# Effects of essential oil components exposure on biological parameters of *Caenorhabditis elegans*

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**Abstract**

The extensive use of essential oil components in an increasing number of applications can substantially enhance exposure to these compounds, which leads to potential health and environmental hazards. This work aimed to evaluate the toxicity of four widely used essential oil components (carvacrol, eugenol, thymol, vanillin) using the *in vivo* model *Caenorhabditis elegans*. For this purpose, the $LC_{50}$ value of acute exposure to these components was first established; then the effect of sublethal concentrations on nematodes' locomotion behaviour, reproduction, heat and oxidative stress resistance and chemotaxis was evaluated. The results showed that all the components had a concentration-dependent effect on nematode survival at moderate to high concentrations. Carvacrol and thymol were the two most toxic compounds, while vanillin had the mildest toxicological effect. Reproduction resulted in a more sensitive endpoint than lethality to evaluate toxicity. Only pre-exposure to carvacrol and eugenol at the highest tested sublethal concentrations conferred worms oxidative stress resistance. However, at these and lower concentrations, both components induced reproductive toxicity. Our results evidence that these compounds can be toxic at lower doses than those required for their biological action. These findings highlight the need for a specific toxicological assessment of every EOC application.

**Keywords:** nematode, toxicity, carvacrol, thymol, eugenol, vanillin

## 1. Introduction

Essential oils are oily aromatic liquids obtained from different plant material structures (Hyldgaard et al., 2012) that consist in a complex mixture of compounds, including terpenoids, esters, aldehydes, ketones, acids and alcohols (Ramos et al., 2013). The biological functions of essential oils have been related to their content in phenolic components. The two isomeric monoterpenoids carvacrol and thymol, and the phenylpropenes eugenol and vanillin, are widely studied essential oil components (EOCs) (Hyldgaard et al., 2012). All four are widely distributed in several aromatic plants and its products. Carvacrol and thymol are primary components of thyme and oregano oils; eugenol is the main active compound of the oil extracted from clove and is also found in other plant sources such as basil, cinnamon or pepper; and vanillin is the main constituent of vanilla beans (Jadhav et al., 2009; Kachur and Suntres, 2020; Khalil et al., 2017). They are common flavouring agents in fragrances, cosmetics and food products, and are also used for other purposes in various industries like pharmaceutical, dentistry or agriculture (Kachur and Suntres, 2020; Memar et al., 2017; Nejad et al., 2017; Priefert et al., 2001).

Carvacrol, thymol, eugenol and vanillin display antimicrobial activity against foodborne and food spoilage bacteria, moulds

and yeasts (Hyldgaard et al., 2012; Kachur and Suntres, 2020; Tippayatum and Chonhenchob, 2007). They also have a demonstrated high antioxidant potential at low concentrations (Fujisawa et al., 2002; Oliveira et al., 2014), which minimises the oxidation of lipid components in foods (Yanishlieva et al., 1999). Because of their antimicrobial and antioxidant activity, these components have been proposed as natural preservatives for a wide variety of food products (Kachur and Suntres, 2020). Other beneficial properties reported for pharmaceutical and medical applications include analgesic, anti-inflammatory, antimutagenic and anticarcinogenic potentials (Bezerra et al., 2016; Khalil et al., 2017; Lee et al., 2014; Salehi et al., 2018; Sharifi-Rad et al., 2018; Sisakhtnezhad et al., 2018), as well as a protective effect against different metabolic disorders, such as diabetes mellitus, obesity, renal or gastrointestinal diseases, among others (Al-Naqeb et al., 2010; Nagoor Meeran et al., 2017).

As flavourings, EOCs are employed at low concentrations because of their strong flavour, while higher concentrations may be required to accomplish sufficient activity to be used in other applications (Maisanaba et al., 2015). However, evidence reveals that moderate to high concentrations of EOCs may cause toxicological effects. *In vitro* studies show that carvacrol, thymol and eugenol present cytotoxic effects in a dose-, frequency- and duration-dependent manner on different cell

lines, including the human osteoblastic cell line U2OS, the colon carcinoma cell line Caco-2, the human hepatoma cells HepG2, different human skin cells, and the mouse lymphoma cells V79 (Ho et al., 2006; Llana-Ruiz-Cabello et al., 2014a; Prashar et al., 2006; Slamenová et al., 2007). Several authors have suggested that the cytotoxic effect of these components on eukaryotic cells consists in inducing apoptosis by direct mitochondrial pathway activation (Bakkali et al., 2008; Yin et al., 2012). Some studies have investigated the mutagenic and genotoxic potentials of these components. Although the *in vitro* genotoxic potential of carvacrol and eugenol has been reported using V79, Caco-2, and mouse lymphoma cells (Llana-Ruiz-Cabello et al., 2014b; Maisanaba et al., 2015; Maralhas et al., 2006), these results are still limited. *In vivo* toxicity studies are scarce, and potential adverse effects after acute and prolonged exposure to carvacrol, thymol and eugenol have appeared in different species (Andersen, 2006; Nejad et al., 2017). In humans, it is known that exposure to carvacrol, thymol and eugenol is able to cause allergic reactions because skin irritation, ulcer formation, dermatitis or reduced healing have been reported (Kamatou et al., 2012; Salehi et al., 2018). The toxicological effects of vanillin have been less described, but this compound is considered to have a low cytotoxic potential because only high concentrations (mM range) reduce cell viability in a dose- and time-dependent manner (Fuentes et al., 2021; Oliveira et al., 2014).

