Chemical and Process Engineering: New Frontiers, 2024, 45(1), e55
DOI: 10.24425/cpe.2024.148555

# A comparative study of thyme (*Thymus vulgaris* L.) essential oils and thymol – differences in chemical composition and cytotoxicity

Aurelia Fijałkowska[1], Aneta Wesołowska[2], Rafał Rakoczy[2], Magdalena Jedrzejczak-Silicka[1]*

[1] West Pomeranian University of Technology, Szczecin, Laboratory of Cytogenetics, Klemensa Janickiego 29, 71-270 Szczecin, Poland
[2] West Pomeranian University of Technology, Szczecin, Institute of Chemical Engineering and Environmental Protection Process, Piastow 42, 71-065 Szczecin, Poland

*** Corresponding author, e-mail:** mjedrzejczak@zut.edu.pl

**Article info:**
Received: 29 August 2023
Revised: 15 December 2023
Accepted: 21 December 2023

**Abstract**
The chemical composition of commercial thyme oils, freshly hydrodistilled EO (essetntial oil) from dried thyme herb and thymol, the main thyme oil constituent, were analyzed in the aspect of possible cytotoxic effect against MCF-7 breast cancer and normal L929 mouse fibroblast cell lines.
Based on the GC-MS analysis, it was found that the commercial essential oils revealed similarities in their chemical composition. The content of main components such as thymol, linalool and α-pinene was almost equal. Interestingly, the EO obtained by hydrodistillation from *Thymi herba* showed considerable differences in the percentage content of some main constituents. The reason for the differences may be caused by the intraspecific chemical variability of *T. vulgaris* L. Four types of tested EOs can be classified as a 'thymol' chemotype, with thymol as the predominant compound.
The thymol alone and the freshly hydrodistilled EO demonstrated the highest cytotoxic effect against used cell lines. The difference in $IC_{50}$ values suggests more sensitive L929 cells are more sensitive in both the CCK-8 assay (except EOs Kawon) and the NRU assay.

**Keywords**
thyme, thymol, essential oil composition, cytotoxic/irritating effect, gas chromatography-mass spectrometry

## 1. INTRODUCTION

Breast cancer, the most frequent malignancy in women, is a global problem, which needs multimodal treatment and novel drugs (Harbeck et al., 2018). The main causes that limit the success of treatment in aggressive cancer cases are resistance toward anticancer drugs and their side effects. Essential oils isolated from plants can change the metabolism of cancer cells in very low doses, therefore they are being considered a promising agent adiuvant for anticancer therapy as a way to defeat side effects and the high cost of chemotherapy approaches in breast cancer (Blowman et al., 2018).

A literature survey indicated that some essential oil constituents have shown significant anticancer capabilities in combination with chemotherapy agents (Lesgards et al., 2014). For example, the combination of geraniol with 5-fluorouracil significantly reduces (53%) colon tumour volume in mice, while 5-fluorouracil alone had no effect (Lesgards et al., 2014). Geraniol together with simvastatin significantly inhibits the proliferation of liver cancer cells. Interestingly, these products applied separately are not effective (Polo et al., 2011). Limonene in combination with docetaxel increases oxidative stress in human prostate cancer cells and activates apoptosis (Rabi and Bishayee, 2009).

Essential oil constituents, such as terpenoids and phenolic compounds, also show their efficiency when used alone, without conventional chemotherapy or radiotherapy treatments (Lesgards et al., 2014). Thymol and its isomer carvacrol show similar curative properties on liver, colon and lung cancer (Slamenová et al., 2007). Results obtained by Kang and coworkers (Kang et al., 2016) indicated that thymol suppressed cell growth, and inducted apoptosis by causing morphological changes, generation of intracellular reactive oxygen species and depolarization of mitochondrial membrane potentials in human gastric carcinoma. Thymoquinone activates apoptosis in colon cancer cells (Gali-Muhtasib et al., 2008). This compound is also active in lung, liver and breast cancer cells (Attoub et al., 2012). Several researchers documented molecular mechanisms of apoptosis induced by eugenol in skin tumours (Shin et al., 2007), leukaemia (Yoo et al., 2005), gastric tumours (Manikandan et al., 2010) and breast cancer (Vidhya and Devaraj, 2011).

Many essential oils (*Salvia officinalis* L., *Laurus nobilis* L., *Origanum compactum Bentham* L., *Helichrysum gymnocephalum, Artemisia capillaris, Mentha spicata* L., *Lavandula angustifolia* Mill., *Matricaria chamomilla* L., *Rosa damascena* Mill., *Ocimum basilicum* L., *Thymus vulgaris* L.) were evaluated for their anticancer activity (Lesgards et al., 2014).

The highest cytotoxicity against three human cancer cell lines (PC-3 prostate, A-549 lung and MCF-7 human mammary carcinoma) showed thyme EO (Zu et al., 2010).

Thyme (*Thymus vulgaris* L.), a member of the *Lamiaceae* family, is an aromatic plant commonly used to flavour sauces, stews and soups (Torras et al., 2007). This evergreen herb possesses omnidirectional properties demonstrated in Table 1.

Table 1. Thyme essential oil and thymol biological activity (Islam et al., 2019).

| Therapeutic properties | References |
|---|---|
| Antispasmodic, antiseptic, expectorant, carminative, antitussive | Dapkevicius et al., 2002 |
| Oxidative stress and cancer cell death | Satooka et al., 2012 |
| Apoptosis of cancer cell | Salehi et al., 2018a |
| Antiproliferative effects on cancer cells | Salehi et al., 2018b |
| Antioxidant and antiapoptotic activity | Cicvarek et al., 1964 |
| Protective activity | Maclagan et al., 1974 |
| Anti-inflammatory/ immunomodulatory effects | Kang et al., 2016 |
| Antigenotoxic effects | Habtemariam and Lentini, 2018 |

*Thymi herba* contains tannins, flavonoids, triterpene compounds and up to 2.5% of EO (Anžlovar et al., 2014). The essential oil isolated from leaves and flowering tops of thyme possesses antimicrobial, antifungal, antioxidant and anticancer activities (Sertel et al., 2011). Thyme oil contains mainly thymol (23–60%), $\gamma$–terpinene (18–50%), *p*–cymene (8–44%), carvacrol (2–8%), and linalool (3–4%) (Duke, 1992). The chemical composition of EOs depends on several factors such as the environment, growing region and cultivation practices (Hudaib and Aburjai, 2007). The chemical composition (the constituents of the EOs) determines the biological properties and applications of thyme oil in medicine (Basch et al., 2004).

The main goal of this study was to determine cytotoxic effect of thyme oils (commercially available as well as essential oil obtained from *Thymi herba* by hydrodistillation) and the main thyme oil constituent – thymol on human MCF-7 breast cancer and normal L929 cell lines. Additionaly, the relation of the cytotoxicity towards human MCF-7 breast cancer and normal L929 cell lines and chemical composition was evaluated.

## 2. MATERIALS AND METHODS

### 2.1. Essential oils, Thymi herba and thymol

Three thyme essential oils were purchased from the manufacturers (Bamer batch number 840003, Senti Oils batch number 542362 and Ecospa batch number 4347401, Poland). Particular EOs and thyme herb were selected due to description of particular products available on the company website. The chemical compositions of EOs were determined by gas chromatography-mass spectrometry (GC-MS). Also, the chemical composition of essential oil isolated by hydrodistillation from dried thyme herb (*Thymi herba* was purchased from the herb company of Kawon, Poland) was analyzed.

The plant material (30 g) was subjected to hydrodistillation for 2 hours in a Clevenger-type apparatus, according to the method recommended by European Pharmacopoeia (2010). The collected essential oil (EO) was separated from water and dried over sodium sulfate. Three replicates were carried out. The overall yield of the EO was 1.42% (v/w) established on a dry basis.

The thymol crystals were purchased from the company of POCH, Poland.

### 2.2. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Thyme essential oils were analyzed by GC-MS using a Hewlett Packard 6890 GC coupled with a Hewlett Packard 5973 Mass Selective Detector. The gas chromatograph was equipped with a fused silica HP-5MS (30 m $\times$ 0.25 mm, film thickness 0.25 µm) capillary column. Helium was used as the carrier gas at a flow rate of 1 mL/min. The EOs (20 mg) were diluted in dichloromethane (1.5 mL) and 1 µL of the solution was injected. The inlet temperature was set at 280 °C with a split injection mode for a split ratio of 5:1. The oven temperature program was as follows: the initial temperature of the column was 40 °C (held for 5 minutes); then increased to 60 °C at a rate of 30 °C/min, next to 230 °C at a rate of 6 °C/min (kept constant for 10 minutes), and finally to 280 °C at a rate of 30 °C/min. The oven was held at this temperature for 5 minutes. The total running time for a single sample was about 51 minutes.

