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Comparison of chemical composition and antimicrobial activity of essential oils obtained from wild and cultivated *Thymus zygis*

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The present study aims to examine the chemical composition of *T. zygis* essential oils, collected in the wild from the northern region of Morocco during the flowering phase. Additionally, their antimicrobial activities have been evaluated. Hydro-distillation was used to extract the essential oil, and gas chromatography-mass spectrometry was employed for chemical characterization. In parallel, two distinct assays were used to measure antioxidant activity: the Ferric Reducing Antioxidant Power (FRAP) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. Fumigation and repellency were employed to measure insecticidal activity. The results showed that thymol (36.24%), linalool (11.49%), and o-cymene (7.54%) were the main compounds in wild *T. zygis*, while thymol (57.42%) and α-pinene (7.60%) were the main compounds in cultivated *T. zygis*. DPPH free radical scavenging capacity and reduction power were used to quantify antioxidant activity. In the 2,2-diphenyl-1-picrylhydrazyl assay, essential oils of wild *T. zygis* (TZS-EO) exhibited the highest antioxidant capability, with a median inhibitory concentration (IC_{50}) value of 28.449 ± 0.424µg/mL. In the Ferric Reducing Antioxidant Power test, the highest activity was recorded in essential oils of domesticated *T. zygis* (TZD-EO), with a median inhibitory concentration (IC_{50}) value of 65.393 ± 0.899µg/mL, with inhibitory percentages of 83.86 ± 0.00% and 82.30 ± 2.71%, and CMIs of 0.746 ± 0.00µg/mL against *Aspergillus niger*, respectively. TZS-EO and TZD-EO exhibited encouraging antifungal activity against the tested strains. Concerning the fungicidal effect, demonstrated fungicidal effects against all tested fungal strains. Both oils exhibited insecticidal activity against adult *Callosobruchus maculatus*, resulting in absolute mortality at 20µL/L after 2 days of exposure.

Keywords: Antimicrobial activity, Antioxidant activity, Chemical composition, Essential oil, Insecticidal action, *Thymus zygis*

INTRODUCTION

Essential oils are intricate blends of biologically active substances that have long been used as scent additives and as components of numerous commercial products. In fact, the growing demand from the food, cosmetic, and pharmaceutical industries highlights their significance even more. The Lamiaceae family comprises some 7,000 species in 252 genera. Widely used in the Mediterranean region, it is renowned for its medicinal properties, which have been exploited for centuries (Gormez et al., 2015). The *Thymus* genus, a member of the Lamiaceae family (Cutillas et al., 2018). Comprises approximately 350 species of living plants, shrubs, subshrubs, and aromatic herbs. Many of which are unique to the Mediterranean region and are native to northern Africa and Europe (Benabid, 2000). There are 21 species in Morocco, 12 of which are endemic (Benabid, 2000; Fennane & Tattou, 2005). One of these species, *T. zygis* subsp., is found in Morocco's mountainous regions (Yakoubi et al., 2014).

The fragrant, colorful, bushy, and highly polymorphous *T. zygis* plant can reach a height of 10 to 40 cm and features several thin, compressed, dressed branches that are brown or white velvet (Nieto, 2020). *Thyme* leaves are quite small and vary greatly in shape depending on the cultivar. Each species has a slightly different scent (Talovskaya et al., 2018). The flowers are axillary and gathered at the tips of the branches to form a terminal node type (Nieto, 2020). This is a live plant with short or long inflorescences tied to distant and interrupted stalks. small bracts and bracteoles; corolla may or may not surround the calyx. Spring through summer is when the flowering season occurs.

The *Thymus* species is widespread in the Mediterranean region, with a concentration in the northern equatorial regions and along the coast of Greenland, which are the cold, temperate, and hilly parts of the ancient world (Giweli et al., 2013), It is found in Siberia, Northern Europe, the Canary Islands, Madeira, and the Azores regions, as well as the northern portion of Africa (Morocco, Algeria, Tunisia, and Libya) (Morales, 1997). Moroccan wooded clearings and pastures in the low and medium mountains, which are mostly located in the High Atlas, Middle Atlas, and North Atlantic Morocco, as well as along the Mediterranean coast, are home to *T. zygis* thanks to their semi-arid, cool, and humid bioclimate (Fennane et al., 2007). These plants possess a remarkable capacity to adapt to extreme climatic conditions, such as temperature fluctuations and water shortages. Traditional medicine has made use of several *Thymus* species; *T. zygis* is one of the most commonly used species in various regions of Morocco, where its leaves are used as a spice (Schött et al., 2017). Additionally, *T. zygis* is recommended for use in tinctures, powders, infusions, rhymes, carminatives, anti-diarrhea, and the treatment of diabetes, influenza, as well as respiratory, digestive, and gastric disorders (Benlamdini et al., 2014; El Hafian et al., 2014; Hachi et al., 2016; El Brahimi et al., 2022). In the High Atlas Oriental region, *T. zygis* leaves are used as a decoction and carminative to treat diarrhea and colds (Benlamdini et al., 2014). It is also recommended to use *T. zygis* to treat intestinal disorders and influenza (El Hafian et al., 2014; El Azzouzi & Zidane, 2015). According to a 2016 study by Hachi et al. in the Middle Atlas region, four species of *Thymus*, including *T. zygis*, were used to treat diabetes with their stem leaves (Hachi et al., 2016). Research conducted in the Taza region demonstrates that *T. zygis* is used to treat digestive disorders and fevers (El Brahimi et al., 2022).

T. Zygis essential oils are used in the culinary and pharmaceutical industries (Moldão-Martins et al., 1999), as well as in the cosmetic and perfume industries (Stahl-Biskup, 1991). Its chemical characteristics and abundance of phenols or terpene alcohols vary depending on the chemotype (Stahl-Biskup, 1991). These natural compounds also possess strong antibacterial and antioxidant properties, and they hold significant socioeconomic value in the field of biopharmacological research. Monoterpenoid hydrocarbons predominate in the chemical makeup of the essential oil (EO) of *T. zygis*, which was collected at the height of flowering in Khenifra, Middle Atlas (Tantaoui-Elaraki et al., 1993). Aerial portions of *T. zygis* were gathered at different phenological stages (before, during, and after flowering) in three Moyen Atlas regions, and their chemical composition was analyzed (Yakoubi et al., 2014).

A study in Morocco showed quantitative and qualitative variations in EO composition. Carvacrol was the predominant component

in samples collected in the Timahdite and El Hajeb regions, except for the sample collected in the El Hajeb region after flowering, which contained p-cymene (40.26%). In addition, thymol was the primary component of the essential oils (EO) collected before and during flowering in the Azrou region (Yakoubi et al., 2014). This study aimed to investigate the main compounds in the essential oils of wild and domestic *T. zygis* and to study their antioxidant and antibacterial activities.

MATERIALS AND METHODS

Plant material

In the Province of Taza, in the northern part of Morocco, more precisely in the commune of Bouchfaa (coordinates: 35°12'200.100'' N, 5°18'017.900'' W) (Figure 1), aerial parts and grain samples of *T. zygis* were collected in their natural habitat in 2019. The plants have been verified and catalogued by botanist Amina Bari, who has assigned them the reference number 2018Bou/Tz05112 (El Brahimi et al., 2023). Subsequently, samples were presented to the Herbarium of the Department of Biology, Laboratory of Biotechnology, Environment, Agri-food, and Health (LBEAS) at Sidi Mohamed Ben Abdellah University, Faculty of Sciences, Fez, Morocco. The domesticated species was obtained by germinating seeds collected from wild plants in the study area. After collection, the seeds were sorted by hand, sown in trays placed in a greenhouse maintained at room temperature, and followed through to flowering. The aerial parts of both wild and domesticated plants were then harvested and dried for two weeks in the dark at room temperature. Finally, they were stored in bags in the dark

Essential oil extraction

The aerial sections of *T. zygis*, originating from either wild or cultivated plants, have been gathered and allowed to air dry. Next, 200 g of each aerial part were finely chopped and placed into a round-bottom flask containing 770 ml of distilled water. The resulting essential oils (EO) were extracted using a Clevenger-style device through the hydrodistillation process. In anticipation of their future use, the extracted essential oils have been stored at 4°C (El Brahimi et al., 2023).