In recent years, toxicological research has focused on searching for alternatives to mammalian animal models for the *in vivo* screening of chemicals because these assays are expensive and time-consuming, and they involve ethical concerns (Gao et al., 2018; Xiong et al., 2017). The free-living nematode *Caenorhabditis elegans* is one of the most intensively studied animals ever since it was proposed as an experimental model organism (Brenner, 1974). Its use has extended to many research areas, including toxicology, where it has been utilised as an alternative *in vivo* animal model to evaluate the potential toxic effects of chemicals for human health and the environment (Nagar et al., 2020; Wang et al., 2009; Yang et al., 2015). The popularity of *C. elegans* is because of the many advantages it offers as a model organism, including small body size, short life cycle, efficient reproduction, and easy and inexpensive maintenance (Hunt, 2017; Kumar and Suchiang, 2020). It also presents a simple anatomy with a defined number of cells. At the same time, these cells are organised into simple tissues and organs that bear similarities with human structures (Gonzalez-Moragas et al., 2015). Many cellular and molecular processes are also conserved between worms and humans given the presence of homologous genes and proteins in both species (Kumar and Suchiang, 2020; Leung et al., 2008). Another major feature of C. elegans for toxicity testing consists in the wide range of biological parameters and molecular markers that can be studied after toxicant exposure to obtain molecular- and

whole organism-related information (Wu et al., 2019). However, this model also presents some limitations since C. elegans lack of particular mammalian organs, molecular pathways or a circulatory and an adaptive immune system (Hunt, 2017). Moreover, variations in the experimental conditions can significantly alter toxicological responses for multiple generations, making good C. elegans culture practice mandatory for obtaining reliable results (Hunt, 2017). Still C. elegans may develop a relevant role for predictive toxicology.

Previously, Lanzerstorfer et al. (2020) evaluated the impact of rosemary, citrus and eucalyptus essential oils on acute, developmental and reproductive toxicity in C. elegans and found severe toxic properties at already low concentrations. Also, some EOCs have been studied using C. elegans as a model to evaluate their anthelmintic activity against plant and animal parasitic nematode species (Abdel-Rahman et al., 2013; Marjanović et al., 2018). However, to the best of our knowledge, the effect of EOCs' exposure on different biological C. elegans parameters to predict their potential toxicity has not yet been reported. This work aimed to evaluate the acute toxicity of carvacrol, eugenol, thymol and vanillin, and to investigate the effect of sublethal concentrations on different biological parameters, using the *in vivo* model *C. elegans*.

## 2. Materials and Methods

### *2.1. Chemicals*

Carvacrol (≥ 98% w/w), eugenol (≥ 98%), thymol (98.5% w/w), vanillin (≥ 98% w/w), sodium azide ($NaN_3$) and 30% hydrogen peroxide ($H_2O_2$) solution (w/w) were obtained from Sigma-Aldrich (Spain). Dimethyl sulfoxide (DMSO), NaOH, sodium hypochlorite (NaClO), and all the other reagents used to prepare Nematode Growth Medium (NGM) agar (3 g/L NaCl, 2.5 g/L peptone, 17 g/L agar, 1 M potassium phosphate buffer [25 mL], 1 M $CaCl_2 \cdot 2H_2O$ [1 mL], 1 M $MgSO_4 \cdot 7H_2O$ [1 mL], and [1 mL] 5% cholesterol in ethanol), K-medium (32 mM KCl, 51 mM NaCl) and M9 buffer (3 g/L $KH_2PO_4$, 6 g/L $Na_2HPO_4$, 5 g/L NaCl, 1 ml 1 M $MgSO_4$) were purchased from Scharlab (Spain), except for cholesterol (95% w/w), which was supplied by Acros Organics (Spain). All the chemicals used in this study were of standard reagent grade.

Stock solutions of carvacrol, eugenol, thymol and vanillin (2.5 M) were completely dissolved in DMSO and remained frozen until used. The final tested EOCs concentrations were prepared in K-medium (final DMSO concentration ≤ 0.6% (v/v)).

### 2.2. *C. elegans strain and maintenance*

Wild-type Bristol (N2) *C. elegans* worms were grown on NGM agar plates seeded with a lawn of *Escherichia coli* strain OP50 as feed and were left under standard conditions (Brenner, 1974).

Regular subculturing was performed to avoid crowding and to maintain the *C. elegans* population. For all the experiments, adult hermaphrodites were synchronised to the same larval stage using an alkaline hypochlorite solution (1 M NaOH, 5% NaClO) and cultured at 20ºC in the dark for 3 days after the late L4 stage was reached.

### *2.3. Lethality test and $LC_{50}$ estimation*

The lethality assays for the different EOCs were performed in 24-well plates in a final volume of 1 mL of liquid media in the presence of food (10 µL *E. coli* suspension at 1,000 FAU). About 30 age-synchronised L4 worms were transferred to each well, which contained serial dilutions of carvacrol (0.47-2.5 mM), eugenol (0.94-5 mM), thymol (0.47-2.5 mM), or vanillin (2.25-12 mM) in K-medium. 1.5. The concentration range used during the toxicity tests were selected according to previous *in vitro* studies (Fuentes et al., 2020) and preliminary tests. The negative controls (0.6% DMSO in K-medium) were included in triplicate on each plate. Plates were incubated at 20ºC in the dark for 24 h. After exposure, the number of live and dead worms was recorded by visual inspection under a dissection microscope. Nematodes were considered dead if they did not respond to stimulus when touched with a metal wire. Results were expressed as the percentage of live worms of the total number of nematodes in each well (survival rate). Data were presented as the mean (SEM) of three independent experiments.

The lethal concentration ($LC_{50}$) value for each EOC was calculated by a non-linear regression test using version 8.0.1 of the GraphPad Prism software (GraphPad Software, USA).

### *2.4. Effect of sublethal concentrations on biological parameters*

Based on the $LC_{50}$ values obtained from the lethality test experiments, three sublethal concentrations ($LC_{50/2}$, $LC_{50/4}$, $LC_{50/8}$) were set to further study the effect of EOCs on different nematode biological parameters. For this purpose, the L4 synchronised worms were exposed to sublethal concentrations of EOCs in K-medium in the presence of food (10 µL E. coli suspension at 1,000 FAU), and incubated in the dark at 20ºC for 24 h. After exposure, nematodes were washed with M9 buffer and transferred to new plates to evaluate the different endpoints. In addition, the chemotactic response to EOCs was determined to evaluate the attractive or repellent potential of these components for *C. elegans*. All the conditions were tested in at least three independent experiments with three replicates per condition.

#### *2.4.1. Locomotion behaviour*

Locomotion behaviour was assessed on the same plates employed for the reproduction assays by examining the tracks produced while worms moved on the agar surface by an image analysis. Images of agar surfaces were taken after the reproduction assays with a CMOS camera Moticam 3+ (Motic,

Hong Kong) connected to a BA310E microscope (Motic, Hong Kong) at the10X magnification. These were taken in RGB (red, green and blue) at the 2048x1536 resolution, and stored in the JPEG format. Then images were transformed to a grey-scale and the tracks formed while nematodes moved on agar wells were quantified by imaging segmentation. By this method, pixels' areas were attributed to tracks or background according to a 145-255 grey-level threshold (Buckingham and Sattelle, 2008). The results were expressed as a percentage of tracks area from the total well (%). The image analysis was carried out with the Fiji image processing package (Schindelin et al., 2012) using the *threshold* and *measure* functions.

