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REVIEW OF JULIEN ANTIH'S THESIS :

Chemical composition and in vitro antibacterial effects of vapours of essential oils from plants recommended by the European Medicines Agency against respiratory infections

Objectives of thesis: The aim of this study was to assess the antibacterial activity of the vapour of five EOs hydrodistilled from aromatic plants species approved by HMPC of the EMA for the treatment respiratory infections and analyse their chemical compositions.

The specific objectives of the study are as follow:

i. Evaluation of the in vitro growth-inhibitory effect of EOs against bacterial pathogens causing pneumonia in liquid and vapour phase using broth microdilution volatilization method.

ii. Characterization of the chemical composition of T. vulgaris EO obtained from 3 different commercial suppliers using dual column/dual detector GC-MS.

iii. A comparative headspace-GC-MS analysis of chemical composition of samples obtained by HS-SPME and HS-GTS techniques from *T. vulgaris* EO vapours released from liquid and solid matrices.

REVIEW

Julien's ANTIH thesis entitled « Chemical composition and *in vitro* antibacterial effects of vapours of essential oils from plants recommended by the European Medicines Agency against respiratory infections » aims to evaluate antimicrobial activity of EOs used in european herbal medicine to treat respiratory tract infections, as inhalated therapies, combining evaluation of these EOs in both liquid and vapour phase. The thesis is divided into 3 sections : literature review / material and methods / Results and discussion.

I-Literature Review section :

A first bibliographic part describes the epidemiology of lower respiratory tract infections in paediatric population, emphasing on the most frequents bacteria involved : *Haemophilus influenzae*, *Streptococcus*

pyogenes, and *Staphylococcus aureus*, and conventional prescribed antibiotic treatments, both by oral and inhalated route.

After a general presentation of EOs (vegetal sources, production, phytochemical composition, analytical methods), a short review of antibacterial effect of EOs highlights differences of antibacterial activity between main phytochemical groups represented in EOs such as oxygenated mono- and sesquiterpens, phenylpropanoids, and small volatil fatty acids.

As herbal medicine is recognized and regulated by the European medicine Agency (EMA), Julien ANTIH choosed to focus on 5 EOs beneficiating of a validated indication for the treatment of lower respiratory tract infections in paediatric population : *Eucalyptus globulus*, *Thymus vulgaris*, *Mentha* x *piperita*, *Foeniculum vulgare*, and *Pimpinella anisum.* Bibliographical data describe the antibacterial activity of these 5 EOS on the 3 selected bacteria, and the identified compounds showing the highest antibacterial activity. Few studies dealing with the antibacterial effect of the EOs in vapour phase, are also presented. However, the poor available literature in this field highlights a lack of scientific data regarding the characterization of the antibacterial effect of EOs under their vapour phase.

Reviewer's comments on the literature review :

The literature review allows a good understanding of the thesis hypothesis, and objectives. However, a more detailed literature review focusing, in a specific chapter, on the studies on the EOs' vapour phase, would have been interesting. Particularly a detailed description and comparison of the few published technics used to determine the in vitro antibacterial effects of EOs' vapour phase would have been appreciated.

II-Material and Methods section :

In this section, after the description of the vegetal starting material (dried parts of the 5 selected species) provided by 3 suppliers, the extraction of EO by hydrodistillation using Clevenger apparatus is described.

- For antibacterial assays, strains and media are well characterized. A detailed description of the broth microdilution volatile (BMV) assay using 96-well microtiter plates with a specific covering system is presented with corresponding schemes of the organization of tested plates, and determination of MIC values using MTT dye. MIC are expressed in µg/ml for the liquid phase starting from 8 to 1024 µg/ml and in µg/cm3 for the vapour phase but from 2 to 256 µg/cm^3.

