GC/MS Analysis, Cytotoxicity, and Antimicrobial Properties of Six Moroccan Essential Oils Traditionally Used for COVID-19 Prevention

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Abstract

The COVID-19 pandemic has reignited interest in traditional medicinal plants as potential therapeutic agents. This study examines the chemical composition, cytotoxicity, and antimicrobial activity of essential oils from six Moroccan medicinal plants: *Eucalyptus globulus, Artemisia absinthium, Syzygium aromaticum, Thymus vulgaris, Artemisia alba*, and *Santolina chamaecyparissus*, commonly used by the Moroccan population for COVID-19 prevention. The chemical composition of each essential oil was determined using gas chromatography-mass spectrometry (GC-MS) to identify key compounds. Cytotoxicity was evaluated on the Vero E6 cell line, frequently used in SARS-CoV-2 research, using the neutral red assay, with oil concentrations ranging from 25 to 100 µg/mL. Antimicrobial activity was tested against multidrug-resistant strains such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans*, and *Bacillus subtilis* using the disc diffusion method. The GC-MS analysis revealed significant components such as spathulenol (15%) and caryophyllene oxide (7.67%) in Eucalyptus globulus, and eugenol (54.96%) in *Syzygium aromaticum*. Cytotoxicity assays indicated that higher concentrations of essential oils significantly reduced cell viability, with *Thymus vulgaris* showing the highest IC_{50} (8.324 µM) and *Artemisia absinthium* the lowest (18.49 µM). In terms of antimicrobial activity, *Eucalyptus globulus* exhibited the strongest effect with a 20 ± 0.00 mm inhibition zone against *Bacillus subtilis*, while both *Syzygium aromaticum* and *Artemisia herba-alba* showed a 12.25 ± 0.1 mm inhibition zone against the same strain. These findings suggest that these essential oils have significant therapeutic potential, particularly in combating antimicrobial resistance and cytotoxic effects on viral cell lines. Further research is necessary to explore their mechanisms of action and ensure their safety for therapeutic use.

1. INTRODUCTION

Since the Middle Ages, aromatherapy has been employed in traditional pharmacopoeia for a wide range of applications, including cancer treatment, anti-inflammatory, antibacterial, and antiviral activities, as well as insecticidal, medicinal, and cosmetic uses [1]. Essential oils derived from these plants contain a complex mixture of volatile compounds such as monoterpenes, sesquiterpenes, phenolic derivatives, and aliphatic compounds which are believed to contribute to their therapeutic effects [2].The chemical composition of these oils can vary significantly based on the plant species, geographic origin, and extraction methods [2]. To this day, the importance of aromatic oils remains significant, with increased understanding of their mechanisms of action on various biological and medicinal activities, such as cytotoxicity and antimicrobial effects.

Morocco, like many other countries, has a long history of using medicinal aromatic plants for curative and preventive purposes. Its Mediterranean location and diverse climatic conditions enrich its vegetation and plant species. In addition, the herbal market in Morocco has a good knowledge of the benefits and medicinal uses of plants, which means that the majority of Moroccans turn to consuming different plants at the height of the Covid-19 pandemic, not only because of the lack of treatment, but also because they are cheaper and more available [3]. Moreover, numerous ethnobotanical studies conducted during the pandemic in all of the kingdom's prefectures provide valuable insights into traditional knowledge and practices relating to the use of these plants, serving as a basis for scientific investigation. The aromatic medicinal plants examined in this study, including *Thymus vulgaris L., Eucalyptus globulus*, and *Artemisia absinthium L.*, are among the most widely distributed and used by the Moroccan population for treating respiratory ailments. For instance, *Artemisia absinthium* and *Thymus vulgaris L.* are commonly consumed with tea by Moroccans during the winter season. On the other hand, cytotoxic effects were observed *in vitro* against bacteria and eukaryotic cells, causing membrane damage, depolarization of mitochondrial membranes by decreasing the membrane potential, coagulation of the cytoplasm, and damage to cellular lipids and proteins, as well as exerting prooxidant activity [1]. These cytotoxic properties can disrupt the cellular function of

microorganisms, making essential oils effective as antiseptic and antimicrobial agents for personal hygiene and air purification [1]. This attribute makes essential oils promising candidates for research against multidrug-resistant bacteria. Indeed, aromatic plants have demonstrated notable activity in various studies [4–11]. The rise of multidrug resistance, combined with the adverse effects of current antibiotics on public health and the lack of new effective antibiotics on the horizon, underscores the urgent need to explore alternative treatments [12].

Currently, the COVID-19 pandemic, caused by the novel coronavirus SARS-CoV-2, has highlighted the urgent need for effective treatments and preventive measures. Various studies have reported the promising anti-COVID-19 properties of essential oils from plants such as *Mentha pulegium, Mentha microphylla, Mentha vilosa, Mentha thymifolia, Illicium verum, Syzygium aromaticum, Citrus limon*, and *Pelargonium graveolens*. These essential oils have demonstrated a selectivity index (SI) of more than four against SARS-CoV-2 [13]. The vaporous nature of essential oils gives them an advantage in targeting the respiratory system, where SARS-CoV-2 primarily infects. Eucalyptol, for example, has shown a direct virucidal effect by interfering with the binding of SARS-CoV-2 spike proteins to ACE2 receptors. This compound tends to concentrate in the lungs, particularly in the lower respiratory tract, where it may exert a local virucidal effect [14][15].

The cytotoxicity of essential oils may correlate with their antiviral activity, as previous research has demonstrated a direct relationship between high cytotoxicity and strong anti-SARS-CoV-2 effects. For example, essential oils such as *Syzygium aromaticum, Cymbopogon citratus, Citrus limon, Pelargonium graveolens, Origanum vulgare*, and *Matricaria recutita* exhibited high antiviral activity, which was accompanied by high cytotoxicity. In contrast, *Zingiber officinalis, Melaleuca alternifolia*, and *Rosmarinus officinalis* showed lower cytotoxicity and correspondingly lower anti-SARS-CoV-2 activity [16].

This study offers new insights into the chemical composition, cytotoxicity, and antimicrobial activity of essential oils from Moroccan medicinal plants, including *Artemisia absinthium L., Syzygium aromaticum, Artemisia herba-alba, Eucalyptus globulus, Thymus vulgaris L.*, and *Santolina chamaecyparissus*. Although these plants have been investigated in prior research, our study reveals notable differences in their chemical profiles and their corresponding cytotoxic and antimicrobial effects. By identifying their chemical compounds and establishing non-cytotoxic concentrations, we provide valuable data that can guide further exploration of these essential oils for potential antiviral or anti-COVID-19 applications. Notably, this research includes *Artemisia absinthium L., Santolina chamaecyparissus*, and *Artemisia herba-alba* tested on the Vero E6 cell line for the first time, offering essential insights into the safe and effective use of these oils in therapeutic contexts.

2. MATERIAL AND METHODS

2.1. Plant collection.

Aromatic medicinal herbs (*Artemisia absinthium L., Syzygium aromaticum, Artemisia herba-alba*, and *Eucalyptus globulus*) were procured from a local herb store in Rabat, Morocco, in March 2022. The medicinal plants were identified morphologically with the assistance of Professor Khamar Hamid from the Botany Department at the Scientific Institute of Mohammed V University in Rabat, Morocco. Additional plant species, including *Santolina chamaecyparissus* and *Thymus vulgaris L.*, were provided in May 2022 by the National Agency for Medicinal and Aromatic Plants (ANPMA) in Taounate, Morocco. This study utilized the aerial parts of *Artemisia absinthium L., Santolina chamaecyparissus, Artemisia herba-alba, Eucalyptus globulus*, and *Thymus vulgaris L.*, as well as the seeds of *Syzygium aromaticum*, to accurately reflect their traditional usage by the Moroccan population.

2.2. Extraction of essential oils.

The fresh aerial parts were dried at room temperature in a dark, open drying room for ten days. After drying, they were pulverized into small pieces using a Willy mill and labeled. Essential oils from six species were prepared using a conventional hydrodistillation method in a Clevenger-type apparatus, following a slightly modified version of the method described by [17]. Specifically, 200 g of each sample was accurately weighed and combined with 1L of distilled water in a 2 L flask connected to a Clevenger-type apparatus with a Dean-Stark distillation tank. The mixture was heated to 100°C for 3 hours, as per the European Pharmacopoeia's standard procedure. The concentrated essential oil was then manually separated from the aromatic water and dried over anhydrous sodium sulfate to remove any residual moisture, ensuring the essential oil remained dry. The final extracts were stored in amber-colored bottles at -20°C for subsequent cytotoxicity testing.