2.4.2. *Reproduction*

Reproduction was evaluated by the brood size endpoint. The pre-exposed worms were individually transferred to NGM agar 24-well plates (1 nematode/well), seeded with *E.coli* OP50, and incubated at 20ºC for 72 h. Parent worms were daily transferred to new wells in identical plates until the end of the assay. Brood size was determined by counting under a BA310E microscope (Motic, Hong Kong) the number of larvae in all the stages and the number of eggs laid per adult worm at the end of the experiment (72 h).

*2.4.3. Stress resistance*

The effect of EOCs exposure on *C. elegans* resistance against thermal and oxidative stress was evaluated by two different survival assays. Thermal stress resistance was evaluated by exposing pre-treated young adult worms to 38ºC for 90 min (approx. 30 worms/well). The oxidative stress resistance assays were performed according to Acosta et al. (2018) with modifications. The pre-exposed worms to the sublethal concentrations of EOCs were transferred to K-medium 24-well plates (approx. 30 worms/well) containing 2 mM of $H_2O_2$ and were incubated at 20ºC for 5.5 h. After exposure to both stress factors, plates were incubated at 20ºC for 2 h. Next the number of live and dead nematodes was determined by the touch-provoked method, and the survival rates were calculated as the percentage of live worms of the total number of animals per well. Data were presented as the mean (SEM) of three independent experiments.

*2.4.4. Chemotaxis*

The chemotaxis assays were conducted in 60 mm-diameter Petri plates containing 6 mL of NGM agar. One EOC was analysed separately in each plate. For this purpose, plates were divided into four equal quadrants and a circular mark was made in the centre of each quadrant, which was equidistant between them and the centre of the plate. Five μL of the tested compound or K-medium were placed on the agar surface over the centre of two perpendicular circular marks. Sodium azide (0.25 M) was

used in all the quadrants to paralyse nematodes for counting purposes. In the centre of the plate, 5 µL of M9 buffer containing about 30 synchronised worms were placed. Then plates were sealed with Parafilm® and incubated in the dark at 20ºC for 90 min. After this time, the number of nematodes present in each quadrant was counted and the chemotaxis index was calculated as CI = A−B/A+B, where A is the number of worms at both test quadrants and B is the number of worms at both controls (K-medium). Evaluations were made for each concentration at least in triplicate.

### *2.4.5. Prediction of $LD_{50}$ values*

$LD_{50}$ values were calculated from the experimental $LC_{50}$ obtained for each EOC during the lethality assays using the regression function described at ICCVAM (2006):

$$Log\,(LD50) = 0.439 * \log(LC50) + 0.621$$

### *2.5. Statistical analyses*

Minimum sample size for each endpoint was selected to ensure normal distribution of data. Only L4 adults' hermaphrodites were used in the experiments. The statistical analysis was performed using Statgraphics Centurion XVI (Statpoint Technologies, Inc., Warrenton, VA, USA). The differences observed in the nematodes exposed to test solutions and the control were analysed by the Student's t-test for paired samples. Differences were considered significant at $p \leq 0.05$.

## 3. Results

### *3.1. Lethality test and $LC_{50}$ estimation*

The results of the lethality assays for the different EOCs are shown in Figure 1. All the compounds exhibited a dose-dependent effect on the mortality of adult nematodes after 24 h of exposure. The data from the concentration-response curves were used to calculate the $LC_{50}$ values. Carvacrol had the highest toxicological effect on *C. elegans*, and its $LC_{50}$ value was 1.10 (0.07) mM after 24 h exposure. This value slightly increased for thymol, which obtained an $LC_{50}$ value of 1.32 (0.03) mM. With eugenol, *C. elegans* was found to be approximately 2-fold less sensitive to this compound than to carvacrol and thymol, and the $LC_{50}$ value was 2.08 (0.21) mM. Moreover, while carvacrol and thymol induced significant lethal effects at concentrations above 0.47 mM (Fig. 1a and 1c), mortality only increased significantly for eugenol at concentrations above 1.37 mM (Fig. 1b). Vanillin showed the lowest acute toxic effect on young adult nematodes of all the different analysed EOCs, with an $LC_{50}$ of 5.84 mM (Fig. 1d), which was approximately 5-fold less toxic than carvacrol and thymol, and 3-fold less toxic than eugenol.

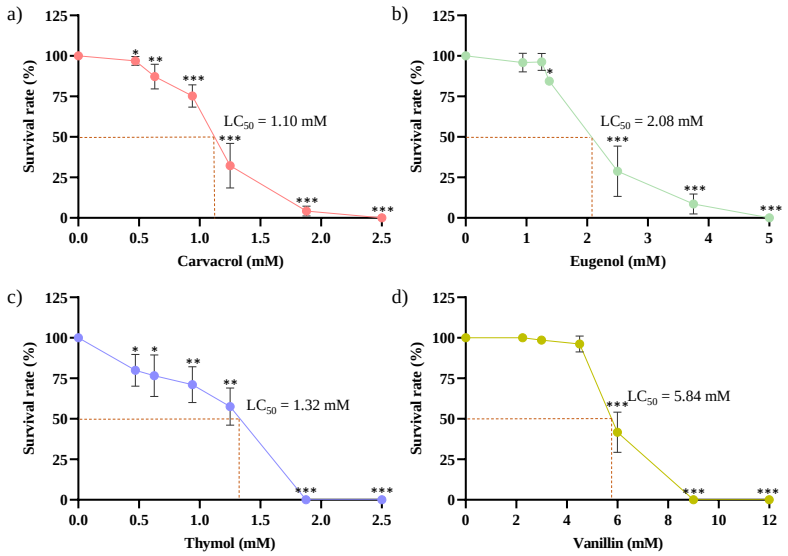


**Figure 1.** Concentration-response curves of the nematodes exposed to carvacrol (a), eugenol (b), thymol (c) and vanillin (d) for 24 h. Each point represents the mean mortality percentage (SEM) in at least three independent experiments, and each was performed in triplicate. (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$ indicates significant differences compared to the control according to the Student's t-test.

