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RESEARCH ARTICLE

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Essential oils of Moldavian *Thymus* species: Chemical composition, antioxidant, anti-*Aspergillus* and antigenotoxic activities

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Abstract

Thymus species are important aromatic, medicinal and culinary plants with a significant economic value. This study evaluated for the first time the chemical composition and *in vitro* bioactivities of the essential oils (EOs) from five Moldavian *Thymus* species (*T. vulgaris*, *T.* × *citriodorus*, *T. calcareus*) and cultivars (*T. vulgaris* 'Faustini', *T. citriodorus* 'Aureus'). The main compounds in *Thymus* EOs were: thymol in *T. vulgaris* and *T. calcareus* EOs (55.44% and 55.45%, respectively), lavandulol in *T.* × *citriodorus* EO (54.27%), and geraniol in *T. citriodorus* 'Aureus' and *T. vulgaris* 'Faustini' EOs (60.31% and 31.45%, respectively). *T. vulgaris* and *T. calcareus* EOs showed the most potent antioxidant activities (EC_{50}=0.003 mg/mL in ABTS radical cation scavenging assay) and exhibited significant inhibitory effects against aflatoxin-producing *Aspergillus flavus* fungus (MIC=0.25 μL/mL). At doses that provided micromolar concentrations of thymol, *T. vulgaris* and *T. calcareus* EOs acted genoprotective at preventive and interventional levels against H_2O_2-induced genomic damage in V79 cells, the former being more active (6.21% and 5.52% *vs*. 25.13% and 7.26% tail DNA in pre- and post-treatment protocols, respectively). The genoprotective effects may be ascribed to antioxidant potential and, possibly, to stimulation of DNA repair processes. The Moldavian *Thymus* species are valuable resources of bioactive EOs for pharmaceutical and food industries (*T. vulgaris*, *T.calcareus*) but also for flavor industry and perfumery (*T.* × *citriodorus*, *T. citriodorus* 'Aureus', *T. vulgaris* 'Faustini').

KEYWORDS

antigenotoxicity, bioactivity, chemical composition, essential oils, *Thymus* species

1 | INTRODUCTION

Genus *Thymus* L. comprises about 250 species of perennial, aromatic and medicinal plants and subshrubs native to Europe, North Africa and Asia.[1] They are widely used as culinary herbs, flavouring and food preservative agents, and they have numerous significant applications in pharmaceutical, food and cosmetic industries, and also in perfumery. Various biological properties have been reported for thyme species, such as: antioxidant, antimicrobial, anti-inflammatory, antinociceptive, spasmolytic, expectorant, or antitumor.[1–3] The

essential oils (EOs) are largely responsible for the bioactivity and non-therapeutic uses of *Thymus* species. *Thymus vulgaris* EO is among the world's top ten Eos.[4] Due to multiple and valuable uses, there is a growing demand for *Thymus* Eos.[5] Thus, there is a considerable interest concerning the chemistry, biological effects and safe use of *Thymus* EOs. Although an extensive research has been performed mainly with reference to chemical composition and biological activity, to the best of our knowledge, no data has been reported for the EOs of *Thymus* species from Republic of Moldova.

The present study aimed to evaluate the chemical composition of EOs isolated from three *Thymus* species (*T. vulgaris* L., *T.* × *citriodorus* (Pers.) Schreb. and *T. calcareus* Klokov & Des.-Shost.) and two cultivars (*T. vulgaris* 'Faustini' and *T. citriodorus* 'Aureus') from Republic of Moldova. The antioxidant properties and antifungal activities against *Aspergillus flavus*, an important aflatoxin-producing fungus, were also determined. Besides, the genoprotective potential of *T. vulgaris* and *T. calcareus* EOs against DNA damage induced by H_2O_2 in Chinese hamster lung fibroblast V79 cells, was assessed. The data on chemistry and biological properties of *T. calcareus* and *T. citriodorus* 'Aureus' EOs, regardless of geographical area, are reported here for the first time.

2 | EXPERIMENTAL

2.1 | Chemicals and media

Alkane standard solution C_8-C_{20}, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), butylated hydroxyanisole (BHA), potassium ferricyanide, ferric chloride, dimethyl sulfoxide (DMSO), sodium lauryl sarcosinate, normal and low melting point agarose were purchased from Sigma-Aldrich (Steinheim, Germany). H_2O_2 was from Fluka (Steinheim, Germany). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-dip henyl-2H-tetrazolium bromide (MTT) and tween 80 were supplied by Merck (Darmstadt, Germany). Thymol and ethidium bromide were obtained from Carl Roth (Karlsruhe, Germany). Trichloroacetic acid and potassium persulfate were from Riedel-de Haën (Seelze, Germany). Dulbecco's Modified Eagle's medium (DMEM), phosphate buffered saline (PBS), fetal bovine serum, streptomycin and penicillin were from Biochrom AG (Berlin, Germany). Ultrapure water was obtained using an Ultra Pure Water System type Ultra Clear TWF UV (SG Water, Barsbüttel, Germany). Ringer powder was purchased from Scharlau (Barcelona, Spain). RPMI 1640 medium was from Biochrom GmbH (Berlin, Germany).

2.2 | Plant material

Aerial parts of *T. vulgaris*, *T.* × *citriodorus*, and two cultivars, *T. vulgaris* 'Faustini' and *T. citriodorus* 'Aureus', were collected at the full flowering stage from specimens grown in the Botanical Garden of Chisinau (Academy of Sciences of Moldova, Republic of Moldova) in July 2015. The plants were cultivated in ecological conditions. *T. calcareus* was collected at the full flowering stage from spontaneous flora, in the same year. The aerial parts were dried separately at room temperature (20-23 °C) in dark. Voucher specimens of each species/cultivar (TV1/Bot/2015; TC1/Bot/2015; TVF1/Bot/2015; TCAu1/Bot/2015; TCa1/Bot/2015) were deposited at the herbarium of the Botanical Garden of Chisinau (Academy of Sciences of Moldova, Republic of Moldova).

2.3 | Essential oils isolation

100 g of each plant material was powdered, mixed with 1000 ml of doubly distilled water and subjected to hydrodistillation for 3 hr in a modified Clevenger-type apparatus. Two extractions were performed for each plant material. The EOs were dried over anhydrous sodium sulfate and stored in sealed glass vials at 4 °C until use. The yield of extraction was expressed as volume (ml) of EO per 100 g of plant dry matter (v/w).

2.4 | GC and GC/MS analyses

Gas chromatography analysis of EOs was performed on an Agilent 6890N gas chromatograph equipped with a flame ionization detector (FID) and a 5975 inert XL mass selective detector (MSD) working at electron ionization energy of 70eV. Two HP-5MS capillary columns (30 m × 0.25 mm i. d., 0.25 μm film thickness), were used, one for each detector. Volumes of 0.2 μL of EO were simultaneously injected in each GC column. The oven temperature was programmed from 60 to 250 °C at 4 °C/min and then from 250 to 300 °C at 10 °C/min; the final temperature was maintained for 7.5 min. The split ratio was of 1:20 in GC-FID analysis and of 1:50 in GC-MS analysis. The front inlet temperature was set at 250 °C. Helium was used as carrier gas at a flow rate of 1 ml/min. Mass spectra were acquired in the scan mode, in the mass range of 15-450 *m/z*.

