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On the mode of action of *Origanum vulgare* spp. *hirtum* methanolic extract and essential oil on *Chlamydomonas reinhardtii*

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Abstract

Aim: To reveal whether methanolic extract and essential oil from *Origanum vulgare* subsp. *hirtum* in doses causing even low levels of mortality in aphids, would have harmful effects on plants-genotoxic, mutagenic and/or DNA damaging. **Materials and methods:** Aerial parts of *Origanum vulgare* ssp. *hirtum* from the *ex-situ* collection of IBER, BAS during flowering were collected. Extraction and isolation procedures, as well as GC/MS analysis of essential oil and methanolic extract were performed by standard protocols. The components were identified by comparing their relative retention times to the retention times of authentic standards, and with mass spectra with the NIST. **Test system:** *Chlamydomonas reinhardtii* strain 137 C+ (WT). **Endpoints:** "clonal" assay, the test of "visible mutations", constant field gel electrophoresis. **Statistics:** GraphPad Prism version 6.04 (San Diego, USA) and One-way Analysis of Variance ANOVA with multiple comparisons using the Tukey method. **Results:** A good correlation was observed between chemical composition of essential oil and methanolic extract, and their mode of action. Our genotoxic and double strand breaks results demonstrated mild genotoxic and statistically non-significant DNA damaging potential of methanolic extract and concentration-dependent well - expressed genotoxic and DNA damaging potential of essential oil. A good relationship between increased double strand breaks levels and decreased survival might be related to one of the main constituents of essential oil, suspected to be carvacrol. No mutagenic effect for ME and EO was found. **Conclusion**: Well-expressed toxic/genotoxic capacity of essential oil, as well as its capacity to damage DNA inducing double strand breaks, but the absence of mutagenic potential, could be considered as a good reason to recommend *Origanum vulgare* subsp. *hirtum* essential oil as a promising candidate for purposes of "green" technologies.

Keywords
Cell survival, *Chlamydomonas reinhardtii*, DSBs, mutations, *Origanum vulgare* spp. *hirtum* methanolic extract, *Origanum vulgare* spp. *hirtum* essential oil

Introduction

For decades, the application of chemical / synthetic pesticides has been the most effective and common tool for weed, and pests control in agriculture. Unfortunately, their long-term use negatively affects the environment and biota, including human health (Gill and Garg 2014; Chu and Karr 2017; Böcker et al. 2019) due to the low biodegradability of most of them, and their ability to accumulate in the basic environmental matrices (Ali et al. 2019). On the other hand, many target organisms have increased their resistance to certain groups of pesticides that has provoked the development and release of new groups of chemical compounds (Heap 2021). As of 28 October 2021, the International Herbicide Resistant Weed website reported that 266 weed species (153 dicots and 113 monocots) were resistant to 164 different herbicides (Heap 2021). These alarming data "force" the scientific community to look for an environmentally-friendly solution for successful control of weed populations (Gnanavel 2015; Stankovic et al. 2020), and increased target specificity, as well as rapid degradation of the active substance (Cordeau et al. 2016).

Plants-based bioactive compounds with pesticide and/or herbicidal potential have been the focus of scientists for at least three decades (Balandrin and Klocke 1988; Gerwick and Sparks 2014; Fouad et al. 2015; Bona et al. 2016; Della Pepa et al. 2019; Elshafie et al. 2019; Gruľová et al. 2020) due to their chemical composition (Araniti et al. 2018; Jankowska et al. 2018; Lins et al. 2019).

Till now, the question of whether and how plants' essential oils or/and extracts could be effectively used for the purposes of "green agro chemistry" is ongoing. New information has been gathered about their insecticidal and inhibitory activity on seed germination and weed seedling growth (Matković et al. 2018; Nikolova and Berkov 2018; Stankovic et al. 2020), but information about their mutagenic, and/or DNA damaging effects are scarce (Karpouhtsis et al. 1998; Llana-Ruiz-Cabello et al. 2018).