2.3. Gas Chromatography–Mass Spectroscopy Analysis.

The essential oils were analyzed by gas chromatography-mass spectrometry (GC-MS) using a Shimadzu GC-2010 gas chromatograph coupled to a Shimadzu GCMS-QP2010 Ultra mass detector, operating with electron ionization at 70 eV. Sample injections (1 µL) were performed using an AOC-20i autosampler, and a 30 m × 0.25 mm i.d. capillary column with a 0.25µm film thickness Teknokroma TRB-5 (95% dimethyl-diphenylpolysiloxane, 5%) was used. The operating conditions were as follows: split ratio of 20:1, injector temperature at 300°C, transfer line temperature at 250°C, and initial column temperature set at 70°C. The temperature was then ramped to 290°C at a rate of 6°C/min. Full scan mode (m/z 35–450) was used for detection. The identity of compounds was determined by comparing electron ionization mass spectra and retention data with those in the Wiley 229 and NIST 17 Mass Spectral Databases. All extracts (4 µg/µL) were dissolved in 100% DCM for injection.

2.4. Cytotoxicity assay of essential oils

2.4.1. Vero -E6 cells.

The potential effect of essential oils on cell morphology and viability was investigated in vitro using the Vero-E6 cell line, which has been shown to be highly sensitive to the SARS-CoV-2 virus. The cell line corresponds to green monkey kidney fibroblast cells (Cercopithecus aethiops) in continuous culture (ATCC-CRL 1586)/Passage:3, provided by the CNR Lyon-France.

2.4.2. Culture medium and preparation of plates.

The culture medium used for maintaining and growing the cells was Dulbecco's Modified Eagle's Medium (DMEM), modified with Earle's salts and non-essential amino acids. This medium was buffered with 7.5% sodium bicarbonate to a pH of 6.90 ± 0.1 under CO_2. Sterile fetal bovine serum (FBS) (10%) was added to this DMEM medium. Vero-E6 cells were cultured as monolayers in tissue culture flasks (75 – 25 cm²) under standard conditions of 37°C ± 1°C, 90% ± 5% humidity, and 5.0% ± 1% CO_2/air. Cell morphology was monitored daily using a phase-contrast microscope to ensure optimal growth conditions. After reaching 80% confluence, the cells were detached from the flasks with 0.5% trypsin diluted in PBS. For the experiment, a cell suspension of 3.105 cells/mL was prepared and added to 96-well microtiter plates (200 µl per well). The plates were then incubated for 24 ± 2 hours under the same conditions to allow for cell recovery and adhesion, forming a monolayer of less than 50% confluence.

2.4.3. Preparation of essential oils.

To prepare the essential oils, 10 µL of each oil (equivalent to 1 mg) was solubilized in a mixture of two emulsifiers, DMSO/TWEEN 20, at a concentration of 2% (v/v). This mixture was optimized to ensure complete solubilization and improve the diffusion of volatile molecules, while minimizing toxicity to the cells. The resulting homogeneous stock solution for each essential oil was then prepared for assay. Each stock solution was further diluted in culture medium

(without FBS) using a titration plate. The initial dilution was 1/10, followed by a series of double dilutions over a range of concentrations from 100 µg/mL to 0.39 µg/mL.

2.4.4. Cytotoxicity using the neutral red colorimetric assay (NR).

The cytotoxicity of the selected essential oils was evaluated at the National Hygiene Institute in Rabat, Morocco using the Neutral Red Uptake (NRU) assay, based on the method described by [18] with some optimizations. The NRU assay utilizes the neutral red dye, a weak cationic dye that readily penetrates cell membranes by passive non-ionic diffusion. The dye accumulates in lysosomes, where it binds to anionic and phosphate groups in the lysosomal matrix via electrostatic and hydrophobic interactions.

Essential oils were tested at various concentrations, ranging from 100 to 0.39 µg/mL, in quadruplicate for each well. Two control columns were included: one containing the respective solvent and the other containing untreated cells. Immediately after adding the oils, adhesives were applied to the plates to prevent contamination by vapors from high doses. The plates were incubated for 48 hours at 37°C in a 5% CO_2 atmosphere. After the incubation period, the cells were rinsed with pre-warmed D-PBS to remove any residual oil. The cells were then incubated for 3 hours in a neutral red medium, prepared in DMEM at a concentration of 40 µg/mL, to allow the formation of NR crystals indicating cell viability. Following the 3-hour incubation, the NR medium was removed, and the cells were rinsed with D-PBS before adding NR Desorb solution consisting of 50% ethanol 96%, 49% deionized water, 1% glacial acetic acid to extract the NR dye from the cells. The plates were shaken for 10 minutes to ensure thorough extraction, and absorbance measurements were taken immediately at 540 nm ± 10 nm using a microplate reader (Multiskan EX, Thermo Scientific).

Cell viability was calculated using the following formula:

% Cell viability = (OD of essential oils / OD of control cells) ×100

where OD refers to Optical Density, control cells refer to solvent-treated cells, and OD of essential oils refers to the treated cells.

2.5. Antimicrobial activity

2.5.1. Microbial Strains.

The six strains used in this study were chosen for their multidrug-resistant profiles. These include two Gram-negative strains, *Escherichia coli ATCC 25922* and *Pseudomonas aeruginosa ATCC 27853*, three Gram-positive strains, *Staphylococcus epidermidis ATCC 12228, Bacillus subtilis ATCC 6633*, and *Staphylococcus aureus ATCC 25923*, as well as one yeast strain, *Candida albicans ATCC 10231*.

2.5.2. Preparation of the inoculums.

Before conducting antimicrobial tests, the microbial strains were subcultured overnight at 37°C for 24 h (Bacteries) and 48h (fungi) in nutrient agar favorable to their growth. Bacterial strains were cultured on Mueller-Hinton Agar (MHA), while yeast strain was cultivated on Potato Dextrose Agar (PDA). Well-isolated colonies of the bacterial species were collected using a sterile platinum loop and suspended in sterile saline solution. The optical density of the suspensions was adjusted to a range of 0.4 to 0.6 at 405 nm using a spectrophotometer, corresponding to a concentration of 10^6-10^7 CFU/mL [10].

2.5.3. Disk diffusion method on agar

To evaluate the sensitivity of the microbial strains to the different essential oils, a disk diffusion method was employed [11]. Briefly, 10 µL of diluted essential oil samples (20 mg/mL, dissolved in 10% DMSO/ 0.5% Tween 20) were applied to sterile filter paper discs with a diameter of 6 mm. The discs were then placed on Mueller-Hinton Agar (MHA) previously inoculated with the test microorganisms using a sterile loop. For fungal strains, Potato Dextrose Agar (PDA) was used. A disk with an equivalent volume of 10% DMSO/ 0.5% Tween 20 served as the negative control. Positive controls included commercially available antibiotic discs (BIO-RAD), such as gentamicin (10 µg), nalidixic acid (30 µg), ampicillin (10 µg), spectinomycin (100 µg), penicillin (6 µg), fluconazole (30 µg), and itraconazole (30 µg). The Petri dishes were incubated at 4°C for 2 hours to facilitate compound diffusion. Subsequently, the diameters of the inhibition zones were measured in millimeters after 24 hours of incubation at 37°C for bacteria and 48 hours at 28°C for yeast.

The results are expressed as the diameter of the inhibition zone and are categorized according to the sensitivity of the strains to the extracts, as follows [9]:

- **Not Sensitive (−) or Resistant**: Diameter < 8 mm
- **Sensitive (+)**: Diameter between 9 and 14 mm
- **Very Sensitive (++)**: Diameter between 15 and 19 mm
- **Extremely Sensitive (+++)**: Diameter > 20 mm

2.6. Data Analysis

Relative cell viability was expressed as a percentage of the vehicle control (VC). The mean Neutral Red Uptake (NRU) values from four replicates per concentration were calculated, with blank values subtracted. The IC50 value was determined by fitting a non-linear regression curve to the normalized data using GraphPad Prism software version 9.1.0, with results reported as the mean ± S.D. Statistical differences between experimental and control values were analyzed using one-way ANOVA followed by Tukey's post hoc test, with a significance level set at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Essential oil chemical composition