### *3.2. Effect of sublethal concentrations on biological parameters*

Three different concentrations of each compound were calculated according to the acute toxicity tests (Table 1) and used to evaluate the effect on the behavioural endpoints of nematodes: locomotion, reproduction, stress resistance, and chemotaxis.

**Table 1.** Sublethal concentrations of carvacrol, eugenol, thymol and vanillin assayed for the assessment of different biological parameters.

| | Concentration (mM) | | |
|---|---|---|---|
| **EOC** | **$LC_{50/8}$** | **$LC_{50/4}$** | **$LC_{50/2}$** |
| **Carvacrol** | 0.14 | 0.28 | 0.55 |
| **Eugenol** | 0.26 | 0.52 | 1.04 |
| **Thymol** | 0.17 | 0.33 | 0.66 |
| **Vanillin** | 0.73 | 1.46 | 2.92 |

*3.2.1. Locomotion behaviour*

Figure 4 shows that displacement of *C. elegans* on agar surfaces was greater for the worms treated with EOCs compared to the untreated controls. Moreover, worm motility was concentration-dependent and tended to be determined by the analysed compound. Monoterpenoids carvacrol and thymol enhanced motility with increasing compound concentrations, while lower concentrations of phenylpropenes eugenol and vanillin gave higher displacement percentages compared to the control. A 0.55 mM carvacrol concentration increased worm motility by 12% 24 h post-exposure, while all the thymol sublethal concentrations enhanced worm locomotive behaviour. The lowest tested thymol concentration (0.17 mM) increased the area

covered by worms by 11%, and the 0.33 mM and 0.66 mM thymol concentrations enhanced motility by 23% and 26%, respectively. For eugenol, the 0.26 mM and 0.52 mM concentrations respectively increased motility by 17% and 13%, while no differences were observed for the control at the lowest analysed concentration. Finally, a maximum increase of 23% was recorded for vanillin compared to the control for the 0.73 mM concentration, but this rise remained at 13% at both the lower concentrations.

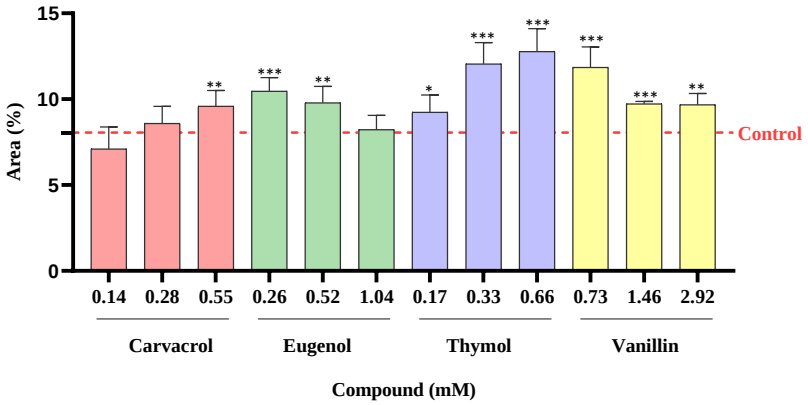


**Figure 4.** Area (%) of the tracks generated on the surface of agar wells by the pre-exposed worms to sublethal carvacrol, eugenol, thymol and vanillin concentrations for 24 h. Bars represent the mean (SEM) of at least three different experiments, each carried out in triplicate. (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$ indicates significant differences compared to the control by the Student's t-test.

Moreover, exposure to sublethal EOCs concentrations altered nematodes' rhythmic locomotory pattern. Figure 5 shows the representative micrographs of the locomotion tracks generated by the control nematodes or those worms acutely pre-exposed to different thymol concentrations on agar surfaces. The control worms produced shorter and less curved sinusoidal tracks, while the EOCs pre-exposed worms made more undulatory waves with increased curvatures, which suggests that undulating body shortens during crawling. This observation shows that exposure to the sublethal concentrations of these components not only enhances motility, but also modifies locomotor patterns in *C. elegans*.

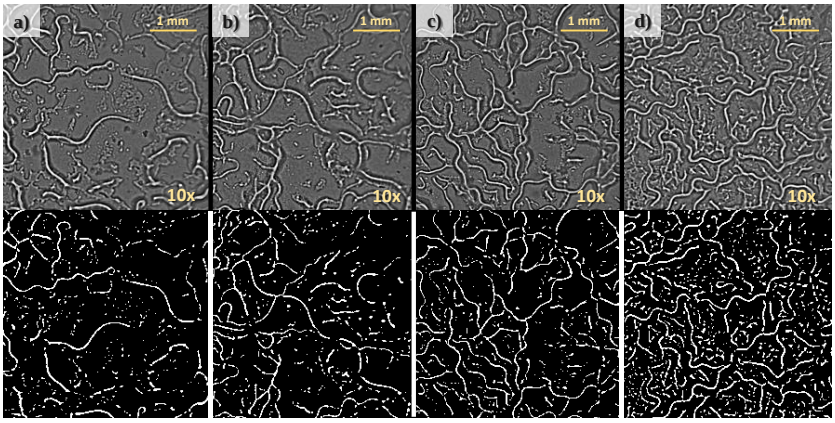


**Figure 5.** Tracks formed after worms crawled freely on a bacterial lawn on agar wells. The control (a) and pre-exposed

nematodes to increasing thymol concentrations for 24 h: 0.17 mM (b), 0.33 mM (c) and 0.66 mM (d).

### *3.2.2. Reproduction*

Depending on the tested concentration, carvacrol and eugenol exposure affected *C. elegans* reproduction (Figure 3). The strongest effect was for carvacrol, which significantly reduced the total brood size by 43% and 42% after exposure to the 0.28 mM and 0.55 mM concentrations, respectively. Moreover, the number of eggs laid per worm for the nematodes pre-exposed to the lowest carvacrol concentration (0.14 mM) decreased compared to the untreated controls. Eugenol also compromised nematodes' reproductive capability as pre-exposure for 24 h to a 1.04 mM concentration of this compound also resulted in fewer worms and eggs, and a smaller total brood size, and fewer eggs were also observed at 0.52 mM. Unlike carvacrol and eugenol, no reproductive toxicity was observed after exposure to sublethal thymol and vanillin concentrations (Figure 3c and Figure 3d, respectively).

