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Assessment of the insecticidal activity of five essential oils used against the subterranean termite *Reticulitermes lucifugus* (Rossi) (Blattodea, rhinotermitidae) in laboratory

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Abstract
Among *Reticulitermes* genus the most abundant species living in Italy is *Reticulitermes lucifugus* Rossi. This termite is very dangerous to wooden structures of artistic heritage and buildings. The traditional control of *R. lucifugus* is very difficult resulting in negatively impact on the environment, therefore the use of phytochemical, such as essential oils (EOs), could lead to an alternative to synthetic insecticides, with advantages in reducing environmental risks. In this work we investigated the insecticidal activity of EOs of *Citrus bergamia* Risso, *Syzygium aromaticum* L., *Foeniculum vulgare* Mill., *Pelargonium odoratissimum* L., and *Origanum vulgare* L. by inhalation against *R. lucifugus* workers. It is the first study to examine the efficacy of EOs on this termite species. Results showed that after 24 hours of treatment exposure, mortality occurred in all treatments and was dose-dependent, increasing when the treatment dose increased. At the lowest dose (2.5 µl/l), oregano, clove, and geranium EOs resulted in mortality of more than 80%. Oregano EO was the most active already at low doses, while fennel EO was the least effective. 24 hours after the treatment, the lowest DL50, obtained by the dose-response analysis, was detected for oregano EO (1.21 µl/l), followed by those of EOs of clove (1.28 µl/l), geranium (1.73 µl/l), fennel (2.20 µl/l), and bergamot (2.88 µl/l). This study highlighted the possibility of using these EOs in the termite control for the preservation of wooden structures.

Keywords: Bergamot, clove, fennel, geranium, inhalation effects, oregano

1. Introduction
The Mediterranean termite *Reticulitermes lucifugus* (Rossi) (Blattodea, Termitoidae, Rhinotermitidae) is widespread in Europe, especially in Spain and Italy [30, 10, 45], where is the most abundant species of the *Reticulitermes* genus [17]. This species, like other Subterranean termites, is an important pest in the damage of the wooden structures of historical and artistic heritage, and modern and ancient buildings [31, 32, 55, 20, 45].
The termite live in colonies of several tens of thousands of individuals. The most numerous are workers while soldiers represent a small amount [50]. The life stages involve the formation of young workers which develop into workers, or soldiers, or complementary or substitute royal forms [21]. *R. lucifugus* produces nests in the ground and attacks the old plants or decaying wood from which it can move to wooden materials of buildings. The control of *R. lucifugus* is very difficult, because even the new formulations of chemicals have not led to improvements in the protection of the environment and indigenous species [49]. EOs of plants could be a valid alternative to chemical pesticides, as they have long been used against economically important pests with satisfactory results [29, 37]. In fact, the advantages of their use are the low cost, biodegradability and specificity towards the target species [6]. In applications against pests of many crops and stored products, EOs showed insecticidal, repellent, deterrent, growth regulatory, antivector, and antifeedant activity [22, 46, 9, 44, 2, 12]. However, many of this studies concern Coleoptera and Diptera species [38, 37, 35], and those on termites interest a low number of termite species [3, 56]. Most termite control studies have been carried out on the use of chemicals, because the experiments on biological control in the field have not achieved satisfactory outcomes [13].

In our study we wanted to improve the knowledge on the control of *R. lucifugus* by using these natural products to evaluate a pest control at low environmental impact. Specifically, we evaluated the insecticidal activity of *Citrus bergamia* Risso (bergamot), *Syzygium aromaticum* L. (clove), *Foeniculum vulgare* Mill. (fennel), *Pelargonium odoratissimum* L. (geranium), and *Origanum vulgare* L. (oregano) EOs by inhalation against workers under laboratory conditions. This is the first study to examine the efficacy of EOs against *R. lucifugus*.

