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GC/MS profiling, insecticidal performances and acetylcholinesterase inhibition of Tunisian *Thymus capitatus* (L.) essential oil for sustainable stored grain pest management

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Abstract: *Sitophilus oryzae* L. commonly known as the rice weevil is a challenging pest of stored products causing serious economic losses. The management of this pest in stored products is based on the use of synthetic insecticides. Nevertheless, the excessive use of chemicals induced several side effects. Essential oils (EOs) have become a sustainable alternative for stored product pest management due to their entomotoxic performances. Accordingly, this study aimed to investigate the fumigant toxicity of *Thymus capitatus* L. EOs and its active component carvacrol against *S. oryzae* adults in cereal grain substrate during 10 days of storage under semi-industrial setup. Besides, Thyme EOs chemical composition and its acetylcholinesterase inhibition on *S. oryzae* adults were screened. GC-MS analysis revealed that Tunisian Thyme EOs was a carvacrol chemotype representing 78.35% of the total oil. Results showed that EO and carvacrol possessed fumigant activities towards *S. oryzae*adults with LC_{50} values of 991.962 and 274.195 µl/L air over 10 days of storage, respectively. Meanwhile, carvacrol exhibited a strong acetylcholinesterase activity with value of 96.26 after 72 hours of exposure. Results highlighted fumigant performances of *T. capitatus* EOs and carvacrol, especially carvacrol, as a promising candidate for sustainable grain pest management.

1. Introduction

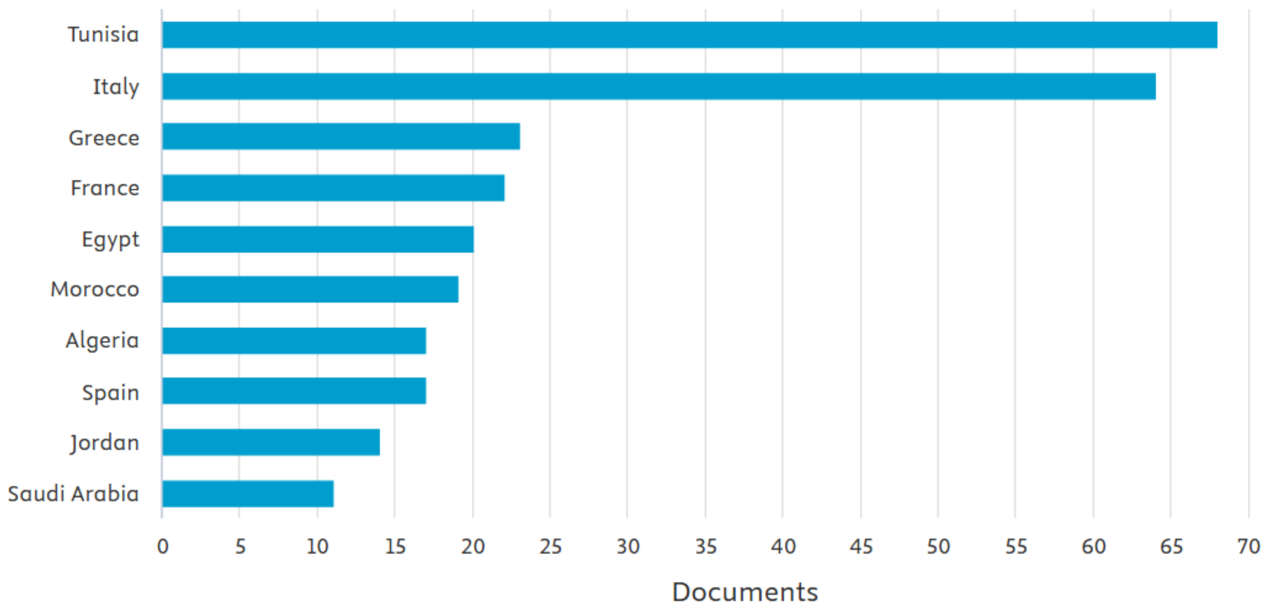
Stored products such as cereal wheat grains constitute a key agricultural commodity and a vital source of carbohydrates. Nevertheless, they face significant challenges, particularly from insect pests crop destruction that cause substantial losses leading to decline quantity and quality (Paul *et al*., 2020). The rice weevil (*Sitophilus oryzae* L.) is a primary destructive pest which adults feed directly on the whole grains. So, this feeding activity not only deteriorate both the quality and quantity of the grains, but also increased both temperature and humidity levels in the stored grains intensifying damages (Irabagon, 1959), (Stathers *et al.*, 2020) *S. oryzae* induces severe economic losses in developing countries especially those of the Mediterranean climate region estimated between 30 to 50% of production (Mehalaine*et al.*, 2021).

