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FORMULATION AND TESTING OF INNOVATIVE ESSENTIAL OIL-BASED NANO-INSECTICIDES AGAINST STORED PRODUCT PESTS

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INDEX

ABSTRACT

In Italy, the legislation regulating the use of synthetic pesticides has become more stringent in recent years with the consequent banning of several active ingredients. In the agro-food sector, pesticides are commonly used also within the food industries and, thus, they can potentially contaminate food. For these reasons, it is important to find new eco-friendly pest control tools.

Among the proposed new tools, essential oils (EOs) have been widely studied for their recognized antimicrobial, insecticide, antioxidant and antifungal activity. Essential oils can be a viable alternative to synthetic pesticides due to their reasonable cost and general wide availability.

It is not easy to handle and store EOs because they are easily subject to oxidative processes and they are thermosensitive; in this scenario, the present PhD thesis has focused on developing EO-based nanoemulsions to increase their insecticidal activity and to make the final formulations more user-friendly.

Eight EOs have been selected and their chemical composition has been characterized by GC-MS analysis. Subsequently, the nano-insecticides were formulated using the spontaneous emulsification process of oils in water, assisted by a surfactant. To further reduce the size of the lipidic micelles, an ultrasonic homogenizer was used.

The insecticidal effectiveness and the repellent action of the developed nanoformulations have been evaluated against *Tribolium confusum,* a cosmopolitan pest of milling industry. The toxicity against adult beetles has been verified by applying EOs as cold aerosol inside plexiglass boxes connected to an Air Delivery System. To assess the repellence of the nanoformulations, a sodium polyacrylate-based gel was developed to convey and gradually release the volatile constituent of the EOs and prolong their effectiveness toward adult *T. confusum*. Toxicity tests were also performed on *Drosophila melanogaster,* a model pest damaging fruit in post-harvest, administering the nanoformulations to adult flies as fumigants in hermetically sealed arenas.

Results from the trials highlighted that, among the eight developed EO-based nanoformulations, the most promising were the garlic and anise-based nanoemulsions, which can cause high toxicity and repellence against both *D. melanogaster* and *T. confusum*.

To understand the mechanisms regulating the insecticidal activity, the most promising EO nanoformulations were selected and a gene expression analysis focused on the nervous system was performed on *D. melanogaster*. The target genes whose expression was evaluated were *AChE, Gabat, Tbh, ADH, AANAT, GstS1, Mgstl* and *Vha68-2*, which were reported in the literature as genes commonly involved in the nervous system. The expression of the genes *Cyp6a2, Cyp6a8, Cyp6a19, Cyp6a23, Cyp6g1, Cyp6g2, Cyp6t3* and *Cyp12d1* was also evaluated; according to literature, these genes, afferent to the CP450 system, are involved in detoxification and resistance to insecticides.

The analysis of gene expression demonstrated that anise EO inhibited both the acetylcholine and octopamine systems, while garlic EO of garlic did not directly affect the main enzymes related to the nervous system, although its toxicity is probably due to an interaction with the acetylcholine system through an allosteric action.

The results of the present study are promising because the insecticidal activity of EO-based nanoformulations has been proved even at very low concentrations. Furthermore, useful information about the mechanism of action of these EOs have been provided, although further investigation is needed to definitely identify the target sites of the two best performing essential oils.

RIASSUNTO

In Italia, la normativa che regola l'uso dei pesticidi di sintesi è diventata negli ultimi anni più stringente con la conseguente messa al bando di numerosi principi attivi. Nel comparto agroalimentare, i pesticidi sono comunemente utilizzati anche all'interno dell'industria alimentare e quindi potenzialmente possono contaminare gli alimenti. Per questi motivi è importante trovare nuovi strumenti di disinfestazione eco-compatibili.

Tra i nuovi strumenti di interesse, gli oli essenziali (OE) sono stati ampiamente studiati per la loro riconosciuta attività antimicrobica, insetticida, antiossidante e antimicotica. Gli oli essenziali possono essere una valida alternativa ai pesticidi di sintesi grazie al loro costo ragionevole e alla generale ampia disponibilità.

Non è semplice maneggiare e stoccare gli OE poiché questi sono facilmente soggetti a processi ossidativi e sono termosensibili. In questo contesto, il presente lavoro di tesi si è occupato di sviluppare delle nano-formulazioni a base di OE volte ad incrementarne l'efficacia e a rendere la formulazione insetticida maggiormente user-friendly.

Sono stati selezionati otto OE e di questi ne è stata caratterizzata la composizione chimica tramite analisi GC-MS. Successivamente i nano-insetticidi sono stati formulati sfruttando il processo di emulsificazione spontanea degli oli in acqua, coadiuvato dall'impiego un surfattante. Per ridurre ulteriormente la dimensione delle micelle lipidiche è stato utilizzato un omogeneizzatore ultrasonico.

L'efficacia insetticida e l'azione repellente delle nano-formulazioni sviluppate sono state valutate nei confronti di adulti di *Tribolium confusum,* un infestante cosmopolita delle industrie molitorie. La tossicità è stata verificata applicando gli OE come nebbia fredda (cold aerosol) all'interno di box di plexiglass connessi ad un Air Delivery System. Per valutare la repellenza delle nano-formulazioni, è stato elaborato un gel a base di poliacrilato di sodio, allo scopo di veicolare e rilasciare gradualmente la costituente volatile degli OE prolungandone l'efficacia. Su *Drosophila melanogaster,* un insetto modello che danneggia

numerosi frutti in post-raccolta, sono stati effettuati test di tossicità ma, a differenza del trattamento effettuato su *T. confusum,* le nano-formulazioni sono state somministrate alle mosche adulte come fumiganti in arene con chiusura ermetica.

Tra le 8 nano-formulazioni a base di OE sviluppate, i risultati più promettenti sono stati ottenuti con le nanoemulsioni a base di OE di aglio e di anice, per i quali è stata osservata un'ottima tossicità e repellenza sia nei confronti di *D. melanogaster* che di *T. confusum*.

Per avere una conoscenza più approfondita dei meccanismi che regolano l'azione insetticida, sono stati selezionati gli OE più promettenti ed è stata effettuata una analisi dell'espressione genica focalizzata sul sistema nervoso utilizzando *D. melanogaster* come modello. I geni target di cui è stata valutata l'espressione sono *AChE, Gabat, Tbh, ADH, AANAT, GstS1, Mgstl* e *Vha68-2*, riportati in letteratura come geni comunemente coinvolti nel sistema nervoso. Si è valutata anche l'espressione dei geni *Cyp6a2, Cyp6a8, Cyp6a19, Cyp6a23, Cyp6g1, Cyp6g2, Cyp6t3* e *Cyp12d1* che sono riportati in letteratura come geni afferenti al sistema CP450, il quale è coinvolto nei processi di detossificazione e resistenza agli insetticidi.

L'analisi dell'espressione genica ha evidenziato che l'OE di anice causava un'azione inibitoria sia sul sistema dell'acetilcolina che dell'octopamina, mentre l'OE di aglio non determinava una chiara azione diretta sui principali enzimi del sistema nervoso; probabilmente, la tossicità riscontrata era da imputare ad un'azione allosterica che interagisce con il sistema dell'acetilcolina.

I risultati del presente studio sono promettenti poiché le formulazioni hanno mostrato una buona attività insetticida anche a bassissime concentrazioni di olio essenziale. Questo studio ha fornito informazioni utili anche riguardo i meccanismi di azione di questi OE, sebbene ulteriori approfondimenti siano necessari per comprendere e identificare chiaramente i siti target dei due oli essenziali selezionati.

1. INTRODUCTION

Stored products are constantly attacked by pest species, which can cause relevant product damages, with an estimated production loss around 40% when appropriate control programs are not assessed (Boyer *et al.*,2012).

The second half of the 20th century witnessed the birth and sudden development of chemical control against insects, following the discovery of insecticidal properties of numerous groups of organic compounds. In recent decades, the use of insecticides, characterized by high toxicity not only against insects but also against other organisms, has shown its duplicity of action, highlighting positive effects compared to pest control in agriculture, as well as serious negative consequences on the biotic and abiotic environment such as:

- soil and water pollution;
- increased toxicological risk for humans and vertebrate animals;
- significant depletion of populations of entomophages and pollinators;
- appearance of insecticide-resistant phytophagous strains;
- mass multiplication of new phytophages over the damage.

The main synthetic insecticides used were chlororganic, phosphorganic, carbamates, pyrethrins, pyrethroids, polysulphides and various products of different groups and plant origin. At present, almost all these active ingredients have been banned by the current legislation, although the use of phosphorganics, pyrethrins, some products of different groups and those of vegetable derivation is still allowed (Isman, 2020; Giraev *et al.*, 2017). These control tools have been used for their undoubted effectiveness, but, in recent years, more attention has been paid to the problems associated with these insecticides, such as the risk for human health, the toxicity to non-target organisms (both upper vertebrates and insects useful for humans or important for the ecosystem) and the environmental pollution (Yu, 2008).

In Italy, the law regulating the use of pesticides has become stricter in the last decade, banishing a lot of active ingredients, thus promoting research about new and alternative pest control tools. Furthermore, the use of pesticides is generally

limited inside the food industry; for this reason, it is important to find new eco-compatible pest control tools to apply also during post-harvest.

The growing concerns about synthetic pesticides have prompted the development of alternative, eco-sustainable and safe control strategies. In order to achieve this goal, nature itself has been investigated and a possible solution has been found by investigating popular traditions in which plant derivatives are used to protect the stored food reserves from pests (Hassanali *et al.*, 1990). Insecticidal formulations obtained from botanicals are generically considered less toxic to humans, and more eco-sustainable due to their biodegradability (Rosenthal, 1986). However, traditional plant protection products should be implemented to be effectively used also under operative conditions in production plants, by developing alternative control techniques based on organic or plant products with low environmental impact.

In the last years, new approaches to pest control have been developed, involving natural and environmentally friendly pesticides, as well as applying the hurdle concept (i.e. the obstacle theory), which theorizes that the synergic effects of more products and techniques may improve their efficacy against pests.

Among plant-derived products, essential oils (EOs) have been extensively studied due to their acknowledged antimicrobial, insecticidal, antioxidant and antifungal activity. In this scenario, EOs are promising tools for Integrated Pest Management (IPM) programs for stored product pests. Furthermore, EOs can be a valuable alternative to synthetic pesticides due to their reasonable cost and general widespread availability. Essential oils are constituted by a blend of different class of substance. Manly two fraction are identified, one “volatile”, who can represent from the 85% to the 99% of the essential oil and one “not volatile” between the 1% and 15% (Stevanović, *et al.*, 2018). The volatile fraction of essential oils is the most studied since its compounds usually are bioactive against a series of undesired pests and microbes. The volatile compounds can be classified into four main groups: terpenes, benzene derivatives, hydrocarbons and other miscellaneous compounds (Tripathi *et al.*, 2003). Every essential oil is a very complex various of different class of molecules; EO can contain about 20–60 components at quite different concentrations but usually they present two or three major components at higher

concentrations (20–70%) (Bakkali *et al.*, 2008). Usually, the main constituent of the essential oil is responsible of is insecticidal, antifeedant or repellent activity and it is known that other minor compounds can enhance its effectiveness. On the other hand, the mechanisms of action against insects as well as the synergistic (or the antagonist) effects of these compounds have not been thoroughly studied (Hummelbrunner *et al.*, 2001; Nerio *et al.*, 2010). Some studies on the physiological action of EOs, reported a neurotoxic effect against insects, affecting acetylcholine esterase and octopamine (Coats *et al.*, 1991; Kostyukovsky *et al.*, 2002), but no conclusive information about the mechanism of action of these botanicals is still available. In vertebrates the octopamine has a marginal role and the lack of octopamine receptors in humans increases the interest in the use of essential oils as insecticides. However, the bioactivity of an essential oil against insects can vary depending on the variability of its chemical composition; studies observed that mixtures of various monoterpenoids, as well as mixtures of these compounds and synthetic insecticide, can result in synergistic effect against target pests (Gaire *et al.* 2020; Abbassy *et al.*, 2009). The variability of the composition is due to different factors exogenous (seasonality, ecotypes, climate, etc.) and endogenous (site of production of the EOs in the plant, the age and genetic characteristics of the plants) (Barra, 2009). For this purpose, the chemical characterization of the EOs represents a key factor to understand the role of the individual components and, consequently, to standardize the insecticide formulations.

In the present thesis work, we have investigated if and how insecticidal nanoformulations obtained from plant extracts can be considered a valid tool in order to prevent and contain pest infestations in production facilities. In this research project new EO-based nanoformulations aimed to increase the already known insecticidal and/or repellent activity and the stability of the compound were developed. In addition, EO-based nanoformulations were developed to maximize their effectiveness and reduce the cost for the treatments. Furthermore, through biomolecular technique, as Real Time qRT-PCR, the mode of action of selected EOs was investigated by quantifying the gene expression of the target sites.

1.1. Phytosanitary products

According to EC Reg. 1107 of 2009, plant protection products are chemicals used to control pests. These are defined as products, in the form in which they are supplied to the user, containing or consisting of active substances, safeners or synergists, intended to:

(a) protect plants or plant products against all harmful organisms or to prevent their effects;

(b) affect the life processes of plants but which are substances other than nutrients;

(c) preserve plant products;

d) destroy unwanted plants or parts of plants;

e) control or prevent unwanted plant growth (Official Journal of the European Union, 2009).

Plant protection products include insecticides, herbicides, fungicides, insecticides, nematicides, rodenticides, etc. (Official Journal of the European Union, 2009).

1.2. The importance of phytosanitary products

Plant protection products are used to protect agricultural crops and crop products from pest attack, preventing considerable product losses. According to the National Research Council (2000), if the use of plant protection products were abolished in the United States of America, there would be considerable economic losses for the entire national agricultural sector (Yu, 2008). Globally, about 1/3 of crops used for food purposes are destroyed by pests during the various phases of product management, from growth to storage. The greatest losses are found mainly in developing countries; in Latin America, for example, annual product losses are around 40% (Ware and Whitacre, 2004). Damage to crops varies depending on the cultivar and pest, but in many cases, without the use of plant protection products, some crops would be impossible to harvest or would have little commercial value (Yu, 2008).

Table 1 show that, in Italy, in the last decade there has been a decrease in the use of synthetic plant protection products in favour of an increase in organic products.

The reduction in the use of plant protection products is mainly due to the new European directives aimed at reducing the use of such products. Research in recent years has developed several solutions to the limitation of effective control tools, that, although individually sometimes proved to be poorly effective, through their synergistic effect can maximize the effectiveness of control programs by developing a multi-step approach (Wisniewski *et al.*, 2016). This type of approach remains difficult to manage and the results are not optimal yet, since it does not reach the same efficacy of chemical plant protection treatments (Wisniewski *et al.*, 2016). In this historical moment of transition from chemical to biological control, the multi-step protocol is, however, proposed as one of the best alternatives (Wisniewski *et al.*, 2016).

Tables 2 show that in the last decade the use of many families of non-organic active ingredients has decreased significantly, mainly due to the intervention for regulations that have banned their use.

Table 1 Pesticides and traps distributed for agricultural use, by category (in tons, unless otherwise specified). Italy (ISTAT, 2020)

Year	Fungicides	Insecticides and acaricides	Herbicides	phytosanitary products and various active principles	Other phytosanitary products	Total	Traps (number)
2003	81765	33497	30569	11877	303	158012	625.787
2004	80751	29902	25143	18256	335	154387	888.842
2005	82439	29307	25746	18480	425	156398	868.004
2006	75891	27036	26542	19182	344	148995	701.919
2007	77956	27291	27502	20328	336	153412	919.675
2008	79659	22174	25869	21766	469	149937	1.095.010
2009	73147	27542	25680	20694	411	147474	863.489
2010	67708	28160	28129	19912	-	143908	728.354
2011	69891	27571	24086	20876	-	142425	664.862
2012	64359	26872	24241	18770	-	134242	590.615
2013	54987	22829	23490	16968	-	118273	600.585
2014	65315	22284	24209	18170	-	129977	474.460
2015	69538	23746	23255	19517	-	136055	583.106
2016	61015	21857	22636	18604	-	124112	191.337
2017	54537	22410	21066	18796	-	116809	244.644
2018	53729	20645	20258	18293	-	114396	312.836

Table 2 Active ingredients contained in plant protection products, by category and family of insecticides and acaricides (in tons). Italy (ISTAT, 2020)

Year	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018
Chlororganic	139	116	117	81	39	14	16	-	-	-	-	-	-	-	-	-
Carbamates	571	540	514	505	620	272	71	59	54	58	43	69	64	65	80	65
Urea derivatives	33	28	29	25	33	31	51	43	23	21	10	7	5	3	4	3
Phosphorganics	2609	2068	2144	2249	2209	1798	1741	1710	1760	1569	1265	1327	1418	1222	1084	821
Nitrogen/sulphur/organic nitrogen halo-hydrocarbons	201	269	222	181	191	147	152	125	115	97	40	20	11	12	12	11
Other insecticides and acaricides	232	231	226	207	310	319	352	340	352	344	335	360	507	431	466	398
Total	3783	3252	3252	3249	3401	2581	2383	2276	2303	2089	1693	1783	2006	1733	1647	1298
Inorganic compounds	951	607	580	569	529	578	565	351	354	269	375	96	111	78	71	27
Oils	8.035	7.837	7521	7.073	6571	5264	4828	5415	4804	4193	3916	3563	4042	3813	3489	3869
Vegetable derivatives and similar synthetic	45	55	533	565	626	682	110	121	117	136	161	150	135	147	152	180
Total allowed in organic farming	8710	*8428*	*8050*	7593	7072	5822	5371	5747	5140	4599	4.452	3.808	4.288	4.039	3.711	4.076

The new tendency to reduce the use of synthetic chemicals represents a major challenge in pest management. Indeed, this method has proved to be, compared to biological control, economically less expensive, simpler for the operators, generally it can guarantee a quick control of the infestation and can be the only valid means available in cases of emergency. Obviously, chemical control has a series of disadvantages such as the possible selection of resistant strains, the adverse effects of non-target species, some risks for the operators, the presence of residues on food and the environmental contamination. All these disadvantages have given rise to a substantial European legislation that limits the use of synthetic chemicals, triggering a growing trend of substitution of the chemical active ingredients with others of natural origin.

1.3. Actual law and regulation

With the law n.26 of January 5, 1955, concerning the approval and implementation of the Convention for the establishment of the European and Mediterranean Plant Protection Organization (EPPO), signed in Paris on April 18, 1951, one of the requests of the International Convention is answered at European level by establishing this important control body. This body was established with the task of:

- Acting as an advisor to member states on technical, administrative and legislative measures necessary to prevent the introduction and spread of enemies and diseases of plants and plant products;
- Helping the member states to implement these measures where necessary;
- Coordinating and encourage campaigns at international level against pests of plants and plant products;
- Obtaining reports from member states on the existence, appearance or proliferation of pests and diseases of plants and plant products;
- Making these reports known to the member states;
- Ensuring the exchange of information on national legislation concerning the quarantine of plants and other measures concerning the free movement of plant products.

EPPO focuses its activities on the unification and simplification of phytosanitary regulations and certificates, facilitating cooperation in research on all issues related to plant pests and plant products, encouraging the creation of operational protocols useful for the application of the principles of control, thus facilitating the exchange of scientific reports, establishing a system of documentation and publishing in the desired form documents intended for propaganda, technical or scientific progress.

In recent decades, crop protection practices have changed a lot and many active ingredients have been banned from European markets. This represents a serious problem for agriculture due to the difficulty of satisfactorily controlling pests. One of the main objectives of EPPO is the development of proper crop protection management methods in EPPO regions, encouraging Integrated Pest Management, i.e. the use and integration of all pest control techniques in order to keep populations below an economic damage threshold and at the same time minimize the use of phytosanitary products.

In Europe, Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 establish *"a framework to achieve a sustainable use of pesticides by reducing the risks and impacts of pesticide use on human health and the environment and promoting the use of integrated pest management and of alternative approaches or techniques such as non-chemical alternatives to pesticides*" (Official Journal of the European Union, 2009).

The above-mentioned Directive has been implemented in Italy by Legislative Decree no. 150 of August 14, 2012, whose provisions have been implemented taking into account the precautionary principle by limiting or banning the use of plant protection products in specific circumstances or areas due to a potential danger to human or animal health or the environment. With the Legislative Decree 150, the Technical and Scientific Council on the sustainable use of plant protection products was established and developed a National Action Plan for the use of plant protection products that must be updated every five years.

With the introduction of the Plan, were introduced mandatory training courses for the operators, to obtain the authorization to use plant protection products in real conditions; these courses are mandatory regardless of the toxicity of the product

used for disinfestation. This approach is fundamental to constantly update consultants and operators, in order to minimize the risks associated with treatment application and to avoid the revocation of the authorization to use synthetic products.

Legislative Decree 150 regulates the introduction of compulsory integrated pest management, encouraging the application of techniques for the prevention and monitoring of infestations and infections, the use of biological means of pest control, the use of appropriate cultivation practices and the use of phytosanitary products that present the least risk to human health (Official Gazette of the Italian Republic, 2012).The National Action Plan was implemented by the Ministerial Decree of 22 January 2014 (Official Gazette of the Italian Republic, 2014).

The formulations used for pest control operations (pesticides), rodents (rodenticides) and viruses and bacteria (disinfectants) must be authorized and registered by the Italian Ministry of Health. The Italian legislation identifies two distinct categories of formulations, one including formulations intended for agricultural use and / or food, the other formulations intended for civil use. The use of phytosanitary formulations is regulated by Regulation 1107/2009, and with the implementation of Directive 91/414/EEC the authorization process of these substances has taken into account the concept of risk for the environment and has implemented an upstream prevention strategy, requiring for new substances also the compliance with certain environmental requirements.

The definition Medical-Surgical Presidium (PMC) includes all those products that show on the label an activity that can be traced back to the definition in article 1 of D.P.R. 392/ 1998. The above mentioned D.P.R. regulates the procedure of production authorization and marketing authorization of disinfectants and substances marketed as germicide or bactericide, insecticides for domestic and civil use, insect-repellents, topicides and ratticides for domestic and civil use. With the Directive 98/8/EC, implemented in our legislation with the Legislative Decree n.174/2000, it regulates the placing on the market of biocidal products, defined as

- "*any substance or mixture, in the form in which it is supplied to the user, consisting of, containing or generating one or more active substances, with the intention of destroying, deterring, rendering harmless, preventing the*

action of, or otherwise exerting a controlling effect on any harmful organism by any means other than mere physical or mechanical action;

- *any substance or mixture, generated from substances or mixtures which do not themselves fall under the first indent, to be used with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action.*

The use of a phytosanitary formulation or a PMC/biocide is linked to the kind of disinfestation/disinfection activity. The choice must be made on the basis of the definition of the formulations provided by the respective regulatory laws, since in Article 1 of the above-mentioned article it is specified which are the intended uses; if there is the need to treat plants, plant products or primary or processed foods, the use of a phytosanitary formulation is correct in other cases it is correct to use a PMC/biocide. This is due to the differences in the registration procedures. Indeed, for both it is mandatory to provide, in addition to the target species and the characteristics of the substances contained, the types of risks involved, but for plant protection it is essential to also provide the results of tests attesting the risks and consequences of the use of a given formulation on a specific plant or fruit. Hence, the prohibition to use a PMC/biocide in the presence of products intended for food and feed.

When using PMC/biocide formulations, however authorized for that use, or plant protection products within food industries for defined disinfestation interventions, the directives of the hygiene package, specifically EC Reg. n.852/04 must also to be followed by food operators to ensure that primary products are protected from contamination, paying great attention that the environments, or machinery, have been emptied of processing products or, alternatively, that any products present are disposed of and not used in production.

1.4. Environment and phytosanitary products

Synthetic chemicals, due to their high toxicity and non-biodegradable nature, are a cause of concern for human health, as they are known to concur to environmental pollution, resulting from their use due to the permanence of residues in soil, water and crops (Campolo *et al.*, 2013).

Synthetic plant protectants have been widely used to control agricultural, food, household and veterinary-medical pests. Depending on the method of application and target species, the active ingredients can generally reach the soil, which acts as a reservoir of residues, and from the soil they can spread to other components of the environment (Figure 1).

Residues once in the soil can:

- be absorbed by plants and enter the food chain where they are concentrated in animal fats,
- be transported by the wind in the form of steam at considerable distances and then condensed with dust and rain to re-precipitate into the ground again,
- reach and pollute the groundwater sources.

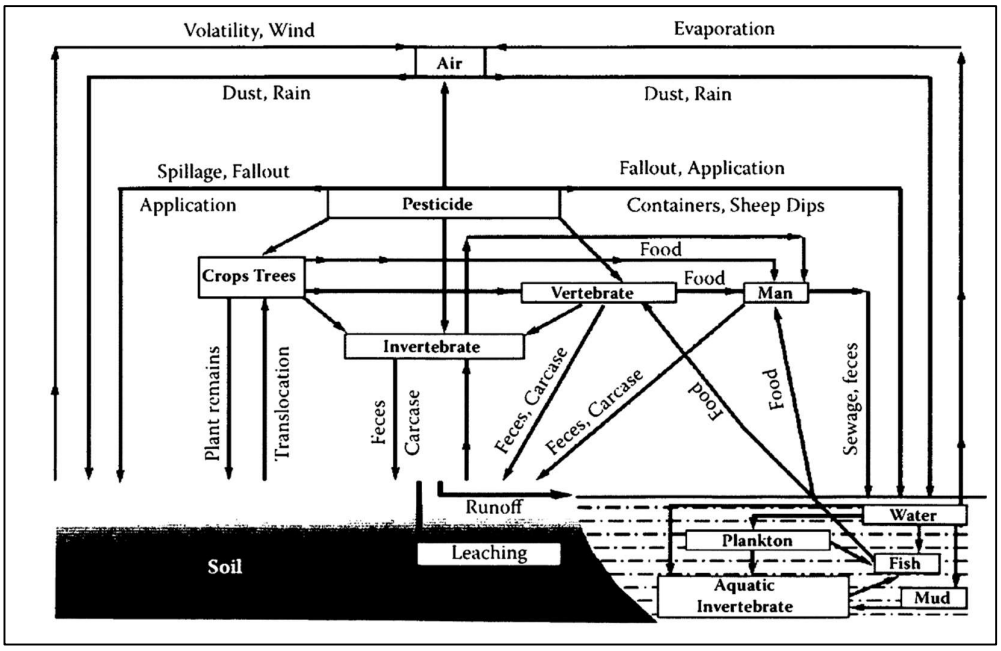

Figure 1 Pesticide cycle in the environment (Edwards, C.A., Critical Review Environment. Control, 1,1, 1970)

The soil is a dynamic biotic and abiotic system; the synthetic substances deposited in it can have different ways of absorption by the mineral clay and the organic component of the soil matrix. The persistence in the soil of specific chemicals is influenced by many factors such as: the nature of the active ingredient itself, soil type, soil pH, temperature and degradation capacity of the microbial flora (Yu, 2008).