The retention indices were calculated both from the GC-FID and GC-MSD data, using as reference a standard solution of *n*-alkanes (C_8-C_{20}) analyzed under the same chromatographic conditions. Qualitative identification was also achieved by comparing the recorded mass spectral data of each chromatographic peak with the standard spectra stored in the NIST 14 MSD database, accepting a quality of recognition above 75%, as well as by comparing the calculated retention indices with those reported in literature and NIST database.[6] The relative percentages of the compounds were obtained from the FID peak areas without applying the correction factors[7]. The analysis was carried out two independent times, and average values were reported.

2.5 | DPPH radical scavenging assay

DPPH radical scavenging activity was assessed as previously described.[7] Initially, the EOs were diluted in methanol in concentrations ranging from 80 to 0.156 mg/ml. 1 ml of each EO dilution was mixed with 1 ml of DPPH methanolic solution (0.004%, w/v) and further incubated for 30 min in dark at room temperature, following which the absorbance was measured at 517 nm. BHA was the

positive control. The DPPH radical scavenging activity (%) was calculated using the formula: $100\times(A_C - A_S)/A_C$, where A_C and A_S are the absorbances of DPPH radical in the control and in the presence of EOs/BHA, respectively. The results were expressed as EC_{50} values (concentrations giving half-maximal response) calculated by linear interpolation between values above and below 50% activity.

2.6 | ABTS radical cation scavenging assay

ABTS radical cation scavenging activity was evaluated as described by Re et al. with minor changes.[7,8] ABTS radical cation solution was prepared by incubating ABTS (7 mM) with potassium persulfate (2.45 mM) for 12-16 h in dark followed by dilution with methanol to an absorbance of 0.70 ± 0.02 at 734 nm (equilibration at 23±2 °C). Several dilutions of each EO were prepared in methanol with concentrations ranging from 80 to 0.156 mg/ml. The reaction was initiated by mixing 1.98 mL of ABTS radical cation solution with 0.02 ml of each EO dilution. The absorbance was read at 734 nm after 6 min reaction time. BHA was used as positive control. ABTS radical cation scavenging activity (%) and EC_{50} values were calculated as described in 2.5.

2.7 | Reducing power assay

The reducing power was assessed according to Ferreira et al. with some modifications[9]. The EOs were diluted in methanol in concentrations varying from 600 to 2.5 mg/ml. Each dilution (50 μL) was mixed with 1% potassium ferricyanide (1.25 ml) and 0.2 M phosphate buffer, pH 6.6 (1.2 ml), followed by incubation at 50 °C for 20 min. Then, 10% trichloroacetic acid (1.25 ml) was added. The mixture was further centrifuged at 3,000 rpm for 10 min. The upper layer (1.25 ml) was mixed with ultrapure water (1.25 ml) and 0.1% ferric chloride (0.25 ml). The absorbance at 700 nm was measured after 10 min. BHA was the positive control. The results were expressed as EC_{50} values (concentration of sample giving an absorbance of 0.5).[9]

2.8 | Anti-Aspergillus flavus assay

A. flavus (MUCL 19006-Belgian Coordinated Collections of Microorganisms) was cultured on 2% malt yeast agar (MYA2) medium slant at 4 °C. For conidia production, the fungus was cultivated on potato dextrose agar (PDA) medium at 28 °C for 7 days. Minimum inhibitory and minimum fungicidal concentrations (MICs and MFCs, respectively; μL/ml) of thyme EOs against *A. flavus* were determined using a broth microdilution method.[10] Initially, the EOs were diluted in a sterile solution of tween 80 (0.001%). Serial doubling dilutions of EOs (0.03-2.00 μL/ml) in RPMI 1640 medium (with L-glutamine and phenol red but without bicarbonate, supplemented with glucose up to 2%) were prepared in 96-well microtiter trays. The stock inoculum was prepared by recovering the conidia with sterile Ringer solution (containing 0.1% tween 80, v/v) from the seventh day culture grown on PDA medium; its turbidity was adjusted to 0.09-0.11 yielding $0.4\text{-}5\times10^6$ conidia/ml. An aliquot (100 μL) of the stock inoculum diluted 1:50 in RPMI was added to 0.1 ml of RPMI 1640 medium containing EO dilutions, followed by incubation for 48 hr at 28 °C in a humid atmosphere. Both sterility (uninoculated medium) and growth (inoculated medium) controls were included. Thymol, a major constituent of thyme EOs, was also tested using the same experimental protocol described for EOs. The MIC values were recorded as the lowest concentrations of EOs/thymol inhibiting the visible growth of *A. flavus*. The MFCs were determined by the spot inoculation of 10 μL from the wells showing no visible fungal growth on PDA medium, followed by incubation for 72 hr at 28 °C. The lowest concentrations of EOs/thymol inhibiting the fungal growth were defined as MFCs.[11]

2.9 | Cell culture

Chinese hamster lung fibroblast V79 cells ATCC® CCL-93 grown in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 μg/ml streptomycin, 100 IU/ml penicillin) were plated in 96-well tissue-culture plates (8×10^3 cells/well) and allowed to attach and grow for 24 hr at 37 °C in a humidified air incubator containing 5% CO_2. Once the cells have reached the confluence in the monolayer stage, they were subjected to viability and genotoxicity/antigenotoxicity assays.

2.10 | Cell viability assay

After 24 hr incubation with EOs in different doses (25-300 μg/ml), the cell viability was assessed by MTT test[12], with minor modifications. Briefly, the cells were washed with PBS, the medium was replaced by fresh growth medium (100 μL/well) and then MTT [10 μL/well from a stock solution of 5 mg/ml (12 mM)] was added. After 3 hr incubation at 37 °C, the formazan dye, generated by the reduction of MTT in living cells, was dissolved in DMSO (100 μL/well) and further quantified at 540 nm. The cell viability (%) was calculated as $100\times(A_S/A_C)$, where A_S and A_C are the absorbances of the formazan dye in the cells incubated with EO dilutions and sham control, respectively.