To ensure a protection of grain food and seeds, chemicals are largely employed inducing pest resistance phenomena and health and environmental side effects due to their large half-life (Mssillou*et al.*, 2022), (Zhou *et al.*, 2024). Bioinsecticides offer various advantages in comparison to chemicals, namely lower toxicity to non-target organisms, greater efficiency at lower concentrations and natural biodegradability (Pang *et al.*, 2020). In order to secure a sustainable food grain protection, there is a need to develop eco-friendly pest management tools. In this context, essential oils (EOs) and their active compounds have been recognized as a novel promising approach to insect pest management in stored product commodities (Isman, 2020), (Jumbo *et al.*, 2022).

Plants belonging to the Laminaceae family, such as thyme, savory and oregano provide active compounds obtained from their essential oil, mainly carvacrol and thymol. These secondary metabolites function as a chemical defense against insect and phytopathogens. In this sense, essential oils derived from these plants are recognized for their strong antimicrobial, insecticidal, and acaricidal properties, making them highly effective in natural pest management approaches. So, active compounds like thymol, carvacrol, p-cymene and, γ-terpinene were renewed for their biological activities (El Ouariachi *et al.*, 2011), (Nabavi *et al.*, 2015), (Rajhi *et al.*, 2021).

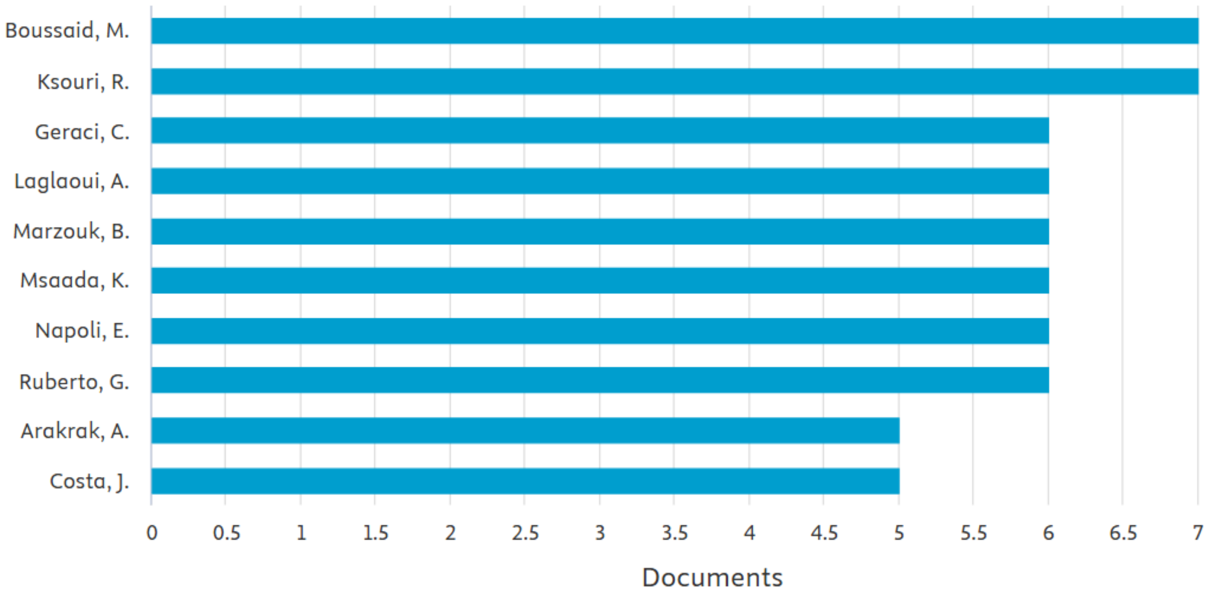
Several studies demonstrated that essential oils (EOs) disturb the nervous, respiratory, and digestive insect systems via enzyme activity inhibition like acetylcholinesterase (AChE), peroxidase (POD), and carboxylesterase (CarE) (Zhang *et al.*, 2019), (Boumezzourh *et al.*, 2023). In this regard, EOs and its active compound carvacrol interfere with the nervous system of insects. So, AChE inhibition leads to an accumulation of acetylcholine at synaptic sites, causing continuous nerve stimulation, paralysis, and eventual death of the pest (Anderson *et al.*, 2012). Similarly, AChE inhibition plays a key role in disrupting insect nerve conduction.

