Industrial Crops & Products 114 (2018) 81–86

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Aniseed (*Pimpinella anisum* L.) essential oil reduces pro-inflammatory cytokines and stimulates mucus secretion in primary airway bronchial and tracheal epithelial cell lines

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ARTICLE INFO

Keywords:
Pimpinella anisum
Essential oil
Anethole
Anti-inflammatory
Expectorant
Normal bronchial and tracheal epithelial cells (HBEpC/HTEpC)

ABSTRACT

*A*niseed (*Pimpinella anisum* L., Apiaceae) is a medicinal and aromatic plant widely cultivated in the Mediterranean area and used in foodstuffs, as ingredient of famous liqueurs, confectionery and bakery products. Its essential oil is one of the most selled on the market and used on an industrial level. In addition, aniseed is exploited as an herbal remedy to threat respiratory disorders. However, little information is available about its effect on respiratory tissues during inflammation. In the present work, we evaluated the anti-inflammatory activity and the effect on mucin secretion of aniseed essential oil in primary airway bronchial and tracheal epithelial cells (HBEpC and HTEpC, respectively) in a model of lung inflammation induced by lypopolysaccharide (LPS). The aniseed essential oil was obtained by hydrodistillation of the fruits from plants cultivated in central Italy, and analysed by gas chromatography-mass spectrometry (GC–MS). HBEpC and HTEpC lines were treated with LPS and then incubated with 0.3% of aniseed essential oil. Levels of pro-inflammatory cytokines, IL-8 and IL-1 beta were assessed by RT/PCR and Western Blot analysis. Muc5ac protein release was determined in culture medium of HBEpC- and HTEpC- LPS-treated lines by Western Blot analysis. Aniseed essential oil showed a very high level of (*E*)-anethole (97.9%). At non-toxic doses, it significantly decreased the expression levels of IL-1 and IL-8 and increased the Muc5ac secretion in LPS-treated HBEpC and HTEpC lines. These results provide new evidence supporting the ethnobotanical use of aniseed in the treatment of respiratory diseases. In particular, aniseed essential oil showed a significant anti-inflammatory effect on both HBEpC and HTEpC cells together with mucus hypersecretion.

1. Introduction

Inflammatory lung pathologies including several kinds of disorders such as asthma, emphysema, cystic fibrosis, and chronic obstructive pulmonary disease have become some of the most prevalent diseases in Europe (Weigand and Undem, 2012). During an inflammatory response, such cytokines as interleukin-6 (IL-6), IL-1, IL-8, IL-12, IL-18 and tumor necrosis factor (TNF) are released. The nuclear factor κB (NF-κB) is involved in regulating the expression of various genes encoding these pro-inflammatory cytokines and chemokines, together with growth factors and cyclooxygenase-2 (COX-2). In addition, inducible nitric oxide synthase (iNOS) and COX-2 stimulate the production of pro-inflammatory mediators (Hanada and Yoshimura, 2002; Makarov, 2000). Conventional therapy includes inhaled anti-inflammatory drugs such as glucocorticoids, which suppress the inflammatory process, but due to several undesired side effects, alternative treatments are needed (Rhen and Cidlowski, 2005). Moreover, during infection and inflammatory processes, the airway mucosa increases the mucus secretion. Mucus is made up of a polymeric matrix of large, oligomeric, gel forming glycoproteins, called mucins such as Muc5b and Muc5ac (Davies et al., 1999). Afterwards, mucus is cleared by ciliary movement and cough. During heavy infections, the activity of mucous cells seems to be reduced (Davies et al., 1999). Expectorant remedies are those substances capable of decreasing the adhesivity of secretions and increasing the airway hydration, which is often altered during inflammation (Rubin, 2007).

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https://doi.org/10.1016/j.indcrop.2018.01.076
Received 11 October 2017; Received in revised form 4 January 2018; Accepted 28 January 2018
Available online 16 February 2018
0926-6690/

Various medicinal and aromatic plants have been demonstrated to exhibit anti-inflammatory activity by decreasing the expression of pro-inflammatory cytokines (IL-1 beta, IL-6), COX-2 and iNOS (Chien et al., 2008; Krishaswamy, 2008; Marinelli et al., 2016).