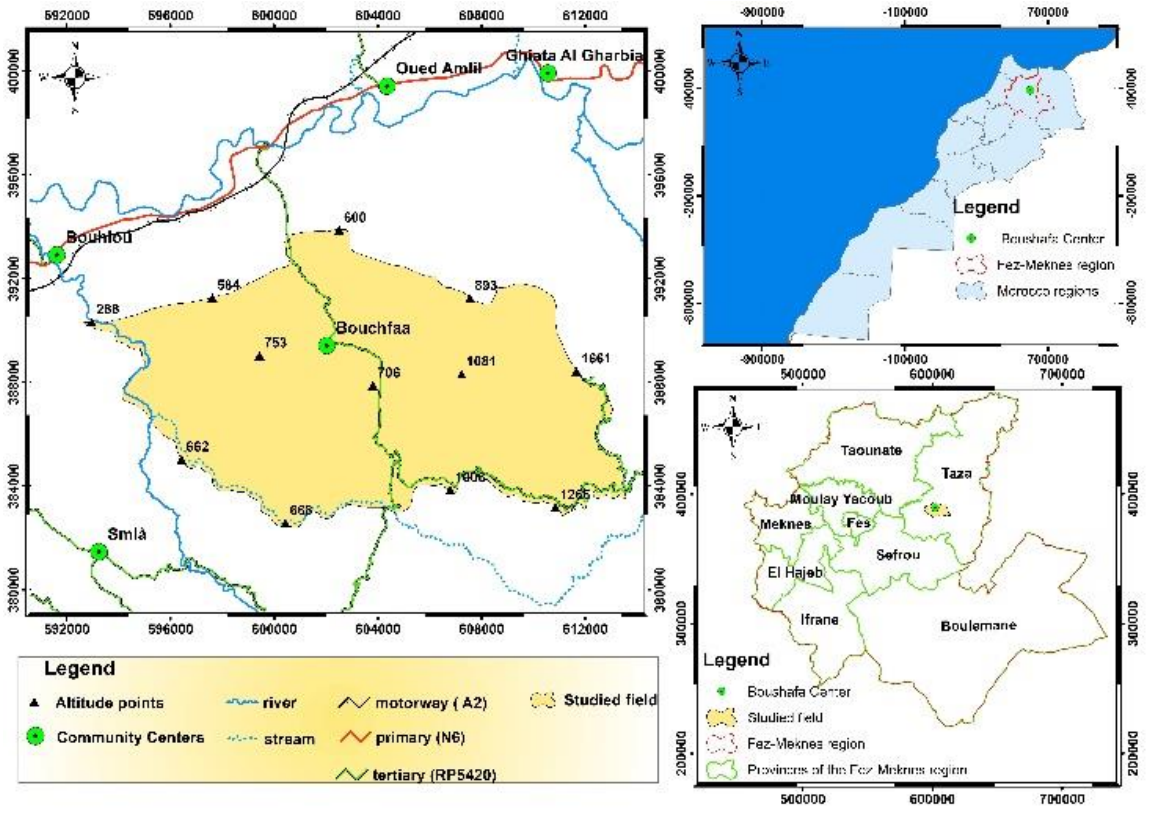


Figure 1. Geographical map of the commune Bouchfâa of Taza province (El Brahimi et al., 2023)

Gas chromatography-mass spectrometry analysis

Gas-phase chromatography coupled to a mass spectrometer was used to identify the various chemical components present in essential oils (El Brahimi et al., 2023). Gas chromatography/mass spectrometry (GC/MS) was used to analyze the constituents of *T. zygis* essential oil, regardless of whether it was derived from wild or domesticated plants. An Agilent Technologies MS model 5973 was connected to an Agilent 19091S-433 HP-5MS column, which was 30meters long, 0.25mm in internal diameter, and had a 0.25µm stationary phase thickness. Helium was used as the carrier gas at a flow rate of 0.9mL/s. The injector and detector temperatures were set to 250°C and 260°C, respectively. The oven temperature was programmed to range from 60 to 300°C, increasing by 10°C per minute, and then held at 300°C for 20min.

Antioxidant activity of essential oils of *Thymus zygis*

2,2-diphenyl-1-picrylhydrazyl (DPPH) method

With slight modifications, the antioxidant capacity of essential oils (EO) was assessed using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method, following the technique described by Wang et al. (2008).

To prepare a DPPH solution, dissolve 4 milligrams of DPPH in 100mL of methanol. Test tubes containing 100µL of diluted hexane essential oil (EO) in methanol were mixed with varying amounts (750µL) of hexane extracts ranging from 0.1 to 2mg/mL and ascorbic acid (vitamin C) at concentrations ranging from 0.1 to 2mg/mL. Then, 1.5mL of the DPPH solution was added. Following a 30min incubation period in the dark at room temperature, the absorbance (OD) was measured at 517nm. The following equation was used to calculate the inhibition percentages (%):

$$\text{Antioxidant activity (\%)} = \frac{(\text{OD control} - \text{OD sample})}{\text{DO control}} \times 100$$

The absorbance of the control (OD control) represents the control's optical density, while the absorbance of the sample (OD sample) corresponds to the experimental optical density. Using quercetin as a positive control, the median inhibitory concentration (IC_{50}) was determined by plotting an inhibition curve corresponding to the oil content.

Ferric reducing antioxidant power (FRAP) method

With slight adjustments, the method described by Shams Moattar et al. (2016) was used to conduct the regression test. Briefly, 2.5mL of *T. zygis* essential oils (from wild or domesticated plants) were mixed with 2.5mL of phosphate buffer (pH 6.60) and 2.5mL of potassium ferricyanide (1%). After a 20min incubation at 50°C, 2.5mL of 10% trichloroacetic acid ($C_2HCl_3O_2$) was added to the mixture, which was then centrifuged for 10min at 3000 rpm (Shams Moattar et al., 2016). The resulting solution was combined with 2.5mL of water and 0.50mL of $FeCl_3$ (0.1%). The Median inhibitory concentration (IC_{50}) was measured by plotting absorbance at 700nm against the corresponding sample concentration. The experiment was carried out in triplicate, and mean values ± SD for IC_{50} were recorded.

Antimicrobial activity of essential oils of *Thymus zygis*

Tested microbial strains

The antimicrobial properties of *T. zygis* essential oils were assessed against four fungal strains (*Aspergillus niger* MTCC282; *Aspergillus flavus* MTCC9606; *Candida albicans* ATCC10231; and *Fusarium oxysporum* MTCC9913) and four bacterial strains (*Escherichia coli* K12; *Staphylococcus aureus* ATCC6633; *Bacillus subtilis* DSM6333; and *Proteus mirabilis* ATCC29906). These samples were obtained from the Laboratory of Biotechnology, Environment, Agri-food, and Health of the Faculty of Sciences Dhar El Mahraz, Sidi Mohammed Ben Abdellah University, Fez, Morocco, and from the Bacteriology Laboratory of Hassan II University Hospital, Fez, Morocco.

Disk diffusion test

The El Barnossi et al. (2020) procedure was followed to evaluate the antimicrobial activity of *T. zygis* essential oils using the disk diffusion method. Petri dishes filled with Mueller-Hinton medium were inoculated with *Aspergillus niger* MTCC282, *Aspergillus flavus* MTCC9606, *Fusarium oxysporum* MTCC9913, and four bacterial strains, and *Candida albicans* ATCC10231 was added (El Barnossi et al., 2020). 20µL of *T. zygis* essential oils were then impregnated onto sterile 6-mm-diameter paper disks and placed in the center of each Petri dish. The inoculation of Petri dishes with fungi, including *Candida albicans*, was done at 30°C for fungal cultures and at 37°C for bacterial cultures.

According to Elegbede & Lateef (2019) and El Barnossi et al. (2020), the inhibition diameters and percentages were measured after 24h for the four bacteria, 48h for *Candida albicans*, and 7 days for *Aspergillus niger*, *Aspergillus flavus*, and *Fusarium oxysporum* (Elegbede & Lateef, 2019; El Barnossi et al., 2020).

Determination of the minimum inhibitory concentration

The procedure described by Sarker et al. (2007) was followed to determine the minimum inhibitory concentration (MIC) of *T. zygis* essential oil as compared to the four bacterial and four fungal strains. This was done using the microdilution technique (Sarker et al., 2007). The microplates were prepared under aseptic conditions, and each sterile 96-well microplate was meticulously labeled. The first column of the plate was then pipetted with 100µL of hexadecyltrimethylammonium bromide (HE) solution in 10% (v/v) dimethyl sulfoxide (DMSO) at a concentration of 10% (v/v). To the other wells, 50µL of sterile malt extract (ME) was added to bacterial strains, whereas 50µL was added to the fungal ones.