*T. capitatus*is widely studied as shown in literature collected from Scopus. 272 documents as indicated from 1958 to now and **Scheme 1** indicates the ten most interested countries in *T. capitatus*.



Scheme 1. The ten Countries affiliations in the collected papers.

The Mediterranean countries as Tunisia (68 documents), followed by Italy (64), Greece (23), France (22), Egypt (20), Morocco (19), Algeria (17), Spain (17), Jordan (14) and Saudi (11) have the most published papers. **Scheme 2** indicated that the majority of most published papers are Tunisians as Boussaid, Ksouri, Marzouk, Mssaada. Laglaoui is from Morocco. We notice also that the most cited paper is on the Sardinian reaching 900 citations (Cosentino *et al*., 1999)



Scheme 2. The ten most authors

Meeting the current interest in alternative botanical-based pesticides, this work is focused on the investigation of the chemical composition of *T. capitatus* EOs and their fumigant activities in comparison with its active compound carvacrol towards *S. oryzae* in cereal grain substrate during 30 days of storage under semi-industrial set-up. Moreover, a detoxifying enzyme inhibition was carried out to determine the mode of action of Thyme EO on *S. oryzae*.

2. Methodology

2.1 Chemicals

Carvacrol (98%) was obtained from Sigma-Aldrich (Darmstadt, Germany) and was used without further purification.

2.2 Plant material

Thymus capitatus L. aerial parts were collected during the flowering stage in July 2023 from the region of Haouaria(Northeastern of Cape Bon, at the entrance to the Gulf of Tunis, Nabeul, Tunisia) (latitude 36° 31' 38.7" North; longitude 10° 45' 06.6" East, Mediterranean climate). A voucher specimen was preserved in the Laboratory of Plant Protection LPV at the National Institute of Agronomic Research of Tunisia under the name THYTU2023. The plant material was air dried in a well-ventilated area, protected from direct sunlight, and stored in canvas bags at room temperature (25°C).

2.3 Thyme Essential Oil extraction

A total of 500 g of the dried leaves were deposited in a Clevenger-type distillation apparatus in 10 L of distilled water were hydrodistilled for 3 hours using a Clevenger apparatus at the Materials

Molecules and Applications Laboratory of the Preparatory Institute for Scientific and Technical Studies. The essential oils were collected and stored in hermetically sealed amber bottles at 4 °C in a refrigerator.

2.4 Insect rearing

The insecticidal potential of *T. capitatus* EOs was evaluated towards the rice weevil: *Sitophilus oryzae* in cereal grain substrate. Insect adults were collected from the rearing colony maintained in the Laboratory of Plant Protection (LPV) in National Institute of Agronomic Research of Tunisia. Insects were cultivated in glass jars filled with maize grains kept in a rearing room maintained at (25± 1) °C, with a relative humidity of (65± 5) % and a photoperiod of 15 hours light and 9 hours dark. Insect adults aged 0 to 7 days were chosen to investigate fumigant toxicity trials.

2.5 Gas chromatography-mass spectrometry analysis

GC-MS analysis of *T. capitatus*EO was performed using an Agilent 7890A gas chromatograph coupled with an Agilent 5975C inert XL mass spectrometer operating in electron impact ionization mode at 70 eV. Analyte separation was achieved with a 30 m long, 0.25 mm inner diameter HP-5MS capillary column coated with a stationary phase of 5% phenyl methyl silicone and 95% dimethylpolysiloxane, with a film thickness of 0.25 µm. The oven temperature was initially set at 40 °C for 1 minute, then ramped to 100 °C at 8 °C/min and held for 5 minutes. It was further increased to 200 °C at 10 °C/min, held for 3 minutes, and finally ramped to 300 °C at 12 °C/min. Helium was used as the carrier gas at a flow rate of 1 mL/min with a split ratio of 60:1. The mass spectrometer scanned the m/z range of 40 to 300, with a scan time of 1 second per cycle.

2.6 Fumigant activity

The evaluation of insecticidal activity was undertaken through fumigant toxicity test

***In vitro* fumigant bioassay with bulk thyme EO on cereal substrate after 10 days of storage**

The fumigant toxicity of bulk thyme essential oil against *S. oryzae* on a cereal grain substrate was evaluated following the method of (Bachrouch *et al*., 2023). Disks of Whatman No. 1 filter paper (2 cm in diameter) were impregnated with different doses of the oil and attached to the screw cap of a 720 mL glass bottle, allowing them to hang inside. The tested oil concentrations were 163.63, 245.45 and 408.09 µL/L air. 50 insect adults were placed in each glass bottle. Each concentration, along with a control, was tested in triplicate. Mortality was recorded after 10 days of storage, and all data replicates were subjected to probit analysis according to the Finney method.