Among traditionally used plants, *Pimpinella anisum* L., well known as aniseed (with the local name 'anice verde'), is an annual herb belonging to the Apiaceae family and native to the Mediterranean area (Italy, Spain, Greece, Turkey, North Africa, and the Middle East) (Pignatti, 1982) and cultivated on the worldwide scale (Sayed-Ahmad et al., 2017). The plant part used on an industrial level is represented by fruits, also called 'seeds' or 'aniseeds', which are schizocarps exuding a yellowish, aromatic and sweet oil (2–6%) (Leung and Foster, 2003). The latter is widely available on the market with a price ranging from 7 to 9 €/kg (Lubbe and Verpoorte, 2011). Currently, Spain, Turkey and China are the largest producers of aniseed (Sayed-Ahmad et al., 2017).

Aniseed is widely used in foodstuffs, as ingredient of alcoholic (e.g., brandy and liqueurs) and non-alcoholic beverages (e.g., herb tea), confectionery (e.g., cakes, candies) and bakery products (e.g., cookies, donuts, bread, 'maritozzi'), and in oral care products (e.g., toothpaste, mouthrinse). Notably, the aniseed-based liqueurs are differently named according to the geographic origin: i.e. 'anisetta', 'sambuca', 'mistrà' in Italy; 'anisette' and 'pastis' in France; 'ouzo' and 'mastik' in Greece; 'raki' in Turkey; 'arak' in Siria and Lebanon (Leung and Foster, 2003).

Aniseed enjoys a good reputation in the traditional medicine as digestive, carminative, diuretic, tranquillizer, galactogogue and expectorant agent (Tirapelli et al., 2007; Al-Bayati, 2008; Andallu and Rajeshwar, 2011; Sayed-Ahmad et al., 2017). In particular, aniseed is an ingredient of traditional teas used to protect the mucous layer in the hypopharynx tract and to give antispasmodic, secretolytic and antibacterial effects (Müller-Limmroth and Fröhlich, 1980). On the basis of the long-standing traditional use of aniseed, the Committee on Herbal Medicinal Products of the European Medicines Agency (EMA) approved its use as a treatment for mild indigestion and as an expectorant for coughs associated with colds (ema.europa.eu/Find medicine/Herbal medicines for human use). However, there is a lack of clinical evidence to substantiate these uses.

Aniseed essential oil, also known as '*Anisi aetheroleum*', is endowed with antioxidant, antifungal and antimicrobial properties (Kosalec et al., 2005; Gulcin et al., 2005). Furthermore, it showed anticonvulsant effects in mice (Pourgholami et al., 1999) and neuroprotective (Karimzadeh et al., 2012), antiulcer (Al Mofleh et al., 2007), and insecticidal (Pavela, 2014; Benelli et al., 2017) effects. Its marker compound is (*E*)-anethole (1-methoxy-4-(prop-1-enyl) benzene) (75–90%), a phenylpropanoid formed through the shikimate pathway via the amino acid phenylalanine; it is soluble in organic solvents but poorly soluble in water. Recently, the anti-inflammatory activity of (*E*)-anethole has been reported in a mouse model of LPS-stimulated acute lung injury (ALI). Pretreatment with anethole (250 mg/kg) reduced the numbers of pro-inflammatory macrophages and neutrophils, as well as the production of pro-inflammatory mediators such as TNF-α, MMP-9 and NO (Kang et al., 2013).

Given the long-standing traditional use of *P. anisum* as an expectorant agent, we decided to investigate the anti-inflammatory activity of the essential oil as well as its effect on mucin secretion in primary airway (bronchial/tracheal) epithelial cells (HBEpC/HTEpC) in a model of lung inflammation induced by LPS. In such a situation, the exposure to LPS has been demonstrated to increase the expression of several pro-inflammatory mediators such as IL-1beta and TNF-α in airway epithelial cells (Li et al., 1997, Marinelli et al., 2016).