2. Materials and Methods

Essential oils (EOs)

For producing essential oils (EOs), we used bergamot flavedo (*Citrus bergamia* Risso Fam. Rutaceae), buds of cloves (*Syzygium aromaticum* L. Fam. Mirtaceae), fennel seeds (*Foeniculum vulgare* Mill. Fam. Ombrellifereae), leaves and flowers of geranium bourbon (or fragrant geranium) (*Pelargonium odoratissimum* L. Fam. Geraniaceae), and leaves and inflorescences of oregano (*Origanum vulgare* L. Fam. Labiatae). All EOs were distilled in hexane by the Soxhlet extractor, except bergamot EO which was obtained by cold pressing according to the method described by Ferhat *et al*. [15]. EOs were used 100% pure at concentrations of 20, 10, 5, 2.5, 1 µl/l with 2% Tween® 20 (Sigma Aldrich) in distilled water (2 ml) per each treatment. Only Tween® 20 and distilled water were used in the control. EOs were tested against worker termites by inhalation.

Termites

The individuals of *R. lucifugus* were obtained from a field of Palermo district (N 38° 6′ 25.72″; E 13° 21′ 2.22″) (Sicily, Italy), using a colony kept in fir tablets (10 cm×10 cm×1 cm) inside open bottom containers buried at 30 cm depth. To perform the bioassays, a container was removed from the soil and brought into the laboratory (25 °C, 60% R.H.), and termites were collected from the tablets. To be sure to use in experiments individuals at the same development stage, workers with dark brown and distended abdomen were selected.

Bioassays

Bioassays were carried out in laboratory (25±1 °C, 60-70% RH and 16:8 L/D photoperiod) evaluating by inhalation the insecticidal activity of EOs. Experimental glass jars (6 cm diameter × 3 cm high) containing a filter paper disk (Whatman No. 1) attached to the screw cap were used. Initially, the filter papers were sprayed by means a micropipette with 1 ml of EO test solution, and Tween® 20 and distilled water only as control. After solvent evaporation, the filter papers were introduced into the jars. Five worker termites were added in each jar. Six replicate were made for each dose of treatment and for the control, using groups of 30 individuals per replication. The glass jars were placed in a glass box (70×35×35 cm) darkened by black cardboard, with two containers of deionised distilled water. After 24 hours from the treatment, each jar was observed and the number of alive and dead termites was detected.

Statistical analysis

To analyse the data, ANOVA (one-way analysis) and paired t test were done. The post hoc analysis was applied using the Duncan Multiple Range test to detect significant differences at P=0.05 level. All analyses was performed using Statistica 7.0 for Windows Package [54]. Correction of mortality data for the control response, was made using the Abbott's formula [1]. 24 hours after treatment exposure, for each EO, the LD50 (Lethal Dose: dose killing half test sample) was calculated by dose-response analysis interpolating the percentage of mortality (expressed in probits) and EO doses [16].

3. Results and Discussion

In this study we highlighted the insecticidal effects on *R. lucifugus* by inhalation of bergamot, clove, fennel, geranium and oregano EOs, tested for the first time against this termite. Results showed that all EOs 24 hours after the treatment caused termite mortality and their effectiveness was dose-dependent, increasing as the dose increases. Mortality was also observed in the control with an average value of 1.9% (Figure 1).