***In vitro* fumigant bioassay with carvacrol on cereal substrate after 10 days of storage**

The fumigant toxicity of carvacrol was evaluated following the method described by (Bachrouch *et al*., 2023). Carvacrol was tested at concentrations of 163.63, 245.45, and 408.09 µL/L air. 50 insect adults were placed in each glass bottle each concentration, along with a control, was tested in triplicate. Mortality was recorded after 10 days of storage, and all data replicates were analyzed using probit analysis according to the Finney method.

2.7 Acetylcholinesterase inhibitory activities

The inhibitory effect of bulk *T. capitatus* essential oil on AChE activity was evaluated *in vivo*. 50 adults were treated in three replicates with three progressive doses of essential oil: 15, 25 and 35 µL. Then, samples from each treated dose were homogenized in phosphate buffer (pH 7) using a Teflon glass tissue homogenizer. The homogenates were centrifuged at 10.000 rpm for 10 minutes at 4 °C, and the resulting supernatants were used as the enzyme source for determining AChE activity according to the method of (Ellman *et al*., 1961). In brief, sodium phosphate buffer (pH 8.0) and enzyme solution (30 µL) were mixed, followed by the addition of 60 µL of Ellman's reagent (DTNB). The reaction was initiated by adding 60 µL of acetylthiocholine iodide as the substrate. AChE activity was measured based on the formation of the colored 5-thio-2-nitrobenzoate anion, produced by the reaction of DTNB with thiocholine, released by substrate hydrolysis. The absorbance of the colored product was measured at 412 nm. Protein content in the homogenate was quantified using bovine serum albumin (BSA) as the standard. AChE inhibition (%) was calculated with the following equation:

$$\text{AChE inhibition (\%)} = \frac{C-I}{C} x100$$

Where *C* is the control activity and *I* is the activity of the treatment.

Data analysis

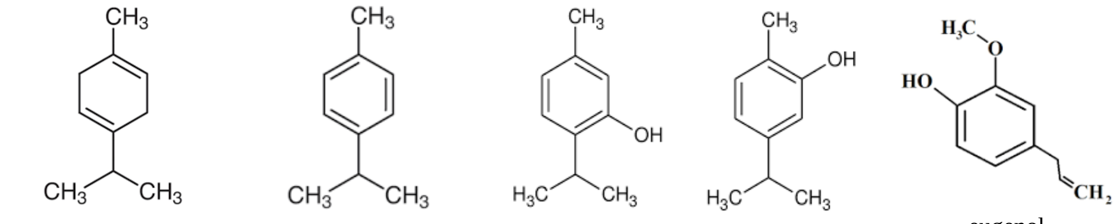
Statistical analysis was performed using one-way analysis of variance (ANOVA) in SPSS software version 20 (IBM Corporation, New York, USA). Duncan's multiple range test was applied to determine significant differences among means at the 0.05 level.

3. Results and Discussion

This work aims to explore chemical composition and fumigant performances of EOs extracted from Tunisian *T. capitatus* and its active compound carvacrol towards *S. oryzae* in cereal grain substrate. GC-MS analysis demonstrated that *T. capitatus* EOs was rich in carvacrol, a compound renewed for its antibacterial, antioxidant and entomotoxic proprieties (Taibi *et al*., 2024). In this sense, the inhibitory effect of bulk thyme EO on acetylcholinesterase (AChE) activity against *S. oryzae* was assessed to determine toxicity mechanism. Our study offers novel insights regarding the efficacy of *Thymus capitatus* (L.) EOs and carvacrol as wheat protectants against rice weevil adults and toxicity mechanism. These finding could be valuable to design new bioinsecticides efficient in stored product pest management in milling commodities.

GC-MS profiling

The extraction yield of *T. capitatus* EOs was 1.57% ± 0.26 (w/w). As shown in **Figure 1**. Data underlined a predominance of oxygenated monoterpenes (83.16%), whereas monoterpene hydrocarbons and sesquiterpene hydrocarbons represent a percentage of 14.59% and 1.91% of the total EO, respectively. Then,GC-MS analysis of Thyme EOs was characterized by the predominance of carvacrol representing a percentage of 78.35% of the total EOs. Other significant compounds were revealed p-cymene (6.08%) and γ- terpinene (4.63%). Meanwhile, thyme EO contained also thymol and eugenol with percentage of 0.28% and Eugenol 0.35% respectively. In this regard, (Annaz *et al*., 2023) demonstrated that *T. capitatus* EO was characterized by the predominance of carvacrol with a percentage of 78%. Moreover, our findings are in accordance with those obtained by (Bachrouch *et al*., 2022) who demonstrated that EOs extracted from *T. capitatus* areal parts from the Krib region(crape from the Siliana mountain areas of Tunisia)was rich in carvacrol according a percentage of 78.34% in thyme EO.



