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Commercial thyme essential oil as natural beverage preservative and molecular docking study on its mode of action against *Saccharomyces cerevisiae*

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Abstract: The present investigation explored the possible use of *Thymus vulgaris* essential oil (TVEO) as a beverage antifungal preservative instead of chemical ones. The chemical profile of TVEO exposed carvacrol (60.47%) as the predominant compound. The antifungal properties of TVEO were assessed on various food spoilage yeast and mould species using two tests. TVEO showed a powerful antimicrobial effect against all the fungal strains at the three volumes of essential oil (EO) used (i.e. 10, 20, and 30 µL). The minimum inhibition concentration (*MIC*) of TVEO was also evaluated and ranged from 0.0625% to 0.015% (*v*/*v*). Furthermore, the potency of TVEO as a beverage antimicrobial preservative was tested at four distinct concentrations (0.6, 1.25, 4, and 6 µL·mL^{-1}) against *Saccharomyces cerevisiae* alone and combined with medium heating (70 °C for 2 min) in a real food matrix (Orangina® drink) for eight storage days. TVEO exhibited a significantly higher preservative effect than chemical preservatives (sodium benzoate and potassium sorbate). Lastly, a molecular docking examined the mechanism of action of carvacrol against two crucial enzymes in *S. cerevisiae* viability [ERG2 (sterol C8-isomerase) and ERG3 (sterol C5-desaturase)] compared to a chemical preservative (potassium sorbate). The two ligands highly interacted with the two target enzymes. However, carvacrol achieved a better score than potassium sorbate against ERG2 and ERG3, with binding energy of –10.19 kcal·mol^{-1} and –11.73 kcal·mol^{-1}, respectively. Our results open up the perspective of using TVEO as a natural food preservative.

Keywords: *Thymus vulgaris*; natural food preservative; carvacrol; Orangina® drink

Food deterioration and intoxication caused by microbial pathogens are major issues, particularly in the industrial world, leading to substantial economic losses. The *Saccharomyces cerevisiae* strains are among the microbial pathogens linked to foodborne illnesses (Forsythe et al. 2004), and it's also a usual beverage contaminant (Recca et al. 1952). This yeast strain releases fermented products with undesirable tastes attributable to the release of CO_2, ethanol, and residues from other fermentation products (Tajchakavit et al. 1998).

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The use of chemical agents is the most common method to avoid the deterioration of foodstuffs (Olmedo et al. 2013). Nevertheless, there is an increasingly negative reputation of chemical additives among consumers due to their harmful effects and the emergence of microbial resistance to these substances (Loureiro and Ferreira 1993). Hence, plant-derived antimicrobial agents are emerging as an alternative to chemical ones (Harvey 2008). *Thymus vulgaris* (thyme), or 'garden thyme', is a *Labiatae* subshrub broadly distributed and indigenous to southern Europe. Traditionally, *Thymus vulgaris* essential oil (TVEO) was applied as an antibacterial and flavouring agent in food and confectionery products (Mandal and DebMandal 2016) Also, TVEO is generally recognised as safe (GRAS) by the Food and Drug Administration (FDA 2009) and didn't show any sign of toxicity even at 5 000 $mg \cdot kg^{-1}$ (Abdelli et al. 2017).

Essential oils (EOs) are frequently employed in the agro-food and cosmetic industries to prevent microbial contaminations (Fratianni et al. 2010). However, despite their high potential, their commercial utilisation remains limited (Board et al. 1991) due to the substantial EO volume required to produce an equal influence on food preservation (Smid and Gorris 1999). These EOs quantities can have a negative sensory attribute consequence by altering the food's texture and aroma by exceeding the tolerable consumer threshold (Nazer et al. 2005; Liang et al. 2011). Therefore, the association of EOs with classic preservation methods like heat process is interesting in reducing volumes used while maintaining the same protective effect (Tyagi et al. 2014).

In the present research, the chemical composition of TVEO was determined using gas chromatography with downstream flame ionisation detector (GC-FID) combined with gas chromatography-mass spectrometry (GC-MS). Next, the *in vitro* antifungal potential of TVEO was evaluated against a large panel of food spoilage fungal strains. Afterwards, the authors evaluated the preservative effect of TVEO against a food spoilage yeast strain (*S. cerevisiae*) in a well-known commercial citrus drink (Orangina®) and compared it with the antifungal effect of chemical preservative. The preservative effect of TVEO was also tested in association with mild heating. Finally, as a primary cause of beverage contamination, the authors focused on yeasts and fungi. Ergosterol creates a crucial part of their membranes (Jordá and Puig 2020). The last enzymes in the chain of ergosterol biosynthesis and also crucial enzymes ERG2 (sterol C8-isomerase) and ERG3 (sterol C5-desaturase) were used in the molecular docking study. In the latter, carvacrol was used as a ligand since it is the predominant compound of TVEO. Especially since carvacrol carries out its fungicidal action by disrupting the biosynthesis of ergosterol and the integrity of the cell membrane (Ahmad et al. 2011).