2. Materials and methods

2.1. Plant material

Schizocarps of *P. anisum* were collected in Castignano (Ascoli Piceno, Marche, Italy, N 42°56′25″08; E 13°37′29″64, altitude 475 m a.s.l.) in September 2013 and dried at room temperature. The plant was identified by a member of our group (F. Maggi) using the available literature (Pignatti, 1982), and the nomenclature was checked against The Plant List database (www.theplantlist.org). A voucher specimen was deposited in the *Herbarium Universitatis Camerinensis* of the School of Biosciences and Veterinary Medicine (University of Camerino, Italy) with reference codex CAME 28168, and archived using the anArchive system for botanical data (anArchive system, http://www.anarchive.it).

2.2. Isolation of essential oil

Forty g of dry schizocarps were hydrodistilled in a Clevenger type apparatus (2 L volume) using 800 mL of distilled water for 2 h following the indications of the Pharmacopoeia. The essential oil was stored in a vial sealed with teflon septa in the dark at −20 °C before chemical analysis and biological experiments. The oil yield was estimated on a dry weight basis ($n = 3$).

2.3. Chemical analysis

Aniseed essential oil was analysed by GC–MS using an Agilent 6890N equipped with a 5973N mass spectrometer. For separation of compounds, an HP-5 MS capillary column (5% phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 μm film thickness; J and W Scientific, Folsom, CA) was used with helium as the carrier gas (1 mL/ min). The temperature programme selected was as follows: 5 min at 60 °C, 4 °C/min up to 220 °C, 11 °C/min up to 280 °C, held for 15 min, for a total run of 65 min. Mass range was 29–400 *m/z*. Mass spectra were recorded at 70 eV. The oil sample was diluted 1:100 in *n*–hexane and the volume injected was 2 μL (three replicates). Data were analysed by using MSD ChemStation software (Agilent, Version G1701DA D.01.00) and the NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library v. 2.0. The identification of essential oil components was performed by comparison of retention indices, calculated using a C_8–C_{30} series of *n*-alkanes (Sigma-Aldrich, Milan, Italy) and mass spectra of unknown peaks with those contained in the commercial libraries WILEY275, NIST 08, ADAMS and FFNSC2 as well as those in a homemade library. Furthermore, the identity of (*E*)-anethole was confirmed by comparison with analytical standard, which was purchased from Sigma-Aldrich. Percentage values of essential oil components were obtained from the peak areas in the chromatogram without the use of correction factors.

2.4. Cell lines

Human Bronchial/Tracheal Epithelial Cells (HBEpC/HTEpC) are derived from the surface epithelium of normal human bronchi/trachea (Sigma-Aldrich). HBEpC/HTEpC were grown in Bronchial/Tracheal Epithelial Cell Growth medium (Sigma-Aldrich), following the manufacturing protocol. This serum-free medium, which was fully supplemented with growth factors, trace elements, and antibiotics, was specifically designed to promote attachment, spreading and proliferation of HBEpC/HTEpC in culture. Media was changed every 48 h until cells were 90% confluent. All cell lines were maintained at 37 °C with 5% CO_2 and 95% humidity.

2.5. Reagents

The aniseed essential oil was properly diluted in DMSO and used at a concentration of 1 mg/mL. LPS (Sigma-Aldrich), the major component of the outer membrane of Gram-negative bacteria, was diluted in sterilized water at concentration of 2 mg/mL for each experiment.

2.6. MTT assay

Four × 10^4 HBEpC/HTEpC cells/ml were seeded in 96-well plates.

After 24 h, compounds or vehicles were added. At least four replicates were used for each treatment. At 72 h post-treatments, cell viability was assessed by 3-[4,5-dimethylthiazol- 2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich) assay. MTT (0.8 mg/mL) was added to the wells and left for 3 h, then the supernatant was removed and the pellet was solubilized with DMSO. The absorbance of the samples against a background control (medium alone) was measured at 570 nm using an ELISA reader microliter plate (BioTek Instruments, Winooski, VT, USA).