The complexity of the essential oils was identified and quantified by chromatography coupled with mass spectrometry (GC-MS) analysis. Our findings revealed differences when compared to previous analyses, highlighting variations in the chemical profiles and potential implications for their use. In the absinthe oil analyzed, 51 compounds were identified, representing 99.81% of the total compounds. The results showed that the oil is rich in α-thujone **(3)** (29.02%) and camphor **(4)** (24.34%), with notable amounts of chamazulene (6.92%) and (-)-4-terpineol (3.68%) (Table 1). The essential oil obtained by hydrodistillation of dried wormwood has a dark blue color with a strong odor. The composition of *Artemisia absinthium* essential oil has been highly variable, resulting in the identification of at least nine distinct chemotypes [19]. Thujone, a bicyclic monoterpene, is an important compound mentioned in several references [3–8]. The presence of β-thujone isomer has been mentioned to be higher than his isomer α-thujone **(3)** in the oil [7]. An Iranian study on wormwood revealed a difference of 23.8% β-thujone and 18.6% α-thujone **(3)** [20]. In another study on a Serbian plant, β-thujone was the main chemotype, accounting for 63.4% of the oil extracted from the aerial plant herb, while alpha was only 0.4%[21]. Contrary to expectations, our study revealed that α-thujone **(3)** was present at a remarkably high level of 29%, while β-thujone was detected at only 1.67%. Surprisingly, another Moroccan study proved that *Artemisia absinthium* essential oil was rich in 3,3,5-trimethylcyclohexene (27.93%), which was absent in our study, and camphor **(4)** (22.50%), a level similar to what we

found, but no α-thujone and β-thujone were detected in the sample [22]. Camphor **(4)** (structure shown in Fig. 1) was also found in the oil of wild plants collected in Italy and Iran, at concentrations of 17.1% and 14.8%, respectively[23], [24]. The chemotypic variations of *Artemisia absinthium* are qualitatively and quantitatively significant. These differences are observed in various regions of Europe, where the plant grows natively, as well as in regions where it has been introduced. At least 17 major compounds were identified in the plant oil across Europe, including myrcene, sabinene, sabinyl acetate, epoxyocimene, chrysanthenol, chrysanthenyl acetate, linalool, chamazulene and β-pinene. Indeed, cis-epoxyocimene was the main essential oil compound in all samples collected in Teruel, Spain, with a concentration of (49.3–71.5%), followed by cis-chrysanthenyl acetate (7.6–18%) and linalool (0.7–10.4%) [7, 8]. It has also been reported as a dominant compound in Italy (24.6%) [9], France (54.4%) [10], Tajikistan (17.9%) [11]. However, our sample was devoid of cis-epoxycimene, as well as chrysanthenol, chrysanthenyl acetate and β pinene. Studies on Tunisian and Turkish oils reported higher concentrations of chamazulene, which is responsible for the oil's blue color, with concentrations of 39.9% and 17.8%, respectively [9, 10]. This is significantly higher compared to our sample and another Moroccan sample collected in Marrakech, which had a chamazulene concentration of 8.02% [11]. Another interesting compound identified in our sample is arborescin, a sesquiterpene compound scarcely found in wormwood oil from other geographical regions. A Moroccan study has confirmed also the presence of this molecule at 6.82% [11].

Table 1
Chemical composition of *Artemisia absinthium L.* (boldface indicate the major compound).

N_0	Compound[a]	Ret.Time (min)	Relative content (%)	Molecular formula
1	Sabinene	5109	1.64	C10H16
2	β-Myrcene	5291	2.58	C10H16
3	γ-Terpinene	6740	1.32	C10H16
4	*trans* Sabinene hydrate	7038	1.54	C10H18O
5	Linalool	7566	1.06	C10H18O
6	β- Thujone	7883	1.67	C10H16O
7	**α-Thujone**	**8153**	**29.02**	C10H16O
8	**Camphor**	**8920**	**24.34**	C10H16O
9	**(-)-4-Terpineol**	**9578**	**3.68**	C10H18O
10	Caryophyllene	15147	1.23	C15H24
11	Germacrene-D	16504	2.65	C15H24
12	3,6-Dihydrochamazulene	17111	1.52	C14H16
13	Elemol	17898	1.53	C15H26O
14	**Chamazulene**	**21772**	**6.92**	C14H16
15	Arborescin	26180	2.18	C15H24
16	*geranyl*-α-terpinene	26491	2.14	C15H24

[a] Molecules higher than 1%

The chemical composition of *Eucalypthus globulus labill* identified 67 compounds, representing 99.26% of the total oil (Table 2**).** Our oil analysis detected spathulenol **(2)** (structure shown in Fig. 1) as an abundant component with an amount of 15%, while the predominant compound, 1,8-cineole or eucalypthol, the best-known molecule in eucalypthus oil, recorded only 4.52%. These results contradict another Moroccan study conducted in the province of Ouejda in Morocco, where the results showed a percentage of 79.85% for 1,8-cineole, while no trace was detected for spathulenol **(2)** [25]. An Algerian study also has confirmed the presence of 1.8-cineole with a high percentage (55.29%) and a low percentage of spathulenol **(2)** (7.44%)[26]. On the other hand, other molecules were detected in our study as major compounds and were absent in other works[17–19] such as caryophyllene oxide **(1)** (7.67%), trans-caryophyllene (7.33%) and farnesol (7.52%). In addition, monoterpene hydrocarbons were also strongly present in our sample, such as p-cymene (6.29%), β-phellandrene (4.2%), α-pinene (3.6%) and limonene (1.26%), which is consistent with findings from publications [19, 20], although with slight quantitative variations.

Table 2
Chemical composition of *Eucalyptus globulus*.

N_0	Compound[a]	Ret.Time (min)	Relative content (%)	Molecular formula
1	α-Pinene	4473	3.6	C10H16
2	**p-Cymene**	**6086**	**6.29**	C10H14
3	Limonene	6169	1.26	C10H16
4	β-Phellandrene	6241	4.2	C10H16
5	**1,8-Cineole**	**6277**	**4.52**	**C10H18O**
6	Linalool	7567	1.02	C10H18O
7	4-Terpineol	9580	2.33	C10H18O
8	Crypton	9810	1.82	C10H16O
9	α-Terpineol	9909	3.95	C10H18O
10	Copaene	14088	1.08	C15H24
11	**trans-Caryophyllene**	**15154**	**7.33**	**C15H24**
12	Neolloocimene	16059	2.39	C15H24
13	**Spathulenol**	**18624**	**15**	**C15H24O**
14	**Caryophyllene oxide**	**18773**	**7.67**	**C15H24O**
15	Viridiflorol	19012	2.23	C15H26O
16	Isoleptospermone	19164	1.14	C15H22O3
17	*cis*-Eudesm-6-en-11-ol	19228	1.08	C15H26O
18	Aromadendrane-4,10-diol	19726	1.59	C15H26O2
19	**Farnesol**	**21172**	**7.52**	**C15H26O**
20	Valerenal	21791	1.18	C15H22O
21	(1S,4R,5S)-1-Methyl-4-(prop-1-en-2-yl)spiro[4.5]dec-7-ene-8-carbaldehyde	22188	2.15	C15H24O

[a] Molecules higher than 1%

The chromatographic profile of *Syzygium aromaticum* oil (Fig. 2) revealed 16 compounds, representing 99.72% of the total oil content (Table 3). The main compound is eugenol **(9)** (54.96%), followed by trans-caryophyllene **(10)** (29.18%). These molecules are the best-known in clove essential oil in various studies [13–16]. In addition to these two molecules mentioned, we confirmed the presence of α-humulene (3.79%) which was detected at only 0.19% in reference [15] and eugenol acetate (5.41%) which was found in trace amounts in reference [14]. However, two molecules were interestingly found in our sample, α-cubebene (1.34%) and α-copaene (1.9%), which are absent from the publications cited above.

Table 3
Chemical composition of *Syzygium aromaticum.*

N_0	Compound[a]	Ret.Time (min)	Relative content (%)	Molecular formula
1	α-Cubebene	13369	1.34	C15H24
2	**Eugenol**	**13604**	**54.96**	C10H12O2
3	α-Copaene	14099	1.9	C15H24
4	**trans-Caryophyllene**	**15172**	**29.18**	C15H24
5	α-Humulene	15968	3.79	C15H24
6	Eugenol acetate	17115	5.41	C12H14O3
[a] Molecules higher than 1%				

The characterization of *Thymus vulgaris* essential oil via GC-MS analysis revealed a total of 40 compounds, collectively accounting for 100% of the oil's composition. Among these, 14 compounds were found to have concentrations greater than 1%, with individual percentages ranging from 1.28–33.33% (Table 4). The major component identified was thymol (**5**), an active monoterpene, representing 33.33% of the essential oil. This was followed by significant quantities of its terpenic hydrocarbons precursors, *p*-cymene (**6**) (25.87%) and γ-terpinene (7.21%). Additionally, a noteworthy concentration of carvacrol (5.23%) was observed, which is another phenolic monoterpene related to thymol **(5)**. Comparison of these results with those reported by C.Jianu [29] reveals a reverse order of abundance for these compounds: *p*-cymene was reported at 8.41%, γ-terpinene at 30.90%, and thymol **(5)** at 47.59% while no carvacrol was detected. This divergence highlights the influence of harvest time, whose concentration of these components exhibited synchronized patterns of variation over the course of the vegetative cycle, bearing in mind that γ-terpinene is the precursor of *p*-cymene **(6)** and the latter at the same time as thymol **(5)** and carvacrol [30]. In addition to thymol **(5)**, which contributes to the quality of thyme oil, there are other chemotypes that characterize other regions, such as the linalool chemotype in France, the geraniol chemotype and the sabinene hydrate chemotype in Serbia [31]. Furthermore, camphor has been demonstrated as a chemotype of thyme essential oil of eastern Morocco at a concentration of 39.39%, which was not detected in our study [32]. Spanish thyme, endemic to the Iberian Peninsula, has been characterized by a 1,8-cineole chemotype that was recorded at 2.39% in our analysis [30].

