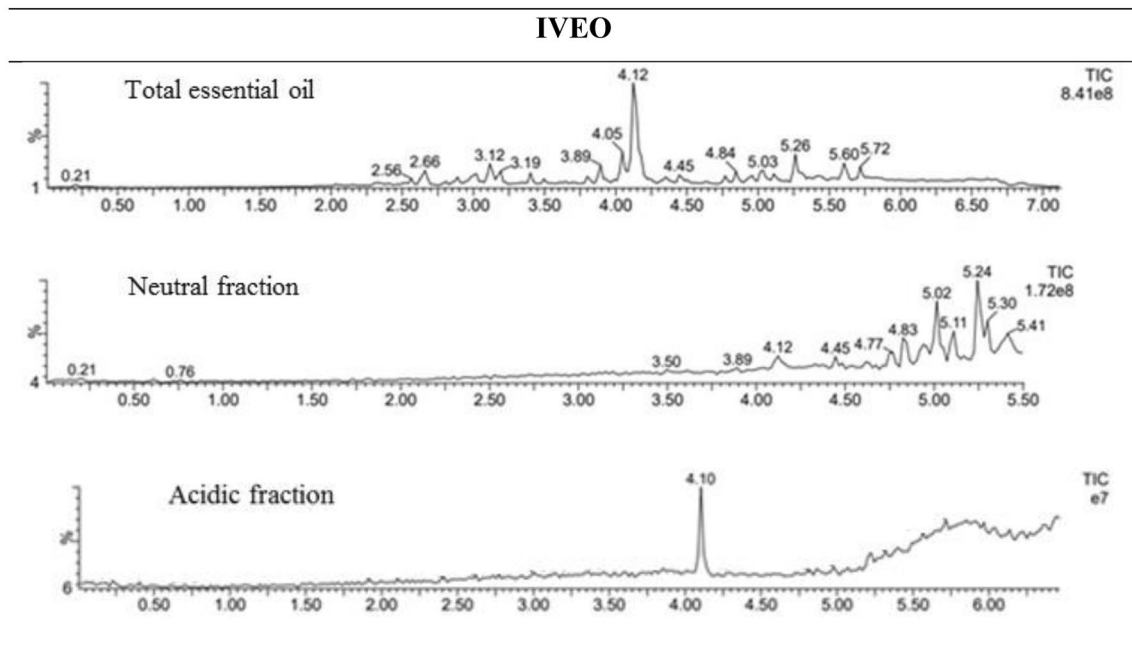
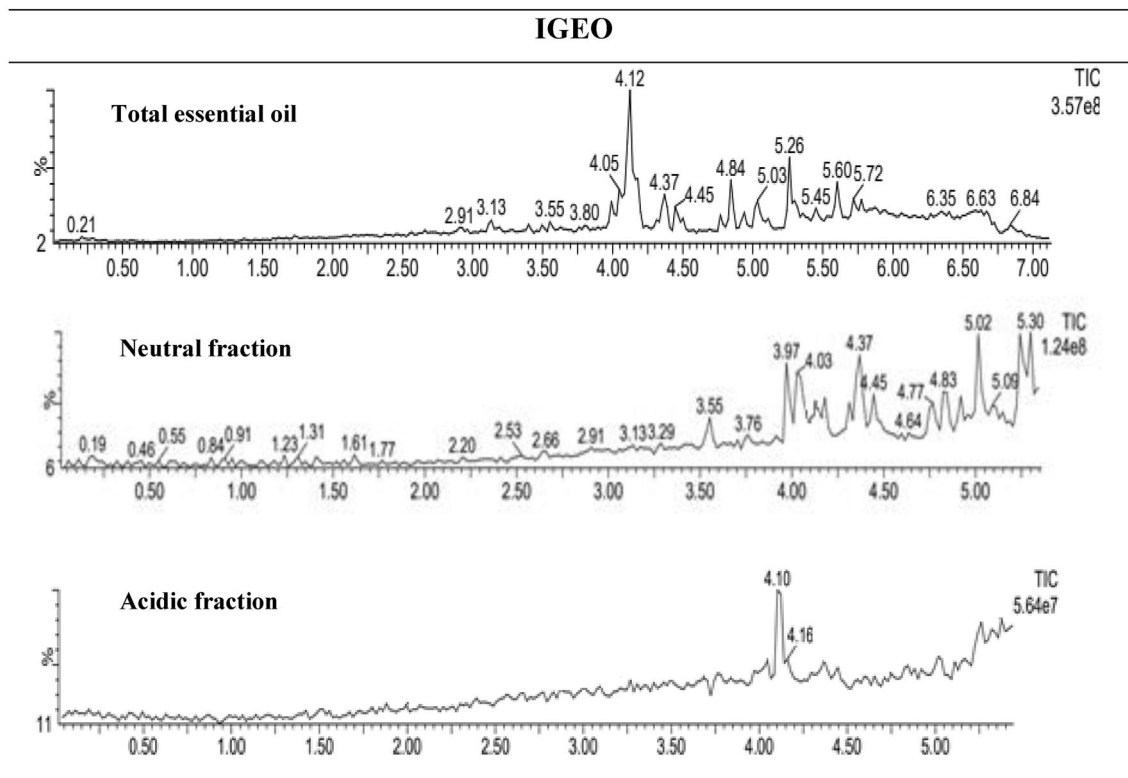


