ITEMIZED RESPONSE TO THE REVIEWER'S COMMENTS

Reviewer: prof. PharmDr. Karel Šmejkal, Ph.D.

- **Formal comments**: Some minor graphical things, missing interpunctions etc., not decreasing the value of work. Some Figures possess caption above the picture (better bellow), but it is maybe because of the different version of wordfile processing.
- **Response**: with aim to improve minor graphical and formatting errors, all proposed comments have been accepted and the captions of pictures have been moved and anchored bellow the respective figures.

- **Question 1:** During GC analysis, some compounds with relatively high molecular weight like sterols and some fatty acids were observed. I would not expect these compounds observed simple in GC, don't they need derivatization for evaporation in GC injector?
- **Response:** Higher molecular weight constituents like sterols and fatty acids are highly polar and less amendable for GC-MS. These constituents were not expected as major compounds and therefore, no derivatization has been performed prior to analysis. However, especially in case of CO_2 extracts, such constituents have been determined. In case of *A. kravanh* CO_2 extract, where fatty acids constituted most of the sample, derivatization followed by GC-MS analysis appears to be a promising approach for further investigation of chemical profile.
- Furthermore, following sentence has been added to the results section **(pg. 58)** **"**although no derivatisation step has been performed prior to analyses".

- **Question 2:** Can you please describe the derivatization techniques for analysis of fatty acids (FAs) by GC?
- **Response:**
    - **Derivatization:** A procedure performed with aim to change the analyte properties for a better separation and for enhancing the method sensitivity. In case of fatty acids, derivatization is an essential step because of their low volatility and high boiling points.
    - **Some common derivatization techniques include:**
    - **a) Methylation:** Most common derivatization method for FAs, converting into their respective FA methyl esters (FAMEs). FAMEs are more volatile and stable than FAs in their native form and thus more suitable for GC-MS analysis. Partial steps include dissolving FAs in methanol and adding a catalyst (HCl, H_2SO_4, boron trifluoride BF_3, diazomethane and its safer alternative trimethylsilyl diazomethane TMSD).
    - **b) Silylation:** Such technique involves introduction of a silyl group into the FA molecule, which helps to protect polar groups and increase volatility. The reaction replaces a reactive hydrogen atom in COOH molecule with a silyl group. Silylation generally consists of hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) as derivatizating agents. Other reagents such as *N*-trimethylsilylimidazole (TMSI), *N*-methyl-*N*-trimethylsilylacetamide

(MSA), *N*-trimethylsilyldiethylamine (TMSDEA), *N*-trimethylsilyldimethylamine (TMSDMA), *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), *N*,*O*-*bis*(trimethylsilyl)acetamide (BSA) and *N*,*O*-*bis*(trimethylsilyl) trifluoroacetamide (BSTFA) can also be used for silylation.

- **c) Alkylation:** Derivatization method resulting in formation of other alkyl esters than methyl esters (usually ethyl or propyl esters).
- **d) Esterification with use of diazomethane:** Diazomethane is a reagent converting FAs unto their methylesters. (Topolewska et al., 2015)

Furthermore, following recommendation has been added to the discussion section regarding to *A. kravanh* CO_2 extract: **(pg. 107)**
"To confirm and further analyse the fatty acid profile of *A. kravanh* CO_2 extract, derivatization prior to analysis is highly encouraged to be conducted. Suggested methods for derivatization include methylation, silylation and alkylation (Topolewska et al., 2015)"

- **Question 3:** Similarly, equilin was detected in *C. cassia* CO_2 extract (in *C. verum* also in essential oil), is this a plant compound? Is the identification reliable?
- **Response:** Equilin is naturally occurring estrogenic sex hormone found in horses, more specifically in urine of pregnant mares. In our investigation, such compound was detected in *C. cassia* CO_2 extract and *C. verum* both EO and CO_2 extract, with higher occurrence in case of CO_2 extracts.
- Detection of such compound was based on comparison of MS spectra and RI was unfortunately not available in the literature. Therefore, inaccurate identification is likely. Compound could be present from contamination (very unlikely), or phytoestrogenic compound with similar spectra could have mimicked equilin.