Table 4
Chemical composition of *Thymus vulgaris.*

N_0	Compound[a]	Ret.Time (min)	Relative content (%)	Molecular formula
1	Amyl vinyl carbinol	5,127	1.78	C7H14O
2	β-Myrcene	5,294	1.82	C10H16
3	α-Terpinene	5,920	1.28	C10H16
4	**p-Cymene**	**6,099**	**25.87**	C10H14
5	1,8-Cineole	6,279	2.39	C10H18O
6	**γ-Terpinene**	**6,746**	**7.21**	C10H16
7	Linalool	7,568	3.34	C10H18O
8	L-Borneol	9,438	2.26	C10H18O
9	4-Terpineol	9,582	2.03	C10H18O
10	Isothymol methyl ether	10,812	1.41	C11H16O
11	**Thymol**	**12,053**	**33.33**	C10H14O
12	**Carvacrol**	**12,246**	**5.23**	**C10H14O**
13	Caryophyllene	15,155	1.44	C15H24
14	Caryophyllene oxide	18,761	2.19	C15H24O

[a] Molecules higher than 1%

As for *Artemisia Herba-alba*, 50 compounds were detected in the GC/MS analysis, representing 96.73% of the total oil (Table 5). Among these compounds, davana ether **(11)** was found to be the main compound with a concentration of 14.48%. In a Tunisian study [33], it was detected as three isomers while a Spanish study [34] identified it as two isomers. In another Moroccan study, davanone was identified as the chemotypic compound of the essential oil, followed by davana ether **(11)**. This contrasts with our findings, where davanone was present at a concentration of only 3.21% [35]. This suggests the presence of specific and advanced biosynthetic pathways in the plan. Other davanone derivatives such as nordavanone (2.19 %) and davaa-furan (1.31 %) have als been detected; these compounds are a valuable ingredient in perfumery and aromatherapy and are generally isolated from the essential oil of *Artemisia pallens*. In addition to the aforementioned sesquitepenes, three oxygenated monoterpenes were found with different proportions: (+)-2-bornanone **(4)** known as camphor **(4)** 6.02% and α- and β-thujones (4.09% and 4.31%, respectively). The authors of a comparative study [36] detected the presence of the same compounds with a quantitative difference, with β-thujone being higher than α-thujone **(3)**, while the Spanish samples did not contain traces of the latter [34]. Three other oxygenated monoterpenes were recognized as fingerprints of this essential oil in addition to those mentioned above: chrysanthenone, cis chrysanthenyl acetate and 1,8-cineole as published in [28–30] where they were classified as major compounds compared to the low traces found in our sample (cis chrysanthenyl acetate (0.97%) and 1,8-cineole (0.67%)) (see full table in the appendix). This may be explained by the difference in harvesting time, since camphor plays a role in the chrysanthenone biosynthesis pathway.

Table 5. Chemical composition of *Artemisia helba-alba*.

N_0	Compound[a]	Ret.Time (min)	Relative content (%)	Molecular formula
1	Cyclohexanebutanal, 2-methyl-3-oxo-, *cis*-	4653	1.41	C11H18O2
2	Decane, 2-cyclohexyl-	6140	1.41	C16H32
3	*cis*-Arbusculone	6594	1.17	C15H24O2
4	**β-Thujone**	**7884**	**4.31**	**C10H16O**
5	**Thujone**	**8132**	**4.09**	**C10H16O**
6	**(+)-2-Bornanone**	**8905**	**6.02**	**C10H16O**
7	Nordavanone	10530	2.19	C10H14O2
8	*Cis* jasmone	14504	1.97	C11H16O
9	Davana furan	14735	1.31	C11H16O
10	**2-Butyl-5-methyl-3-(2-methylprop-2-enyl) cyclohexanone**	15883	2.49	C14H24O
11	**Davana ether 1**	**16789**	**14.48**	**C13H22O2**
12	Artedouglasia oxide C	17474	1.46	C15H22O2
13	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a α, 4a α-,7 β, 7a β,7b α)]-	18610	2.91	C15H24O
14	Caryophyllene oxide	18755	1.36	C15H24O
15	**1-((1R,2R,3R)-2-(3-Isopropylfuran-2-yl)-3-methylcyclopentyl) ethanone**	18944	1.5	C13H20O2
16	3-Methyl-but-2-enoic acid, 1,7,7-trimethyl-bicyclo [2.2.1] hept-2-yl ester	19146	2.1	C15H24O2
17	Humulenol-II	19564	1	C15H26O
18	**2-Propenal, 3-(2,6,6-trimethyl-1-cyclohexen-1-yl)-**	**20291**	**5.58**	C12H18O
19	Davanone	20656	3.21	C14H22O

[a] Molecules higher than 1%

Santolina chamaecyparissus essential oil was also studied, yielding 70 compounds, containing 99.98% of the oil (Table 6). The predominant compound observed was longiverbenone **(7)** (nootkatone) with a proportion of 18.15%, followed by artemisia ketone (**8**) (15.58%). In comparison with the results published by the authors of [39], they detected artemisia ketone **(8)** as the main compound with a proportion close to 15.65%, while nookatone recorded only 6.97%. According to numerous revised articles, various chemotypes from different regions have been noted to be totally devoid of nootkatone **(7).** For instance, in Turkey, artemisia ketone **(8)** was found to comprise 38.1%[32]; in Tunisia, 1,8-cineole (12.94%) and β-eudesmol were present but absent from our sample [41]. In Algeria, camphor **(4)** and cubenol were reported to represent 31.1% and 17.0%, respectively [42]. In Spain, camphor **(4)** (25.19%) and *allo*-aromadendrene (19.04%) were also found to be absent from our essential oil[43]. In Italy, *p*-cymene **(6)** (15.33%) and β-phellandrene (12.75%) were identified and compare with our results of 1.3% and 2.73%, respectively [44]. In Egypt, caryophyllene oxide was observed as the main compound, constituting 3.62% [45]. Other compounds known as the fingerprints of this plant were detected in different proportions, such as α-curcumene (4.82%), spathulenol **(2)** (4.41%) and epizanone (3.19%). These intraspecies variations in essential oil profiles could be explained by various

factors such as the phenological stage, seasonal changes, and specific harvesting and processing methods. A complete list of all identified compounds, their characteristics can be found in the supplementary materials (Appendix).

Table 6
Chemical composition of *Santolina chamaecyparissus.*

N_0	Compound[a]	Ret.Time (min)	Relative content (%)	Molecular formula
1	β- Myrcene	5293	1.28	C10H16
2	*Para* cymene	6085	1.3	C10H14
3	β-Phellandrene	6239	2.73	C10H16
4	**Artemisia ketone**	**6669**	**15.58**	C10H16O
5	*cis*-Chrysanthemyl alcohol	8803	1.67	C10H18O
6	(+)-2-Bornanone	8905	2.11	C10H16O
7	*endo*-Borneol	9432	1.17	C10H18O
8	α-Longipinene	13552	1.96	C15H24
9	γ-Curcumene	16288	1.55	C15H22
10	**α-Curcumene**	**16376**	**4.81**	**C15H22**
11	n-Pentadecanol	16464	1.4	C15H32O
12	Germacrene-D	16505	1.79	C15H24
13	δ-Cadinene	17228	1.52	C15H24
14	Caryophyllene oxide	17735	1.49	C15H24O
15	**Spathulenol**	**18611**	**4.41**	**C15H24O**
16	Epizizanone	18763	3.19	C15H22O
17	β-Oplopenone	19157	1.46	C15H22O
18	(+)-*trans*-caran-*cis*-3-ol	19216	1.82	C10H18O
19	(2R,3R,4aR,5S,8aS)-2-Hydroxy-4a,5-dimethyl-3-(prop-1-en-2-yl)-2,3,4,4a,5,6-hexahydronaphthalen-1(8aH)-one	19998	2.77	C15H24O2
20	**Longiverbenone**	**20123**	**18.15**	C15H22O

[a] Molecules higher than 1%

3.2. Cytotoxicity of essential oils

The cytotoxicity of essential oils was tested on the Vero E6 cell line, a model frequently used for SARS-CoV-2 research, with cells incubated at different doses. After 48 hours, cell viability was determined using the neutral red test. Specific essential oils exhibited significant cytotoxic activity as a function of concentration, ranging from 0.39 to 100 µg/mL, as shown in the Fig. 3. Results are presented as mean cell viability percentages compared to vehicle control cells, representing 100% viability. Each graph corresponds to a specific essential oil, with different concentrations. Two additional sets of bars represent baseline cell viability (untreated cells) and cells treated with

DMSO/TWEEN 20, serving as controls to assess the impact of the solvents and natural cell growth, respectively. A one-way ANOVA was performed to determine the statistical significance of the differences observed between the cytotoxic effects of each concentration of essential oil and the controls. The analysis shows that high concentrations of oils, such as 100, 50, and 25 µg/mL, significantly decrease cell viability compared to the vehicle control, with P values < 0.05. Notably, essential oils at concentrations between 100 and 12.5 µg/mL showed a marked reduction in cell viability, reaching as low as 17% of control levels, which is significantly lower than the effects observed with Vehicle controls (approximately 95% viability). Less concentrated solutions, from 12.5 to 0.3 µg/mL, in all samples showed no statistically significant difference from controls (**Figure.4**). The IC_{50} values, which indicate the concentration at which the essential oil reduces cell viability by 50%, also showed notable variation in the cytotoxic potency of the six essential oils analyzed. *Thymus vulgaris L.* and *Eucalyptus globulus* exhibited potent IC_{50} values of 8.324 and 8.363 µM, respectively. *Santolina chamaecyparissus, Syzygium aromaticum*, and *Artemisia herba-alba* followed with IC_{50} values of 11.27, 12.17, and 13.69 µM, respectively. The inhibitory potential of *Artemisia absinthium* was intriguing but not as strong as that of the other plants, with an IC50 value of 18.49 µM.