Mass spectra were recorded at 70 eV at a scan mode from $m/z$ 50 to 500. The transfer line temperature was 280 °C. The ion source and quadrupole temperatures were 230 °C and 150 °C, respectively. The quantitative data were expressed as the relative percentage of the oil components calculated from the GC peak areas without using correction factors.

For all essential oil samples, the GC-MS analysis was performed in triplicate.

### 2.3. Identification of essential oil constituents

Qualitative analysis of the essential oil constituents was based on the comparison of their calculated retention indices (relative to n-alkanes $C_7$–$C_{30}$; Supelco Park Bellefonte, PA USA on the HP-5MS column) with those reported in NIST Chemistry WebBook and the literature (Babushok et al., 2011),

and by comparison of their mass spectra with those of authentic standards (thymol, carvacrol, *p*–cymene, *β*–pinene, purchased from Sigma–Aldrich) as well as those from MS Libraries (NBS75K.L and NIST 2002).

## 2.4. Cell lines and cell culture conditions

The effect of the thyme oils and thymol was analyzed on two chosen cell lines – mouse L929 fibroblast (ATCC®CCL-1 ™) and MCF-7 human breast adenocarcinoma (ATCC®HTB-22™).

The L929 and MCF-7 cells were seeded into 96-well plates (Corning Inc., Corning, NY, USA) at the density of $3 \times 10^3$ (into the 96-well plate for CCK-8, LDH and NRU assays) and $3.5 \times 10^4$ (into 24-well plates for cellular morphology analysis) per well and were maintained (for 24-hour before cells treatment) at standard culture conditions at 37 °C, 5% $CO_2$, 95% relative humidity (RH) as was described elsewhere (Jedrzejczak-Silicka et al., 2017; Jedrzejczak-Silicka, 2017; Jędrzejczak-Silicka et al., 2020). The complete DMEM culture medium (Dulbecco's Modified Eagle Medium, High Glucose, Corning Inc., Corning, NY, USA) was supplemented with 10% fetal bovine serum (FBS, heat-inactivated, Corning Inc., Corning, NY, USA), 2 mM L-glutamine (Corning Inc., Corning, NY, USA), 50 IUmL$^{-1}$ penicillin and 50 μg·mL$^{-1}$ streptomycin (Corning Inc., Corning, NY, USA) and amphotericin B (2.5 μg·mL$^{-1}$; Sigma–Aldrich, St. Louis, MO, USA). The maintained cultures were monitored every 24 hours with a Nikon TS-100 microscope (NIS Elements F Package, camera Nikon DS-Fi1, Nikon, Melville, NY, USA). All the analyses were conducted in three independent experiments.

## 2.5. Experimental treatment

After 24 hours of cell seeding, five different final concentrations – 50, 100, 250, 500 and 1000 μg·mL$^{-1}$ of essential oils and thymol were prepared in DMEM culture medium (with 0.1% of DMSO independently forming EO's concentration) and added to cell cultures. Additionally, three control cultures were prepared – the first one ('w DMSO control') – cells were treated with the equivalent volume of DMSO solution (0.1%), the second one ('w/o DMSO control') – cells cultured in standard DMEM medium in the absence of EO and DMSO (to evaluate the effect of DMSO on cellular metabolism) and the third one – the positive control – L929 and MCF-7 cells incubated with the camptothecin solution (final concentration 6 μM). Cell lines were incubated with three thyme commercial EOs, one thyme essential oil isolated from dried thyme herb by hydrodistillation and thymol for 48 h.

## 2.6. Phase contrast microscopy analysis

Firstly, the morphology of the L929 and MCF-7 cell lines exposed to EOs and thymol at different concentrations: 50, 100, 250, 500 and 1000 μg·mL$^{-1}$ and the control samples for both cell lines (after 48-hour cultures) were analyzed by phase contrast inverted Nikon TS-100 microscope (objective lens – CFI Achromat DL 10X, N.A. 0.25, W.D. 6.2 mm, Ph1) and Nikon DS-Fi1 camera (5-megapixels; acquisition software – NIS Elements F Package Ver. 4.00.06 – Nikon, Melville, NY, USA) at 100× magnification.

## 2.7. CCK-8 assay – determination of cell viability

The effect on the relative cell viability of L929 and MCF-7 cell lines after 48-hour exposition was determined using the Cell Counting Kit-8 (CCK-8; Sigma–Aldrich, St. Louis, MO, USA). CCK-8 assay (using WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt)) proceeds via the conversion of tetrazolium salt into coloured formazan by living cells. The bioreduction of the WST-8 is possible due to active cellular dehydrogenases, thus the amount of the coloured formazan is proportional to the number of metabolically active cells. The CCK-8 solution (10 μL per well) was added to each well and incubated for 2 hours at 37 °C. After incubation, the absorbance was recorded at 450 nm (with a reference wavelength at 650 nm), according to the manufacturer's instructions, on a Sunrise Absorbance Reader (Sunrise, Tecan, Männedorf, Switzerland) as was described elsewhere (Aleksandrzak et al., 2019). All the analyses were conducted in three independent experiments.

The effect of EOs and thymol on cellular metabolic activity was calculated using the following Formula (1):

$$\text{Relative viability from CCK-8 assay (\%)} = \left(\frac{\text{sample } A_{450-650\text{ nm}}}{\text{positive control } A_{450-650\text{ nm}}}\right) \cdot 100 \qquad (1)$$

where $A$ is absorbance.

## 2.8. LDH assay – determination of lactate dehydrogenase leaking

The effect of the thyme EOs and thymol on cellular membranes (after 48 h culture) was also evaluated using the LDH CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) as was described elsewhere (Aleksandrzak et al., 2019). The lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released upon cell lysis, thus the higher the cellular membrane damage the higher the LDH level determined in cell cultures. The LDH release was determined by the absorbance measured at 490 nm using a microplate spectrophotometer (Sunrise, Absorbance Reader, Tecan, Männedorf, Switzerland) according to the manufacturer's instructions. All the analyses were conducted in three independent experiments.

The relative cell viability was determined according to the equation described by Verrax and Buc (2011) and expressed as 100% minus the ratio of released LDH activity to the total activity of LDH using Equation (2):

$$\text{LDH released (\%)} = 100 - \left(\frac{A_{490}\text{ nm of treated and untreated cells} - A_{490}\text{ nm of control}}{A_{490}\text{ nm of maximum of untreated cells} - A_{490}\text{ nm of control}} \cdot 100\right) \quad (2)$$

where $A$ is absorbance.

## 2.9. Neutral red uptake assay

Moreover, the biological activity of the thyme EOs and thymol was analysed using neutral red uptake (NRU) assay (In Vitro Toxicology Assay Kit, Neutral Red based, Sigma–Aldrich, St. Louis, MO, USA). The neutral red assay is based on the measuring of living cells that are able to take up the neutral red dye by active transport and incorporate the dye into lysosomes. After the 48-h exposition of L929 and MCF-7 cells to EOs and thymol the cells were washed with phosphate-buffered saline (1xDPBS) (Corning Inc., Corning, NY, USA). Then, DMEM medium containing 10% of neutral red dye was added to the cultures and incubated at standard culture conditions for 3 hours. Lately, cells were washed with DPBS and Neutral Red Assay Solubilization Solution (Sigma–Aldrich, St. Louis, MO, USA) was added to cells to release the incorporated NR and the absorbance at 540 nm was measured (the background absorbance of multiwell plates at 690 nm) using Tecan Sunrise microplate reader (Sunrise, Tecan, Männedorf, Switzerland) as was described elsewhere (Aleksandrzak et al., 2019). All the analyses were conducted in three independent experiments. The cell viability was calculated using Eq. (3):

$$\text{Neutral red uptake assay (\%)} = \left(\frac{\text{sample } A_{540-690\text{ nm}}}{\text{positive control } A_{540-690\text{ nm}}}\right) \times 100 \quad (3)$$

where $A$ is absorbance.

## 2.10. Statistical analysis

The composition data presented in this study are given as the mean values ± standard deviation (SD). The statistical analyses were determined by one-way analysis of variance (one-way ANOVA) using Fisher's Least Significant Difference (LSD) and a two-way ANOVA using Tukey's honest significant difference test (HSD). The $p$–values 0.05 were considered significant and are represented by different small letters (Table A1–A8, Appendix). The statistical analyses were performed using *STATISTICA* 13.3 (StatSoft Inc., Tulsa, OK, USA).

The $IC_{50}$ values for each test were calculated by non-linear regression analysis (Sebaugh, 2011) using Equation (4) (GraphPad Prism, San Diego, CA, USA), as follows:

$$y = d + \frac{(a-d)}{1 + \left(\frac{x}{c}\right)^b} \quad (4)$$

where:

$y$ – the cellular response,

$x$ – the concentration of ASA,

$a$ – the lower asymptote (lower plateau),

$d$ – the upper asymptote (upper plateau),

$b$ – the slope factor,

$c$ – the concentration of ASA, that corresponds to the response midway between $a$ and $d$.

