

Article

# In Vitro Antifungal and Topical Anti-Inflammatory Properties of Essential Oil from Wild-Growing *Thymus vulgaris* (Lamiaceae) Used for Medicinal Purposes in Algeria: A New Source of Carvacrol

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Received: 9 May 2020; Accepted: 2 June 2020; Published: 3 August 2020



**Abstract:** The aim of this study is to investigate the *Thymus vulgaris* essential oil (TVEO) as an antifungal agent in aromatherapy and/or as an active ingredient in the prevention or management of topical inflammatory diseases. The chemical composition of TVEO was determined with gas chromatography and revealed the presence of 25 compounds. Carvacrol was found to be the major component (56.8%). Antifungal action of TVEO was determined in vitro by using different methods. By the disc diffusion method, TVEO showed more potent antifungal activity against *Candida* strains than the positive control. The diameter of inhibition zone (DIZ) varied from 34 to 60 mm for *Candida* yeasts. Significantly higher antifungal activity was observed in the vapor phase at lower quantities. *Candida albicans* and *C. parapsilosis* were the most susceptible strains to the oil vapor with DIZ varying from 35 to 90 mm. The minimum inhibitory concentrations (MIC) of yeast were determined with an agar dilution method and revealed that MIC varied from 0.3 to 0.15  $\mu\text{L/mL}$  for yeast species. The topical anti-inflammatory potential of TVEO was also explored in vivo with the croton oil-induced ear edema assay. TVEO exhibited a potent anti-inflammatory effect at all doses (100, 10 and 2 mg/kg), which were statistically similar ( $p > 0.05$ ) to the positive control. This activity was also confirmed at the cellular level with histopathology analysis. Our results suggest the potential application of this carvacrol-rich TVEO in the prevention and management of fungal infections and topical inflammation and deserve further investigation for clinical applications. Furthermore, while the mode of action remains mainly undetermined and should be studied.

**Keywords:** natural antifungal; topical anti-inflammatory; carvacrol-rich essential oil; *Thymus vulgaris*; chemotype

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## 1. Introduction

The number of fungal infections has been increasing in recent years due to the increasing number of high-risk patients, particularly immunocompromised hosts. *Candida albicans* is the most prevalent cause of human fungal infections [1,2]. Further, microscopic fungi (molds) are omnipresent strains with a huge ability to colonize numerous kinds of substrates and to expand under extreme environmental situations. Molds and their airborne particles in the environment are known as probable contributory agents to a variety of diseases in humans: airway infections, irritation of respiratory mucous membranes, acute and chronic damage of respiratory organs and mycotoxicoses [3].

Despite the introduction of new antifungal treatments, they are restricted in number. The rise of fungal resistance to classical drugs, the high treatment costs, and the reality that most existing antifungal molecules have only fungistatic properties, are good reasons to explore novel strategies [4]. Furthermore, the fungal contagion commonly induces irritation, and free radicals are released from the cells of the immune system during the inflammatory progression. Inflammation is a series of host-produced defensive responses against environmental stimulation. The cycle involves growing blood circulation and endothelial dysfunction consisting in liquid and inflammatory intermediaries (i.e., cytokines, eicosanoids and reactive oxygen species (ROS)) aggregation in inflammatory cells. By secreting anti-inflammatory cytokines, the immune cell stimulates feedback channels to suppress pro-inflammatory signaling cascades and sustain homeostasis and normal tissues. Anti-inflammatory medications have frequent adverse effects, such as gastrointestinal complaint and organ toxicity [5]. Consequently, the exploration of natural products and molecules with fewer side effects is increasingly essential for the pharmaceutical industry.

Because various skin disorders are connected with fungal infection-stimulated inflammation, the presence of antibacterial, antifungal, anticancer, antioxidant and anti-edematous effects may give an explanation to the efficiency of some plant-derived essential oils (EOs) and their pure compounds in the management of these disorders. This information also shows that microbial inhibitory effect and inflammatory inhibition process are routine objectives for safe and natural phytochemicals research and investigation. In addition, aromatic herbs and medicinal plants act as antioxidant and anti-irritation drugs by reducing free radicals, increasing antioxidant fortifications or obstructing the discharge of pro-inflammatory substances. Additionally, in various areas of the globe, spices and phytochemicals continue to have the main parts in human health care, predominantly in pastoral zones [6,7].

The genus *Thymus* comprises 300–400 species, several of which are utilized in folk medicine. *Thymus vulgaris*, belonging to the Lamiaceae family, is a small scented perennial herb, predominantly found in the Mediterranean region, North Africa and Southern Europe. In alternative medicine, flowering parts and leaves of *Thymus* species have been widely used as herbal tea, tonic, antitussive, carminative and antimicrobial, as well as for treating colds. It has been suggested that a fraction of these properties is related to the EO [7–9]. Therefore, there is substantial interest in the chemical composition of *Thymus vulgaris* essential oil (TVEO) and its pharmacological properties. There are many reports that confirm the potential of EOs to suppress or remove bacterial and fungal infections, promote heart protection and, among other advantages, reduce inflammation in the body. Different studies also recorded the anti-inflammatory activity of the characteristic compounds of thyme EO such as carvacrol and thymol. For example, the thymol tends to reduce inflammation and infection. The anti-inflammatory and antimicrobial properties of thymol make it useful for dental hygiene according to new findings. The substance is a component in a variety of oral care products [10]. TVEO is generally recognized as safe (GRAS) and allowed as a food additive for internal consumption by the US Food and Drug Administration (FDA) [8].

Numerous reports have revealed that thyme EOs, particularly those of *Thymus vulgaris*, have antiseptic action, with the phenol type being the main active compound. The limited amount of these phenols in the environment is one of the reasons why *Thymus* oils containing carvacrol and thymol have been of immense importance for some time. However, the major inconvenience of using EO in the liquid phase is that they are more efficient as an antifungal when tested in culture, and so elevated doses are necessary to bring about the similar result. Numerous methodologies have been projected to decrease EO quantity. One of them is the use of EO in vapor phase to diminish the requisite amount [11,12].

To the best of our knowledge, no methodical reports comparing antifungal activity (in liquid and vapor phase) of TVEO are available. In the present work, we investigated the antifungal activity of TVEO against 8 yeast species and 8 filamentous fungi isolated from recurrent mucocutaneous fungal infections in order to study the use of TVEO as an antifungal agent for skin diseases. The topical anti-inflammatory activity of TVEO was evaluated with croton oil-induced ear edema followed by histology examinations. We also evaluated the major chemical composition of TVEO with gas chromatography-mass spectrometry (GC-MS). These results should help to clarify the application of this medicinal plant.

## 2. Materials and Methods

### 2.1. Materials

#### 2.1.1. Thyme Essential Oil Extraction

The TVEO used in our study was a commercial sample produced by steam distillation (SD) in industrial conditions (a stainless steel alembic) and was purchased from Ziphee-Bio company of essential oils (Bouira, Algeria) on May 2014. The SD process consists of passing water vapor at a low pressure throughout a boiler containing aromatic plants. The steam apparently captured the EO micro-pockets that were enclosed in the plant; it then passed through a cold-water refrigerated serpentine to condense into a liquid. Upon exit, the EO was obtained and the floral water (hydrosol) was in turn separated according to different densities using a Florentine vase.