These findings (Fig. 3) can be compared with previous research[16] that highlighted the potent anti-SARS-CoV-2 and cytotoxic properties of some essential oils, including *Thymus vulgaris L., Eucalyptus globulus*, and *Syzygium aromaticum.* In that study, cytotoxicity was assessed using the XTT assay on HeLa cells expressing human angiotensin-converting enzyme 2 (HeLa ACE-2), similar to Vero E6 cells in terms of anti-COVID-19 research. The reported CC_{50} values were 23.9 µg/mL for Eucalyptus globulus, 2 µg/mL for Thymus vulgaris, and 0.42 µg/mL for *Syzygium aromaticum.* These values contradict our findings, where *Thymus vulgaris* and *Syzygium aromaticum* exhibited lower cytotoxicity. This discrepancy suggests potential variations due to differences in cell lines, which include distinct genetic backgrounds, proliferation rates, metabolic activities, cellular targets, drug transport mechanisms, or essential oil composition. In particular, the same study identified thymol as a chemotype of *Thymus vulgaris* with a higher proportion of carvacrol (25.5%) compared to 5.23% in our study. For *Syzygium aromaticum*, they recorded a higher concentration of eugenol (87.9% vs. 54.96% in our sample). Eugenol and carvacrol have been documented for their strong cytotoxicity [46]. However, in another study, carvacrol and thymol did not show significant cytotoxicity on Vero 76 cells, another cell model for SARS-CoV-2 research, with IC_{50} values of 455 and 508.60 µg/mL, respectively [47], just as for eugenol tested at concentrations (15–250 mg/mL) on Vero cells in [48], no cytotoxic effects was observed. Additionally, the chemical composition variance of *Eucalyptus globulus* was notable, with the previous study [16]showing a high concentration of 1.8-cineole (86.62%) compared to our characterization, which showed spathulenol **(2)** as the major compound (15%), while 1.8-cineole was only 4.52%.

However, according to the findings presented in [47], S.Ćavar Zeljković et al., have demonstrated the lack of cytotoxic effect of a single monoterpene, 1.8-cineole, on Vero 76 cells, taking into account that interactions between monoterpenes could increase their cytotoxicity on human cells [49], it was even found that the combined effect was more important than the individual chemical effects. This is due to the theory that other tiny molecules have the ability to alter the activity of the main constituents, which may improve the dispersion of the essential oil [42].

Subsequently, the same study [16] has applied an interesting chemometric study using PDA analysis to demonstrate the role of functional group of essential oils compounds on antiviral and cytotoxicity effect. They have proven that hydrocarbons, ketones and ethers decreased or appeared to decrease anti-SARS-CoV-2 activity and cytotoxicity, while the opposite was held for aldehydes, phenols, alcohols and esters. This may explain the variance in toxicity of our Eucalyptus oil, which was rich in spathulenol **(2)** (an alcohol), whereas the one in the previous study was rich in 1.8-cineole (an ether). The same theory can explain the cytotoxic effect of the thymol and carvacrol (phenol groups) identified in Thymus vulgaris oil. Additionally, thymol has the ability to disrupt cell membranes and induce apoptosis

in a variety of cancer cell lines, as documented in the literature (Braga et al., 2006). Vero cells may be subject to comparable mechanisms, indicating the potentially far-reaching efficacy of these essential oils in combating enveloped viruses such as SARS-CoV-2, the causative agent of COVID-19. Moreover, the cytotoxic effect of thyme oil on the HeLa and MCF-7 cell lines was measured, and the results showed IC_{50} values of 34.66 ppm and 27.66 ppm, respectively [50] showing lower cytotoxicity compared to the IC_{50} found in our study on Vero E6 cells (8.324 µM) and on HeLa-ACE-2 in [16]. On the other hand, other studies on eucalyptus oil have indicated potential cytotoxicity in cancer cell lines such as HEK293t, SW48 and HepG2 [51]. However, the same study also showed low cytotoxicity in fibroblast cells, with an IC_{50} of 5%, comparing to the epithelial cell Vero E6 used in our study.

Continuing with the cytotoxicity of our plant's essential oils, *Santolina chamaecyparissus L.*, commonly known as cotton lavender, which is undervalued, has been traditionally used for its medicinal properties in the Mediterranean region, including its antiseptic, anti-inflammatory, and insect-repellent effects[52]. However, we have found a lack of activity in terms of antiviral and anticancer effects, particularly for its essential oil extract. One study conducted by E.Elsharkawy et al., on HepG2 and A549 cells demonstrated a 38.9% reduction in cell viability at 25 µg/mL and 11.8% at 100 µg/mL, respectively [53]. They suggested that α-bisabolol and caryophyllene oxide are responsible for this cytotoxicity because they are major compounds in its oil, but no studies have proven this conclusively. Conversely, another study on A549 cells estimated an IC_{50} between 200–240 µg/mL[54], and identified artemisia ketone **(8)** and camphor **(4)** as chemotypes in their sample. Additionally, they evaluated the affinity between these major molecules and Ezrin, an oncogenic protein, using docking studies, showing strong binding which contributes to the downregulation of this protein's expression and therefore inhibited cell motility. Contradictorily, these statements were in discordance with our findings that the IC_{50} evaluated was more toxic at 11.27 µg/mL. In addition to the variance of cell lines and methods assay used, the qualitative and quantitative chemical variance may also be a cause of this cytotoxicity difference, where we have detected a significant concentration of nootkatone **(7)** (18.15%) which is scarcely identified in Santolina oil. Nootkatone **(7)**, or longiverbenone **(7)**, has shown similar cytotoxicity on retinoblastoma cells with an IC_{50} of 10.2 µM [55] and also exhibited an inhibitory effect with IC_{50} values of 4.27 – 27.6 µg/mL against human promyelocytic leukemia HL-60 cells [56]. Moreover, it has been proven to play a principal role in inhibiting cell cycle arrest, activating the generation of endogenous reactive oxygen species, mediating autophagy, and suppressing the NF-κB signaling pathway[57]. In recent investigations of SARS-CoV-2 research, nootkatone has been underlined as a possible potent inhibitor of SARS-CoV-2 main proteases using a bioinformatics approach [58]. Thus, the cytotoxicity on Vero E6 cells spurred further in vitro antiviral investigation.

Artemisia herba alba and *Artemisia absinthium* are revered, owing to their plethora traditional uses in Ayurveda [59]. In this work, *Artemisia herba alba* oil performed an inhibitory effect with an IC_{50} of 13.59 µM. This value can be compared with the findings reported in[60], where they evaluated different parts of the plant's oil, showing a variance in IC_{50} values: 15 µg/mL for leaves oil and 36 µg/mL for capitulum oil on the P815 cell line. Additionally, they also reported an IC_{50} of 20 µg/mL for capitulum oil and 26 µg/mL for leaves oil on the BSR cell line. They suggest that the difference in chemical composition between plant parts influences biological activity, as they identified widdrene, α-bulnesene with significant proportions in leaves, which is not the case in capitulum oil where Verbenol and β-guaiene (not found in leaves) were noticed as significant compounds. It should be noted that these molecules were not detected in our sample. These statements support the theory that the nature, concentration and synergism of the molecules affect the variance of cytotoxicity, not to mention the sensitivity of the molecular characteristics of the cell lines tested to these molecules.