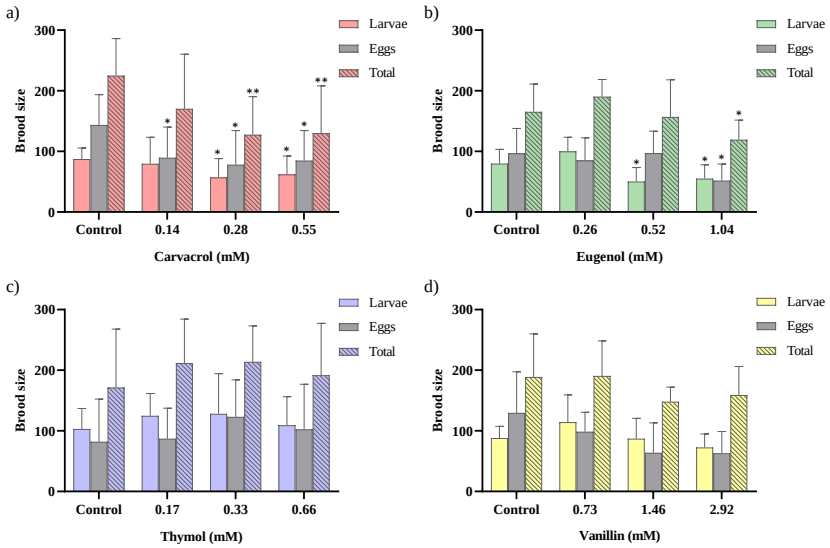


**Figure 3**. Effects on *C. elegans* brood size of exposure to carvacrol (a), eugenol (b), thymol (c) and vanillin (d) for 24 h. Bars represent the mean (SEM) from at least three different experiments, each carried out in triplicate. (*) $p \leq 0.05$; (**) $p \leq 0.01$ indicates significant differences compared to the control by the Student's t-test.

### *3.2.3. Stress resistance*

The effect of EOCs exposure on *C. elegans* resistance against two abiotic factors was analysed. To evaluate thermotolerance, pre-treated worms were exposed to 38ºC for 1.5 h and then the survival rate was determined. As shown in Figure 2a, no significant effect on heat stress resistance of worms was observed ($p > 0.05$), except for the lowest assayed eugenol concentration. At this concentration (0.26 mM), the mean life

span increased by 42% compared to the untreated control worms, and higher doses did not further increase survival.

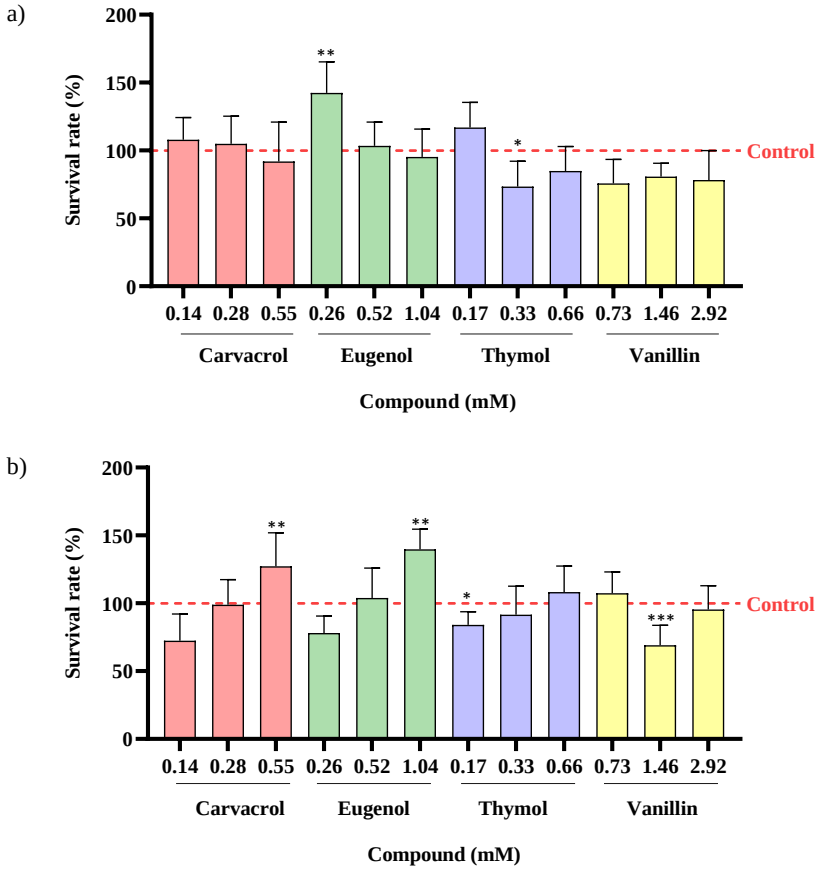


**Figure 2.** Survival rates after heat stress (a) and oxidative stress (b) of the nematodes pre-exposed to different concentrations of EOCs for 24 h. Each bar represents the mean survival rate (SEM) in at least three independent experiments, each carried out in triplicate. (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$

indicates significant differences compared to the control by the Student's t-test.

Oxidative stress was measured by the hydrogen peroxide resistance assay. As Figure 2b reflects, a general trend to higher survival rates values at rising concentrations appeared for the different EOCs. However, only carvacrol and eugenol at the highest tested concentrations conferred worms oxidative stress resistance. Pre-exposure to both compounds for 24 h significantly increased the survival rates by 27% and 40% compared to the controls, respectively. Neither lower carvacrol and eugenol concentrations, nor any of tested thymol and vanillin concentration, enhanced the antioxidant resistance of nematodes.

*3.2.4. Chemotaxis*

The present work tested the chemotactic responses of *C. elegans* to carvacrol, eugenol, thymol and vanillin to evaluate the attractive or repellent effect of these components on worms. The compounds with a chemotaxis index of $\geq 0.2$ are considered attractants, those with a chemotaxis index between 0 and 0.2 are described as weak attractants, while the molecules presenting a chemotaxis index $\leq -0.02$ are considered to be repellent (O'Halloran and Burnell, 2003). All four EOCs were attractive for nematodes at all the tested concentrations (Figure 6).