MATERIAL AND METHODS

Materials

Thymus vulgaris L. essential oil was acquired from the 'Ziphee-Bio' company of EOs (Lakhdaria, Bouira, Algeria).

Composition of Orangina® drink

Carbonated water, citrus fruits concentrate 11%, sugar, pulp 2%, citric acid, orange peel extract, preservatives (potassium sorbate and sodium benzoate), and natural orange flavour.

Fungal species

Five isolated yeast (*Candida albicans, C. parapsilosis, C. tropicalis, C. glabrata,* and *Saccharomyces cerevisiae*) and four mould species (*Aspergillus fumigatus*, *A. niger, A. flavus*, and *Fusarium* sp.) were used. All strains were identified by Laboratory of Mycology of PASTEUR Institute (DelyBrahim, Algiers, Algeria) and all cultures were preserved in SDA (Sabouraud Dextrose Agar) at 4 °C until use.

Chemical composition of *Thymus vulgaris* essential oil (TVEO)

Gas chromatography with downstream flame ionisation detector (GC-FID). The GC-FID analysis of TVEO were conducted using a Clarus 500 Perkin Elmer (Perkin Elmer, France) system outfitted a flame ionisation detector (FID) and two Capillary columns (50 m × 0.22 mm, film thickness 0.25 μm), BP-1 (polydimethylsiloxane) and BP-20 (polyethylene glycol). The oven temperature was increased from 60 °C to 220 °C at 2 °C per min before being isotherm for 20 min. The carrier gas was pure helium, flowing at a rate of 8 $mL \cdot min^{-1}$. The injection was carried out with a split and a volume of 1/60 μL and 0.5 μL, respectively.

Gas chromatography-mass spectrometry (GC-MS). The GC-MS of TVEO were performed using Clarus SQ8S Perkin Elmer TurboMass detector (quadripole), with a direct linkage to Perkin-Elmer Autosystem XL (Perkin Elmer, France), outfitted with a BP-1 (polydimethylsiloxane) fused-silica capillary column [60 m × 0.22 mm i.d. (inner diameter), film thickness 0.25 μm]. The carrier gas was helium with

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a flowing debit of 1 mL·min^{-1}. The oven temperature was raised from 60 °C to 230 °C at a rate of 2 °C per min, then isotherm for 45 min.

Agar disc diffusion test. TVEO's antifungal properties were assessed using Kirby disc diffusion test. In brief, a fresh microbial suspension was prepared in sterile saline solution (0.9% NaCl) and spread on culture plates. Filter paper discs (Sigma Aldrich, USA) were then placed in the centre of the inoculated plates, and three different amounts (10, 20, and 30 µL) of TVEO were deposited on the discs. Hexamidine® (0.1%) (Isopharma, Algeria) and Nystatin solution (1%) served as positive controls for yeast and mould strains, respectively. Finally, all plates were incubated at 25 °C and 37 °C for 72 h and five days for yeasts and mould strains, respectively. The zones of inhibition (*ZOI*) were measured and expressed in millimetres.

Volatilisation diffusion test. The antifungal potential of TVEO's vapour phase was evaluated using volatilisation diffusion test. The technique involves taking around 50 µL of the microbial suspension and spreading it on SDA Petri dishes. Filter paper discs were laid on the inside surface of the upper lid of the Petri dishes, and three different amounts of TVEO (10, 20, and 30 µL) were then deposited. Finally, the plates were immediately turned over and incubated at 37 °C for 72 h, and five days for yeasts and mould strains, respectively.

Determination of minimum inhibitory concentration (MIC). TVEO's *MIC* was determined using the macrodilution technique. An EO volume of 250 µL was diluted in 50 mL of SDA culture media to obtain a solution at 0.5% (*v*/*v*), followed by its successive dilution to half. A volume of 25 mL was taken from each dilution, transferred to medium plates, and inoculated with fresh microbial suspensions. The poisoned food technique (Grover and Moore 1962) was used for mould strains. The technique consists of cutting a mycelia disc (6 mm diameter) from a fresh mycelia culture, and then deposing it in the centre of Petri dishes. Finally, all the plates were incubated for 72 h and five days for yeast and mould strains, respectively. The *MIC* is the smallest EO concentration where no perceptible microbiological growth appears.

The preservative effect of *Thymus vulgaris* essential oil (TVEO) against *Saccharomyces cerevisiae* in citrus drink (Orangina®)

The preparation of citrus drink (Orangina®) inoculated with Saccharomyces cerevisiae. The preservative effect of TVEO against food spoilage was tested based on the method of Tyagi et al. (2014) with some modifications. For this experiment, we chose the well-known citrus drink (Orangina®), which was purchased from Orangina® Company (Algeria). The drink samples were mixed with a fresh solution of foodborne yeast (*S. cerevisiae*) strain at 10^4 CFU·mL^{-1} (CFU – colony forming unit), then transferred to sterile glass vials of 100 mL and stored at 4 °C.