Although *Artemisia absinthium* oil exhibited lower cytotoxicity in our study with an IC_{50} of 18.49 µM, it still demonstrated potent cytotoxicity compared to findings observed in[61] and [62]. In their studies, significant

sensitivity was observed in SK-MEL-5 (melanoma), HCT116 (colorectal adenocarcinoma), A549, and H292 (adenocarcinoma and squamous nonsmall cell lung cancer) cell lines at 100 μM of two fractions isolated from absinthium oil extract rich in *trans*-caryophyllene and/or germacrene [61]. Conversely, according to[62], it was reported that the essential oil exhibited weak cytotoxicity against three cancer cell lines (A-431, MCF7, and PANC-1) at 100 μM, with davanone constituting 56.07% of the total oil. The higher cytotoxicity observed in our study can be attributed to the significant amount of thujone (29.02%) found in our oil, compared to the slight traces reported in the aforementioned studies. Thujone is well-known as a toxic monoterpene, as demonstrated in research[63] on MRC-5, HT-29, and HCT116 cell lines, with IC_{50} values of 2.2, 2.8 and 1μM, respectively. In general, the main constituents of essential oils reflect their biophysical and biological properties, with the extent of their effects depending on their concentration, whether they are tested individually or in the essential oil blend [60].

3.3. Antimicrobial activity

The antimicrobial efficacy of the essential oils, as presented in Table 7, was compared to that of standard antibiotics and antifungal agents (Table 8) across various microbial strains, using inhibition zone diameters as the primary metric for evaluation. For *Escherichia coli*, *Eucalyptus globulus* demonstrated sensitivity with an 11 mm inhibition zone, categorizing it as Sensitive (+), though it was less effective than Gentamicin and Nalidixic acid, which both had inhibition zones of 18 mm, classifying them as Very Sensitive (++). Pseudomonas aeruginosa showed resistance to all essential oils tested (inhibition zone < 8 mm), with only Gentamicin providing minimal sensitivity (+) with a 10 mm zone, this resistance aligns with *P.aeruginosa*'s well-documented challenge in treatment due to its robust resistance profiles. These results align with previous findings indicating that the genus Eucalyptus exhibits limited activity against Gram-negative strains, with Minimum Inhibitory Concentrations (MIC) ranging between 1000–2000 μg/ml, which is significantly higher than the 200 μg/mL concentration used in our study [8]. Generally, Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* are known for their higher resistance, likely due to their outer lipopolysaccharide membrane[64]. This membrane acts as a physical barrier, hindering the penetration of lipophilic compounds, including essential oils, thereby reducing their efficacy. Furthermore, these bacteria possess a key mechanism known as efflux pumps. These pumps actively recognize and expel a variety of substances from the cell, including not only antibiotics but also other lipophilic noxious compounds that might otherwise enter the cell [65]. Eucalyptus globulus showed extreme sensitivity (+++) against Bacillus subtilis with a 20 mm inhibition zone, closely matching the 20 mm zone observed with Nalidixic acid. Moderate sensitivity (++) was observed with *Syzygium aromaticum* and *Artemisia herba-alba* against *Bacillus subtilis*. These results align with those of Prabuseenivasan et al., who identified Bacillus subtilis as the most susceptible strain to all tested essential oils across four different concentrations (1:1, 1:5, 1:10, and 1:20) [6]. For *Staphylococcus aureus, Thymus vulgaris L*. demonstrated moderate sensitivity (+) with a 15 mm inhibition zone, though this was significantly less than the extreme sensitivity (+++) observed with Penicillin and Ampicillin, which produced 30 mm and 31 mm zones, respectively. Our findings reinforce the observation that Gram-positive bacteria are generally more sensitive to essential oils than Gram-negative bacteria. This increased sensitivity is likely due to the structural differences in their cell walls. Gram-positive bacteria possess a thick peptidoglycan layer that is more permeable to essential oils and antimicrobial agents [5]. In contrast, Gram-negative bacteria have an outer membrane that acts as a more substantial barrier, limiting the penetration of these compounds. The essential oils showed little to no activity against Staphylococcus epidermidis, with Gentamicin providing only moderate sensitivity (+) with a 12 mm inhibition zone. This pathogen is known to readily acquire antibiotic resistance via horizontal gene transfer, particularly exchanging resistance genes with other Staphylococcus species, including Staphylococcus aureus [7]. In the case of *Candida albicans*, *Santolina chamaecyparissus* exhibited slight sensitivity (+) with an 11.75 ± 0.1 mm zone, which was less effective compared to the extreme sensitivity (+++) indicated by Itraconazole with a 19 mm zone. These findings contrast with those of Lisin et al.,[4] who reported that *Candida albicans* was highly sensitive to various essential oils,

including *Thymus vulgaris* (both borneol and linalool types), *Thymus serpyllum*, several Eucalyptus species (*Eucalyptus smitii, Eucalyptus globulus, Eucalyptus radiata*, and *Eucalyptus polybractea*), *Eugenia caryophyllus* (bud), and *Rosa damascene*. The reduced effectiveness observed in this study may be attributed to differences in the chemical composition or concentration of the essential oils used. Additionally, intrinsic resistance mechanisms of Candida albicans, such as biofilm formation or adaptive responses, could have played a role in diminishing the antifungal activity. On the other hand, our results indicated that the solvent used for diluting the essential oils (10% DMSO/ 0.5% Tween 20) demonstrated no antibacterial activity against any of the tested bacterial strains. Overall, although some essential oils demonstrated antimicrobial activity, their effectiveness largely fell within the lower sensitivity categories and did not reach the levels of sensitivity observed with conventional antibiotics and antifungal agents. This highlights their limited potential as alternatives for treating resistant microbial strains. Furthermore, variations in the activity of essential oils across different bacterial groups are anticipated due to the structural and functional differences in bacterial membranes. Additional research is needed to better understand the interactions between these essential oils or their key compounds and various bacterial strains.

Table 7
Antibacterial activities of the essential oils

Essential oils	**Inhibition Zone (mm) for Reference Strains[a]**					
	Negative Gram (-)		**Positive Gram (+)**			**Yeast strain**
	Escherichia coli	***Pseudomonas aeruginosa***	***Bacillus subtilis***	***Staphylococcus aureus***	***Staphylococcus epidermidis***	***Candida albicans***
Eucalyptus globulus	11.25 ± 0.6	NA	20 ± 0.00	NA	15 ± 0.7	NA
Thumus vulgaris L.	NA	NA	NA	15.05 ± 0.7	NA	NA
Artemisia absinthium L.	NA	NA	NA	NA	NA	NA
Syzygium aromaticum	NA	NA	12.75 ± 0.1	NA	NA	NA
Artemisia helba-alba.	NA	NA	12.25 ± 0.1	12.25 ± 0.1	NA	NA
Santolina chamaecyparissus	NA	NA	10.00 ± 0.6	NA	NA	11.75 ± 0.1

[a]Diameter of the inhibition zone (mm), including a 6 mm disc, tested at a concentration of 0.2 mg/disc. NA indicates no activity. Values (mean ± SD, $n = 3$).

Table 8
Inhibition Zone Values (mm) for Reference Antibiotics and Antifungal Agents. Values (mean ± SD, *n* = 3).

Microbial Strains	Fluconazole 30µg/mL	Gentamicin 10µg/mL	Nalidixic acid 30µg/mL	Itraconazole 30µg/mL	Penicilin 6µg/ mL	Ampicillin 10µg/mL
Escherichia coli	Nt	18.25 ± 0.3	18 ± 0.00	Nt	10.62 ± 0.3	NA
Pseudomonas aeruginosa	Nt	10.25 ± 0.6	NA	Nt	NA	NA
Bacillus subtilis	Nt	NA	20.05 ± 0.00	Nt	15.25 ± 0.6	12.00 ± 0.00
Staphylococcus aureus	Nt	20 ± 0.1	NA	Nt	30.75 ± 0.2	31.05 ± 0.4
Staphylococcus epidermidis	Nt	12.06 ± 0.4	10.25 ± 0.25	Nt	NA	NA
Candida albicans	12 ± 0.00	Nt	Nt	19 ± 0.00	Nt	Nt

NA: Not active, Nt: Not tested.

4. CONCLUSION AND FURTHER DIRECTION

In summary, this study evaluated the cytotoxic effects of various essential oils extracted from aromatic medicinal plants commonly used in Morocco for COVID-19 prevention and treatment on Vero E6 cells. *Thymus vulgaris L., Eucalyptus globulus*, and *Santolina chamaecyparissus* exhibited the most potent cytotoxic activities. Variations in cytotoxicity compared to previous research were attributed to differences in chemical composition, cell lines, and assay methodologies. Key compounds such as thymol, carvacrol, eugenol, and thujone were significant contributors to the observed effects.

The assessment of antimicrobial activity against a range of multidrug-resistant bacterial and fungal strain revealed variable effectiveness. Differences in strain biology and essential oil chemistry were highlighted, demonstrating the limited potential of these oils as alternatives to traditional treatments for resistant strains. Further investigation is necessary to understand the mechanisms of action of essential oils and their active compounds, and to optimize their use in antimicrobial therapies.