A series of dilutions was performed using a multichannel pipette, then each well was filled with 30µL of microbial suspension from each strain. The minimum inhibitory concentration (MIC) was determined following an incubation period of 24h for bacteria, 48h for *Candida albicans*, and 7 days for *Aspergillus niger*, *Aspergillus flavus*, and *Fusarium oxysporum* at 37°C and 30°C, respectively (Sarker et al., 2007; Chebaibi et al., 2016). This is accomplished either directly by monitoring the growth in the wells or by applying the colorimetric method together with the addition of 0.2% (w/v) and resazurin at 1.68mg/mL (Sarker et al., 2007; Chebaibi et al., 2016).

Insecticidal power of *Thymus zygis* essential oils by fumigation

The effectiveness of *T. zygis* essential oils as insecticides in the vapor phase was evaluated through fumigation tests in 1-liter jars. The initial process involved impregnating 3 cm² pieces of Whatman paper with different concentrations of essential oils (4.0, 8.0, 12.0, 16.0, and 20.0µL/L of air). Attaching these impregnated papers to the inside of the jar lids was done to prevent direct contact with insects. The next step was to introduce ten pairs of the insect pest *C. maculatus* aged 0-48 hours into each jar, while the control group did not contain essential oils. For further observation, the jars were placed in the incubation chamber (Baghouz et al., 2022). In this context, Abbott's formula (Abbott, 1925), was applied to calculate the observed mortality rate :

$$\mathbf{Pc} = \left(\frac{\boldsymbol{Po - Pt}}{\mathbf{100 - \boldsymbol{Pt}}}\right) \times \mathbf{100}$$

where, Pc: Corrected mortality, Po: Observed mortality, Pt: Control mortality.

Insecticidal power of *Thymus zygis* essential oils by repellency

The repellency of essential oils (EOs) on adult *C. maculatus* was evaluated using the method provided by McDonald (McDonald et al., 1970). Whatman No. 1 filter paper discs, 8 cm in diameter and split into two equal parts, were used. The first half was exposed to 4.0, 12.0, 16.0, and 20.0µL/cm² of each essential oil, diluted with 0.5mL of acetone. The same volume of acetone was used to treat the second half (control group). Each box was filled with five pairs of adult *C. maculatus* after the sides had been air-dried and adhered together. After 30min, the insects on both faces were recorded. All tests were conducted three times. The percentage of repellency (PR) was calculated based on the formula below:

$$\mathbf{PR}(\%) = \left(\frac{\mathbf{N - NT}}{\mathbf{N + NT}}\right) \times \mathbf{100}$$

where, RP: Percentage of repulsion, N: Number of individuals present in the area treated with acetone only, NT: Number of insects present in the area treated with essential oil mixed with acetone.

Molecular docking of wild and domesticated *Thymus zygis* essential oils for their biological activities

Molecular docking studies were performed using AutoDock Vina 1.5.6 software (Trott & Olson, 2010). This software tool was used to evaluate the possibility of different modes of interaction with the ligand and the biological sites involved. The 3-D crystal structure of Crystal Structure of Recombinant Human Acetylcholinesterase in Complex with Donepezil (PDB ID: 4EY7) to explore the mechanism of antioxidant activity and the crystal structure of Crystal Structure of Staphylococcus aureus nucleoside diphosphate kinase complexed with ADP (PDB ID: 3Q8U) also the structure of beta-ketocyl-[acyl carrier protein] synthase I in complex with Thiolactomycin (PDB ID: 1FJ4) to study the mechanism of antibacterial activity, the proteins were downloaded in PDB

format from the RCSB protein database (www.rcsb.org) and the studied compounds were downloaded from the zinc database (Shaw et al., 2014). The protein was then optimized by adding Geister and Kollman charges, as well as polar hydrogen atoms, and removing water molecules and all heteroatoms. It was then saved in PDBQT format. The energy of the proteins was lowered using an AutoDock technique, and polar hydrogen atoms were added. The structures of the chemical compounds (ligands) were drawn, optimized, and saved in mol2 format, which was then converted to PDBQT format. To identify binding sites, the grid was generated around the active site of each protein using the grid center of x= -14.10, y= -43.83, z= 27.66 for the 4EY7 and x= 6.44, y= -18.96, z= 0.26 for 1FJ4, also x = -16.71, y= 28.54, z= -17.48 for the 3Q8U and with a common grid size: X = 20, Y = 20, Z = 20 with a spacing of 0.375 ˚A. Ligand binding affinity expressed in the kcal/mol unit was scored as a negative classification. With the help of Biovia Discovery Studio Visualizer 4.0 (DSV 4.0) (BIOVIA, 2023), protein-ligand interactions were viewed in 2D and 3D and studied.

Statistical data

The experimental mean in triplicate ± standard deviation was used to analyze the results. ANOVA, or two-way analysis of variance, was employed to identify significant variations in the means. Furthermore, Tukey's multiple comparison tests were run with GraphPad Prism 8.0.1 at a significance level of P< 0.05.

RESULTS

Extraction yield and chemical composition of wild and cultivated *Thymus zygis* essential oils

Table 1 presents the essential oil yields for the essential oils of wild and domesticated *T. zygis* in this study, which were 3.80% and 2.95%, respectively. The chemical composition of TZS-EO and TZD-EO is summarized in Table 2. The chemical composition of TZS-EO and TZD-EO is outlined in Table 2. GC-MS analysis identified a total of 37 compounds in TZS-EO (Figure 2A), whereas only 8 compounds were detected in TZD-EO (Figure 2B).

Table 1. The EO yields of *T. zygis,* wild (TZS-EO) and cultivated (TZD-EO)

	Yield %
TZS-EO	3,80
TZD-EO	2,95

Antioxidant activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) test

The free radical scavenging activity (DPPH) of wild and domesticated *T. zygis* plants (Figure 3) shows a significant difference in the oil extracts from each plant. The inhibition values increase rapidly for concentrations between 0 and 200µg/mL, then stabilize at higher concentrations (above 1000µg/mL). The analysis of the curves reveals that TZS-EO exhibits a stronger effect than TZD-EO, demonstrating a greater potential to neutralize free radicals. The maximal inhibition percentage is observed at a concentration of 1000µg/mL for TZS-EO and 80.588 ± 0.337% (P< 0.0001 vs quercetin) for TZD-EO. The IC50 values further indicate significant differences between the two oil extracts (P< 0.0001). TZS-EO shows a marked superiority with an IC50 value of 28.449 ± 0.424µg/mL (P< 0.05 vs quercetin), compared to 89.055 ± 3.791µg/mL (P< 0.0001 vs quercetin) for TZD-EO (Table 3).

Ferric Reducing Antioxidant Power (FRAP) test

The analysis of the absorbance curves (FRAP) for the essential oil extracts from *T. zygis* (Figure 3) reveals similar profiles for both TZS-EO and TZD-EO, with absorbance increasing to a maximum value of 0.75 ± 0.009 for TZD-EO and 0.729 ± 0.008 for TZS-EO at a concentration of 1000 µg/mL. As indicated by the IC50 values, the concentrations required for the two oil extracts to achieve similar effects are significantly different (P< 0.0001). Additionally, TZD-EO shows a notable superiority, with an IC50 value of 53.829 ± 0.401 µg/mL (P < 0.05 vs. Quercetin), compared to 74.647 ± 2.404 µg/mL (P< 0.0001 vs. Quercetin) for TZS-EO (Table 3).

Antibacterial activity

The antibacterial activity of *T. zygis* essential oils, both wild (TZS-EO) and cultivated (TZD-EO), was evaluated using the disk diffusion method, which measures the sensitivity of microbial strains to essential oils. As shown in Table 3, both TZS-EO and TZD-EO demonstrated significant antibacterial activity against all tested bacterial strains. The data analysis revealed significant differences in the inhibition zones of TZS-EO and TZD-EO, as well as differences depending on the bacterial strain tested (Figure 4). When tested against four different bacterial strains, the antibacterial effect of the essential oils from domesticated plants was significantly stronger than that of the wild plants. The most promising inhibitory effect was observed against *Staphylococcus aureus* ATCC6633, with

inhibition zones of 48± 1.73% for TZD-EO and 41.67± 0.58% for TZS-EO. This was followed by *Escherichia coli* K12, with inhibition diameters of 44.33± 1.15 and 34.67± 2.31; *Proteus mirabilis* ATCC29906, with inhibition diameters of 42 ± 0.00 and 39.33± 1.15; and *Bacillus subtilis* DSM6333, with inhibition diameters of 34.33 ± 0.58 and 21.67± 0.58 for TZD-EO and TZS-EO, respectively.