#### 2.1.2. Yeast and Fungal Strains

Six clinical *Candida* strains comprising *C. albicans*, *C. parapsilosis* and *C. tropicalis*; four *Aspergillus* clinical strains including *A. niger*, *A. terreus*, *A. flavus* and *A. fumigatus*; and one *Penicillium* sp. and one *Mucor* sp. strains were used. Such isolates were isolated from patients (Institut Pasteur d'Algérie, Algiers, Algeria) with mucocutaneous infections. The fungal isolates were identified using standard microbiology methods and stored in Sabouraud dextrose agar with Chloramphenicol (SDA).

#### 2.1.3. Animals

Male Swiss mice (weighing 25–30 g) were provided by the Central animal house of the National Laboratory of Pharmaceutical Control (LNCPP, Algiers, Algeria). The animals were housed in standard cages at  $24 \pm 4$  °C under a 12 h/12 h light/dark cycle with free access to a standard commercial diet and water ad libitum. Prior to the experiments, the animals were fasted overnight, with water provided ad libitum. The experimental protocols were conducted in accordance with directives approved by current institutional guidelines for animal treatment (88-08/1988) and approved by the Council of the European Union (2010/63/EU) on the protection of animals used for scientific purposes.

#### 2.1.4. Drugs and Chemicals

The following drugs and chemicals were used: Isomedine<sup>®</sup> 0.1% (Hexamidine dermal solution, Isopharma, Algiers, Algeria), Voltarène<sup>®</sup> Emulgel<sup>®</sup> 1% gel (diclofenac diethylammonium, Novartis Sante Familiale, Rueil-Malmaison, France), SDA (Institut Pasteur d'Algérie, Algiers, Algeria) and filter

paper discs (9 mm in diameter, Schleicher and Schull GmbH, Dassel, Germany). Diclofenac sodium, bovine serum albumin (BSA), tween 80 and Alsever's solution were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

## 2.2. Methods

### 2.2.1. Chemical Composition of Essential Oil Determined by GC-MS Analysis

Investigation of *Thymus vulgaris* volatile oil was done with GC-MS. Analytical GC-MS was done on a Shimadzu GC-17A gas chromatograph coupled with a Shimadzu QP-5050A mass spectrometer detector (Shimadzu Corporation, Kyoto, Japan) with a Tracsil Meta.X5 (95% dimethylpolysiloxane and 5% diphenylpolysiloxane) column (60 m × 0.25 mm, 0.25 µm film thickness). The oven temperature program was 45–230 °C (2 °C/min). The injector temperature was 230 °C; the carrier gas was helium, adjusted to a linear velocity of 30 cm/s; the splitting ratio was 1:200 and the detector temperature was 250 °C. The individual peaks were recognized by comparing their retention index (RI), as well as by comparing their mass spectra with the NIST 2002 (National Institute of Standards and Technology, Gaithersburg, MD, USA) the Wiley 6.0 library (New York, NY, USA) mass spectral database and literature [13].

### 2.2.2. Antifungal Activity of Essential Oil In Vitro

The antifungal activity of TVEO in liquid and vapor phases was investigated using disc diffusion and disc volatilization methods, respectively [14]. TVEO was tested against a panel of microorganisms (*Candida* and filamentous fungal strains). The positive antifungal activity was recognized by the presence of measurable zones of inhibition after a 72 h incubation period.

#### Disc Diffusion

The inoculum of each strain to be tested was prepared with fresh cultures by suspending the microorganisms in sterile saline (0.9% NaCl). In the first step, the antifungal potential of TVEO was investigated with the disc diffusion method. hexamidine (1%) was used to control the sensitivity of clinically isolated fungal and yeast strains. In addition, it has been reported that hexamidine is used as a topical antiseptic in pharmaceutical products, has a broad antifungal property and are also used topically to treat some minor infections and mucocutaneous disorder [15]. All these indications motivated us to use hexamidine as a positive control. Filter paper discs (9 mm diameter) were impregnated with 3 different volumes (20, 40 and 60 µL per disc) of TVEO and placed on the inoculated plates (SDA). After maintaining at room temperature for 30 minutes, the plates were incubated under aerobic conditions for 72 h. Antifungal activity was assessed by measuring the diameter of the growth-inhibition zone (DIZ) in millimeters (including disc diameter of 9 mm) for the test strains and comparing to the controls [14].

#### Vapor Diffusion

The antifungal activity of TVEO in vapor phase was investigated with the disc volatilization technique at three different quantities (20, 40 and 60 µL per disc) [14,16]. In brief, solidified medium was inoculated over the surface of the SDA with 0.1 mL suspension of the fungal strains under study and allowed to dry. A paper disc (9 mm diameter) was laid on the inside surface of the upper lid and 20 µL of TVEO was positioned on each disc. The plate inoculated was instantly inverted on top of the lid and covered with parafilm to avoid escape of TVEO vapor. Plates were incubated for 72 h. The fungal inhibitory action of TVEO was calculated by measuring the DIZ of microorganism growth above the disc. Blank discs served as negative control.

### Determination of Minimum Inhibitory Concentration (MIC) by Agar Dilution

The MIC of EO was determined using the agar dilution assay [14]. Briefly, a series of twofold dilutions of TVEO ranging from 2 to 0.06  $\mu\text{L}/\text{mL}$  were prepared in SDA. For enhancing the oil solubility, Tween-80, 0.5% (*v/v*) was added into the agar. Plates were dried at room temperature prior to inoculation with 10  $\mu\text{L}$  of cell suspension of the tested strains. All the plates were incubated in duplicate for each concentration for 48 h. Plates with Tween-80, but without any EO were used as control. Observation of the plates for microbial growth was done at 12 h intervals until 48 h of incubation. The MIC values were determined as the lowest concentration of oil inhibiting the visible growth of each yeast on the plate agar.

### 2.2.3. Hemolytic Activity Using Red Blood Cell (RBC) System Cellular Model In Vitro

This assay allows the quantification of adverse effects of EOs on the plasma membrane of red blood cells, and the consequent release of hemoglobin (hemolysis), which enables the determination of the irritation degree of the EOs. The human venous blood samples were freshly collected and put into test tubes containing anticoagulant (EDTA-Na2 10.0%). For the calculation of  $H_{50}$  (effective concentration that causes 50% of hemolysis), TVEO was diluted (triplicates) to different concentrations (1 to 10  $\mu\text{L}/\text{mL}$ , in phosphate buffered saline (PBS), then, 50  $\mu\text{L}$  of blood was added and the tubes were homogenized and incubated for 90 min at room temperature. Samples were centrifuged at 2000 rpm for 5 min, and the supernatant was removed to measure the absorbance (540 nm) against the blank (100% buffered solution containing red blood cells) [17].

