



The role of dilution mediums in studies of fumigant insecticidal activity of essential oils

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Received: 27 February 2020 / Revised: 8 May 2020 / Accepted: 25 May 2020 / Published online: 3 June 2020
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Abstract

The essential oils (EOs) are volatile plant extracts that are commonly considered as potential biopesticides. They present promising properties as relatively safe and potent insecticides, primarily delivered via fumigation. Such specificity of the EOs has led to the growing interest of researchers and, subsequently, to an increasing body of publications. The studies focusing on assessing the insecticidal action of EOs frequently require testing the effects of several dilutions of a given EO. To this end, researchers employ a range of solvents differing widely between the studies. While informative, the standardization concerning either tested oil or pest species is lacking in these studies. The presented study herein aimed to test whether the solvent used could affect the observed insecticidal activity of tested EO. As the model setup for studying the possible influence of solvents on the insecticidal effect of EOs, two EOs: mint and rosemary were chosen. The EOs were applied in fumigation assay against three economically important species of stored product pests: *Callosobruchus maculatus*, *Sitophilus oryzae*, and *Tribolium castaneum*. Each EO was tested in three concentrations—diluted with a range of solvents; dimethyl sulfoxide, acetone, methanol, ethanol, paraffin (mineral) oil, ultrapure water with 0.1% polysorbate 20 (TWEEN® 20). The results confirm the hypothesis that the applied solvent could significantly alter the observed efficiency of tested EO. Such a result is of crucial value for conducting credible meta-analysis as well as for establishing a high standard of reproducibility.

Keywords Solvent · Dilution medium · Insecticide · *Callosobruchus maculatus* · *Sitophilus oryzae* · *Tribolium confusum*

Key message

- The essential oils insecticides research lacks standardization regarding solvent used for diluting the oils.
- The article investigated the potential impact of the commonly used solvent on the insecticidal effectiveness of two essential oils.
- The mortality assessment was conducted on three model species of the stored products pests, namely: *Callosobruchus maculatus*, *Sitophilus oryzae* and *Tribolium confusum*.

- Tested solvents alone did not exhibit insecticidal action.
- The solvent in which essential oil is diluted could significantly affect its observed insecticidal properties.

Introduction

Pesticides are natural or synthetic chemicals extensively used in contemporary agriculture as a control agent of pests and diseases. According to a report by the US Environmental Protection Agency, more than two million tons of pesticides are currently used worldwide each year, and this value is constantly significantly increasing (Atwood and Paisley-Jones 2017). After the herbicides, insecticides are the largest group among pesticides (29.5%) (De et al. 2014). Due to their extensive use, these substances permeate the soil, water, and air, eventually entering and accumulating in trophic chains, thus, posing a considerable risk to non-target organisms, including vertebrates (Taylor et al. 2002). In order to

Communicated by C.G. Athanassiou.

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counteract this problem, there is a growing emphasis on reducing the use of commonly applied pesticides in favor of biopesticides, other natural plant products, and physical methods of insect infestation control (Navarro and Navarro 2018). Among these methods, essential oils (EOs), which are mixtures of volatile secondary metabolites produced by plants, are becoming increasingly popular (Bakkali et al. 2008).

According to the scientometric analysis of publications from the Web of Science, conducted in a similar way as Isman and Grieneisen (2014), an increasing number of articles describe the potential use of EOs as insecticides (Fig. 1). That may be caused by, among other things, comparably low vertebrates toxicity, high availability, relatively low price, and reduced probability of generation of resistance in targeted insects (Koul et al. 2008; Arena et al. 2018). Many of these studies (44.05%) focus on fumigation toxicity to determine the effect of EOs' vapors on the mortality of a specific insect species, and due to the relatively low LC_{50} and the sizes of the used experimental systems, most of the authors dilute essential oils in various substances (i.e., acetone, methanol, ethanol) (Mohamed and Abdelgaleil 2008; Dayaram and Khan 2016; Manju et al. 2018). However, to the author's best knowledge, no studies have yet been carried out to determine whether those substances affect the observed insecticidal properties of essential oils.