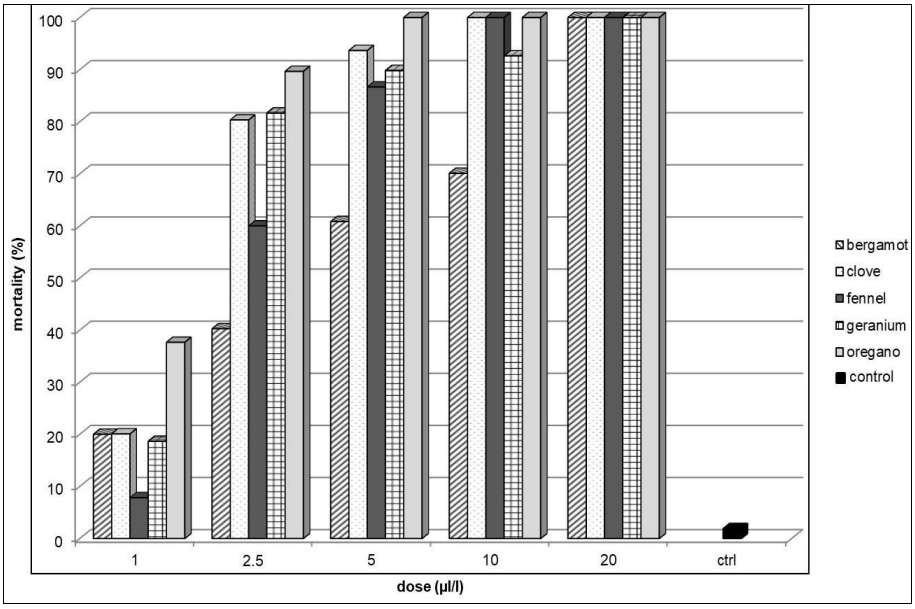

Fig 1: Mortality (%) of *R. lucifugus* due to inhalation toxicity after 24 hours of exposure to treatment with EO of bergamot, clove, fennel, geranium, and oregano tested at different doses (µl/l) in laboratory

Among the species belonging to the genus *Reticulitermes*, the toxicity of EOs has been tested against only a few species such as *R. flavipes*, *R. virginicus* [43], *R. speratus* [47, 27, 40] and *R. dabieshanensis* [57]. Generally, EOs obtained from different parts of the plant determine termiticidal effects, for example, EOs of *Tagetes erecta*, *Mentha spicata*, *Calocedrus macrolepis*, *Cryptomeria japonica*, *Chamaecyparis* species, *Melauleuca* species, *Allium sativum*, *Eucalyptus globules*, *E. citrodera*, *Thujopsis* species, *Taiwania cryptomerioides*, *Cinnamomum osmophloeum*, *Ephedra* species, *Nepeta cataria*, *Eugenia caryophyllata* and *Cymbopogon citratus* have insecticidal activity against termites [56, 36, 57].

Bergamot EO has important biological and healthy activities and has shown an insecticidal effect against Dipterans (*Musca domestica*, *Aedes albopictus*) and Lepidopterans (*Plodia interpunctella*) [28, 24, 11], but is poorly studied against termites. In our experiments this EO showed insecticidal effects against *R. lucifugus* causing mortality ranging from a minimum of 17% at the dose of 1µl/l to a maximum of 100% at the dose of 20µl/l. Statistical analysis showed significant differences between test (treatment doses) and control and between treatment doses (F=259.80, df=5, p<0.001). Post hoc analysis highlighted that each treatment dose was statistically different from the control, and that the differences were not significant between treatments at dose of 5 µl/l and dose of 10 µl/l only (Table 1).

Table 1: Mean mortality (X±SD) of *R. lucifugus* workers and post hoc analysis (Duncan's test) after 24 hours treatment exposure at different doses (µl/l) of EOs

Plant EO	Treatment Doses					Control
	1 µl/l	2.5 µl/l	5 µl/l	10 µl/l	20 µl/l	Tween+ water (2 ml)
Bergamot	20.0±2.8 a	40.3±3.2 b	60.8±8.5 c	70.1±8.2 c	100.0±0.0 d	1.9±0.6 f
Clove	20.1±4.1 a	80.3±10.5 b	93.7±9.6 bc	100.0±0.0 c	*	1.9±0.6 d
Fennel	7.8±3.9 a	60.0±8.3 b	86.7±17.2 bc	100.0±0.0 c	*	1.9±0.6 a
Geranium	18.7±5.7 a	81.7±16.8 b	89.8±9.9 b	92.7±9.9 b	100.0±0.0 b	1.9±0.6 c
Oregano	37.6±12.7 a	89.7±9.4 b	100.0±0.0 b	*	*	1.9±0.6 c

Within each row different letters indicate statistically significant differences (p<0.001).
(*) the dose was not used because the previous dose had already caused 100% mortality in all tests.