The absence of hemolysis (blank control) or total hemolysis (positive control) was determined by replacing the EO solution with an equal volume of PBS or distilled water, respectively. The results were determined by comparing the percentage of hemolysis with the positive control (100% hemolysis), and the experiments were performed in triplicate. The results were compared with a tube in which the cells were completely lysed by distilled water (positive control). The hemolytic activity of each sample was calculated by the formula:

$$\text{Hemolysis (\%)} = \left( \frac{\text{Abs sample}}{\text{Abs control}} \right) \times 100 \quad (1)$$

### 2.2.4. In Vitro and In Vivo Anti-Inflammatory Activities

#### Inhibition of Denaturation of Albumin In Vitro

The capability of TVEO to inhibit the denaturation of bovine serum albumin (BSA) was investigated by method as reported by Akinwunmi et al [18]. Typically, different concentrations (8–0.5  $\mu\text{L}/\text{mL}$ ) of TVEO were prepared and the volumes were adjusted to 2.5 mL with 0.85% NaCl. After which 0.5 mL of BSA (5 mg/mL) was added. The mixture was incubated at 37 °C for 20 min and further incubated at 55 °C for 30 min. The tubes were cooled and 2.5 mL of PBS (0.5 M, pH 6.3) was added. The turbidity was measured spectrophotometrically at 660 nm. The assay was carried out in triplicates and the standard (sodium diclofenac) was used in place of the TVEO. Percentage inhibition of BSA denaturation was evaluated as follows:

$$\% \text{ Inhibition} = \left( \frac{\text{Abs Control} - \text{Abs Test}}{\text{Abs Control}} \right) \times 100 \quad (2)$$

#### In Vivo Topical Anti-Inflammatory Activity

The topical anti-inflammatory activity of TVEO was evaluated as inhibition of the croton oil-induced ear edema in mice of 6–8 weeks of age [19]. Cutaneous inflammation was induced by the application of croton oil (10  $\mu\text{L}$  per mouse) in acetone to the inner surface of the left ear. The right ear received an equal volume of vehicle. TVEO (100, 10 and 2 mg/kg), diclofenac diethylammonium 1%

and vehicle were topically applied to the left ear 1 h before croton oil application. At the maximum of the edematous response, mice were sacrificed and a plug (6 mm<sup>2</sup>) was removed from the treated (left) and the untreated (right) ears. Edema was measured as the weight difference between the two plugs. As a reference, the non-steroidal anti-inflammatory drug (NSAID) diclofenac diethylammonium was used. Data are expressed as the mean  $\pm$  SD weight of the ears. Anti-inflammatory activity was expressed as percentage of edema weight reduction in treated mice in comparison to the untreated (control) group and calculated by the following formula:

$$I(\%) = \left(1 - \frac{\Delta W_t}{\Delta W_c}\right) \times 100 \quad (3)$$

where I (%) = percent inhibition of edema,  $\Delta W_t$  is the change in weight of ear tissue in the treated mice, and  $\Delta W_c$  is the change in weight of ear tissue in the control mice.

### Morphologic Analysis of Mouse Ear Tissue

Inflammatory response was checked and monitored with measurement of edema formation and microscopic observation. For morphologic examination of cutaneous inflammation, biopsies from control and treated ears of animals were collected at the end of the experiment. Samples were fixed in 10% neutral buffered formalin, routinely processed, and sectioned at 6  $\mu$ m using a microtome (Leica RM 2125RT, Nussloch, Germany). Sections were stained with Hematoxylin & Eosin (H&E) and length was evaluated using light microscopy. Tissues were observed with a light microscope (Olympus CX41) and graded as mild (+), moderate (++) or severe (+++) for inflammation phase. Infiltration and polymorphonuclear (PMN) cells' accumulations were also assessed [20].

#### 2.2.5. Statistical Analysis

Mean values of treated groups were compared with those of a control group and analyzed using statistical methods. Data of the ear edema of each mouse are reported as mean  $\pm$  standard deviation (SD). Comparison between different groups was conducted with one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test. Differences with  $p < 0.05$  between experimental groups were considered statistically significant. Statistical data analysis was performed using probit analysis method using XLStats 2013 software (Pros statistical software, Addinsoft, Paris, France).

## 3. Results

### 3.1. Chemical Composition of Thyme Essential Oil

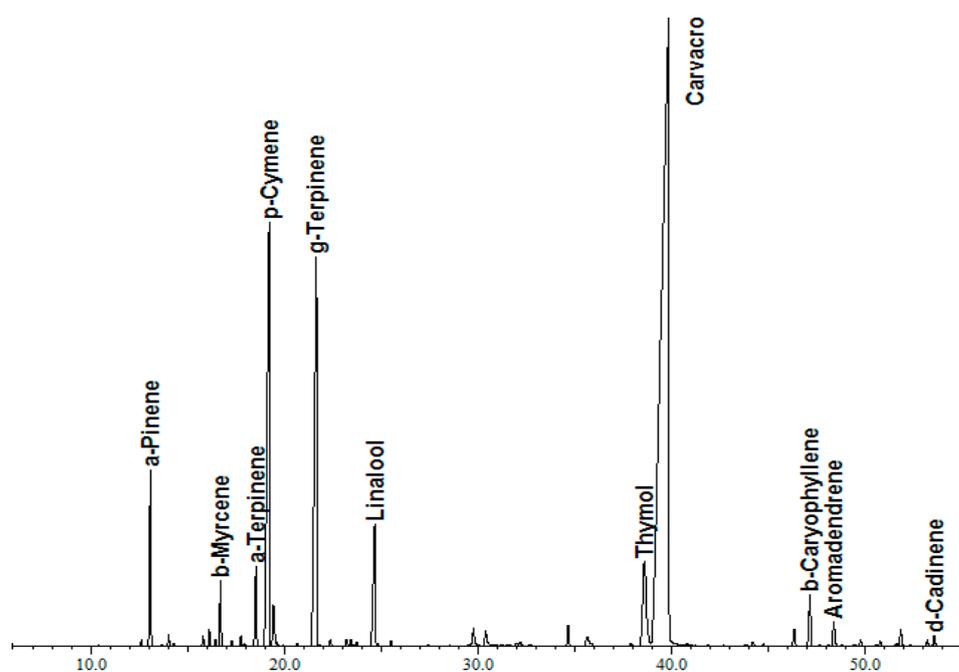
TVEO from fresh plant material had a yellow color and a strong camphor smell. The qualitative and quantitative compositions of TVEO, analyzed with GC-MS, are shown in Table 1 and Figure 1. Twenty five components of the volatile oil were identified. TVEO was composed of high amounts of carvacrol (56.8%), followed by *p*-cymene (12.8%),  $\gamma$ -terpinene (11.17%) and thymol (3.99%) (Figure 2). The amount of all other components of TVEO was less than (4%). TVEO showed a high content of oxygenated monoterpenes (65.44%) and low amounts of sesquiterpene hydrocarbons (2.82%) and oxygenated sesquiterpenes (0.52%).

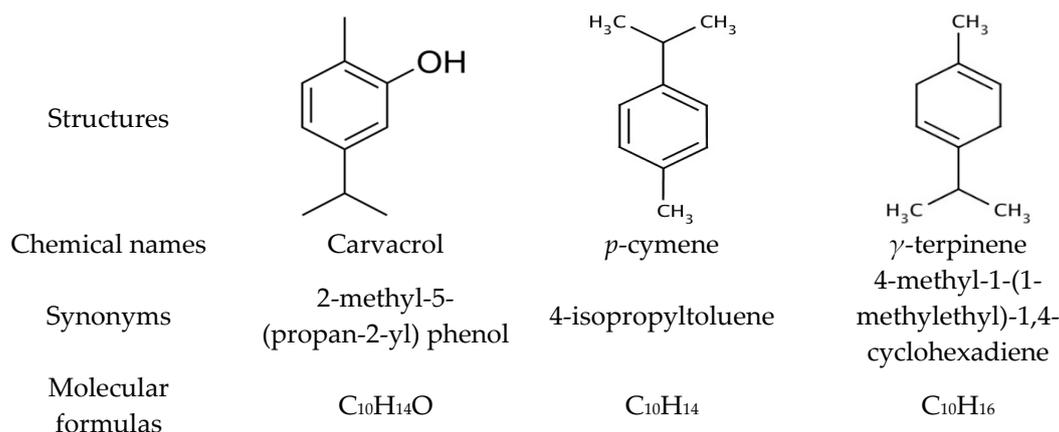
It has been reported that TVEO is a mixture of monoterpenes. The main compounds of this oil are the natural terpenoid carvacrol and its phenol isomer thymol, which have antimicrobial, antioxidant, expectorant and antispasmodic effects [21]. *Thymus* EO is the vital commercial product obtained by distillation of the flowering tops and fresh leaves of *T. vulgaris*. Its principal compounds are from the phenols (thymol and carvacrol). The phenols are the main chemical constituents of TVEO, thymol being the most commonly used for medicinal uses, but carvacrol, an isomeric phenol, dominates in some EOs. Cymene and pinene are present in the TVEO, as well as a small amount of menthone [8].