Table 2. Chemical composition of essential oils in wild and cultivated *T. zygis* identified by gas chromatography-mass spectrometry (GC-MS). (TZS-EO: Essential oil of wild *T. zygis*, TZD-EO: Essential oil of cultivated *T. zygis*, RI: Retention index, CT: Calculate, LT: Literature, (-) absence)

Compounds	RI		TZS-EO		TZD-EO	
	CT	LT	R.T	Area %	R.T	Area %
α-Pinene	939	939	7.899	0.30	7.901	7.60
Camphene	954	954	8.373	0.14	8.375	2.86
1-octen-3-ol	980	980	9.177	0.71	-	-
3-Octanone	985	985	9.337	0.14	-	-
3-Octanol	993	992	9.691	0.16	-	-
o-Cymene	1026	1026	10.570	7.54	-	-
trans-3-Caren-2-ol	1011	1011	10.710	0.07	-	-
Eucalyptol	1031	1031	10.813	0.16	-	-
Linalool, oxide	1048	1048	11.956	0.38	-	-
1-Nonen-3-ol	1086	1085	12.221	0.22	-	-
1,2-Oxidolinalool	1072	1072	12.435	0.35	-	-
Linalool	1096	1096	12.835	11.49	-	-
l-Menthone	1162	1162	14.518	0.68	-	-
Isoborneol	1160	1160	15.007	2.83	15.000	5.42
4-Carvomenthenol	1263	1263	15.257	1.31	-	1.31
4-isopropylbenzyl alcohol	1162	1162	15.402	0.58	-	0.58
(1S)-1,3,3-trimethylnorbornan-2-ol	1173	1173	15.666	0.41	-	0.41
cis-Dihydrocarvone	1192	1192	15.741	0.28	-	0.28
(+)-isodihydrocarvone	1214	1214	15.940	0.16	-	0.16
(-)-cis-Carveol	1226	1225	16.350	0.16	-	0.16
Carvacrolmethylether	1244	1244	16.637	0.15	-	0.15
Pulegone	1237	1237	16.962	1.37	-	1.37
Piperitone oxide	1256	1256	17.334	0.25	-	0.25
Carvacrol	1299	1299	18.073	1.60	-	1.60
Thymol	1290	1290	18.373	36.24	-	57.42
p-Cymen-7-ol	1290	1290	18.464	0.20	-	0.20
o-Thymol	1290	1290	18.614	27.23	-	27.23
Piperitenone	1343	1343	19.677	0.11	-	0.11
Rotundifolone	1459	1459	20.246	0.54	-	0.54
Caryophyllene	1466	1464	21.897	0.30	-	0.30
4-ethyl-2-methoxy-6-methyl-phenol	1548	1548	22.231	0.31	-	0.21
(-)-Spathulenol	1578	1578	25.974	0.30	-	0.20
Caryophyllene oxide	1465	1467	26.160	2.50	-	2.50
Isoaromadendrene epoxide	1455	1455	28.238	0.53	-	0.53
Alloaromadendrene oxide-(1)	1625	1625	28.629	0.27	-	0.22
D-Limonene	1029	1029	-	-	10.715	1.52
Camphor	1141	1140	-	-	14.260	19.82
L-alpha-Terpineol	1187	1188	-	-	15.658	5.35
Hydrocarbon monoterpenes			**12.17**		**-**	
Oxygenated monoterpenes			**82.26**		**99.99**	
Hydrocarbon sesquiterpenes			**-**		**-**	
Oxygenated sesquiterpenes			**3**		**-**	
Others			**2.54**		**-**	
Total (%)			**99.97**		**99.99**	

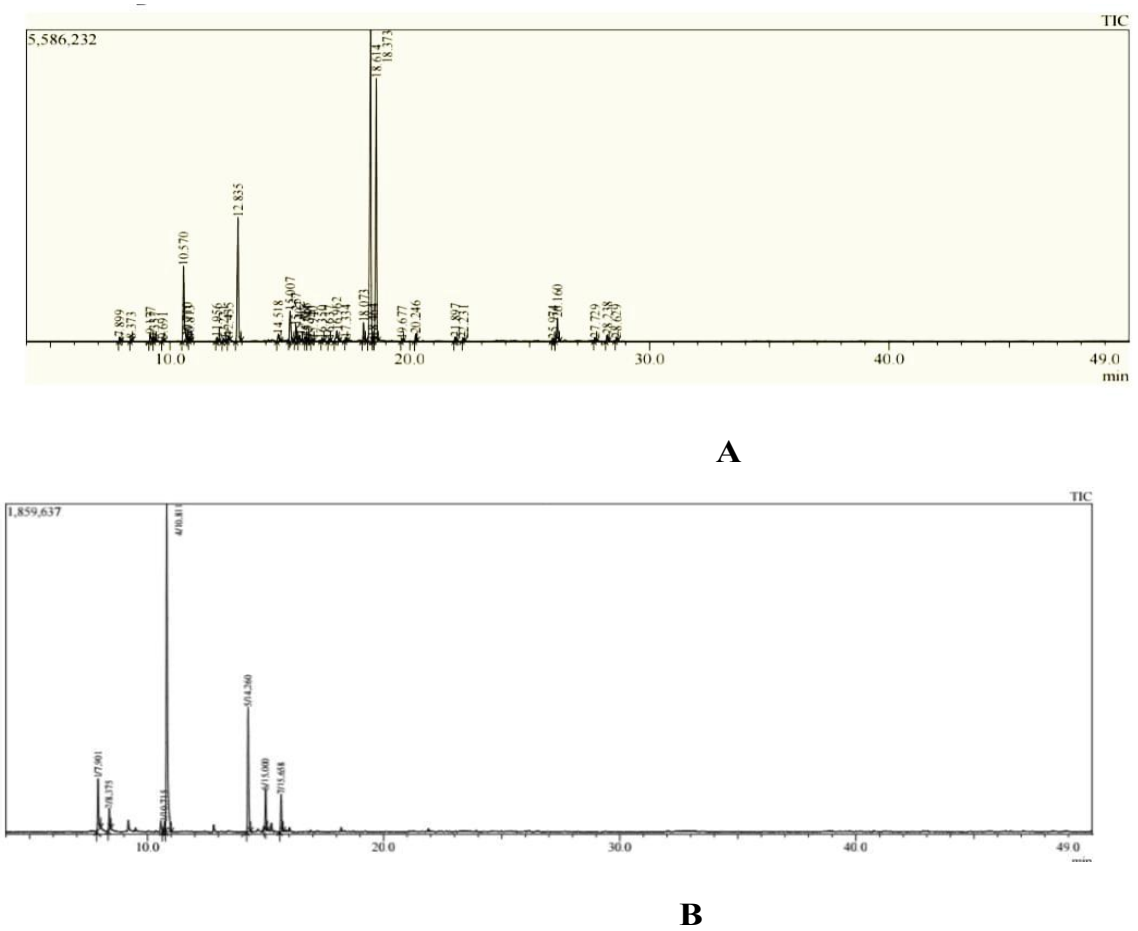


Figure 2. Chromatograms of TZS-EO (A) and TZD-EO (B)