# 3. RESULTS

## 3.1. Chemical composition of commercial thyme essential oils

Considering four thyme oils together, a total of seventy-seven different compounds were identified: 54 for Kawon (99.44% of the total oil), 47 for Bamer (99.73% of the oil), 40 for Senti (99.87% of the oil) and 42 for Ecospa (99.77% of the oil) (Table 2; Figure A1–A4).

In the EO obtained by hydrodistillation from thyme herb (Kawon) dominated thymol (48.23%), γ–terpinene (11.85%), *p*–cymene (10.58%) and carvacrol (5.62%). Other compounds found in significant amounts were linalool (2.76%), α–terpinene (2.52%) and β–caryophyllene (1.86%). The main constituents found in commercial thyme oil offered by Bamer company were thymol (33.61%), *p*–cymene (27.03%), α–terpineol (11.23%), linalool (6.54%), α–pinene (3.60%), γ–terpineol (2.51%) and β–caryophyllene (1.98%). In the thyme oil offered by Senti Oils company thymol (33.73%), *p*–cymene (28.89%), α–terpineol (13.77%), linalool (6.45%), α–pinene (3.69%), γ–terpineol (2.57%) and β–caryophyllene (1.75%) were the major components. Similarly, thyme oil offered by Ecospa company was rich in thymol (33.28%), *p*–cymene (27.85%), α–terpineol (14.03%), linalool (6.56%), α–pinene (3.70%), γ–terpineol (2.70%) and β–caryophyllene (1.82%).

Interestingly, γ–terpinene (11.85%) was found only in the oil isolated from thyme herb by hydrodistillation in a Clevenger apparatus. Trace amounts of γ–terpinene (0.03%) were present in commercial thyme oil from Ecospa.

Oxygenated monoterpenes (60.35–64.30%), monoterpene hydrocarbons (30.09–37.04%) and sesquiterpene hydrocarbons (2.24–3.56%) dominated in the analyzed oils. Oxygenated sesquiterpenes (0.04–0.81%) were present in very low amounts (Table 3).

Table 2. Chemical composition of the essential oil obtained by hydrodistillation from thyme herb (Kawon) and commercially available thyme oils (Bamer, Senti and Ecospa).

| No. | Compounds[a] | $R_t$ [min] | RI[b] | Kawon | Bamer | Senti | Ecospa |
|---|---|---|---|---|---|---|---|
| 1. | Methyl α–methylbutanoate | 5.45 | 783 | 0.25 | –* | – | – |
| 2. | (Z)-3-Hexen-1-ol | 7.30 | 856 | 0.04 | – | – | – |
| 3. | α–Thujene | 9.02 | 925 | 0.76 | – | – | – |
| 4. | **α–Pinene** | **9.19** | **932** | **0.85** | **3.60** | **3.69** | **3.70** |
| 5. | **Camphene** | **9.56** | **947** | **0.43** | **1.19** | **1.20** | **1.21** |
| 6. | *trans*-*p*–Menthane | 10.23 | 973 | – | – | 0.05 | 0.05 |
| 7. | β–Pinene | 10.28 | 975 | 0.23 | 0.38 | 0.43 | 0.42 |
| 8. | 1-Octen-3-ol | 10.35 | 978 | 0.81 | – | – | – |
| 9. | 3-Octanone | 10.46 | 982 | – | 0.06 | 0.06 | 0.05 |
| 10. | 6-Methyl-5-hepten-2-one | 10.56 | 986 | 0.06 | – | – | – |
| 11. | **β-Myrcene** | **10.65** | **990** | **1.64** | **1.35** | **1.42** | **1.45** |
| 12. | 3-Octanol | 10.76 | 994 | 0.09 | – | – | – |
| 13. | (E,Z)-2,4-Heptadienal | 10.89 | 999 | – | – | – | 0.03 |
| 14. | α–Phellandrene | 10.98 | 1003 | 0.30 | 0.09 | 0.09 | 0.12 |
| 15. | **α-Terpinene** | **11.31** | **1016** | **2.52** | **0.05** | **0.06** | **0.07** |
| 16. | o-Cymene | 11.44 | 1021 | – | 0.21 | – | – |
| 17. | ***p*–Cymene** | **11.55** | **1025** | **10.58** | **27.03** | **28.89** | **27.85** |
| 18. | **D-Limonene** | **11.62** | **1028** | **0.57** | **0.96** | **0.97** | **1.17** |
| 19. | Eucalyptol | 11.69 | 1031 | 0.71 | 0.70 | 0.66 | 0.94 |
| 20. | (Z)-β-Ocimene | 11.86 | 1038 | 0.03 | 0.15 | 0.15 | 0.23 |
| 21. | (E)-β-Ocimene | 12.09 | 1047 | 0.08 | – | – | – |
| 22. | **γ-Terpinene** | **12.37** | **1058** | **11.85** | **–** | **–** | **0.03** |
| 23. | *cis*-Sabinene hydrate | 12.59 | 1067 | 0.81 | – | – | – |
| 24. | α–Terpinolene | 13.12 | 1088 | 0.25 | 0.07 | 0.14 | 0.13 |
| 25. | 2-Phenyl-2-propanol | 13.20 | 1091 | – | 0.05 | 0.03 | 0.05 |
| 26. | **Linalool** | **13.40** | **1099** | **2.76** | **6.54** | **6.45** | **6.56** |
| 27. | Fenchol | 13.76 | 1114 | – | 0.22 | 0.06 | 0.06 |
| 28. | β–Thujone | 13.96 | 1122 | 0.09 | 0.06 | 0.04 | 0.05 |
| 29. | 10-Northuja–2-ene-4-one | 14.03 | 1125 | – | 0.07 | 0.08 | 0.09 |
| 30. | **(Z)-*p*–2,8-Manthadien-1-ol** | **14.26** | **1135** | **–** | **1.06** | **0.08** | **0.08** |
| 31. | Pinocarveol | 14.40 | 1140 | 0.06 | – | – | – |
| 32. | **Camphor** | **14.56** | **1147** | **0.16** | **1.06** | **0.63** | **0.65** |
| 33. | (+)-2-Bornanone | 14.61 | 1149 | 0.24 | – | – | – |
| 34. | *p*–Menthone | 14.77 | 1156 | 0.33 | – | – | – |
| 35. | Isoborneol | 14.83 | 1158 | – | 0.68 | 0.53 | 0.55 |
| 36. | (Z)-β-Terpineol | 14.99 | 1165 | – | 0.31 | 0.20 | 0.20 |
| 37. | **Borneol** | **15.05** | **1167** | **1.20** | **1.38** | **1.27** | **1.34** |
| 38. | Menthol | 15.21 | 1174 | 0.11 | – | – | – |
| 39. | Isomenthol | 15.26 | 1176 | 0.18 | – | – | – |
| 40. | **Terpinen-4-ol** | **15.32** | **1178** | **1.05** | **0.42** | **0.08** | **0.07** |
| 41. | *p*–Cymen-8-ol | 15.50 | 1186 | 0.10 | 0.08 | 0.04 | 0.04 |
| 42. | **α–Terpineol** | **15.67** | **1193** | **0.27** | **11.23** | **13.77** | **14.03** |