The aim of the current study was to investigate the potential impact of the most commonly used dilution mediums (DM) on the acute toxicity of two EOs on three model species of the stored products pests [namely: *Callosobruchus maculatus* (F.), *Sitophilus oryzae* (L.) and *Tribolium confusum* (Herbst)]. These specific species were selected due to having the highest number of publications reporting EOs' effect on them, and many reports describe research on the same insect species but use different DM. Therefore, we

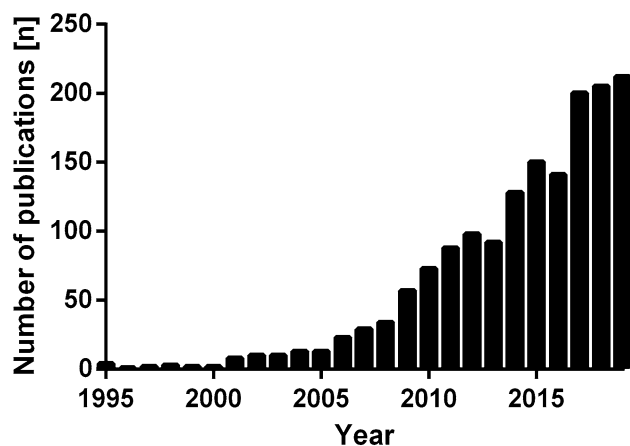


Fig. 1 Bibliometric analysis for query "Essential oil insecticide". <http://apps.webofknowledge.com/> date of access: 20.02.2020

hypothesized that the DM used could significantly affect the observed insecticidal properties of tested EOs. Such discrepancy in methods could pose a crucial, but previously omitted, factor affecting the entire branch of studies on EOs.

Materials and methods

Insects rearing

Cowpea weevils, *C. maculatus*, were reared on mung beans (*Vigna radiata*) obtained from local vendors. 1-week-old, unsexed adults were used. Confused flour beetles, *T. confusum*, were reared in the plastic terrarium (ExoTerra "S" 16.5 × 11 × 13cm) on oat bran obtained from local vendors. Rice weevils, *S. oryzae*, were reared in a plastic terrarium (ExoTerra "S" 16.5 × 11 × 13cm) on barley groats obtained from local vendors. All the insects were reared in the constant conditions of 31 ± 1 °C, relative humidity $50 \pm 10\%$, and the photoperiodic regime of 12/12 h light/dark.

Used substances

Analytical grade, undiluted methanol, ethanol (96%), and acetone were obtained from P.P.H. STANLAB Poland. Dimethyl sulfoxide (DMSO) was purchased from POCH S.A. Poland, while paraffin oil was obtained from P.H.U. Agola Poland. For all water solutions, Tween 20 (Sigma-Aldrich) and ultrapure water (Millipore SimPak1 Purification system) were utilized. All of the EOs used in the assessments were water distilled and provided by Naturalne Aromaty sp. z o.o.

Fumigation toxicity bioassays

Mortality was assessed in five repetitions for each species (each repetition consisted of ten unsexed individuals; $n = 1350$, sum of all individuals- $\Sigma_n = 4050$). Each replication consisted of two EOs (selected based on previous pilot studies) diluted in six solvents, namely: dimethyl sulfoxide (DMSO), acetone, methanol, ethanol, paraffin (mineral) oil, and ultrapure water with 0.1% polysorbate 20 (TWEEN® 20). Diluted in the following proportions 1.6 µl; 0.8 µl; 0.4 µl of oils to 3.4 µl; 4.2 µl; 4.6 µl of solvents, obtaining 5 µl of test solutions (Fig. 2). For the positive control groups, pure EO's (5 µl) were used and, respectively, for the negative control, pure solvents (5 µl) and untreated insects were applied. 5 µl of test solutions were applied on the cotton pad attached to the cover of the container and left for 5 min undisturbed to allow the evaporation. Insects were then put into the 50 ml plastic containers with tight-fitting lids. Dead beetles were counted and removed after 24 and 48 h. Insects were considered dead when no movement for 1 h was observed. All