The dose-response analysis obtained by interpolating the mortality rate (transformed in probits) and the EO doses after 24 hours from the treatment (Figure 2) showed LD50= 2.88 µl/l.

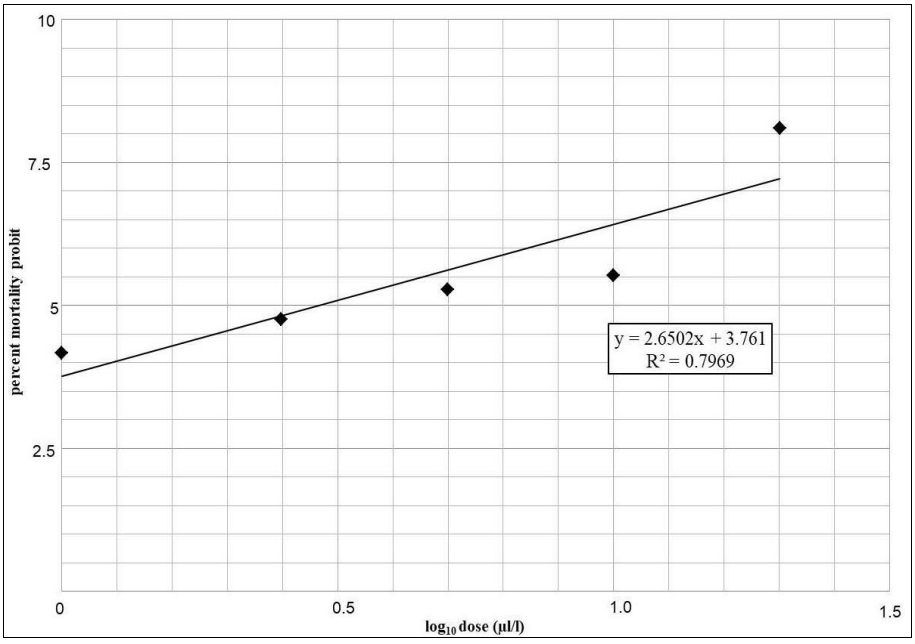

Fig 2: Dose-response analysis on *R. lucifugus* mortality (probit) after 24 hours of treatment exposure at different doses (µl/l) of bergamot EO in laboratory

Clove EO is reported to have good efficacy against many pests [23, 25, 53, 7, 34]. A strong termiticidal activity of clove has been reported against the Japanese termite *R. speratus*, *Odontotermes assamensis* and *Microcerotermes eugnathus* [40, 39, 48]. In our experiments, mortality caused by clove EO treatment, ranged from a minimum of 14% at the dose of 1µl/l to a maximum of 100% at the dose of 5µl/l. Mortality reached 100% in all replicates at the dose of 10µl/l. Significant differences were found between test (treatment doses) and control and between treatment doses (F=272.66, df=4, p<0.001). Each treatment dose was statistically different from the control, while there were significant differences between the dose of 1µl/l and the others, and between dose 2.5 µl/l and dose 10 µl/l (Post hoc analysis, P=0.005) (Table 1).

The dose-response analysis (Figure 3) showed LD50= 1.28 µl/l after 24 hours of treatment exposure.