2.7. Gene expression analysis

Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Milan, Italy). Next, the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, PA) was used to synthesize cDNA according to the manufacturer's instructions. Quantitative real-time polymerase chain reactions (qRT-PCR) for IL-1beta, IL-8 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were performed, using PrimePCR™ PCR Primers (Bio-Rad, Hercules, CA) and the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). For PCR analysis, iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) was used. All samples were assayed in triplicates in the same plate. Measurement of GAPDH levels was used to normalize mRNA contents, and gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method.

2.8. Western blot analysis

HBEpC and HTEpC lines, treated with LPS, aniseed essential oil (0.3%) alone and in combination for 3 h, were lysed and twenty micrograms of the lysates were separated on a SDS polyacrylamide gel, transferred onto Hybond-C extra membranes (GE Healthcare). For protein secretion analysis, medium proteins were precipitated by a modified TCA protocol and then processed as described above. Blots were blocked with 5% low-fat dry milk in PBS-Tween 20, immunoblotted with mouse monoclonal anti-Muc5ac (1:500, Thermo Scientific, Rockford, IL), anti-IL-1 beta (1:100, Novus, Littleton, CO), anti-IL-8 (2 µL/mL, R&D System, Minneapolis, MN) and anti-GAPDH (1:8000, Origene, Rockville, MD) antibodies (Abs) overnight and then incubated with HRP-conjugated anti-mouse secondary Ab (1:2000, Cell Signaling Technology, Danvers, MA) for 1 h. Peroxidase activity was visualized with the LiteAblot ®PLUS or TURBO (EuroClone, Milan, Italy) kit and densitometric analysis was carried out by a Chemidoc using the Quantity One software (Bio-Rad).

2.9. Statistical analysis

The data represent the mean with standard deviation (SD) of at least 3 independent experiments. The statistical significance was determined by Student's *t*-test and by one way ANOVA.

3. Results

3.1. Composition of aniseed essential oil

The essential oil of *P. anisum* used in this study came from the 'Castignano' eco-type (Ascoli Piceno, central Italy) and exhibited a 2% yield. GC–MS analysis showed a significant (*E*)-anethole content (97.9%) (Table 1, Fig. 1). It is worth noting that the levels of (*E*)-anethole found in this study were significantly higher than those previously reported in samples from several European countries (Orav et al., 2008; Tabanca et al., 2006). This may be due to the favourable edaphic and climatic conditions of the cultivation area (Iannarelli et al., 2017). Among the minor components, we detected propenylphenols such as methyl chavicol (1.7%) and (*Z*)-anethole (0.1%), and pseudoisoeugenols such as (*E*)-pseudoisoeugenyl 2-methylbutyrate (traces). Terpenoids were only represented by γ-himachalene (0.3%).

Table 1
Composition of aniseed essential oil.

N.	Component[a]	RI Exp.[b]	RI Lit.[c]	%[d]	ID[e]
1	methyl chavicol	1195	1195	1.7 ± 0.8	RI,MS
2	(*Z*)-anethole	1250	1249	0.1 ± 0.0	RI,MS
3	(*E*)-anethole	1288	1282	97.9 ± 1.5	RI,MS,Std
4	γ-himachalene	1468	1481	0.3 ± 0.0	RI,MS
5	(*E*)-pseudoisoeugenyl 2-methylbutyrate	1840	1841	tr[f]	RI,MS
	Total identified (%)			100.0	

[a] Compounds are listed in order of their elution from a HP-5MS column.
[b] Linear retention index on HP-5MS column, experimentally determined using homologous series of C_8–C_{30} alkanes.
[c] Linear retention index taken from Adams (2007) or NIST 08 (2008) and literature (for compounds 19, 47, 58 and 62).
[d] Relative percentage values are means of three determinations ± SD.
[e] Identification methods: std, based on comparison with authentic compound; MS, based on comparison with WILEY, ADAMS, FFNSC2 and NIST 08 MS databases; RI, based on comparison of calculated RI with those reported in ADAMS, FFNSC 2 and NIST 08.
[f] tr, % below 0.1%.

