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Evaluations of thyme extract effects in human normal bronchial and tracheal epithelial cell lines and in human lung cancer cell line

Marinelli Oliviero [a, 1], Iannarelli Romilde [a, 1], Morelli Maria Beatrice [b], Valisi Matteo [c], Nicotra Giovanna [c], Amantini Consuelo [d], Cardinali Claudio [b], Santoni Giorgio [a], Maggi Filippo [a, **], Nabissi Massimo [a, *]

[a] School of Pharmacy, University of Camerino, Camerino, MC, Italy
[b] Dept. Experimental Medicine, University Sapienza, Rome, Italy
[c] Epo s.r.l., Istituto Farmochimico Fitoterapico, Milano, Italy
[d] School of Biosciences and Veterinary Medicine, Camerino, MC, Italy

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ABSTRACT

Thyme (*Thymus vulgaris*) is used traditionally to prepare herbal remedies possessing expectorant, mucolytic, antitussive and antispasmodic properties.

The aim of the present study was to investigate the effects of a standardized hydroalcoholic extract of thyme on primary human airway (bronchial/tracheal) epithelial cell lines in a model of lung inflammation induced by LPS. In addition, the effects of thyme extract on human lung cancer cell line (H460) were analysed. Thyme extract showed significant anti-inflammatory properties by reducing the NF-κB p65 and NF-κB p52 transcription factors protein levels followed by the decrease of pro-inflammatory cytokines (IL-1 beta and IL-8), and Muc5ac secretion in human normal bronchial and tracheal epithelial cells. Moreover, the extract showed cytotoxic effects on H460 cancer cells, modulated the release of IL-1 beta, IL-8 and down-regulated NF-κB p65 and NF-κB p52 proteins.

Taken together, these results substantiated the traditional uses of thyme in the treatment of respiratory diseases. Thyme extract might be an effective treatment of chronic diseases based on inflammatory processes when hypersecretion of mucus overwhelms the ciliary clearance and obstructs airways, causing morbidity and mortality. Moreover thyme extract, evaluated in H460 lung cancer cell line, demonstrated to induce cell cytotoxicity in addition to reduce inflammatory cell signals.

1. Introduction

Inflammatory lung pathologies have become one of the most prevalent diseases in Europe and are relevant cause of morbidity, mortality and health care expenditure. Many lung diseases are of inflammatory origin and the symptoms of these disorders are mediated by allergen-induced inflammatory reaction in the airways [1]. Whereby, the degranulation of resident mast cells and the release of pro-inflammatory mediators from lung epithelial cells, trigger and increase vascular permeability and infiltration of activated inflammatory cells [2]. As a consequence, chronic inflammation and destructive structural changes occur in the airways [3,4]. Despite the recommended therapy is based on inhalative anti-inflammatory drugs such as glucocorticoids that suppress the inflammatory processes and bronchodilators that act on reversing muscle contractility [5], the use of traditional herbal remedies in the treatment of respiratory diseases is widely adopted by world population [6]. In this regard, one of the most used herbal drugs is definitely thyme, ecompassing several species belonging to the genus *Thymus* L. (Lamiaceae family) that are used throughout the

Abbreviation: Abs, Antibodies; HBEpC/HTEpC, Bronchial/tracheal epithelial cells; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GCH, goblet cell hyperplasia; IL-1 beta, interleukin-1 beta; LPS, lipopolysaccharide; PI, propidium iodide; TNF-α, tumor necrosis factor-alpha; NF-κB, Nuclear factor-κB.

* Corresponding author. University of Camerino, Section of Experimental Medicine, School of Pharmacy, via Madonna delle Carceri 9, 62032 Camerino, MC, Italy.
** Corresponding author. University of Camerino, School of Pharmacy, via Sant'Agostino 1, 62032 Camerino, MC, Italy.
E-mail addresses: filippo.maggi@unicam.it (M. Filippo), massimo.nabissi@unicam.it (N. Massimo).

[1] Contributed equally to this work.

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world in the traditional medicine as well as in the food, cosmetic and pharmaceutical industries [7]. Actually, the most important species of the genus used in therapeutic dosage forms is *Thymus vulgaris* L. This species is used as a spice in many regions of the world and is one of the most important herbal drugs used as antibacterial agent [7-9]. In the traditional medicine, the flowering aerial parts of thyme are used to prepare infusions and decoctions possessing expectorant, mucolytic, antitussive and antispasmodic properties [10-13]. The most important bioactive compounds of thyme are phenols (thymol and carvacrol) [7], phenolic acids (rosmarinic and caffeic acids) and flavonoids (luteolin, quercetin and apigenin derivatives) [14].

Given the traditional uses of thyme in the treatment of respiratory diseases, during which the inflammatory process plays a major role, in this work we have evaluated whether a commercial standardized thyme extract could mediate inflammatory cytokines regulation. For the purpose, we studied the effects of thyme extract on primary human airway (bronchial/tracheal) epithelial cells (HBEpC/HTEpC) in a model of lung inflammation induced by lipopolysaccharide (LPS). Indeed, exposure to LPS has been demonstrated to increase expression of several pro-inflammatory mediators, such as interleukin-1 beta (IL-1 beta) and tumor necrosis factor-alpha (TNF-α) [15], and mucin gene expression with mucus production in airway epithelial cells [16,17]. Furthermore, since inflammation signals often support lung oncogenesis [18], the effects of thyme extract on human lung cancer cell line (H460) were investigated. Since NF-kB pathway is considered a prototypical pro-inflammatory signaling pathway and an upstream regulator of mucin gene expression [19], we evaluated thyme extract activity in regulating the canonical and non-canonical NF-kB pathways, with the aim to identify the potential mechanisms by which thyme extract reduces the expression of pro-inflammatory signals in all cell line tested.