Many active ingredients are synthesized trying to obtain formulations with the best possible stability, but, subjected to environmental processes (i.e., hydrolysis and photolysis), these are converted into physiologically active compounds. The amount of active ingredient detected in a matrix can be considered an insufficient indicator to measure its degradation in soil because it can undergo to significant changes. As an example, the active ingredient Aldicarb forms two metabolites, both significantly more mobile in soil than the original product, which are more difficult to detect (Hornsby, 1996). The interactions occurring between the environment and active ingredient can be considered bidirectional because the environment, through metabolic or photodegradative processes, alters the product that can affect non-target species or interact negatively with the ecosystem.

Solvents, emulsifiers and other ingredients, included in the formulation of a plant protection product to maximize its effectiveness, are classified as inert but can create unexpected problems. For example, the reaction between the active ingredient organ phosphate dimethoate and the solvent 2-methoxyethanol greatly increases the toxicity of dimethoate in mammals without substantially increasing its insecticidal activity (Casida and Sanderson 1961).

Plant protection products vary widely for their toxicity against non-target organisms and for their environmental impact. The most important and accurate assessments on the effects on non-target species have been made on mammals, with the addition of information obtained from birds, fish and bees. The problems of acute toxicity of synthetic plant protectants against mammals are mainly related to organophosphates and methylcarbamates, with similar results against birds. Regarding fish, most of the insecticides with neurotoxic action have a very high toxicity against aquatic organisms, similarly to some herbicides and fungicides (Casida, 2012).

Bees generally are not more sensitive than other insects, but the colony collapse disorder and the general reduction of their populations pose great concerns in agriculture (Hardstone and Scott 2010). In this scenario neonicotinoids are very concerning because, since their introduction in the 1990s, they showed a great efficacy as insecticides against several key pests, leading neonicotinoids to quickly become the most commonly used class of insecticides worldwide (Jeschke *et al.*, 2011). Their great efficacy is countered by crucial negative implications; as an example, neonicotinoids degrade slowly and remains in the environment for months, or even years, after the application (Hopwood *et al.*, 2012). Thus, non-target and/or untreated plants growing in treated or contaminated soils may absorb chemical residues even remaining from the previous year (Hopwood *et al.*, 2012). Another critical characteristic of neonicotinoids is that they can be translocated inside plant tissues, and can also reach plant pollen and nectar, which are important food sources for flower-visiting insects such as bees. The ingestion of neonicotinoid can cause severe side-effects to non-target organisms and even small can be highly toxic, causing mortality and/or behavioural alterations, and can contribute, together with other factors, to colony collapse disorder of bees' populations (Cressweel, 2011). This case is emblematic to understand the importance of developing and investigating new ecofriendly solutions for pest control.

The reduced use synthetic chemical has been incentivized by a series of various factors, including research and scientific findings, as well as legal actions (Casida, 2012), such as:

- the progress in production of plant protectants and the increased knowledge of their chemistry and their toxicity;
- the banning of many synthetic chemical;
- the development of new biochemical targets;
- the increased use of genetically modified crops that reduce the amount and variety of applied pesticides;
- the great emphasis of legislations on environmental protection and product biodegradability;
- the development of complex integrated pest and plant protection product management systems.

1.5. Essential oils

The essential oils (EOs) are complex mixtures and are obtained by extraction from plant material rich in aromatic compounds, i.e. the "essences" that are produced by plants for various functions, that can sometimes be also waste products of the food industry. The essential oils play various functions, including allelopathy, antibiotics, attraction of pollinators and intermediaries of energy reactions (Dhifi *et al.*, 2015).

EOs are composed of complex mixtures of various substances. There are two fractions, one "volatile", which can represent from 85 to 99% of the substance and the other "non-volatile" which varies between 1 and 15% (the non-volatile fraction is absent in oils obtained by distillation) (Stevanović *et al.*, 2018). The knowledge of the chemical-analytical characteristics of EOs has been allowed by the development of instrumental techniques of analysis, especially chromatography; gas chromatography, in particular, has allowed to aknowledge the quantitative and qualitative composition of the volatile fraction of oils. Through gas chromatography, analyzing the enantiomers of the constituents of the volatile part, it is possible to determine the plant origin of the essential oil and recognize any mixtures with synthetic compounds. In the past, for the study of the composition of the non-volatile fraction has been usually used the thin layer chromatography (TLC), while today the most suitable technique is high performance liquid chromatography (HPLC). The non-volatile component consists of hydrocarbons, fatty acids, sterols, carotenoids, waxes, coumarins, psoralenes and flavonoids.

EOs do not have in the plant a specific site or organs of production, and they can be synthetized both internally (secretory glands allocated inside the plants) as externally (secretory glands placed on the plant surface) (Svoboda *et al.*, 2003). The plant species capable to elaborate the constituents of essential oils, are called aromatic plants and are worldwide distributed. More than 17000 species of aromatic plants are known but they belong to a limited number of family: Myrtaceae, Lauraceae, Rutaceae, Lamiaceae, Asteraceae, Apiaceae, Cupressaceae, Poaceae, Zingiberaceae and Piperaceae. Among the known EOs, only 300 have an economic relevance for their use in pharmaceuticals, cosmetics, perfumes and have a potential as pesticide (Tripathi *et al.*, 2003).

Four methods of extraction of the EOs are recognized in the European Pharmacopoeia and the International Standard Organization on Essential oils (ISO 9235:2013). Hydro-distillation, steam distillation, dry distillation and mechanical processes are used to extract the EOs from raw plant material. After extraction, to clean the sample, a filtration, decantation, and centrifugation process can be done without any significant change in EO composition (Campolo *et al.*, 2018).

The reckless use of synthetic pesticides, given their high toxicity and non-biodegradability, causes approximately 100 billion dollars of damage every year (Koul *et al.*, 2008). As previously mentioned, in recent years we have tried to remedy this by focusing research on the development of alternative control techniques, trying to obtain insecticides from derivatives obtained from plants (Rosenthal, 1986).

Among plants extracts, the interest for EO has been renewed starting from the 1990s with the demonstration of their fumigant and contact insecticidal activity on several pest (Campolo *et al.*, 2018). Despite all these promising properties the EOs present a big challenge, due to their high volatility, poor water solubility and high tendency to oxidation (Turek *et al.*, 2013). Furthermore, the composition can change in relation with the phenological stage and the geographical origin (Barra, 2009).

1.6. Nanotechnology e nanosciences

Nanoscience born at the end of 50s, precisely in the 1959 when the physicist Richard Feynman formulated for the first time the concept of nanoscience (Feynman, 1960) in the speech entitled "There's plenty of room at the bottom - An invitation to enter a new field of physics", he assumed that in the future we could build devices of various nature acting directly on the position of atoms. For the first time the term nanotechnology appeared in 1974 in the article "On the basic concept of nano-technology" by Taniguchi and he said that "Nano-technology mainly consists in the processing of separation, consolidation, and deformation of materials by one atom or one molecule". To exist the nanoscience uses the know-how of different disciplines; to develop and understand this science we need to merge from

quantum physics to supramolecular chemistry, from materials science to molecular biology. The aim of nanotechnology is to exploit and apply the methods and the knowledge derived from nanoscience.

Nanotechnology finds application in virtually all production sectors. The greatest interest has mainly focused on biology (nanosensors and manipulators of biological matter), on medicine (markers, detectors, distributors of drugs), on new materials, information, computation and quantum (quantum computing, memories, flexible organic LED displays), and approaches with which individual molecules can be manipulated superficially on a material, and atoms.

1.6.1. Nanomaterials

With the term "nanomaterials" (NMs) has been identified particulate nanostructures, that do not have a standard shape, but which have at least one dimension on the "nano" scale, less than 100 nanometers (nm). The shape of the NM can be spherical, tubular, filamentous or irregular and can be formed from various materials that can exist in dispersed, molten, aggregated or agglomerated form. The particular properties of NMs are due to the fact that they follow physical laws that are found between classical and quantum physics and this is due to their size.

Nanoparticles (NPs) are atomic or molecular aggregates with particular chemical-physical properties, which make them exploitable in different application fields, such as food, agriculture, medical sectors. The definition of NP varies depending on the material, the field of use and application. Overall, particles with smallest dimensions, between 10-20 nanometers, can be considered actual NPs. although particles ranging between 1 nm and 1 μm are usually called nanoparticles. (Yokoyama, 2012) Commonly the NPs, because of their size, have properties and characteristics different from those of the original chemical species. This is due to NPs larger exposed surface area, with the same mass, compared to the macro-particles of the same original material, that can exponentially increase their chemical and biological reactivity.

A NM can be of natural origin, such as those produced by natural combustion processes (volcanoes, spontaneous fires) or anthropogenic origin (Stern *et al.*,

2008). In the last case, should be done a distinction between those produced involuntarily (vehicular traffic, incinerators, industry and domestic heating) and those produced voluntarily. The voluntary produced NMs belong to the artificial NMs that is specifically produced by nanotechnology to perform technological purposes to various levels and in various scientific and industrial fields (Borm *et al.*, 2006).

A commonly used classification divides the nanomaterials in 4 groups:

I. NMs of carbon, mainly composed of carbon, usually in the form of empty spheres, ellipsoids or tubes;

II. NMs of metals, which include quantum dots, nanogolds, nanosilver and oxides of metals such as titanium dioxide (TiO_2);

III. dendrimers and polymers of nano size, consisting of branched units. These can be used for the selective and controlled transport of drugs, markers and oligonucleotides as they contain internal cavities in which they can be included molecules;

IV. NMs composites, obtained by combining solids of different nature and often made up of a matrix (metallic, polymeric or ceramic) that is reinforced with nano-sized particles. This union allows to obtain hybrid systems with intermediate mechanical, thermal and electrical properties at those of the individual constituents and therefore more resistant, lighter, less sensitive materials to corrosion.

1.6.2. NM synthesis

The essence of nanotechnology is to build new materials and objects on a nanoscale system and to achieve this aim various production techniques are required, grouped in two main strategies, the "Top-down" and "Bottom-up". Conceptually, the Top-down approach consists in starting from a bulk material by obtaining the nanostructure by progressive removal of matter. What has always characterized this production strategy has been the reproducibility, reliability and complexity of the objects that can be obtained, on the other hand they are the techniques with the greatest energy impact and that produce the greatest production waste. The Top-down production strategy through the use of precision engineering and lithography

techniques has allowed the production of most of the available electronic and optical devices (Whatmore, 2006).

The Bottom-up strategy consists in the creation of more complex structures starting from the assembly of single atoms or molecules; this is normally achieved by the natural manifestation of interaction between the constituent elements, which are thus organized to form the desired structure. According to these strategies there are basically only three techniques to create NM: chemical synthesis, self-assembly, and "positional assembly" (Niemeyer, 2001).

The chemical synthesis allows to obtain nanostructures in two ways:

- the first consists in producing and manipulating the bulk material until the desired structure is obtained;
- the second way is to produce the elements making up the structure at a higher level of organization than the various bulk materials (molecules), which will then be assembled to form the nanostructure using one of the two remaining Bottom-up production strategies.

Obviously with the self-assembly process atoms and molecules spontaneously organize themselves to obtain nanostructures; this happens because they can establish characteristic local physical and chemical interactions that guide their recognition and assembly. It is a reversible process, which can be controlled by an appropriate component design, the environment in which the process takes place, and the forces that guide it.

The "positional assembly" technique refers to the direct manipulation of atoms, molecules or aggregates to form the nanostructure sought after, precisely according to the ideas of Feynman. This technique can be used to create structures on a surface using Scanning Probe Microscopy (SPM), or in the three dimensions using optical tweezers technology; however, there are still some extremely laborious techniques that are not applicable at industrial level.

1.6.3. Nanoemulsion

There is a growing interest in the development of nanoemulsion, since their production requires low energy methods, their implementation is simple and low expensive equipment is required.

An emulsion is a biphasic dispersed system, rarely polyphasic, formed by two immiscible phases. Emulsions consisting of liquids are thermodynamically unstable formulations, in which one of the two liquids is dispersed in the other in the form of globules or spherical droplets. The most common liquids used to prepare emulsions in the food industry are oils and water. As mentioned above, emulsions are traditionally biphasic and can be of type W/O (water in oil) or O/W (oil in water), and they have droplet size in a range between 100 to 600 nm. In absence of surfactants (i.e., substances capable of reducing surface and interfacial tension between liquids, solids and gases allowing them to mix), the system is highly unstable because the two phases tend to break down.

Nanoemulsions have small droplet size and are kinetically stable colloidal systems. They have enhanced functional properties in comparison to conventional emulsions. The composition and structure of the nanoemulsions can be controlled for the encapsulation and effective delivery of bioactive lipophilic compounds.

A O/W nanoemulsion produced with a bioactive EO is more effective because reducing the droplet size the effectiveness of the oil can increase. Emulsions and nanoemulsion are metastable systems and thus they have the tendency to break down over time. This tendency is due to different destabilization mechanisms, such as gravitational separation, coalescence, flocculation, and Ostwald ripening (McClements, 2015). Furthermore, the smaller size of the droplets in nanoemulsions typically gives them better stability to gravitational separation and droplet aggregation than conventional emulsions (Komaiko *et al.*, 2016). The separation rate due to gravitation can by described by Stokes' Law, an expression for the force of viscous friction to which a sphere in laminar motion is subjected with respect to a fluid, the velocity of the droplet is determined by gravity (g), particle radius (r), the difference in density between the continuous and dispersed phases ($\Delta\rho$), and the shear viscosity of the continuous phase (η):

$$V = \frac{2gr^2(\Delta\rho)}{9\eta}$$

The smaller diameter of the droplets in a nanoemulsions corresponds to greater stability against gravitational phenomena. Furthermore, Brownian motion effects can oppose the gravitational force, so due to the reduced droplet size can also inhibit movement (Komaiko *et al.*, 2016).

In the last years the formulation of several novel nanopesticide was observed, and some of these formulations showed a higher efficacy compared also to traditional commercial pesticide (Kah and Hofmann, 2014).

1.7. Food facilities pests

Numerous health and hygiene problems are almost entirely related to the trophic activity of insects infesting food, damaging it and modifying its integrity, both during storage and in the subsequent phase of transformation into final products (Olsen, 1995). Only in some cases, favoured by thermal hygrometric conditions of the environments and products, mites' infestations can also occur (Peace, 1983). The activity of synanthropic vertebrates, rodents and birds, even if quantitatively lower compared to insects, is qualitatively very relevant for food contamination; as an example, it is sufficient that only one hair of murids is found in food to eliminate the entire production batch from market (Holah, 2014).

The damage caused within a milling plant by pests, which live at the expense of the food, can be considerable. The infestation of insects and mites of wheat or flour, during both milling and storage, can occur in many circumstances, sometimes difficult to predict (Mohandass *et al.*, 2007; Stejskal *et al.*, 2014). In addition to the damage caused by the quantitative losses of the food, pests can contaminate the flour with manure, excrement, faeces, faeces, exuviae, hair and with their own body, generating serious health and hygiene problems and causing repulsion and serious damage to the consumer's image for the company. Experimental studies on the vector capacity of insects have repeatedly shown that many of those which infest edible products can carry pathogens to food from external sources of contamination (Holah, 2014). Furthermore, arthropod fragments can cause digestive disorders, abrasions, small lesions to the villi and intestinal mucosa of humans, with

manifestations of diarrhea; moreover, fragments of insect exoskeleton can generate allergic reactions, even intense, such as dermatitis and asthma crisis, caused by chitin (Hubert *et al.*, 2018).

The protection of post-harvest products is challenging especially for vegetable and fruits, IPM programs represent the best option to manage pests below an economic injury level. The current legislative measures regimenting the standard for food sanitary quality and hygiene in international exchange establish that food or feed product destined for trade must be free of arthropod pests (WTO,2020). To archive this result, physical control methods represent the best option and these methods of control can be active or passive.

The active methods have a direct activity on the pest and products and require energy, the most used are thermal shock (e.g., heat, cold), electromagnetic radiation (e.g., microwaves, radio frequencies, ionizing radiations, UV), mechanical shock and pneumatic (e.g., blowing or vacuum). Passive methods (e.g., traps, airtight or hermetic storage, barriers) do not require further energy to achieve desired effect (Vincent *et al.*, 2003).

1.7.1. Food facilities arthropods pests

In order to summarize food protection context, below some bio-ethological notes on the main Arthropods pests are provided (Table 3).

Table 3 Main species of arthropods infesting the production facilities.

ORDER	FAMILY	SPECIES	COMMON NAME
THISANURA	Lepismatidae	*Lepisma saccarina*	silverfish
PSCOCOPTERA	Liposcelidae	*Liposcelis sp.*	booklouse
BLATTODEA	Blattidae	*Blatta orientalis*	black cockroach
	Blattelidae	*Blattella germanica*	german cockroach
COLEOPTERA	Anobiidae	*Lasioderma serricorne*	tobacco beetle
	"	*Stegobium paniceum*	bread beetle
	Bostrichidae	*Rhyzopertha dominica*	australian wheat weevil
	Cucujidae	*Cryptolestes ferrugineus*	flat grain beetle
	Dryophthoridae	*Sitophilus oryzae*	rice weevil
	"	*Sitophilus granarius*	grain weevil
	Dermestidae	*Trogoderma granarium*	khapra beetle
	Silvanidae	*Oryzaephilus surinamensis*	saw-toothed grain beetle
	Tenebrionidae	*Tenebrio molitor*	yellow mealworm beetle

ORDER	FAMILY	SPECIES	COMMON NAME
	Tenebrionidae “ “ Trogossitidae	*Tribolium castaneum* *Tribolium confusum* *Gnatocerus cornutus* *Tenebroides mauritanicus*	red flour beetle confused flour beetle broad-horned flour beetle cadelle beetle
LEPIDOPTERA	Gelechiidae Pyralidae	*Sitotroga cerealella* *Ephestia kuehniella* *Plodia interpunctella*	angoumois grain moth mill moth indian meal moth
DIPTERA	Drosophilidae	*Drosophila melanogaster*	common fruit fly
ACARINA	Acaridae	*Acarus Siro*	cereal mite

1.7.2. Target insect species

***Drosophila melanogaster* (Meigen)**

Kingdom: Animalia
Subkingdom: Bilateria
Infrakingdom: Protostomia
Superphylum: Ecdysozoa
Phylum: Arthropoda
Subphylum: Hexapoda
Class: Insecta
Subclass: Pterygota
Infraclass: Neoptera
Superorder: Holometabola
Order: Diptera
Suborder: Brachycera
Family : Drosophilidae
Subfamily: Drosophilinae
Genus: *Drosophila*
Subgenus: *Sophophora*
Species: *Drosophila melanogaster* Meigen, 1830

Drosophila melanogaster is an important insect belonging to the Diptera order (abundantly distributed all over the world with more than 125,000 species), and to the genus *Drosophila*, whose members are often called "small fruit flies" vinegar or wine flies. This genus contains more than 1,500 species.

Life cycle

Drosophila melanogaster (fruit or vinegar fly) is an insect about 3 mm long, easily visible around fermenting substrate.

Females (Figure 2) can lay up to 600 eggs inside fruit or other organic materials. The eggs, whose diameter is about 0.5 mm, hatch 24 hours after laying. The resulting larvae grow for 5 days, using microorganisms that decompose the fruit and the sugars in the fruit to feed themselves. Then the larva becomes a pupa, spending another 5 days consuming the accumulated energetic reservoirs to carry out the metamorphosis, at the end of which the adult fly emerges. Females mate about 12 hours after the emergence, accumulating the male semen in spermathecas, to use it afterwards to fertilise the eggs. The entire life cycle lasts few weeks and varies manly according to the ambient temperature (Arias and Dahmann, 2008).

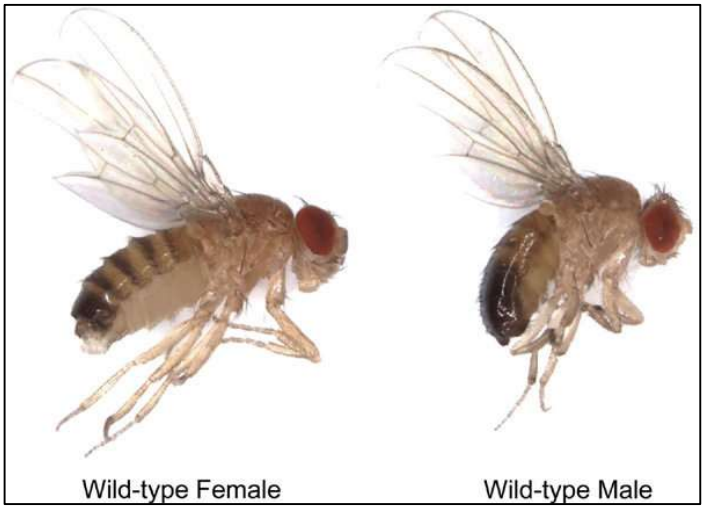

Figure 2 Male and female adult specimens of Drosophila melanogaster

Model system

D. melanogaster has played a crucial role in the last century in the development of biology. In the beginning has been chosen because convenient to investigate the evolutionary theory but soon became the central element in research program dealing with nature and function of the genes (Arias and Dahmann 2008). The main factor that contributed to this success are the rapid generation time, the ease and robustness of breeding, and the low maintenance cost. This insect has been intensively studied in biology and has been selected as a model system,

demonstrating to be a helpful tool in the investigation of biological processes in higher eukaryotes, including humans (Adams *et al.*, 2000)

The use of insecticides, despite is problematic, is still important in the pest management industry, especially for crop pests and vectors. A clear understanding of the physiological processes involved in the interaction insect/insecticide will help to develop more effective and safer approaches for pesticide use. *D. melanogaster* is a key model insect also for toxicology studies (Scott and Buchon, 2019).

Indeed, this organism has been used to study several diseases and various noxious substances (Aralby at al., 2016; Pandey *et al.*, 2011, Gonzalez, 2013), as well as to investigate insecticide resistance (Daborn *et al.*, 2012), mode of action (Schneider, 2000) and toxicological impact (Sharf *et al.*, 2006).

Insecticide resistance is an important threat for pest management and control, especially considering disease vectors (Karunamoorthi and Sabesan, 2013) and invasive alien pest species (Campos *et al.*, 2014; Wan and Yang, 2014). In this scenario, *D. melanogaster* has been a key tool in the investigation of insecticide resistance imposing itself as a genetic model for resistance studies (Daborn *et al.*, 2007; le Goff *et al.*, 2003; Daborn *et al.*, 2012; Ffrench-Constant, 2013).

The growing demand for eco-friendly insecticides has risen the attention about the development of bioactive formulations from botanicals, including EOs, which seem a promising alternative. For this reason, several studies have been already carried out on *D. melanogaster* evaluating toxicity (Franzios *et al.* 1997; Karpouhtsis *et al.*, 1998; Lazutka *et al.*, 2001; Pavlidou *et al.*,2004), repellence (Anggraeni *et al.*,2018). and the mode of action (Enan, 2001; Enan, 2005; da Cunha *et al.*, 2015; Pinho *et al.* 2014; Riaz *et al.* 2018) of various EOs, in order to identify suitable green insecticides. The toxic activity of several EO constituents has been also evaluated toward *D. melanogaster* (Zhang *et al.* 2016; Enan, 2005), investigating their mode of action of these compounds (Enan, 2005).

In this context, *D. melanogaster* can represent the key model species also to evaluate the efficacy and to assess the risks of nanopesticide (Demir, 2020). As an example, Araj *et al.* (2015) investigated the toxicity of silver and sulphur nanoparticle on this model insect. This study was intended as a screening program,

and the nanoparticles were tested on larval, pupal, and adults of *D. melanogaster,* demonstrating that silver nanoparticles were highly effective on egg deterrence, as well as on larvae, pupae, and adults' mortality (Araj *et al.*, 2015).

Further studies are needed to clarify the mode of action of EOs and EO components toward *D. melanogaster* adults. Since the genome of *D. melanogaster* is completely mapped, genetic and biochemical approaches to identify the target sites of EOs can be easier with this model species.

Damages

D. meleanogaster flies most commonly feed on fruit and other sugary substances. *D. melanogaster* is an important fruit pest, especially cherries and berries (Lin *et al.*, 2014). This pest is attracted by fermenting substrate and often are considered human commensals. This pest is common in the proximity of wineries, since they are attracted by fermenting plant materials. Although it cannot cause direct economic damage to fruit production, for wine facilities the presence of *D. melanogaster* can be greatly risky concerning product contamination. Indeed, adult flies are highly attracted by the fermenting wines, and they can fall inside the tanks and vats drowning inside the fermenting wine or vinegar (Günther and Goddard, 2019).