- **Question 4:** Some compounds can come from contamination, like styren and dimethylsulfoxid in *C. cassia*, or are present naturally?
- **Response:** DMSO and styrene were detected in a headspace above the mixture of *C. cassia* buds EO and HM broth at a c= 256 μg/ml.
  - **Styrene:** occurs naturally in small amounts in some plants and foods (cinnamon). It can also be formed during biodegradation of naturally occurring compounds with similar structures (cinnamyl acetate, cinnamyl alcohol, cinnamic acid) (Cao et al., 2018).
  - **DMSO:** Small amount of DMSO is utilized for dissolving EOs prior to their mixing with a growth media (MH broth). In this case we suspect that detected DMSO comes from this preparation step.

Mgr. Pavlína Kyjáková, Ph.D.

- **Recommended corrections**:
- **Query 1**: All chromatograms need to be edited so that there are units on axes x and y (time and response of the detector).
- **Response**: All chromatograms that were part of the grouped figures (Fig. 11 and 12) have been corrected and have both axes with units present.

- **Query 2**: In the time series headspace sampling, Figure 8, page 78, it would be more informative to keep the scale on axis y in the same range, thus more comparable to each other. In Figure 9, I wonder if the comparison would not work better in peak areas, instead of relative percentages.
- **Response**: For the time reasons, changes have not been reflected in the thesis graphs, but will be shown at the defence. Furthermore, Fig. 10 containing a HS heatmap has been added for a better visualization of contents of 11 main constituents in the headspace over time (**pg. 80**).

- **Question 1:** "Subsequent time series of headspace sampling by solid phase microextraction and analysis of vapours above the mixture of growth medium and *C. cassia* fruits EO revealed a notable decrease in content of (*E*)-cinnamaldehyde in the headspace". What is the explanation for the decrease? You tested this only with the EO. Do you plan to test CO_2 extract in this respect?

- **Response:** with aim to explain the gradual decrease in the content of (*E*)-cinnamaldehyde in the headspace, we proposed the partially responsible mechanism. Most likely, (*E*)-cinnamaldehyde can pass back to the liquid medium, similarly to thymoquinone, previously reported by Novy et al. (2014). In this study, thymoquinone vapours also inhibited bacterial growth of *S. aureus* in wells not originally inoculated with this compound, but adjoining wells containing such constituent. We suggest similar phenomenon for (*E*)-cinnamaldehyde, which can result in the decrease of the content in the headspace over time.
    - EO from *C. cassia* fruits was tested, because it was the most potent and active sample in the antimicrobial assay which obtained the lowest MIC. Since CO_2 from the same material was not so effective, its headspace analysis was not performed.

- **Question 2:** Page 56: I need more information/explanation for Table 3. What is the difference between "not tested" and "not determined" and why were some of the extracts not tested?
- **Response:** In order to clarify the difference between not tested and not determined, additional information has been added to Table 3 footnote as follows:
- "NT: not tested (in main table body: this type of extract was not obtained for lack of material reason and thus not tested, in positive ATB control: this ATB is not used as a control for such bacterial strain), -: Not determined =>1024 μg/ml with no further MIC specification"

- **Question 3:** Page 94, in table 10, chemical composition of *P. nigrum* 'Kampot'. Observed retention index 2018, identified as "Heptadec-14-enal". Can you please share the identification details? How was the position of double bond determined?
- **Response:** Heptadec-14-enal was detected in small amounts (0.122 %) in *P. nigrum* 'Kampot' CO_2 extract during HP-5 column analysis. Constituent was identified solely based on comparison of NIST ms spectra, RI was not available in the literature and the position of double bond was not exactly determined.

doc. Ing. Adéla Fraňková, Ph.D.

- **Question 1:** The influence of the environment, maturity and plant variety on the chemical composition of extracts is mentioned in your work quite often. Did you any have chance to collect this information for tested plants?
- **Response:** Unfortunately, this information was not gathered during our experiments, as most of the samples were purchased. However, this presents an excellent opportunity for future research. Additionally, studying the collection of tropical spice species in relation to the dry and rainy seasons offers an intriguing research focus.