2.11 | Genotoxicity/antigenotoxicity assays

For the evaluation of the genotoxic potential, V79 cells were exposed to thyme EOs (25 μg/ml) for 60 min. To investigate the potential protective effects of thyme EOs against the genotoxic agent H_2O_2, pre- and post-treatment protocols were used. In the first, V79 cells were pre-treated with EOs (25 μg/ml) for 60 min. Then, the medium was removed, the cells were washed with PBS and treated with H_2O_2 (50 μM) for 30 min. In the post-treatment protocol, V79 cells were first exposed to H_2O_2 (50 μM, 30 min) and then to EOs (25 μg/ml, 60 min). In both protocols, controls exposed only to H_2O_2 (50 μM, 30 min) or EOs (25 μg/ml, 60 min) were also included. At the end of the treatment, the medium was removed and the cells were detached by trypsinization and resuspended in fresh medium for the Comet assay.[13,14]

2.12 | Alkaline single-cell gel electrophoresis assay (Comet assay)

In brief, an aliquot of cell suspension (200 μL, aprox. 40,000 cells) embedded in 1000 μL of 1% low melting agarose was spread on 1% normal melting agarose precoated slides. After solidification of the gel, the slides were plunged into the freshly prepared cold lysis solution (1.2 M NaCl, 100 mM Na_2EDTA, 0.1% sodium lauryl sarcosinate, 0.26 M NaOH, pH>13), lysed overnight (4 °C in dark) followed by three washes with electrophoresis solution (0.03 M NaOH, 2 mM Na_2EDTA, pH≈12.3) and transfer to a horizontal gel electrophoresis tank. The electrophoresis was performed under dimmed light at 0.6 V/cm for 25 min. After washing with water, the slides were stained with ethidium bromide (20 μg/ml, 30 s) and examined with a Nikon Eclipse 600 fluorescence microscope. The DNA damage (% tail DNA) was quantified by ImageJ OpenComet plugin.[13,15,16]

2.13 | Statistical analysis

The antioxidant assays were performed in triplicate and the results were expressed as mean ± standard deviation. In anti-*Aspergillus flavus* assay, the MIC and MFC values were obtained from three independent experiments performed in triplicate. In the cell viability and genotoxicity/antigenotoxicity assays, five replicates were performed for each concentration; the results were expressed as mean ± standard error of mean. Student's *t*-test was performed for the statistical comparison between treatments and control. The differences were considered statistically significant at $p < 0.001$, $p < 0.01$ and $p < 0.05$.

3 | RESULTS AND DISCUSSION

3.1 | Essential oils yield and chemical composition

Hydrodistillation of the aerial parts of *Thymus* species and cultivars gave EOs with yields ranging from 1.31 to 3.15% (Table 1). The chemical composition of thyme EOs is summarized in Table 1. The constituents are listed in the order of elution on HP-5MS capillary column; their retention indices and percentages are also reported. In *T. vulgaris* EO, 63 compounds were identified accounting for 91.78% of total EO composition. Thymol (55.44%), *m*-cymene (11.88%), γ-terpinene (5.74%) and *o*-cymen-5-ol (5.14%) were the major compounds. The chemical profile of *T. vulgaris* EO is characterized by the predominance of the aromatic terpene fraction (72.58%), represented mainly by monoterpene phenols (60.58%) (Table 1). A large number of studies on the chemical composition of *Thymus* EOs have been performed and *T. vulgaris* has been the most intensively investigated species. Thymol chemotype has been the most frequently reported chemotype for *T. vulgaris* from different geographic areas: Balkan Peninsula (17.4-80.4%),[17] Egypt (32.33%),[18] Spain (57.7%),[19] Iran (40.97%).[20] These findings are consistent with the present study reporting the thymol chemotype for Moldavian *T. vulgaris*. The analysis of *T. vulgaris* 'Faustini' EO enabled the identification of 53 compounds (93.92% of EO). Geraniol (31.45%), nerolidol (10.05%), geranial (9.14%), neral (7.05%), germacrene D (5.61%), linalool (4.92%) and nerol (3.75%) were the main components of *T. vulgaris* 'Faustini' EO. The oxygenated monoterpenes constituted the bulk of *T. vulgaris* 'Faustini' EO (69.07%) whereas the oxygenated sesquiterpenes were the second most abundant compounds (10.79%) (Table 1). Data on the chemical composition of *T. vulgaris* 'Faustini' EO are scarce. To the best of our knowledge, only one study investigated the chemistry of *T. vulgaris* 'Faustini' EO from Italy reporting thymol as its main component (about 50%).[21] The Moldavian 'Faustini' cultivar contained low amounts of thymol (0.22%); instead, it was a rich source of geraniol, a compound that, together with geranial and neral, contributes to the pleasant floral, lemon-like scent of EO. With respect to *T.* × *citriodorus* EO, 54 compounds were identified representing 94.99% of total EO composition. Lavandulol (54.27%), geranial (12.25%), neral (9.00%) and nerol (3.88%) were the major constituents. Oxygenated monoterpenes predominated in Moldavian *T.* × *citriodorus* EO (83.87%) being primarily responsible for the green, rosy and lemon-like fragrance of EO (Table 1). Data from literature show that *T.* × *citriodorus* EOs are commonly characterized by the presence of geraniol (up to 60%), geranial (8.2%), neral (5.5%) and nerol (2.8%) as the most important constituents[22,23]. On the contrary, Wu et al. have identified borneol (28.82%) and thymol (14.43%) as the most abundant compounds in *T.* × *citriodorus* from China[24]. The compositional diversity of *Thymus* EOs is well-known, the genus *Thymus* being characterized by a high chemical polymorphism. Both environmental and genetic factors cause this chemical variability. The presence of lavandulol (monoterpenol) as the major component in *T.* × *citriodorus* EO is reported for the first time in this paper. The Moldavian *T.* × *citriodorus* (lemon thyme) can be considered an important source of this valuable odorant compound. On the other hand, geranial and neral, lemon-scented components, are also found in Moldavian thyme in similar levels with those reported in literature. In *T.* × *citriodorus* 'Aureus' EO there have been detected 33 components (98.72% of EO), geraniol (60.31%), geranial (9.26%), neral (7.15%) and thymol (3.45%) being the most abundant. Oxygenated monoterpenes (81.59%) represented the predominant fraction of the EO, their level being comparable with that found in *T.* × *citriodorus* EO (83.87%). On the contrary, the aromatic fraction was higher in *T.* × *citriodorus* 'Aureus' EO than in *T.* × *citriodorus* EO (7.53% *vs.*1.05%) (Table 1). To the best of our knowledge, there are no literature data on the chemical composition of *T.* × *citriodorus* 'Aureus' and *T. calcareus* EOs. In total, 44 compounds were identified in *T. calcareus* EO representing 96.16% of total EO composition, thymol (55.45%), γ-terpinene (13.38%), *o*-cymene (8.32%) and linalool (4.86%) being predominant. Similar to *T. vulgaris* EO, the aromatic terpene fraction predominated in *T. calcareus* EO (66.73%). The monoterpene hydrocarbons were the second most abundant group of components in *T. calcareus* EO, their level being higher than in *T. vulgaris* EO (17.69 *vs.* 9.24%). In addition, *T. calcareus* EO was richer in oxygenated monoterpenes than *T. vulgaris* EO (8.41 *vs.* 4.95%) (Table 1).