γ- terpinene 4.63% | p-Cymene 6.08% | Thymol, Carvacrol 0.28%-78.35% | eugenol 0.35%

In this regard,similarly, several researchers have focused on the biological properties of carvacrol, emphasizing its antimicrobial, antioxidant, antiviral, and therapeutic activities (Nascimento *et al.*, 2020). In this sense, carvacrol exhibits insecticidal activities, producing adverse effects on a wide range of pest insects, including larval mortality and growth inhibition(Liu *et al.*, 2023). GC/MS composition of EOs is influenced by geographic region, plant physiologic stage, and pedoclimatic conditions (Chbel*et al.*, 2022).

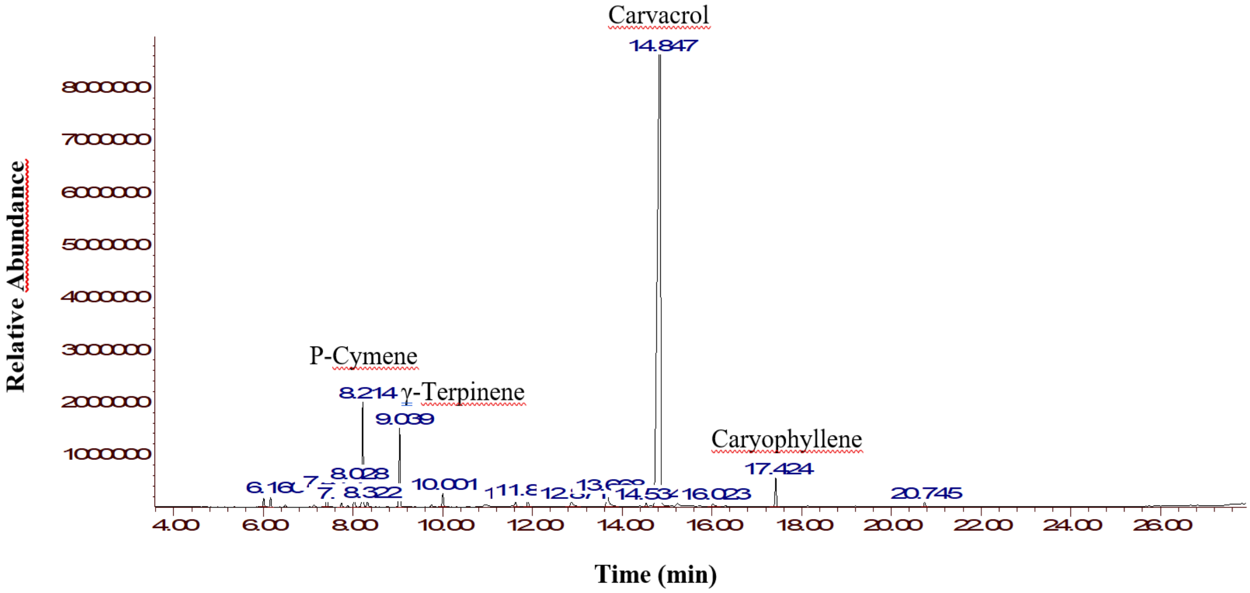


Figure 1: GC/MS spectrum of *Thymus capitatus* L. essential oil from the region of Haouaria (Nabeul, Tunisia)

Fumigant toxicity

Significant mortality was recorded towards the rice weevil *S. oryzae* on cereal grain substrate over 10 days of storage demonstrating a concentration-depend fumigant effect for both thyme EO and its active compound carvacrol. **Figure 2** demonstrated that for Thyme EOs, at the highest concentration, 409.09 µL/L air, a mortality rate of 23.3% was achieved after 10 days of exposure, while the lowest concentration, 163.63 µL/L air led to a mortality rate of 6.7% only. Regarding the effectiveness of the active compound of thyme EO carvacrol, results are illustrated in **Figure 2**. The same trend was observed for carvacrol. At 163.63 µL/L air, a mortality rate of 8.6% was recorded. While, a concentration of245.45 µL/L induces 15.3% of insect mortality. Finally, at the highest concentration of 409.09 µL/L air, mortality reached 100% after 10 days of storage. Notably, carvacrol exhibited higher activity than *T. capitatus* EO after 10 days of storage at the same concentration for the highest concentration (409.09 µL/L air) reaching a mortality rate of 100% (**Figure 2**). The fumigant toxicity of bulk thyme EO and its major compound, carvacrol, was evaluated using Probit analysis

(Finney,1971). The calculated LC_{50} values for thyme EO and carvacrol were 991.962 and 274.195 µL/L air, respectively (**Table 1**).