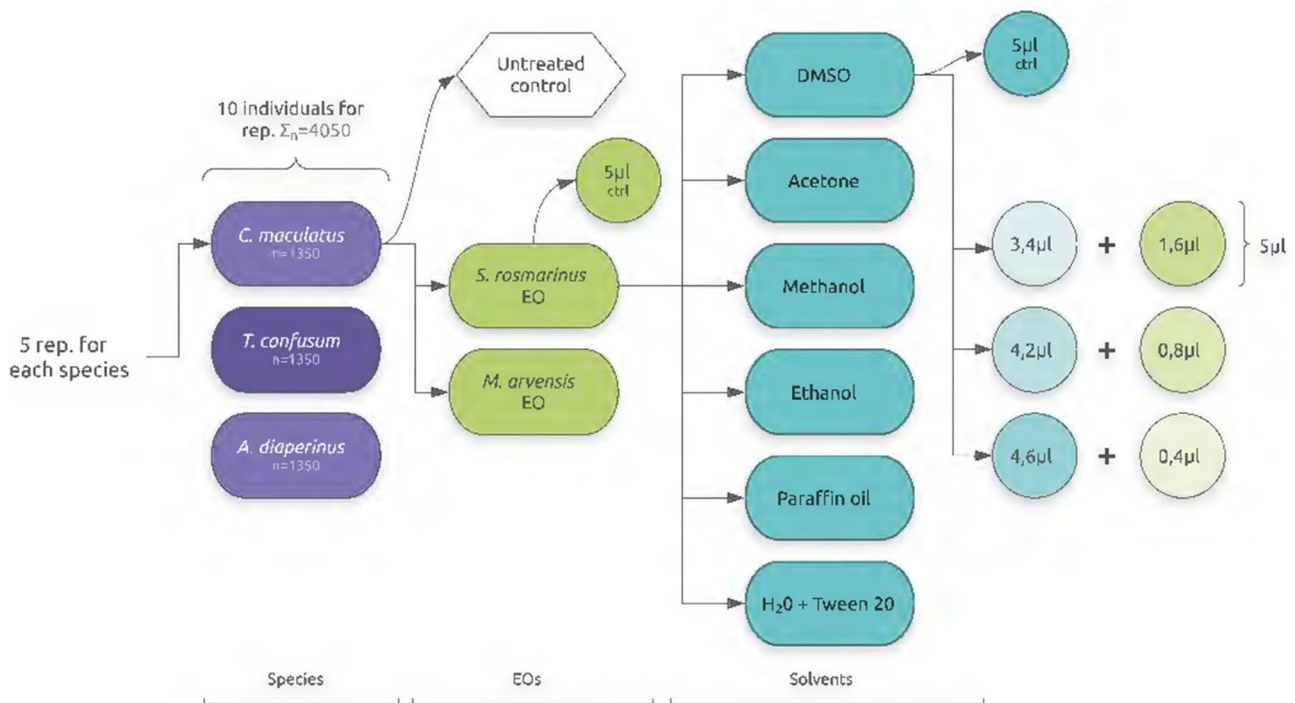


Fig. 2 Experimental procedure workflow. The single path on the flowchart represents one experimental variant

tests were carried out under the same conditions as during the rearing.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism v6 software for Windows. Heatmaps were prepared using plot.ly (<https://chart-studio.plot.ly/>), experimental procedure flowchart was prepared with Lucidchart. Additionally, the multivariate analysis with the classical clustering method (paired method, Euclidean similarity index) was conducted using PAST v4 (Hammer et al. 2001). For all the obtained data, comparison tests between independent sampled groups on a single, non-normally distributed continuous variable were performed (Kruskal–Wallis with Dunn’s multiple comparisons test, $p < 0.05$). Due to a large number of used substances, many control tests had to be carried out (effect of solvents on insect mortality). Statistical analysis of the effects showed that there were no statistically significant differences between the control groups (Table 1). Therefore, the baseline control (without solvent) was used as a reference. To avoid confusion, the baseline control is abbreviated as “Ctrl” while the solvent controls groups are abbreviated, respectively, as “Ctrl_{DMSO}”, “Ctrl_{Acetone}”, “Ctrl_{Oil}”, “Ctrl_{Ethanol}”, “Ctrl_{Tween0.1%}”, “Ctrl_{Methanol}”.