These findings support the need for continued research to explore the therapeutic potential of these essential oils in treating COVID-19 and other viral infections. Future studies should include comprehensive in vitro and in vivo analyses to better understand the antiviral mechanisms and validate the efficacy of these oils against SARS-CoV-2. Our laboratory is currently investigating the antiviral properties of these essential oils, which may lead to novel, plant-based therapeutic strategies for combating viral infections.

Declarations

Availability of data and materials

The datasets supporting the results of this article are included within the article itself and the Supplementary Materials.

Informed Consent Statement

Not Applicable.

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Ethical Approval

Ethical approval was not applicable for this article.

Conflicts of Interest

The authors declare no conflict of interest.

Author contributions

Conceptualization, Bouchaib Bencharki, José Francisco Quílez del Moral, and Houda Zaher; methodology, Houda Zaher, Azucena Gonzalez-Coloma, and José Francisco Quílez del Moral; software, Azucena Gonzalez-Coloma and Houda Zaher; validation, Houda Zaher, Sanae Lemrabet, José Francisco Quílez del Moral, and Bouchaib Bencharki; formal analysis, Sanae Lemrabet; investigation, Houda Zaher; resources, Azucena Gonzalez-Coloma, Sanae Lemrabet, and José Francisco Quílez del Moral; data curation, Houda Zaher; writing—original draft preparation, Houda Zaher; writing—review and editing, Sanae Lemrabet and José Francisco Quílez del Moral; visualization, Bouchaib Bencharki and José Francisco Quílez del Moral; supervision, José Francisco Quílez del Moral and Bouchaib Bencharki. All authors have read and agreed to the published version of the manuscript.

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Figures

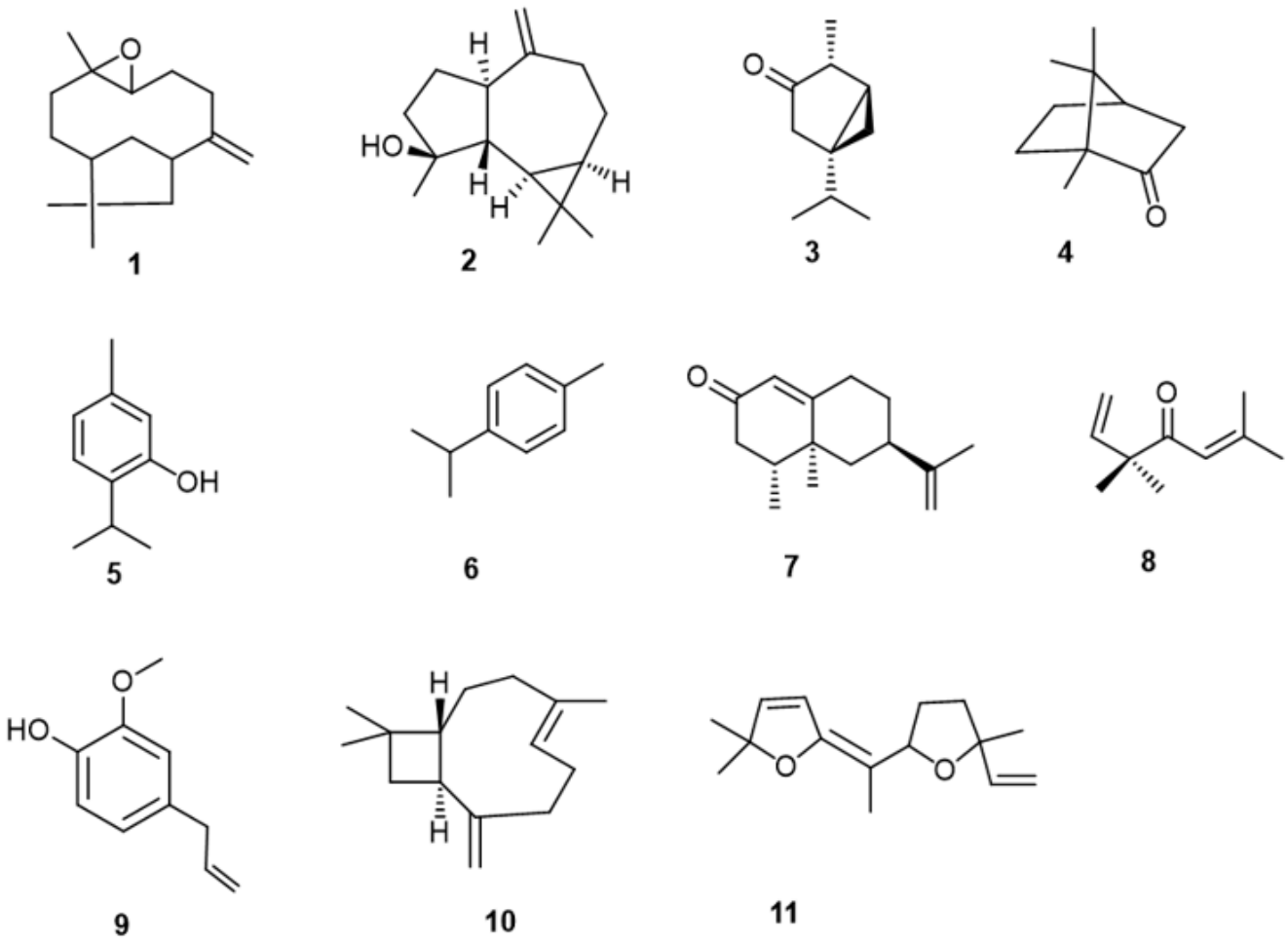

Figure 1

Major Compounds Identified in Essential Oils of Each Plant.

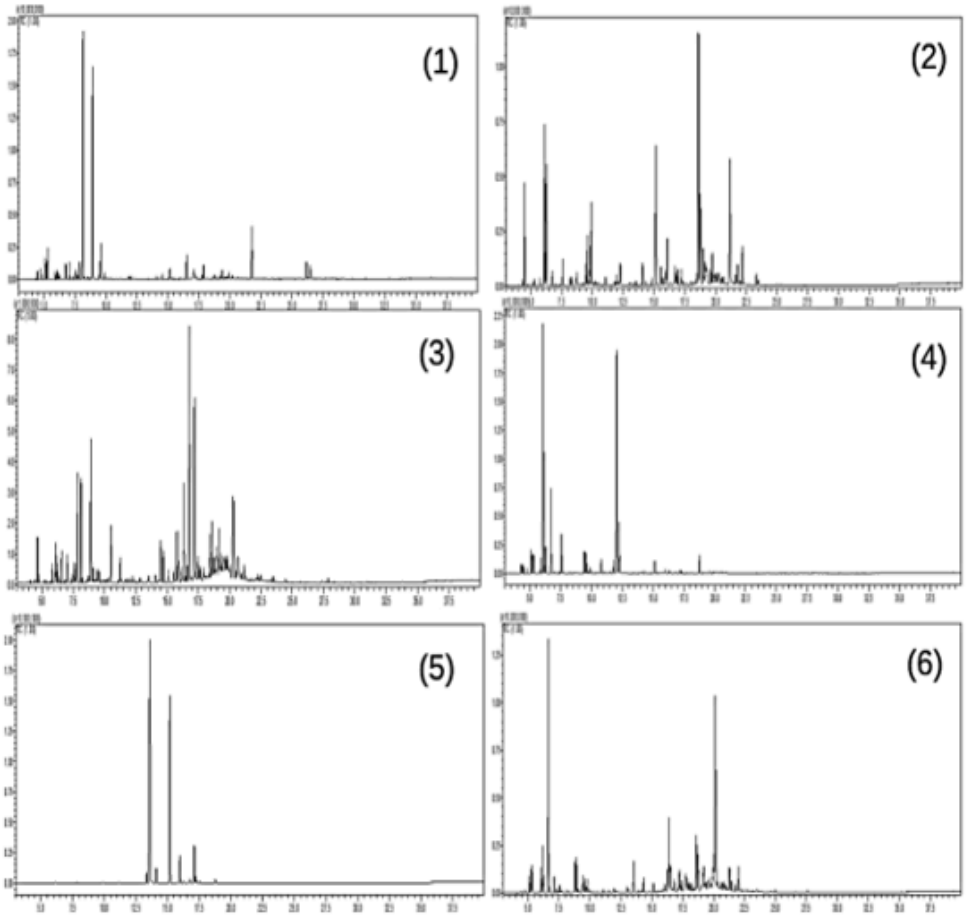

Figure 2

Gas chromatography chromatograms were obtained for the essential oils extracted from the following plants: **(1)** *Artemisia absinthium L.*;**(2)** *Eucalypthus globulus;* **(3)** *Thymus vulgaris*; **(4)** *Syzgium aromaticum*; **(5)** *Artemisia alba-helba ;* **(6)** *Santolina chamaecyparisses.*