Action of Thymus vulgaris essential oil. The inoculated drinks were mixed with TVEO and Tween 80 (0.5%) at four concentrations (0.6, 1.25, 4, and 6 µL·mL^{-1}). Yeast viability was monitored for eight days of storage at 4 °C, with sampling at 0th, 2nd, 5th, and 8th day. Pasteurised beverages without TVEO were the negative control, whereas those containing chemical preservatives were the positive control.

Action of Thymus vulgaris essential oil associated with mild heating. The inoculated vials were mixed with TVEO and Tween 80 (0.5%) solution at four concentrations (0.6, 1.25, 4, and 6 µL·mL^{-1}). Then, the drinks were heated (70 °C for 2 min) and maintained at 4 °C for 8 days, with sampling at 0th, 2nd, 5th, and 8th day. Each test was repeated three times. All samples have undergone a 10^{-2} dilution in saline solution (0.9% NaCl), filtered, spread on SDA plates, and incubated for 48 h or 72 h at 25 °C. The microbial load was expressed by the variation in log CFU·mL^{-1} of the inoculated foodborne yeast (*S. cerevisiae*) strain in the function of storage time expressed in days.

Molecular docking. In molecular docking study, carvacrol was selected and used as a ligand, since it is the major compound of TVEO. While, potassium sorbate was employed as a comparison ligand, as it is widely used in the food industry as a preservative, including in beverages. Molecular docking investigated the interaction between the selected ligands against two enzymes: C-8 sterol isomerase and Delta (7)-sterol 5(6)-desaturase communally named ERG2 and ERG3, respectively. The chosen enzymes are involved in ergosterol biosynthesis in the *S. cerevisiae* strain. Their 3D structures were retrieved from the alphaFOLD prediction structure database under the codes C7GRE9 and P32353 for ERG2 and ERG3, respectively. The possible binding sites of the target enzymes were predicted using Castp software (version 3.0; http://sts.bioe.uic.edu/castp/index.html?201l). Whereas, the ligands employed for the docking study were downloaded from PubChem as an SDF file and converted to a PDB file using Open Babel software (version 2.0; Sibi et al. 2013). The molecules preparation and docking study was realised using Argus Lab software (version 4.0.1; Thompson 2004).

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The different binding modes between the bound complexes were observed through Biovia Discovery Studio Visualizer software (version 2021).

Statistical study

Triplicates of each trial were run. The findings were presented as the mean value and standard deviation (mean ± SD). Antifungal results and the preservative effect of the EO test in a real food matrix were analysed using the Kruskal-Wallis test, followed by Dunn's multiple comparison tests. The difference was fixed at ($P \leq 0.05$) level of significance. All the analyses were carried out using GraphPad Prism statistical software (version 8.00).

RESULTS AND DISCUSSION

Chemical characterisation of *Thymus vulgaris* essential oil

Twenty-eight compounds were identified, representing 97.92% of TVEO's total composition. The latter is mainly composed of oxygenated monoterpenes (74.54%), with carvacrol (68.20%) as the predominant compound, followed by the p-cymene (8.12%) and the -terpinene (5.73%) (Table 1).

Carvacrol-rich TVEO has already been documented in countries like Algeria (56.8%), Switzerland (up to 49.37%), Brazil (45.5%), and Jordan (up to 86.1%) (Hudaib et al. 2007; Iten et al. 2009; Boukhatem et al. 2020). In contrast, a TVEO from the Iberian region (Spain) exposed a different profile from ours, identifying 1.8-cineole (up to 68.5%) as the main compound (Llorens-Reddy et al. 2016).

The antifungal activity of *Thymus vulgaris* essential oil

TVEO showed a dose-dependent effect (10, 20, and 30 µL) illustrated by the total inhibition of all fungal strains *ZOI* at the higher volume used. TVEO was significantly more effective against yeast species than the positive control (Hexamidine 0.1%) ($P < 0.0001$). Also, TVEO exhibited the predominant effect (*ZOI* = 85 mm) against *S. cerevisiae* and *C. albicans* and all mould strains at the three volumes used with a superior effect than the positive control (Nystatin 1%) (Table 2).

Our findings didn't correlate with those of Galovicová et al. (2021), who exposed moderate effect of TVEO against *C. albicans*, *C. tropicalis*, and *C. glabrata* with *ZOI* does not exceed 13 mm. In contrast, our results are regular with those of Gucwa et al. (2018), which exposed the potent anti-yeast action of TVEO against 259 clinical species of *C. albicans* and *C. glabrata*. By the way, it is not surprising that TVEO exhibit a great antifungal potential due mainly to its phenolic compounds including carvacrol and thymol (Inouye et al. 2001).