Table 2 *continued on next page*

Table 2 continued

| No. | Compounds[a] | $R_t$ [min] | RI[b] | Kawon | Bamer | Senti | Ecospa |
|---|---|---|---|---|---|---|---|
| 43. | **γ–Terpineol** | **15.81** | **1198** | **0.06** | **2.51** | **2.57** | **2.70** |
| 44. | *trans*–Carveol | 16.25 | 1217 | 0.05 | – | – | – |
| 45. | **Thymol methyl ether** | **16.64** | **1234** | **1.02** | **–** | **–** | **–** |
| 46. | Carvacrol methyl ether | 16.86 | 1244 | 0.84 | – | – | – |
| 47. | Thymoquinone | 17.11 | 1255 | – | 0.03 | – | – |
| 48. | Piperitone | 17.16 | 1257 | – | 0.09 | – | – |
| 49. | Geranial | 17.48 | 1271 | – | 0.06 | 0.02 | – |
| 50. | Borneol acetate | 17.86 | 1287 | 0.22 | – | – | – |
| 51. | **Thymol** | **18.03** | **1294** | **48.23** | **33.61** | **33.73** | **33.28** |
| 52. | Tridcane | 18.18 | 1301 | – | 0.20 | – | – |
| 53. | Isocarveol | 18.25 | 1304 | – | 0.02 | – | – |
| 54. | **Carvacrol** | **18.31** | **1307** | **5.62** | **0.27** | **0.10** | **0.06** |
| 55. | Undecanal | 18.37 | 1310 | – | – | – | 0.04 |
| 56. | Methyl geranate | 18.78 | 1328 | – | 0.04 | – | – |
| 57. | Thymol acetate | 19.39 | 1356 | 0.19 | 0.10 | 0.04 | 0.04 |
| 58. | Cycloisosativene | 19.76 | 1373 | – | 0.03 | – | – |
| 59. | α–Longicyclene | 19.85 | 1377 | – | 0.09 | 0.03 | 0.03 |
| 60. | α–Copaene | 19.92 | 1380 | 0.07 | 0.06 | 0.05 | 0.05 |
| 61. | β–Bourbonene | 20.12 | 1389 | 0.04 | – | – | – |
| 62. | Sativene | 20.26 | 1396 | – | 0.06 | – | – |
| 63. | Longifolene | 20.62 | 1413 | – | 1.04 | 0.17 | 0.17 |
| 64. | (E)-β–Damascone | 20.72 | 1418 | – | 0.03 | 0.02 | 0.03 |
| 65. | **β-Caryophyllene** | **20.89** | **1425** | **1.86** | **1.98** | **1.75** | **1.82** |
| 66. | β–Copaene | 21.06 | 1434 | 0.03 | 0.06 | 0.04 | 0.04 |
| 67. | (E)-Geranylacetone | 21.49 | 1455 | – | 0.07 | 0.03 | 0.04 |
| 68. | α–Caryophyllene | 21.60 | 1460 | 0.08 | 0.24 | 0.20 | 0.21 |
| 69. | γ–Muurolene | 22.03 | 1480 | 0.16 | – | – | – |
| 70. | Germacrene D | 22.16 | 1487 | 0.05 | – | – | – |
| 71. | α–Muurolene | 22.41 | 1499 | 0.09 | – | – | – |
| 72. | Bicyclogermacrene | 22.50 | 1503 | 0.08 | – | – | – |
| 73. | γ–Cadinene | 22.80 | 1518 | 0.20 | – | – | – |
| 74. | δ–Cadinene | 22.98 | 1528 | 0.33 | – | – | – |
| 75. | Caryophyllene oxide | 24.23 | 1591 | 0.41 | 0.14 | 0.05 | 0.04 |
| 76. | Isospathulenol | 24.96 | 1630 | 0.10 | – | – | – |
| 77. | τ–Cadinol | 25.31 | 1648 | 0.30 | – | – | – |
| | **Total identified** | | | **99.44** | **99.73** | **99.87** | **99.77** |
| | **Number of compounds identified** | | | **54** | **47** | **40** | **42** |
| | Monoterpene hydrocarbons | | | 30.09 | 35.08 | 37.04 | 36.38 |
| | Oxygenated monoterpenes | | | 64.30 | 60.50 | 60.35 | 60.74 |
| | Sesquiterpene hydrocarbons | | | 2.99 | 3.56 | 2.24 | 2.32 |
| | Oxygenated sesquiterpenes | | | 0.81 | 0.14 | 0.05 | 0.04 |
| | Others | | | 1.25 | 0.45 | 0.19 | 0.29 |

[a] Compounds are listed in order of their elution from an HP-5MScapillary column
[b] Retention indices relative to $C_7$–$C_{30}$ alkanes on a HP-5MScolumn
–* not detected

Table 3. Statistical analysis of the content of main constituents identified in the investigated thyme essential oils.

| Constituents (*factor I*) | (*factor II*) | | | | |
|---|---|---|---|---|---|
| | Kawon | Bamer | Senti | Ecospa | Mean |
| $\alpha$–Pinene | 0.85 ± 0.081 | 3.60 ± 0.274 | 3.69 ± 0.115 | 3.70 ± 0.035 | 2.96 |
| Camphene | 0.43 ± 0.032 | 1.19 ± 0.091 | 1.20 ± 0.040 | 1.21 ± 0.050 | 1.01 |
| $\beta$–Myrcene | 1.64 ± 0.100 | 1.35 ± 0.102 | 1.42 ± 0.061 | 1.45 ± 0.081 | 1.46 |
| $\alpha$–Terpinene | 2.52 ± 0.110 | 0.05 ± 0.006 | 0.06 ± 0.000 | 0.07 ± 0.010 | 0.67 |
| *p*–Cymene | 10.58 ± 0.238 | 27.03 ± 0.464 | 28.89 ± 2.109 | 27.85 ± 1.369 | 23.59 |
| D-Limonene | 0.57 ± 0.040 | 0.96 ± 0.046 | 0.97 ± 0.064 | 1.17 ± 0.074 | 0.92 |
| $\gamma$–Terpinene | 11.85 ± 0.153 | – | – | 0.03 ± 0.001 | 5.94 |
| Linalool | 2.76 ± 0.301 | 6.54 ± 0.082 | 6.45 ± 0.420 | 6.56 ± 0.244 | 5.58 |
| (Z)-*p*–2,8-Menthadien-1-ol | –* | 1.06 ± 0.02 | 0.08 ± 0.010 | 0.08 ± 0.010 | 0.41 |
| Camphor | 0.16 ± 0.106 | 1.06 ± 0.275 | 0.63 ± 0.051 | 0.65 ± 0.051 | 0.62 |
| Borneol | 1.20 ± 0.086 | 1.38 ± 0.015 | 1.27 ± 0.115 | 1.34 ± 0.142 | 1.30 |
| Terpinen-4-ol | 1.05 ± 0.089 | 0.42 ± 0.010 | 0.08 ± 0.006 | 0.07 ± 0.006 | 0.41 |
| $\alpha$–Terpineol | 0.27 ± 0.049 | 11.23 ± 0.148 | 13.77 ± 0.700 | 14.03 ± 0.332 | 9.83 |
| $\gamma$–Terpineol | 0.06 ± 0.015 | 2.51 ± 0.020 | 2.57 ± 0.208 | 2.70 ± 0.157 | 1.96 |
| Thymol | 48.23 ± 1.965 | 33.61 ± 1.032 | 33.73 ± 0.699 | 33.28 ± 0.278 | 37.21 |
| Carvacrol | 5.62 ± 0.370 | 0.27 ± 0.015 | 0.10 ± 0.050 | 0.06 ± 0.038 | 1.52 |
| $\beta$–Caryophyllene | 1.86 ± 0.140 | 1.98 ± 0.023 | 1.75 ± 0.097 | 1.82 ± 0.126 | 1.85 |
| Mean | 5.33 | 5.89 | 6.04 | 5.65 | |
| $LSD_{\alpha=0.05}$ for *factor I* | 18.83 | | | | |
| $LSD_{\alpha=0.05}$ for *factor II* | n.s. | | | | |
| $LSD_{\alpha=0.05}$ for interaction *factor I* × *factor II* | 0.54 | | | | |

± standard deviation ($n = 3$), n.s. – not significant, –* – not detected

## 3.2. Biological activity of thyme EOs and thymol

### 3.2.1. Morphological cell analysis

The L929 cell line exposed to commercial oils – Bamer, Ecospa and Senti did not demonstrate a change in morphology and the number of cells in cultures (Fig. 1) even in the highest EO concentrations (at 500 and 1000 μg·mL$^{-1}$). In contrast, the most visible effect of tested factors was observed under a light microscope for the concentration of 500 and 1000 μg·mL$^{-1}$ of freshly hydrodistilled EO (Kawon). Cells exposed to hydrodistilled EO at concentrations of 500 μg·mL$^{-1}$ demonstrated changes in morphology (such as shrinkage/cell spherical shapes) and in the total number of cells, whereas the highest dose (1000 μg·mL$^{-1}$) tested on L929 cells caused cell death (Fig. 1). In the case of thymol solution subtle changes in cell cultures were found at 250 μg·mL$^{-1}$ concentration (Fig. 1). The number of cells was not affected, but cells demonstrated shrinkage. Only a few cells adhered to the surface of the culture dish and exhibited untypical morphology. Most cells were dead after 48 hours of treatment. The highest concentrations of thymol (500 and 1000 μg·mL$^{-1}$) evoked cell death (Fig. 1).

In the case of the MCF-7 cell line, it was found that commercial oils (Bamer and Ecospa) evoked cellular response to the highest concentration (1000 μg·mL$^{-1}$) that demonstrated changes in morphology – spherical shapes of cells were observed (Fig. 2). The same situation was noticed for Senti commercial oil at the highest dose (1000 μg·mL$^{-1}$ concentration) where few cells exhibited spherical shapes whereas the major part of cell population showed unchanged adhesion ability but untypical morphology. When cells were exposed to a freshly hydrodistilled EO (Kawon) at a concentration of 500 μg· mL$^{-1}$ cells demonstrated shrinkage and spherical shapes without changes in total cell number. The highest dose (1000 μg·mL$^{-1}$) of Kawon hydrodistilled oil evoked cell death. The strongest effect observed under a light microscope was found in the case of thymol. At the concentration of 250 μg·mL$^{-1}$ about 50% of cells exhibited spherical shapes, but the total number of cells was unchanged. At the concentration of 500 μg·mL$^{-1}$ few living cells were found in the population and all demonstrated shrinkage, whereas at the concentration of 1000 μg·mL$^{-1}$, all cells were dead and formed cell aggregates in culture medium (Fig. 2).