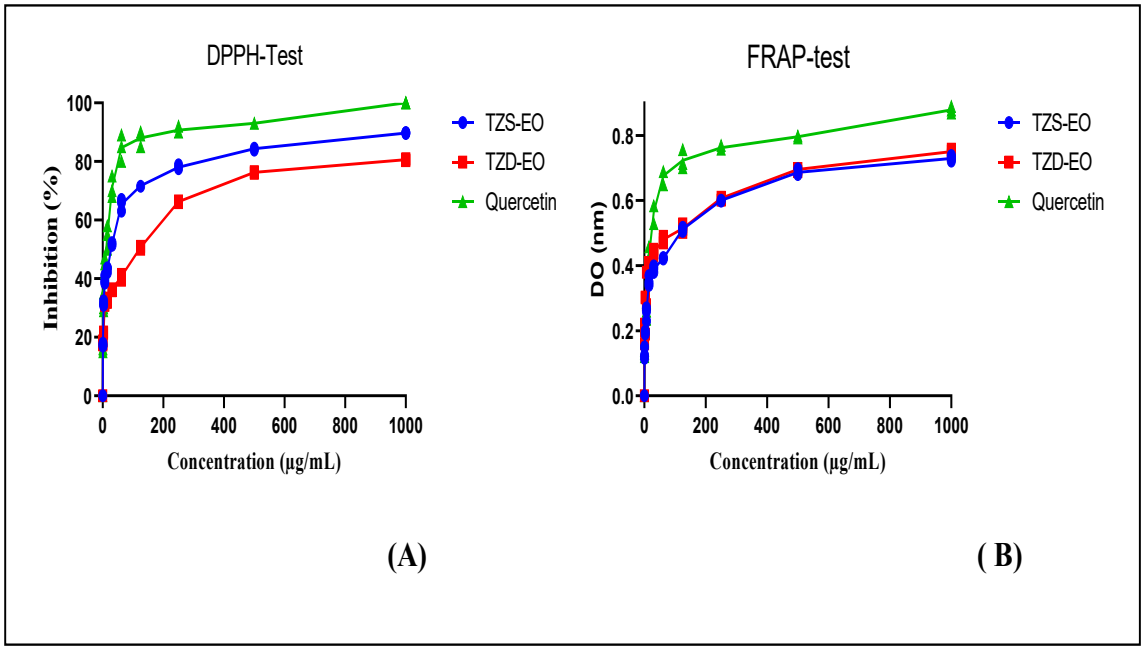
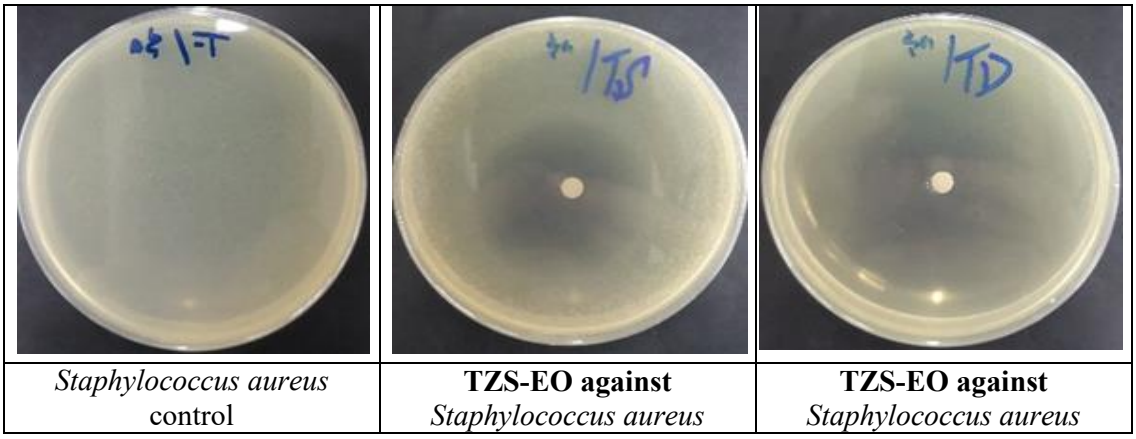


Figure 3. Percentage DPPH and FRAP inhibition of wild and cultivated *T. zygis* species

Table 3. Antibacterial activity and MICs of TZS-EO; EO of wild T. zygis. TZD-EO; cultivated *T. zygis* EO

		TZS-EO	TZD-EO	Streptomycin
Staphylococcus aureus ATCC6633	Inhibition diameter (mm)	41.67 ± 0.58[a]	48± 1.73[b]	11 ± 1.00 [c]
	MIC (µg/mL)	0.74 ± 0.00 [a]	0.74 ± 0.00 [a]	1.56 ± 0.00 [b]
Escherichia coli K12	Inhibition diameter (mm)	34.67± 2.31[a]	44.33± 1.15 [b]	0.00 ± 0.00 [c]
	MIC (µg/mL)	1.49 ± 0.00 [a]	0.74 ± 0.00 [b]	-
Bacillus subtilis DSM6333	Inhibition diameter (mm)	21.67 ± 0.58[a]	34.33± 0.58 [b]	0.00 ± 0.00 [c]
	MIC (µg/mL)	2.98 ± 0.00 [a]	1.49 ± 0.00 [b]	-
Proteus mirabilis ATCC29906	Inhibition diameter (mm)	39.33± 1.15[a]	42± 0.00[b]	0.00 ± 0.00 [c]
	MIC (µg/mL)	0.74 ± 0.00 [a]	0.74 ± 0.00 [a]	-

Means (± standard deviation, n= 3) designated by the same letter in the same row did not indicate a significant difference according to Tukey's multiple range tests at P< 0.05.



Staphylococcus aureus control | **TZS-EO against** *Staphylococcus aureus* | **TZS-EO against** *Staphylococcus aureus*

Figure 4. Antibacterial activity of TZS-EO and TZD-EO against *Staphylococcus aureus*

Antifungal activity

The studied *Thymus zygis* essential oils (TZS-EO and TZD-EO) exhibited a potent antifungal effect against the tested strains. Table 4 and Figure 5 summarize the inhibition percentages and minimum inhibitory concentrations of TZS-EO and TZD-EO against known fungal pathogens. Both oils demonstrated strong antifungal activity, with inhibition percentages reaching 70.00± 0.00% for *Fusarium oxysporum*, *Aspergillus flavus*, *Aspergillus niger*, and *Candida albicans* ATCC10231. For TZD-EO, the inhibition percentage was 70.00± 0.00%, compared to 59.67± 1.15% for TZS-EO. The MIC values for both oils were 0.746± 0.00µg/mL for *F. oxysporum*, *A. flavus*, and *A. niger*, except for TZD-EO against *A. niger* MTCC-82, where the MIC was 1.49± 0.00µg/mL. For *C. albicans* ATCC10231, the MIC was remarkably low at 0.186± 0.00µg/mL for both oils. These results highlight the strong antifungal properties of both TZS-EO and TZD-EO, with TZD-EO showing slightly greater effectiveness against certain fungal strains.

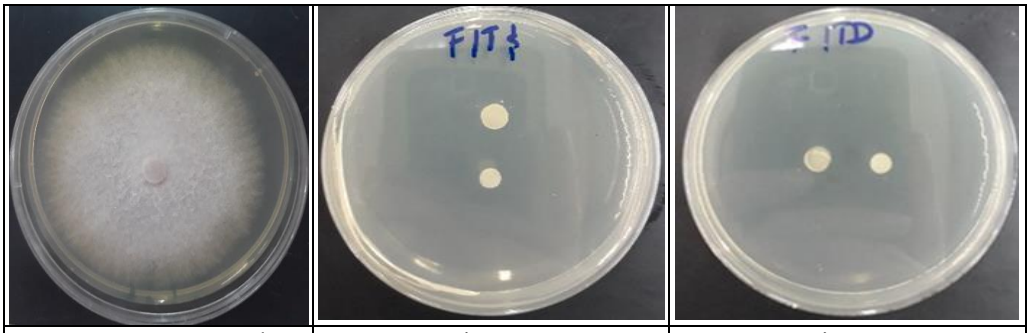
Insecticidal activity by fumigation

The results of the fumigation bioassay for wild and cultivated *T. zygis* essential oils (TZS-EO and TZD-EO) indicate that the insecticidal efficacy of these products depends on the oil dose, plant origin, and exposure time (Table 5). Our tests demonstrated that both oils were effective in eliminating *Callosobruchus maculatus* adults. TZS-EO exhibited greater potency than TZD-EO, achieving 100% insect mortality at a high dose (20.0µL/L) after 48h of exposure. In contrast, TZD-EO achieved complete mortality at a dose of 16.0µL/L after 72h of exposure. This study also evaluated the lethal concentration (LC_{50}) of the oils. The findings revealed that TZS-EO was more effective than TZD-EO at killing the test pests. After 24h of exposure, the LC50 for TZS-EO was 4.17µL/L of air, while TZD-EO had an LC50 of 6.55µL/L. Similarly, after 48 hours of exposure, TZS-EO had an LC50 of 1.80µL/L of air, compared to 3.37µL/L for TZD-EO (Table 6). These results highlight the superior insecticidal efficacy of TZS-EO over TZD-EO under the tested conditions.