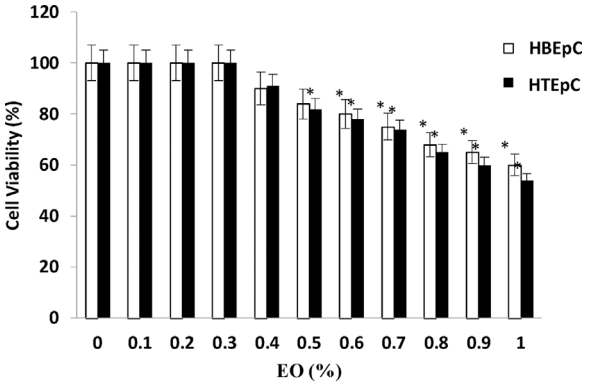

Fig. 1. Effects of aniseed essential oil in HBEpC and HTEpC cells. Cell viability was determined by MTT assay. HBEpC and HTEpC cells were treated for 72 h with different doses (0–1%) of aniseed essential oil. Data shown are expressed as mean ± SD of three separate experiments. *$p < .01$ vs 0% EO.

Noteworthy, (*E*)-pseudoisoeugenyl 2-methylbutyrate is considered a chemotaxonomic marker of *P. anisum* (Tabanca et al., 2006). The European Pharmacopoeia (EP) defined 0.5–5% as an optimal percentage range in essential oil for the metabolite methyl chavicol, which is a restricted substance occurring in flavourings and food ingredients with flavouring properties (Cachet et al., 2015), and indicated for (*Z*)-anethole the range of 0.1–0.4% (European Pharmacopoeia, 2005). The concentrations for methyl chavicol and (*Z*)-anethole in our sample fell within these ranges.

3.2. Cytotoxic effect in HBEpC and HTEpC lines

The cytotoxic effects of aniseed essential oil on HBEpC and HTEpC cell lines were evaluated by MTT assay. Cells were treated with different doses (0–1%) of aniseed essential oil and cell viability was evaluated after 72 h (Fig. 2). Given that aniseed essential oil did not significantly reduce cell viability (up to 0.3%) in either cell line, it was decided to use the higher non-cytotoxic dose (0.3%) for the next experiments.

3.3. Effects on IL-1 beta and IL-8 genes expression

LPS (20 mg/l) was added to the incubation medium to stimulate immune responses. An increase of IL-1 beta and IL-8 transcripts in LPS-treated cells, compared with non-treated cells, was found by qRT-PCR analysis (Fig. 2). Next, the LPS-stimulated HBEpC and HTEpC cells were

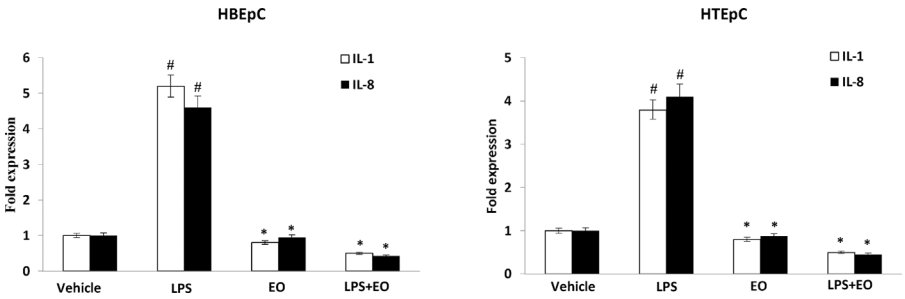

Fig. 2. Effect of aniseed essential oil on the LPS-induced IL-1beta and IL-8 gene expression in HBEpC and HTEpC cells. The expression of IL-1 and IL-8 mRNA was measured using real-time PCR. HBEpC and HTEpC cells were treated for 3 h with LPS (20 mg/l) and aniseed essential oil (final concentration 0.3%), alone or in combination. Relative gene expression normalized to GAPDH mRNA levels was calculated considering vehicle samples as calibrator. Values are mean ± SD of three different experiments. *$p < .05$ vs LPS, #$p < .05$ vs vehicle.