Table 1. Chemical composition of *Thymus vulgaris* essential oil

No.	Compound	RI^a	RI^p	%
1	α-thujene	925.38	1 021.79	0.45
2	α-pinene	933.22	1 019.19	0.63
3	camphene	946.41	1 070.24	0.16
4	sabinene	964.41	1 021.79	0.46
5	octan-3-ol	973.29	1 115.68	0.12
6	myrcene	983.31	1 165.43	1.79
7	α-phellandrene	999.93	1 169.86	0.21
8	d-3-carene	1 007.75	1 153.00	tr
9	α-terpinene	1 011.70	1 185.52	1.45
10	p-cymene	1 014.46	1 276.00	8.12
11	limonene*	1 023.33	1 205.08	0.23
12	1.8-cineole*	1 023.33	1 214.31	0.31
13	e-b-ocimene	1 038.66	1 254.08	tr
14	γ-terpinene	1 051.05	1 249.57	5.73
15	trans-sabinene hydrate	1 056.88	1 465.16	0.44
16	linalool	1 086.59	1 549.21	2.77
17	borneol	1 152.37	1 701.96	0.75
18	terpineol-4	1 164.63	1 603.22	1.22
19	α-terpineol	1 174.24	1 609.75	0.10
20	thymyl acetate	1 270.00	1 876.29	0.30
21	thymol	1 272.62	2 204.20	0.14
22	carvacrol	1 281.89	2 216.00	68.20
23	carvacryl acetate	1 346.66	1 876.29	0.31
24	β-caryophyllene	1 419.44	1 597.83	3.45
25	α-humulene	1 452.07	1 669.58	0.11
26	β-bisabolene	1 502.51	1 728.04	0.23
27	γ-cadinene	1 516.13	2 277.78	tr
28	caryophyllene oxide	1 571.97	1 979.13	0.24
Total identified				97.92
Monoterpene hydrocarbons				19.35
Oxygenated monoterpenes				74.54
Sesquiterpene hydrocarbons				3.79
Oxygenated sesquiterpenes				0.24

The apolar column provides compound elution; percentages were determined on apolar column, with the exception of those with * (polar column); RI^a – retention indice on apolar column; RI^p – retention indice on polar columns; tr – traces

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Table 2. Antifungal effect of *Thymus vulgaris* essential oil

Fungal species	*ZOI* (mm)[a] – volume of TVEO (µL per disc)								
	Agar disc diffusion test			Volatilisation diffusion test			Positive control[b]		
	10	20	30	10	20	30	10	20	30
Yeast species									
Candida albicans	85.0 ± 0.0	85.0 ± 0.0	85 ± 0	85.0 ± 0.00	85.0 ± 0.00	85.0 ± 0.0	16.0 ± 2.6	17.7 ± 0.6	17.7 ± 0.6
Candida parapsilosis	40.3 ± 1.5	63.3 ± 2.9	85 ± 0	46.7 ± 5.89	53.7 ± 2.93	64.0 ± 3.6	27.0 ± 0.0	33.3 ± 0.6	37.0 ± 1.0
Candida tropicalis	62.7 ± 0.6	85.0 ± 0.0	85 ± 0	52.7 ± 3.2.4	64.7 ± 4.50	71.3 ± 3.2	33.0 ± 1.0	34.0 ± 0.0	37.4 ± 1.2
Candida glabrata	68.0 ± 3.5	85.0 ± 0.0	85 ± 0	53.3 ± 4.00	60.0 ± 5.00	70.0 ± 5.0	34.7 ± 2.0	38.3 ± 1.2	45.7 ± 0.6
Saccharomyces cerevisiae	85.0 ± 0.0	85.0 ± 0.0	85 ± 0	85.0 ± 0.00	85.0 ± 0.00	85.0 ± 0.0	33.3 ± 0.6	35.3 ± 1.0	40.7 ± 0.5
Mould species									
Aspergillus fumigatus	85.0 ± 0.0	85.0 ± 0.0	85 ± 0	85.0 ± 0.00	85.0 ± 0.00	85.0 ± 0.0	17.0 ± 1.7	19.0 ± 0.6	22.0 ± 4.5
Aspergillus niger	85.0 ± 0.0	85.0 ± 0.0	85 ± 0	85.0 ± 0.00	85.0 ± 0.00	85.0 ± 0.0	40.0 ± 0.6	43.0 ± 2.7	45.0 ± 6.4
Aspergillus flavus	85.0 ± 0.0	85.0 ± 0.0	85 ± 0	85.0 ± 0.00	85.0 ± 0.00	85.0 ± 0.0	31.0 ± 0.0	34.0 ± 0.0	35.0 ± 4.7
Fusarium sp.	85.0 ± 0.0	85.0 ± 0.0	85 ± 0	85.0 ± 0.00	85.0 ± 0.00	85.0 ± 0.0	12.3 ± 0.6	18.3 ± 0.6	21.3 ± 2.5

[a] The *ZOI* comprises the disc diameter (9 mm); [b] antiseptic solution (Hexamidine 0.1%) and Nystatin solution (1%) were the positive controls for yeast and mould species respectively; *ZOI* – zone of inhibition; TVEO – *Thymus vulgaris* essential oil

Indeed, carvacrol is one of TVEO's most effective compounds against fungal species due to its hydrophobic characteristics. These properties allow it to attach to the lipophilic region of the cell membrane and solubilise it, which is likely to result in fungal death (Yan et al. 2021). Likewise, the hydroxyl functional group in carvacrol may provoke the production of free radicals in oxidative phosphorylation process, which can lead to microbial cell death (Kawhena et al. 2021).