Results

Baseline and all solvent control groups did not cause increased mortality and did not differ significantly from each other (Table 1), unlike the positive control groups (pure EOs), which differed significantly and caused relatively high mortality (Table 2).

The analysis of insect mortality after 24 h indicates differences between the species, used solvent, type, and volumes of EOs. In the case of *S. oryzae*, there were no differences in groups treated with rosemary EO, whereas in the case of *T. confusum*, there were no differences were observed in groups treated with mint EO. In *T. confusum*, differences were observed in the groups treated with 1.6 µl of rosemary EO solution (ethanol, methanol and oil). In the case of *C. maculatus*, significant differences were found in the groups treated with solutions of 1.6 µl of mint and rosemary EOs (acetone, methanol, ethanol, and oil) and 0.8 µl of rosemary (ethanol) (Fig. 3).

After 48 h in the *S. oryzae* groups treated with rosemary EO solutions, no differences were observed. In the other groups, the effect similar to that after 24 h was observed except for increased mortality in *S. oryzae* (1.6 µl mint EO with oil solvent) and *T. confusum* (0.8 µl rosemary EO) groups. A significant increase in mortality was observed in the *T. confusum* group treated with mint EO solutions in ethanol, methanol, and oil. Additionally, *C. maculatus* groups

Table 1 Mean mortality of three beetle species (*C. maculatus*, *S. oryzae* and *T. confusum*) after 24 and 48 h in the negative control groups

	Mortality [%]					
	<i>C. maculatus</i>		<i>S. oryzae</i>		<i>T. confusum</i>	
	24 h	48 h	24 h	48 h	24 h	48 h
<i>Negative control</i>						
Ctrl	0a	0a	0a	0a	10a	10a
Ctrl _{DMSO}	0a	0a	0a	10a	0a	0a
Ctrl _{Acetone}	0a	0a	0a	0a	0a	0a
Ctrl _{Oil}	0a	0a	0a	0a	0a	0a
Ctrl _{Ethanol}	0a	0a	10a	10a	0a	0a
Ctrl _{Tween}	10a	10a	0a	0a	0a	0a
Ctrl _{Methanol}	0a	0a	10a	10a	0a	0a

Means followed by the same letter do not significantly differ between the control groups from the same time point ($p < 0.05$, Kruskal–Wallis with Dunn’s multiple comparisons test)

Table 2 Mean mortality of three beetle species (*C. maculatus*, *S. oryzae* and *T. confusum*) after 24 and 48 h in the positive control groups

	Mortality [%]					
	<i>C. maculatus</i>		<i>S. oryzae</i>		<i>T. confusum</i>	
	24 h	48 h	24 h	48 h	24 h	48 h
<i>Positive control</i>						
Ctrl _{Mint}	70a	70a	60a	60a	40a	50a
Ctrl _{Rosemary}	100b	100b	0b	0b	100b	100b

Means followed by the same letter do not significantly differ between the control groups from the same time point ($p < 0.05$, Mann–Whitney test)