Table 4. Antifungal activity and minimum inhibitory concentrations (MICs) of TZS-EO and TZD-EO

		TZS-EO	TZD-EO	Fluconazole
Candida albicans ATCC10231	Inhibition diameter (mm)	59.67± 1.15 [a]	70.00 ± 0.00 [b]	0.00 ± 0.00 [c]
	MIC (µg/mL)	0.186 ± 0.00 [a]	0.186 ± 0.00 [a]	-
Aspergillus niger MTCC282	Inhibition diameter (%)	70.00± 0.00 [a]	70.00± 0.00 [a]	18.46 ± 2.02 [b]
	MIC (µg/mL)	0.746 ± 0.00 [a]	1.49 ± 0.00 [b]	7.125 ± 0.00 [c]
Aspergillus flavus MTCC9606	Inhibition diameter (%)	70.00± 0.00 [a]	70.00± 0.00 [a]	0.00 ± 0.00 [b]
	MIC (µg/mL)	0.746 ± 0.00 [a]	0.746 ± 0.00 [a]	-
Fusarium oxysporum MTCC9913	Inhibition diameter (%)	70.00± 0.00 [a]	70.00± 0.00 [a]	30.77 ± 0.58 [b]
	MIC (µg/mL)	0.746 ± 0.00 [a]	0.746 ± 0.00 [a]	3.125 ± 0.00 [b]

Means (± SD, n= 3) with similar letters on the same line indicate a significant difference according to Tukey's multiple tests at P< 0.05.



F. oxysporum control | TZS-EO against *F. oxysporum* | TZD-EO against *F. oxysporum*

Figure 5. Antifungal activity of TZS-EO and TZD-EO against *Fusarium oxysporum* MTCC9913.

Table 5. Effect of wild and cultivated *T. zygis* EOs on *C. maculatus* mortality.

EOs	Doses	Exposure time (h)			
	(µL/L)	24h	48h	72h	96h
TZS-EO	4	53.33 ± 5.77	75 ± 8.66	98.4 ± 2.88	100 ± 0
	12	56.66 ± 7.63	80 ± 10	100 ± 0	100 ± 0
	16	70 ± 10	96.66 ± 5.77	100 ± 0	100 ± 0
	20	98.33 ± 2.88	100 ± 0	100 ± 0	100 ± 0
	Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0
TZD-EO	4	43.33 ± 7.63	58.33 ± 7.63	85 ± 5	100 ± 0
	12	56.7 ± 5.77	68.33 ± 2.88	88.33 ± 7.63	100 ± 0
	16	60 ± 10	78.33 ± 7.63	100 ± 0	100 ± 0
	20	76.66 ± 7.64	96.66 ± 5.77	100 ± 0	100 ± 0
	Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Table 6. Lethal concentration, and chi-square (_2) values of wild and cultivated *T. zygis* EOs by fumigation test on *C. maculatus* adult

	Days	LC_{50} (µl/L)	95% CI	LC_{90} (µl/L)	95 % CI	*df*	*χ2*
TZS-EO	24	4.17	---	76.74	---	2	2.01
	48	1.80	0.002-4.047	11.04	6.043-52.506	2	2.27
	72	1.04	---	2.37	---	2	0.002
	96	---	---	---	---	---	---
TZD-EO	24	6.55	---	113.53	---	2	0.97
	48	3.37	0.059 -6.274	27.061	14.930-1176.315	2	3.43
	72	1.27	0-3.280	6.60	0.891-17.128	2	2.712
	96	---	---	---	---	---	---

Insecticidal activity by Repellency

The repellent activity of TZD-EO and TZS-EO against *C. maculatus* adults was assessed. TZD-EO demonstrated repellent activity at a concentration of 12µL/L, effectively repelling more than 60% of the insects within 30 minutes of application. In contrast, TZS-EO exhibited repellent activity against *C. maculatus* adults at concentrations of 16µL/L and higher. According to McDonald's (1970) classification, TZD-EO was considered moderately repellent to adult *C. maculatus*, with a mean percentage repellency (PR%) of 55%. In comparison, TZS-EO showed stronger repellency against this pest, with a PR% of 61.25% (Table 7).

Molecular docking of wild and cultivated *Thymus zygis* essential oils for their biological activities

The docking results obtained from Table 8 illustrate the docking scores of Caryophyllene oxide against the studied proteins. From Figure 6, we can see that Caryophyllene oxide forms one conventional hydrogen bond with residue HIS447, from the active site of Recombinant Human Acetylcholinesterase (4EY7), and that ligand showed the best docking score among the other studied compounds with a binding energy of -8.5 kcal/mol. The analysis of the molecular docking of Caryophyllene oxide with *Staphylococcus aureus* nucleoside diphosphate kinase (3Q8U) protein showed that the ligand formed a hydrophobic Pi-Alkyl interaction with HIS 52, whose binding energy is -6.2kcal/mol (Figure 7). Figure 8 shows that Caryophyllene oxide has a binding energy of -8.0kcal/mol manifested in hydrophobic Pi-Alkyl interaction with PHE 392 from synthase I in complex with Thiolactomycin (1FJ4). In sum, Caryophyllene oxide has a good binding pose with all the studied proteins, having a binding energy from -6.2 kcal/mol to -8.5kcal/mol.

Table 7. Repellent effect of wild and cultivated *T. zygis* EOs against *C. maculatus*

EOs	Dose (µL/ cm²)				Average rate of repulsively (%)	Repulsively class
	4	12	16	20		
TZS-EO	50 ± 11.54	60 ± 16.34	65 ± 10	70 ± 11.55	61.25	Repulsive
TZD-EO	45 ± 10	50 ± 11.54	60 ± 16.33	65 ± 19.15	55	Moderately Repulsive

Table 8. Docking results of "*Thymus zygis*" in active site of acetylcholinesterase (4EY7) *E. coli* beta-ketoacyl-[acyl carrier protein] synthase(PDB: 1FJ4)), and *S. aureus* nucleoside diphosphate kinase (PDB: 3Q8U) by using auto dock-vina

	Antioxidant activity		Antibacterial activiy			
	PDB ID : 4EY7		PDB ID : 3Q8U		PDB ID : 1FJ4	
	Docking score (kcal/mol)	predominant type of interaction	Docking score (kcal/mol)	predominant type of interaction	Docking score (kcal/mol)	predominant type of interaction
α-PINENE	-6.7	Hydrophobic	-5.0	Hydrophobic	-6.2	Hydrophobic
CAMPHOR	-6.7	Hydrophobic	-4.9	Hydrophobic	-5.9	Hydrophobic
CAMPHENE	-6.6	Hydrophobic	-5.1	Hydrophobic	-5.8	Hydrophobic
O-CYMENE	-7.1	Hydrophobic	-6.1	Hydrophobic	-6.0	Hydrophobic
LINALOOL	-6.5	Hydrophobic	-5.5	Hydrophobic	-6.1	Hydrophobic
ISOBORNEOL	-6.5	Hydrophobic	-4.6	Hydrophobic	-6.0	Hydrophobic
O-THYMOL	-7.4	Hydrophobic	-6.1	Hydrophobic	-6.6	Hydrophobic
THYMOL	-7.4	Hydrophobic	-6.1	Hydrophobic	-6.5	Hydrophobic
CARYOPHYLLENE OXIDE	-8.5	Conventional hydrogen bond	-6.2	Hydrophobic	-8.0	Hydrophobic