Tribolium confusum (Du Val)

Kingdom: Animalia
Subkingdom: Bilateria
Infrakingdom: Protostomia
Superphylum: Ecdysozoa
Phylum: Arthropoda
Subphylum: Hexapoda
Class: Insecta
Subclass: Pterygota
Infraclass: Neoptera
Superorder: Holometabola
Order: Coleoptera
Suborder: Polyphaga
Infraorder: Cucujiformia
Superfamily: Tenebrionoidea
Family: Tenebrionidae
Genus: *Tribolium*
Species*: Tribolium confusum* Jaquelin Du Val, 1868

Tribolium confusum, is an insect belonging to the order Coleoptera (the largest order in the class Insecta with over 250,000 described species); it belongs to the family Tenebrionidae (worldwide-distributed polyphagous beetle including around 15,000 species) and to the genus *Tribolium,* which includes numerous pests of cereal and cereal derivatives.

Life cycle

Belonging to the wider order of the animal kingdom, Coleoptera, *T. confusum* is part of the Tenebrionidae family, which contains the largest number of beetles harmful to food.

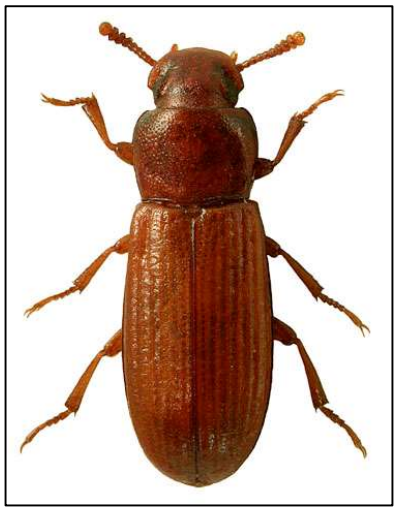

Figure 3 Adult specimen of Tribolium confusum

The adult (Figure 3) measures 3-4 mm in length and is characterised by a variable colouring, from brownish red to dark brown. Anatomically very similar to *Tribolium castaneum* Herbst from which it is distinguished by two morphological characteristics, the different conformation of the antennae, progressively thickened from the base to the apex, and from the front corners of the protruding prothorax.

The lifespan is estimated at 1-1.5 years for females, 2 years for males. The female lays 350-400 eggs. The development cycle lasts about 25 days, at 30 °C and 70% R.H. (optimal environmental conditions). It is also able to withstand low levels of R.H., down to 10% if the temperature is constant at about 22°C.

Damages

Due to its trophic activity, it prefers powdery and starch-rich foodstuffs such as flour, semolina and bran, but it is also characterised by a high polyphagia which allows it to feed on other vegetable substrates such as peanuts, copra, castor-oil, sesame, flax, grain legumes, dried fruit, cocoa, chocolate, cassava and grain cereals. The flour affected by the pest tends to take on a pinkish-brownish colour and give off an unpleasant odour caused essentially by glandular secretions. An attack lasting 2-3 months can irremediably alter the flour (Lis *et al.*, 2011). *T. confusum* is considered a secondary feeder of cereal industry, because this species is not able to damage intact kernels, but it can feed on damaged and broken caryopses, as well as on cereals already damaged by primary feeder species. It is a serious pest of milling industries, causing qualitative and quantitative economic losses.

Control

For food products worldwide the legislation demands high standards, and in the food industry the absence of pest contaminant is an important parameter to

archive this standard (Bell, 2014). As an example, for the Italian legislation, food products or raw materials cannot present any trace of insect, including small fragments visible just after appropriate analyses, like the filth-test. Thus, pest control is fundamental in this sector to ensure the marketability of the food.

On the other hand., pest control in food industry cannot rely on synthetic pesticides, which are almost forbidden; furthermore, chemical treatment (i.e. for the few allowed active principles) should be limited to restricted areas, to avoid that the application could directly contaminate also processed food or packaging (Faustini, 2006).

Fumigants have been the most used formulations for decades, and they have been applied using a damage threshold evaluation; usually, in flour mills and chocolate factories one treatment *per* year at least is performed. Methyl bromide was the most effective insecticides used, but it is also a highly toxic compound; furthermore, it has great environmental impact on atmospheric ozone layer, reason why it has been banished in several countries. Phosphine is an excellent fumigant, commercially formulated as solid tablets, which can be apply across many types of storage structure, whether a silo, a bag stack, pad storage, a bunker, rail cars, a shipping container, a large-bulk shipload, or a flour mill (Chaudhry, 2000); its application is consistently limited just by its corrosive ability on electronic components. Nowadays, phosphine is the only effective active principle against stored product pests, although an increasing number of researches pointed out the insurgence of resistance to this chemical in many insect pest species (Nayak *et al.*, 2020). The resistance problem has been aggravated due mostly to the lack of suitable alternatives, in term of price, ease of application, proven effectiveness against a broad pest spectrum, compatibility with most storage conditions, and international acceptance as a residue-free treatment (Nayak *et al.*, 2020).

Integrated Pest Management (IPM) programs are not easy to correctly design, but these are the most appropriate solution for pest management in food industry (Trematerra, 2013). IPM are applied combining a selection of tools and methods used to control the risks (economical, environmental and for health) balancing their use to archive a sustainable productivity (Bell, 2014). In a successful IPM program the training of the staff is crucial (Bartosik, 2010) as the early detection of the pests.

Thus, a correct monitoring plan should be done throw visual inspection (e.g. the products, the spaces and the production equipment) or traps to evaluate the risk of infestation (Trematerra and Lassard, 2015). Several different trapping systems (e.g. sticky papers, baited trap) are available but pheromones traps are particularly important especially for the selectivity. Aggregation pheromones are usually produced by beetles of the families Bostrichidae, Cucujidae, Curculionidae and Tenebrionidae, and can be used to monitor their presence inside a food facility.

Packaging can also be included as a tool in IPM program. Its effectiveness depends by the material used and the technique of sealing; standard carton designs generally provide little protection against stored product insects (Mullen *et al.*, 2012), but also repellent packaging has been implemented to deter insect infestation (Hou *et al.*, 2004).

Physical Control Methods (PCM) in IPM programs are valuable and ecological treatment but generally expensive and limited (Bell, 2014). Among PCM, heat treatment offers a control effectiveness similar to chemical fumigation (Campolo *et al.*, 2013). The main problem of this kind of treatment is to achieve the heating requirements and the correct heat source deployment to obtain in the structure the required uniform temperature needed to kill the pests. Moreover, higher temperatures can damage the structural or electronic components of the buildings, while lower ones cannot fulfil the elimination of the pests in some areas (Arcidiacono, 2015). Cold treatment may also be used to sanitize food and buildings; however, it requires a very prolonged exposure to be effective thus, this treatment usually is limited to cold storage areas for incoming or finished products (Arthur and Phillips, 2003).

Irradiation is another alternative PCM tool that allow to directly treat pallet load, ensuring the absence of pesticide residues. Gamma rays from the isotope cobalt-60 may be legally used for food irradiation in several counties (Hallman, 2011), nevertheless, consumer acceptance and the costs has limited their use.

Among PCM tools, modified atmospheres are also very limited due to the difficulty to seal the structures for a correct application. This technique is limited to silos treatment and packaging (Conyers and Bell, 2007). The most effective modified atmospheres are those with reduced rate of oxygen (O_2), or those with

increased amount of carbon dioxide (CO_2) or nitrogen (N_2) (Riudavets *et al.*, 2009). The absence of oxygen, as well as the presence of high doses of carbon dioxide and nitrogen, interfere with insect respiration, causing their death; however, stored product beetles can survive several days in absence of respiration.

Despite any possible insecticidal treatment, pest populations can be reduced but their eradication is impossible. For this reason, a correct monitoring and control program is crucial for an appropriate pest management, avoiding contamination and excessive loss of products.

Tenebrionidae are among the main pests of stored products and the control of these pests in the production facility has great relevance. IPM protocols, integrating different approaches and several tools, are the best option to archive this result. Eos can represent new useful active ingredients for biopesticides also in product protection against *T. confusum*. Indeed, promising studies with anise (Isikber *et al.*,2009; Tunc *et al.*, 2000), artemisia (Hashemi and Safavi,2013), fennel (Li *et al.*,2011), garlic (Isikber *et al.*,2009; El-Aziz *et al.*, 2009), lavender (Martynov *et al.*,2019), mint (El-Aziz *et al.*, 2009), rosemary, (Isikber *et al.*, 2006; Tunc *et al.*, 2000; Sener *et al.*, 2009), sage (Sener *et al.*, 2009; Abdellaoui *et al.*, 2017), and several other EOs have been carried out, aiming to investigate both toxicity and repellence of these botanical against *T. confusum*.

2. MATHERIALS AND METHODS

The aim of this research project was to develop alternative valuable eco-friendly tools for IPM programs that can be used during post-harvest in food production facilities. To archive this result, several EO-based nanoformulations were elaborated and their toxicity and repellent potential were evaluated. Furthermore, through biomolecular techniques, as Real Time qRT-PCR, the project aimed to investigate the mode of action of selected EOs by quantifying the gene expression of the target sites.

First, a chemical characterization of eight EOs was performed by GC-MS analysis; this step is fundamental for the standardization of the formulates, because different extraction techniques, different phenological stage or plant organs can provide different EO compositions.

Second, the EOs were formulated using the principle of spontaneous emulsification of oils in water, assisted by a surfactant and ultrasonic homogenization, to obtain nanoemulsions. The quality of the developed nanoformulations was evaluated by Dynamic Light Scattering technique, to measure their droplet dimension, polydispersity index and the surface charge.

Third, repellence and acute toxicity were assessed against the major pest of milling industries, *T. confusum.* For repellence trials, a sodium polyacrylate-based gel was developed to gradually release the volatile constituents of EOs and prolong its effectiveness. Acute contact/inhalation toxicity of the developed nanoformulations was verified in toward adults of *T. confusum* by cold aerosol treatments.

Then, the fumigant acute toxicity of nanoemulsions were evaluated also against *Drosophila melanogaster*, a model organism for genetic studies. The most effective EO-based nanoformulations were selected for further investigations about their mode of action.

Lastly, to clarify the mechanisms regulating the insecticidal action, a gene expression analysis focused on the nervous system was performed using *D. melanogaster* adult flies, investigating several putative target sites, including the

Cytochrome P450 system, which is involved in detoxification and resistance to insecticides.

2.1. Rearing of insects

Tribolium confusum

Unsexed adults of *T. confusum* of 3-15 days has been used for the experimental trials. The original colony was collected from a local milling plant (Melito Porto Salvo, RC). Insects were reared for several generations in glass container for food use and fed with wheat flour mixed with brewer's yeast (10:1, w:w) in the laboratories of General and Applied Entomology of the Department of Agriculture of the University *Mediterranea* of Reggio Calabria. The breeding was maintained under controlled thermo-hygrometric conditions (25±1 C° 70±5% R.H.) with a photoperiod of 16h:8h (L: D). To obtain adults of the same age, about 100 unsexed adults were placed inside 5 l glass containers each provided with 500 g of non-infested rearing medium previously frozen for 24h at -20°C to ensure the absence of previous infestations. After 2 days the specimens were removed and the newly emerged adults (2–8 days old) were used in the trials. Insects were collected from cultures using a 450-μm sieve (Technotest; Modena, Italy) and a mouth aspirator.

Drosophila melanogaster

D. melanogaster wild type strains fly stocks were obtained from the Bloomington Drosophila Stock Center (Bloomington Drosophila Stock Center, BDSC) reared for several year in the Eri Biotecmed section of the Faculty of Biology of the University of Valencia. Unsexed adults of flies of 3-5 days have been used for the experimental trials. The flies were reared in a plastic bottles 5.5 × 12 cm containing approximately 50 mL of standard medium (agar 1% w/v, yeast 4.8% w/v, Soy flour 1% w/v, corn flour 4,8% w/v, sugar 4% w/v, propionic acid 0,4% w/v, Ethanol 0,8 % w/v, nipagin 0,3% w/v). They were maintained at 12:12 h (light/dark cycle), and constant temperature and humidity (25 ± 1 °C, 60% R.H., respectively).

2.2. Essential oil GC-MS analysis

The choice of the essence has been done in relation of the proved efficacy observed in bibliography against the target pests.

Commercial EOs of *Allium sativum* (Amaryllidaceae) (Garlic), *Artemisia vulgaris* (Asteraceae) (Artemisia), *Foenicum vulgare* (Apiaceae) (Fennel), *Lavandula angustifolia* (Lamiaceae) (Lavender), *Mentha piperita* (Lamiaceae) (Mint), *Pimpinella anisum* (Apiaceae) (Anise), *Rosmarinus officinalis* (Lamiaceae) (Rosemary) and *Salvia officinalis* (Lamiaceae) (Sage) were obtained from the University of Catania (Catania, Italy).

The constituents of EOs were analysed using gas chromatography–mass spectrometry (GC–MS) technique. GC–MS analyses were performed with a Thermo Fisher TRACE 1300 gas chromatograph equipped with a MEGA-5 capillary column (30 m x0.25 mm; coating thickness=0.25 μm) and a Thermo Fisher ISQ LT ion trap mass detector (emission current: 10 microamps; count threshold: 1 count; multiplier offset: 0 V; scan time: 1.00 s; prescan ionization time: 100 μs; scan mass range: 30–300 m/z; ionization mode: EI). The following analytical conditions were employed: injector and transfer line temperature at 250 and 240 °C, respectively; oven temperature programmed from 60 to 240 °C at 3 °C min−1; carrier gas, helium at 1 mL min−1; injection, 0.2 μL (10% hexane solution); split ratio, 1:30.

2.3. Nanoemulsions

The EO nanoemulsions were prepared following Giunti *et al.* (2019). To produce the nanoemulsion the spontaneous emulsification process, which occurs when an organic phase and an aqueous phase were mixed, was used. A mixture (3:1 w:w) of each EO and Tween 80® [Polyoxyethylene (20) sorbitan monooleate, Sigma Aldrich, Munich, Germany] was stirred for 30 min.. To realize a homogeneous emulsified phase, after this step double-distilled water was added dropwise to this mixture (4:1 respectively) and stirred for 60 min. This rough emulsion was sonicated for 5 min using an UP200ST ultrasonic immersion homogenizer (Hielsher©, Teltow, Germany) at 100 W power to optimize its physical

characteristics and reduce micellar dimension. The composition of the developed oil in water nanoemulsion was by 5% Tween 80®, 15% EO and 80% water.

2.4. Qualitative analysis of nanoemulsions

To assess qualitative analyses, as the droplet surface charge, indicated by the zeta potential (ζ) values, and the droplets dimension, expressed in terms of Z-average size (d) and polydispersity index (PDI) a Dynamic Light Scattering (DLS) instrument (Zetasizer Nano, Malvern®) was used. PDI and d analysis was done in cuvette, model DTS0012 in polystyrene latex. The nanoemulsion was eluted in bi-distillated water 1/200, 1 ml of this solution was taken, the temperature of the analysis was set at 25°C. For each sample, three replicates of fourteen cycles were provided.

ζ analysis has been done putting 730 µl of solution in the cuvette model DTS1070 in polystyrene latex, the temperature of the analysis has been set at 25°C. For each sample, three replicates of fourteen cycles were provided.

2.5. Gel from EO-based nanoemulsion

To produce the EO-based gels a simple hydrophilic gel was created; agarose was used to give a stronger texture and sodium poliacrilate to maintain hydrated the gels. A mixture of water and agarose (0.8%) was stirred and heated until the complete melting of the gel. When melted, the EO nanoformulations were added and immediately after was added the sodium poliacrilate (0.4%), and then the mixture was stirred for 30 seconds. To avoid EO degradation, immediately after the stirring, the mixture was put in a plastic mold in an ice-bath, to quickly reduce the temperature.

2.6. Bioassays

2.6.1. Repellence of gels against *Tribolium confusum*

To determine the repellent activity of EO nanoemulsions, an area preference bioassay was set up. Insects were placed inside a rectangular plexiglass box covered with filter paper (Whatman n°1). In the short side of the arena 5 g of flour were placed on each side. Then, in one side 1g of EO-based gel was put. A gel made with a mixture of water and 5% Tween 80® was used as control.

Unsexed adults (30 beetles, 5-10 days old) were placed in the central area of the arena. Plexiglas box were covered with nylon meshes to prevent the odour saturation of the arena. The arenas were maintained at 25 ± 1 °C, 50 ± 5% R.H., under constant light condition.

The gels were obtained using the following proportion of EO nanoformulations:

- 80%, 40%, 20% 10%, 5%, 2.5%, 1.25%, 0.625%, 0.313%, 0.156%, 0.078%, 0.039%, 0.02%, 0.01%, 0.005% for Anise EO
- 80%, 40%, 20% 10%, 5%, 2.5%, 1.25%, 0.625%, 0.313% for Artemisia EO
- 80%, 40%, 20% 10%, 5%, 2.5%, 1.25%, 0.625%, 0.313%, 0.156%, 0.078%, 0.039% for Fennel EO
- 5%, 2.5%, 1.25%, 0.625%, 0.313%, 0.156%, 0.078%, 0.039%, 0.02%, 0.01% for Garlic EO
- 80%, 40%, 20% 10%, 5%, 2.5% for Lavender EO
- 80%, 40%, 20% 10%, 5%, 2.5%, 1.25%, 0.625%, 0.313% for Mint EO
- 80%, 40%, 20% 10%, 5%, 2.5%, 1.25%, 0.625%, 0.313%, 0.156% for Rosemary
- 80%, 40%, 20% 10%, 5%, 2.5%, 1.25%, 0.625%, 0.313%, 0.156% for Sage

In every comparison, 1 g of gel containing the EO nanoemulsions was evaluated against *T. confusum*. Thus, the actual amount of EO present in the gel dispenser and used in repellence bioassays were:

- 120, 60, 30, 15, 7.5, 3.75, 1.875, 0.938, 0.469, 0.235, 0.117, 0.059, 0.029, 0,015, 0.007 mg of Anise EO

- 120, 60, 30, 15, 7.5, 3.75, 1.875, 0.938, 0.469 mg of Artemisia EO
- 120, 60, 30, 15, 7.5, 3.75, 1.875, 0.938, 0.469, 0.235, 0.117, 0.059 mg of Fennel EO
- 7.5, 3.75, 1.875, 0.938, 0.469, 0.235, 0.117, 0.059, 0.029, 0,015 mg of Garlic EO
- 120, 60, 30, 15, 7.5, 3.75 mg of Lavender EO
- 120, 60, 30, 15, 7.5, 3.75, 1.875, 0.938, 0.469 mg of Mint EO
- 120, 60, 30, 15, 7.5, 3.75, 1.875, 0.938, 0.469, 0.235 mg of Rosemary EO
- 120, 60, 30, 15, 7.5, 3.75, 1.875, 0.938, 0.469, 0.235 mg of Sage EO

For every EO*dose combination, 3 replicates with 30 unsexed *T. confusum* adults each were provided. Insects were used only once. The number of insects on the two halves of the arena (i.e., treated or not) was recorded after 24 h and 48h from the beginning of the exposure. The percent repellence (PR) for every EO nanoemulsions and for every considered time was calculated by the formula: $PR(\%)= [(Nc - Nt)/(Nc + Nt)] \times 100$ where Nc is the number of insects in the control half and Nt the number of insects in the treated one.

2.6.2. Acute toxicity by cold aereosol against *Tribolium confusum*

The insecticidal activity of the EOs against the unsexed adults of *T. confusum* was determined by evaluating the acute toxicity of cold aerosol treatments using the method of Giunti *et al.* (2019). Four replications of 20 unsexed adults have been tested for every EO*dose combination.

Trials were carried out under laboratory conditions at 25 ± 1 °C, 50 ± 5% R.H. with a photoperiod of 16h:8 h (L:D). The tested adults were placed inside a Perspex cage (30 × 30 x 30 cm), presenting on one side a hole (height from base 15 cm; 14 mm Ø) where an aerosol borosilicate-glass ampule (GammaDis Farmaceutici s.a.s., Civitanova Marche, Italy) was plugged. A solution containing different concentrations of the EOs was placed inside the ampule, which was connected to an air delivery system, blowing purified air at 2 L min^{-1} constant flow. The air flow was turned off when the ampule was almost empty (i.e., residues inside the glass ampoule < 0.1 mL) and the tested insect were leave inside the treated cage. After

24h of exposure, tested insects were removed from the cage, then gently placed in a clean glass Petri dish with rearing medium (wheat flour mixed with yeast (10:1, w:w). The mortality was recorded at 24 h and 168 h from the beginning of the cold aerosol treatment to account also for delayed mortality caused by the treatment.
The EO nanoemulsion was diluted with distilled water to obtain the following concentrations:

- 15% (i.e. pure EO nanoemulsion), 7.5%, 3.75%, 1.88%, 0.94%, 0.47% and 0.235% Anise EO
- 15% (i.e. pure EO nanoemulsion), 7.5%, 3.75%, 1.88%, 0.94%, 0.47% and 0.235% Artemisia EO
- 15% (i.e. pure EO nanoemulsion), 7.5%, 3.75%, 1.88%, 0.94% and 0.47% Fennel EO
- 15% (i.e. pure EO nanoemulsion), 7.5%, 3.75%, 1.88%, 0.94%, 0.47% and 0.235%, 0.118% and 0.059% Garlic EO
- 15% (i.e. pure EO nanoemulsion), 7.5%, 3.75%, 1.88%, 0.94%, 0.47% and 0.235% and 0.118% Lavender EO
- 15% (i.e. pure EO nanoemulsion), 7.5%, 3.75%, 1.88%, 0.94% and 0.47% Mint EO
- 15% (i.e. pure EO nanoemulsion), 7.5%, 3.75%, 1.88%, 0.94%, 0.47% and 0.235% and 0.118% Rosemary EO
- 15% (i.e. pure EO nanoemulsion), 7.5%, 3.75%, 1.88%, 0.94% and 0.47% Sage EO

Inside the glass ampulla, 4 mL of every diluted EO nanoemulsions were inserted and applied against *T. confusum*. Thus, the actual EO concentrations tested in the cages were:

- 22.22, 11.11, 5.56, 2.78,1.39, 0.69 mg of Anise EO /L of air
- 22.22, 11.11, 5.56, 2.78,1.39, 0.69 mg Artemisia EO/L of air
- 22.22, 11.11, 5.56, 2.78 and 1.39 mg Fennel EO/L of air
- 22.22, 11.11, 5.56, 2.78,1.39, 0.69, 0.35 and 0.17 mg Garlic EO/L of air
- 22.22, 11.11, 5.56, 2.78,1.39, 0.69 and 0.35 mg Lavender EO/L of air
- 22.22, 11.11, 5.56, 2.78 and 1.39 mg/L mg Mint EO/L in air

- 22.22, 11.11, 5.56, 2.78,1.39, 0.69 and 0.35 mg Rosemary EO/L of air
- 22.22, 11.11, 5.56, 2.78 and 1.39 mg Sage EO/L of air

Control trials were carried out using formulations of Tween 80® in water at the same concentrations tested in the EO nanoemulsions. Additional control trials using just double-distilled water were performed.

2.6.3. Fumigation toxicity against *Drosophila melanogaster*

The insecticidal activity of the EO nano-insecticides against unsexed *D. melanogaster* adults was assessed by fumigant bioassay using the fumigation bioassay method of Scharf *et al.* (2006) with few modifications. Three replications of 30 unsexed adults, less than 1 week old, were carried out for every EO*dose combination.

Flies were briefly anesthetized with ether inside the rearing vials, and, using a camel-hair brush and a 5 × 5 cm sheet of rice paper, 30 flies were moved to a 0.5 L glass jars. Each vial was previously provided with a 0.5 × 0.5 × 0.5 cm block of rearing diet, that was dried on a paper towel to remove excessive moisture. Immediately after insect release, the jars were capped with a modified metal lid with a hook supporting a filter paper. Cellulose-based 2-cm diameter No. 1 Qualitative (Whatman; Florham Park, NJ, USA) filter papers were used. In a fume hood, the filter papers were previously treated with EO nanoemulsion dilutions or with 5% Tween80 water solution as control. The mortality was checked after 24 h. Flies were counted as dead only when they showed no movement. All bioassays were conducted in climatic chamber at 25 ± 1°C, 65 ± 5% R.H. with a photoperiod of 16h:8h (L:D).

The EO nanoemulsions were diluted with distilled water to obtain the following concentrations:

- 15%, 7.5%, 3.75%, 2.813%, 1.875%, 1.406%, 0.938% of Anise EO
- 15%, 7.5%, 5.625%, 3.75%, 2.813%, 1.875% of Artemisia EO
- 15%, 7.5%, 3.75%, 2.813%, 1.875%, 1.406%, 0.938% of Fennel EO
- 7.5%, 3.75%, 1.875%, 0.938%, 0.469%, 0.234%, 0.117%, 0.059%, 0.029% of Garlic EO

- 15%, 11.25%, 7.5%, 5.625%, 3.75%, 2.813%, 1.875% of Lavender EO
- 15%, 7.5%, 3.75%, 1.875%, 0.938% of Mint EO
- 15%, 13.125%, 11.25%, 9.375%, 7.5%, 6.563% of Rosemary EO
- 15%, 13.125%, 11.25%, 9.375%, 7.5%, 6.563%, 5.625%, 3.75% of Sage EO

Every filter paper was treated with 0.1 mL of EO nanoemulsion dilutions; thus, the following doses of EO were applied inside the jars against *D. melanogaster*:

- 30, 15, 7.5, 5.625, 3.75, 2.813, 1.875 mg Anise EO/L of air
- 30, 15, 11.25, 7.5, 5.625, 3.75 mg Artemisia EO/L of air
- 30, 15, 7.5, 5.625, 3.75, 2.813, 1.875 mg Fennel EO/L of air
- 15, 7.5, 3.75, 1.875, 0.938, 0.469, 0.234, 0.117, 0.059 mg Garlic EO/L of air
- 30, 22.5, 15, 11.25, 7.5, 5.625, 3.75 mg Lavender EO/L of air
- 30, 15, 7.5, 3.75, 1.875 mg Mint EO/L of air
- 30, 26.25, 22.5, 18.75, 15, 13.125 mg Rosemary EO/L of air
- 30, 26.25, 22.5, 18.75, 15, 13.125, 11.25, 7.5 mg Sage EO/L of air

2.7.RNA extraction and cDNA synthesis

RNA was extracted from the head and the body of 3-7 days old, unsexed adults of *D. melanogaster,* fumigated with selected EOs nanoemulsion using the correspondent LC_{95}; three replicates were performed. Each sample was composed of RNA extracted from 25 whole bodies. Samples were homogenized using liquid nitrogen and a pestle, and total RNA was extracted using RNAzol® RT (Sigma) following the manufacturer's protocol. One microgram of RNA was added to Maxima™ H Minus cDNA Synthesis kit with dsDNase (Thermo) following the manufacturer's protocol.