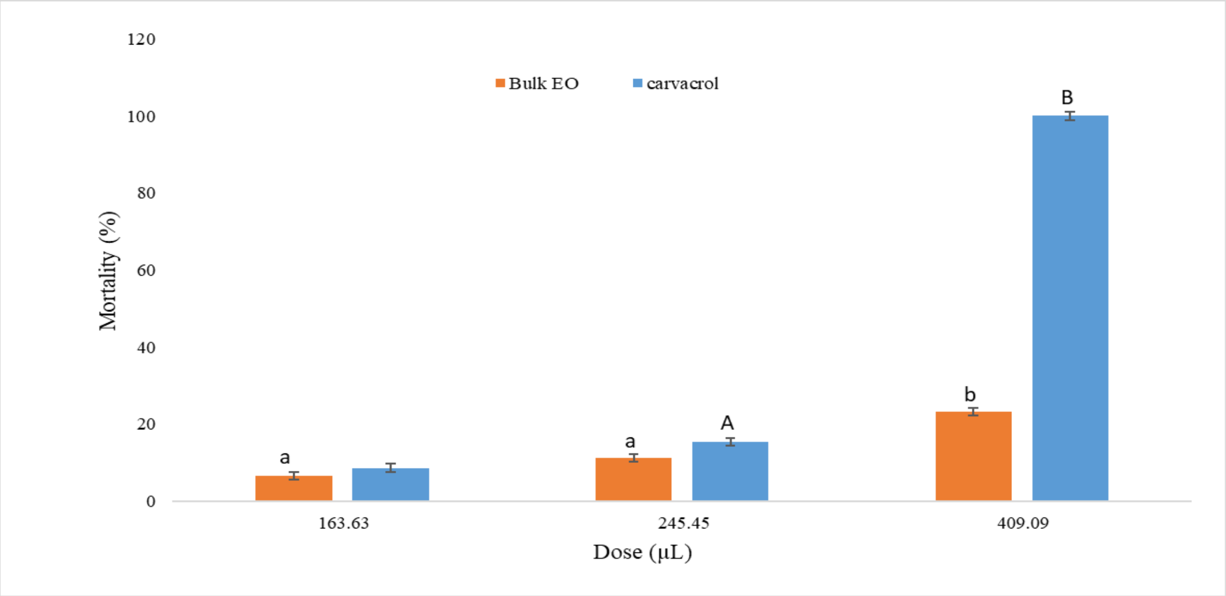


Figure 2: Mortality induced by Bulk Thyme EO and its active compound carvacrol *against S. oryzae*in grain cereal substrate after 10 days of exposure (different letters indicate significant differences (at P<0.05) among thyme EOs for each concentration (lowercase letters) and among carvacrol for each concentration (uppercase letters). Each value is the mean ± SD of three replicates

Table 1: Lethal concentration (LC) of *T. capitatus* essential oil and carvacrol after 10 days of exposure using probit analysis at p< 0.05

	LC^{a}_{50}	LC^{b}_{90}	Slope± SEM	df	Chi-square χ^2
Thyme EOs	991.962	5631.006	0.92 ± 0.924	2	3.841
Carvacrol	274,195	383.380	8.804± 5.134	2	3.841

[a] Units LC_{50}= µL/L of air,[b]LC_{50} applied for 7 days at 25 °C
χ^2= Chi square; df = degrees of freedom.

In this regard, carvacrol displayed significantly greater fumigant response than thyme EOs. Statistical analysis showed a significant difference in insect mortality for both Thyme EOs and carvacrol. Particularly, carvacrol exhibited the strongest significance (P<0.05 and F= 397.705) for insect mortality in comparison with thyme EOs (P<0.05 and F=16.63). Our investigation revealed the fumigant effect of EOs derived from *T. capitatus* and its active compound carvacrol. Both act as a fumigant and toxic agent towards *S. oryzae* adults on cereal grain substrate. Furthermore, toxicity mechanism of *T. capitatus* EOs was undertaken via acetylcholinesterase inhibition. The key conditions to ensure grain storage management are to secure the grains from rodents and insects and to ovoid damage induced by micro-organisms activities (Moboladeet *al*., 2019). Chemical control methods are based on the use of pyrethroids and organophosphates. Nevertheless, they displayed side effects for human, animals and environment. Consequently, several studied focused on ecofriendly grain protectants methods as alternative.
This research provides data on the impact of Thyme EOs and carvacrol including their fumigant toxicity and EOs acetylcholinesterase inhibition. Our work emphasizes the high carvacrol rate in