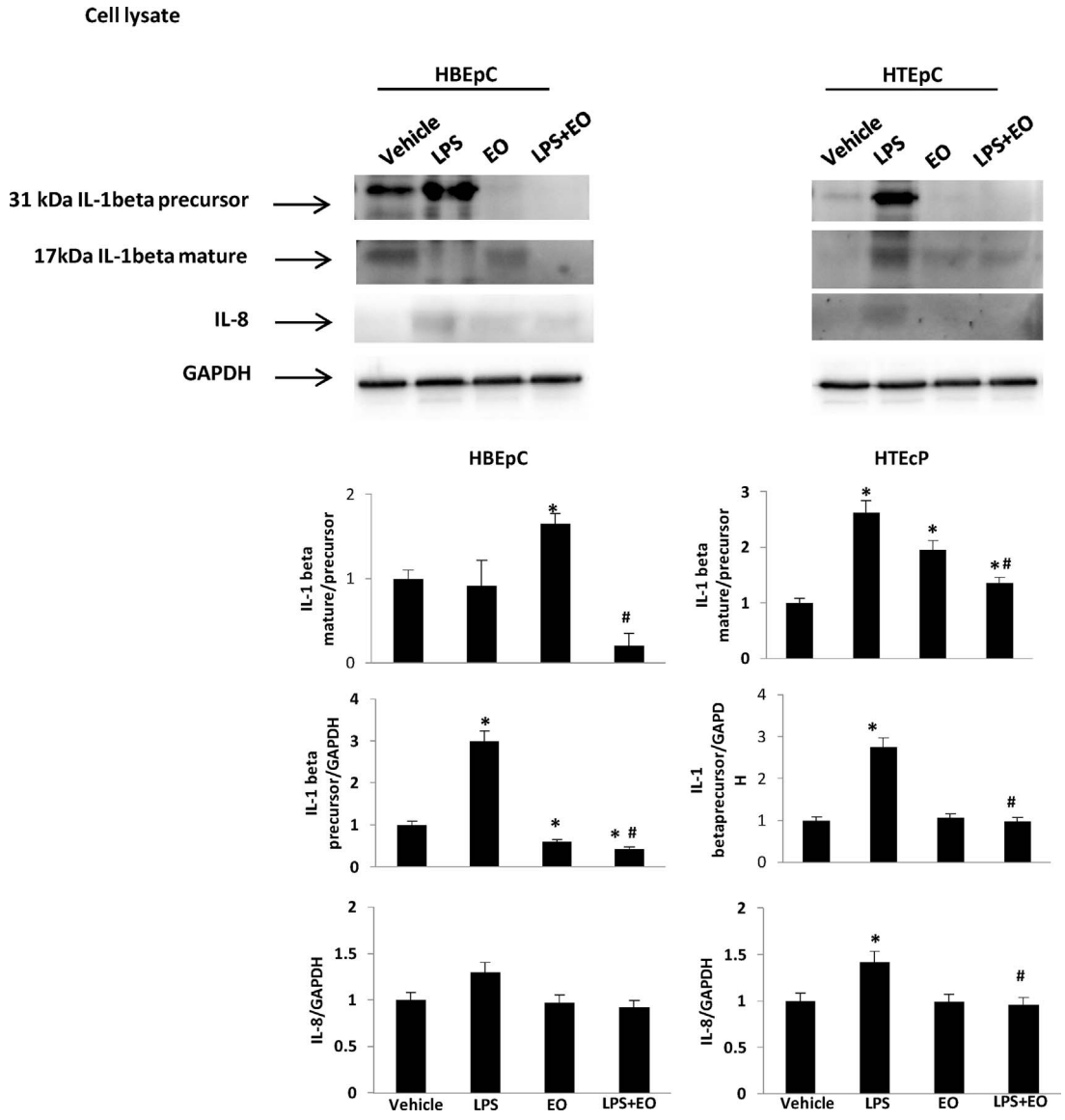

Fig. 3. Effects of aniseed essential oil on the LPS-induced IL-1beta and IL-8 production in HBEpC and HTEpC cells. HBEpC and HTEpC were treated for 24 h with LPS (20 mg/l), aniseed essential oil (final concentration 0.3%) alone or in combination. The production of IL-1beta, IL-8 was evaluated, in cell lysates, by western blot analysis. GAPDH was used as loading control. Immunoblots are representative of at least three different experiments. Densitometric values are the mean ± SD of three experiments. * $p < .05$ treated vs vehicle, # $p < .05$ LPS or EO.