Volatilisation diffusion test

TVEO exhibited a *ZOI* against fungal strains correlated to the increasing volume used (10, 20, and 30 µL). Further, the EO showed pronounced efficacy against all yeast species, with a predominant effect against *S. cerevisiae* and *C. albicans* that were totally inhibited (*ZOI* = 85 mm) at all the amounts of EO used. The *ZOI* of TVEO against *C. parapsilosis*, *C. tropicalis*, and *C. glabrata* ranged from 46 mm to 74 mm from the lowest to the highest volume used. Finally, TVEO totally inhibited the four mould strains, regardless of the volume used (Table 2).

As a whole, our TVEO showed a better antifungal effect with the agar disc than the volatilisation diffusion test ($P = 0.01$). Yet, TVEO demonstrated the same degree of inhibition against *C. albicans*, *S. cerevisiae*, and mould strains for both methods.

Even if the used protocol influence quantification of the EO antimicrobial, our results considerably conflict prior studies showing that the TVEO vapour phase display superior antifungal properties than the liquid phase (Inouye et al. 2001; Tullio et al. 2007; Fisher et al. 2008). As a hypothesis, the poor aldehyde compound presence in our EO can explain these differences. Since EOs containing high aldehyde content exhibit the higher inhibitory effect in vapour state, followed by those containing alcohol, ketone, and ether (Inouye et al. 2001).

The minimum inhibitory concentrations (*MIC*) of *Thymus vulgaris* essential oil

The essential oil (EO) presented a *MIC* value of 0.015% (*v*/*v*) against the five yeast strains. Besides, The *MIC* of TVEO against mould strains varied from 0.0625% to 0.015% (*v*/*v*), with *A. fumigatus* exhibiting the lowest and *Fusarium* sp. displaying the highest one (Table 3).

Despite the difference of methodology between our study and others, our findings surpass those found by other authors. In this context, Gucwa et al. (2018), found a TVEO's *MIC* value of 0.625% (*v*/*v*) against *C. albicans* and *C. glabrata* strains. Further, Tullio et al. (2007) reported an EO's *MIC* value of 0.25% (*v*/*v*) against two mould strains, namely *A. flavus* and *A. fumigatus.*

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Table 3. The minimum inhibitory concentrations of *Thymus vulgaris* essential oil against selected yeast and mould strains

Fungal species	*MIC* % (*v*/*v*)
Yeast species	
Candida albicans	0.0150
Candida parapsilosis	0.0150
Candida tropicalis	0.0150
Candida glabrata	0.0150
Saccharomyces cerevisiae	0.0150
Mould species	
Aspergillus fumigatus	0.0150
Aspergillus niger	0.0300
Aspergillus flavus	0.0300
Fusarium sp.	0.0625

MIC – minimum inhibitory concentration

The preservative effect of *Thymus vulgaris* essential oil against *S. cerevisiae* in citrus drink (Orangina®)

Action of Thymus vulgaris essential oil. TVEO exhibited a total antifungal growth inhibition on the highest concentration used (6 $\mu L \cdot mL^{-1}$) on the second day and during the whole storage period. While the EO concentration of 4 $\mu L \cdot mL^{-1}$ showed total growth inhibition beginning from the eighth day of storage. Nevertheless, the lowest EO concentration (0.6 $\mu L \cdot mL^{-1}$) didn't show a considerable inhibition of growth during the eight days of storage when compared to the positive and the negative controls ($P > 0.99$) (Figure 1A).

Similarly, Shabnum and Wagay (2011) evaluated the antifungal potential of TVEO on the preservation of strawberries infected with *Botrytis cinerea* and *Rhizopus stolonifer* species that induced the apparition of grey mould and deterioration of the fruit. After two weeks of preservation, they reported a reduction in decay correlated with an increase in TVEO concentration. Additionally, a maximal reduction of the infection (more than 70%) was reported at the highest EO concentration. These findings are certainly attributed to TVEO phenolic monoterpenes, which affect fungal cells by altering the cellular organelles, including the mitochondria, and the plasma membrane (Helal et al. 2006).

In fact, to ensure the microbial safety of the beverages, using only EO might influence the product's overall sensory characteristics. Hence, we must investigate the impact of the association of TVEO with mild heat treatment to try to reduce the EO amounts used in food preservation.

Action of Thymus vulgaris essential oil associated with mild heating. TVEO exhibited a total inhibition at the two highest (6 $\mu L \cdot mL^{-1}$ and 4 $\mu L \cdot mL^{-1}$) EO concentrations beginning from the second and fifth day of storage, respectively. Further, these two concentrations showed significantly higher inhibitory effects against *S. cerevisiae* than positive and negative controls ($P < 0.0001$). In contrast, the two lowest EO concentrations (0.6 $\mu L \cdot mL^{-1}$ and 1.25 $\mu L \cdot mL^{-1}$) showed no higher inhibition than the positive and negative control ($P > 0.99$). Our findings also revealed that the thermal treatment didn't show significantly better results than his absence ($P > 0.05$) (Figure 1B).