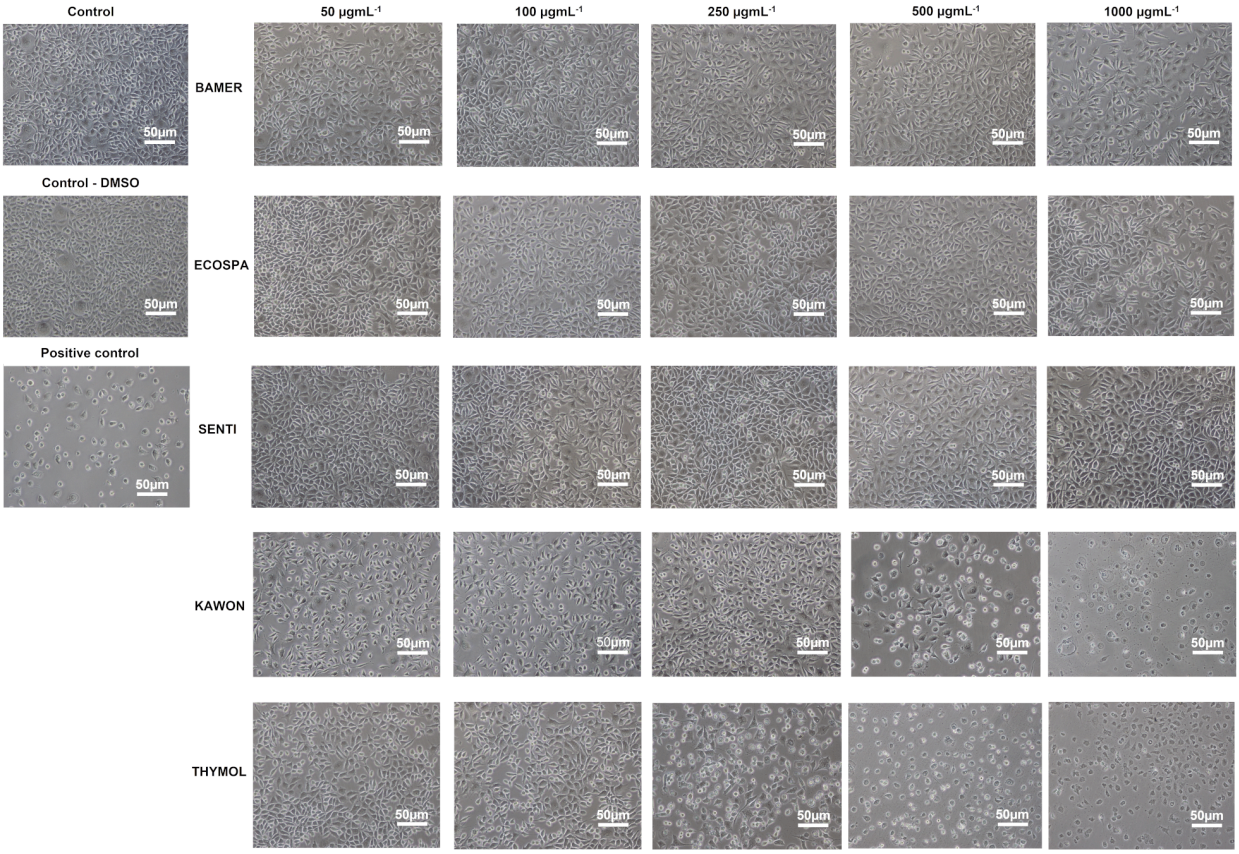


Figure 1. The morphology of the L929 cell line after 48-hour exposition to essential oils and thymol.

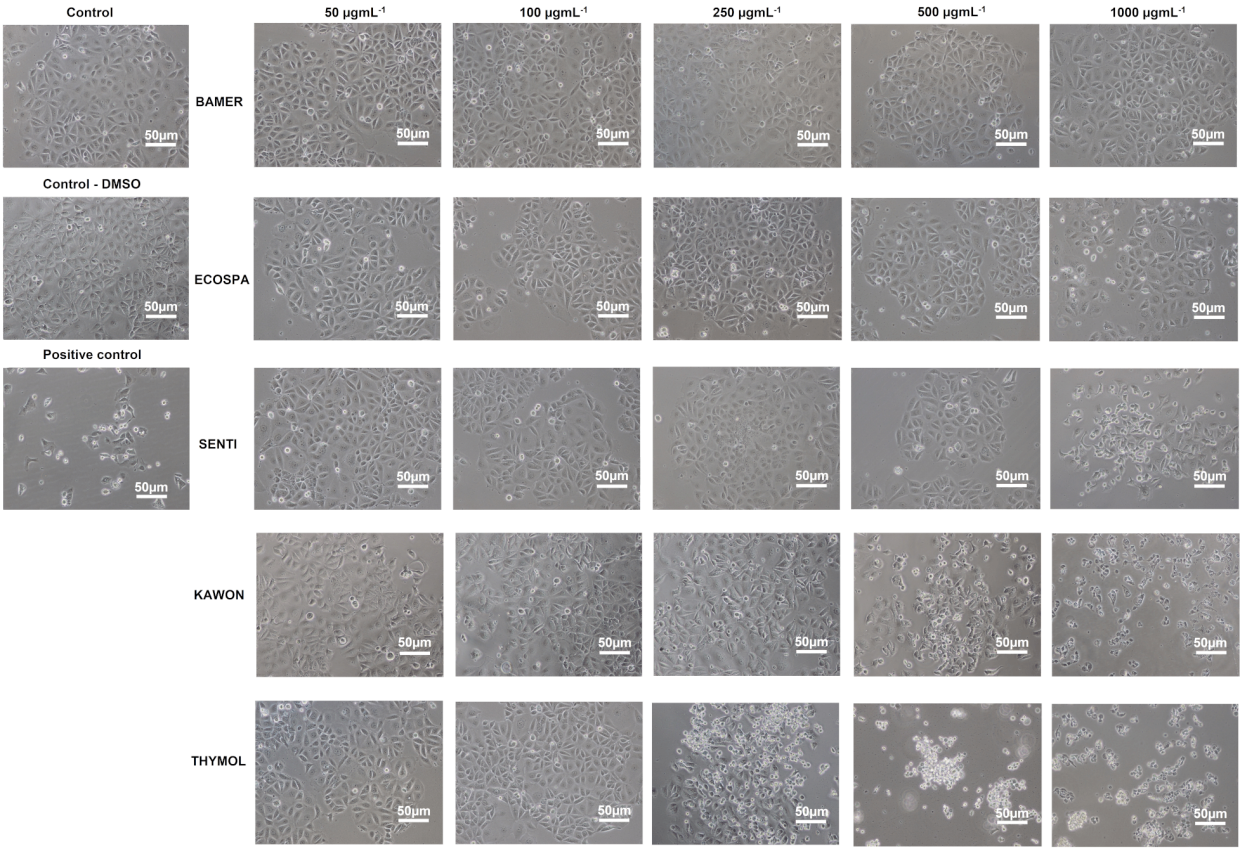


Figure 2. The morphology of the MCF-7 cell line after 48-hour exposition to essential oils and thymol.

3.2.2. Analysis of the cytotoxicity based on cell viability

The biological effect of EOs and thymol was also evaluated using CCK-8, LDH leakage and NRU assays. Results obtained from the CCK-8 assay for the L929 cell line exhibited the strongest effect of freshly hydrodistilled oil and thymol. Obtained results confirmed morphology observation. In addition, the concentration in the range of 50 to 500 $\mu g \cdot mL^{-1}$ of the commercial EOs did not reduce relative cell viability significantly with the lowest value of cell viability equal to 80% (Fig. 3A). The highest concentration of Bamer and Senti commercial oils (1000 $\mu g \cdot mL^{-1}$) reduced cell viability at the higher level, whereas the Ecospa EO demonstrated the weakest action on cells with the reduction of cell viability to 60% after 48-hour exposition to 1000 $\mu g \cdot mL^{-1}$ concentration (differences considered as significant at a level of $p < 0.05$; Table A1–A3). The biological action of freshly hydrodistilled oil and thymol evoked the highest reduction of cell viability at a concentration of 500 $\mu g \cdot mL^{-1}$ 1 and 1000 $\mu g \cdot mL^{-1}$ (Fig. 3A; differences considered as significant at a level of $p < 0.05$; Table A4–A5). The effect of EOs was dose-dependent.