**Table 1.** Chemical profile of essential oil from thyme essential oil extracted by steam distillation and analyzed with gas chromatography.

N°	RI <sup>b</sup>	Retention Time (min)	Compound <sup>a</sup>	%	
1	926	13.09	$\alpha$ -Pinene	2.80	
2	940	14.03	Camphene	0.19	
3	969	15.81	$\beta$ -Pinene	0.18	
4	974	16.13	1-Octen-3-ol	0.27	
5	983	16.71	$\beta$ -Myrcene	1.05	
6	1000	17.75	$\alpha$ -Phellandrene	0.16	
7	1010	18.54	$\alpha$ -Terpinene	1.49	
8	1020	19.22	<i>p</i> -Cymene	12.8	
9	1023	19.44	Limonene	0.79	
10	1040	20.68	<i>cis</i> -Ocimene	0.04	
11	1054	21.65	$\gamma$ -Terpinene	11.17	
12	1095	24.67	Linalool	3.06	
13	1164	29.76	Borneol	0.47	
14	1173	30.40	Terpinen-4-ol	0.42	
15	1231	34.66	Carvacrol Methyl Ether	0.37	
16	1244	35.64	Pulegone	0.44	
17	1285	38.60	Thymol	3.99	
18	1303	39.84	Carvacrol	56.79	
19	1396	46.32	$\alpha$ -Gurjunene	0.37	
20	1408	47.14	$\beta$ -Caryophyllene	1.13	
21	1427	48.37	Aromadendrene	0.61	
22	1497	51.83	Leden	0.40	
23	1500	53.20	$\gamma$ -Cadinene	0.14	
24	1506	53.57	$\delta$ -Cadinene	0.23	
25	1562	57.15	Spathulenol	0.15	
				Oxygenated monoterpenes	65.44
				Monoterpene hydrocarbons	30.67
				Sesquiterpene hydrocarbons	2.82
				Oxygenated sesquiterpenes	0.52
				Total	99.51

<sup>a</sup> Compounds listed in order of elution from an DB-5 nonpolar column. <sup>b</sup> RI (retention index) calculated on the DB-5 column relative to C6–C17 n-alkanes.

**Figure 1.** Part of the chromatograph of thyme essential oil analyzed with Gas Chromatography-Mass Spectrometry. (X-axis in minutes).



**Figure 2.** Chemical structures of major components identified in *Thymus vulgaris* essential oil.

Our results disagree with those reported by Jordan et al. [22], who studied the oil composition of thyme in Spanish *Thymus vulgaris*. The main compounds identified were eucalyptol, followed by terphenyl acetate, borneol,  $\beta$ -pinene, linalool,  $\alpha$ -terpineol and camphor. Phytochemical studies have reported the occurrence of  $\alpha$ -pinene, *p*-cymene and terpinene in thyme EO [21]. *Thymus* species EO was the subject matter of numerous studies conducted in the last period. It was confirmed that TVEO contained thymol (44.1–58.1%), *p*-cymene (9.1–18.5%),  $\gamma$ -terpinene (6.9–18.9%) and carvacrol (2.4–4.2%). Current results are different from other earlier findings on the volatile oil of thyme (*T. longicaulis*) grown in some European countries (Italy and Serbia), which revealed  $\alpha$ -terpenyl acetate, geraniol and *p*-cymene as the principal chemical constituents of these EOs [23].

Certain reported species have been characterized by the presence of thymol with or without a small amount of its isomer carvacrol, such as *Thymus ciliatus*, *T. transcaspicus* and *T. dreatensis*. A little described thyme species have a small concentration of thymol and no carvacrol such as *Thymus aureopunctatus* and *T. serpyllum*. In contrast, few thyme species contain elevated quantities of carvacrol and small amounts of thymol, such as *Thymus caramanicus* and *T. pubescens* [8].

### 3.2. Antimicrobial Activity

#### 3.2.1. Disc–Diffusion Assay

The antifungal activity of TVEO was evaluated in vitro at three different volumes. The DIZ for yeast and filamentous fungi are given in Tables 2 and 3, respectively.

**Table 2.** Susceptibility of yeast strains to classic antiseptic and to *Thymus vulgaris* essential oil.

	Diameter of Inhibition Zone (mm) <sup>a</sup>						Positive Control HEX <sup>c</sup>
	Disc Diffusion Method			Vapor Diffusion Method			
	Quantity of TVEO ( $\mu$ L per disc)						
	20	40	60	20	40	60	
<b>Yeast Strains</b>							
<i>Candida albicans</i> (Ca1)	34	40	50	40	50	85	29
<i>Candida albicans</i> (Ca2)	29	35	49	35	65	85	33
<i>Candida albicans</i> (Ca3)	- <sup>b</sup>	19	27	12	18	28	20
<i>Candida tropicalis</i>	55	55	60	50	70	85	22
<i>Candida parapsilosis</i> (Cp1)	35	35	44	45	35	50	26
<i>Candida parapsilosis</i> (Cp2)	-	22	24	16	25	33	15
<i>Trichosporon</i> sp.	-	13	13	-	-	-	16
<i>Rhodotorula</i> sp.	25	32	33	-	15	16	22

<sup>a</sup> Diameters of inhibitory zone values are given as mean (mm). <sup>b</sup>—no activity. <sup>c</sup> HEX (Hexamidine, 1%) was used a positive reference standard for fungal strains. TVEO—*Thymus vulgaris* essential oil.

**Table 3.** Susceptibility of filamentous fungal strains to *Thymus vulgaris* essential oil.

Filamentous Fungal Strain	Diameter of Inhibition Zone (mm) <sup>a</sup>						Positive Control HEX <sup>c</sup>
	Disc Diffusion Method			Vapor Diffusion Method			
	Quantity of TVEO (μL/disc)						
	20	40	60	20	40	60	
<i>Aspergillus terreus</i>	55	60	75	45	65	85	33
<i>Aspergillus flavus</i> (Af 1)	40	50	45	45	75	85	19
<i>Aspergillus flavus</i> (Af 2)	35	44	50	36	68	85	26
<i>Aspergillus niger</i> (An 1)	35	35	35	35	42	50	26
<i>Aspergillus niger</i> (An 2)	- <sup>b</sup>	24	30	25	33	39	33
<i>Aspergillus fumigatus</i>	45	45	45	42	48	65	35
<i>Mucor</i> sp.	35	40	40	35	40	58	12
<i>Penicillium</i> sp.	30	50	45	40	42	45	29

<sup>a</sup> Diameters of inhibitory zone values are given as mean (mm). <sup>b</sup>—no activity. <sup>c</sup> HEX (Hexamidine, 1%) was used a positive reference standard for fungal strains. TVEO—*Thymus vulgaris* essential oil.