Fig. 2 Chromatograms(LC-MS) of total essential oil, neutral fraction and acidic fraction of *I. viscosa*



**Fig. 3** Chromatograms(LC-MS) of total essential oil, neutral fraction and acidic fraction of *I. graveolens*

5.03 and 5.26 min and which were amplified in the chromatogram of the neutral fraction, the peak of  $\alpha$ -costic acid disappeared while it was intense in the chromatogram of the acidic fraction. This confirms the good separation of  $\alpha$ -costic acid from total IVEO by the acid-base treatment. The same was observed for the  $\beta$ -costic acid which has been separated from the neutral fraction of IGEO. According to the chromatogram shown in Fig. 3, the peak at 4.12 min corresponded to  $\beta$ -costic acid and was confirmed by its mass spectrum given in the supplementary Figure S4 which also gave a parent peak at  $m/z$ : 234.

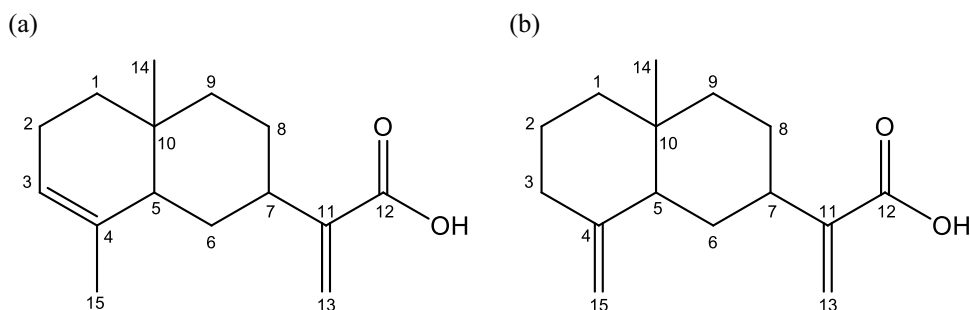
The identification of  $\alpha$ -costic acid (Fig. 4a) and  $\beta$ -costic acid (Fig. 4b) in each acidic fraction was verified by  $^1\text{H}$  NMR. As described in the literature by Sofou et al. [34] and Moeini et al. [36], the  $^1\text{H}$  NMR spectrum of  $\alpha$ -costic acid

showed clearly the presence of the most significant protons  $\text{H}_2$ -13 at 6.22, 5.59 ppm, H-3 at 5.31 ppm, Me-15 at 1.62 ppm and Me-14 at 0.75 ppm. The same was observed for the  $^1\text{H}$  NMR spectrum of  $\beta$ -costic acid which indicated the presence of a methyl group at 0.75 ppm and four olefinic protons, indicating the presence of two double bonds in the carbon framework, at 6.22 and 5.59 ppm for  $\text{H}_2$ -13 and at 4.60 ppm and 4.31 ppm for  $\text{H}_2$ -15.

### 3.4 Anti-inflammatory Activity of the Acid and Neutral Fractions of IGEO and IVEO

After carrying out the cytotoxicity test of the acidic and neutral fractions on the cells of macrophages (results given in supplementary Figure S5) of IVEO et IGEO, the