treated with 0.3% aniseed essential oil for 3 h to evaluate its effect in reducing LPS-induced IL-1 beta and IL-8 gene expression. The results indicated that 0.3% of aniseed essential oil was able to reduce IL-1 beta and IL-8 transcript levels in LPS-treated cells (Fig. 2). Furthermore, we found that the 0.3% oil dose alone was able to reduce the IL-1 beta and IL-8 basal mRNA levels in both cell lines (Fig. 2).

3.4. Effects on IL-1 beta and IL-8 protein levels

After 3 h of incubation, LPS stimulated the increase of IL-8 and IL-1 beta protein levels, as assessed by western blot analysis (Fig. 3). Moreover, LPS-stimulated HBEpC and HTEpC cells treated with aniseed essential oil showed a significant reduction of IL-8 production compared to LPS-treated cells in both cell lines. Regarding IL-1 beta, there

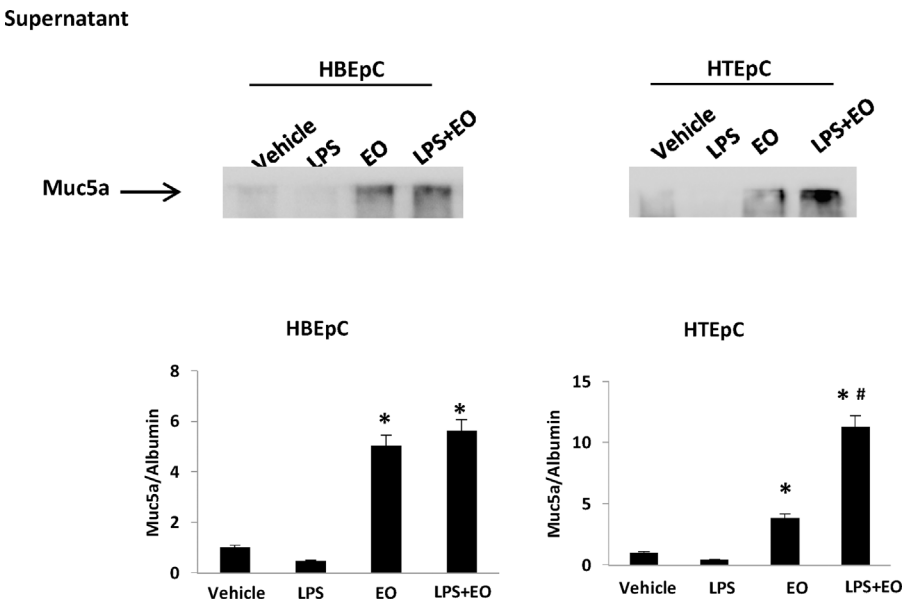

Fig. 4. Effects of aniseed essential oil on the LPS-induced MUC5ac secretion in HBEpC and HTEpC cells. The production of MUC5ac was measured using the culture supernatant of HBEpC and HTEpC cells stimulated for 24 h with LPS (20 mg/l) and anise essential oil (final concentration 0.3%), alone or in combination. Immunoblots are representative of at least three different experiments. Albumin levels were used as loading control. Densitometric values are presented as the mean ± SD of three experiments. * $p < .05$ treated vs veihcle, # $p < .05$ vs LPS or EO.

was a significant increase in the protein levels of the IL-1 beta precursor (31 kDa) and mature (17 kDa) forms after LPS treatment in both cell lines, whereas a reduction of the protein levels in the two forms was measured in LPS plus essential oil treated cells. Furthermore, essential oil alone was able to reduce both IL-8 and IL-1 protein levels compared with non-stimulated cells in both cell lines.