2.7.1. Primer design

Primers for α-Tubulin used as housekeeping has been found in literature (Ponton *et al.*, 2011).

Primers for Tyramine β hydroxylase (*Tbh*), Gamma-aminobutyric acid transaminase (*Gabat*), Acetylcholinesterase (*AChE*), Alcohol dehydrogenase (*ADH*), Arylalkylamine N-acetyltransferase (*AANAT*), Glutathione S transferase S1 (*GstS1*), Microsomal glutathione S transferase (*Mgstl*), Vacuolar H[+] ATPase 68 kDa subunit 2 (*Vha68-2*), Cytochrome P450 6a2(*Cyp6a2),* Cytochrome P450 6a8 (*Cyp6a8),* Cytochrome P450 6a19 (*Cyp6a19),* Cytochrome P450 6a23 (*Cyp6a23),* Cytochrome P450 6g1 (*Cyp6g1),* Cytochrome P450 6g2 (*Cyp6g2),* Cytochrome P450 6t3 (*Cyp6t3*) and Cytochrome P450 12d1(*Cyp12d1*) (Table 4) were designed by the Primer3 software based on *D. melanogaster* sequences from the GenBank.

Table 4 Primers information and polymerase chain reaction (PCR) efficiencies

GENE		PRIMER SEQUENCE (5'-3')	EFF%	PRODUCT SIZE(BP)	SEQUENCE ACCESSION NUMBER(S)
Tub84	Forward	TGTCGCGTGTGAAACACTT	105.24	96	NM_057424.4
	Reverse	AGCAGGCGTTTCCAATCTG			
AChE	Forward	TCAGAACCAGCAGCAAATCG	87.84	131	X05893.1
	Reverse	TGCTTGTGCGGTGTGTAAAG			
ADH	Forward	TCAAGCGCGATCTGAAGAAC	88.05	96	NM_001032099.2; NM_001032098.2; NM_001032097.2; NM_001032096.2; NM_001032095.2; U07641.1
	Reverse	TGACGGTCACCTTTGGATTG			
AANAT	Forward	AAATGGAGGACGCATTGACC	98.27	126	Y07964.1; NM_206212.2; NM_079115.3
	Reverse	AGGTCTTGAGCATGGCTATCAC			
Gabat	Forward	TGCAAGAAGAATGGCATCGC	105.62	125	NM_001300196.1; NM_140911.4; NM_168829.3; NM_001170008.1
	Reverse	TTGCTGAAGGTCACCACATC			
GstS1	Forward	AAGTTGGTCACCCTGAATGC	82.22	145	NM_001274111.1; NM_166217.3; NM_079043.3; NM_166216.2
	Reverse	AGTTCATGTAGTCGGTGATGCC			
Mgstl	Forward	ATCGTCCACACACTGGTCTAC	90.28	138	NM_001298578.1; NM_079957.4
	Reverse	AGACCTATGTGCTCAGAAGGC			
Tbh	Forward	ACGTTCGCCGACATTTATGC	84.61	111	Z70316.3
	Reverse	ATCTCGTGCAGCTTTGTGTC			
Vha68-2	Forward	TCATCGACTTCTACGACATGGC	84.66	96	NM_001273496.1; NM_167724.3; NM_001273495.1;
	Reverse	AATGTTGCCCATTGCCTCAC			

GENE		PRIMER SEQUENCE (5'-3')	EFF%	PRODUCT SIZE(BP)	SEQUENCE ACCESSION NUMBER(S)
					NM_001259086.2; NM_165022.2; NM_165021.2
Cyp6a2	Forward	TCGGCATTGAGTGTAACACG	107.00	150	NM_078904.2
	Reverse	TCATGCGCATTCTCAACCTG			
Cyp6a8	Forward	ACAGTCTGCGCGATGAAAAG	92.18	99	NM_079025.5
	Reverse	AGCTGCGCATAAAGCCATTC			
Cyp6a19	Forward	TTCGCGTGGTCGATGAAAAG	109.91	113	NM_137157.4; NM_001299505.1
	Reverse	GCATTTCAGGCCAAAAGCAC			
Cyp6a23	Forward	AATCGGTAATTGCGCCTTCG	104.04	83	NM_137156.3
	Reverse	TCGCCCTTTTTCCGATTGTC			
Cyp6g1	Forward	AGTGGCCATGGCATATCAAC	93.21	122	NM_001299406.1; NM_136899.4; AY081960.1
	Reverse	AGTTGCGCACTGAGTGATTC			
Cyp6g2	Forward	TGTTCGAGTTCAACCTGCTG	127.94	124	NM_136900.2
	Reverse	ATGGTCTTGCGCAGAAAACG			
Cyp6t3	Forward	AGACTATGCGCGCTATATGAGG	115.70	137	NM_136901.2
	Reverse	AAGCAACAAAGTCCGGATGC			
Cyp12d1	Forward	TAATGCCCGGAATGTTTGGC	108.10	148	NM_136791.6; NM_001299375.1; NM_206090.2
	Reverse	ACATCTGGTCGAACGTGTTC			

2.7.2. Quantitative Real-Time Polymerase Chain Reaction (PCR)

qRT-PCR was carried out on 2ng of cDNA template with Fast EvaGreen® qPCR Master Mix (Biotium) following the manufacturer's protocol and using specific primers (Table 4). For reference gene Tubulin (housekeeping, Ponton *et al.*, 2011), qRT-PCR was carried out on 0.2ng of cDNA. Thermal cycling was performed with Step One Plus Real Time PCR System (Applied Biosystems). Three biological replicates and three technical replicates per biological sample were carried out. Reactions were executed in triplicate.

2.8. Statistical analysis

Statistical processing of the data was conducted with the software: IMB SPSS statistic 23, Microsoft® Excel® per Microsoft 365 MSO and Relative Expression Software Tool-384 (REST-384©) calculation Software for the Relative Expression in real-time PCR using Pair Wise Fixed Reallocation Randomisation Test ©.

In repellence assays, PR data calculated after 24 h and 48h were subjected to probit analysis in order to calculate the median repellent concentration (RC_{50}) and 95% repellent concentration (RC_{95}) of the tested EO. RC values were considered significantly different when their respective 95% fiducial limits (FL) did not overlap.

In cold aerosol and fumigation trials, the efficacy of the tested formulation was corrected for control mortality using Abbotts formula (Abbott, 1925) and probit analysis was used to estimate the median lethal concentration (LC_{50}) and 95% lethal concentration (LC_{95}). In fumigation trials, probit analysis was performed on mortality data recorded at 24 h of exposure and in cold aerosol trials with mortality data recorded at 24 h and 168 h from exposure. LC values were considered significantly different when their respective 95% fiducial limits (FL) did not overlap.

Statistical differences ($P<0.05$) among repellence/mortality rates were also distinctly analysed using General Linear Model (GLM) with one fixed factor (i.e., dose) followed by a post-hoc test (Tukey-Kramer HSD) for every EO and time of exposure.

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3. RESULTS AND DISCUSSION

3.1. GC–MS chemical characterization of essential oil

The GC-MS analyses of EOs used to develop the nano-insecticides allowed to identify the compounds which constituted these plant extracts.

Anise

In anise EO, thirty-two compounds were identified representing 98.16% of the oil (Table 5). This EO was almost entirely composed by phenylpropene (91.93%), followed by monoterpenes (3.08%). The main constituents were (E)-anethole (86.54%), estragol (3.88%), α-pinene (1.14%), linalool (1.84%) and limonene (1.13%).

The analysed EO was composed also by several sesquiterpenes, although with a low relative percentage (sesquiterpenes 0.91%, oxygenated sesquiterpenes 0.12%).

Artemisia

Thirty-three compounds representing 90.77% of the oil (Table 5) were identified. The artemisia EO was mainly composed by oxygenated monoterpene (82.52%), followed by monoterpenes (7.24%). The main constituents were α-thujone (27.13%), camphor (21,45%), β-thujone (13.25%), chrysantenyl acetate (4.68%) and eucalyptol (4.09%).

The EO contained several sesquiterpenes and aromatic hydrocarbon, although with a low relative percentage (sesquiterpenes 0.61%, aromatic hydrocarbon 0.40%).

Fennel

GC–MS analyses allowed to identify twenty-six compounds representing 97.82% of the oil (Table 5). The fennel EO constituents was almost for half represented by phenylpropene (45.19%), followed by monoterpenes (41,37%). The main

constituents were (E)-anethole (40.58%), limonene (30.84%), fenchone (9.88%), α-phellandrene (2.68%) and estragol (2.40 %). A number of sesquiterpenes, although with a low relative percentage (0.34), were also detected and identified.

Garlic

In garlic EO, seventy-two compounds, representing 99.68% of the oil (Table 5), were identified by GC-MS analysis. This oil was mainly composed by sulphur compounds (84,75). The main constituents were Diallyl disulfide (29.66%), Diallyl trisulfide (21.50%), Diallyl tetrasulfide (13.19%), Diallyl sulfide (10.69%) and Allyl methyl trisulfide (3.22%).

Lavender

The GC–MS analyses allowed to identify forty-one compounds in lavender EO; the identified substances represented the 97.45% of the oil (Table 5). The lavender EO was almost entirely composed by oxygenated monoterpene (89.87%), followed by sesquiterpene (4.33%). The main constituents were linalool (38.13%), linalyl acetate (33.42%), camphor (6.68 %), eucalyptol (5.51 %) and borneol (2.25%).

The analysed EO was composed also by several oxygenated sesquiterpenes and ester, although with a low relative percentage (sesquiterpenes 0.22%, aromatic hydrocarbon 0.39%).

Mint

Thirty-one compounds were identified in mint EO; these compounds accounted for 95.37% of the oil (Table 5). This oil was mainly composed by oxygenated monoterpene (80,43%), followed by monoterpene (9.27%). The main constituents were (-) menthol (38.63%), menthone (18.47%), isomenthone (6.78 %), limonene (6.11%) and (-) neomenthol (4.89 %).

The analysed EO contained also several oxygenated sesquiterpenes, although with a low relative percentage (0.1%).

Rosemary

In rosemary EO twenty-eight compounds were identified by GC-MS analysis; these substances represented the 98.53% of the EO constituents (Table 5). The rosemary essential oil was mainly composed by oxygenated monoterpene (68.34%), followed by monoterpene (27.68%). The main constituents were eucalyptol (52.58%), camphor (11.84%), α-pinene (11.78%) ortho-cymene (4.18%).and camphene (2.82 %).

The analysed EO contained also several oxygenated sesquiterpenes, although with a low relative percentage (0.11%).

Sage

GC–MS analyses of sage EO allowed to identify twenty-three compounds which represented the 97.42% of the oil (Table 5). The sage essential oil was mainly composed by oxygenated monoterpene (56.19%), followed by monoterpene (33.45%). The main constituents were α-pinene (19.18%), eucalyptol (19.08%), α-thujone (16.18%), camphor (12.64%) and camphene (4.44%).

The analysed EO revealed also several oxygenated sesquiterpenes, although with a low relative percentage (0.11%).

Table 5 GC-MS analysis of the selected EO

Component	RI[a]	*Pimpinella anisum*	*Artemisia vulgaris*	*Foeniculum vulgare*	*Allium sativum*	*Lavandula angustifolia*	*Mentha piperita*	*Rosmarinus officinalis*	*Salvia officinalis*
allyl methyl sulfide	699				0.62				
dimethyl disulfide	739				0.05				
hexan-2-one	797				0.02				
4-Methylthiazole	822				0.01				
allyl isopropyl sulfide	826				0.01				
furfural	832				0.01				
1,2-dithiolane	842				0.03				
unknown 1	851		0.13						
diallyl sulfide	857				10.69				
allyl propyl sulfide	872				0.03				
allyl methyl disulfide	914				2.60				
tricyclene	924		0.32						
α-thujene	928					0.04			0.21
methyl propyl disulfide	930				0.01				
α-pinene	934	0.57	0.58	2.33		0.57	1.03	11.78	19.18
2-ethoxythiazole	944				0.01				
methyl (E)-1-propenyl disulfide	947				0.11				
camphene	947		4.04	0.28		0.14		2.82	4.44
(E)-2-hexenal	952				0.02				
verbenene	953		0.15						
3H-1,2-dithiolene	957				0.16				
dimethyl trisulfide	967				0,06				
benzaldehyde	969				0,04				
sabinene	971	0.04	0.62	0.11		0.14	0.39	0.17	
β-pinene	974	0.14	0.09	0.88		0.20	1.24	4.06	0.14
3-(methylthio)-1-propanol	979				0.01				
1,2,4-trimethylbenzene	986		0.20						
β-myrcene	989			1.04					
myrcene	989	0.08	0.13			0.27	0.50	1.10	0.90

Component	RI[a]	*Pimpinella anisum*	*Artemisia vulgaris*	*Foeniculum vulgare*	*Allium sativum*	*Lavandula angustifolia*	*Mentha piperita*	*Rosmarinus officinalis*	*Salvia officinalis*
α-phellandrene	1002	0.29 ±	0.08	2.68				0.34	0.09
δ-3-carene	1010	0.16		0.28				0.35	0.09
1,2,3-trimethylbenzene	1014		0.20						
α-terpinene	1014			0.36				1.79	0.67
p-cymene	1014	0.04	0.96	1.70		0.27			2.23
m-cymene	1018	0.10							
ortho-cymene	1019							4.18	
β-phellandrene	1025	0.32	0.14						
limonene	1026	1.13		30.84			6.11		
eucalyptol	1026		4.09			5.51		52.58	19.08
(Z)-β-ocimene	1024			0.50		0.63			
cis-β-ocimene	1033							0.07	
(E)-β-ocimene	1044					0.31			
allyl isopropyl disulfide	1050				0.16				
γ-terpinene	1054	0.16	0.29	0.17		0.10		0.84	1.26
trans-Sabinene hydrate	1063					0.11			
1-(Methylthio)-3-pentanone	1067				0.01				
linalool oxyde	1069					0.06			
fenchone	1075			9.88					
diallyl disulfide	1080				29.66				
terpinolene	1085	0.05	0.14	0.20		0.07		0.18	0.41
unknown 2	1089		1.10						
allyl (Z)-1-propenyl disulfide	1093				0.22				
α-thujone	1093		27.13						16.18
linalool	1094	1.84		0.59		38.13		0.43	
allyl (E)-1-propenyl disulfide	1099				0.01				
β-thujone	1103		13.25						4.42
chrysanthenone	1109		6.12						
(Z)-β-terpineol	1118		0.14						
camphor	1129	0.03	21.45	0.1±		6.68		11.84	12.64
trans-pinocarveol	1135		0.93						

Component	RI[a]	*Pimpinella anisum*	*Artemisia vulgaris*	*Foeniculum vulgare*	*Allium sativum*	*Lavandula angustifolia*	*Mentha piperita*	*Rosmarinus officinalis*	*Salvia officinalis*
unknown 3	1135							0.07	
allyl methyl trisulfide	1138				3.22				
menthone	1140						18.47		
pinocarvone	1149		0.18						
isomenthone	1149						6.78		
isoborneol	1153					0.38			
4-methyl-1,2,3-trithiolane	1156				0,95				
menthofurane	1156						1.02		
unknown 4	1158							0.18	
(-) neomenthol	1159						4.89		
borneol	1161		0.66			2.25		1.45	2.59
methyl (E)-1-propenyl trisulfide	1166				0.01				
(-) menthol	1170						38.63		
terpinene-4-ol	1171	0.14	0.66	0.13		1.67		0.42	
4,5-dimethyl-2-propylthiazole	1174				0.01				
hexyl butyrate	1181					0.39			
α-terpineol	1184	0.11				0.26	0.58	0.93	
estragol	1187	3.88		2.40					
3-vinyl-4H-1,2-dithiine	1189				0.03				
α-phellandrene epoxide	1195			0.18					
4H-1,2,3-trithiine	1200				0,03				
allicin (diallyl thiosulfinate)	1208				0,02				
2 vinyl-4H-1,3-dithiine	1215				0,05				
pulegone	1224						1.10		
4,5-dimethyl-2-butylthiazole	1226				0.07				
s-(+)carvone	1226						0.68		
p-anisaldehyde	1235	0.52		0.51					
piperitone	1237						0.85		
(Z)-anethole	1243	0.23		0.21					
linalyl acetate	1252					33.42			
chrysantenyl acetate	1256		4.68						

Component	RI[a]	*Pimpinella anisum*	*Artemisia vulgaris*	*Foeniculum vulgare*	*Allium sativum*	*Lavandula angustifolia*	*Mentha piperita*	*Rosmarinus officinalis*	*Salvia officinalis*
allyl isoproyl trisulfide	1266				0.06				
isomenthyl acetate	1270						0.15		
(E)-anethole	1274	86.54		40.58			2.97		
4-(hydroxymethyl)-1,2-dithiepane	1278				0.01				
(-)-bornyl acetate	1279							0.44	1.11
isobornyl acetate	1279.5		0.44						
lavandulyl acetate	1282					1.11 ±			
sabinyl acetate	1283		0.25						
4-methyl-1,2,5-trithiepane	1285				0.05				
diallyl trisulfide	1303				21,50				
allyl propyl trisulfide	1314				0,12				
(E)-3,5-diethyl-1,2,4-trithiolane	1342				0.05				
δ-elemene	1343					0.03			
eugenol	1346			1.03					
allyl methyl tetrasulfide	1357				0,01				
5-methyl-1,2,3,4-tetrathiane	1367				0,06				
geranyl acetate	1371					0.19			
(Z)-3,5-diethyl-1,2,4-trithiolane	1374				0.29				
eucarvone	1376		1.01						
α-ylangene	1377							0.04	
(-)-α-copaene	1381					0.11			
2-heptyl thiophene	1381				1.32				
α-copaene	1382	0.05	0.18					0.14	
daucene	1386					0.06			
β-bourbonene	1389						0.24		
β-elemene	1395						0.13		
unknown 5	1407					0.05			
longifolene	1412							0.11 ±	0.42
α-cedrene	1418					0.03			
unknown 6	1419	0.05							

Component	RI[a]	*Pimpinella anisum*	*Artemisia vulgaris*	*Foeniculum vulgare*	*Allium sativum*	*Lavandula angustifolia*	*Mentha piperita*	*Rosmarinus officinalis*	*Salvia officinalis*
β-caryophyllene	1425	0.30	0.19			2.16	1.25	1.90	3.49
3,6-dimethyl-1,2,5-trithiepane	1428				0.02				
β-cubebene	1433						0.07		
unknown 7	1439					0.20			
α-bergamotene	1439	0.32	0.15						
4-ethyl-2,3,5-trithia-6-octene	1444				0.33				
β-farnesene	1454	0.02				0.58			
α-humulene	1459					0.24	0.09		3.73
4,6-dimethyl-1,2,5-trithiepane	1460				0.06				
α-elemene	1474					0.05			
γ-muurolene	1479					0.06	0.03		
germacrene D	1484		0.37			0.36	0.42		
δ-guaiene	1485								0.04
germacrene B	1501		0.06				0.11		
α-farnesene	1503	0.05							
α-bisabolene	1508	0.06				0.13			
β-cadinene	1511							0.01	
γ-muurolene	1515	0.01							
γ-cadinene	1515					0.14	0.03		
β-sesquiphellandrene	1521					0.13			
δ-cadinene	1524	0.05					0.08	0.20	
diallyl tetrasulfide	1544				13,19				
(E)-nerolidol	1558	0.06							
allyl methyl pentasulfide	1573				0.01				
(+) spathulenol	1579						0.03		
caryophyllene oxyde	1582					0.10	0.07	0.11	0.10
7-methyl-4,5,8-trithia-1,10-undecadiene	1583				0,33				
4-ethyl-6-methyl-1,2,3,5-tetrathiolane	1588				0,05				
6-methyl-4,5,8-trithia-1,10-undecadiene	1592				1,21				

Component	RI[a]	*Pimpinella anisum*	*Artemisia vulgaris*	*Foeniculum vulgare*	*Allium sativum*	*Lavandula angustifolia*	*Mentha piperita*	*Rosmarinus officinalis*	*Salvia officinalis*
2-thiopheneacetamide, N-propyl-	1622				0,06				
4-methyl-1,2,3,5,6-pentathiepane	1649				0,18				
t-muurolol	1654	0.06							
6-ethyl-4,5,7,8-tetrathianonane	1658				0,39				
2-thiopheneacetamide, N-isobutyl-	1663				0,24				
foeniculin	1666	1.06		0.46					
α-bisabolol	1678					0.12			
hexathiepane	1680				0,29				
diallyl pentasulfide	1755				1,03				
allyl methyl hexasulfide	1781				0,47				
8-methyl-4,5,6,9-tetrathia-1,11-dodecadiene	1815				5,54				
diallyl hexasulfide	1897				0,22				
allyl methyl heptasulfide	1922				0,16				
2-methyl-1,3-benzothiazole	1957				0,36				
5-ethyl-7-pentyl-1,2,3,4,6-pentathiepane	2005				0,32				
cyclooctasulfur	2044				0,08				
9-methyl-4,5,6,7,10-pentathia-1,12-tridecadiene	2051				0,68				
8-methyl-4,5,6,7,10-pentathia-1,12-tridecadiene	2056				0,68				
Monoterpene		**3.08**	**7.24**	**41.37**		**2.64**	**9.27**	**27.68**	**33.45**
Oxygenated monoterpene		**2.12**	**82.52**	**10.92**		**89.87**	**80.43**	**68.34**	**56.19**
Sesquiterpene		**0.91**	**0.61**	**0.34**		**4.33**	**2.6**	**2.4**	**7.68**
Oxygenated sesquiterpene		**0.12**				**0.22**	**0.1**	**0.11**	**0.10**
Phenylpropene		**91.93**		**45.19**			**2.97**		
Ester						**0.39**			
Sulphur compounds					**99.36**				
Aromatic hydrocarbon			**0.40**						
Total		**98.16**	**90.77**	**97.82**	**99,68**	**97.45**	**95.37**	**98. 53**	**97.42**

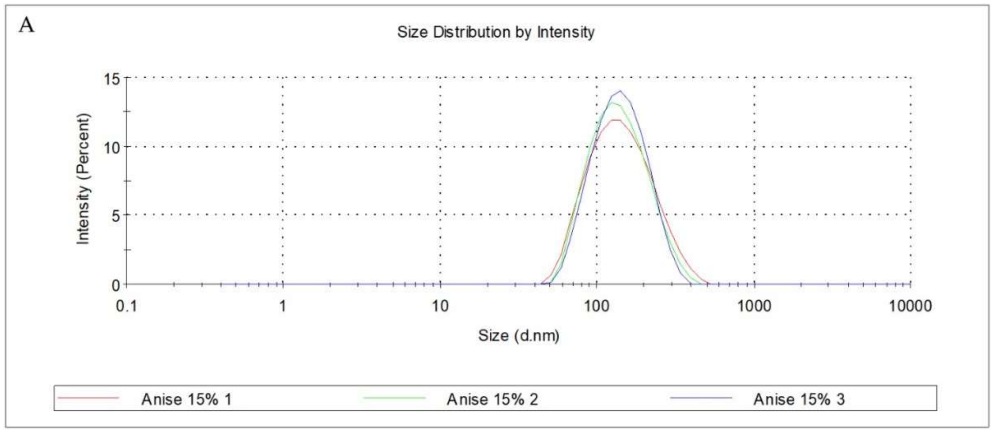
3.2. Qualitative analysis of nanoemulsions

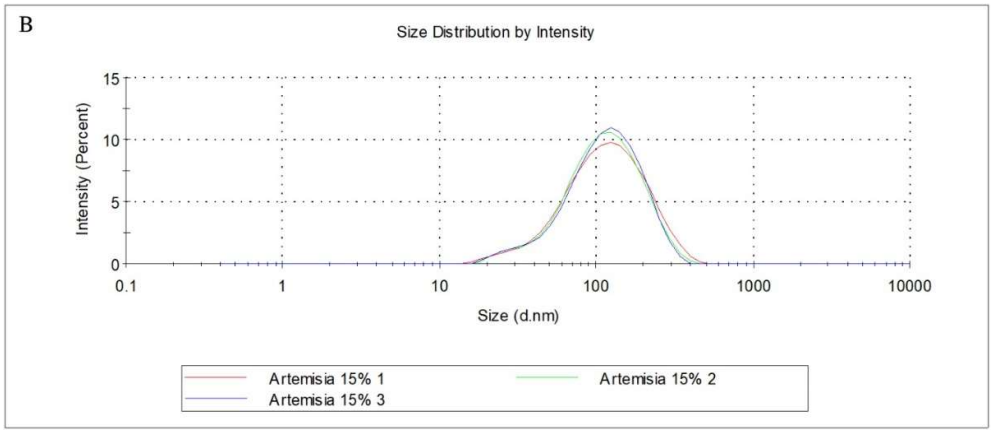
Overall, all the developed formulations presented an average micelle size within the nanoscale range (95.01-144.3 nm), a low polydispersity index (0.146-0.248 PdI) and a negative surface charge (10.81-23.8 mV). All the results related to the physical characteristics of the developed nanoemulsions are reported in the table 6.