**Fig. 4** Chemical structures of  $\alpha$ -costic acid (a) and  $\beta$ -costic acid (b)

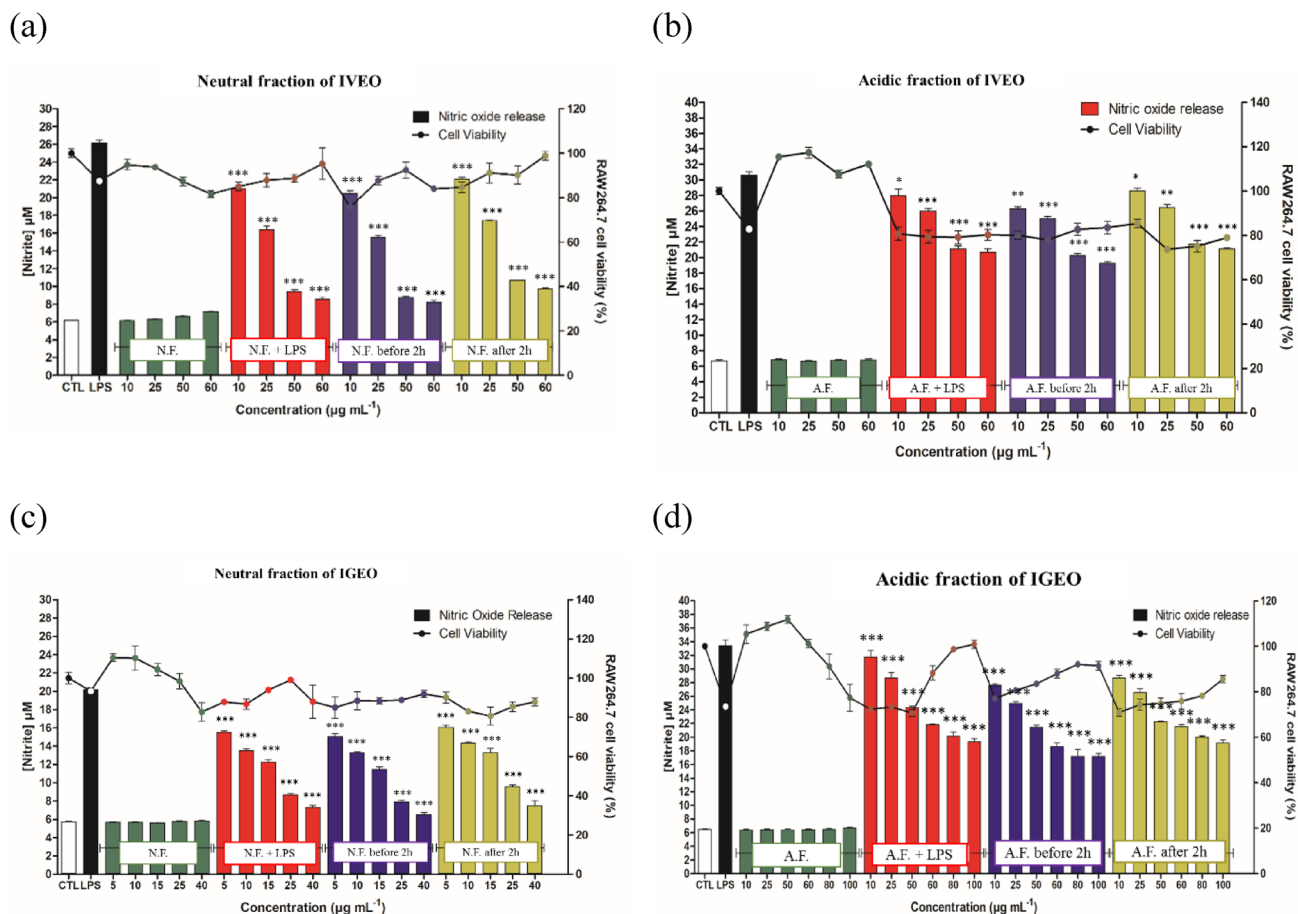


anti-inflammatory activity was evaluated for ranges of concentrations from 10 to 60  $\mu\text{g mL}^{-1}$  for the fractions of IVEO, from 5 to 25  $\mu\text{g mL}^{-1}$  for the neutral fraction of IGEO and from 10 to 100  $\mu\text{g mL}^{-1}$  for its acidic fraction. The quantity of nitrites was determined by extrapolation on the calibration curves (supplementary Figure S6) and Fig. 5 represents both the quantity of nitrites produced by the RAW264.7 macrophage cells and the cell viability as a function of the concentration of the acidic and neutral fractions of both EOs.

It is important to note that the dose-dependent inhibitory effect of the neutral fractions (Fig. 5a and c) is more interesting than that of the acidic fractions (Fig. 5b and d) for the two essential oils (IVEO and IGEO) without affecting the cell viability. As shown in Fig. 5a and c, the neutral fraction of IGEO is more active in the inhibitory effect on NO production than that of IVEO under three conditions; the amount of NO produced by macrophage cells stimulated by LPS in the case of the neutral fraction of IVEO and that of IGEO were  $26.23 \pm 0.47 \mu\text{M}$  and  $20.22 \pm 0.15 \mu\text{M}$ , respectively. The quantity was reduced between  $8.27 \pm 0.21$

$\mu\text{M}$  and  $9.78 \pm 0.11 \mu\text{M}$  by the first at a concentration of 60  $\mu\text{g mL}^{-1}$  and between  $12.38 \pm 0.42 \mu\text{M}$  and  $14.80 \pm 0.44 \mu\text{M}$  by the second at a concentration of 40  $\mu\text{g mL}^{-1}$ . The best result is always obtained for the condition in which the addition of the extract is 2 h before cell stimulation with LPS. These results indicated that these fractions exert a preventive effect rather than a curative effect, which is in accordance with the result obtained for the total EOs of *I. viscosa* and *I. graveolens* (Fig. 1a and b).