2. Materials and methods

2.1. Cell lines

Human Bronchial/Tracheal Epithelial Cells (HBEpC/HTEpC) are derived from the surface epithelium of normal human bronchi/trachea (Sigma Aldrich, Milan, Italy). HBEpC/HTEpC were grown in Bronchial/Tracheal Epithelial Cell Growth medium (Sigma Aldrich), following the manufactoring protocol. Both cell lines were used between passages three and six. Aliquots of passage three, for both cell lines, were frozen in liquid nitrogen and cultured until passage six. Medium was specifically designed to promote attachment, spreading and proliferation of HBEpC/HTEpC in culture. This medium was fully supplemented with growth factors, trace elements, and antibiotics. Bronchial/Tracheal Epithelial Cell Growth Medium is serum-free. Media were changed every 48 h until cells were 90% confluent. H460 cells (carcinoma; large cell lung cancer; ATCC, LGC Standards, Milan, Italy) were grown in RPMI1640 medium (Lonza, Milan, Italy), supplemented with 10% foetal bovine serum (FBS), 2 mM/L glutamine, 100 IU/ml penicillin, 100 mg streptomycin and 1 mM sodium pyruvate. All cell lines were maintained at 37 °C with 5% CO_2 and 95% humidity.

2.2. Reagents

A commercial standardized thyme hydroalcoholic extract (thymol 0.3% w/w) was kindly provided by EPO s.r.l., Istituto Farmochimico Fitoterapico (Milan, Italy). Thyme extract was prepared in ethanol, sonicated and filtered (20 μm filter). Aliquots were prepared and stored at −20 °C for single use. Lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, was purchased from Sigma Aldrich and prepared as fresh for each experiment. LPS was diluted in sterilized water at concentration of 2 mg/ml.

2.3. Sulforhodamine B (SRB) assay

Cells were seeded at a density of 3×10^4 cells/ml in 96-well plates. After 24 h of incubation, extracts (up to 0.6% final concentration) or vehicles were added. Six replicates were used for each treatment. At the indicated time point, cell lines were fixed with cold trichloroacetic acid (TCA), stained by 0.4% SRB dissolved in 1% acetic acid. Bound stains were subsequently solubilized with 10 mM Trizma, and the absorbance was read on the microplate reader at a wavelength of 520 nm using an ELISA reader microliter plate (BioTek Instruments, Winooski, VT).

2.4. Cell cycle analysis

Cells, at a density of 3×10^4 cells/ml, were incubated with the appropriate thyme extract (0.3%) for up to 72 h. Cells were fixed by adding ice-cold 70% ethanol for 1 h and then washed with staining buffer (PBS, 2% FBS and 0.01% NaN3). Next, cells were treated with 100 μg/ml ribonuclease A solution (Sigma Aldrich), incubated for 30 min at 37 °C, stained with 20 μg/ml propidium iodide (PI) (Sigma Aldrich) for 30 min at room temperature and finally analysed by flow cytometry using linear amplification.

2.5. Apoptosis assays and PI staining

Cell death was evaluated using Annexin V-FITC and PI staining followed by biparametric FACS analysis. Cells, at a density of 3×10^4 cells/ml, were treated with thyme extract (0.3%) for a maximum of 72 h and then incubated with 5 μl Annexin V-FITC (Enzo Life Sciences, Farmingdale, NY, USA) and 20 μg/ml PI (Sigma Aldrich) for 10 min at room temperature. The percentage of positive cells determined over 10,000 events was analysed on a FACScan cytofluorimeter using the CellQuest software. PI-stained cells were also evaluated by fuorescent microscope analysis.

2.6. DNA fragmentation assay

Electrophoresis of DNA extracts was performed to assess DNA fragmentation as a criterion for necrosis and apoptosis. Briefly, cells, at a density of 3×10^4 cells/ml, were treated with thyme extract (0.3%) and genomic DNA was extracted using a DNA extraction kit (Qiagen, Hilden, Germany). The purified samples were then subjected to electrophoresis on a 1.25% agarose gel, and DNA was stained with ethidium bromide. Ultraviolet spectroscopy at 302 nm was used to obtain the results.

2.7. Western blot analysis

HBEpC, HTEpC and H460 cell lines, treated with LPS, thyme extract alone (0.3%) and in combination for 3 h, were lysed and 20 μg of the lysates were separated on a SDS polyacrylamide gel, transferred onto Hybond-C extra membranes (GE Healthcare), 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), anti−NF−kB p65 (1:500, Santa Cruz, Dallas, TX), anti−NF−kB p52 (1:500, Santa Cruz) and anti-glyceraldehydes-3-phosphate dehydrogenase (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.