TABLE 1 Chemical composition of *Thymus* species EOs

Compound	MI[a]	KI[b]	Composition (%)[d]					
			KI[c]	*T. vulgaris* EO	*T. vulgaris* 'Faustini' EO	*T. × citriodorus* EO	*T. citriodorus* 'Aureus' EO	*T. calcareus* EO
α-Thujene	1, 2	931	931	0.30±0.01	0.24±0.01	0.03±0.00	-	0.44±0.02
α-Pinene	1, 2	939	940	0.41±0.02	0.32±0.01	0.09±0.00	0.20±0.01	0.34±0.01
α-Fenchene	1, 2	950	951	0.02±0.00	-	-	-	-
Camphene	1, 2	953	952	0.07±0.00	0.16±0.00	0.16±0.00	-	0.07±0.00
β-Thujene	1, 2	968	968	0.87±0.04	-	-	-	1.17±0.04
1-Octen-3-ol	1, 2	975	976	0.75±0.03	0.38±0.01	0.58±0.02	0.57±0.02	-
β-Pinene	1, 2	980	979	0.16±0.00	0.13±0.00	0.07±0.00	0.18±0.00	0.11±0.00
3-Octanone	1, 2	985	984	0.03±0.00	-	1.32±0.06	1.16±0.05	0.05±0.00
β-Myrcene	1, 2	992	993	-	0.21±0.01	0.03±0.00	-	-
3-Octanol	1, 2	995	995	0.02±0.00	0.21±0.01	0.80±0.03	0.71±0.03	-
2-Carene	1, 2	1001	1001	1.04±0.04	0.10±0.00	0.01±0.00	-	1.42±0.05
α-Phellandrene	1, 2	1003	1003	0.14±0.00	-	-	-	0.18±0.00
3-Carene	1, 2	1007	1007	0.05±0.00	-	0.01±0.00	-	0.06±0.00
(E,E)-2,4-heptadienal	1, 2	1009	1010	-	-	0.02±0.00	-	-
4-Carene	1, 2	1012	1011	-	-	0.01±0.00	-	0.15±0.00
o-Cymene	1, 2	1021	1020	-	-	-	-	8.32±0.33
m-Cymene	1, 2	1022	1023	11.88±0.32	-	-	-	-
p-Cymene	1, 2	1026	1026	-	0.12±0.00	0.10±0.00	2.69±0.06	-
Isosylvestrene	1, 2	1027	1027	-	-	-	-	0.25±0.01
Limonene	1, 2	1030	1030	0.31±0.01	0.20±0.00	0.03±0.00	0.21±0.00	-
Eucalyptol	1, 2	1031	1031	0.61±0.03	1.82±0.09	0.29±0.01	0.37±0.01	0.50±0.02
(*Z*)-Ocimene	1, 2	1038	1037	0.04±0.00	1.66±0.05	0.02±0.00	-	0.06±0.00
(*E*)-Ocimene	1, 2	1048	1047	-	0.54±0.02	-	-	-
γ-Terpinene	1, 2	1061	1062	5.74±0.20	0.04±0.00	0.09±0.00	1.20±0.04	13.38±0.53
2-Ethylidene-6-methyl-3,5-heptadienal	1, 2	1066	1066	-	-	0.03±0.00	-	-
cis-Sabinene hydrate	1, 2	1070	1071	0.43±0.02	-	-	-	0.57±0.02
α-Terpinolene	1, 2	1087	1088	0.16±0.00	-	-	-	-
Linalool	1, 2	1098	1097	0.88±0.04	4.92±0.16	0.66±0.03	-	4.86±0.19
cis-Rose oxide	1, 2	1108	1108	-	-	0.01±0.00	-	-
cis-Menth-2-en-1-ol	1, 2	1119	1123	0.02±0.00	-	-	-	-
Chrysanthenone	1, 2	1125	1125	-	0.02±0.00	-	-	-
3-Cyclohexen-1-carboxaldehyde, 3,4-dimethyl	1, 2	1130	1130	-	-	0.04±0.00	-	-
Camphor	1, 2	1145	1145	0.58±0.02	1.12±0.04	-	0.08±0.00	-
cis-β-Terpineol	1, 2	1147	1147	0.03±0.00	1.08±0.04	-	-	-
Nerol oxide	1, 2	1151	1150	-	-	0.08±0.00	0.07±0.00	-
Borneol	1, 2	1165	1166	0.32±0.01	1.08±0.04	1.23±0.04	1.22±0.04	0.39±0.01
Lavandulol	1, 2	1170	1170	-	0.89±0.03	54.27±0.52	-	-
Terpinen-4-ol	1, 2	1176	1177	1.04±0.04	0.74±0.02	0.10±0.00	0.31±0.01	0.78±0.03
α-Terpineol	1, 2	1190	1190	0.22±0.01	2.05±0.07	0.12±0.00	0.16±0.00	0.18±0.00
cis-Piperitol	1, 2	1195	1196	0.03±0.00	-	-	-	0.06±0.00
trans-Dihydrocarvone	1, 2	1200	1202	0.04±0.00	-	0.03±0.00	-	0.04±0.00
Nerol	1, 2	1229	1229	0.03±0.00	3.75±0.16	3.88±0.15	1.65±0.04	-

(*Continues*)

TABLE 1 Continued

Compound	MI[a]	KI[b]	Composition (%)[d]					
			KI[c]	*T. vulgaris* EO	*T. vulgaris* 'Faustini' EO	*T. × citriodorus* EO	*T. citriodorus 'Aureus' EO*	*T. calcareus EO*
Thymol-methyl-ether	1, 2	1235	1233	0.07±0.00	-	0.29±0.01	0.43±0.02	0.02±0.00
Neral	1, 2	1237	1237	0.06±0.00	7.05±0.33	9.00±0.35	7.15±0.07	0.05±0.00
Carvone	1, 2	1241	1241	0.05±0.00	-	-	-	0.02±0.00
Isothymol-methyl-ether	1, 2	1244	1244	-	-	0.07±0.00	0.65±0.02	-
Geraniol	1, 2	1252	1252	0.33±0.01	31.45±0.63	-	60.31±0.57	0.65±0.02
Methyl nerolate	1, 2	1265	1265	-	-	0.07±0.00	-	-
Geranial	1, 2	1269	1270	-	9.14±0.19	12.25±0.36	9.26±0.08	-
Bornyl acetate	1, 2	1285	1285	-	0.02±0.00	0.01±0.00	-	-
Thymol	1, 2	1297	1297	55.44±0.62	0.22±0.01	-	3.45±0.10	55.45±0.48
Geranyl formate	1, 2	1305	1304	-	0.21±0.01	0.20±0.01	-	-
o-Cymen-5-ol	1, 2	1336	1336	5.14±0.19	-	0.59±0.02	0.31±0.01	2.93±0.14
α-Cubebene	1, 2	1348	1348	0.06±0.00	-	-	-	0.03±0.00
Thymyl acetate	1, 2	1357	1357	0.05±0.00	-	-	-	0.01±0.00
Neryl acetate	1, 2	1365	1364	0.16±0.00	0.81±0.04	0.11±0.00	-	0.26±0.01
Ylangene	1, 2	1368	1368	0.02±0.00	-	-	-	-
Geranyl acetate	1, 2	1370	1370	-	2.60±0.14	0.67±0.02	0.67±0.02	-
α-Bourbonene	1, 2	1374	1376	-	0.04±0.00	-	-	-
Copaene	1, 2	1380	1382	-	0.06±0.00	-	-	0.02±0.00
β-Bourbonene	1, 2	1384	1385	0.04±0.00	0.96±0.04	0.35±0.01	0.16±0.00	0.03±0.00
β-Elemene	1, 2	1387	1387	-	0.15±0.00	-	-	-
β-Cubebene	1, 2	1390	1389	0.02±0.00	0.08±0.00	-	-	0.01±0.00
cis-Jasmone	1, 2	1393	1394	0.01±0.00	-	-	-	-
α-Gurgujene	1, 2	1401	1401	-	-	0.02±0.00	-	-
β-Caryophyllene	1, 2	1418	1420	1.53±0.07	1.64±0.08	2.49±0.12	2.53±0.12	2.20±0.11
Neryl propionate	1, 2	1430	1430	0.02±0.00	0.03±0.00	-	-	-
α-Bergamotene	1, 2	1432	1433	-	0.04±0.00	0.02±0.00	-	-
Elixene	1, 2	1437	1437	-	-	0.01±0.00	-	-
Aromadendrene	1, 2	1440	1440	-	-	-	0.08±0.00	-
α-Humulene	1, 2	1452	1452	0.18±0.00	0.06±0.00	0.21±0.01	0.04±0.00	0.07±0.00
Geranyl propionate	1, 2	1447	1448	-	-	-	0.13±0.00	-
Eudesma-4(14), 11-diene (β-Selinene)	1, 2	1465	1464	0.01±0.00	-	-	-	-
δ-Muurolene	1, 2	1468	1468	0.14±0.00	-	-	-	-
β-Cadinene	1, 2	1475	1474	0.04±0.00	-	-	-	0.12±0.00
γ-Muurolene	1, 2	1477	1477	0.30±0.01	0.06±0.00	-	-	-
α-Muurolene	1, 2	1480	1480	0.07±0.00	-	-	-	-
Germacrene D	1, 2	1487	1487	-	5.61±0.22	0.55±0.02	0.14±0.00	0.05±0.00
β-Ionone	1, 2	1490	1489	0.02±0.00	-	-	-	-
Linalyl isovalerate	1, 2	1494	1494	-	0.26±0.01	0.16±0.00	-	-
α-Amorphene	1, 2	1495	1495	0.44±0.02	0.05±0.00	-	-	-
β-Bisabolene	1, 2	1505	1505	-	-	1.99±0.09	2.07±0.09	-
α-Farnesene	1, 2	1507	1507	0.05±0.00	0.36±0.01	-	-	-