In the case of the acidic fraction, the quantity of NO produced by the macrophage cells stimulated by LPS was  $30.64 \pm 0.75 \mu\text{M}$  and  $33.42 \pm 2.03 \mu\text{M}$ . The quantity of NO decreased respectively to  $19.30 \pm 0.30 \mu\text{M}$  by the acidic fraction of IVEO for a concentration of 60  $\mu\text{g mL}^{-1}$  and to  $17.21 \pm 0.64 \mu\text{M}$  by the acidic fraction of IGEO for a concentration of 100  $\mu\text{g mL}^{-1}$ , when they were added 2 h before LPS stimulation. This confirms that the neutral fractions of IVEO and IGEO are more active than their acidic fractions that mainly contain  $\alpha$ -costic acid and  $\beta$ -costic acid respectively.



**Fig. 5** The anti-inflammatory activity of the neutral (a and c) and acidic (b and d) fractions of IVEO and IGEO in RAW264.7 macrophage cell line. Data presented as the mean  $\pm$  SEM of three inde-

pendent experiments. \*Statistically significant difference from the LPS group.  $P < 0.05$ , \*\* $P < 0.001$ , \*\*\* $P < 0.0001$ , nothing: not significant

Therefore, it turns out that the resulting anti-inflammatory activity of the total oil, is mainly due to the combination of minority compounds present in the neutral fraction without neglecting the “contributing” effect of  $\alpha$ -costic acid and  $\beta$ -costic acid.

On the other hand, this interpretation could explain the good inhibitory effect of NO production of ICEO which rather contain neutral compounds.

Although many volatile substances may participate in the anti-inflammatory activity, monoterpenes have been largely recognized as natural molecules with anti-inflammatory effects [15]. Previous studies have demonstrated the inhibitory effect of certain volatile compounds on NO production in activated macrophages RAW264.7 cells induced by LPS. We can cite the studies made by Tung and collaborators [37, 38] who proved the anti-inflammatory activity of certain compounds present in the studied EOs such as caryophyllene oxide, borneol, bornyl acetate, eugenol, *T*-cadinol and (*E*)-nerolidol. Other researchers have demonstrated the ability of carvacrol to inhibit the production of nitrites [39]. Consequently, the high percentages of inhibition obtained with IGEO might be explained by the presence, in large quantities, of borneol (8.1%), caryophyllene oxide (4.8%), *T*-cadinol (3.6%) and bornyl acetate (3.4%). On the other hand, the anti-inflammatory activity of ICEO could be explained by the presence of carvacrol (3.0%), *T*-cadinol (3.6%) and bornyl acetate (0.8%) while that of IVEO can be explained by its caryophyllene oxide (1.8%), eugenol (0.7%), bornyl acetate (0.2%) and (*E*)-nerolidol (1.2%) content. However, these promising activities may not necessarily be due to the major components, but rather arise from the synergic effect between several components.

## 4 Conclusion

In the present work, we have studied, for the first time, the in vitro anti-inflammatory activity of essential oils of three *Inula* species: *Inula viscosa*, *Inula graveolens* and *Inula crithmoides*, through the inhibition of NO production in LPS-activated RAW264.7 macrophages in three conditions. Generally, there was a dose-dependent relationship in inhibiting NO production for all treatments. The three essential oils showed a preventive effect more significant than the curative effect **while** *Inula graveolens* essential oil was slightly more active than the other **two** oils. The evaluation of the anti-inflammatory activity of the major compounds ( $\alpha$ - and  $\beta$ -costic acids) present in *Inula viscosa* and *Inula graveolens* essential oils, revealed that the anti-inflammatory potential exerted by these oils, is not mainly due to the major compounds, but rather to the synergetic effect between all the compounds. The next step will consist in the fractionation of the neutral extract in order to isolate and identify

the main compounds implicated in the anti-inflammatory activity. Finally, we can deduce that the studied essential oils could be used as anti-inflammatory agents for the treatment of various inflammatory pathologies.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s42250-023-00641-3>.

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**Author Contributions** NG and SD-D provided the plant materials. NG analyzed the results of this study and wrote the manuscript. L.M.A.A performed the biological experiments and analyzed the results. AM, CM, NB and SD-D supervised the study. All authors revised the manuscript. All authors have read and approved the final manuscript.

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## Declarations

**Conflict of Interest** The authors declare no conflict of interest.

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