In the same line, Belletti et al. (2010) tested the antifungal preservative potential of three EO monoterpenes (β-pinene, geranial, and linalool) in orange juice inoculated with a wild *S. cerevisiae* strain and associated with moderate heat processing (55 °C for 15 min). The authors reported that the heat treatment significantly enhanced the preservative effect of the beverage, certainly by making it easier for hydrophobic chemicals to reach the cytoplasmic membrane of the yeast, which is where biomolecules exert their deadly effects (Prashar et al. 2003). The EOs associated with heat treatment created a highly advantageous synergy in which higher temperatures during storage increase the level of volatiles in the vapour phase, improving anti-yeast activity and food preservation (Tyagi et al. 2014).

Molecular docking. The docking scores expressed as kcal/mol were largely negative for the two ligands against ERG2 and ERG3 isolated from *Saccharomyces cerevisiae.* These results demonstrate the high interaction of the selected ligands with the two enzymes. However, carvacrol achieved a higher docking score against ERG2 (10.27 $kcal \cdot mol^{-1}$) than potassium sorbate (8.16 $kcal \cdot mol^{-1}$). Comparable results were observed against ERG3, where carvacrol showed a score of −11.73 $kcal \cdot mol^{-1}$, while potassium sorbate displayed a score of −7.84 $kcal \cdot mol^{-1}$. The docking scores proved that carvacrol inhibits ergosterol biosynthesis, which undoubtedly lead to *S. cerevisiae* cell death. The interactions and binding sites of ligands are displayed in Figures 2 and 3.

Due to the hydrophobicity of EO compound's, carvacrol established only hydrophobic interactions against ERG2. These interactions belong mainly to that of Van der Waals established with the following amino acid residues: ALA88 (alanine), ASN86 (asparagine), ALA91, PHE109 (phenylalanine), THR119 (threonine), HIS118 (histidine), GLN135 (glutamine), HIS154, ALA172, PHE186. Whereas against ERG3,

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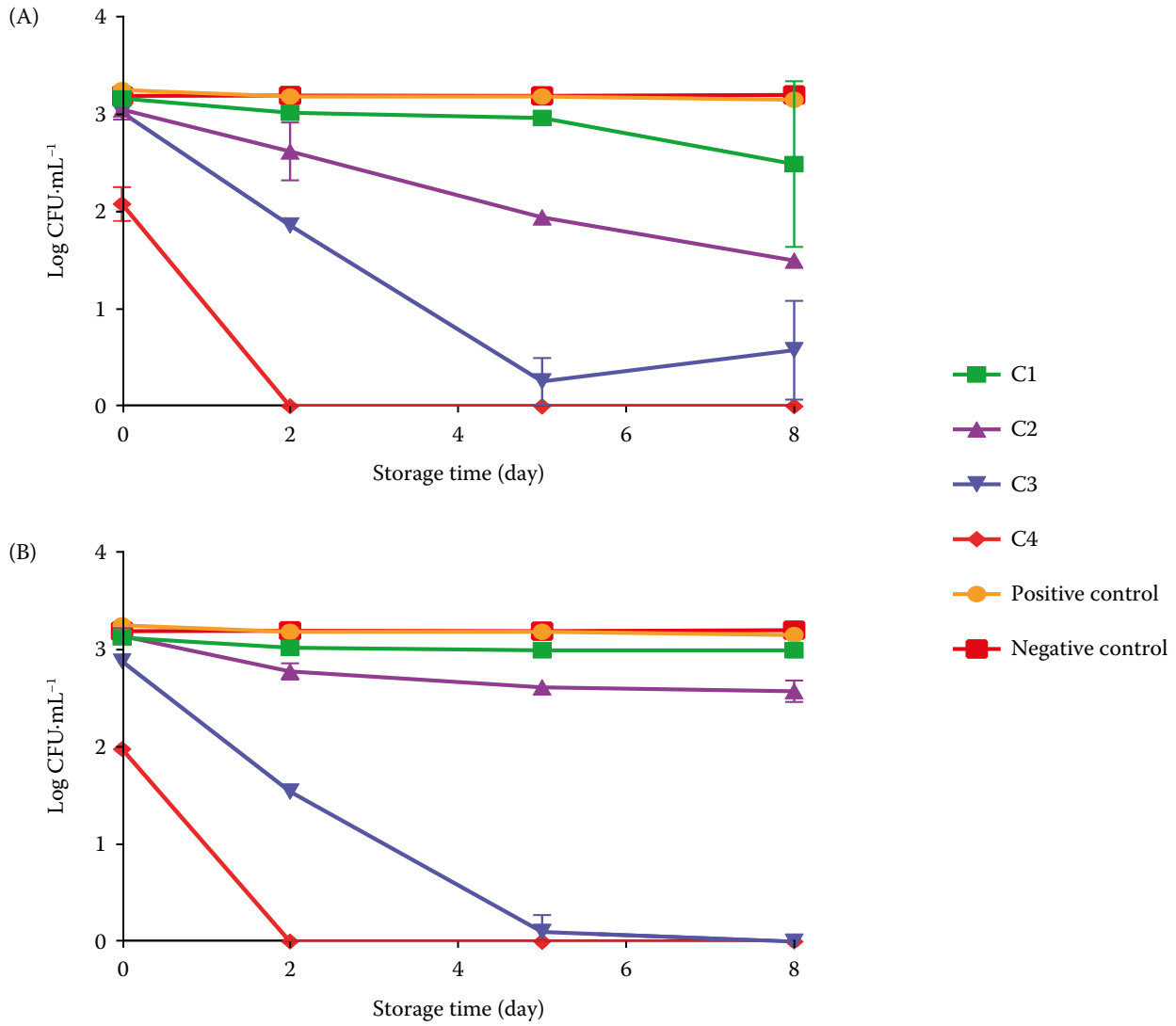