(*Continues*)

TABLE 1 Continued

Compound	MI[a]	KI[b]	Composition (%)[d] KI[c]	*T. vulgaris* EO	*T. vulgaris* 'Faustini' EO	*T. × citriodorus* EO	*T. citriodorus* 'Aureus' EO	*T. calcareus* EO
Bicyclo [4.4.0] dec-1-ene, 2-isopropyl-5-methyl-9-methylene	1, 2	1510	1510	-	-	0.03±0.00	-	-
Cadina-1(2), 4-diene	1, 2	1512	1513	0.04±0.00	-	-	-	0.01±0.00
Geranyl isobutyrate	1, 2	1517	1517	-	0.03±0.00	-	-	-
Δ-Cadinene	1, 2	1520	1520	0.54±0.02	0.21±0.01	0.13±0.00	0.07±0.00	0.34±0.01
γ-Cadinene	1, 2	1524	1524	0.21±0.01	0.20±0.01	0.08±0.00	-	-
Nerolidol	1, 2	1531	1531	0.01±0.00	10.05±0.14	-	-	0.12±0.00
α-Cadinene	1, 2	1536	1537	0.09±0.00	-	-	-	0.05±0.00
Caryophyllene oxide	1, 2	1561	1561	0.31±0.01	0.13±0.00	0.74±0.02	0.28±0.01	0.19±0.00
Geranyl butyrate	1, 2	1570	1570	0.09±0.00	-	0.81±0.04	0.21±0.01	0.05±0.00
Spathulenol	1, 2	1582	1582	0.01±0.00	0.49±0.02	0.08±0.00	-	-
γ-Eudesmol	1, 2	1630	1631	0.14±0.00	-	-	-	-
α-Cadinol	1, 2	1653	1653	-	0.12±0.00	0.04±0.00	-	-
Cadalene	1, 2	1674	1674	0.01±0.00	-	-	-	-
2-Pentadecanone-6,10,14-trimethyl	1, 2	1848	1848	-	0.01±0.00	-	-	-
Monoterpene hydrocarbons				9.24	3.60	0.57	1.79	17.69
Oxygenated monoterpenes				4.95	69.07	83.87	81.59	8.41
Sesquiterpene hydrocarbons				3.72	9.52	5.88	5.09	2.97
Oxygenated sesquiterpenes				0.47	10.79	0.86	0.28	0.31
Aromatic compounds				72.58	0.34	1.05	7.53	66.73
Non-terpenoid aliphates				0.80	0.60	2.76	2.44	0.05
Others				0.02	-	-	-	-
Total identified (%)				**91.78**	**93.92**	**94.99**	**98.72**	**96.16**
Yield (%)				**2.50±0.35**	**3.10±0.28**	**1.61±0.12**	**1.31±0.09**	**3.15±0.21**

[a]MI, Method of identification: 1-Retention index calculated from the series of n-alkanes (C8-C20); 2-Retention index from NIST database;
[b]Retention indices relative to C8–C20 n-alkanes calculated on HP-5MS capillary column; [c]Retention indices reported in literature;
[d]Values are the mean ± standard deviation of two independent experiments.

3.2 | Antioxidant activity

A major trend in the food industry nowadays is the high demand for natural antioxidants as food preservatives. Synthetic antioxidants, used for more than 50 years, seem to have a poor safety profile. The most common ones, BHA and BHT (butylated hydroxytoluene), have shown to be carcinogenic in animals and therefore, their use is restricted in many countries.[25] Lipid oxidation is one of the major causes of food deterioration that might occur during storage and processing. EOs isolated from different *Thymus* species, including *T. vulgaris*, were found to inhibit lipid peroxidation in different experimental models.[18,26] In the present study, the antioxidant potential of thyme EOs was screened by free radical scavenging and ferric ion reduction assays and compared to BHA. As shown in Table 2, there are significant differences in the antioxidant activity of EOs in all three assays. *T. vulgaris* EO was the most potent in scavenging DPPH and ABTS free radicals and reducing ferric ion (EC_{50} = 0.147, 0.003 and 0.041 mg/ml, respectively), followed by *T. calcareus* EO (EC_{50} = 0.369, 0.003 and 0.051 mg/ml, respectively). Thymol, a phenolic monoterpene, the major constituent of both EOs, is undoubtedly responsible for their higher antioxidant activity. Thymol was found to be a potent antioxidant in numerous *in vitro* assays including free radical scavenging, reducing power and anti-lipid peroxidation assays.[27,28] Similar to other phenolic antioxidants, thymol quenches free radicals by hydrogen donation thus forming phenoxyl radicals. Several structural features underlie the strong antioxidant properties of thymol, namely the phenolic hydroxyl and alkyl groups (in *o*- and *m*-positions). The latter have inductive effects and therefore, increase the electron density of the phenoxyl radicals stabilizing them and enhancing the antiradicalar potency.[27] Although *T. vulgaris* and *T. calcareus* EOs had similar thymol contents (55.44 and 55.45%, respectively) (Table 1), they scavenged free radicals and reduced