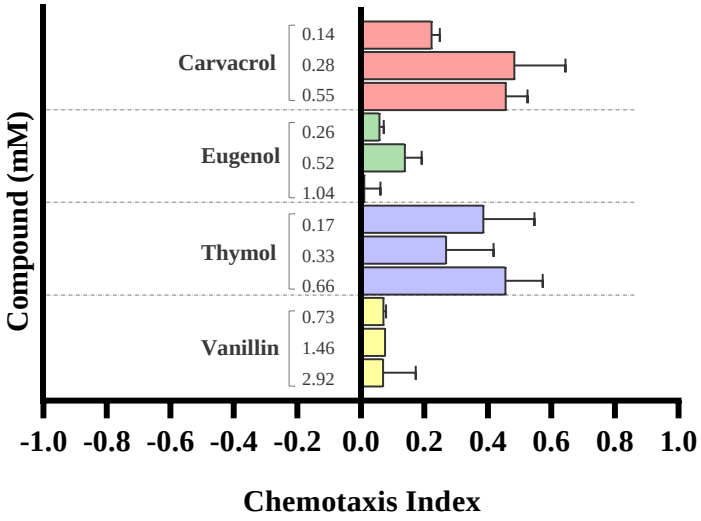


**Figure 6**. Chemotaxis indices of the wild-type *C. elegans* exposed to carvacrol, eugenol, thymol and vanillin. Bars represent the mean (SEM) of at least three different experiments, each carried out in triplicate (n > 200 worms) for each condition.

In wild-type *C. elegans*, we found that carvacrol and thymol had a potent chemoattractant effect, and both were the most attractive components with a maximum chemotaxis index of 0.48 (0.07) and 0.46 (0.11), respectively. A weaker response was detected to eugenol and vanillin as they reached a maximum chemotactic response of 0.13 (0.05) and 0.10 (0.00), respectively, and both were weak attractants.

*3.2.5. Prediction of $LD_{50}$ values*

$LD_{50}$ values were calculated from the experimental $LC_{50}$ obtained for each EOC during the lethality assays using the regression function described at ICCVAM (2006). The predicted $LD_{50}$ values were compared to the oral $LD_{50}$ reported for rats by the European Chemicals Agency (ECHA, 2021). As depicted in Table 2, estimated $LD_{50}$ values displayed a significant correlation with the values reported for rats since the toxicity ranking between *C. elegans* and rodents was maintained. As in nematodes, carvacrol is the most acutely toxic EOC for rats, followed by thymol, eugenol and lastly, vanillin.

**Table 2.** Comparison of predicted $LD_{50}$ values and Rat $LD_{50}$ values obtained from the ECHA toxicological information for the different EOCs.

| Compound | Predicted $LD_{50}$ (mg/kg) | $LD_{50}$ Rat (mg/kg) |
|---|---|---|
| Carvacrol | 655 | 810 |
| Eugenol | 946 | > 2000 |
| Thymol | 709 | 980 |
| Vanillin | 1380 | 3978 |

## 4. Discussion

Carvacrol, eugenol, thymol and vanillin, are authorised as flavouring agents with no specific restricted use in Regulation

(EC) 1334/2008 (EC, 2008), and are approved as Generally Recognized as Safe (GRAS) compounds by the US Food and Drug Administration (FDA, 2021). They are also considered safe by the Joint FAO/WHO Expert Committee on Food Additives as "No safety concern at current levels of intake when used as a flavouring agent" has been established for these components (JECFA, 2021). However, despite these EOCs being considered safe as a food additive when consumed in commonly employed quantities, their increasing and extensive use in a wide range of different applications means that consumers' exposure to these components can be substantially enhanced. Moreover, in addition to their pure form, these compounds are part of complex mixtures in multiple essential oils and spices where cumulative and countervailing effects can occur. Consequently, there is concern about adverse health effects related to exposure to high doses (Kamatou et al., 2012). Indeed the need for comprehensive toxicological studies using animal models to support the safety of these components has been suggested (Nagoor Meeran et al., 2017). Accordingly, this study aimed to investigate the toxicological properties of these four EOCs *in vivo,* using the model system *C. elegans*. For this purpose, the $LC_{50}$ value of acute exposure to these components was first established; then the effect of sublethal concentrations on different nematodes' biological parameters was evaluated.

Based on the lethality assays, carvacrol was the most toxic compound for *C. elegans*, followed by thymol, eugenol, and lastly by vanillin. These results about the descending toxicity ranking of the EOCs are consistent with those reported by different authors who evaluated the anthelmintic potential of phenolic terpenes against *C. elegans* as a model for parasitic nematodes (Abdel-Rahman et al., 2013; Marjanović et al., 2018; Tsao and Yu, 2000). Similarly to our work, the acute toxic effect of carvacrol and, to a lesser extent, that of thymol, have been found to be more potent than the antinematodal effect of eugenol. This effect has been attributed to differences in their chemical structure, such as hydroxyl group position (D'addabbo and Avato, 2021; Park et al., 2007), since molecular structure determine important properties for insecticidal or nematicidal activity including the capability of these molecules to penetrate through the cuticle. Indeed, Gaire et al. (2019) point out the higher lipophilicity of carvacrol and thymol than that of eugenol as a relevant factor determining their higher ability to penetrate insect's cuticle and their related toxicity. By contrast, the underlying toxicological mechanisms seem to be shared for these components. The anthelmintic action of these EOCs has been related to their inhibitory effect on the neuromuscular system of nematodes (Trailović et al., 2015). Despite the molecular mechanisms of the paralysing effects not being completely elucidated, they seem to involve different receptors as main targets. Hernando et al. (2019) studied the effect of

carvacrol, eugenol and thymol on *C. elegans* mutant strains for different worm locomotion-related receptors. These authors found that terpenoid-induced paralysis by inhibiting receptors is involved in muscle contraction and locomotion, such as levamisole sensitive acetylcholine (L-AChR), γ-aminobutyric acid (GABA) type A (UNC-49) receptors and, to a lesser extent, nicotine-sensitive acetylcholine receptors (N-AChRs). They also demonstrated that the inhibitory effect of terpenoids on acetylcholine and GABA neurotransmitters occurs by acting as the negative allosteric modulators of these receptors. Other authors have demonstrated that carvacrol and eugenol reduce the acetylcholine response by the non-competitive inhibition of levamisole sensitive (L-type) nAChRs from *Oesophagostomum dentatum* and a nicotine-sensitive (N-type) nAChR (ACR-16) from *Ascaris suum* (Choudhary et al., 2019; Trailović et al., 2015), which are two relevant porcine parasitic nematodes. The SER-2 tyramine receptor has also been suggested as a target site for carvacrol and thymol in *C. elegans* because both components are able to trigger the signalling cascade downstream from the receptor in those cells expressing wild-type, but not SER-2, mutants (Lei et al., 2010). The SER-2 receptor is also involved in the regulation of muscle contraction and locomotion and in other nematode behavioural processes, such as pharyngeal pumping, foraging behaviour and egg lying (Branicky and Schafer, 2009; Chase and Koelle, 2007; Kagawa-Nagamura et al., 2018; Rex et al., 2004).