Evaluation of DIZ showed that TVEO was active against all the tested strains (Table 2). In our study, we have used yeast and fungal strains isolated from patients with cutaneous fungal infections typically involving the skin, hair and nails. These species could be considered as dangerous for patients with declining immune systems who are also at risk of developing microbial infections. This is the case of patients with HIV/AIDS, people under steroid or chemotherapy treatments.

Essential oil of thyme exhibited significant antifungal activity. TVEO showed little higher antifungal activity against *Candida tropicalis*, *Candida albicans* (Ca1) in comparison with positive control. *Candida tropicalis* and *C. parapsilosis* were the most vulnerable strains to the TVEO. The DIZ was 50 mm and 60 mm. Among filamentous fungi, TVEO was more effective against *Aspergillus terreus* and *A. fumigatus* with DIZ measured at 55 and 45 mm, respectively.

The comparison of our results with those from the literature for other *Thymus* species showed that the antifungal properties of TVEO are markedly stronger than those of other volatile oils distilled from other medicinal plants [24]. Furthermore, the antifungal effect displayed by *Thymus* genus EO has been confirmed by numerous studies [24–26].

Corresponding with our results, Giordani et al. [9] revealed that TVEO appeared to be the most effective, inhibiting all the *Candida* species. It has been confirmed that the combination of TVEO and Amphotericin B, for the treatment of *Candida*, may reduce the efficacious dose of Amphotericin B and thus minimize the side effects of Amphotericin B. Further, the authors reported a synergistic interaction between TVEO and Amphotericin B.

Our findings are in agreement with those reported for another thyme oil rich in carvacrol that showed an antifungal strong action versus *C. tropicalis* and *C. albicans* [9,27]. Essential oil of *Thymus* species has mostly aromatic oxygenated monoterpenes, carvacrol and thymol and their activity is often attributed to these compounds. Numerous papers have confirmed the antifungal effect of the EO and/or the extracts of different varieties of the genus *Thymus* rich in monoterpenic alcohols and/or volatile phenols [28–30].

The significance of the phenolic hydroxyl groups for the antifungal activity of the oxygenated monoterpenes has been reported [27,28]. Other species of the genus *Thymus*, such as *T. zygis* and *T. vulgaris*, with elevated quantities of phenols, also demonstrate a wide spectrum of inhibitory growth against a range of pathogenic filamentous fungi and yeasts, with decreased sensibility to antifungal drugs. However, carvacrol was more active against dermatophyte strains, in a similar manner to the EO [29–31].

Pinto et al. [29] showed that *Thymus* EO alone or in a formulation containing thymol, carvacrol and terpineol was strongly active against various species of fungi and *Candida* strains. The carvacrol

showed strong activity against *C. albicans*, *Trichophyton rubrum* and *Aspergillus flavus*. This study concluded that the EO and its major components may be a novel bio-resource to commercialize various species for specific antimicrobial products to manage human pathogenic diseases.

Numerous EOs have been tested for in vivo and in vitro antimycotic effect and some were revealed to be prospective antiseptic drugs. Their mode of action seems to be primarily on the microbial membrane layer, breaking its arrangement and producing cell death, delaying membrane creation, preventing microbial growth [11].

### 3.2.2. Vapor Diffusion Assay

Comparative investigations of antifungal effects of TVEO were also conducted in the vapor state. DIZ resulting from exposure to TVEO vapors are listed in Tables 2 and 3. As for TVEO in the liquid state, the DIZ due to the oil vapors was also augmented with increasing quantity of the oil. It is interesting to note that for all the tested strains, the DIZ resulting from exposure to TVEO vapors was considerably greater than to that from the same quantity in the liquid phase (Figure 3). This result was evident at both minor (20  $\mu$ L) and more prominently at larger (60  $\mu$ L) quantities. Nevertheless, it is surprising that despite the well documented study on the antifungal effect of TVEO, few reports exist on the biologic activity of EO vapors. *Candida albicans* (Ca1) and *C. parapsilosis* (Cp1) were the most sensitive strains to the EO vapor, and DIZ varied between 40 and 45 mm at 10  $\mu$ L TVEO per disc. Among filamentous fungi, the thyme volatile oil was more effective against *Aspergillus terreus* and *A. fumigatus* with DIZ measured at 45 and 42 mm, respectively. The yeast strains of *C. albicans* (Ca1) and *C. tropicalis* were inhibited entirely by the TVEO vapor at 60  $\mu$ L exposure. Our study evidently exhibits the upper antifungal effectiveness of TVEO vapor. This could be attributed to the difference in the relative composition of the oil and vapor because the latter must be enriched in terms of its volatile chemical compounds.

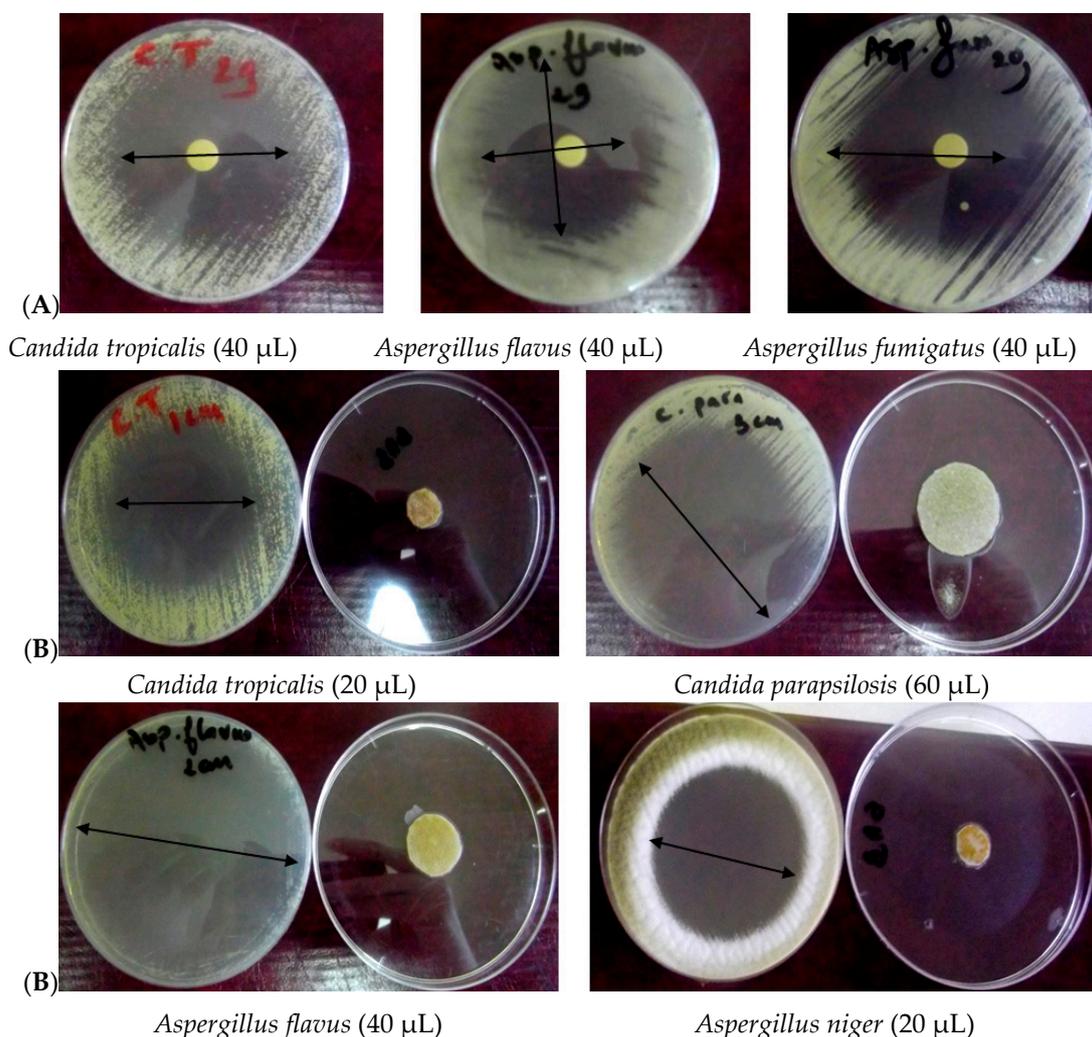
The use of TVEO in vapor phase could have further advantages such as efficiency without needing direct contact, resulting in simplicity of application. Therefore, smaller quantities of TVEO in the vapor phase can be inhibitory to filamentous fungi. Our findings are in conformity with preceding research [32,33]. A study carried out by Goñi et al. [33] revealed that the antimicrobial property of a combination of clove and cinnamon EO in vapor exhibited improved inhibitory effect with a smaller amount in the vapor diffusion method compared to the disc diffusion method. More recently, it has been suggested that, for EO having phenols, alcohols, oxides and esters, the main inhibition came from the vapors, while for EO containing larger amounts of aldehydes, inhibition came from diffusion [12]. An 80% reduction in airborne bacteria and fungi was found when a volatile oil blend of lemongrass and rose-scented geranium was dispersed continuously for 20 h via a fragrance generator dispersing the EO vapor, demonstrating that permanent diffusion is required for a total efficacy [12].