This investigation was based on our previous finding that *Origanum vulgare* ssp. *hirtum* extracts and essential oil negatively affect *Myzus persicae* survival (Parvanova et al. 2020). Here, using *C. reinhardtii* as a plant test system, we aimed to reveal whether methanolic extract and essential oil of *Origanum vulgare* subsp. *hirtum* in doses causing even low levels of aphid's mortality would have harmful effects on plants – genotoxic, mutagenic, and/or DNA damaging.

C. reinhardtii was chosen because it is a robust model test-system in environmental mutagenesis (Chankova et al. 2006, 2013; Dimitrova et al. 2007, 2009, 2014; Chankova and Yurina 2012; Chen et al. 2012; Kopaskova et al. 2012; Jamers et al. 2013; Melegari et al. 2013; Aksmann et al. 2014; Angelova et al. 2014; Boenigk et al. 2014; Chalifour et al. 2014; Almeida et al. 2019; Xu et al. 2019; Todorova et al. 2020).

Materials and methods

Plant materials. Aerial parts of *Origanum vulgare* ssp. *hirtum* were collected during the flowering stage from the *ex-situ* collection of the Institute of Biodiversity and Ecosystem Research (IBER), http://www.iber.bas.bg/sites/default/files/projects/plantscollection/index.html.

Preparation of methanolic extract (ME). Air-dried, ground aerial parts of the species were extracted with methanol by classical maceration for 24 h. After filtration, the organic solvent was evaporated and the resulting crude extract was subjected to further analysis.

Isolation of essential oil (EO). The essential oil was extracted on a Clevenger apparatus by water distillation from 50 g dry plant material in a flask with 500 ml water for 2 h.

GC/MS analysis of EO and ME. For GC/MS analysis, 50 mg of methanolic extract was silylated with 50 µl of N, o-bis- (trimethylsilyl) trifluoroacetamide (BSTFA) in 50 µl of pyridine for 2 h at 50 °C. The spectra were recorded on a Thermo Scientific Focus GC combined with a Thermo Scientific DSQ mass detector as described previously (Berkov et al. 2021). The chromatographic conditions for EO analysis were described by Traykova et al. (2019). The quantities of the compounds have been expressed as the percentage area of the total peaks' area of the chromatogram. The components were identified by comparing their mass spectra and retention indices (RI) to known compounds from the literature, National Institute of Standards and Technology (NIST) and home-made MS databases.

Toxicity/Genotoxicity – a "clonal" assay, based on colony forming ability, was performed (Dimitrova et al. 2007). *C. reinhardtii* WT137C was cultivated at standard conditions – light of 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and t = 25 ± 3 °C to the end of the exponential and the beginning of the stationary phase. Concentrations of ME and EO – 250, 500, 750, 1000 ppm, as well as exposure time, were defined previously. Two negative controls – Sager-Granick liquid medium (SG) and 1000 ppm DMSO as a solvent, and one positive control – Nurelle D at the commercially- recommended concentration of 500 ppm for insects, were used. Both survival fraction (SF) (Bryant 1968) and three levels of lethality were calculated (Lidanski 1988).

Mutagenicity – test of "visible mutations", based on the changes in size, morphology, and pigmentation of surviving colonies, was applied. The method and calculations of a percentage of induced mutant colonies and index of mutagenicity (IM) were described by Dimitrova et al. (2007). When IM < 2.5 – no mutagenic effect is identified, when IM is in the range of 2.5 to 10, the mutagenic effect is mild, and when IM > 10, the mutagenic effect is moderate to strong.

DNA – the damaging potential of both ME and EO was evaluated by CFGE (Constant Field Gel Electrophoresis) (Chankova and Bryant 2002). The advantage of this method is well described by (Chankova et al. 2007). After electrophoresis ended, the gel was visualised in UV light and captured with a digital camera and the GeneSnap programme (SynGene). The images were analysed with GeneTools software (SynGene). The fraction of DNA released (FDR) from the wells was calculated according to Chankova et al. (2009).