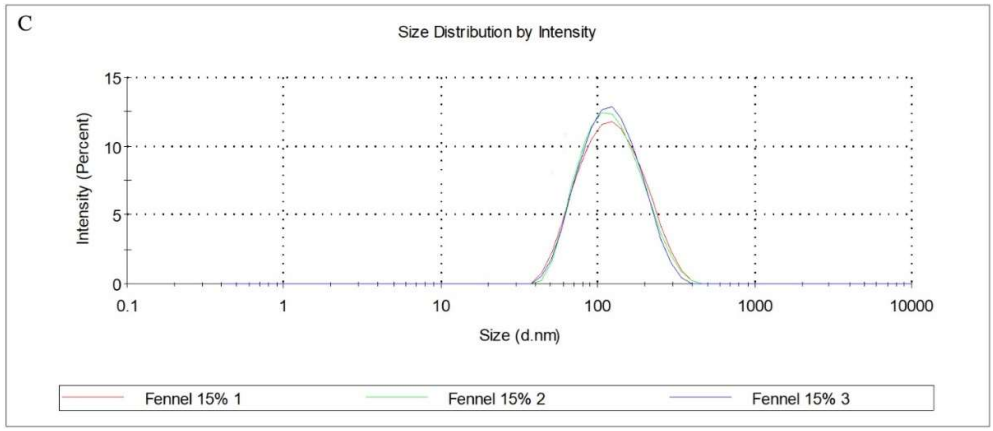
Table 6 Dynamic Light Scattering (DLS) analyses of EOs nanoemulsion. Three replicates were provided for each test. PDI=Polydispersity index; SE=standard error

Essential oils	**Z-average size ± SE (nm)**	**PDI ± SE**	**Zeta potential ± SE (mV)**
Anise	128.23 ±0.37	0.146 ±0.012	-23.8 ±0.27
Artemisia	95.01 ±0.033	0.240 ±0.005	-10.81 ±0.74
Fennel	111.3 ±0.21	0.154 ±0.005	-16.5 ±0.35
Garlic	144.3 ±0.15	0.164 ±0.008	-23.67 ±0.23
Lavender	121.17 ±0.58	0.172 ±0.005	-11.6 ±0.06
Mint	141.53 ±0.26	0.189 ±0.009	-18.4 ±0.76
Rosemary	138.13 ±0.66	0.248 ±0.004	-22.3 ±0.21
Sage	124.87 ±0.09	0.181 ±0.006	-13.27 ±0.20

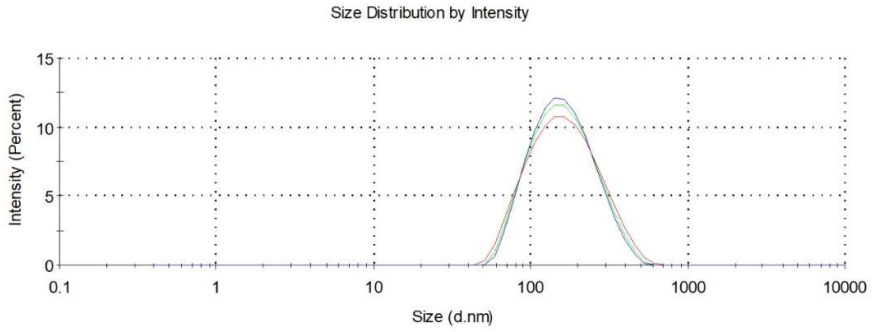
Figure 4 Dimensional values for formulations based on essential oil of anise(A), artemisia(B), fennel(C), garlic(D), lavender(E), mint(F), rosemary(G) and sage(H)





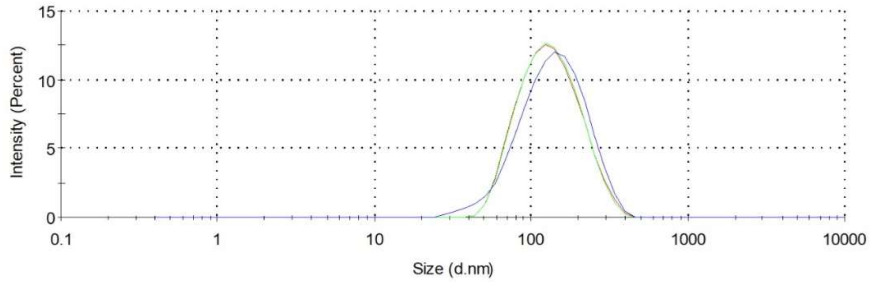


D



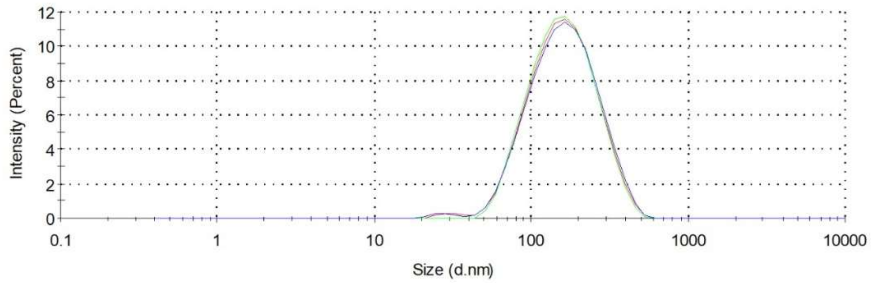
Garlic 15% 1 Garlic 15% 2 Garlic 15% 3

E



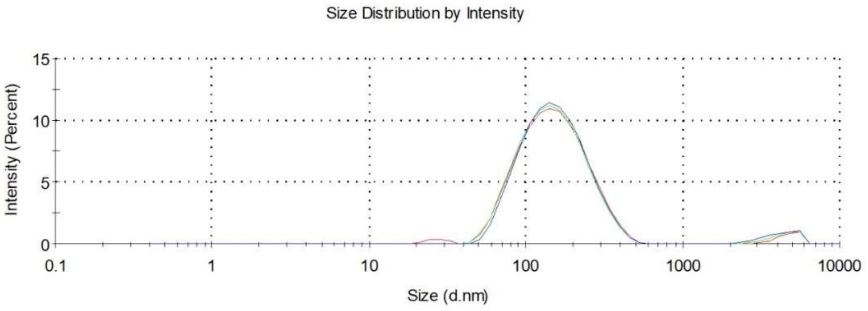
Lavender 15% 1 Lavender 15% 2 Lavender 15% 3

F



Mint 15% 1 Mint 15% 2 Mint 15% 3

G


Size Distribution by Intensity
Intensity (Percent)
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
Rosemary 15% 1
Rosemary 15% 2
Rosemary 15% 3

H

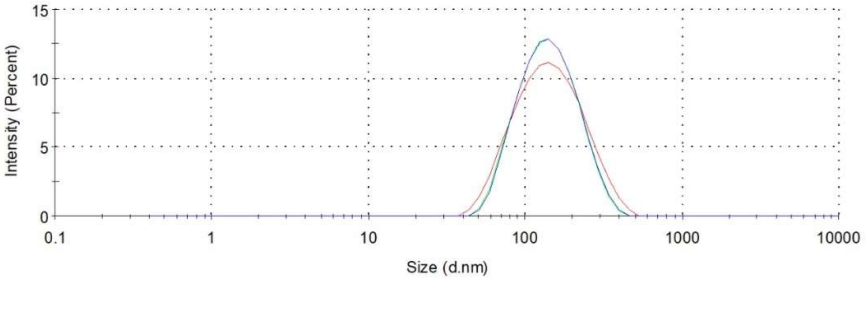
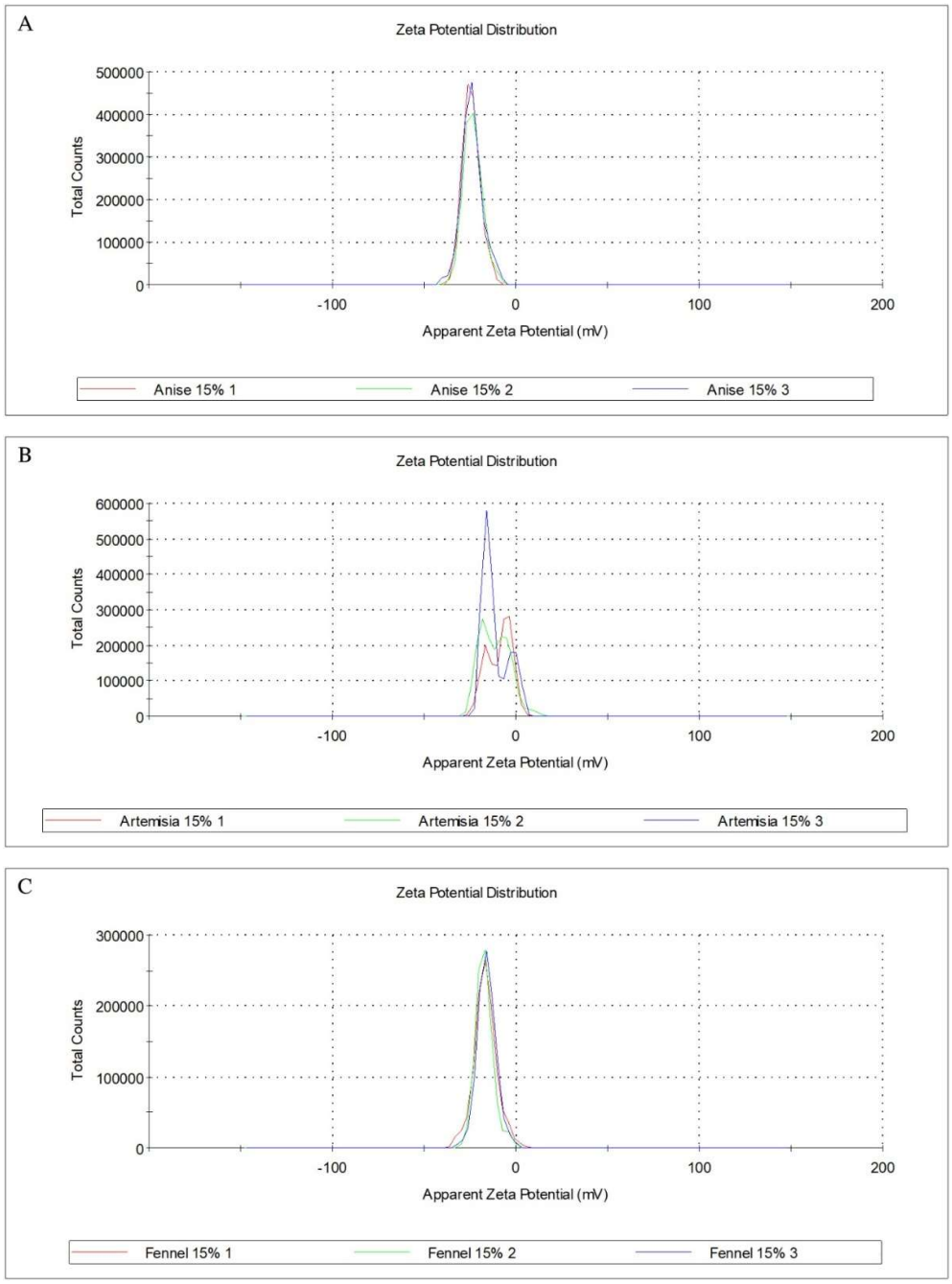
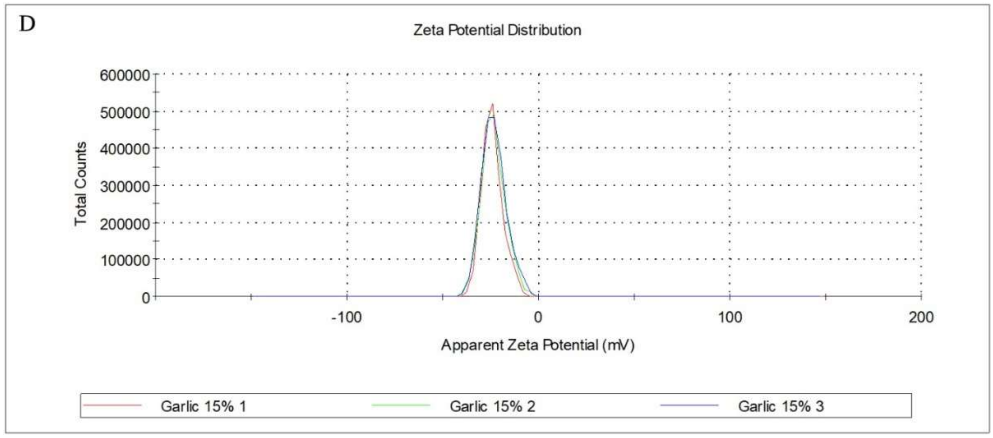
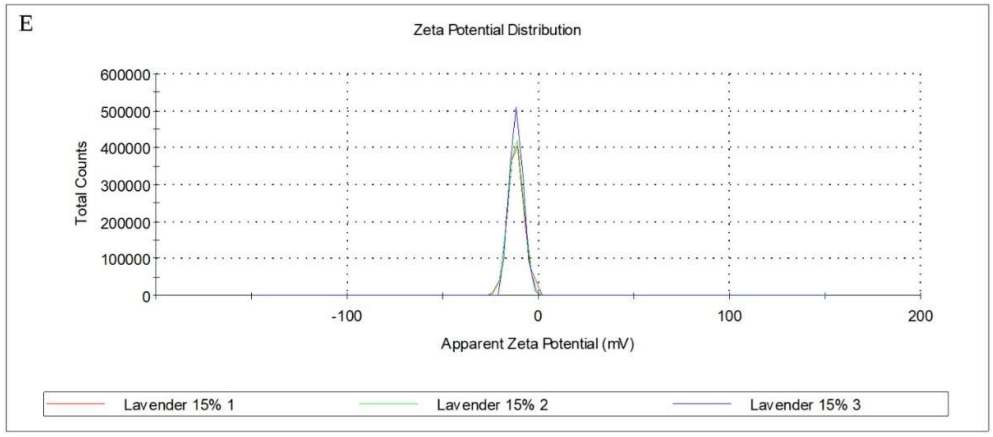
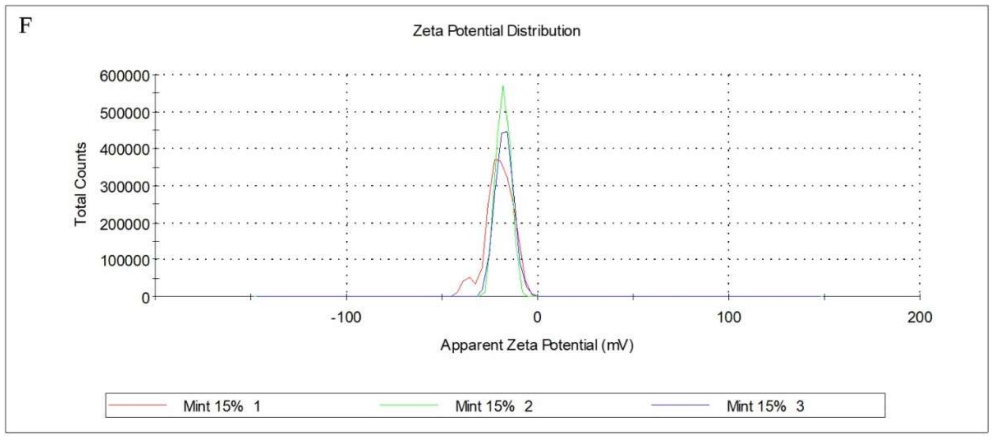

Size Distribution by Intensity
Intensity (Percent)
15
10
5
0
0.1
1
10
100
1000
10000
Size (d.nm)
Sage 15% 1
Sage 15% 2
Sage 15% 3

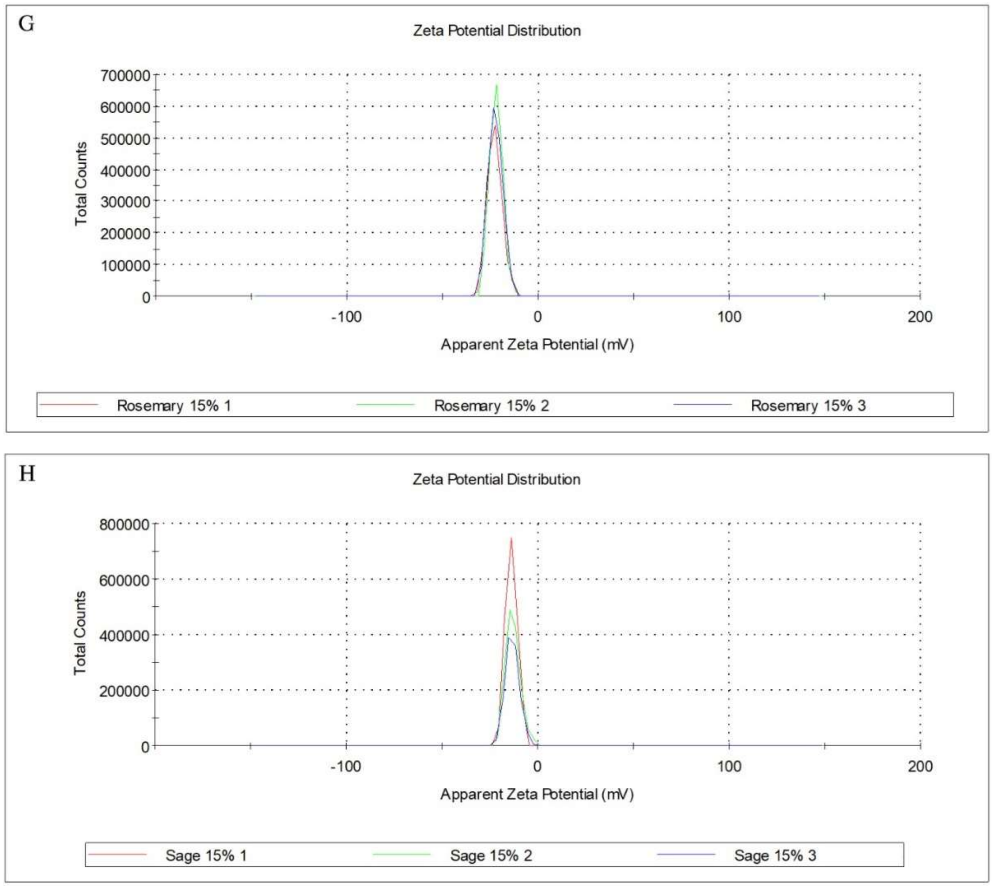
Figure 5 ζ potential for essential oil formulations based on anise(A), artemisia(B), fennel(C), garlic(D), lavender(E), mint(F), rosemary(G) and sage(H)




D
Zeta Potential Distribution
Total Counts
600000
500000
400000
300000
200000
100000
0
-100
0
100
200
Apparent Zeta Potential (mV)
Garlic 15% 1
Garlic 15% 2
Garlic 15% 3


E
Zeta Potential Distribution
Total Counts
600000
500000
400000
300000
200000
100000
0
-100
0
100
200
Apparent Zeta Potential (mV)
Lavender 15% 1
Lavender 15% 2
Lavender 15% 3


F
Zeta Potential Distribution
Total Counts
600000
500000
400000
300000
200000
100000
0
-100
0
100
200
Apparent Zeta Potential (mV)
Mint 15% 1
Mint 15% 2
Mint 15% 3



Among the analysed EO-based nanoemulsions, the anise one had an average size of the droplets of 128.23±0.37 nm; the low values of the polydispersity index (0.146 ±0.012) indicated the size homogeneity of the formulation since few or no aggregates were detected (Figure 4 A). The surface charge (ζ) recorded for this formulation was -23.8±0.27 mV. Artemisia EO-based nanoemulsion had an average size of the droplets of 95.01 ±0.033 nm and among the developed nanoformulations, this one presented the smallest droplet dimensions. Conversely, the polydispersity index (0.240 ±0.005), despite presented good values in terms of size homogeneity, was one of the highest recorded. In any cases no aggregates were detected (Figure 4 B). Also, Artemisia EO-based nanoemulsion exhibited a negative surface charge (ζ) of -10.81 ±0.74 mV.

Fennel EO-based nanoemulsion presented an average size of the droplets of 111.3±0.03 nm and a low polydispersity index (0.154±0.005) indicating the size

distribution homogeneity of the formulation. The surface charge (ζ) reached the value -16.5 ±0.35 mV.

Among the developed formulations, the biggest size of the droplets was recorded in Garlic EO-based nanoemulsion (144.3±0.15 nm) despite the low values of the polydispersity index (0.164 ±0.008) and the high negative surface charge (ζ) (-23.67 ±0.23 mV) guaranteed a good homogeneity and stability, respectively.

The size of the droplets recorded in the Lavender EO-based nanoemulsion was in line with the other formulations developed with Labiatae EOs. In details, the droplet size was 121.17±0.58 nm, whereas the polydispersity index and the surface charge (ζ) were 0.172 ±0.005 and 11.6 ±0.06 mV respectively.

Among the EOs extracted by Labiatae plants, the mint one used to develop the nano-formulation presented the highest size of the droplets (141.53±0.26 nm) whereas both the polydispersity index (0.189 ±0.009) and the negative surface charge (ζ) (-18.4 ±0.76 mV) highlighted a low inhomogeneity and a good stability of the developed formulation. Rosemary EO-based nanoemulsion presented a size of the droplets of 138.13 ±0.66 whereas this formulation was the worst in terms of polydispersity index (0.248±0.004). Finally, Sage EO-based nanoemulsion had a size of the droplets of (124.87 ±0.09 nm), polydispersity index of 0.18±0.006 a negative surface charge (ζ) of -13.27 ±0.20 mV.

The results from the qualitative analyses demonstrated that stable EO-based nanoemulsions with high relative amount of EO (i.e., 15%) were developed using a mixed bottom up/top down process (Donsì and Ferrari, 2016). Several recent studies demonstrated that for nanoemulsions the droplet size decreases with the decrease of the oil:surfactant ratio and that high amount of surfactant could guarantee small droplets' dimension (Gulotta *et al.*, 2014; Li *et al.*, 2017; Saberi *et al.*, 2013; Wang *et al.*, 2009). However, for insecticide formulations, the use of large amounts of surfactants could have negative effect on plants and food (Mirgorodskaya *et al.*, 2020; Niedobová *et al.*, 2019). The chosen surfactant, Tween 80, was able to produce the small droplets. Chang *et al.* (2013) reported differences in the surfactants used for nanoemulsions and obtained the smallest droplets in carvacrol-based nanoemulsions made with a mix of food-grade non-ionic surfactants (Tween 20, 40, 60, 80, and 85). Tweens belong to a class of non-ionic

surfactants derived from sorbitan esters, which are soluble or dispersible in water and are common oil-in-water emulsifiers. Among these surfactants, Tween 80 is one of the most commonly used to develop EO-based insecticides in literature.

Nevertheless, generally the self-emulsifying process alone did not produce nanoemulsions with small droplet size (Lombardo *et al.*, 2020), thus sonication is a viable solution to reduce the dimensions of the micelles and reduce PDI values (Donsì and Ferrari, 2016; Campolo *et al.*, 2020). As an example, ultrasonication for short period (i.e., 3 min) greatly decreased droplet size (coarse: 1420.5 ± 111.0 nm; sonicated: 221.3 ± 0.8 nm) and increased homogeneity (coarse: 0.709 ± 0.04 PDI; sonicated: 0.251 ± 0.00 PDI) when applied to oregano EO nanoemulsion (EO=10%; Tween 80 & lecithin=10%, ratio 3:1) (Lee *et al.*, 2019).

Nanoemulsion qualitative characteristics are considered to mainly depend on the plant-source, since every EO-based formulation showed characteristic values. The good quality of all the developed nano-formulations was supported by the low PDI values (0.15-0.25) recorded. However, the best results were obtained with artemisia EO, whose nanoemulsion was characterized by a size of 95.01 ±0.03 nm, while results for garlic EO-based nanoemulsion presented the highest droplet size (144.3 ±0.15). These differences can be related to the chemical composition of EOs; the molecular weight, polarity and conformation of volatile compounds contained in the EO, as well as the presence of surface-active substances, can alter their water solubility and their capacity to form droplets with Tween 80 (Acevedo-Fani *et al.*, 2015; Ziani *et al.*, 2012). Artemisia EO is mainly constituted of oxygenated monoterpenes, while composition of garlic EO is mainly based on organosulfur compounds.

The droplet dimensions obtained in the present study are consistent with results from previous studies on EO-based nanoemulsions containing lower oil:surfactant ratio (Hashem *et al.*, 2018; Lee *et al.*, 2019; Moghimi *et al.*, 2016; Werdin González *et al.*, 2014). As example, Adak *et al.* (2020) formulated eucalyptus EO nanoemulsions containing from 6 to 10% of EO and EO:Tween 80 ratios ranging from 1:05 to 1:1.5 obtaining dimensions comprised between 150-400 nm. However, when using EO:surfactant ratio similar to those applied in the current study (i.e. around 1:0.3), the authors obtained higher droplet sizes (429.6 nm -318.0

nm), as well as higher PDI values (0.490 - 0.331) for the emulsions containing 8 and 10% of eucalyptus EO, respectively (Adak *et al.*, 2020). High PDI values (around 0.3) were also noted for geranium EO-emulsions [oil:surfactant (i.e. Tween 80) ratios of 1:05 to 1:1], which showed dispersed phase diameters of 79 to 106 nm, respectively, but that were not stable just after 3 days (Jesser *et al.*, 2020).

The developed nanoemulsion remained stable mainly to steric repulsion, considering that Tween 80 is a non-ionic surfactant (Babchin and Schramm, 2012). The presence of a negative surface charges, which depends on the composition of oil, the pH and the electrolytes present in the water phase, may help to stabilize the nanoemulsions (Müller *et al.*, 2001; Tadros *et al.*, 2004). In literature, several EO-based nanoemulsions prepared with non-ionic surfactant presented negative surface charge (Acedo-Carrillo *et al.*, 2006; Fernandes *et al.*, 2014; Giunti *et al.*, 2019; Hashem *et al.*, 2018; Salvia-Trujillo *et al.*, 2015). The negative surface charge of EO-based nanoemulsions could be attributed to the dissociation of ionizable compound of the oils, which can be adsorbed on the droplet surface by the surfactant (Bonilla *et al.*, 2012; Ge and Ge, 2016; Stachurski and Michalek, 1996). Furthermore, also the "incomplete" coverage of the oil core of the droplets by the surfactant can play a role (Li *et al.*, 2016; Martins *et al.*, 2012; Zhao *et al.*, 2010). Hsu and Nacu (2003) demonstrated that an increasing concentration of non-ionic surfactant leads to ζ potential of the nanoemulsion nearest to zero, due to the highest coverage of the droplet surface by the non-charged surfactant molecules.