The response of MCF-7 cells to EOs and thymol demonstrated using CCK-8 assay was also dose-dependent with the highest cytotoxic effect for freshly hydrodistilled oil and thymol. The assay result showed a reduction of cell viability at a concentration of 500 $\mu g \cdot mL^{-1}$ to 22–26% and at a concentration of 1000 $\mu g \cdot mL^{-1}$ to 6-8% in comparison with the control. In contrast, commercial oils demonstrated a slightly higher reduction of cell viability at the concentration in the range of 50 and 250 $\mu g \cdot mL^{-1}$ (differences considered as significant at a level of $p < 0.05$; Table A6–A10).

When cell viability was analyzed using LDH leakage assay the effect of EOs and thymol membrane integrity was minimal at the concentration in the range of 50 and 500 $\mu g \cdot mL^{-1}$ in the case of both cell lines. The highest dose (at a concentration of 1000 $\mu g \cdot mL^{-1}$) of tested factors evoked higher LDH leakage after exposure to Bamer EO, freshly hydrodistilled oil (Kawon) and thymol in L929 cell culture (Fig. 3B). In contrast MCF-7 cells demonstrated the highest LDH leakage (up to 28%) in the presence of hydrodistilled oil (Kawon) at a concentration of 1000 $\mu g \cdot mL^{-1}$ (Fig. 3E).

In the results of the NRU test, the highest relative survival rate for both lines was recorded at a concentration of 50 $\mu g \cdot mL^{-1}$ (Fig. 3C and 3F). The hydrodistilled oil (Kawon) and thymol reduced the L929 cell ability to incorporate neutral red dye to 40% in comparison to the control culture (Fig. 3C). In the range of 250 and 1000 $\mu g \cdot mL^{-1}$ both cell lines showed a dose-dependent response to EOs but MCF-7 cells exhibited the highest reduction of cell viability in the presence of hydrodistilled oil (Kawon) (Fig. 3F; differences considered as significant at a level of $p < 0.05$; Table A6–A10).

Moreover, due to different concentrations of thymol in individual oils (in Kawon 48.23% and from 33.28 to 33.73% in Ecospa, Bamer and Senti EOs) additional comparison was evaluated (Figure A5–A7). Cell cultures were incubated with thymol alone at the concentration corresponding to the content in individual EOs. Comparison of the results obtained from the incubation of L929 cells with EOs to results of L929 cells incubated with thymol showed no significant differences in cell viability (Figure A5–A7) except for Ecospa EO in CCK-8 and NRU assays and Senti EO in the case of LDH assay results. In the mentioned exceptions, thymol alone at the

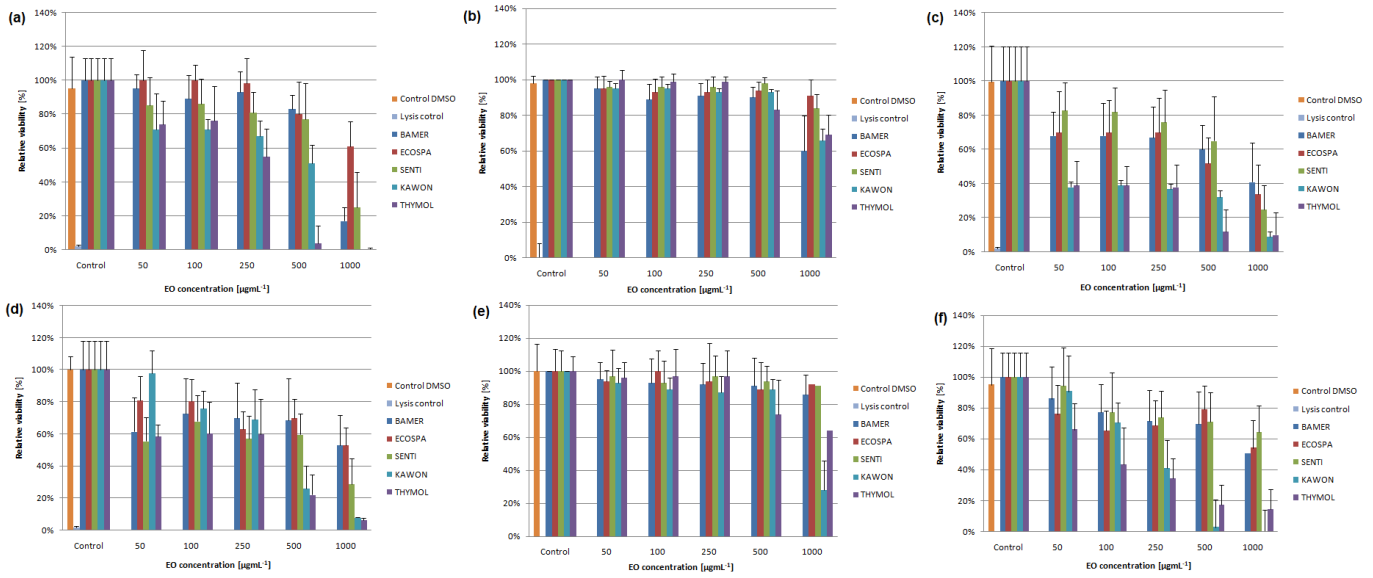


Figure 3. The L929 and MCF-7 cells viability after 48-hour incubation with thyme essential oils and thymol; L929 – CCK-8 results (a); L929 – LDH results (b); L929 – NRU results (c); MCF-7 – CCK-8 results (d); MCF-7 – LDH results (e); MCF-7 – NRU results (f).

adequate concentration caused a higher reduction of cell viability than EO. Similarly, MCF-7 cells incubated with thymol caused a higher reduction of cell viability in NRU assay than all tested EOs. In contrast, results from CCK-8 for Senti and Kawon EOS and LDH for Kawon exhibited a stronger effect of individual EOs than thymol (Figure A5–A6).

#### 3.2.3. Estimating the value of $IC_{50}$

Based on results obtained from CCK-8 and NRU (those two assays showed the greatest impact on cell viability) $IC_{50}$ values were estimated (Tables 4–5). Among all tested EOs and thymol, the thymol solution showed $IC_{50}$ value of $295.00 \pm 52.72$ $\mu g \cdot mL^{-1}$ in CCK-8 assay and $IC_{50}$ value of $65.59 \pm 10.11$ $\mu g \cdot mL^{-1}$ in NRU assay against the L929 cell line. Additionally, the freshly hydrodistilled EO (Kawon) showed cytotoxic activity against the L929 cell line ($43.68 \pm 4.09$ $\mu gmL^{-1}$) in the NRU assay (Table 4). It can be noticed that the L929 cell line indicates greater sensitivity to the thymol in the CCK-8 assay as well as a freshly hydrodistilled EO (Kawon) and thymol in the NRU assay in comparison with the results for the MCF-7 cell line (Table 5). In the case of the MCF-7 cell line, it can be found that the major cytotoxic effects were observed for thymol ($87.18 \pm 28.25$ $\mu g \cdot mL^{-1}$) and freshly hydrodistilled EO (Kawon) ($225.00 \pm 35.33$ $\mu g \cdot mL^{-1}$). Moreover, it should be noticed that the thyme EOs and thymol affected cell activity with varying strength as was presented by $IC_{50}$ values. CCK-8, NRU and LDH assays demonstrate different trends in response to tested EOs and thymol due to different mechanisms which are the basis of the mentioned assays. CCK-8 assay gives information about the reduction of WST-8 (Water Soluble Tetrazolium 8) salt due to cellular dehydrogenase activity and the presence of NADP(H) and NAD(H). On the other hand, NRU assay is based on the ability of cells to incorporate neutral red in lysosomes (Chamchoy et al., 2019, Jedrzejczak-Silicka et al., 2021). In both mentioned assays (CCK-8 and NRU) biological activity of tested freshly hydrodistilled EO and thymol strongly affected cell viability at the highest concentrations. The case of LDH assay is based on LDH release from cells due to cell membrane damage. In our study, we did not observe a reduction of cell viability in LDH assay below 66% in comparison with both (negative and positive) control cultures after exposition to all commercial EOs and thymol. That is why results obtained from the LDH assay were not included in $IC_{50}$ value estimation.