DS: Docking score (kcal/mol); PTI: predominant type of interaction

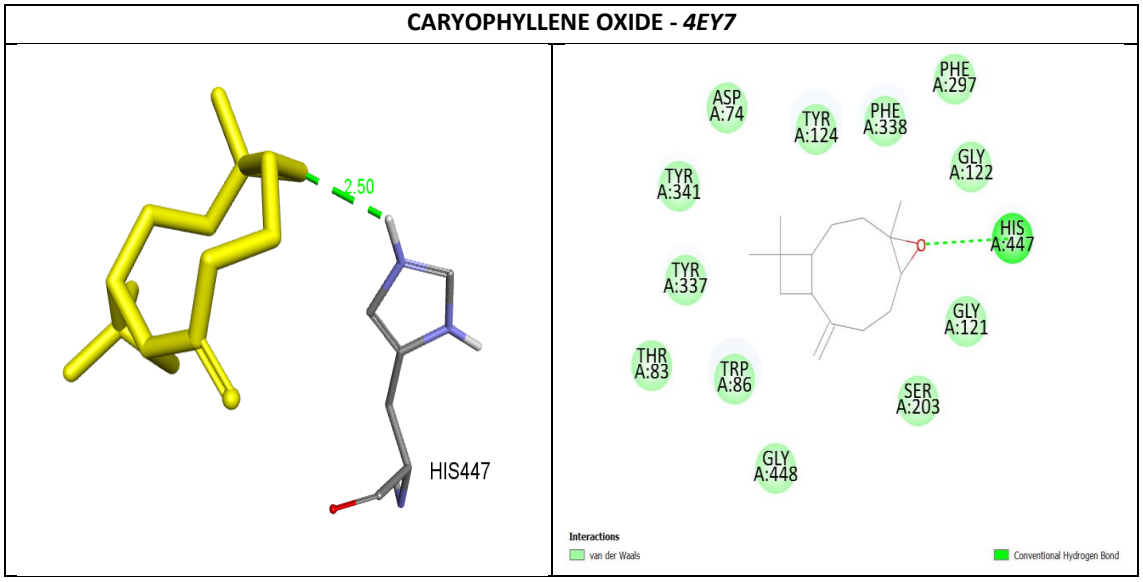


Figure 6. The molecular docking results of Caryophyllene oxide with 4EY7 protein, surfaces around ligand and 2D forms

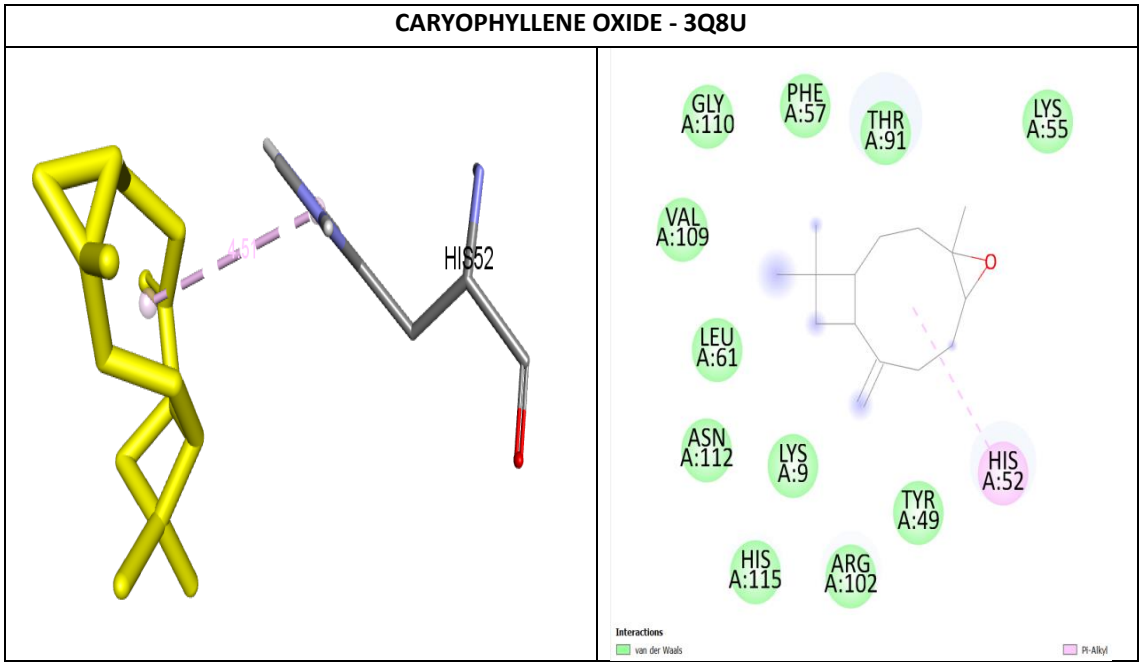


Figure 7. The molecular docking results of Caryophyllene oxide with 3Q8U protein, surfaces around ligand and 2D forms

DISCUSSION

This study examines the chemical composition, antioxidant properties, and biological activities of wild and cultivated *T. zygis*. In our investigation, we extracted 3.80% and 2.95% essential oil from wild and cultivated *T. zygis* plants, respectively. These yields are higher than those reported by Bouymajane et al. (2022) (1.5%) and Radi et al. (2021) (3%) for *T. zygis* collected from the Moyen Atlas region of Morocco.

The *T. zygis* essential oils analyzed in this study show chemical variability, with slight differences in the proportions of major and minor constituents. In TZS-EO, 99.97% of the total composition was identified, comprising mainly oxygenated monoterpenes (82.26%), hydrocarbon monoterpenes (12.17%), and oxygenated sesquiterpenes (3%). The dominant components are o-cymene (7.54%), linalool (11.49%), and thymol (36.24%). In contrast, TZD-EO is composed almost entirely (99.99%) of oxygenated monoterpenes, with α-pinene (7.60%) and thymol (57.42%) the main constituents. Our results are

comparable to those previously reported in various Moroccan regions. Thymol (37.5%) was identified as the main component in our study. This finding aligns with research conducted on the aerial parts of *T. zygis* in the Taza region, where thymol was reported as the main component at a concentration of 37.5% (Zayyad et al., 2014). Similarly, another study on the aerial parts of *T. zygis* in the Khenifra region identified thymol as the main component, but at a slightly higher concentration of 42.5% (Ouknin et al., 2020). However, our results differ from those reported by Yakoubi et al. (2014), who analyzed *T. zygis* collected from three regions in the Moyen Atlas during different phenological stages (pre-flowering, flowering, and post-flowering). In that study, carvacrol (16.07–74.33%) was the predominant constituent, followed by thymol (1.47–32.46%) (Yakoubi et al., 2014). Bouymajane et al. (2022), reported that *T. zygis* collected from the Ifrane region had a chemical composition dominated by p-cymene (25.98 ± 0.07%), thymol (22.64 ± 0.06%), carvacrol (21.28 ± 0.06%), and γ-terpinene (11.01 ± 0.03%). In contrast, Radi et al. (2021) revealed that the main constituents of *T. zygis* essential oils in the Moyen Atlas region were carvacrol (52.20%), o-cymene (23.14%), and thymol (9.68%). Another menu study on the Portuguese *T. zygis* reveals that the main constituents were p-cymene (10.58%), carvacrol (13.00%), and thymol (43.17%) (Coimbra et al., 2022). Thymol was identified as the main component, while carvacrol and cymene were found in significant amounts relative to the other components, as presented in the literature on the EOs of *T. zygis* (Coimbra et al., 2022). Additionally, Gómez et al. (1995) reported that the essential oils of the *Thymus* in the Almeria region contained 38.7% linalool and 18.4% trans-sabinene hydrate. These variations in chemical composition can be attributed to several factors, including environmental conditions, plant physiology, extraction techniques, and drying methods (Zhang et al., 2015; El-Sharkawy, 2018; Ed-Dra et al., 2020).

In terms of antioxidant properties, the essential oil of wild *T. zygis* exhibited the highest DPPH scavenging activity, with an IC_{50} value of 28.449± 0.424µg/mL, compared to 89.055 ± 3.791µg/mL for cultivated *T. zygis*. However, these values are significantly less effective (P< 0.0001) than the positive control, quercetin, which demonstrated an IC_{50} of 17.733± 1.788µg/mL. According to a different study conducted on *T. zygis* collected from the Ifran region, essential oils exhibit antioxidant properties (Bouymajane et al., 2022). Similarly, other studies have reported the antioxidant activity of *T. zygis* essential oils from various regions (Amarti et al., 2011; Salas et al., 2012; Amorati et al., 2013; El-Sharkawy, 2018; Ed-Dra et al., 2020).