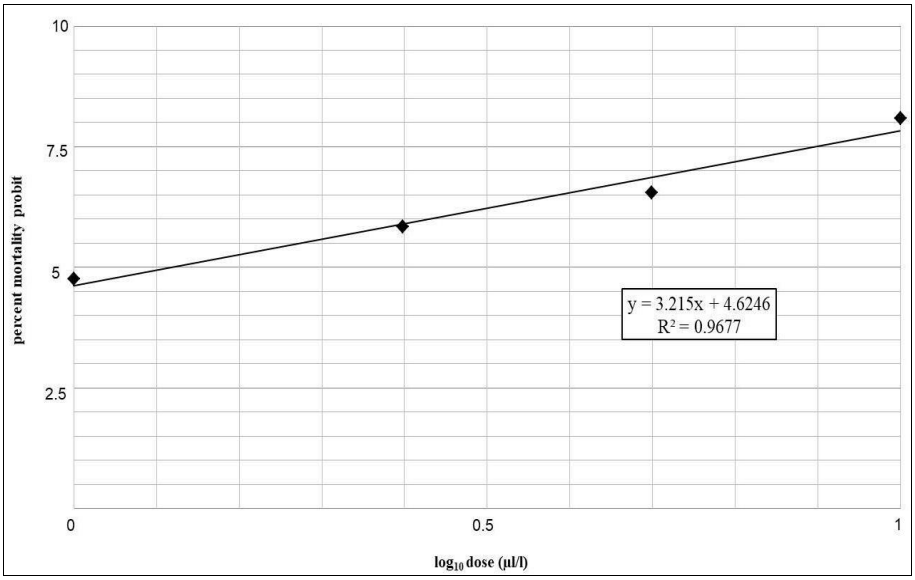

Fig 3: Dose-response analysis on *R. lucifugus* mortality (probit) after 24 hours of treatment exposure at different doses (µl/l) of clove EO in laboratory

Strong insecticidal activity of fennel EO against aphids, beetles and mosquitos has been reported [42, 7]. This EO was also used against termites, such as *Heterotermes indicola* [4]. In our study, after 24 hours of treatment exposure, mortality varied from a minimum of 3% at the dose of 1µl/l to a maximum of 100% at the dose of 5 µl/l. Using the dose of 10µl/l mortality was 100% in all replicates. Statistical analysis highlighted significant differences between test (treatment doses) and control and between treatment doses (F=158.25, df=4, p<0.001). Post hoc analysis showed that differences between each treatment dose and control were highly significant (p<0.001), except for the dose of 1µl/l (P=0.0048). The differences between treatment doses were also significant, and they were due to the dose of 1µl/l compared to the others and the dose of 2.5 µl/l compared to the dose of 10µl/l (Table 1). The dose-response analysis (Figure 4) showed LD50= 2.20µl/l after 24 hours of treatment exposure.