Table 4. $IC_{50}$ values ($\mu g \cdot mL^{-1}$) of essential oils and thymol for CCK-8 assay results (expressed as mean ± SD).

| EOs and thymol | Cell lines (CCK-8 assay) | |
|---|---|---|
| | L929 | MCF-7 |
| Bamer | $603.50 \pm 48.93$ | $> 1000.00$ |
| Ecospa | $> 1000.00$ | $> 1000.00$ |
| Senti | $785.60 \pm 89.45$ | $741.50 \pm 35.25$ |
| Hydrodistilled (Kawon) | $701.02 \pm 127.84$ | $378.10 \pm 18.41$ |
| Thymol | $295.00 \pm 52.72$ | $359.90 \pm 4.56$ |

Table 5. $IC_{50}$ values ($\mu g \cdot mL^{-1}$) of essential oils and thymol for NRU assay results (expressed as mean ± SD).

| EOs and thymol | Cell lines (NRU assay) | |
|---|---|---|
| | L929 | MCF-7 |
| Bamer | $667.70 \pm 32.12$ | $647.63 \pm 40.82$ |
| Ecospa | $468.70 \pm 185.27$ | $578.70 \pm 26.64$ |
| Senti | $623.70 \pm 189.17$ | $> 1000.00$ |
| Hydrodistilled (Kawon) | $43.68 \pm 4.09$ | $225.00 \pm 35.33$ |
| Thymol | $65.59 \pm 10.11$ | $87.18 \pm 28.25$ |

## 4. DISCUSSION

One of the branches of pharmaceutical studies focused its attention on essential oils that can be used omnidirectional, e.g. in aromatherapy due to beautiful aromas, but especially as a product with antibacterial, antifungal and anti-inflammatory properties (Deering et al., 2017). A prime example of widely used EOs are thyme essential oils, which are extracted from the leaves of *Thymus vulgaris* L. Lately, it was found that the EOs from *Thymus vulgaris* L. not only present antibacterial, antifungal, anti-inflammatory properties and antioxidant properties but also apoptotic activity against chosen cancer cell lines, e.g. adenocarcinoma human alveolar basal epithelial cells (A549) (Deering et al., 2017; Niksic et al., 2021). Thus, in the presented study comparison of the main thyme oil constituent between the commercial EOs (Bamer, Ecospa, Senti), freshly synthesized EO from dried thyme herb (Kawon), and thymol was evaluated to explore its cytotoxic effects on chosen cell lines.

Based on Gas Chromatography–Mass Spectrometry (GC–MS) analysis, main differences between analyzed EOs demonstrated the highest number of compounds in Kawon (54 compounds) in contrast to others (Bamer, Ecospa and Senti oils contain 47, 42 and 40 compounds, respectively). Not only differences in the number of compounds were found, but the content of compounds was different and the effect on cells also differed from each other (Table A11). The composition of particular commercial EOs and dried thyme herbs may differ in the main and trace chemical components (as was stated by European Medicines Agency (EMA) (2010) and Niksic et al. (2021) dried thyme herb contains up to 2.5% EO). The reason for mentioned differences may be found in different chemotypes of *T. vulgaris* L. As was found by Granger and Passet (1973) intraspecific chemical variability of *T. vulgaris* L. and has been reported that at least 6 chemotypes of *T. vulgaris* can be found (Thompson et al., 2003), although recent studies

provide information about 20 different chemotypes (Satyal et al., 2016). This kind of interspecific variations have been also found in the case of *Rosmarinus officinalis* (Granger et al., 1973), *Mentha spicata* (Kokkini and Vokou, 1989), and *Origanum vulgare* (Vokou et al., 1993); Thompson et al., 2003) being the effect of different geographical localizations (but also variation during the vegetative cycle, collection time, preparation process and other aspects) (Hudaib and Aburjai, 2007; Jamali et al., 2018) that affect percentage composition of EOs. In other studies mentioned variation was stated to be common in the genus *Thymus*, especially evidence of polymorphic variation in monoterpene production (Stahl-Biskup, 2002). The six chemotypes determined in *T. vulgaris* L. localized in southern France include thymol, carvacrol, terpineol, linalool, geraniol, and thuyanol-4 (all of each are monoterpenes produced from geranyl pyrophosphate) with the higher percentage of linalool (76.2%) and linalyl acetate (14.3%) (Thompson et al., 2003). In contrast, the thyme obtained from Serbia demonstrated the higher presence of geraniol (59.8%) and geranyl acetate (16.7%). In case of *T. vulgaris* L. localized in Bosnia and Herzegovina (Niksic et al. (2021) the main components are thymol (36.7%), *p*–cymene (30.0%), $\gamma$–terpinene (9.0%), carvacrol (3.6%), linalool (2.4%), $\beta$-myrcene (1.50%) and terpinen-4-ol (0.40%). In our study, four EOs were tested and the differences in composition also may be determined by the various geographical localizations where the thyme herba was obtained. From the information obtained from the manufacturer Bamer EO was distilled from *T. vulgaris* L. localized in Spain, Ecospa EO was obtained from *T. vulgaris* L. localized in India, Senti EO was prepared from plants harvested in southern Europe (more detailed information was not available). Finally, Kawon dried herb was harvested in Poland and the main chemical components were as follows: thymol, $\gamma$–terpinene, *p*–cymene, carvacrol, linalool, $\beta$–myrcene and terpinen-4-ol. In contrast Bamer, Ecospa and Senti olis, though were harvested in different regions, demonstrate the same composition of the main components as follows: thymol, *p*–cymene, $\alpha$–terpineol, linalool, $\alpha$–pinene, $\gamma$–terpineol and $\beta$-myrcene. The chemical composition analysis of EOs isolated from *T. vulgaris* L. presented in this study suggests that all four types of EOs can be classified as 'thymol' chemotype, with thymol as the predominant compound. This finding is in agreement with the definition presented by European Medicines Agency (EMA) (2010) and European Pharmacopoeia (2010). Not only herb chemotype influcences the essential oil content and its chemical composition, but also stage of development, plant age, part of plant harvested, harvesting season, environmental factors (e.g. temperature and nutrition) (Marzec et al., 2010; Rios-Estepa et al., 2008). Moreover, drying method (that may activate hydrolytic enzymes, resulting in distinct variations in the ratio of volatile compounds released) (Balladin and Headley, 1999; Venskutonis, 1997), time of hydrodistilation (Wesołowska et al., 2012) and time and condition storage also affect EOs compositon and may be a reason of diffencences between commercial and freshly distilled EOs found in this study (Farahbakhsh et al., 2021).

The effect of EOs or their constituents on different cells was also analyzed in this study. It was stated in many valuable works that EOs act by different mechanisms on human and/or animal cells and can evoke cytotoxic/irritating effects (Table 1) (Blowman et al., 2018; Niksic et al., 2021). According to the research findings, EOs demonstrate anticancer potential against e.g. breast, lung, liver, colon, prostate, and brain cancer (Gautam et al., 2014; Niksic et al., 2021). In our study, the thymol alone and the Kawon – freshly hydrodistilled EO demonstrated the highest cytotoxic effect against used cell lines but in a different manner. The difference in $IC_{50}$ values and % toxicity suggest more sensitive MCF-7 cells (cellular dehydrogenase activity determined via CCK-8 assay) in contact with Kawon – freshly hydrodistilled EO than the L929 cells. Other differences were observed by using NRU assay, and it may be stated that Kawon – freshly hydrodistilled EO and thymol exhibited irritation effect on L929 and affected cell ability to incorporate neutral red in lysosomes. The result obtained using CCK-8 assay based on MCF-7 cell culture is comparable to that of another cytotoxic study presented by Sertel and co-workers (Sertel et al., 2011). In the mentioned study, the cytotoxic effect of EO from *Thymus vulgaris* against the UMSCC1 – human oral cavity squamous carcinoma cell line was demonstrated. It was found that cytotoxic effect (decrease of cell viability to 1.3% determined using XTT assay) was obtained at the concentration of 540.00 $\mu g \cdot mL^{-1}$ of EO with the $IC_{50}$ value equal to 369.55 $\mu g \cdot mL^{-1}$. Moreover, Sertel et al. (2011) showed the effect of thyme EO on cells at the transcriptional level. It was found that thyme EO involves genes that are essential for the cell cycle, cell death and cancer (Figure 4). For example, genes that are crucial in N-glycan biosynthesis might be the target of thyme EO resulting in the growth arrest of cancer cells. Interestingly, the described action is an effect mainly due to thymol presence in EO (Islam et al., 2019; Nagoor Meeran et al., 2017; Sertel et al., 2011).

The presented study corresponds to results obtained in our study in the case of Kawon EO and thymol. It seems that *T. vulgaris* L. EO and/or thymol present proapoptotic potential as was confirmed by the activation of mitochondria–induced apoptosis in two human breast adenocarcinoma cell lines (MCF-7 and MDA-MB-231) (Islam et al., 2019; Kubatka et al., 2019; Nagoor Meeran et al., 2017).