Results from this study highlighted that nanoemulsions are perfect candidates for several applications including pest control.

3.3. Repellence of gels against *T. confusum*

PR values were calculated to assess the efficacy of the treatment. The probit analysis was performed for all the tested EO and according to Pearson Goodness of fit test, PR values from repellence trials fitted the probit curve at 24h (Table 7) and 48h (Table 7). Regarding of the EO nano-formulation used, the anise EO gel showed the highest repellence activity, while lavender one was the less effective to repel *T. confusum* adults. No differences between the PR values recorded at 24h

and 48h were noted within the same treatment (Table 7), suggesting that the repellent activity of the developed EO-base gels can last and remain stable over time (Figure 6-13).

Results highlighted the good repellent activity against *T. confusum* of the majority of the developed nanoemulsions. Except for lavender EO nano-formulations, the RC_{50} values calculated for the others nanoemulsions were very low, compared also to results reported in literature. Unfortunately, when testing the repellence activity, many authors did not calculate RD_{50} values, causing criticisms when comparing the outcomes between different EOs (Campolo *et al.*, 2018). Nevertheless, regarding *T. confusum* the most interesting results were obtained with Pistacia lentiscus (Anacardiaceae) EO with RD_{50}=0.025 µL/cm2 after 24 h, in area preference bioassays in petri dish arena (Bougherra *et al.*, 2015). The results accounted RC_{50} values calculated in different experimental design, in which the repellent was not placed directly on the "walking surface" directly in contact with the insect. Here, gel dispensers were developed to avoid the direct contact of insect with the repellents. This approach was intended to simulate the treatment application in real conditions using dispensers.

Furthermore, this trial aimed to evaluate repellence for longer durations. Usually, repellence of botanicals is assessed using the classic Petri dish arena for area preference and data are collected just after few hours of exposition (Campolo *et al.*, 2018). Indeed, EOs are very volatile and their persistence is very low under this kind of conditions. The formulation of EOs in nanosystems can alter the repellent efficacy of the raw extracts; as an example, *R. dominica* adults were repelled more by the pure EOs than by PEG-EO nanoparticles when used as antifeedants, while for *T. confusum* the PEG nano-formulation improved the deterrent activity of EOs (Werdin González *et al.*, 2014). Indeed, nano-dimensions generally enhance the gradual release of the active compounds (de Oliveira *et al.*, 2014), which, at low concentrations, may not be repellent for target species. Gradual release, thus, can present also adverse effects; nevertheless, the results reported in this study suggested that a more gradual release of EO active principles can boost their efficacy over time.

Table 7 Estimated median repellence concentrations (RC_{50}) of the various EOs nano-formulations on T. confusum adults in the repellency bioassays. Different letters within the same column indicate statistical differences ($p<0.05$).

Essential Oils	**Time**	**RC_{50} (mg)**	**95% fiducial limits**	**Slope+-SE**	**Intercept+-SE**	***χ2* (DF)**	***P***
Anise	24h	0.005a	0.028-0.064	1.246±0.113	1.712±0.165	7.412(4)	0.116
Anise	48h	0.003a	0.024-0.043	0.873±0.106	1.297±0.157	5.509(4)	0.239
Artemisia	24h	1.622c	0.535-3.820	0.977±0.140	1.360±0.146	7.346(3)	0.068
Artemisia	48h	1.262c	1.856-2.794	-0.205±0.069	-0.482±0.073	2.394(3)	0.495
Fennel	24h	0.158b	0.092-0.239	1.475±0.151	1.180±0.119	5.501(3)	0.139
Fennel	48h	0.177b	0.130-0.221	1,471±0.208	1,107±0.129	3.452(2)	0.178
Garlic	24h	0.055a	0.038-0.074	0.787±0.105	0,992±0.127	6.447(4)	0.168
Garlic	48h	0.095ab	0.046-0.212	0.698±0.104	0,715±0.123	6.950(4)	0.139
Lavender	24h	15.389d	11.729-19.683	0.985±0.108	-1,169±0.151	0.658(4)	0.956
Lavender	48h	19.625d	15.130-24.457	1,229±.145	-1,589±0.218	4.555(3)	0.207
Mint	24h	1.083bc	0.205-0.882	0.658±0.139	0.175±0.067	3.216(3)	0.359
Mint	48h	1.601c	0.387-1.215	0.667±0.137	0.064±0.067	4.520(3)	0.211
Rosemary	24h	0.577c	0.249-0.968	1,271±0.147	0.303±0.061	6.633(3)	0.085
Rosemary	48h	0.816c	0.435-1.397	1,334±0.146	0.118±0.060	6.803(3)	0.078
Sage	24h	0.719c	0.370-1.144	1,143±0.112	0.164±0.056	9.066(4)	0.059
Sage	48h	1.985c	1.263-3.510	1,194±0.113	-0.355±0.058	9.264(4)	0.055

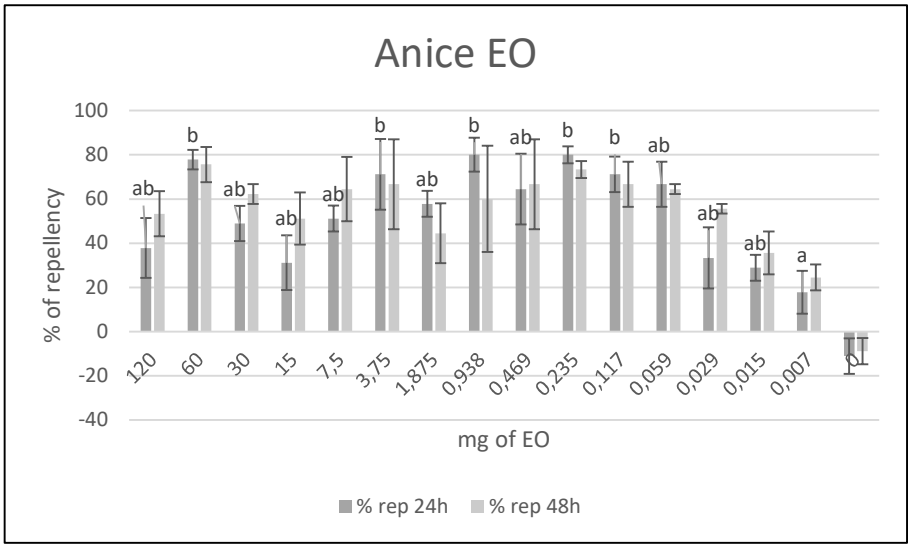


Figure 6 Repellency (%) in T. confusum after exposure (24 h and 48h at 25 °C) to vapours (mg of EO) of anise EO nanoemulsion. Percentages of repellency refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

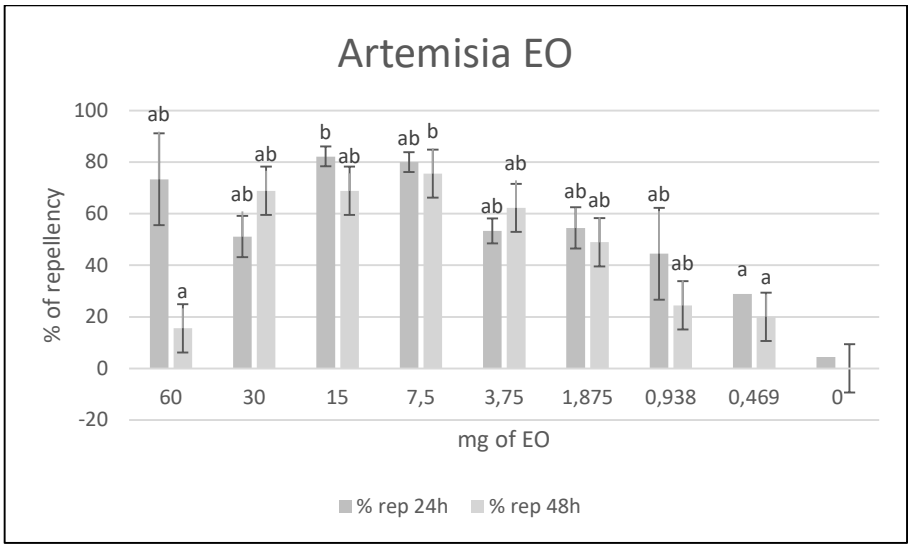


Figure 7 Repellency (%) in T. confusum after exposure (24 h and 48h at 25 °C) to vapours (mg of EO) of artemisia EO nanoemulsion. Percentages of repellency refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

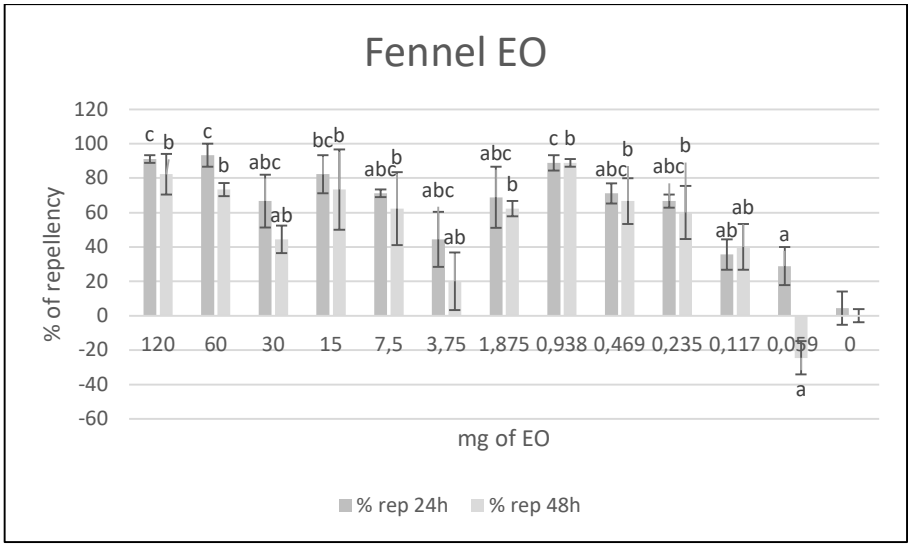


Figure 8 Repellency (%) in T. confusum after exposure (24 h and 48h at 25 °C) to vapours (mg of EO) of fennel EO nanoemulsion. Percentages of repellency refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

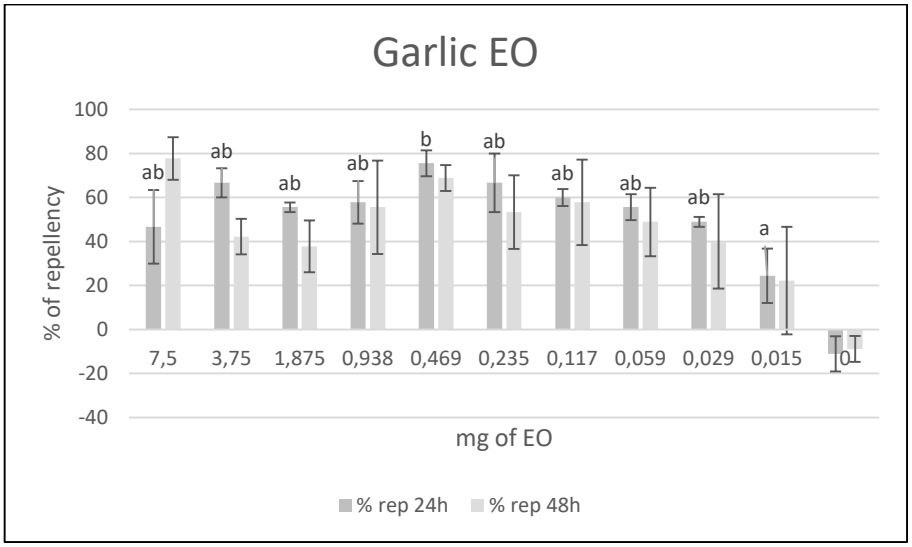


Figure 9 Repellency (%) in T. confusum after exposure (24 h and 48h at 25 °C) to vapours (mg of EO) of garlic EO nanoemulsion. Percentages of repellency refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

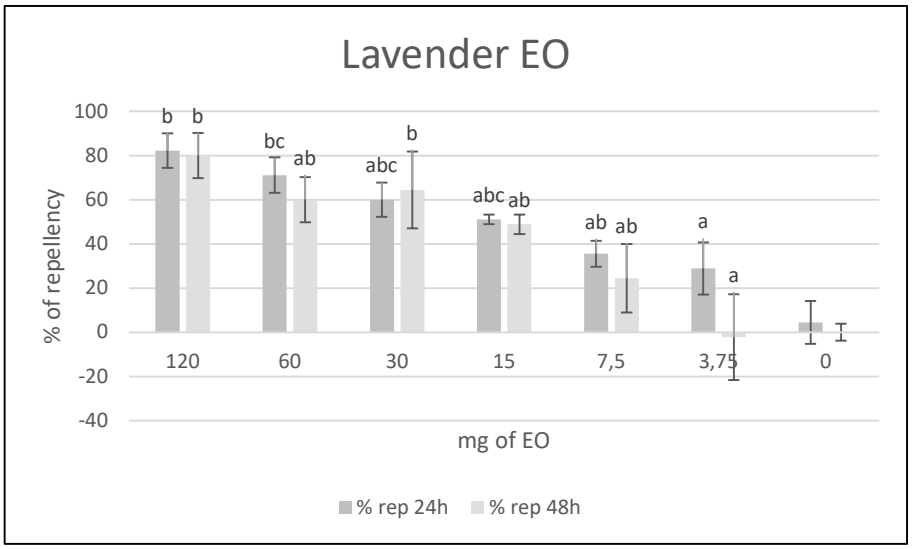


Figure 10 Repellency (%) in T. confusum after exposure (24 h and 48h at 25 °C) to vapours (mg of EO) of lavender EO nanoemulsion. Percentages of repellency refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

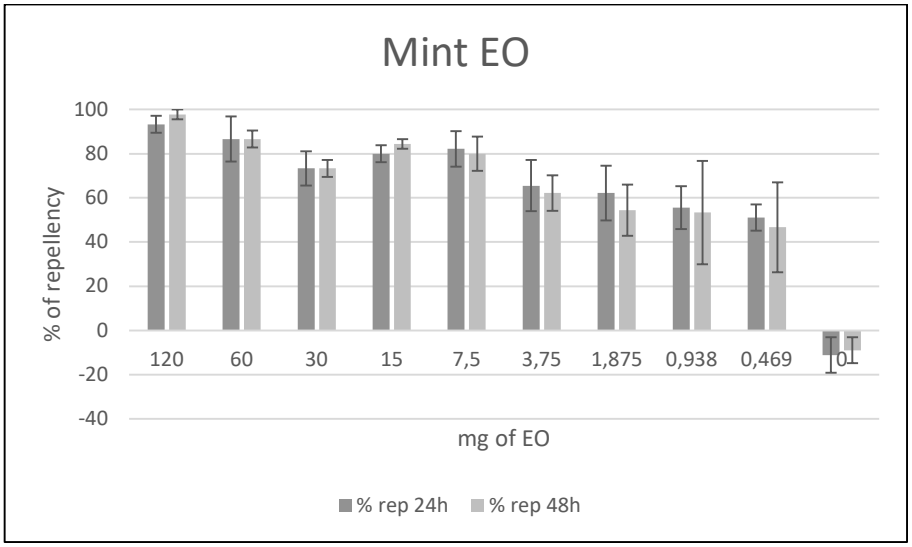


Figure 11 Repellency (%) in T. confusum after exposure (24 h and 48h at 25 °C) to vapours (mg of EO) of mint EO nanoemulsion. Percentages of repellency refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

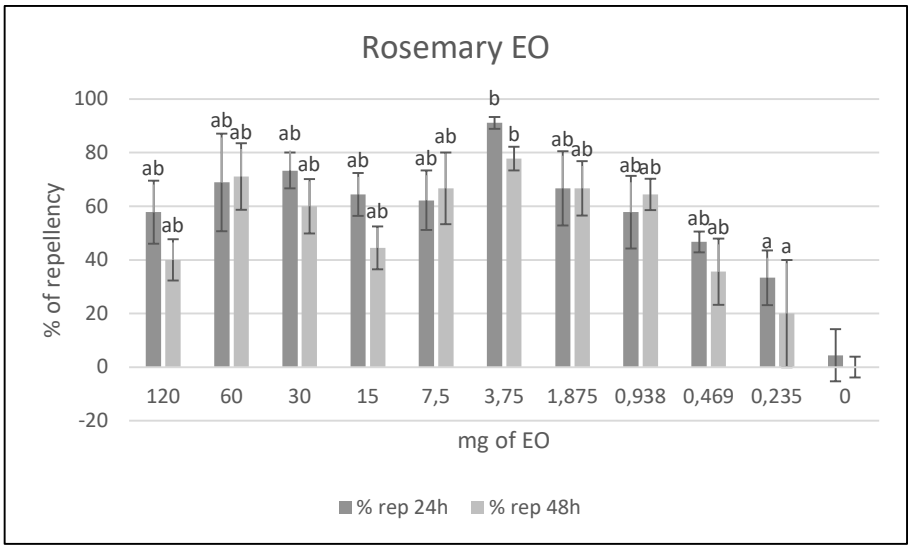


Figure 12 Repellency (%) in T. confusum after exposure (24 h and 48h at 25 °C) to vapours (mg of EO) of rosemary EO nanoemulsion. Percentages of repellency refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

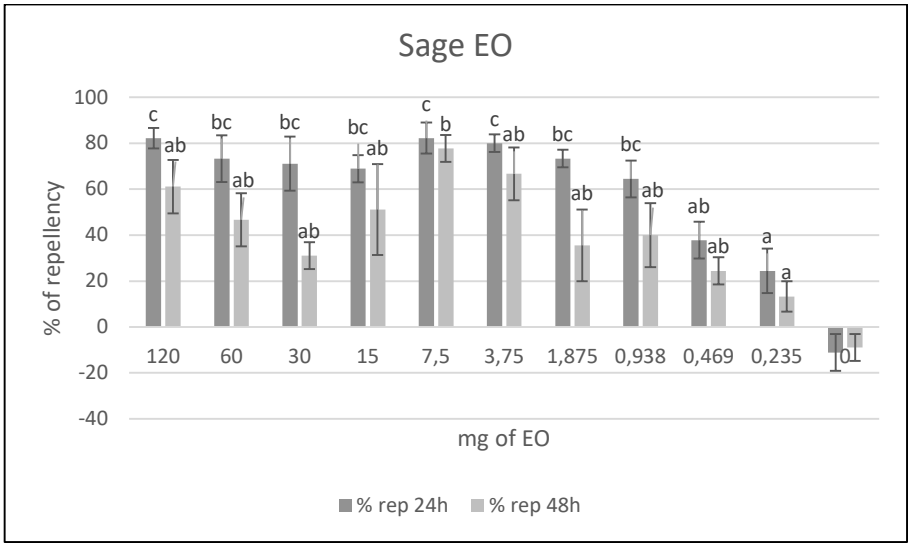


Figure 13 Repellency (%) in T. confusum after exposure (24 h and 48h at 25 °C) to vapours (mg of EO) of sage EO nanoemulsion. Percentages of repellency refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

3.4. Acute toxicity by cold aerosol against *T. confusum*

Mortality values from cold aerosol trials fitted the probit curve (Pearson Goodness of fit test) both at 24h Table 8) and 1 week (Table 8).

Results show that the majority of tested EO can exert an immediate acute toxicity, which cannot increase after 24h (Figure 14-21). In contrast, for three EO-based nanoemulsions, artemisia, mint and sage, the LC_{50} values recorded after 1 week from the exposure were significantly lower respect to those reported just after 24h (Table 8). Thus, for these EO nano-formulation, beside the immediate acute toxicity, an additive toxicity can be noted, suggesting that a delayed mortality induced by the nano-emulsion occurred. In cold aerosol trials against *T. confusum* adults, garlic EO nanoemulsion showed the highest toxic activity, whereas artemisia the lowest one.

The developed nanoemulsion show interesting insecticidal activity against *T, confusum* applied as cold aerosol. Previous research on cold aerosol toxicity of sweet orange (*Citrus sinsnesis*) EO nanoemulsion against the same pest species reported higher LC_{50} values (5.43 mg/mL after 24h) respect to those reported in the present study (Giunti *et al.* 2019); in detail the garlic nanoemulsion were 10-fold more toxic against *T. confusum* adults compared to citrus EO nanoemulsion. The

high efficacy of cold aerosol treatment could be attributable to the different kind of application of the nano-emulsion because, when applied as cold aerosol, EOs could act both as fumigant and contact insecticide. Moreover, mortality recorded in cold aerosol trials is not attributable to Tween 80 or to water saturation of the cage, as previously reported by Giunti *et al.* (2019). Comparing the results from this study with the ones reported for commercial fogging agents, the EO nano-emulsions presented promising insecticidal activity against *T. confusum*. As an example, pyrethrin aerosol (23.4 g formulation/28 m^3 of headspace area) against *T. confusum* determined a mortality rate of 38.9%±4.3 after 7 days from exposure, increasing at 84.8%±3.2 after 14 days (Arthur, 2008).

The toxic activity of EOs against several stored product pests have been demonstrated form many insect species, including the beetle *T. confusum* (for a dedicated review see Campolo *et al.*, 2018). However, the exact mode of action of EOs against this target species has not been clarified yet, but monoterpenes could be able to cause the total breakdown of the nervous system, acting on the octopaminergic system of the insects (Isman, 2000; Price and Berry, 2006). Petrović *et al.* (2019) investigated the fumigant activity of *Carum carvi* L. EO against *T. confusum*, and they noted that this EO can cause also oxidative stress by altering the antioxidative defense system, catalase (CAT), superoxide dismutase (SOD), and glutathione-S-transferase (GST) activities, as well as the level of lipid peroxidation (MDA) and the content of reduced glutathione (GSH) (Petrović *et al.*, 2019). Thus further researches are needed to understand the mechanisms underlying toxicity in this insect species.

In literature, the majority of research on stored product pests evaluated the insecticidal activity of EOs as fumigant or by their direct administration on foodstuff (Campolo *et al.*, 2018). However, these techniques are unfeasible in field operative conditions. For fumigation of EO as such, the need of sealed spaces and the homogeneous distribution in large spaces are quite limiting, while the direct use on foodstuff may alter the qualities and sensory profile of food. In contrast, the treatment of warehouses and buildings using cold aerosol techniques is quite common and easy to handle by the operators from the disinfection sector.

Table 8 Estimated median lethal concentrations (LC_{50}) of the various Eos nano-formulations on T. confusum adults in the aerosol bioassays. different letters within the same column indicate statistical differences ($p<0.05$).