TABLE 2 *In vitro* antioxidant and antifungal activities of *Thymus* species EOs

	Antioxidant activity - EC_{50} (mg/ml)			Anti-*Aspergillus* activity	
EO/Positive control	DPPH radical scavenging assay	ABTS radial cation scavenging assay	Reducing power assay	MIC (μL/ml)	MFC (μL/mL)
T. vulgaris	0.147 ± 0.004	0.003 ± 0.000	0.041 ± 0.000	0.25	0.50
T. vulgaris 'Faustini'	18.607 ± 0.536	0.371 ± 0.007	nd	1.00	1.00
T. citriodorus	34.281 ± 0.653	0.522 ± 0,011	nd	1.00	1.00
T. citriodorus 'Aureus'	1.565 ± 0.045	0.014 ± 0.001	3.057 ± 0.151	0.50	1.00
T. calcareus	0.369 ± 0.007	0.003 ± 0.000	0.051 ± 0.000	0.25	0.50
BHA	6.126 ± 0.014*	1.524 ± 0.008*	3.991 ± 0.032*	-	-
Thymol	-	-	-	0.125	0.25

nd, not determined due to low activity; *, μg/ml. Antioxidant assays: the results are expressed as mean ± SD from three determinations. EC_{50} values (mg/mL or μg/ml) were obtained by linear interpolation between values above and below 50% activity. In reducing power assay6, EC_{50} values were the concentrations that lead to an absorbance of 0.5.

ferric ion with different potencies. According to the EC_{50} values, *T. vulgaris* EO was more potent than *T. calcareus* EO in all antioxidant assays (Table 2). These differences are obviously due to other EO constituents, most probably to cymene derivatives. Cymenes are aromatic compounds with well-known antioxidant effects[29]. They were detected in higher levels in *T. vulgaris* EO (17.02% *vs*. 11.25% in *T. calcareus* EO) (Table 1). Among them, alike thymol (also known as *p*-cymen-3-ol), *o*-cymen-5-ol (5.14% in *T. vulgaris* EO *vs*. 2.93% in *T. calcareus* EO) (Table 1) has a phenolic hydroxyl group and two alkyl (methyl and isopropyl) groups but in *m*- and *p*-positions. As mentioned before, these structural features enhance the antiradical activity.[27]

The antioxidant activity of *T. vulgaris* EOs has been previously reported in literature. Mancini et al. found IC_{50} values of 28.95, 58.25 and 64.93 μg/ml in the DPPH assay for *T. vulgaris* Eos.[30] Other authors reported EC_{50} values of 0.25 mg/ml, 7.57 and 12.60 g/l in the DPPH and anti-lipid peroxidation assays, respectively.[18,31] A Trolox equivalent antioxidant capacity (TEAC) of 2.04 mmol/l Trolox for an EO concentration of 50 g/l[18] and an activity slightly exceeding 6 μmoL of ascorbic acid/g of EO[31] have been reported in the ferric reducing power assay. Due to the different experimental protocols, a comparison of our results with those reported in the aforementioned studies is not feasible.

T. × *citriodorus* EO, containing 54.27% lavandulol, showed the weakest antioxidant effects (EC_{50}= 34.28 and 0.52 mg/mL in DPPH and ABTS scavenging assays, respectively while the ferric ion reduction capacity could not be assessed due to low activity). Higher antioxidant activity was determined for the EOs isolated from the two cultivars, *T. vulgaris* 'Faustini' and *T. citriodorus* 'Aureus' containing geraniol as predominant component (31.45% and 60.31%, respectively) (Table 1). The monoterpene alcohols geraniol and lavandulol are weaker antioxidants than thymol, geraniol being more potent than lavandulol.[32] The higher antioxidant potency of geraniol compared to lavandulol is due to fact that geraniol may lose allylic hydrogens thus neutralizing free radicals.[33] The stronger antioxidant effects exhibited by *T. citriodorus* 'Aureus' EO may be attributed, in large part, to its higher content in geraniol (60.31% *vs*. 31.45% in *T. vulgaris* 'Faustini' EO) and aromatic compounds (7.54% *vs*. 0.35% in *T. vulgaris* 'Faustini' EO) (Table 1). Geraniol, an acyclic monoterpene alcohol, showed strong DPPH scavenging activity; in addition, in cell-based assays, it reduced the lipid peroxidation and generation of reactive species (nitric oxide, reactive oxygen species) and increased the glutathione level and superoxide dismutase activity.[34] Antioxidant aromatic compounds such as thymol and *p*-cymene were detected in higher levels in *T. citriodorus* 'Aureus' EO than in *T. vulgaris* 'Faustini' EO (3.45% *vs*. 0.22% and 2.69% *vs*. 0.12%, respectively) (Table 1).

In terms of antioxidant activity, the investigated EOs can be ranked as follows: *T. vulgaris* EO > *T. calcareus* EO > *T. citriodorus* 'Aureus' EO > *T. vulgaris* 'Faustini' EO > *T.* × *citriodorus* EO (Table 2).

3.3 | Anti-Aspergillus flavus activity

A. flavus is responsible for the contamination of foods and feedstuffs but also for the human invasive aspergillosis and superficial skin infections. *A. flavus* produces many toxic compounds among which aflatoxin B1 is highly carcinogenic, mutagenic and teratogenic to humans and animals. Among natural products, essential oils are very promising antifungal and anti-aflatoxigenic agents. They have great advantages such as effectiveness, low resistance and eco-friendly properties. In the present study, we assayed the antifungal activities of thyme EOs and thymol against *A. flavus* MUCL 19006 standard strain. MIC and MFC values of thyme EOs and thymol are shown in Table 2. *T. vulgaris* and *T. calcareus* EOs, rich in thymol, were the most active against the fungal strain. According to the MIC and MFC values (0.25 and 0.50 μL/ml, respectively), both EOs exhibited similar strong antifungal effects. In addition, *T. citriodorus* 'Aureus', containing 7.53% aromatic compounds alongside a high level of geraniol (60.31%), displayed an important antifungal activity (MIC=0.50 μL/ml, MFC=1.00 μL/ml). Thymol, assayed as single compound, proved

TABLE 3 Viability of V79 cells after 24 hr incubation with *Thymus* EOs

Group	Cell viability (%)
Untreated V79 cells	100±0.00
TVEO25	124.21±21.22
TVEO50	113.41±13.47
TVEO100	104.29±7.50
TVEO200	106.42±16.85
TVEO300	86.03±8.12
TCaEO25	90.95±6.27
TCaEO50	83.31±3.80**
TCaEO100	79.25±7.19*
TCaEO200	79.22±5.83*
TCaEO300	72.14±5.93**

TVEO, *Thymus vulgaris* essential oil; TCaEO, *Thymus calcareus* essential oil; The results are expressed as mean ± standard error of mean of five independent experiments; significant data at *p<0.05 and **p<0.01 compared to untreated V79 cells (t-test)