For vanillin, no significant toxic effects were observed after exposing worms to a 4.5 mM vanillin concentration. This finding demonstrated the low acute toxicological potential of vanillin. Likewise, the effects of long-term vanillin exposure on *C. elegans* have been evaluated by Venkata et al. (2020), who did not find any toxic effects after treating worms with 0.8 mM of vanillin for 4 days. Interestingly, Schmeisser et al. (2013) observed that a vanillin concentration of 1 μM extended *C. elegans* life span, while one of 1 mM had the opposite effect, which reflects a hormetic and non-linear dose-response for this compound. These authors found that exposure to low vanillin concentrations induced a transient increase in ROS formation, followed by a persistent reduction in the steady state. Conversely, high vanillin concentrations did not decrease ROS production in the steady state, but increased ROS formation after a 4-day exposure to produce an excessive ROS load that was responsible for a shorter life span.

Exposure to sublethal concentrations of chemicals may cause behavioural changes that are indicative of developmental and neurological adverse effects. For this reason, sublethal concentrations were used to investigate the effect of each compound on the behavioural endpoints of nematodes: locomotion, reproduction, stress resistance, and chemotaxis.

*C. elegans* locomotes in a rhythmic and undulatory forward and backward fashion by alternating ventral and dorsal bends along

its body (Izquierdo and Beer, 2018). This undulatory movement results freely crawling nematodes carving sinusoidal tracks on the surface of agar plates. In our study, worm motility was measured by directly quantifying those tracks made on the agar surface by the nematodes pre-exposed to EOCs sublethal concentrations or to the control for 24 h. As previously mentioned, terpenoids' antinematodal action has been related to their paralysing effects, which occur because of their interfering activity on muscle contraction and locomotion receptors (André et al., 2017; Shu et al., 2016; Trailović et al., 2015). These receptors control not only *C. elegans*' locomotion, but also other key behavioural processes like pharyngeal pumping or egg laying (Driscoll and Kaplan, 1997; Rand, 2007). According to our results, exposure to sublethal concentrations of carvacrol, eugenol, thymol and vanillin resulted in stimulatory responses of wild-type adult *C. elegans* locomotive behaviour, which suggests that they act in a hormetic-like manner on the neuromuscular system.

Previous healthspan assays have demonstrated that hormesis may enhance locomotion-related behaviours of *C. elegans*, such as bending frequency and pumping rate, by 28.9% (Sun et al., 2020). This phenomenon has also been observed with different constituents of essential oils in other biological parameters like lifespan and fecundity rates in Mediterranean fruit fly (Papanastasiou et al., 2017) or the larvae production of

*Sitophilus zeamais* (Haddi et al., 2015), which suggest disruptions to endocrine, antioxidant or detoxification systems as being the physiological mechanisms responsible for regulating such stimulatory responses (Farias et al., 2020).

*C. elegans* reproduction has been demonstrated as useful endpoint in toxicity testing since a broad spectrum of chemicals disrupt this behaviour (Eom and Choi, 2019; Li et al., 2020; Zhou et al., 2021). Herein, exposure to sublethal concentrations of carvacrol and eugenol affected *C. elegans* reproduction. Similarly, Hernando et al. (2019) found that carvacrol affected egg hatching to a greater extent than eugenol and thymol when *C. elegans* eggs were incubated in the presence of these components for 12 h. In the present study, eugenol and carvacrol reduced brood size as a result of fewer larvae and eggs. Therefore, our results suggest that the effect of pre-exposure to these components on reproduction may be due to other reproductive dysfunctions other than egg-hatching inhibition like alteration of the egg-laying behaviour. Egg-laying results from contraction of specialised muscle cells that open the vulva and compress the uterus, allowing eggs to be deposited (Schafer, 2016). As described for the anthelmintic action and the effects of *C. elegans*' locomotion of the EOCs, a number of serotonin and acetylcholine receptors have been demonstrated to modulate vulval muscle activity (Bradford et al., 2020; Collins et al., 2016; Rand, 2007; Schafer, 2016). Moreover, egg-lying

behaviour may also be indirectly inhibited as a consequence of motility defects deriving from neurotoxicity, since an inverse correlation has been described between egg-laying and locomotion (Schafer, 2016). Other factors related to toxicant-induced reproductive deficits, such as oxidative damage events in gonad and vulva (Wu et al., 2011) during germline development (Rodrigues et al., 2018) or structural changes in the external reproductive organs of nematodes (Andre et al., 2016) may also play a role.

The no reproductive toxicity herein observed after exposure to sublethal thymol concentrations was also found by Shu et al. (2016) when evaluating thymol's safety as an antifungal agent. Exposure to the 0.21 mM and 0.42 mM concentrations for 6 h did not influence the exposed worms' brood size. However, these authors found that a 0.85 mM thymol concentration significantly reduced brood size in *C. elegans* compared to the untreated controls.