In our investigation, the findings from both vapor diffusion and agar diffusion assays were different, and this is in agreement with other studies [14,34]. Even though carvacrol has little volatility and modest solubility, its vapor has been reported to collect in large quantities into agar layers [35]. Chami et al. [36] revealed the high anti-*Candida* potential of carvacrol on experimental oral candidiasis in immunosuppressed animals and interestingly found that the yeast strains disappeared completely from the tongue mucosa in all treated rats whereas many hyphae were found when antifungal drug (nystatin) was used as a positive control.

TVEO clinical use is frequently discouraged because of its probable harsh or caustic effects when used at elevated doses due to its lipophilic character [37]. In fact, it is exactly these problems that could be in part solved by applying their specific volatility at room temperature. Generally, our results suggest the higher potency of TVEO in vapor phase rather than that in solution contact, signifying that it could have distinct advantages including the reduction of effective dose and potential chemical irritation.

The disc diffusion assay is considered inappropriate in measuring the antifungal potential of essential oils because the active volatile molecules are expected to evaporate, mutually with the dispersing solvent, and their nonpolar character stops them from diffusing throughout the agar media.

Consequently, the vapor phase methods are more consistent in evaluating the antifungal activities of TVEO [38,39].



**Figure 3.** Inhibitory effect of *Thymus vulgaris* against fungal strains: disc diffusion (A) versus vapor diffusion (B) methods.

### 3.2.3. Agar Dilution Assay

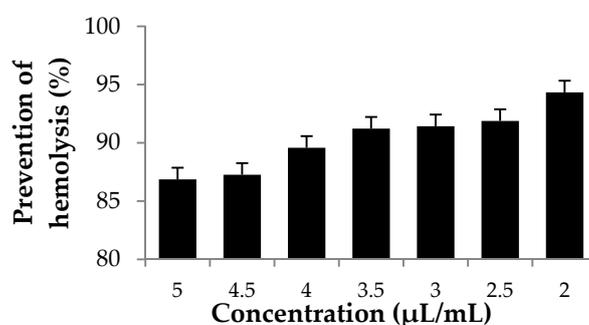
MIC values are shown in Table 4. The oils exhibited a concentration-dependent inhibition of growth. The MIC for fungal strains varied from 0.15 to 0.3 μL/mL. MIC for all *Candida* strains and *Trichosporon* sp. was higher (i.e., 0.3 μL/mL) than *Rhodotorula* sp. (i.e., 0.15 μL/mL); It is logical to consider that the inhibitory effect of TVEO can be related to the presence of volatile phenol (carvacrol). This molecule was found to be the most active component in our study. However, it is problematic to link the antifungal activity of a complex mixture (such as EO) to particular compounds. Some authors suggest that the strong antifungal effect of TVEO could be a result of synergism of major chemical constituents (cymene, terpinene and linalool) in the EO [37,40].

**Table 4.** Minimum inhibitory concentrations (MIC) of *Candida* strains determined with agar dilution method.

Yeast Strain	MIC ( $\mu\text{L/mL}$ )
<i>Candida albicans</i>	0.3
<i>Candida parapsilosis</i>	0.3
<i>Candida tropicalis</i>	0.3
<i>Trichosporon</i> sp.	0.3
<i>Rhodotorula</i> sp.	0.15

### 3.3. Hemolytic Activity Using Red Blood Cell (RBC) System Cellular Model In Vitro

Results of the irritation test showed that TVEO has a high hemolytic activity and a great attention will take with the greatest concentrations (Figure 4). It is necessary to be vigilant for the potential secondary effects of plant therapies because traditional experiments are not very useful when it is question of evaluating the risk. Moreover, administration of the plant by *per os* or others means requires some precautions and the determination of hemolytic effect of the extracts becomes necessary, but not taken only.

**Figure 4.** Effect of thyme essential oil on hyposaline induced hemolysis in red blood cells (RBC). Values are expressed as mean  $\pm$  SD of triplicate experiments.

Several aromatic and medicinal plants contain phytochemical compounds that may have a hemolytic or anti-hemolytic effect on human RBC. Numerous studies show that the membranes of human RBC have varying stability as determined from the mean corpuscular fragility. Phytochemicals can definitely disturb the erythrocyte cell membrane and many natural products have severe adverse effects, such as hemolytic anemia. Hence, most of the commonly used spices and herbs need to be evaluated for their possible hemolytic effect. Erythrocyte cells have been used as a typical cell by a number of researchers for the investigation of interaction of phytochemicals with cell membranes [17].

### 3.4. In Vitro and In Vivo Anti-Inflammatory Activity

#### 3.4.1. Inhibition of Denaturation of Bovine Serum Albumin In Vitro

Denaturation of protein is a recognized source of inflammation. Therefore, as part of the examination to assess the anti-inflammatory mechanism of TVEO, its aptitude to inhibit BSA denaturation was calculated. The inhibitory action of different concentration of TVEO on BSA denaturation is showed in Table 5.

It was detected from this assay that carvacrol rich fraction of TVEO presented a dose-dependent maximum inhibition of denaturation of BSA of 96.35% at 0.5  $\mu\text{L/mL}$  ( $\text{IC}_{50}$  value of  $6.843 \pm 0.830 \mu\text{L/mL}$ ) while a standard anti-inflammatory drug (sodium diclofenac) revealed maximum inhibition of 96.89% at the concentration of 1 mg/mL. Plant extracts such as EOs have been stated to inhibit protein denaturation. Therefore, protection against protein denaturation, which was the central mechanism of action of NSAIDs could play a significant part in the anti-rheumatic action.