Tunisian *T. capitatus* EOs which exceeds 75% enhancing the efficacy of this molecule to act as an entomotoxic agent. Several studies have demonstrated fumigant toxicity of Lamiaceae-derived essential oil towards stored product pests (Yeom *et al.*, 2017). In this sense, our results are in accordance with those obtained by (Eltalawy *et al.*, 2024) who displayed that Both thyme EOs and carvacrol demonstrated significant fumigant toxicity *in vitro* against *T. castaneum*, with LC_{50} values of 168.47 and 106.5 µL/L air for the essential oil and carvacrol, respectively, after 24 hours of exposure. The fumigant toxicity of bulk thyme EO and its major compound, carvacrol, was evaluated using Probit analysis (Finney, 1971). The calculated LC_{50} values for thyme EO and carvacrol were 991.962 and 274.195 µL/L air, respectively (**Table 1**).

Similarly, in laboratory tests, *T. capitatus* EOs was found to be more toxic to *Lasiodermaserricorne* than to *Triboliumcastaneum* when applied through both direct contact and fumigation methods (Bachrouch*et al.*, 2022). Moreover, (Annaz *et al.*, 2023) demonstrated that *Thymus capitatus*EOs presents multifaced effects against *T. castaneum* adults like repellent, antifeedant and contact toxicities. The insecticidal efficacy of *T. capitatus* EO is probably due to its major volatile compound, carvacrol (78.35%), a natural phenolic molecule exhibits extensive insecticidal activity and acts as a fumigant against insects in agricultural and stored product settings (Isman, 2000).

Our findings demonstrated dose-response relationship which exert carvacrol capacity to induce significant toxicity against *S. oryzae* adults. In this regard, (Lima *et al.*, 2011) reported fumigant insecticidal effects of carvacrol and *Lippiasidoides* EO against *Tenebrio molitor*, where carvacrol displayed greater toxicity than *L. sidoides*EO (31.68% carvacrol), with LD_{50} values of 5.53 and 8.04 µL/L air, respectively, after 24 hours of exposure. However, these results diverge from those of (Magierowicz *et al.* , 2018), who demonstrated that the insecticidal activity of *Satureja hortensis* EO was more effective than that of its isolated active component, carvacrol.

Acetylcholinesterase inhibition

Results highlighted that rice weevil adults exposed to progressive doses of thyme EO corresponding to 15, 25, and 35 µL caused significant inhibition of AChE activity, with inhibition rates of 96.26%, 68.76%, and 86.10%, respectively after 72 hours of exposure (**Figure 3**).

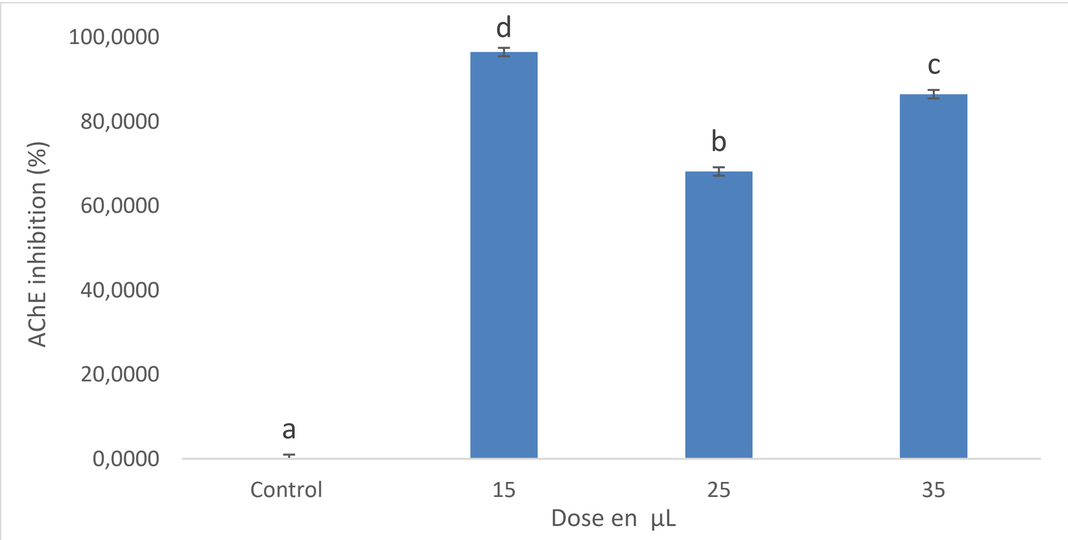


Figure 3: *In vivo* acetylcholinesterase activity of *T. capitatus* essential oil exposed to increasing doses of the oil