Data analysis – All presented data are averages from at least three independent experiments. Statistical data processing was performed with GraphPad Prism version 6.04 (San Diego, USA) and One-way Analysis of Variance (ANOVA) with multiple comparisons, using the Tukey method to compare the results of different treatments.

Results

Chemical composition – carvacrol (74.34%), *p*-cymene (9.46%), *γ*-terpinene (10.66%), *α*-pinene (1.73%), *β*-pinene (1.34%) and carvacrol methyl ether (1.23%) were identified as the main components of essential oil. The other components were presented in quantities of less than 1%.

In the methanolic extract, various primary metabolites as fructose (10.32%), glucose (11.65%), sucrose (10.51%), organic acids – succinic (0.60%), malic (1.09%) and linolenic acid (0.78%) were found. Phenolic acids – 4(p)-hydroxybenzoic (0.53%) and vanillic (0.22%), rosmarinic acid (6.06%), terpenoids – carvacrol (15.67%), caryophyllene (0.40%), flavonoids – catechin (0.23%) 6-hydroxyflavone glycoside (1.49%) were identified as the main secondary metabolites.

Toxicity/Genotoxicity – as the first step of our investigation, we had to clarify two points: whether Nurelle D, chosen as a positive control at the recommended commercial dose for aphids control, would have a detrimental effect on the model plant cells and whether DMSO, as a solvent, would affect *C. reinhardtii* cells negatively. As seen in Fig. 1, no statistically significant decrease in the cells survival fraction after the treatment with DMSO and Nurelle D was found, compared to the negative control SG ($P < 0.05$).

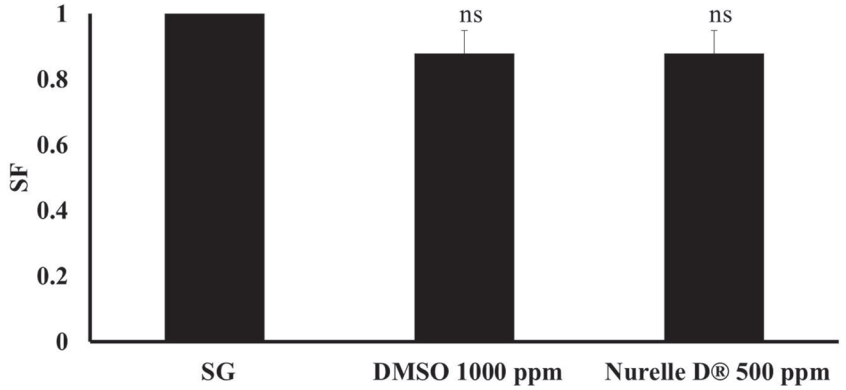

Figure 1. Cells survival fraction in negative and positive control samples of *C. reinhardtii* strain 137C. Mean values from at least three independent experiments. Error bars represent standard errors of mean values. The statistical significance of the differences is presented as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns – no significant difference.

Analysing curves in Fig. 2, statistically significant reduction of cell survival after the treatment with both highest concentrations of ME 750 ppm (SF = 0.25 ± 0.08) and 1000 ppm (SF = 0.00026 ± 0.08) was shown. In the range of 250 ppm and 500 ppm, some plateau was formed. Better expressed dose-effect was obtained for EO (Fig. 2) at a concentration of 250 ppm or above (SF = 0.51 ± 0.09; SF = 0.34 ± 0.11 and SF = 0.09 ± 0.02) (*** $p < 0.0001$).One-way ANOVA analysis reveals statistically significant differences between: SG vs. 250 ppm EO; SG vs. 500 ppm EO; SG vs. 750 ppm EO; SG vs. 1000 ppm EO; SG vs. 750 ppm ME; SG vs. 1000 ppm ME (***, $p < 0.001$).