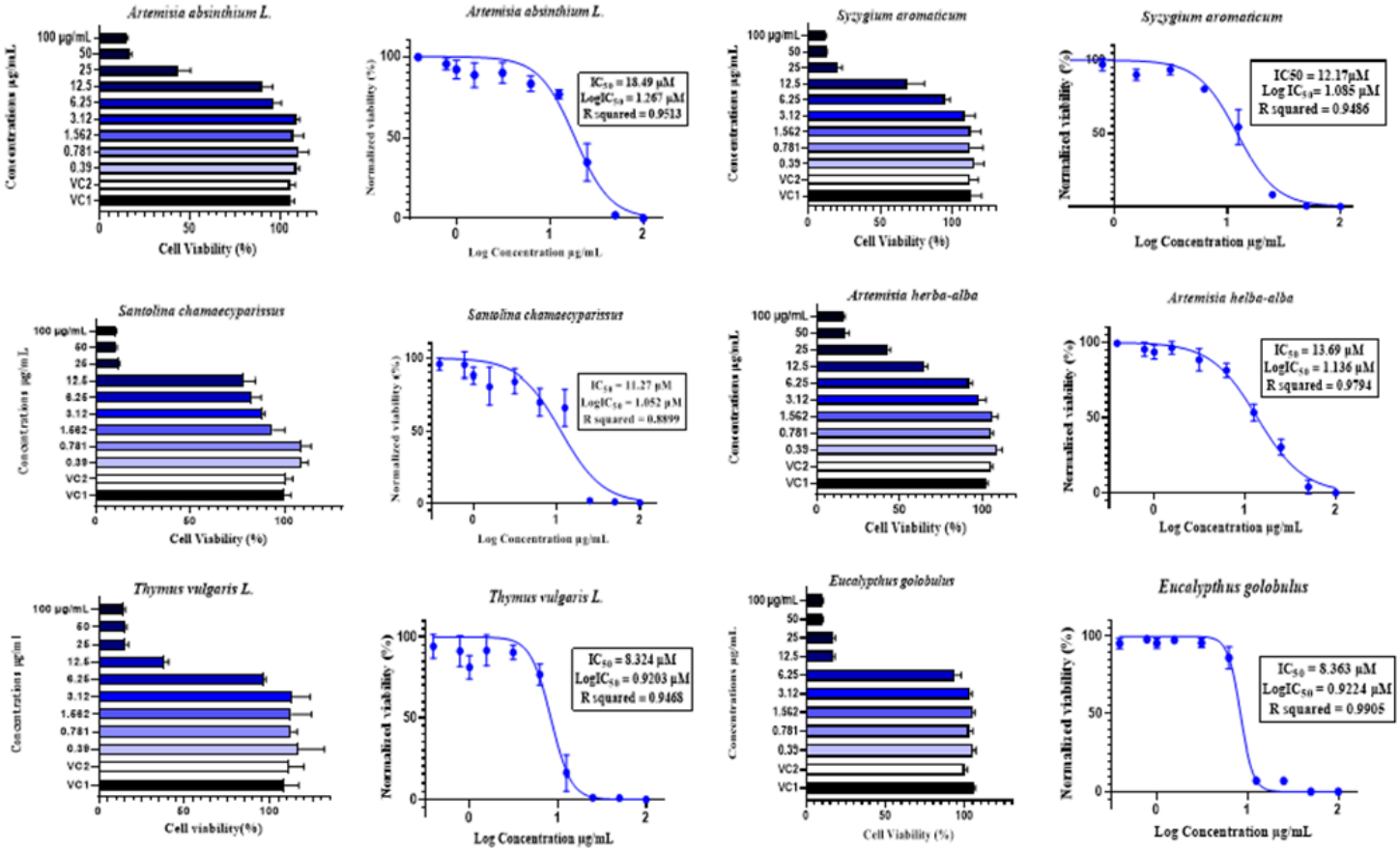

Figure 3

Cytotoxicity of plant essential oils (EOs) on Vero E6 cells was assessed. Cells were treated with EOs at concentrations ranging from 0.39 µM to 100 µM. Cytotoxicity was determined using the neutral red uptake assay, with results expressed as a percentage of cell viability compared to Vehicle Control 1 (VC1: untreated cells) and Vehicle Control 2 (VC2: DMSO/TWEEN 20 treated cells). Data are expressed as mean ± SD, *n*=4. The half-maximal inhibitory concentration (IC_{50}) was calculated by normalizing the data and applying non-linear regression analysis. Comparisons between different concentrations and controls were performed using one-way ANOVA, with statistical significance set at $p < 0.05$.

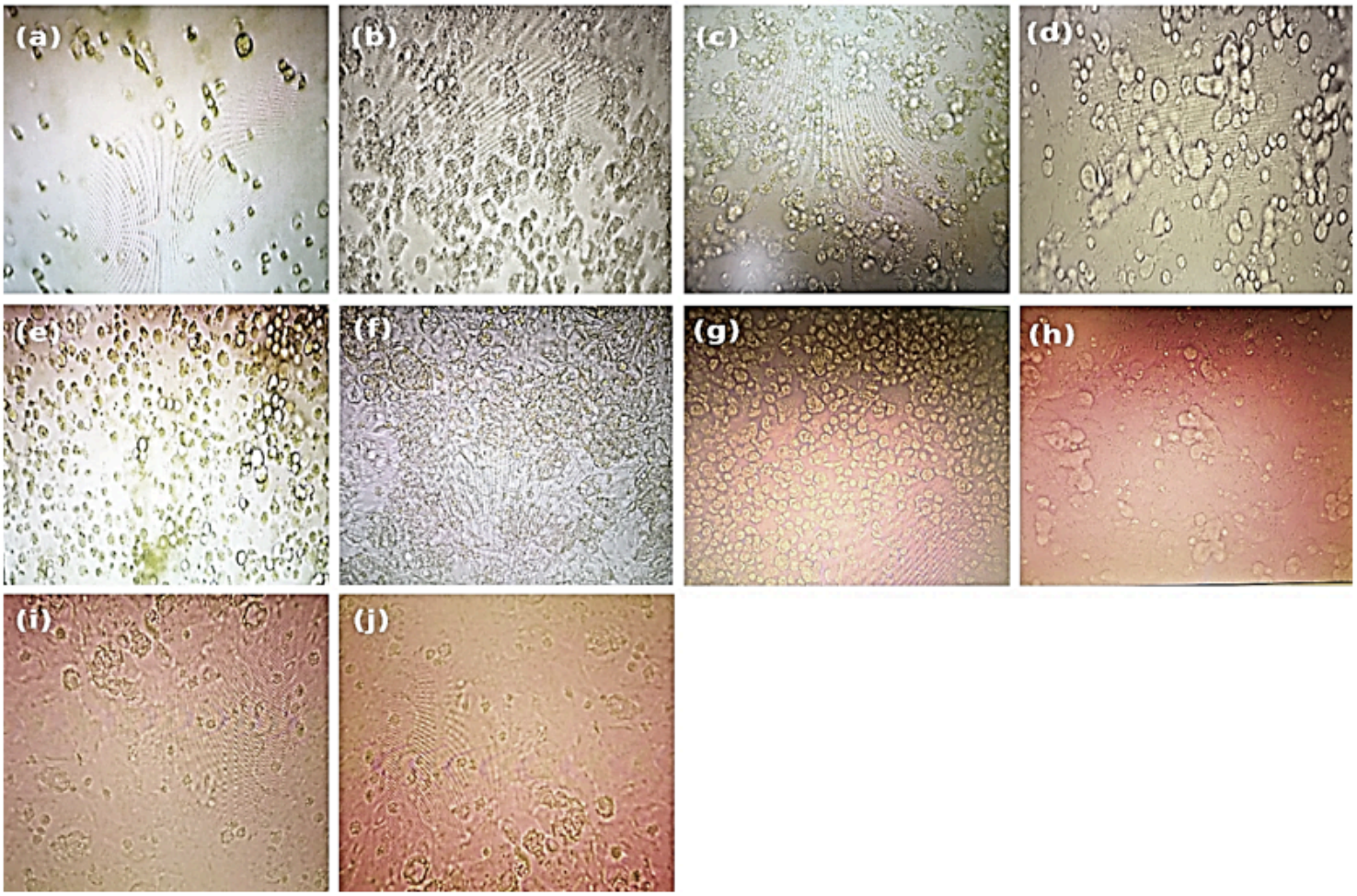

Figure 4

Microscopic evaluation of monolayers revealed dose-dependent cytotoxic effects, with particular attention to cell rounding and other prominent morphological alterations compared to control cells after 48 ± 2 hours of incubation with the treatment concentrations, prior to performing the neutral red assay. The treatments were as follows: **(a)**100 µg/mL, **(b)** 50 µg/mL, **(c)** 25 µg/mL, **(d)** 12.5 µg/mL, **(e)**6.25 µg/mL, **(f)** 3.12 µg/mL, **(g)** 1.562 µg/mL, **(h)** 0.781 µg/mL, **(i)** 0.39 µg/mL, and **(j)** untreated control cells.

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