The toxicity mechanism of EOs compounds on insects and arthropods is primarily due to their interaction with their nervous system receptors as well as their neurotoxic proprieties. Previous studies demonstrated that monoterpenes and sesquiterpenes induced insect toxicity via affecting the respiratory system. Moreover, these bioactive compounds can penetrate the insect body through cuticle (Adjou *et al*, 2019). Our results are in accordance with those reported by (Plata-Rueda *et al*., 2021) demonstrating that1,8-cineole and carvacrol, possess acetylcholinesterase inhibitory effect on insects. In this regard, (Anderson *et al*., 2012) also reported AChE inhibition by carvacrol towards houseflies, ticks, and cockroaches. Furthermore, carvacrol has been shown to inhibit AChE by interacting with nicotinic acetylcholine receptors (Lu *et al*., 2019).

In this regard, the mode of action of EOs derived from Lamiacae family has been reported by (Bachrouch *et al*., 2023) who indicated a potential action mechanism for the peppermint EO (*Mentha pulegium*), with menthone being the major component inhibiting AChE activity *in vivo* against *Triboliumcastaneum*. Thus, our study revealed significant fumigant effectiveness of Thyme EO against *S. oryzae* could be attributed to its remarkable ability to inhibit AChE, which is enhanced by its high carvacrol content. Similarly, (Saad *et al*., 2018) examined the effects of monoterpenes and phenylpropene compounds on acetylcholinesterase (AChE), highlighting the significant inhibitory effect of menthone on this enzyme.Previous studies showed that EOs tend to inhibit AChE more effectively than their individual volatile components. For example, a synergy between the terpenoids in *Salvia lavandulaefolia* EO has been reported by (Tong *et al*, 2012). It is important to note that the impact of minor compounds should not be overlooked in the context of EO activity. The study undertaken by (Essoung *et al*. , 2020) suggests that these minor components might play a significant role in the overall activity of the EO, contributing to a synergistic effect.The observed divergence in inhibition percentages for various EO doses suggests that AChE inhibition is not the primary action of carvacrol. It is plausible that carvacrol acts as a positive allosteric modulator of GABA receptors in insects, binding to GABA-associated chloride channels on postsynaptic neurons. This interaction disrupts the functioning of GABA synapses, leading to antagonistic effects on the insect nervous system, as described by (Tong *et al*., 2012). Our observations in *S. oryzae* revealed symptoms indicative of neurotoxic activity, such as hyperactivity of the limbs and abdomen, convulsions, and tremors, ultimately leading to total insect mortality.

Our findings indicated that rice weevil adults exposed to increasing doses of thyme essential oil (15, 25, and 35 μL) exhibited significant reduction in AChE activity. The inhibition rates were 96.26%, 68.76%, and 86.10%, respectively, following 72 hours of exposure. In this sense, a careful examination of the data suggests that AChE inhibition is more prominent at lower EO volumes, decreasing at higher volumes due to a saturation effect. At higher concentrations of essential oil, the active sites may become saturated, limiting its inhibitory effect. These observations align with the findings of (Anderson *et al*., 2012), who reported a maximal AChE inhibition of only 57% by carvacrol, even at high exposure concentrations.

Conclusion

Our study was focused on the evaluation of *T. capitatus* EOs and its major component carvacrol on rice weevil adults in cereal grain substrate under semi-industrial conditions during 10 days of storage. GC/MS profiling highlighted that Thyme EOs was rich in carvacrol with 78.35% of the total oil. The present observation showed that both *T. capitatus* and carvacrol exhibited considerable fumigant toxicity against *S. oryzae*, demonstrating superior performance for carvacrol. The neurotoxic action of thyme EO was elucidated by a substantial inhibition of acetylcholinesterase (AChE) in *S. oryzae*, with inhibition rates reaching over 96%. Overall, our research highlights *Thymus capitatus* EO as a promising bio-insecticide with dual mechanisms of neurotoxicity and enzyme inhibition, paving the way for future applications in sustainable agriculture. The EO's multifactorial impact on insect physiology provides a solid foundation for its use as a natural alternative to conventional insecticides, benefiting both crop protection and environmental conservation.

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