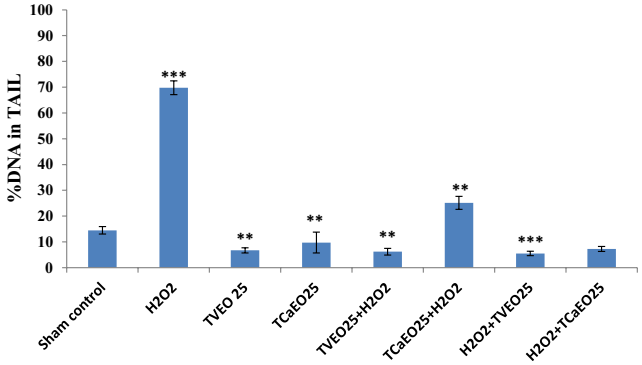

FIGURE 1 Tail DNA (%) in V79 cells after 60 min pre- and post-treatment with thyme EOs (25 μg/ml). TVEO, *Thymus vulgaris* essential oil; TCaEO, *Thymus calcareus* essential oil. The results were expressed as mean ± standard error of mean of five replicates. ***p<0.001 and **p<0.01 when comparing the effects of *Thymus* species EOs with H_2O_2-treated cells and H_2O_2-treated cells with sham control (t-test) [Colour figure can be viewed at wileyonlinelibrary.com]

to be more active than all EOs (MIC = 0.125 *vs*. 0.25-1.00 μL/ml, MFC = 0.25 *vs*. 0.50-1.00 μL/ml).

Many studies have highlighted strong anti-*Aspergillus* properties and inhibitory effects on aflatoxin production of EOs from different *Thymus* species. However, there are no data on the antifungal effects of *T.* × *citriodorus*, *T. calcareus* and *T. vulgaris* 'Faustini' EOs and our study firstly reported on this issue. Klaric et al. showed that *T. vulgaris* EO containing 33% thymol exerted a strong fungicidal activity on *A. flavus* isolated from damp dwellings (MIC=9.35 μg/ml).[35] In addition, Kohiyama et al. reported that *T. vulgaris* EO (40.6% borneol, 19.9% α-terpineol) inhibited *A. flavus* growth (MIC=250 μg/ml) and aflatoxin production.[36] With respect to *T. vulgaris* EO (density=0.92 g/ml, at 20°C), we found a MIC value close to that reported by Kohiyama et al. (230 *vs*. 250 μg/ml).[36] The differences that occur between the reported values can be explained by the sensitivity of the fungal strain, methodology and chemical composition of *Thymus* EOs. The strong antifungal profile of thyme EOs is mainly related to their content in monoterpene phenols such as thymol. Among the volatile constituents, the phenols are considered to be the most potent antifungal agents. These compounds alter the microbial cell membrane integrity causing the loss of essential cytoplasmic constituents, impair the fungal enzyme systems and interfere with the cell wall synthesis.[37]

3.4 | Cytotoxic activity

A toxicity screening of thyme EOs in V79 cells was performed using the MTT assay. V79 cells have a high sensitivity to different chemicals and represent a well-established cell model for the study of DNA damage induced by genotoxicants. Besides, the selection of V79 cells was also based on the fact that the inhalation is one of the main routes of EOs administration in humans. As *T. vulgaris* and *T. calcareus* EOs showed the most potent antioxidant effects, they were selected for further cell-based studies (cytotoxicity and antigenotoxicity/genotoxicity assays). *T. vulgaris* EO (25-200 μg/ml) did not alter the viability of V79 cells but it showed an important reduction in cell viability (over 20%) in a concentration of 300 μg/ml. A decline in the survival of V79 cells (16.69-27.86%) was noticed after treatment with medium and high concentrations of *T. calcareus* EO (50-300 μg/ml). Only a low concentration of *T. calcareus* EO (25 μg/ml) did not significantly alter the viability of V79 cells (9.05% reduction in cell viability) (Table 3).

3.5 | Antigenotoxic/genotoxic activity

The assay investigated the capacity of *T. vulgaris* and *T. calcareus* EOs to protect DNA against H_2O_2-induced damage. Deleterious effects of H_2O_2 against genomic stability primarily result from the generation of highly reactive hydroxyl radicals *via* the iron-mediated Fenton reactions. Hydroxyl radicals affect directly DNA causing DNA strand breakage, oxidation of DNA bases and sugar modification.[13] The effects of H_2O_2, thyme EOs and their combinations have been assessed using the Comet assay. The extent of DNA damage is illustrated by the most common attribute of the comet, namely comet tail (% DNA in tail) (Figure 1). Longer tails of comets indicate an increased DNA damage. The genoprotective effects of thyme EOs were examined at preventive and interventional levels. In this respect, two experimental approaches were applied: pre-treatment (60 min) with EOs prior to the exposition to H_2O_2 and post-treatment (60 min) with EOs after exposition to genotoxicant. Based on the results from MTT assay, we used both essential oils in concentration of 25 μg/ml. Treatment of V79 cells with H_2O_2 (50 μM, 30 min) induced a greater rate of migration of DNA into the comet tail in comparison to the sham control (69.80±2.69 *vs*.14.49±1.43%) (Figure 1).

In both types of treatment, *Thymus* EOs significantly reduced H_2O_2-induced DNA damage in V79 cells (Figure 1). *T. vulgaris* EO