Several plant essential oils rich in different monoterpenes have a protective effect against thermal and oxidative stress in the *in vivo* model *C. elegans* (Kamireddy et al., 2018; Link et al., 2016; Pandey et al., 2018; Rathor et al., 2017; Rodrigues et al., 2018; Yu et al., 2014; Zhang et al., 2021). The molecular mechanism responsible for their enhanced stress resistance activity has been related to different biological markers, such as decreased ROS production, altered expression patterns of

important stress-response genes like sod-3 and gst-4, or the nuclear translocation of FOXO transcription factor DAF-16 from the cytoplasm. Unlike these studies, Piao et al. (2020) found that the essential oil of flesh fingered citron and its main components (d-limonene, γ-terpinene, α-pinene, β-pinene) shorted nematodes' lifespan as a result of increased oxidative stress. The differences in the biological effects between essential oils would depend on the nature and the relative content of their constituents, which could vary not only between plant species, but also due to environmental and growth factors (Yu et al., 2014), which means that studying their individual components is necessary. Our results show that only the highest sublethal carvacrol and eugenol concentrations resulted in increased survival rates as a consequence of their antioxidant effect. Thus, a narrow range of concentrations exists between antioxidant and antinematodal effects, and attention should be paid when using bioactive concentrations of these components because of their potential toxicological implications.

*C. elegans* has a highly developed chemosensory system that consists of neurons in the amphid, phasmid and inner labial organs that are able to sense chemicals from the environment (Bargmann, 2006). Indeed, this nematode responds to a wide spectrum of both water-soluble and volatile chemicals (O'Halloran and Burnell, 2003). Chemosensory signals are associated with food searches, the rapid avoidance of noxious

conditions or mating, and can stimulate different behaviours, including chemotaxis (Margie et al., 2013). The present work tested the chemotactic responses of *C. elegans* to carvacrol, eugenol, thymol and vanillin to evaluate the attractive or repellent effect of these components on worms. Volatile molecules can be detected within the nanomolar range, with nematodes being more sensitive to volatile compounds than to water-soluble chemicals (Bargmann, 2006). In our work, the smallest amounts of compounds were effective attractants to *C. elegans* and no significant differences were observed in worms' response to higher concentrations. The attractant effect of these components has been previously reported in different *Meloidogyne* nematode species (Oka, 2021). Surprisingly, worms were attracted to these aromatic compounds despite their nematicidal activity. Indeed, the two components displaying the greatest nematicidal action in the lethality assays were carvacrol and thymol, which also exhibited the most potent chemoattractant effect. Plant roots are often the storage site for the organic molecules that soil-dwelling organisms may use as nutrients and metabolites. These organisms have evolved to detect and respond to chemical signals for successful foraging purposes (Rasmann et al., 2012). According to Oka (2021), although this "suicidal behaviour" by which nematodes are attracted to nematicidal compounds remains unknown, it may be due to the fact that these chemoattractants act as signals for root finding under natural conditions in the soil matrix, but they

never encounter them at nematicidal concentrations during their evolution.

The acute oral toxicity potential of chemicals is usually determined by the calculation of the median lethal dose ($LD_{50}$) that is the dose that is expected to kill 50% of the test population. This parameter is crucial for hazard and risk assessment purposes, since it helps the industry, regulatory agencies and the international community for the hazard classification and labelling of chemicals and test materials (NIH, 2001). In this work, a regression model was applied for prediction of acute mammalian oral $LD_{50}$ values from the acute toxicity *C. elegans* experimental data. This mathematical model was developed to determine the starting doses for rodent acute oral toxicity tests from basal *in vitro* cytotoxicity data (ICCVAM, 2006) but it has also been successfully used to predict $LD_{50}$ values for different essential oils based on calculated $LC_{50}$ in *C. elegans* (Lanzerstorfer et al., 2020). The predicted $LD_{50}$ values obtained for the four EOCs were compared to the oral $LD_{50}$ reported for rats by the European Chemicals Agency (ECHA, 2021). The results showed that estimated $LD_{50}$ values displayed a significant correlation with the values reported for rats since the toxicity ranking was maintained. Various studies have proposed the use of mutant strains with increased absorption properties during toxicity assessment, since the tough nematode cuticle may prevent from

chemical uptake and lead to an underestimation of effects (Lanzerstorfer et al., 2020; Xiong et al., 2017). However, the predicted $LD_{50}$ values found in our study are lower than those reported by the ECHA. Particularly, small differences were found between predicted and experimental $LD_{50}$ for the two more toxic compounds, carvacrol and thymol, while the two EOCs that showed the mildest toxicity effect, eugenol and vanillin, both exhibited a toxic effect up to 2- fold stronger for *C. elegans* than rats. Li et al. (2013) described that the pH of chemicals could affect the *C. elegans* $LC_{50}$ value, leading to an overestimation of the toxicity effects of acidic chemicals and low correlations with acute toxicity in rodents. In this study, at the higher concentrations tested of EOCs, the pH of K-medium (pH 7.2) was slightly acidified in the case of carvacrol (pH 6.8), thymol (pH 6.9), but a further decrease was observed for eugenol (pH 6.5) and vanillin (pH 5). However, at these ranges of pH no effects on C. elegans survival have been described. Indeed, Khanna et al. (1997) demonstrated that worms survived in K-medium a pH range of 3.1 to 11.9 for 24 h. Therefore, other factors should be responsible for these differences.

## 5. Conclusions

The extensive use of carvacrol, eugenol, thymol and vanillin, either pure or in the form of essential oils and spices, in an increasing number of applications, could pose potential human health and environmental hazards. This study describes the

effect of acute exposure to these four EOCs on different biological *C. elegans* parameters. All the components exhibited a concentration-dependent effect on nematode survival at moderate to high concentrations. Carvacrol and thymol were the two more toxic EOCs, while vanillin had the mildest toxicological effect. The study of acute exposure to sublethal concentrations of these components revealed that they significantly influenced nematode parameters, such as locomotion behaviour and reproduction, at low concentrations. As observed in the stress resistance assays, only the pre-exposure to carvacrol and eugenol at the highest sublethal tested concentrations conferred worms oxidative stress resistance. However, at these low concentrations, both components induced reproductive toxicity, as shown by brood size reduction. Moreover, the correlation found in the acute toxicity ranking of EOCs between nematodes and rodents.

The model organism *C. elegans* was demonstrated as a useful *in vivo* model to evaluate the toxicological properties of EOCs. By comparing different assays, reproduction resulted in a more sensitive endpoint than lethality for evaluating toxicity. However, further studies should be conducted to elucidate the underlying toxic mechanisms involved in the exposure to these compounds. In addition, chronic exposure to these components still needs to be investigated. In summary, our results evidence that these compounds can be toxic at lower doses than those

required for their biological action. These findings highlight the need for a specific toxicological assessment of every EOC application.

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