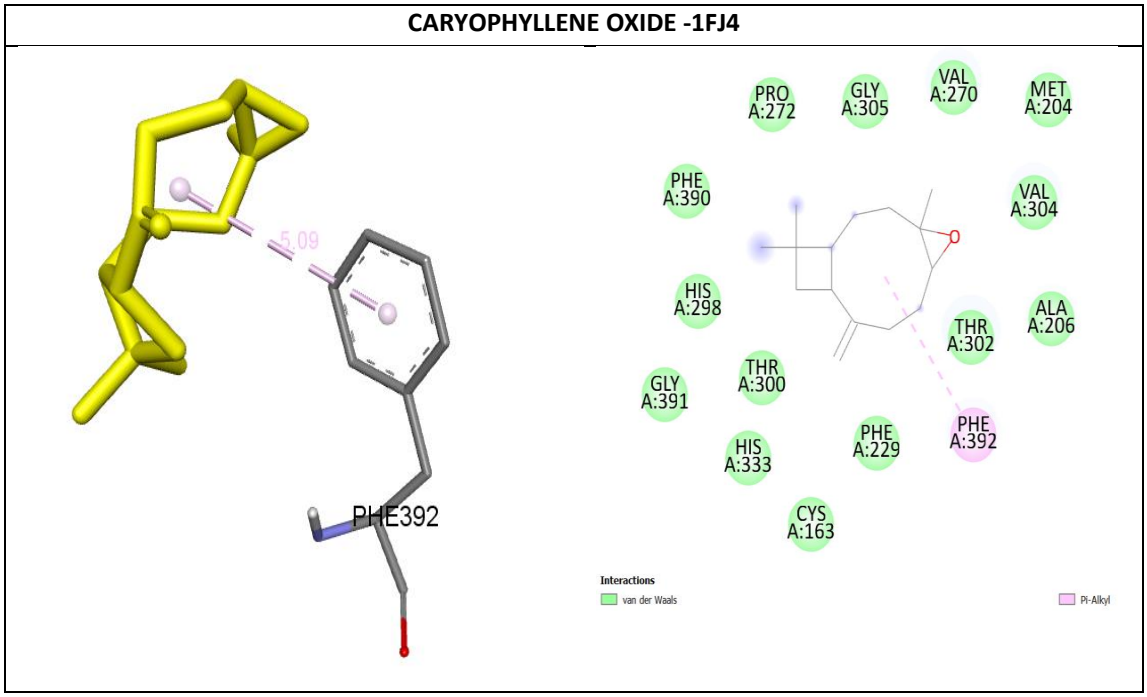


Figure 8. The molecular docking results of Caryophyllene oxide with 1FJ4 protein, surfaces around ligand and 2D forms

Antioxidant activity is closely linked to the chemical composition of essential oils (EO) (Amorati et al., 2013; El-Sharkawy, 2018; Ed-Dra et al., 2020). The significant primary antioxidant properties of *T. zygis* essential oils are mainly attributed to the synergistic effects of key monoterpenes, including p-cymene, thymol, carvacrol, and γ-terpinene (Amarti et al., 2011; Salas et al., 2012). However, factors such as phenological stage, climatic conditions, and the synergistic or antagonistic interactions between EO components can also influence antioxidant activity (Milos & Makota, 2012; Salas et al., 2012). Its effectiveness as a good antioxidant agent has been demonstrated by studies conducted by Milos & Makota (2012) and Rúa et al. (2019). The richness of essential oils of *T. zygis* in thymol and carvacrol enhances its antioxidant power.

Regarding the bactericidal and bacteriostatic effects, TZS-EO and TZD-EO have demonstrated a bacteriostatic effect against all tested bacterial strains. The functioning of the main compounds (alcohols, phenols, aldehydes) and the synergistic effects between the compounds may contribute to the promising antibacterial activity of TZS-EO and TZD-EO against the pathogenic bacterial strains tested. Consequently, the most effective chemical compounds with a broad spectrum of antibacterial action are the phenols (thymol, carvacrol, and eugenol) (Dorman & Deans, 2000; Oussalah et al., 2006).

In terms of fungicidal and fungistatic effects, TZS-EO and TZD-EO were fungicidal against all the fungi tested. The amount of bioactive compounds, such as thymol and carvacrol, in the essential oils can be linked to the considerable antifungal activity demonstrated by TZS-EO and TZD-EO against the pathogenic fungal strains examined. In traditional pharmacopoeia, the *Thymus* species was used against bacteria and mushrooms (Debourgogne, 2021). The essential oils of *T. zygis* showed the highest diameter of inhibition, which were roughly 40mm for *C. albicans*, 18mm for *Condida globrata*, 27mm for *Candida ssp*, 18 mm for *F. solani*, and 40mm for *Aspergillus fischeri*, according to a study conducted by Radi et al. (2021) on strains of fungi (*C. albicans*, *C. globrata*, *Candida spp*., *A. fischeri*, and *F. solani*).

EOs are aromatic compounds with multiple virtues, constituting an essential natural defense system for plants. Several essential oils extracted from plants are known for their insecticidal properties, by fumigation, contact, and repulsion against various insects found in stored food products (Ainane et al., 2023; Tourabi et al., 2023). Generally speaking, there isn't much research on the use of *T. zygis* essential oil to combat food pests, and no studies have been conducted to compare the fumigant and repellent properties of domestic and wild *T. zygis* against the insect pest of Cowpea seeds, *C. maculatus*. The insecticidal activity of *T. zygis* essential oil on insect pests of stored products has been demonstrated by previous studies, such as that carried out by Abdoul-latif et al. (2021), which revealed that *T. zygis* EO is highly toxic to Aphis fabae and has been shown to cause total mortality in adults after 24 hours of exposure at a dose of 1.5µl/mL. And Kim et al. **(**2016**)** showed that the EO of *T. zygis* proved highly toxic to *S. oryzae* adults at a concentration of 25mg/L in air.

The rapid death of *C. maculatus* adults suggests that the insecticidal action of EOs may be primarily due to their fumigant toxicity, which allows them to penetrate the insect's body via its respiratory system (Abdelgaleil et al., 2009; Baghouz et al., 2024). The simultaneous effect of the fumigant and repellent properties of these EOs on different olfactory receptor neurons may be the reason for this. Additionally, monoterpenes, which have a potent neurotoxic effect, could potentially disrupt the inhibition of the enzyme acetylcholinesterase in the central nervous system (Saad et al., 2018).

The impact of these EOs goes beyond repelling and harming the nervous system. They can also disrupt the insect's energy production by inhibiting mitochondria and blocking the function of chemosensory receptors, which are essential for detecting chemicals in the environment (Rösner et al., 2020). The high content of thymol present in the two EOs of Moroccan wild and cultivated *T. zygis* (TG-EO) is mainly responsible for their potent insecticidal properties. Thymol is a volatile monoterpene that disrupts the nervous system of insects, interfering with signal transmission and ultimately leading to insect mortality. This compound has demonstrated effectiveness against a range of insect pests (Paudel et al., 2023). The toxicity of thyme EO is probably due not only to its main composition but also to the presence of specific compounds such as o-cymene, linalool, and caryophyllene, which have proven insecticidal properties. The insecticidal effect of these essential oils may result from a combination of various constituents. These molecules can either reinforce the power of

the essential oil through synergistic interactions or reduce its effectiveness through antagonistic effects (Annaz et al., 2024). Moroccan *T. zygis* EOs exhibit potential as natural alternatives to synthetic pesticides for *C. maculatus* control. While further research is required to validate their effectiveness in practical applications, these oils offer a promising approach to developing environmentally friendly pest management strategies.

CONCLUSION

In the present study, we characterized the chemical composition of the essential oil (EO) from wild and cultivated *T. zygis plants in* the Moroccan commune of Bouchfaâ. Constituents were identified by mass spectrometry coupled with gas chromatography. These analyses identified 37 compounds in the wild *T. zygis* EO, with the main ones being thymol (36.24%), linalool (11.49%), and o-cymene (7.54%). For cultivated *T. zygis* EO, 8 compounds were identified, with thymol (57.42%) and α-pinene (7.60%) as the main constituents. These results highlight the variability in chemical composition between wild and domesticated *T. zygis* plants, suggesting that domestication influences both the quantity and quality of the main compounds. The essential oils TZS-EO (wild) and TZD-EO (cultivated) showed strong antioxidant and antimicrobial properties against all the bacterial and fungal strains tested. A statistically significant difference was observed in the zones of inhibition between the two oils. Furthermore, the increased antimicrobial activity of these essential oils can be attributed to the higher concentration of chemical compounds such as thymol and p-cymene. These results not only support the feasibility of domesticating *T. zygis* but also highlight the potential for optimizing cultural practices to target specific molecules.

Declaration: The authors declare no conflict of interests.

Authors' contributions: Rajae El Brahimi: Writing - original draft, investigation, methodology, conceptualization; Asmae Baghouz: Writing - original draft, formal analysis; El-Mehdi El-Assri: Visualization, methodology; Youssef El-Assri: Data curation, software; Najoua Darkaoui: Resources, software; Oussama Merzouki: Investigation, formal analysis; Amina Bari: supervision, conceptualization; Azeddin El Barnossi: Writing- review & Eediting, supervision, validation.

Ethical approval: Not applicable.

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