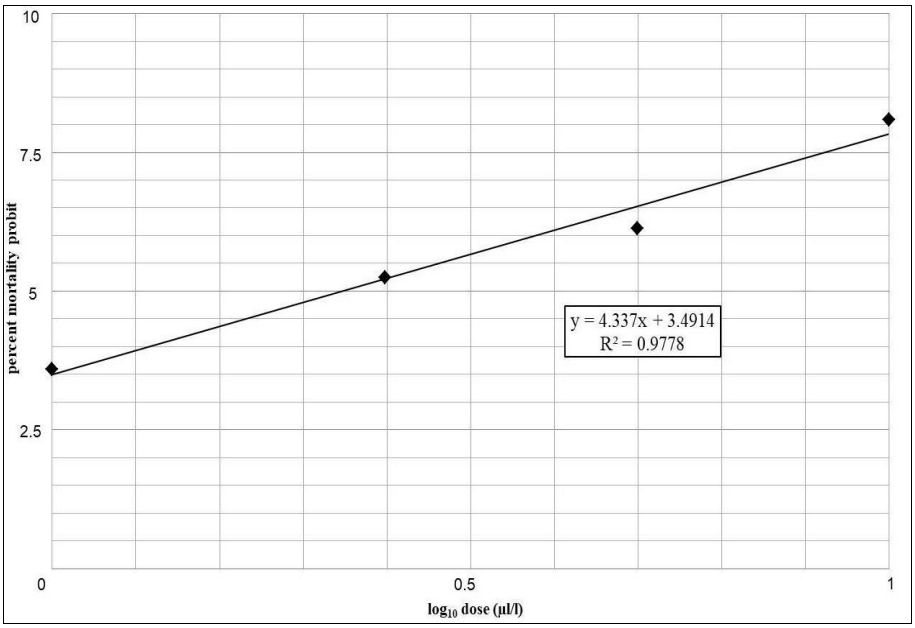

Fig 4: Dose-response analysis on *R. lucifugus* mortality (probit) after 24 hours of treatment exposure at different doses (µl/l) of fennel EO in laboratory

Studies on geranium EO showed insecticidal activity against mosquitos and aphids [5, 8, 34]. Some works reported the efficacy of geranium EOs against the Japanese termite *R. speratus* and *Formosan subterranean* termites [51, 56]. In our experiments, geranium EO caused mortality ranging from a minimum of 10% at the dose of 1µl/l to a maximum of 100% at the doses of 2.5µl/l, 5µl/l, 10µl/l and 20µl/l. Statistical analysis showed significant differences between test (treatment doses) and control and between treatment doses (F=114.55, df=5, P<0.001). Each treatment dose was statistically different from the control, whereas between treatment doses the differences were due only to the dose of 1µl/l compared to the others (Post hoc analysis, P=0.05) (Table 1). The dose-response analysis (Figure 5) showed LD50= 1.73 µl/l after 24 hours of treatment exposure.

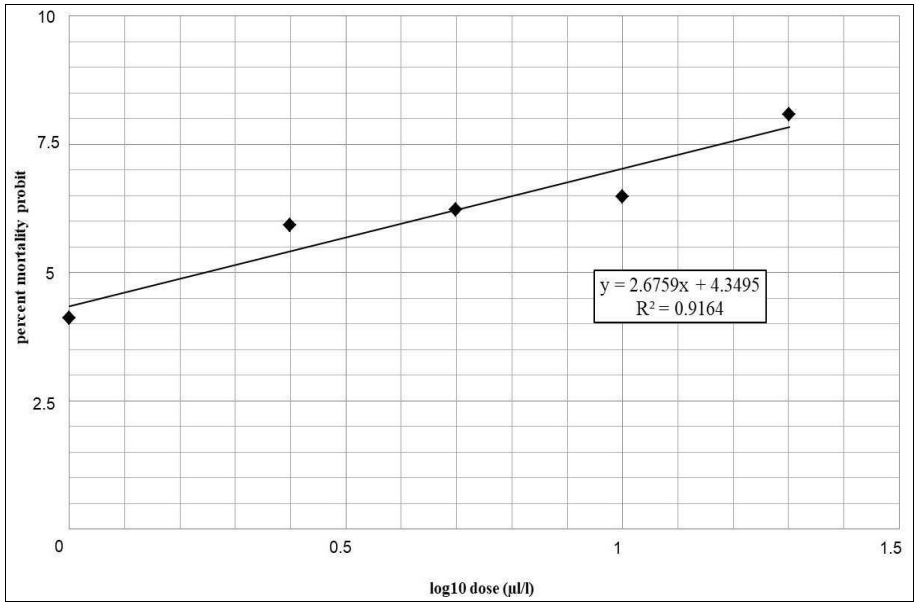

Fig 5: Dose-response analysis on *R. lucifugus* mortality (Probit) after 24 hours of treatment exposure at different doses (µl/l) of geranium EO in laboratory

Effective termiticidal activity of oregano EO has been reported against *Odontotermes assamensis* and *Microcerotermes eugnathus* [39, 48]. In our results mortality caused by treatment with oregano EO ranged from a minimum of 18.9% at the dose of 1µl/l to a maximum of 100% at the dose of 2.5µl/l. Already at the dose of 5 µl/l mortality reached 100% in all replicates. Significant differences were found between test (treatment doses) and control and between treatment doses (F=202.02, df=3, p<0.001). Each treatment dose was statistically different from the control, and significant differences between treatment doses were imputable only to the dose of 1µl/l compared to the others (Post hoc analysis, P=0.05) (Table 1). The dose-response analysis (Figure 6) showed LD50= 1.21 µl/l after 24 hours of treatment exposure.