Reviewer's comment : A general scheme of the principle of the BMV assay would have been appreciated, in order to describe the composition of the well and corresponding flange and show the vapour phase contact zone with the agar in the flange, such as presented in Houdkova et al. 2021[1]. Moreover a detailed explanation of the determination of the dividing factor from 1024µg/ml for the liquid phase to 256 µg/cm^3 for the vapour phase should have been given. (well volume 400 µL…)

[1] Houdkova, M.; Chaure, A.; Doskocil, I.; Havlik, J.; Kokoska, L. New Broth Macrodilution Volatilization Method for Antibacterial Susceptibility Testing of Volatile Agents and Evaluation of Their Toxicity Using Modified MTT Assay In Vitro. *Molecules* **2021**, *26*, 4179. https://doi.org/10.3390/molecules26144179

-For EO's physicochemical analysis : Description of dual column/dual detector GC-MS is clear. Methodology for identification of compounds and determination of the quantitative relative percentage using FID are also clear.

The HS-SPME and HS-GTS used for the characterization of the vapour phase are clearly described and comprehensive general schemes illustrates this part.

Reviewer's comment : incubation times for both equilibrium of the vapour phase and adsoprtion on the SPME matrix should have been assessed by literature data.

III-Results and discussion section :

Extraction yields : The 15 EOs samples for the study were prepared using hydrodistillation process. Extraction yields for each EO is reported. A significant difference between the yields of the 3 suppliers is observed for the same species revealing the heterogeneity of available commercial samples.

Reviewer's comment : As these yields are in accordance with general description of main vegetal samples, they should also have been compared to European Pharmacopeia standards for medicinal vegetal drugs oils which requires minimum amonts of EO (ex *Mentha* x *piperita* dried leaves should contain from 9 ml/Kg (cut) to 12 ml/Kg (whole) (European Pharmacopeia 11.0).

Antibacterial effect : The antibacterial effect of EOs on both liquid (broth) and vapour (agar) phase using the BMV is reported in table 2 for all the 15 samples (3 samples for each of the 5 species). As explained in material and methods MIC are expressed in µg/ml for the liquid phase starting from 8 to 1024 µg/ml and in µg/cm3 for the vapour phase but from 2 to 256 µg/cm3. Antibacterial effect is detected for *E.globulus* and *T.vulgaris* EOs both in liquid and vapour phase.

Antibacterial versus chemical composition : A deeper study comparing antibacterial effect and chemical composition of the EO in liquid and vapour phase is described only for EOs obtaned from *T.vulgaris*. Analysed EO were obtained from a second hydrodistillation on the same batch, but different extraction yields, highlight a lack of reproductibility of the hydrodistillation process. However, chemical composition of the 3 batches of *T.vulgaris* obtained EOs are in accordance with main works for *T. vulgaris* with thymol type, with a slight difference in the γ-terpinene and carvacrol percentages. The use of a dual column/dual detector allows a more precse description of EOs composition.

Antibacterial effect of *T.vulgaris* both in liquid and vapour phase is weak regarding published works (for vapour phase CMI from 128 to 256 µg/cm^3 in this work compared to 0.125µg/cm^3 in the literature). In fact, the head-space study of the chemical composition of the vapour phase with EO diluted in the broth reveals a very low percentage of thymol, whereas *p*-cymene is the most abundant compound, which can explain this low antibacterial effect. Indeed comparison with the Headspace analysis of the EO adsorbed on a solid matrix of cellulose shows a really different chemical profile, with high proportion of thymol. These results are discussed and compared to previous works on the antibacterial evaluation of EOs in vapour phase.

Reviewer's conclusion :

This work describes the application of the BMV antibacterial assay, which allows the simultaneous evaluation of EOs' antibacterial effetc in both liquid and vapour phase, to 5 EOs used to treat respiratory tract infections. The design of the BMW assay, which allows to work on 96wells microplates, is indeed a powerful assay to evaluate the antibacterial effect of the vapour phase of EOs. The originality of this work, is the precise chemical characterization of the vapour phase. In fact, it reveals that vapour composition for *T.vulgaris* EO may vary depending of the matrix (broth/solid cellulose matrix) and thus lead to difference of antibacterial effects.

As there is a lack of data regarding the EOs' vapour phase composition and antibacterial effect during their medicinal use, Julien ANTIH's thesis affords some new elements to have a better understanding on the effectiveness of EOs used through the inhalated route. It may contribute to identify suitable and optimized administration devices to treat respiratory tract infections.

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