Figure 1. *Saccharomyces cerevisiae* strain count of citrus drink (Orangina®) in the function of storage days after adding of different concentrations of *Thymus vulgaris* L. essential oil (A) alone and (B) combined with moderate heat treatment

The drink samples treated with chemical preservatives (sodium benzoate and potassium sorbate) represent the positive control; C – concentration; C1 – 0.6 μL·mL^{-1}; C2 – 1.25 μL·mL^{-1}; C3 – 4 μL·mL^{-1}; C4 – 6 μL·mL^{-1}; CFU – colony forming unit; values of three replicate experiments are expressed as mean ± standard deviation (SD)

the hydroxyl (OH) function of carvacrol allowed the formation of one conventional H-bond established with LYS253 (lysine). Besides, carvacrol performed better than potassium sorbate due probably to the aromatic ring in its structure that allowed additional interactions (Pi-Pi stacked, Pi-alkyl) with the amino acids of the enzymes. To our knowledge, no docking studies have been carried out on carvacrol with ERG2 and ERG3, but they have been done against other ergosterol biosynthesis enzymes. For instance, Akermi et al. (2023) employed molecular docking to investigate the mode of action of carvacrol on lanosterol 14α-demethylase (ERG11) enzyme isolated from *C. albicans*. The outcomes exposed a largely negative energy score (–6.3 kcal·mol^{-1}), which indicates a high interaction of carvacrol with ERG11. Another study explored the mode of action of carvacrol against three enzymes TEM-72 (PDB ID: 3P98), CTXM-9 (PDB ID: 1YLJ), and SHV-2 (PDB ID: 1N9B) from the extended-spectrum β-lactamase family isolated from *Escherichia coli*. The docking exposed a binding energy reaching –5.27 kcal·mol^{-1} due mainly to H-bonds and hydrophobic interactions between carvacrol and the target enzymes (Khan et al. 2020).

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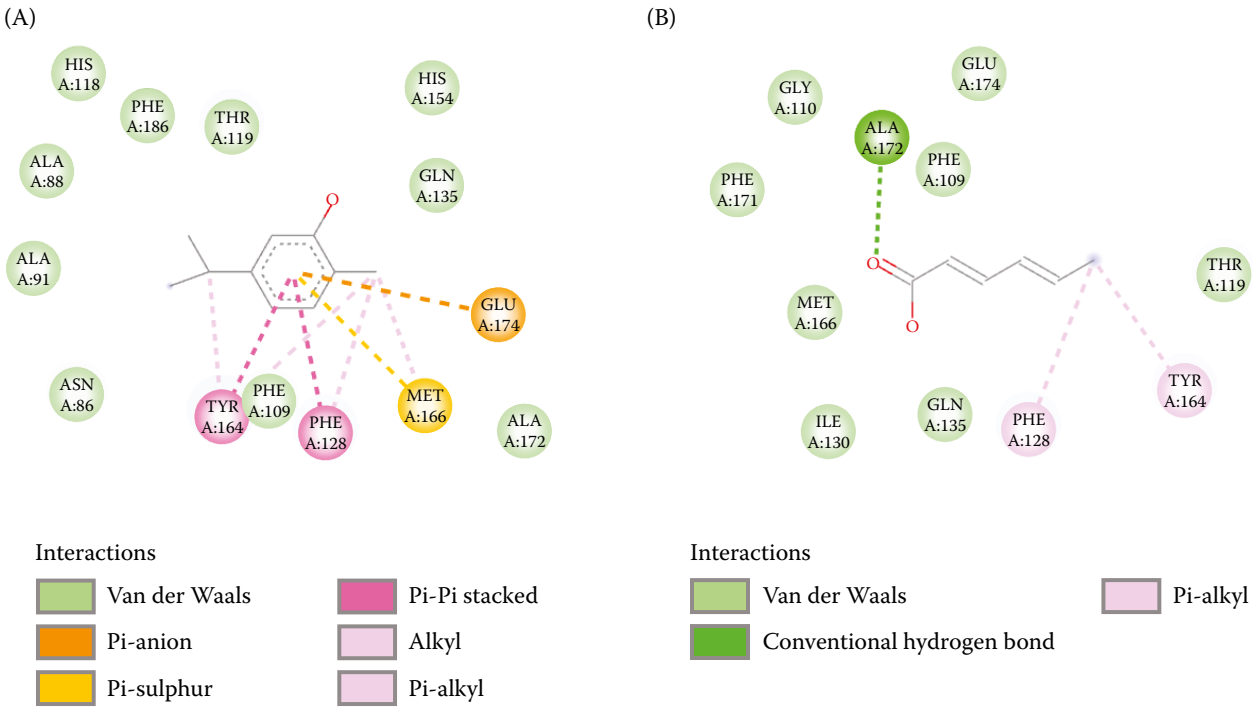