**Table 5.** Effect of Thyme essential oil on heat induced protein denaturation.

Treatment (s)	Dose	Absorbance (660 nm)	% Inhibition of BSA	IC <sub>50</sub>
Control (PBS)		1.288	–	–
TVEO (µL/mL)	8	1.149	10.791	6.843 ± 0.830 <sup>A</sup>
	4	0.066	94.875	
	2	0.047	96.350	
	1	0.047	96.350	
	0.5	0.047	96.350	
Sodium diclofenac (mg/mL)	10	0.165	87.189	8.260 ± 0.943 <sup>A</sup>
	1	0.04	96.894	
	0.1	0.044	96.583	
	0.01	0.043	96.661	

Each value represents mean ± SD. TVEO—*Thymus vulgaris* essential oil. IC<sub>50</sub>—inhibitory concentration 50%, BSA—bovine serum albumin. Means within the same column followed by the same capital letter are not significantly different ( $p > 0.05$ ) in accordance with the ANOVA test followed by post hoc multiple comparison Tukey’s test.

### 3.4.2. In Vivo Topical Anti-Inflammatory Effect

To determine the topical effect of TVEO in vivo, we assessed ear edema inflammation induced by irritant solvent (croton oil) which leads to cell injury and stimulates phospholipase A2, which discharges arachidonic acid from the cell plasma membrane. In vivo croton oil administration to the left ear caused a superficial inflammatory reaction. Topical application of croton oil on the left ears caused noticeable edema as indicated by the augmentation in the ear plug weight of the left ear compared with the untreated right ear (Table 6).

**Table 6.** Thyme essential oil prevents croton oil-induced ear edema in vivo.

Treatment	Dose (mg/kg)	Mean Edema Weight (mg ± SD)	% Edema Inhibition
Control (PBS)		6.41 ± 2.45 <sup>B</sup>	–
TVEO	100	1.98 ± 0.29 <sup>A</sup>	73.00
	10	2.33 ± 0.20 <sup>A</sup>	68.02
	2	2.49 ± 1.15 <sup>A</sup>	65.69
Positive control (diclofenac diethylammonium)		1.82 ± 0.36 <sup>A</sup>	73.52

Results are given as mean (mg) ± SD ( $n = 5$  mice per group). Means within the same column followed by different capital letter are significantly different ( $p < 0.05$ ) in accordance with ANOVA test followed by post hoc multiple comparison Tukey’s test. TVEO—*Thymus vulgaris* essential oil.

In comparison with positive control, TVEO exhibited a similar and effective anti-inflammatory activity in our experimental animal model. Diclofenac diethylammonium produced a 73.52% inhibition of croton oil-induced ear edema, and this effect was statistically not different than that observed with all doses of TVEO (65.69% to 73%). The current research could be considered as the first report to demonstrate that Algerian TVEO has an important and significant topical anti-inflammatory effect in vivo. In agreement with current findings, Abe et al. [41] described that topical administration of volatile oils can prevent the inflammatory indicators of irritation and neutrophil recruitment. Some volatile oils are used as remedial components for topical inflammations such as lesional fungal infections [20].

Several *Thymus* species are medicinal plants of the Mediterranean vegetation, frequently used as flavors and as traditional treatments. They are also stated to have numerous pharmacological properties [34]. In recent times, it has been reported that the alcohol extracts of two *Thymus* species (*T. willdenowii* and *T. broussonettii*) grown in Morocco [42], were responsible for in vivo topical anti-inflammatory effects.

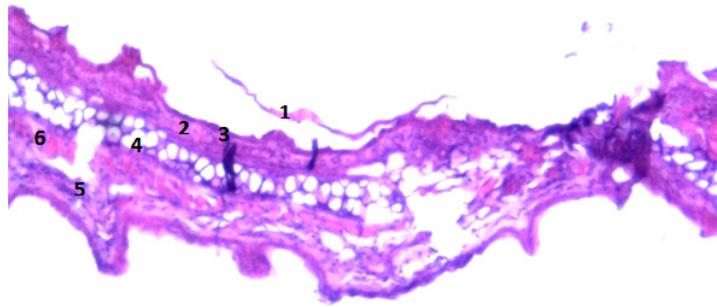
Hotta et al. [43] revealed that the carvacrol in thyme EO was an effective anti-inflammatory agent with cardioprotective capabilities, making it potentially beneficial for people with heart disease. More recently, Abdelli et al. [44] studied the chemical composition of two Algerian TVEO samples and evaluated their in vivo acute anti-inflammatory effect. Both TVEOs were confirmed to have anti-inflammatory properties, significantly reducing the in vivo carrageenan-induced paw edema (400 mg/kg *p.o.*) with a proportion ranged from 50.4% to 58.4%.

### 3.4.3. Examining the Mouse Ear Tissue Morphology

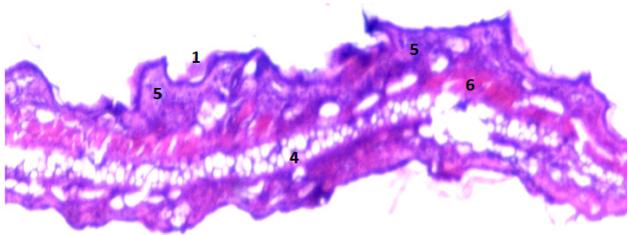
We investigated H&E-stained ear sections from croton oil-induced animals (Figure 5). Croton oil application resulted in a noticeable increase in ear thickness with obvious confirmation of edema, epidermal hyperplasia and inflammatory cell infiltration in the dermis with associated connective tissue disruption (Figure 5E). Microscopic investigation showed the valuable anti-inflammatory activities of the topical application with TVEO. Compared with the control groups, edema was dramatically reduced by the previous topical treatment with TVEO in particular when using a lower dose (Figure 5A). However, by using a larger dose of TVEO (2.5  $\mu$ L/mL), there was a medium inflammation-associated inflammatory cell infiltration (Figure 5C1–3) in the dermis. This may be related to the irritation effect of TVEO at higher concentration.

To the best of our knowledge, this is the first study to reveal that Algerian TVEO possesses a significant topical anti-inflammatory activity at lower concentration, which is confirmed by histopathology examination. By histological comparison, topical application of TVEO decreased ear thickness and associated pathologic indicators to an extent comparable to the positive controls (diclofenac diethylammonium 1%) (Figure 5D). These findings directly demonstrate the properties of TVEO within the target tissue, providing additional confirmation that TVEO ameliorates croton oil-induced contact dermatitis.

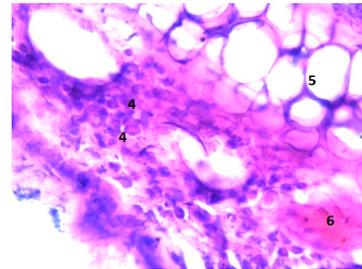
Our histopathology examinations are in agreement with previously published articles about other EOs using the croton oil-induced ear edema assay [20,45]. Further, the present data are in accordance with our previous publications [46], in which histological examination revealed that rose-scented geranium EO decreased the skin inflammatory process in vivo. The anti-inflammatory effect of TVEO can be principally linked to its major chemical constituent, carvacrol. Indeed, several investigations reported anti-inflammatory activities for this phenolic molecule [7,30,36]. In addition, it was shown that thyme EO decreases the release of inflammation mediators such as TNF- $\alpha$ , IL-1 and IL-8 [47]. However, minor molecules, such as *p*-cymene and linalool, can also lead to anti-inflammatory properties. For example, *p*-cymene has been shown to inhibit NF-Kb and MAPK signaling pathways, hence reducing the production of TNF- $\alpha$  and IL-1 $\beta$  [48]. Another minor compound (linalool) prevents the inducible nitric oxide synthase (iNOS) and therefore its proinflammatory effect [49].