Essential Oils	**Time**	**LC_{50} (mg/L)**	**95% fiducial limits**	**Slope+-SE**	**Intercept+-SE**	**$\chi 2$ (DF)**	***P***
Anise	24h	2.561b	1.988-3.239	2.284±0.159	-0.933± 0.098	6.671(4)	0.149
Anise	1w	2.099b	1.833-2.385	2.324±0.166	-0.748±0.094	5.964(4)	0.202
Artemisia	24h	7.462d	6.058-9.496	1.245±0.116	-1.087±0.095	5.617(4)	0.229
Artemisia	1w	4.069c	3.370-4.924	1.343±0.115	-0.818±0.088	2.351(4)	0.671
Fennel	24h	3.764bc	2.699-5.018	2.417±0.187	-1.392±0.139	5.782(3)	0.123
Fennel	1w	3.369bc	2.323-4.561	2.348±0.186	-1.238±0.135	6.115(3)	0.106
Garlic	24h	0.486a	0.381-0.601	1.297±0.150	0.632±.070	2.484(3)	0.478
Garlic	1w	0.325a	0.243-0.408	1.257±0.145	0.394±0.066	0.145(3)	0.986
Lavender	24h	4.476bcd	3.039-7.061	1.180±0.094	0.768±0.070	10.975(5)	0.052
Lavender	1w	2.048b	1.482-2.776	1.362±0.099	0.424±0.065	8.504(5)	0.131
Mint	24h	3.768c	3.298-4.275	2.411±0.190	-1.389±0.139	2.411(3)	0.492
Mint	1w	2.915b	2.527-3.316	2.469±0.205	1.147±0.137	3.219(3)	0.359
Rosemary	24h	6.098cd	4.666-8.651	1.087±0.117	-0.853±0.074	5.703(4)	0.222
Rosemary	1w	4.582c	3.646-6.048	1.175±0.116	-0.777±.072	6.702(4)	0.153
Sage	24h	5.782c	5.146-6.506	2.682±0.196	-2.044±0.162	4.424(3)	0.219
Sage	1w	4.119c	3.665-4.613	2.817±0.209	-1.732±0.152	2.778(3)	0.427

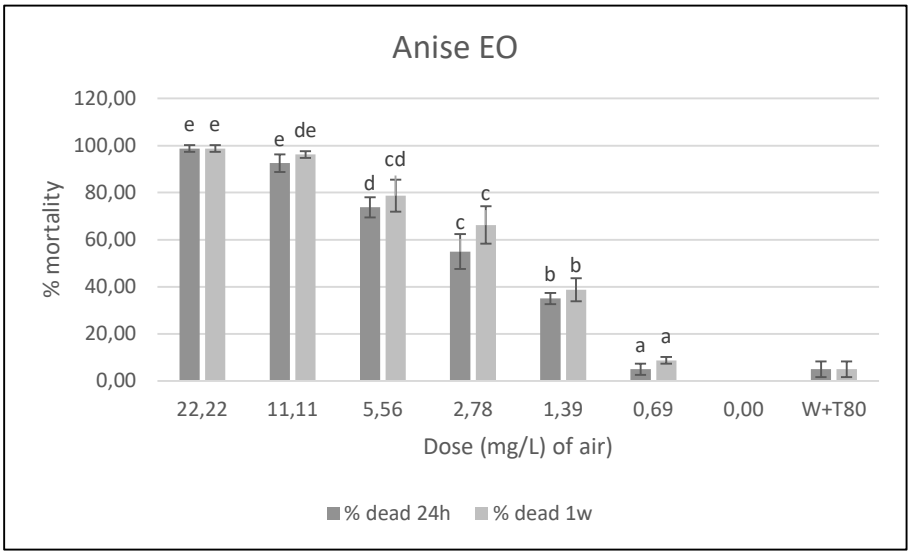


Figure 14 Mortality (%) in T. confusum after exposure (24 h and 1w at 25 °C) to cold aerosol (mg/L) of anise EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the four replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

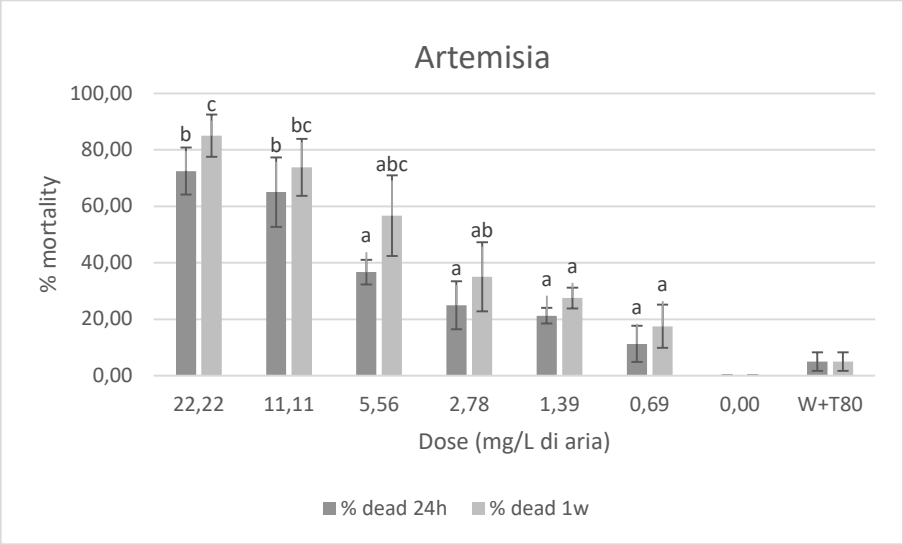


Figure 15 Mortality (%) in T. confusum after exposure (24 h and 1w at 25 °C) to cold aerosol (mg/L) of artemisia EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the four replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

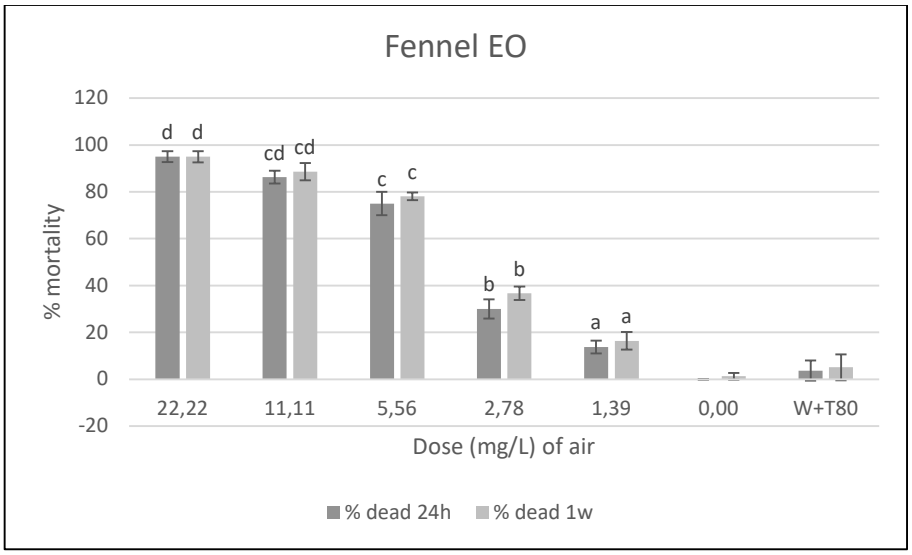


Figure 16 Mortality (%) in T. confusum after exposure (24 h and 1w at 25 °C) to cold aerosol (mg/L) of fennel EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the four replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

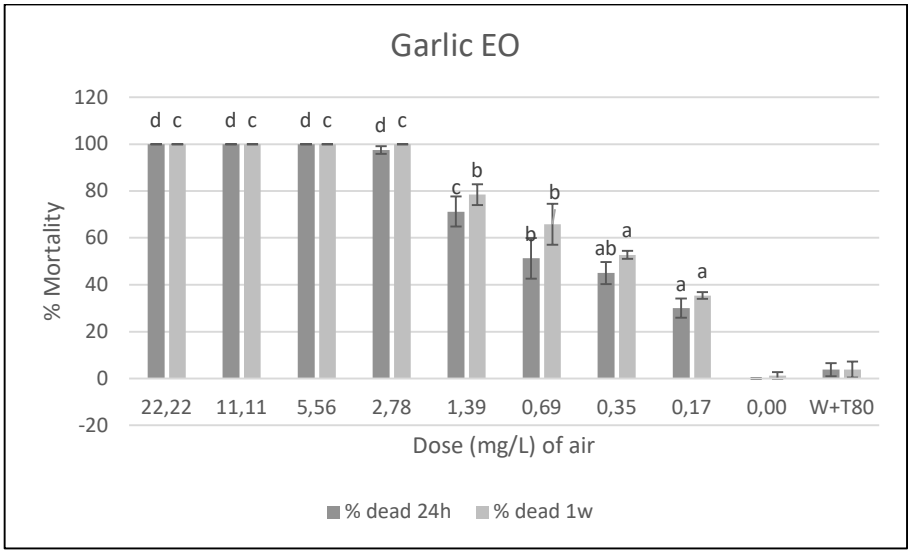


Figure 17 Mortality (%) in T. confusum after exposure (24 h and 1w at 25 °C) to cold aerosol (mg/L) of garlic EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the four replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

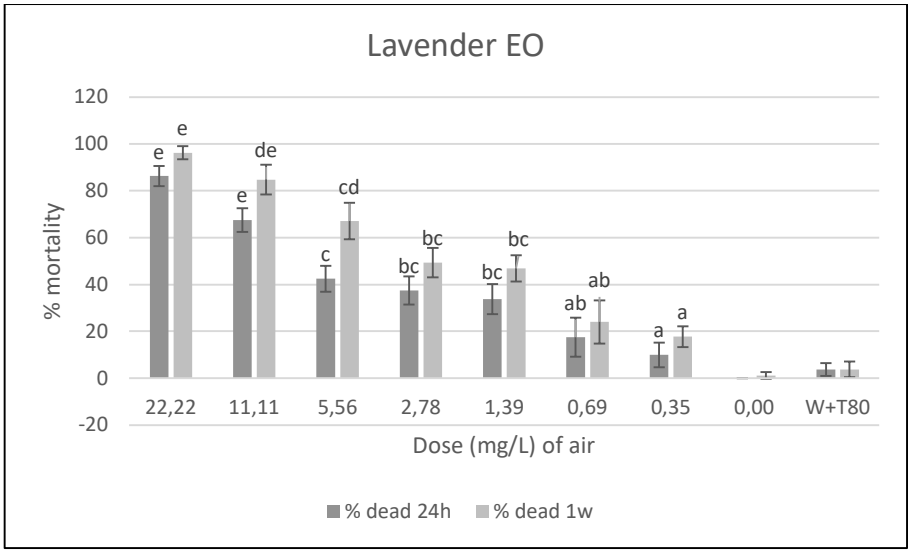


Figure 18 Mortality (%) in T. confusum after exposure (24 h and 1w at 25 °C) to cold aerosol (mg/L) of lavender EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the four replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

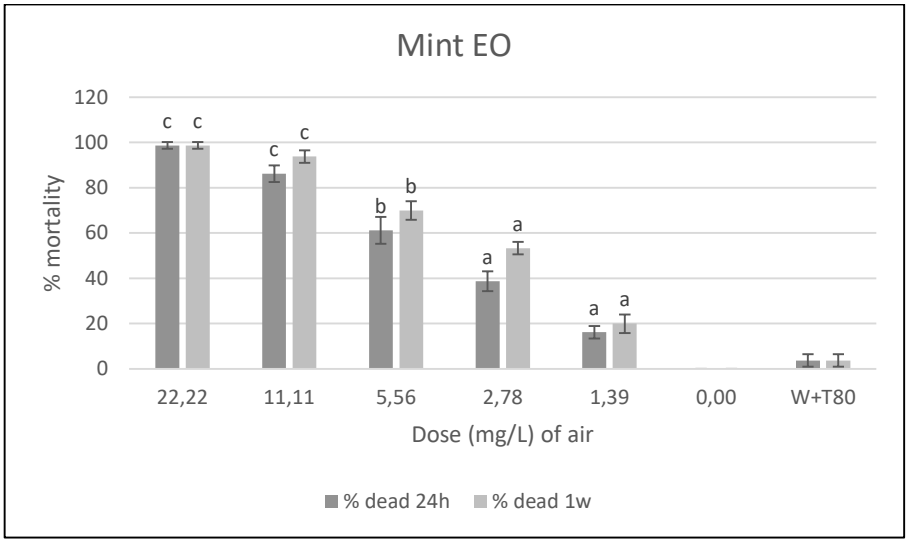


Figure 19 Mortality (%) in T. confusum after exposure (24 h and 1w at 25 °C) to cold aerosol (mg/L) of mint EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the four replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

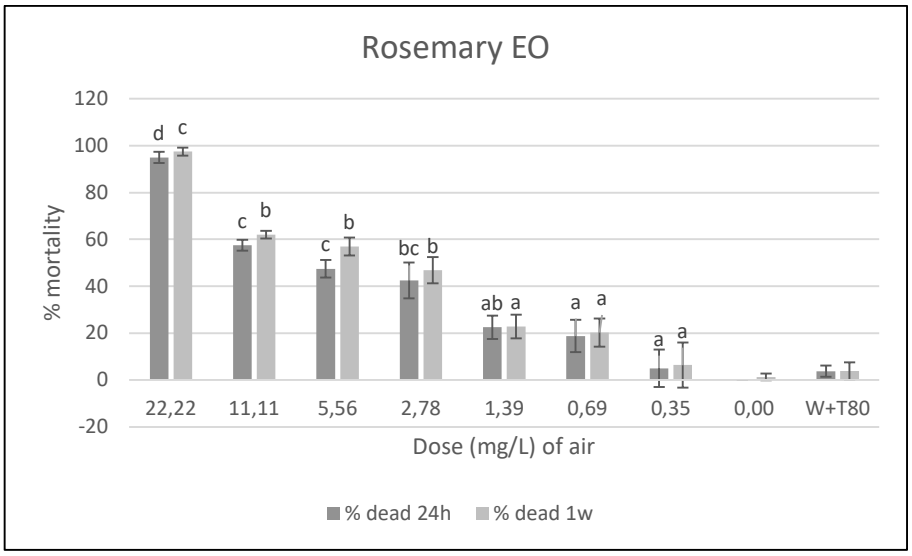


Figure 20 Mortality (%) in T. confusum after exposure (24 h and 1w at 25 °C) to cold aerosol (mg/L) of rosemary EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the four replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

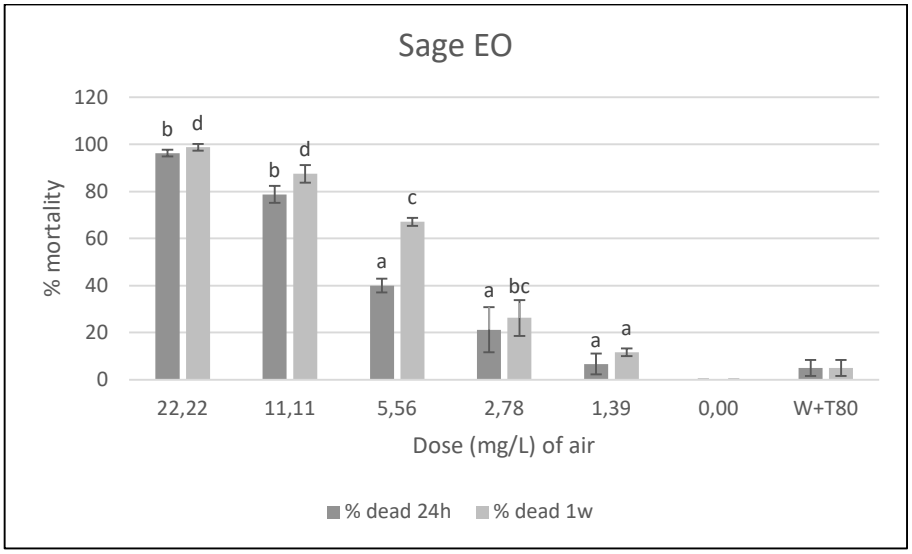


Figure 21 Mortality (%) in T. confusum after exposure (24 h and 1w at 25 °C) to cold aerosol (mg/L) of sage EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the four replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

3.5. Fumigation toxicity against *D. melanogaster*

Mortality values from fumigation trials fitted the probit curve (Pearson Goodness of fit tests) at 24 h for *D. melanogaster* (Table 9). Results reported in Figures 22-29 show that garlic EO nanoemulsion had the highest toxic activity against *D. melanogaster* flies and the LC_{50} value of this nano-formulation was significantly lower compared to those of the other nano-insecticides, since their 95% fiducial limits did not overlap (Table 9). In contrast, the sage EO nanoemulsion provoked the lowest toxicity against the target pest.

Probit analysis was performed to evaluate LC_{95} value (Table 10) and perform a fumigation trials at this concentration with the most promising nanoformulation to evaluate the genic expression of the target gene after the treatment.

Recorded mortality of *D. melanogaster* adults was very low in Tween 80 controls (maximum mortality: 5% ± 3.3) and significant lower respect to EO nano-emulsions. In addition, fumigation with double distilled water caused no mortality.

Dipteran pests (mosquitoes and flies) are the most common insects in human settlements. Furthermore, *Drosophila melanogaster can* be a highly valuable model in meeting today's demands of toxicity studies into nano-based pesticides. EO

toxicity against this fly species recorded in fumigation trials almost reflect exactly those reported for *T. confusum* under cold aerosol treatment. Previous studies about EO insecticidal activity against *D. melanogaster* aimed mostly to determine the mechanism of action of the bioactive botanicals (Enan, 2005; da Cunha *et al.*, 2015; Pinho *et al.*, 2014; Riaz *et al.*, 2018). Flies exposed to EOs in fumigation trials showed, beside to mortality, also sub-lethal effects, as locomotor deficits and signs of oxidative stress (da Cunha *et al.*, 2015; Pinho *et al.*, 2014).

Table 9 Estimated median lethal concentrations (LC_{50}) of the various EOs nanoformulations on D. melanogaster adults in the fumigation bioassays. different letters within the same column of each trial indicate statistical differences ($p<0.05$).

Essential Oils	**LC_{50} (mg/L)**	**95% fiducial limits**	**Slope+-SE**	**Intercept+-SE**	***χ2* (DF)**	***P***
Anise	2.858b	2.685-3.029	6.458±0.502	-7.639±0.609	1.817(3)	0.611
Artemisia	7.763d	7.224-8.345	4.024±0.331	-3.582±0.300	2.532(3)	0.480
Fennel	8.376d	7.104-10.798	2.579±0.343	-2.381±0.235	3.403(3)	0.334
Garlic	0.351a	0.272-0.466	2.827±0.213	1.284±0.129	5.523(3)	0.137
Lavender	10.855d	9.746-12.087	5.876±0.373	-6.085±0.393	10.879(5)	0.053
Mint	5.350c	4.803-5.945	3.178±0.238	-2.315±0.194	1.893(3)	0.595
Rosemary	12.225bcde	1.742-15.188	5.880 ±0.373	-6.090±0.393	7.694(3)	0.053
Sage	15.235e	13.846-16.556	6.458±0.502	-7.639±0.609	8.265(4)	0.082

Table 10 Value of EOs nanoformulations concentration necessary to reach LC_{50} and LC_{95} with confidence limit on D. melanogaster adults in the fumigation bioassays.

Essential Oils	***Exposure Time***	***Probability***	***95% Confidence Limits EOs Dose (mg/ L of air)***		
			Estimate LC	***Lower Bound***	***Upper Bound***
Anise	24h	0.50	2.858	2.685	3.029
Anise	24h	0.95	5.571	5.065	6.306
Artemisia	24h	0.50	7,763	7.224	8.345
Artemisia	24h	0.95	19.895	17.172	24.243
Fennel	24h	0.50	8.376	7.104	10.798
Fennel	24h	0.95	36.372	23.365	76.670
Garlic	24h	0.50	0,351	0.272	0.466
Garlic	24h	0.95	1.431	0.881	2.285

Essential Oils	*Exposure Time*	*Probability*	*95% Confidence Limits EOs Dose (mg/ L of air)*		
			Estimate LC	*Lower Bound*	*Upper Bound*
Lavender	24h	0.50	10,857	9.746	12.087
Lavender	24h	0.95	20.681	17.682	26.169
Mint	24h	0.50	5,350	4.803	5.945
Mint	24h	0.95	17.617	14.778	22.078
Rosemary	24h	0.50	12,225	1.742	15.188
Rosemary	24h	0.95	27.693	22.432	112.574
Sage	24h	0.50	15,235	13.846	16.556
Sage	24h	0.95	27.386	23.694	35.414

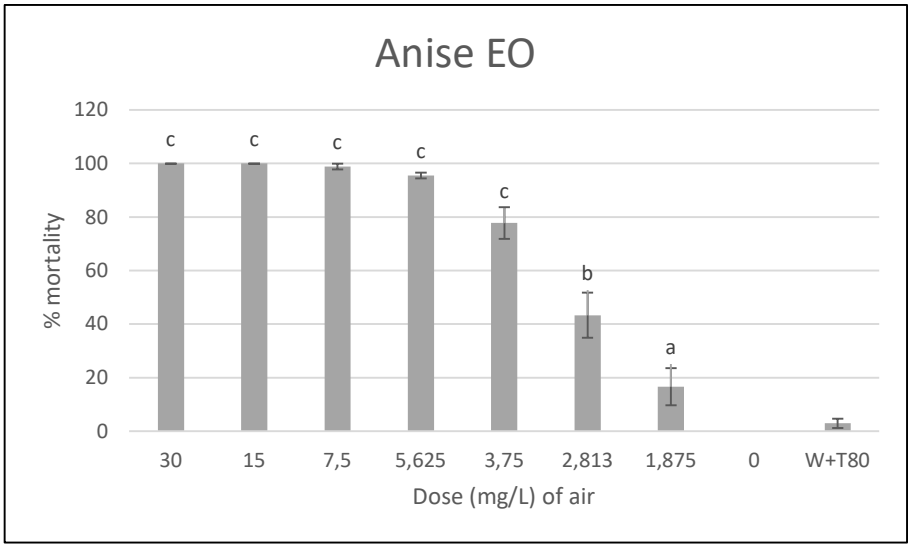


Figure 22 Mortality (%) in D. melanogaster after exposure (24 h at 25 °C) to vapours (mg/L) of anise EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

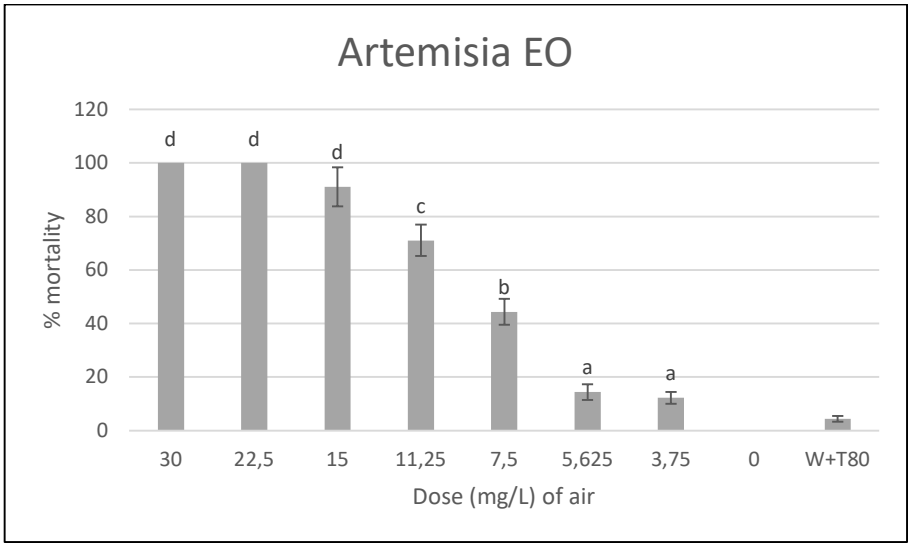


Figure 23 Mortality (%) in D. melanogaster after exposure (24 h at 25 °C) to vapours (mg/L) of artemisia EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

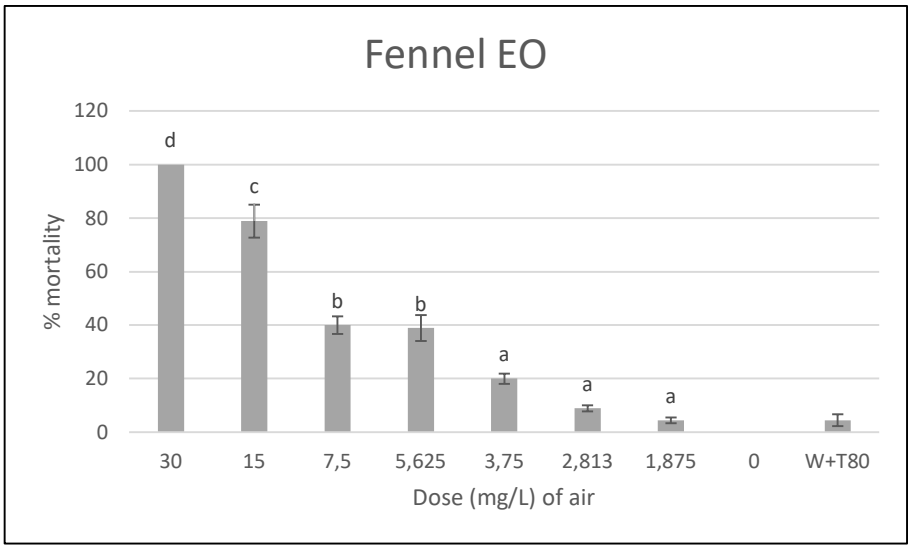


Figure 24 Mortality (%) in D. melanogaster after exposure (24 h at 25 °C) to vapours (mg/L) of fennel EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

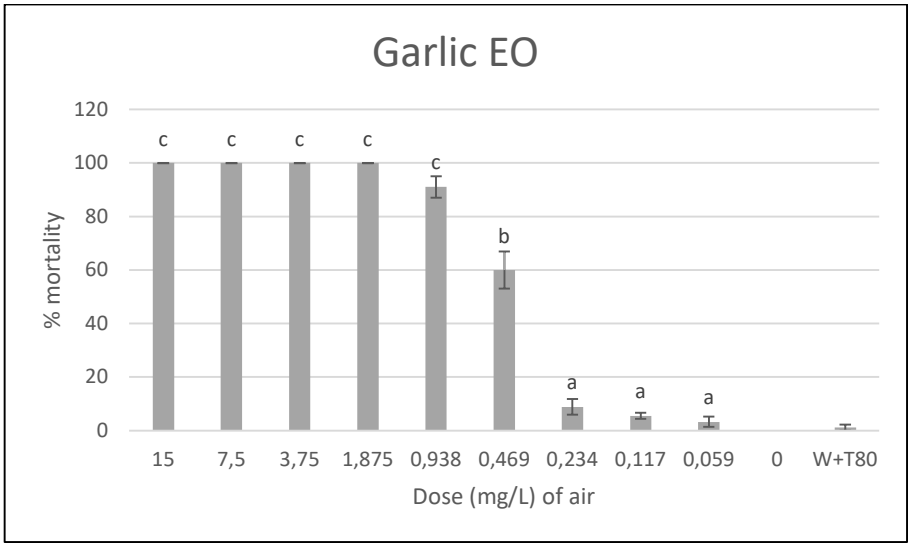


Figure 25 Mortality (%) in D. melanogaster after exposure (24 h at 25 °C) to vapours (mg/L) of garlic EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

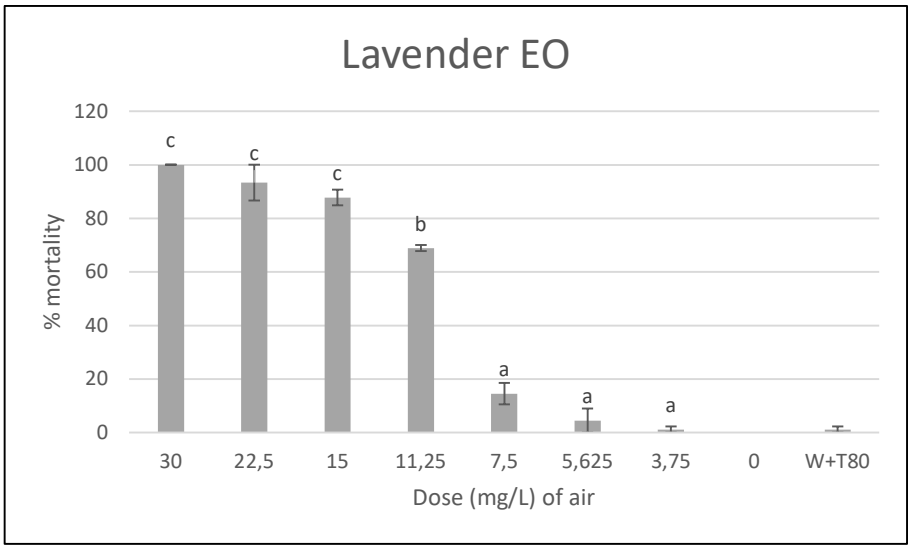


Figure 26 Mortality (%) in D. melanogaster after exposure (24 h at 25 °C) to vapours (mg/L) of lavender EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