Figure 2. 2D diagram visualisation of molecular docking using the Discovery Studio Visualizer for ERG2 with (A) carvacrol and (B) potassium sorbate

ALA – alanine; ASN – asparagine; GLN – glutamine; GLU – glutamic acid; GLY – glycine; HIS – histidine; ILE – isoleucine; MET – methionine; PHE – phenylalanine; THR – threonine; TYR – tyrosine

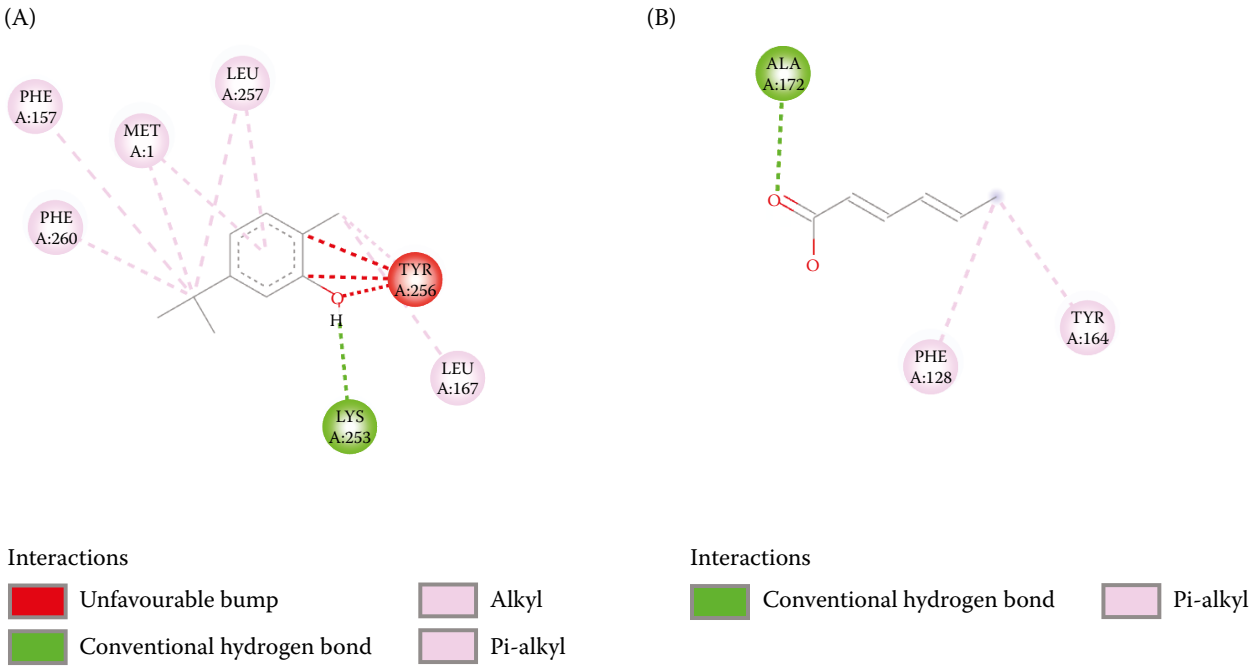

Figure 3. 2D diagram visualisation of molecular docking using the Discovery Studio Visualizer for ERG3 with (A) carvacrol and (B) potassium sorbate

ALA – alanine; LEU – isoleucine; LYS – lysine; MET – methionine; PHE – phenylalanine; TYR – tyrosine

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CONCLUSION

In summary, TVEO showed a powerful antifungal action against a panel of food spoilage fungal strains determined using two different *in vitro* techniques. At small concentration, TVEO showed a significantly better antifungal effect against *S. cerevisiae* in the citrus drink (Orangina®) than chemical preservatives. The docking study revealed the high interaction of TVEO main compound (carvacrol) against two ergosterol biosynthesis enzymes, crucial molecules in *S. cerevisiae* survival. As a result, these results provide an exceptional perspective on the use of TVEO as a natural food preservative instead of synthetic preservatives. Nevertheless, we must confirm the current findings, assess the sensory effects of adding TVEO to food commodities, and consider encapsulation as the final form of commercialisation.

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