3.5. Aniseed essential oil stimulates the release of muc5ac protein from HBEpC and HTEpC cells

After 3 h, LPS treatment caused a reduction in Muc5ac secretion in both cell lines (Fig. 4). Treatment with 0.3% aniseed essential oil induced an increase in Muc5ac secretion in both HTEpC and HBEpC cell lines. The co-treatment of essential oil and LPS did not influence the Muc5ac secretion respect to essential oil-treated cells in HBEpC, while induced an increase in HTEpC cells.

4. Discussion

Though *P. anisum* has been used in traditional medicine as an expectorant, little scientific work has been published about its anti-inflammatory activity on the respiratory system or its effect on mucin secretion. One study reported that aniseed essential oil and phenylpropanoids isolated from different *Pimpinella* species, including *P. anisum,* inhibited the NF-κB dependent transcription induced by PMA (phorbol myristate acetate) in SW1353 cells (human chondrosarcoma cells) (Tabanca et al., 2007). The NF-κB pathway is one of the principal targets able to alleviate the symptoms of several diseases, including inflammatory ones. This mediator controls the expression of several genes in inflammation and carcinogenesis (Bremner and Heinrich, 2001). It has been shown that (*E*)-anethole blocks TNF-induced NF-κB activation, IκBα phosphorylation and degradation, and NF-κB reporter gene expression, and that it is a potent inhibitor of TNF-induced cellular response (Chainy et al., 2000). Thus, the inhibition of NF-kB pathway plays a pivotal role in the neutralization of pro-inflammatory signaling pathways such as those promoting cytokine and chemokine production in airway cell lines (Lawrence, 2009). As a matter of fact, a study in a mouse model of acute and persistent inflammation showed that anethole at a concentration of 250 mg/kg decreased the production of inflammatory mediators IL-6, TNF-α, and NO (Kang et al., 2013). More recently, it has been confirmed that (*E*)-anethole might be effective for prevention and treatment of chronic lung inflammation. In particular, this compound decreased the serum concentrations of pro-inflammatory cytokines such as IL-6 and TNF-α in a mouse model of chronic obstructive pulmonary disease (COPD) after oral administration (125 mg/kg) (Kim et al., 2017).

In line with literature data, our findings demonstrated that aniseed essential oil, containing 97% of (*E*)-anethole, reduces the levels of IL-1 and IL-8 in HBEpC- and HTEpC LPS-treated cells. IL-1 is a cytokine involved in the regulation of immune and inflammatory responses to infections. Il-1 alpha and IL-1 beta are the most studied cytokines. Whereas IL-1α is synthetized as a precursor protein, IL-1 beta is secreted only upon inflammatory signals. In particular, the expression of IL-1 beta is induced by transcription of NF-κB after innate immune cells are exposed to alarmins.

In this study, we also demonstrated that aniseed oil stimulates the Muc5ac secretion in HBEpC and HTEpC cells. Since Muc5ac is secreted by airways epithelial cells and functions as a protective layer, our data suggested that treatment with aniseed oil should protect the mucosa from infection and chemical damage by stimulating Muc5ac, which bind to inhaled microorganisms and particles that are subsequently removed by the mucociliary system. Our results are consistent with those of Coelho-de-Souza et al. (2013) who found that (*E*)-anethole is capable of significantly increasing the production of mucus by the gastric mucosa in mice at 30 and 100 mg/kg. Taken together these results seem to confirm that (*E*)-anethole, and (*E*)-anethole-rich essential oils increase factors involved in the protection of the airway mucosa through neutralization of pro-inflammatory cytokines, increasing the mucus production and stabilizing the surface of the epithelial cells. However, further studies elucidating the precise mechanisms are needed.

In conclusion, these results, although preliminary, confirm the ethnobotanical uses of aniseed in the treatment of airway inflammatory diseases where the beneficial effects are attributable to its major component anethole and encourage further studies on its mode of action on the respiratory system.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by the FAR 2014–2015 (Fondo di Ateneo per la Ricerca, grant No. FPI000044) of the University of Camerino. The authors would like to thank Sheila Beatty for her editing of the English

usage in the manuscript.

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