(A) TVEO treatment (2 mg/kg) (×5). Edema (±); inflammatory cell infiltration (+), inflammation phase (±) in epidermal layer.

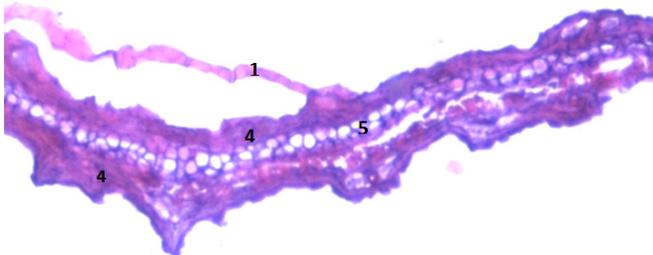


(B1) TVEO treatment (10 mg/kg) (×5)

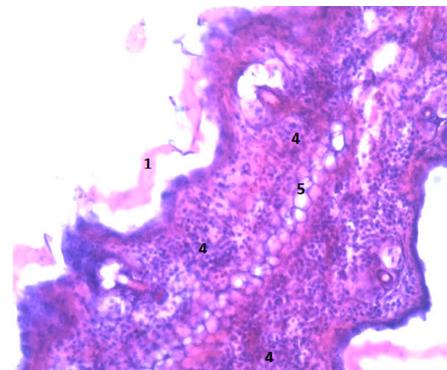


(B2) TVEO treatment (10 mg/kg) (×40)

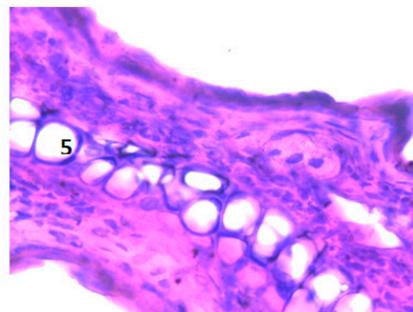
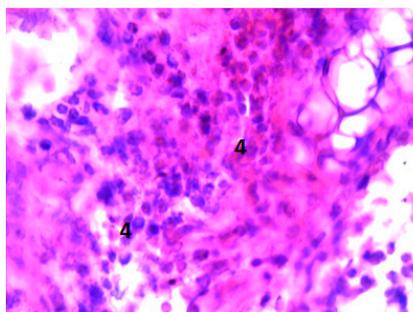
Edema (±); Inflammatory cell infiltration (+), inflammation phase (±) in epidermal layer and muscle.



(C1) TVEO treatment (100 mg/kg) (×5)

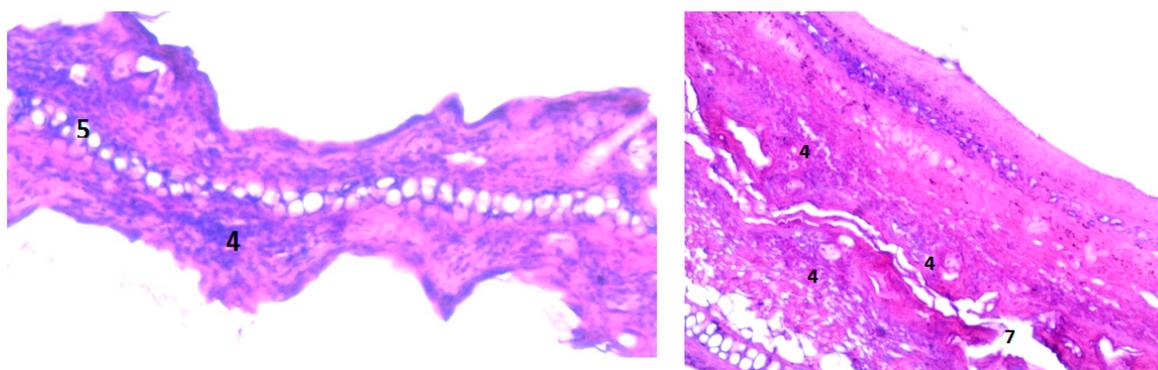


(C2) TVEO treatment (100 mg/kg) (×10)



(C3) TVEO treatment (100 mg/kg) (×40). Inflammatory cell infiltration (+), inflammation phase (±) in dermal layer, cartilage and muscle.

Figure 5. Cont.



(D) Positive control (diclofenac diethylammonium? 1%) (×10). Inflammation phase (±) in epidermal layer.

(E) Negative control (isosaline solution) (×10). Edema (++) and inflammatory cell infiltration (++) in dermal layer, cartilage and muscle.

**Figure 5.** Histological fragments of mice ear biopsies displaying epidermal, dermal and bone layers. H&E stained sections were recorded as minor (+), moderate (++) and great (+++) for edema and inflammatory (polymorphonuclear) cell infiltration. (1) keratin; (2) epidermal layer; (3) epithelium; (4) polymorphonuclear cells infiltration (PMN); (5) cartilage layer; (6) muscle; (7) edema.

#### 4. Conclusions

Our findings demonstrate that TVEO may be a source of antifungal and anti-inflammatory ingredient for the pharmaceutical industry. Our results support the alternative and complementary application of TVEO as an anti-inflammatory agent despite the need for further investigations to better estimate its pharmaceutical potential and understand its mode of action. Further studies are being considered to reveal the molecular targets modulated by TVEO and the major compounds responsible for its antifungal and anti-inflammatory properties.

**Author Contributions:** Conceptualization, M.N.B.; methodology, M.N.B., N.H.E.D., T.S., S.B., A.B.B., H.C., M.R., Y.B., and S.A.M.; software, M.N.B. and M.R.; formal resources, M.N.B., D.K., H.C. and S.A.M.; writing—original draft preparation, M.N.B.; writing—review and editing, M.N.B. and S.A.M. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was supported in part by the Fulbright Program Grant to Mohamed Nadjib Boukhatem and administered by the United States Department of State, Bureau of Educational and Cultural Affairs with the cooperation of the Institute of International Education (USA).

**Acknowledgments:** The authors would like to thank Kelly Keating (The Pharmaceutical Research Institute (PRI), Albany College of Pharmacy and Health Sciences, Rensselaer, NY, USA) for proofreading, constructive criticism and English editing of the manuscript. The authors are profoundly grateful to the “Institut Pasteur d’Algérie” and the “Laboratoire National de Contrôle des Produits Pharmaceutiques” (Algiers, Algeria) for their technical assistance and support.

**Conflicts of Interest:** The authors declare no conflict of interest.

#### Abbreviations

ANOVA	analysis of variance
COX	cyclooxygenase
DIZ	diameter of inhibition zone
EO	essential oil
GC-MS	gas chromatography-mass spectrometry
GRAS	generally recognized as safe
H&E	hematoxylin & eosin
IL-1	interleukin-1
Inos	inducible nitric oxide synthase
MIC	minimum inhibitory concentration

NIST	National Institute of Standards and Technology
NSAID	non-steroidal anti-inflammatory drug
PBS	phosphate buffered saline
PMN	polymorphonuclear cells
RBC	red blood cell
ROS	reactive oxygen species
SDA	sabouraud dextrose agar–chloramphenicol
TNF	tumor necrosis factors
TVEO	<i>Thymus vulgaris</i> essential oil

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