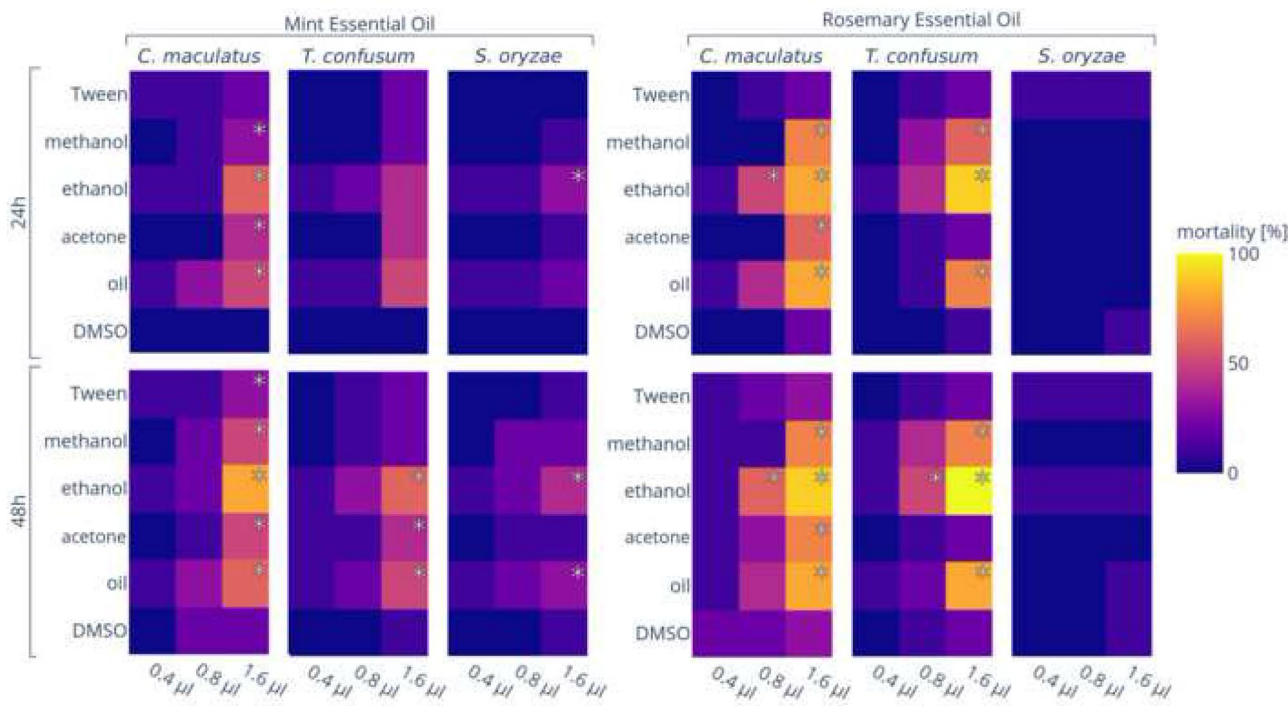


Fig. 3 Mortality of three beetle species (*C. maculatus*, *S. oryzae*, and *T. confusum*) after 24 and 48 h. The matrix illustrates the mortality according to the volume of EOs and the solvents in which they were

diluted (Kruskal–Wallis, Dunn’s multiple comparisons test, $p < 0.05$). The asterisk denotes significant differences between given groups and control groups

treated with 1.6 μl mint EO solution in Tween showed a significant increase in mortality.

To sum up, the increase in mortality after 48 h was mainly observed in 1.6 μl of both EOs in the following solvents: acetone, ethanol, methanol, and oil. No significant effects were observed in insects treated with DMSO. Of all tested insect species, *C. maculatus* was the most sensitive to all the tested EOs, while, respectively, the *S. oryzae* was the least susceptible. Additionally, based on the obtained results, a more effective insecticidal action of rosemary EO, in comparison to the mint EO, can be observed.

Cluster analysis based on all insect mortality results (insect \times EO \times volume) showed solvent grouping into three main clusters. As separate clusters, the analysis indicated that oil and ethanol as one pair (cluster strongly separated from the other two), acetone and methanol as another, and as a third pair DMSO and Tween 1%.

Discussion

EOs are an essential group of substances that can potentially replace commonly used pesticides. This results in a conspicuous trend of an increasing number of publications testing their effectiveness against insect pests. However, the research workflow usually depends on the methodology commonly applied in a particular research institution.

Considering the number of such institutions, the range of potential possibilities becomes very broad. This often results in significant differences in the methodology described in articles from the same field, and the obtained results are then compared with each other in discussions or meta-analyses. This may result in the accumulation of potential errors and misinterpretations of the results, as well as difficulties in the replicability of research. One such discrepancy may be the type of solvent used in EO's research. In this paper, we confirm the hypothesis that the choice of the solvent is of key importance for the effectiveness of EO's action on insects.