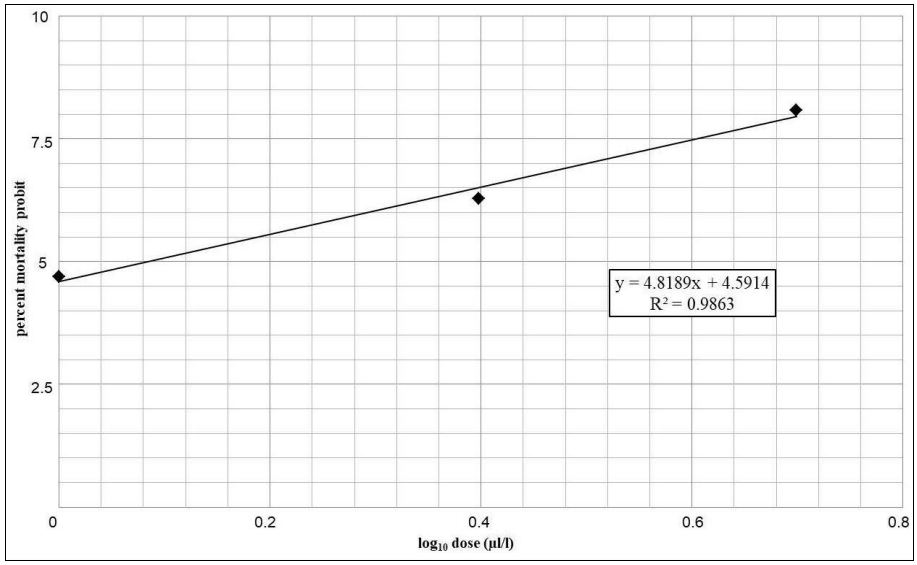

Fig 6: Dose-response analysis on *R. lucifugus* mortality (probit) after 24 hours of treatment exposure at different doses (µl/l) of oregano EO in laboratory

The comparison of the effectiveness of the EOs against *R. lucifugus*, reveals that oregano EO was the most active already at low doses, while fennel EO was the least effective. In fact, already at dose 2.5 µl/l, oregano, clove, and geranium EOs caused a mortality more than 80%. Considering the minimum dose killing half a test sample (Lethal Dose/50) detected for each EOs, the lowest dose was used with oregano (1.21 µl/l) followed by clove (1.28 µl/l), geranium (1.73 µl/l), fennel (2.20 µl/l), and lastly bergamot (2.88 µl/l). This confirms the strong activity of oregano EO, already demonstrated in studies against other insect species. Indeed, several studies reported that oregano EO has remarkable effects on the control of pests such as pentatomids [19], moths [18], stored product insects [26], mosquitoes [33], cockroaches [52], houseflies [41], and aphids [14, 7, 34]. The results achieved with EOs against termites suggest that the implementation of such natural substances can significantly reduce the use of chemical products in the urban environment [49].

4. Conclusion

In this study we have detected the insecticidal activity of five EOs in the control of *R. lucifugus*. Specifically, EOs of

oregano, clove and geranium have good effectiveness, whereas fennel and bergamot EOs have shown lower activity on worker mortality. Furthermore, mortality increased as the EO dose increased. 24 hours after the treatment, the lowest DL50 was found for oregano EO, followed by those of clove, geranium, fennel, and bergamot EOs. Our results highlighted the possibility of using EOs in termite control for the wood conservation. However, further studies are needed for practical applications that can lead to the safeguarding of the artistic heritage and buildings.

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