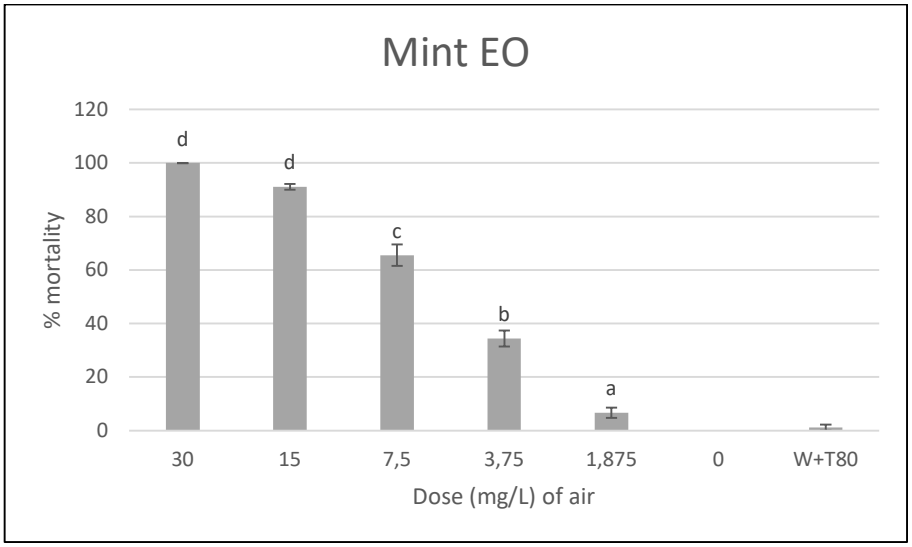


Figure 27 Mortality (%) in D. melanogaster after exposure (24 h at 25 °C) to vapours (mg/L) of mint EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

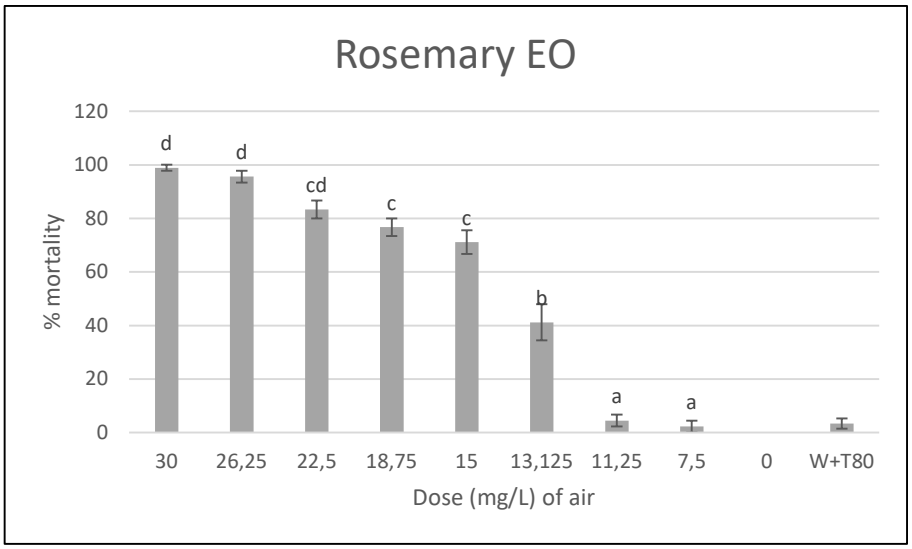


Figure 28 Mortality (%) in D. melanogaster after exposure (24 h at 25 °C) to vapours (mg/L) of rosemary EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

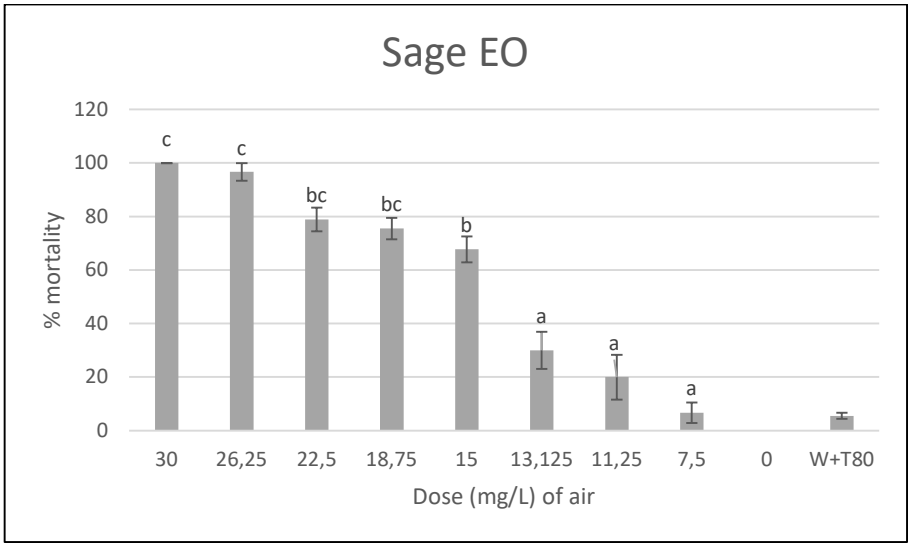


Figure 29 Mortality (%) in D. melanogaster after exposure (24 h at 25 °C) to vapours (mg/L) of sage EO nanoemulsion. Percentages of mortality refer to total number of insects tested in the three replications per dose; black bars indicate standard errors; different letters indicate significant differences among the doses *at the same time (Tukey-Kramer HSD).*

3.6. Evaluation of mode of action for selected nanoemulsions

Relative quantification of the target genes was performed with REST software tool. Expression analysis for the genes *AChE, Gabat, Tbh, ADH, AANAT, GstS1, Mgstl, Vha68-2, Cyp6a2, Cyp6a8, Cyp6a19, Cyp6a23, Cyp6g1, Cyp6g2, Cyp6t3* and *Cyp12d1* showed that the quantitative changes of gene expression in flies treated with the anise OE nano-emulsions were statistically significant for: *AChE* (-2.02-fold, P = 0.016), *Tbh* (-1.724-fold, P = 0.016), *ADH* (-2.181-fold, P = 0.001), *AANAT* (-2.988-fold, P = 0.001), *GstS1* (-4.747-fold, P = 0.031), *Cyp6g1* (-1.577-fold, P = 0.001), *Cyp6a2* (2.217-fold, P = 0.001), *Cyp6a23* (2.312-fold, P = 0.031), *Cyp12d1* (4.239-fold, P = 0.001) and *Cyp6a8* (12.865-fold, P = 0.001). The downregulation of the genes indicated an inhibitory action of acetylcholinesterase *AChE,* Tyramine β hydroxylase *Tbh* and Arylalkylamine N-acetyltransferase *AANAT* (Figure 30). Moreover, the underexpression of the detoxification system involving alcohol dehydrogenase *ADH,* glutathione transferase s1 *GstS1* and *Cyp6g1* has been evidenced. The latter gene (*Cyp6g1*) is particularly relevant, because its overexpression is responsible for the onset of resistance to neonicotinoids (Daborn *et al.*, 2001). Lastly, also a high overexpression of the *Cyp6a8* gene (involved in fatty acid metabolism (Helvig *et al.*,2004)) (Figure 31)

was shown in insects treated with the anise-based nanoformulation, suggesting the priming oxidative stress.

Concerning garlic EO nanoemulsion, the expression analysis of the genes *AChE, Gabat, Tbh, ADH, AANAT, GstS1, Mgstl, Vha68-2, Cyp6a2, Cyp6a8, Cyp6a19, Cyp6a23, Cyp6g1, Cyp6g2, Cyp6t3* and *Cyp12d1* showed that it could not significantly alter the gene expression related to the main enzymes of the nervous system (Figure 32), intuitable because statistically significant downregulation of *AANAT* (0.287-fold, P = 0.046) (involved in major neurotransmitter metabolism) and *GstS1*(-0.325-fold, P = 0.016) (detoxification mechanism). specifically, Glutathione S-transferase S1 (*GstS1*) is important because in specimens of *D. melanogaster* parkin mutant its overexpression suppressed neurodegeneration, while arylalkylamine N-acetyltransferase (*AANAT*) has a crucial role, catalyses the transacetylation from acetyl-CoA to arylalkylamines and inactivate arylalkylamines, such as octopamine, dopamine, and serotonin. A significant overexpression of *Mgstl* (4.587-fold, P = 0.049), a gene involved in the mechanism of detoxification was also noted, probably compensating the underexpression of other genes.

In addition, overexpression of *Cyp6a8* (10,661-fold, P = 0.016*), Cyp6a23* (9,670-fold, P = 0.031), *Cyp6g1* (3,304-fold, P = 0.031), and *Cyp12d1*(3,324-fold, P = 0.016) was detected (Figure 33). *Cyp6a8* is involved in fatty acid metabolism and *Cyp6a23* is involved in oxidoreductive processes and is supposed to be involved in insect hormone metabolism and degradation of synthetic insecticides. Furthermore, *Cyp6a23*, *Cyp6g1*, and *Cyp12d1* are involved in resistance to organophosphates and neocotinoids, and their overexpression can support the hypothesis that there is an allosteric interaction with the acetylcholinesterase receptor.

The fruit fly *Drosophila melanogaster* is a modern model system, with an extensive literature ranging from classical and modern genetics, biochemistry, physiology and complex phenotypes, including toxicology. Several studies showed neurotoxic actions of EOs, causing insect paralysis followed by death [reviewed by (Jankowska *et al.*, 2017)]. The inhibition of acetylcholinesterase (*AChE*) is one of the most investigated mechanisms of action since *AChE* is one of the most

important enzymes in neuro-neuronal and neuromuscular communication in insects. It differs from mammalian enzyme by a single residue, making *AChE* an insect-selective target for insecticides. Some studies reported locomotor inhibition due to EO administration, that can be partially explained by the terpenes activity on acetylcholinesterase (*AChE*) which can cause damage to the locomotor apparatus (da Cunha *et al.*, 2015; Pinho *et al.*, 2014). The insecticidal activity of EOs was suspected to lay on the interaction with *AChE* also for stored product pests (Abdelgaleil *et al.*, 2016; Liao *et al.*, 2016; Nattudurai *et al.*, 2017). These studies on *AChE*-inhibitor activity of EOs were carried out on Coleoptera species, demonstrating the inhibition of *AChE* activity based on I_{50} values (i.e., the concentrations of the tested essential oil that inhibited the hydrolysis of substrate by 50%). However, EOs may present weak or moderate AChE inhibition, as reported by Nattudurai *et al.* (2017), but decrease total esterases (Nattudurai *et al.*, 2017).

Esterases are known to be involved in the detoxification of foreign compounds and allelochemical volatiles, similarly to glutathione S–transferases (*GSTs*) which are known to play a key role for insect detoxification mechanisms, and the neutralization and resistance mechanisms toward synthetic and natural insecticides (Kostaropoulos *et al.*, 2001; Siegfried and Scharf, 2001). Similar to garlic EO nanoemulsion tested in the above-described trials, also other EOs can decrease *GST* activity (Nattudurai *et al.*, 2017). In contrast to these results, Shojaei *et al.* (2017) reported that total esterase activity and mixed function oxidases (*MFOs*) in two Tenebrionidae species, *T. castaneum* and *T. confusum*, was not affected by the administration of *Artemisia dracunculus* (Asteraceae) EO, even at high dosages (LC_{70}). *In D. melanogaster*, the administration of *Eugenia uniflora* boost a significant increase in the activity of *GST* tighter with a strong increase of oxidative stress (da Cunha *et al.*, 2015). Such an effect is confirmed by the increased production of reactive species and accumulation of lipid peroxidation byproducts. In this scenario, the activation of antioxidant signaling pathways is a clear adaptive response to oxidative stress.

The ability of EOs to reduce and suppress the activity of detoxifying enzymes may improve the insecticidal efficacy of EO-based formulations, as well as be

exploited as synergistic ingredient to enhance the efficacy of other insecticides. Indeed, generally insects activate detoxifying enzymes to prevent and counterattack oxidative damage, thus the reduced activity of *GST* and esterase might improve the insecticidal activity of the insecticidal formulation. To obtain a better understanding of the mechanisms associated with the mode of action of EOs, Liao *et al.* (2016) performed for the first time a comparative transcriptome analysis in *S. zeamais,* discovering that the majority of differentially expressed genes were involved in insecticide detoxification and mitochondrial function and they hypothesize that adenosine triphosphatases (*ATPases*), a class of enzymes that catalyse the decomposition of ATP into ADP releasing energy, may be a target for EOs.

Overall, EOs are generally supposed to act as neurotoxic-insecticides and their insecticidal activity is considered species-dependent (Jankowska *et al.*, 2017). In this context, other proposed mechanisms of EO action mode on insects include the inhibition of GABA receptors (*GABArs*) and the alteration of the octopaminergic system. To the best of our knowledge, the ability of EOs to alter *GABArs* has never been proved for insects. On the other hand, modifications of the insect octopaminergic system following EO exposure have been already reported (Enan, 2001). For instance, some EO components may compete with octopamine in binding to its receptor, causing an increase in the level of cAMP and calcium in nervous cells and modifying the neuron activity in *Periplaneta americana* L. (Blattodea: Blattidae) (Enan, 2001). Several studies observed that there was a locomotor inhibition caused by EO on *D. melanogaster* flies (da Cunha *et al.*, 2015; Pinho *et al.*, 2014), which was not linked to changes in the dopaminergic and cholinergic systems but can suggest a potential interaction between EO components and flies neurotransmitters pathway. Furthermore, Enan (2005) demonstrate that monoterpenes from several EOs can alter the receptor binding activity of an octopamine precursor, tyramine receptor (*TyrR*), playing a fundamental role for the insecticidal activity of tested plant EOs. On this basis, it is possible to suggest that the broad-spectrum insecticidal activity of EOs could be attributable to the characteristics of these plant extracts, which are composed by numerous different compounds operating via several modes of action toward insect species.

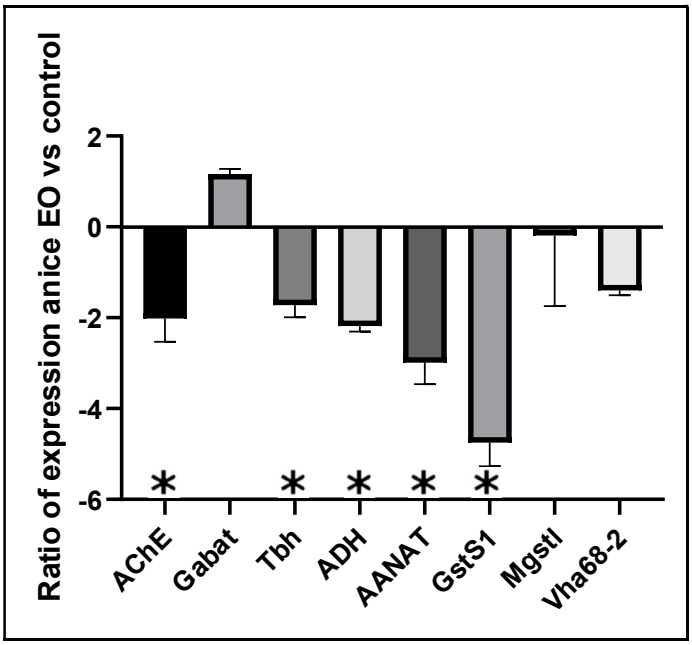


Figure 30 Ratio of relative gene expression (±SD based upon a permuted expression data in REST. Standard error of the mean [SEM] is provided by REST as a precision indicator of the estimated mean ratios of expression and SD was calculated from the SEM) of anise EO vs control. Asterisks indicate significant differences (REST statistical randomization test; P < .05)

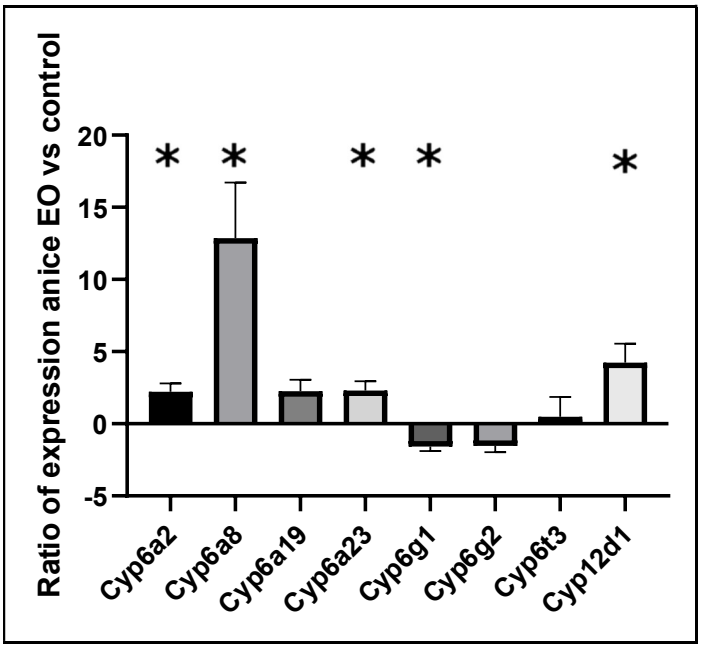


Figure 31 Ratio of relative gene expression (±SD based upon a permuted expression data in REST. Standard error of the mean [SEM] is provided by REST as a precision indicator of the estimated mean ratios of expression and SD was calculated from the SEM) of anise EO vs control. Asterisks indicate significant differences (REST statistical randomization test; P < .05)

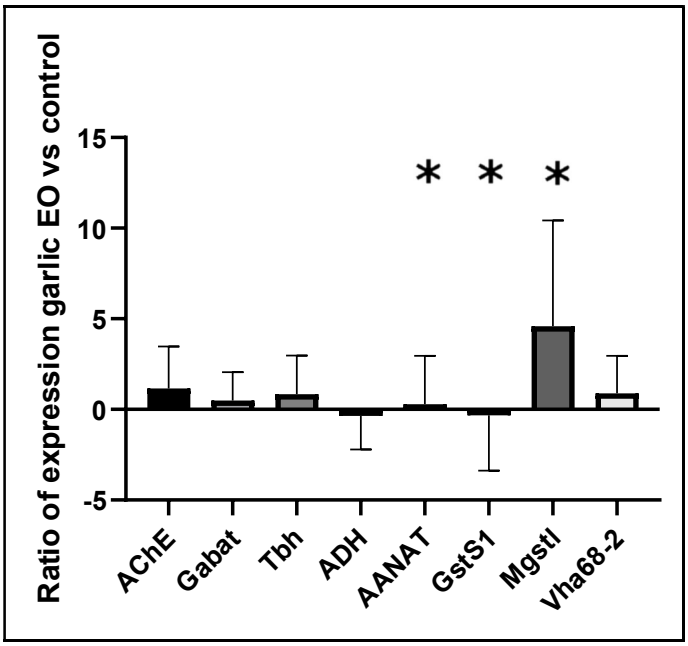


Figure 32 Ratio of relative gene expression (±SD based upon a permuted expression data in REST. Standard error of the mean [SEM] is provided by REST as a precision indicator of the estimated mean ratios of expression and SD was calculated from the SEM) of garlic EO vs control. Asterisks indicate significant differences (REST statistical randomization test; P < .05)

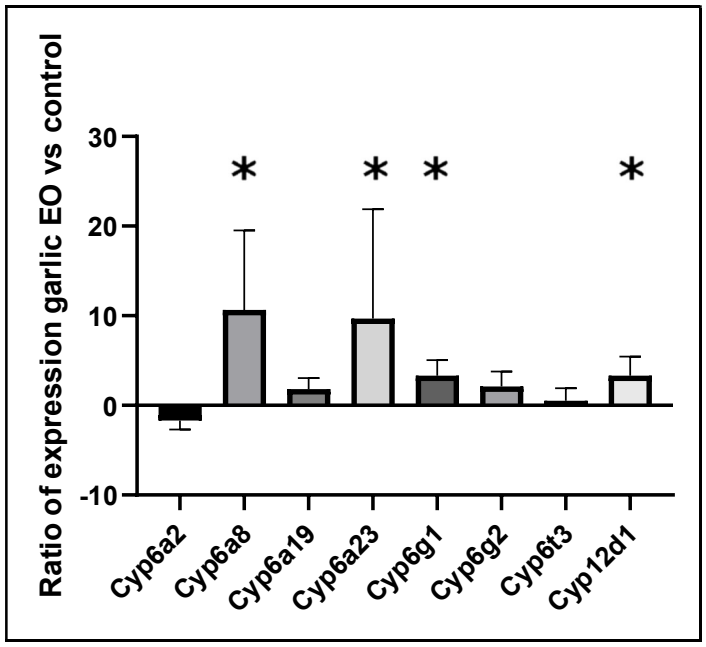


Figure 33 Ratio of relative gene expression (±SD based upon a permuted expression data in REST. Standard error of the mean [SEM] is provided by REST as a precision indicator of the estimated mean ratios of expression and SD was calculated from the SEM) of garlic EO vs control. Asterisks indicate significant differences (REST statistical randomization test; P < .05)

4. CONCLUSIONS AND FUTURE PERSPECTIVES

In the present PhD thesis focused on the development of novel solutions for IPM programs, with reference to formulation of EO-based nanoemulsions and slow-release gels with bioactive insecticidal activity. Eight EOs were selected and used in this study (anise, artemisia, fennel, garlic, lavender, mint, rosemary, sage) and all the nanoemulsions were developed using biodegradable ingredient to ensure the eco-compatibility of the formulations.

Although an impressive increase in the number of publications involving botanical insecticides was recorded from 1980, as highlighted by Isman *et al.* (2011), the use of EOs as insect control tools in stored products still represents a niche compared to other sectors. The increasing interest about essential oils derives from a number of factors such as their widespread availability, relatively low cost, and the belief that plant-borne extracts are non-toxic to humans and pets. Furthermore, EOs usually showed a noticeable dose-dependent acute toxicity (i.e., mortality), which can be risen concerns when the essences used derive from spontaneous non-cultivated plants. Despite the promising results, there are few authorized commercial EO-based insecticide formulations available on the market.

This research aimed to formulate stable nano-insecticides, which can improve the handling of EOs under field conditions. The developed nanoemulsions were physically characterized. The analysis showed, for all the developed formulation, a good dimensional range (95.03-144.6 nm), a low PdI (0.126-0.235 PdI) and a negative surface (9.32-23.9 mV), which together indicated a good quality of the tested nanoemulsions.

In toxicity trials, either via fumigation against *D. melanogaster* or though aerosol nebulization against *T. confusum,* a valuable efficacy for all the tested EOs was noted, but the best performances were observed when the insects were treated with garlic EO. This exerted strong insecticidal effect also at low dosages, provoking 51% of mortality at only 0.69 mg/L of air for *T. confusum* adults after 24h, increasing to 66% after 1 week. In fumigation trials on *D. melanogaster,* similar results were archived, because 60% of mortality was observed at 0.469 mg/L of air. In repellency trials using *T. confusum* adults, a good repellent activity

was reported for all the tested formulations, which was stable up to 48h. The most promising results were observed with anise EO, whose can cause a repellent action even at very low EO concentration.

D. melanogaster was used as model organism to investigate the mechanism of action for the two most promising EO (garlic and anise), which expressed the highest toxicity in fumigation trials against adult flies. The experiment was carried out using RT qPCR analysis and the investigation involved several enzymes of the nervous system and of CP450 system. Results from gene expression revealed that the anise EO affected the expression of several nervous enzymes downregulated the gene *Cyp6g1,* an important enzyme involved in neocotinoid resistance, suggesting a possible synergic effect of the nanoemulsion with this kind insecticides. In contrast, *D. melanogaster* treated with garlic EO nanoemulsion presented a set of differentially expressed genes, which did not clearly identify the site of action, but which supported the idea that nervous system interaction occurred. In detail, the down regulation of *AANAT* and *GstS1* indicated a neurotoxic action, while the upregulation of *Cyp6a8 Cyp6a23, Cyp6g1, Cyp12d1* confirmed the neurotoxic action and supported the hypotheses that garlic EO could act an allosteric action on the *AChE* receptor.

The collected data allow us to conclude that the developed nanoformulation may be potentially used as IPM tools for pest control in post-harvest conditions, since they were effective in controlling and repelling the target pests. Moreover, the scalability of the EO nano-emulsion process is realizable by pesticide industry because this process has been already used to produce some “new generation” insecticides. Aerosol and fogging systems are rising interest among the scientific community as alternative methods to fumigation in commercial food storage facilities (Arthur, 2008; Scheff *et al.*, 2018; Toews *et al.*, 2006). The application of EO nanoformulations as cold aerosol can be a promising method for the sanitation of production areas, warehouses, handling equipment and production machineries from stored product pests. Furthermore, the proposed techniques can be used in combination with other control approaches; as said above, and the administration of nanoformulations can be combined with other insecticides, while the EO-based

gels can integrate the barrier system (e.g., packaging, air barrier) to deter insect feeding, avoiding any kind of residues on food.

Future studies about the mechanisms of action of the EOs against insects are needed to clearly understand the target site for every different EO and to develop effective EO-based insecticides. Indeed, deeper knowledge on this topic may be helpful to estimate the impact of EOs toward non-target species and their safety for consumers. In addition, the effect on the sensory analysis of food treated with these compounds should be evaluated since, although this aspect is a main concern for costumers, it has been often disregarded. Therefore, a multidisciplinary approach, involving also chemists and food technologists, could be a route to develop new EO-based insecticide formulations which could be successfully applied to various sectors. Further research is needed to test the efficacy of the developed nano-insecticides under more realistic operative conditions. In addition, considering the controversial opinions of biopesticides, it would be interesting to evaluate the potential side effects of these compounds on natural enemies used as biocontrol agents and on non-target organisms, as well as their sublethal effects on selected behavioral traits both of target and non-target species.

LIST OF FIGURES

LIST OF TABLES

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