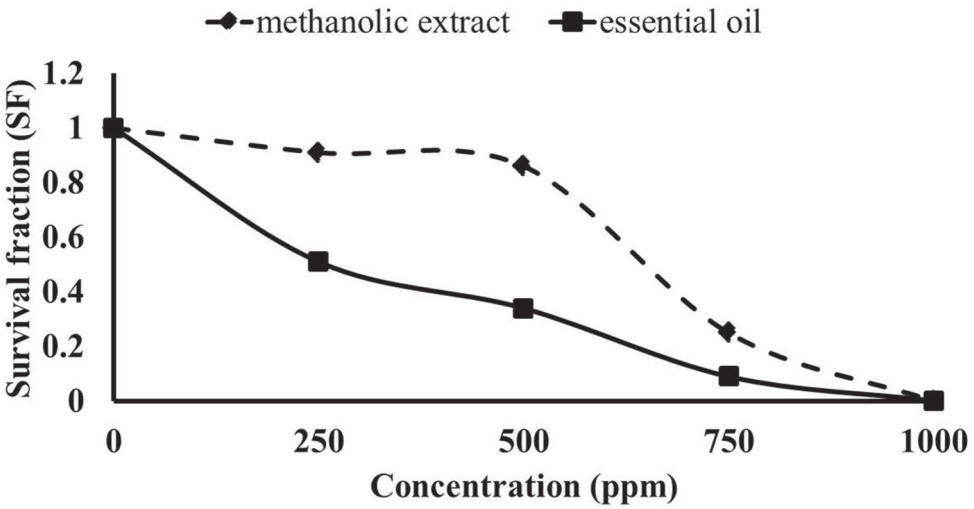

Figure 2. Cells survival fractions (*SF*) after the treatment with *Origanum vulgare* ME and EO. Mean data are from three independent experiments. Error bars represent standard errors of mean values. Where no error bars are evident, errors were equal to or smaller than the symbols.

Further, we had to calculate three levels of lethality – LD_{20}, LD_{50} and LD_{80} as a commonly-used approach for comparing the genotoxic potential of standard mutagens or other chemical/physical factors. The stronger genotoxic potential of essential oil is obvious by data shown in Table 1. Approximately two-fold lower EO concentrations can induce LD_{20}, LD_{50} and LD_{80} comparing with those of ME.

Table 1. LD_{20}, LD_{50} and LD_{80} in strain 137C, measured after the treatment with *Origanum vulgare* spp. *hirtum* ME and EO.

Origanum vulgare subsp. hirtum	**LD_{20}**	**LD_{50}**	**LD_{80}**
Methanolic extract [ppm]	523	634	810
Essential oil [ppm]	< 250	263	588

Mutagenicity – test of "visible mutations"

The next step in our investigation was to reveal whether both ME and EO of *Origanum vulgare* spp. *hirtum* possess some mutagenic potential on *C. reinhardtii*. The level of spontaneous "visible mutations" was 0.136%. Calculated IM clearly demonstrated an absence of mutagenic capacity for DMSO and Nurelle D. No mutagenic capacity of ME and EO was identified (IM < 2).

DNA-damaging potential of ME and EO

Our CFGE results show no DNA damaging capacity of ME. The levels of DSBs, measured after treatment with concentrations in the range of 250 – 1000 ppm, were approximately similar to the levels of spontaneously arisen DSB in the control sample (Fig. 3). One-way ANOVA analysis reveals statistically significant differences between: SG vs. 250 ppm EO; SG vs. 500 ppm EO; SG vs. 750 ppm EO; SG vs. 1000 ppm EO (***, $p < 0.001$); SG vs. 500 ppm ME (*, $p < 0.05$).

A quite different curve was drawn from the DSB levels measured after treatment with EO (Fig. 3). Around two-fold higher levels of DSBs were calculated compared to those in the negative control and the samples treated with methanolic extract. All differences, showing higher values compared to the negative control, were statistically significant. These results demonstrate a stronger DNA-damaging potential of oregano EO than that of ME under our experimental conditions. A well evident correlation was found between the potential of oregano EO to induce DSBs and lower SF measured as a colony-forming ability.