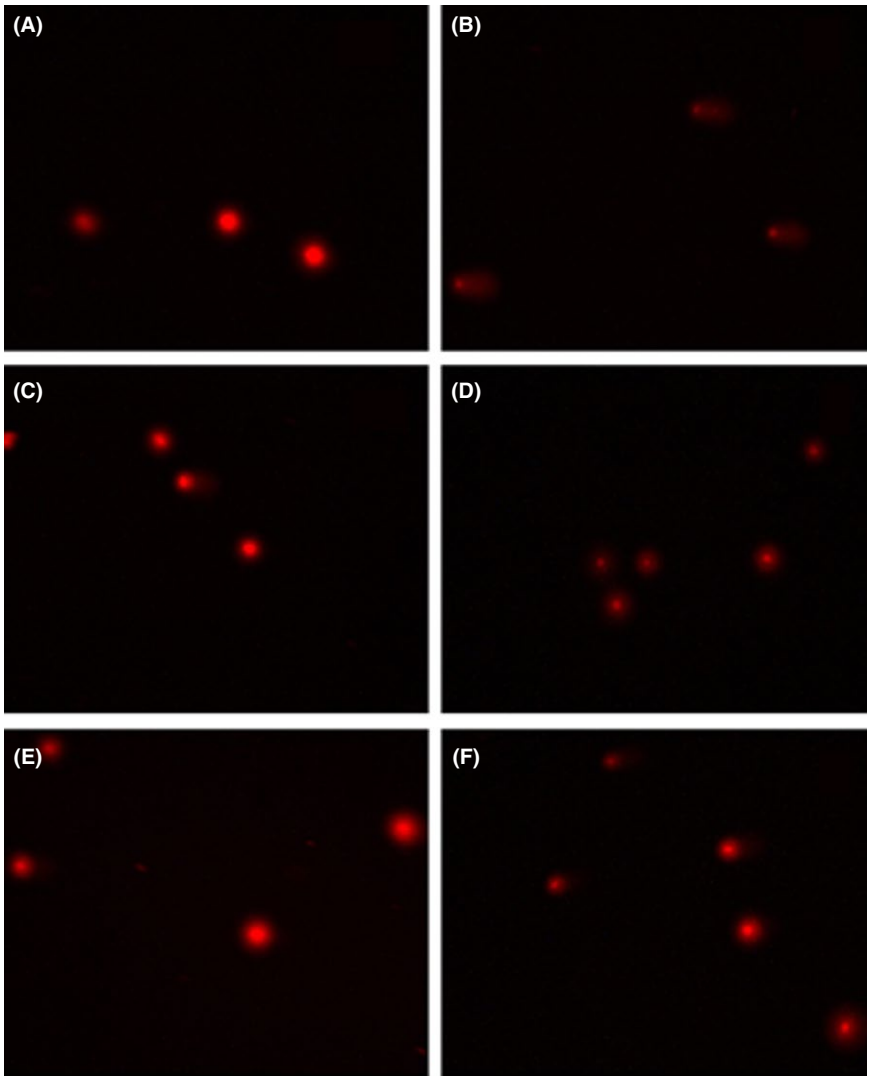

FIGURE 2 Photomicrographs of comet length in normal and treated V79 cells: a) normal; b) exposed to H_2O_2 (50 μM, 30 min); exposed to H_2O_2 (50 μM, 30 min) after 60 min pre-treatment with thyme EOs (25 μg/ml): c) *T. vulgaris* EO, d) *T. calcareus* EO; exposed to H_2O_2 (50 μM, 30 min) and 60 min subsequent treatment with thyme EOs (25 μg/ml): e) *T. vulgaris* EO, f) *T. calcareus* EO [Colour figure can be viewed at wileyonlinelibrary.com]

showed similar pronounced protective effects in pre- and post-treatment protocols (93.79±1.29% DNA in head and 6.21±1.29% DNA in tail *vs.* 94.48±0.88% DNA in head and 5.52±0.88% DNA in tail) while post-treatment with *T. calcareus* EO induced a higher protection than the pre-treatment (92.74±4.32% DNA in head and 7.26±0.95% DNA in tail *vs.* 74.87±2.56% DNA in head and 25.13±2.53% DNA in tail) (Figure 1, Figure 2). Both EOs are devoid of genotoxicity at tested concentration.

Only few studies have investigated the antigenotoxic/genotoxic activities of *Thymus* EOs. Zani et al. showed that *T. vulgaris* EO had no mutagenic or DNA-damaging effects in the Ames test (0.25 and 0.5 μL/plate) or *Bacillus subtilis* rec-Assay (10 and 30 μL).[38] The treatment with *T. kotschyanus* EOs containing carvacrol (5.3-54.2%) and thymol (8.1-28.1%) as main components, protected against DNA-oxidative damage induced by H_2O_2 (100 μM, 30 min) in human normal lymphocytes.[39] The protective activities are mostly attributable to the presence of monoterpene phenols such as thymol and carvacrol, compounds known as powerful antioxidants. Many studies have also revealed the genoprotective properties of short-time exposure to low concentrations (μM) of thymol or carvacrol. Thymol, the major component of *T. vulgaris* and *T. calcareus* EOs, showed the ability to protect various mammalian cells against genotoxicity induced by different agents, such as: H_2O_2,[40] mitomycin C and imidazolquinoline,[41] gamma-radiation[42], or UVA and UVB radiations[43] in a dose-dependent manner. Aydin et al. showed that the concentrations of thymol below 100 μM are genoprotective while the values above these concentrations significantly induced DNA damage in human normal lymphocytes.[41] Also, Ündeğer et al. reported the lack of clastogenic activity for biologically relevant concentrations of thymol (up to 5 μM) in V79 Chinese hamster lung fibroblast cells, whilst 25 μM of thymol produced DNA damaging effects.[28] The same dual behavior has also been reported for other constituents of thyme EOs. Thus, carvacrol and γ-terpinene did not induce genotoxic events at concentrations lower than 50-100 μM in human normal lymphocytes.[41] This dual behavior might be attributed to the pro-oxidant effects of the volatiles, mainly phenolic ones, at high concentrations. In our study, the concentrations of thymol and γ-terpinene in 25 μg/ml *Thymus* EOs (dose used to treat cells), are lower than 1 μM; at these concentrations, we determined genoprotective effects. Different mechanisms of genoprotection can be considered depending on

the experimental design. In the pretreatment protocols, the antigenotoxic activity of *Thymus* EOs can be ascribed to the antioxidant mechanisms such as free radical scavenging and enhancement of endogenous antioxidant status. As we already showed, *Thymus* EOs included in our study have strong free radical scavenging abilities. The superior antioxidant activity of *T.vulgaris* EO compared to *T. calcareus* EO explains, at least in part, its higher antigenotoxic potential. Besides, thymol has been reported to act as a good scavenger of free radicals responsible for DNA injuries, increase both enzymatic (catalase, superoxide dismutase) and non-enzymatic (glutathione) antioxidant responses, and inhibit lipid peroxidation.[42]

In post-treatment experimental protocol, the beneficial effects of *Thymus* EOs might be due to stimulation of DNA repair process. Although these mechanisms are not common for volatile terpenes, Nikolić et al. reported that some monoterpenes such as camphor and eucalyptol, found in thyme EOs, can stimulate DNA repair, acting as bioantimutagens.[44] In addition, Calò et al. suggested that thymol could decrease DNA damage induced by UVB irradiation by enhancing the expression of nucleotide excision repair genes.[43]

4 | CONCLUSION

The present study investigated for the first time the volatiles of Moldavian *Thymus* species (*T. vulgaris*, *T.* × *citriodorus*, *T. calcareus*) and cultivars (*T. vulgaris* 'Faustini', *T. citriodorus* 'Aureus'). The essential oils of Moldavian *T. vulgaris* and *T. calcareus* are important sources of thymol and showed the most potent antioxidant and antifungal activities, and therefore, being of interest to food and pharmaceutical industries. Due to their high content in geraniol and lavandulol, the Moldavian *T.* × *citriodorus* EOs and *T. vulgaris* 'Faustini' EO seem to be particularly valuable for perfumery and flavor industry. Low doses of *T. vulgaris* and *T. calcareus* EOs exhibited remarkable genoprotective effects at preventive and interventional levels against oxidative DNA damage as a result of antioxidant mechanisms but also possible stimulation of DNA repair processes. Further research on *Thymus* EOs is needed for a better understanding of their antigenotoxic mechanisms and effects on genomic stability at chronic exposure. Identification of phytochemicals and extracts that counteract genomic damage is important for the prevention of mutation-related diseases and aging; in addition, lack of genotoxicity is an indicative of a safety profile in administration.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization.

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