- **Question 2:** Antimicrobial activity of several plants was not tested due to the low extract yield. Why did not you buy or collected higher amount of the plants?
- **Response:** With aim to elucidate reasons of the low extract yield and lack of material, each Cambodian plant where EO was not obtained will be discussed during the defence as follows:
    - ***Boesenbergia rotunda*:** Material available at the market was limited and after receiving the sent samples by post, material also came back mouldy.
    - ***Zingiber zerumbet:*** Rhizome was collected from the wild and material available on location was limited. Part of the rhizome also came back mouldy.
    - ***Etlingera littoralis:*** Collection of such material was sort of coincidental since our local guide during an ethnobotanical field work started collecting flowers of this plant for cooking purposes. Since this plant was not pre-selected, but such use was exactly what we were looking for, amount of material I could ask for was limited.
    - ***Kaempferia galanga:*** Material was purchased at the specialised medicinal plant store and the availability was limited.

  Furthermore, following sentence has been added to the results section **(pg. 53)**:
  "In case of *B. rotunda* and *Z. zerumbet*, rhizome got contaminated by mould during the transportation. For *E. littoralis* and *K. galanga*, material available on location was limited".
- **Question 3:** The antimicrobial activity of EO was usually slightly better compared to CO_2 extracts, probably due to the higher content of the main antimicrobial agent (e.g. cinnamaldehyde). Did you consider measuring absolute concentration of the main antimicrobial compounds in CO_2 and EO extracts? This could help to elucidate, whether the other compounds in extract significantly influence the antimicrobial activity.
- **Response:** Although absolute concentration of the main antimicrobial compounds was not measured yet, some patterns can be observed. *C. cassia* fruit EO was the sample with highest content of (*E*)-cinnamaldehyde and the most effective sample regarding to antimicrobial activity. However, in *C. cassia* bark, CO_2 extract was more effective than EO despite lower content of cinnamaldehyde. Such observation could suggest involvement of other compounds present in the extract, also contributing to the antibacterial activity. Furthermore, EOs are chemically very complex and probably other minor constituents, and their ratio can play a role in the antimicrobial activity.

- **Question 4:** The antimicrobial activity of plant EOs against pathogenic bacteria is widely researched. Are there also data how the EOs you tested can influence the human microbiome?
- **Response:** Yes, there are multiple studies and reviews available, just some examples below:
    - **Myers et al. (2009)** investigating the potential of EO use to treat dysbiosis and IBS syndrome. *C. carvi*, *L. angustifolia*, *Trachyspermum copticum* and *C. aurantinum* var *amara* concluded as most potent but with a little detrimental effect on beneficial human microbiota (*Lactobacillus* and *Bifidobacterium* spp.)
    - **Lazar et al. (2022)** reviewing the potential use of EOs and nanoparticles with aim to treat dysbiosis.
    - **Spisni et al. (2020)** reviewing microbial-modulating and antioxidant activity in relationship to colon pathophysiology.
    - **Most common conclusion:** EOs and especially their single molecules are effective multitarget modulators of microbiota. However, determination of specific activities is rather complicated and affected by many factors, e.g. fate of individual compounds within the human body.

- **Question 5:** According to your findings and gained knowledge, can you recommend the best application of the CO_2 extracts and EOs in food industry as antimicrobial agents?
- **Response:** We would recommend a combination of EO or CO_2 extracts vapours in combination with modified atmosphere packaging technique as another hurdle. The best options include incorporation of EOs and CO2 extract into the active packaging with over-time release of active constituents. Furthermore, incorporation into nanoemulsions also presents an interesting research area (Ribeiro-Santos et al., 2017; Sánchez-González et al., 2011; Singh et al., 2022). However, organoleptic acceptance and antimicrobial effect in various food matrices would need to be thoroughly researched before such industrial application in food industry.
- **Furthermore**, more specific recommendation has been added to conclusion section as follows (**Pg.109**): "The most promising approach in food preservation would be incorporation of EOs and CO_2 extract into the active packaging with over-time release of active constituents. Furthermore, incorporation into nanoemulsions also presents an interesting research area (Ribeiro-Santos et al., 2017; Sanchez-Gonzalez et al., 2011; Singh et al., 2022)"

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