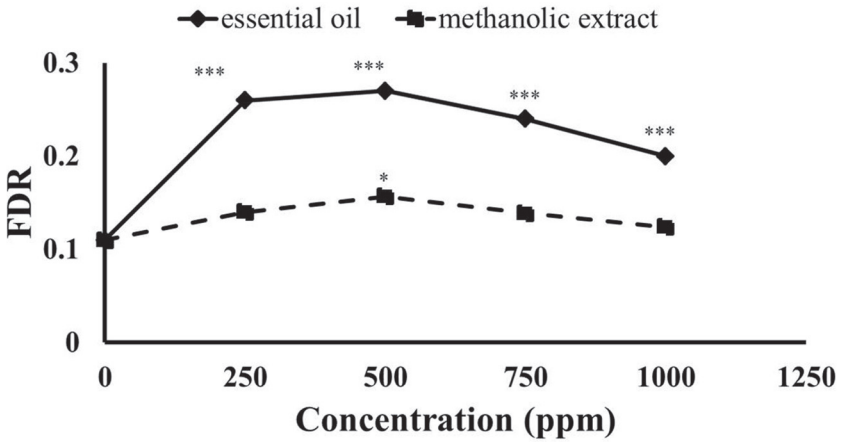

Figure 3. DSBs measured after treatment with different concentrations of ME and EO of *Origanum vulgare* spp. *hirtum*. Mean data are from three independent experiments. Error bars represent standard errors of mean values. Where no error bars are evident, errors are equal to or smaller than the symbols. The statistical significance of the differences is presented as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns – no significant difference.

Discussion

Previously, it was found by us that *Origanum vulgare* ssp. *hirtum* extracts and essential oil can cause *Myzus persicae* mortality depending on the concentrations applied. Here, an attempt was made to clarify whether ME and EO of *Origanum vulgare* subsp. *hirtum*, in doses causing even low levels of mortality in aphids, would have harmful effects – toxic/genotoxic, mutagenic and/or DNA damaging on *C. reinhardtii*, used as a plant test-system.

The better-pronounced capacity of EO vs. ME to decrease *C. reinhardtii* cell survival was demonstrated by comparing the concentrations inducing three levels of lethality – LD_{20}, LD_{50} and LD_{80}. It was calculated that EO is about 1.4–2-fold more genotoxic for algae cells than ME. Till now, a large spectrum of effects of oregano EO has been described – phytotoxic (Ibáñez and Blázquez 2017, 2020; Grul'ová et al. 2020; Abd-ElGawad et al. 2021), antimicrobial (Karaday et al. 2020; Simirgiotis et al. 2020), antifungal (Pušk árová et al. 2017; Saghrouchni et al. 2021); insecticidal (Alkan 2020), anti-plant pathogens (Raveau et al. 2020) etc. The data reported by us have further expanded this spectrum.

Information concerning DNA damaging or mutagenic potential of ME and EO are scarce. Llana-Ruiz-Cabello et al. (2018), using both MN test and comet assay (standard and enzyme-modified), have reported no increased levels of MN and DNA damage. Contrary to this study, our experiments revealed, for the first time, the DNA damaging capacity of oregano EO. It was clarified that the level of DSBs depends on the concentration applied. The good relationship between increased DSBs levels and decreased survival, described by us, might be linked to one of the main constituents of EO, likely to be carvacrol.

Both oregano ME and EO were shown to possess no mutagenic activity in *C. reinhardtii* test-system. Similar findings have been described previously by others (Adam et al. 1998; Karpouhtsis et al. 1998). No mutagenic effect on Aims test and no mutagenic or recombinogenic activity for ME and EO were found, using the Wing Somatic Mutation and Recombination Test (SMART) on *D. melanogaster.*

Conclusion

In this study, mild toxic/genotoxic and statistically non-significant DNA damaging potential of ME and concentration-dependent effects of EO were identified. The differences in the mode of action of EO and ME could be related to differences in their chemical composition. Further experiments are required in order to clarify the effect of main and minor constituents. Well-expressed toxic/genotoxic capacity of EO, as well as its capacity to damage DNA inducing DSBs, but the absence of mutagenic potential, could be considered as a good reason to recommend *Origanum vulgare* subsp. *hirtum* EO as a promising candidate for purposes of "green" technologies.

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