Out of over 40 analyzed papers describing assays in which the EOs were diluted with various solvents, acetone was used in over half. Nevertheless, for each species tested with the usage of acetone, studies involving other solvents were also conducted. This phenomenon is illustrated by the example of studies carried out on *Callosobruchus chinensis* and *Tribolium castaneum* beetles. Acetone was used as a solvent in fumigant toxicity assessment done on *C. chinensis* by Chaubey (2014) and on *S. oryzae* by Mohamed and Abdelgaleil (2008). Although Dayaram's and Khan's (2016) study was conducted on a phylogenetically similar species *C. maculatus*, ethanol was used for the same purpose, while Kim et al. (2003) used methanol in the study on *S. oryzae*. Such a pattern of methodological inconsistency is observed in many reports.

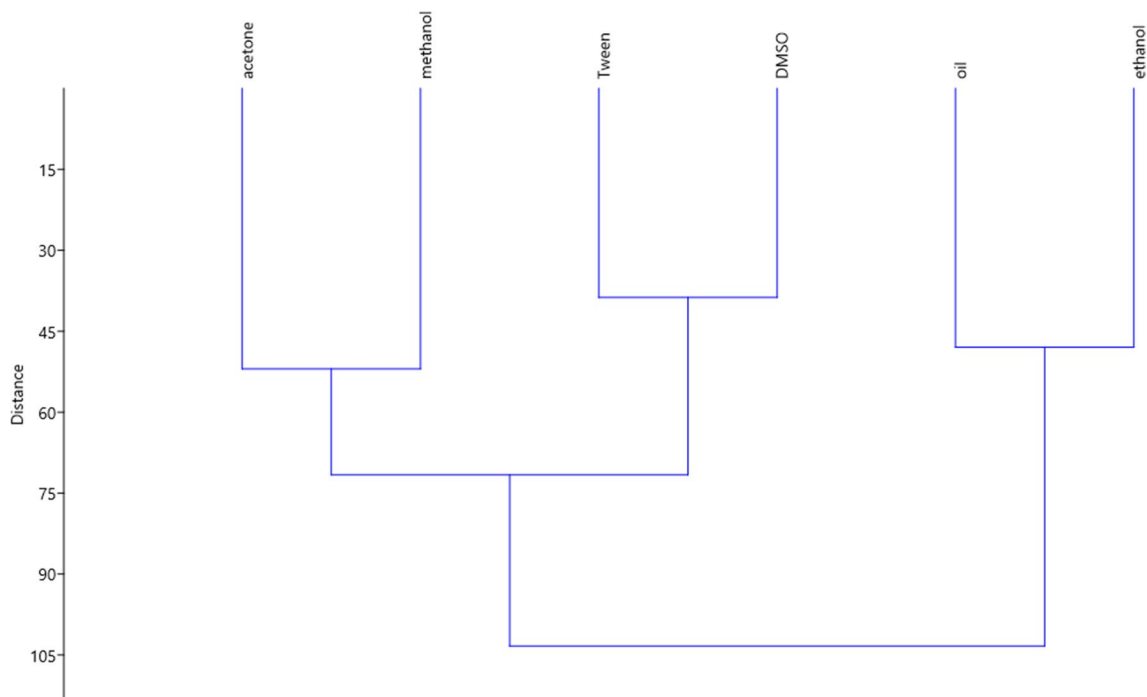


Fig. 4 Clustering of the solvents, based on the toxicity profiles of the used oils (Cohen correlation value 0.7655; Euclidean distance)

The tested solvents alone did not significantly affect insects' mortality; thus, the observed differences in the effectiveness of tested EOs solutions were caused by EO–solvent interactions. This means that all or some constituents of the EO could be retained by the solvents to a varying degree, therefore being released unequally and affecting the treated insects differently. This could be caused by differing volatility of solvents and their polarity. Although discerning, which exact mechanism is responsible for the observed effects requires additional research. Thusly, we also claim that there is no universally applicable solvent, although a potential group of substances with promising properties seem to be volatile organic solvents [as was depicted by the efficiency dendrogram (Fig. 4)]. Therefore, the used substances should be carefully selected for the tested species.

Author contributions

MK and JF conceived and designed research. MK conducted experiments. JK and BŁ analyzed data. MK and BB wrote the manuscript. All authors read and approved the manuscript.

Funding This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals (vertebrates) performed by any of the authors.

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