In the comprehensive and very interesting work of Jamali et al. (2018) effect of thymol, carvacrol, *p*–cymene and $\gamma$–terpinene was analysed on three cell lines – normal L929 and two cancer MDA-MB-231 and MCF-7 cell lines. Moreover, it was found that thymol presented a growth-inhibitory effect on MDA-MB-231, MCF-7 and L929 cells with $IC_{50}$ values 31.20 $\mu g \cdot mL^{-1}$, 27.00 $\mu g \cdot mL^{-1}$ and $>$ 250.00 $\mu g \cdot mL^{-1}$ respectively after 24-hour treatment (results obtained from MTT assay), in a dose-dependent manner. It was also demonstrated that thymol evoked an inhibitory effect on the proliferation of chosen cancer cell lines ($IC_{50}$ level 56.00 $\mu g \cdot mL^{-1}$ for MDA-MB-231 and MCF-7 47.00 $\mu g mL^{-1}$) (Jamali et al., 2018). Moreover, our results obtained from the microscopic analysis were con-

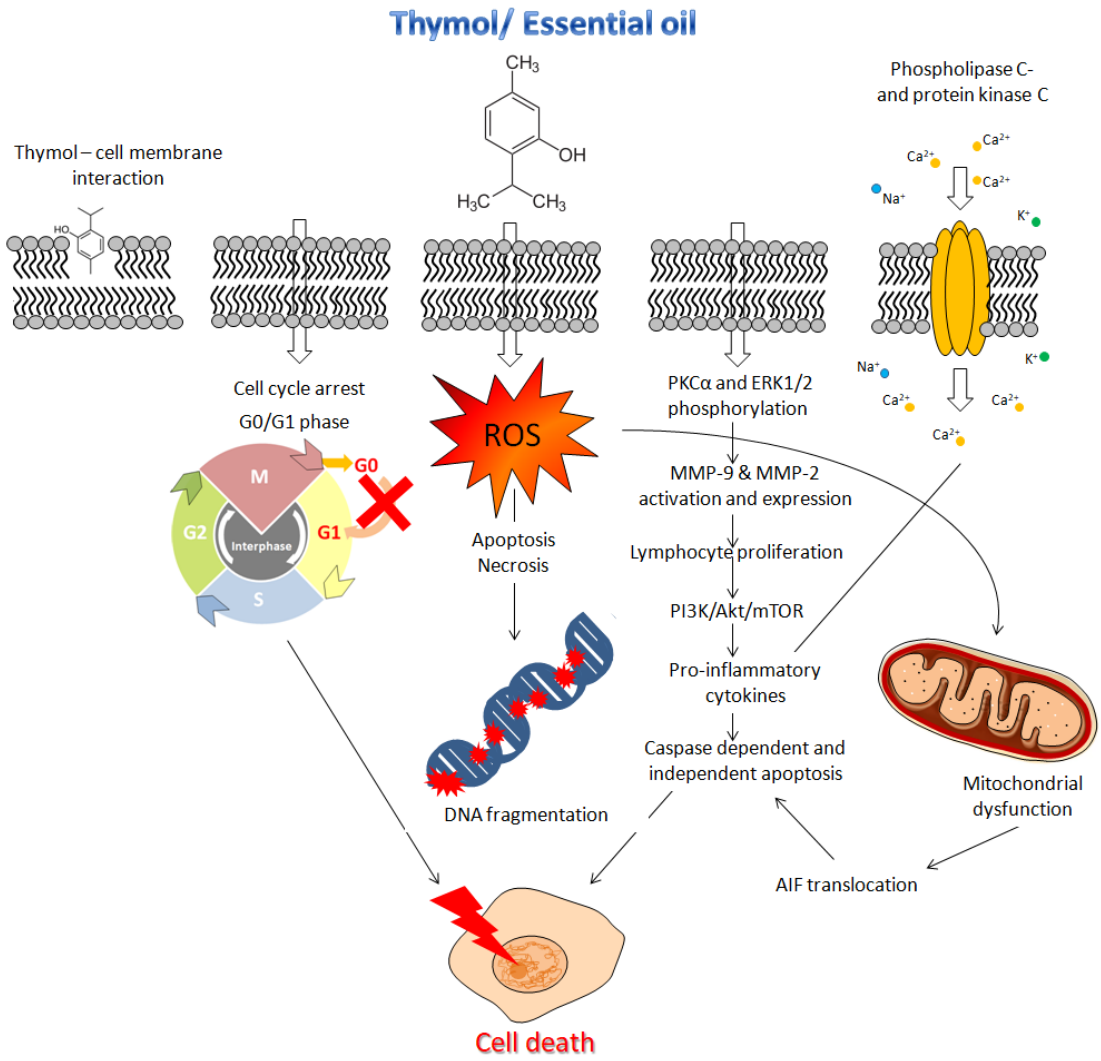


Figure 4. Possible effect and mechanism of thymol on different cancer cell models (Ferreira et al., 2016; Islam et al., 2019; Nagoor Meeran et al., 2017; Sertel et al., 2011).

firmed by Jamali et al. (2018) results where the effect of thymol activity was demonstrated by cell rounding, shrinkage, cytoplasmic vacuolation, detachment and cell floating. As was verified by Jamali et al. (2018) by acridine orange (AO)/ethidium bromide (EtBr) staining mentioned features are specific for apoptosis. Additionally, Deering et al. (2017) stated that thymol-containing and non-thymol-containing thyme (thyme linalool and thyme thujanol) evoke apoptosis in HeLa and MCF7 cancer cells (Deering et al., 2017). This statement is at odds with the results obtained by Jamali et al. (2018); Sertel et al. (2011) and ours.

In our analysis also mouse L929 cell line – the workhorse cell line was used to evaluate the effect of *T. vulgaris* L. EO or thymol. The sensitivity of the L929 cell line differs from the MCF-7 cell line and exhibited inhibition of viability in NRU assay after exposition to thymol (Table 4–5). In contrary human normal bronchial and tracheal epithelial cells treated with thyme extract induced necrotic cell death of H460 cells (lung cancer cell line), but did not affect the viability of normal bronchial and tracheal epithelial cells (Oliviero et al., 2016).

As was noticed by Sikkema et al. (1995) the action of thyme EO might be due to its lipophilic compounds (such as cyclic terpenes and their oxygenated derivatives, *a*–pinene, together with *b*–pinene, limonene, and terpinolene) that can cumulate in cell membranes and evoke leakage of metabolites and enzymes due to higher membrane permeability (Jamali et al., 2018; Sertel et al., 2011; Sikkema et al., 1995). The effect of *a*–pinene was described on *S. cerevisiae* where *a*–pinene affected the cellular integrity and inhibited respiratory activity in the mitochondria of *S. cerevisiae*. Furthermore, *b*–pinene inhibited respiration in both *S. cerevisiae* cells and mitochondria isolated from these cells. This compound was responsible for the inhibition of proton and potassium ion translocation (the passive efflux) by decreasing the transmembrane electrical potential (Uribe et al., 1984; Uribe et al., 1985). At the same time, *b*–pinene did not affect ATPase activity (Uribe et al., 1985). Moreover, analyses focused on liposome model systems confirmed that cyclic terpene hydrocarbons accumulate in

the membrane resulting in a loss of membrane integrity and dissipation of the proton motive force (Sikkema et al., 1995). Additionally, Jamali et al. (2018) studied also the cytotoxic effects of carvacrol and *p*-cymene and demonstrated that both MDA-MB231 and MCF-7 cell lines were more sensitive to carvacrol ($IC_{50}$ 53.00 $\mu g \cdot mL^{-1}$ and 46.50 $\mu g \cdot mL^{-1}$, respectively) than to *p*-cymene ($IC_{50}$ 295.20 $\mu g \cdot mL^{-1}$ and 261.00 $\mu g \cdot mL^{-1}$, respectively) (Jamali et al., 2018). The other studies highlight the effect of *p*–cymene on cell viability, for example in the case of MCF-7 cells Rahman and co-workers (Rahman et al., 2018) noticed the induction of p53 protein expression and limited ability to cell invasion. The activity of *p*–cymene was also determined by the example of mouse melanoma B16-F10 cell cultures (Rahman et al., 2018). On the other hand in the study focused on the MDA-MB-231 metastatic breast cancer cell line it was found that carvacrol was responsible for apoptosis induction due to mitochondrial membrane permeabilization (Arunasree, 2010). This finding corresponds to results obtained by Niksic et al. (2021) in the case of thymol and *p*–cymene, Jamali et al. (2018) in the case of thymol, carvacrol and *p*–cymene and ours in the case of thymol. Both components – thymol and *p*–cymene – are the most abundant in thyme EOs, and thus could be taken into account as possible candidates for therapeutic use (Kubatka et al., 2019; Sertel et al., 2011). Importantly, potential therapeutic properties and role in therapies must always be preceded by a detailed analysis of mechanisms of action on normal and cancer cells (Islam et al., 2019). Mechanisms that are underlying the activity of thyme EO may be caused by the multi-component nature of EOs, but on the other hand, it was found that single components also evoke changes in cell activity. It seems that the key is to answer the question about the mechanism of action, e.g. apoptosis, DNA fragmentation, the antioxidant effect of a single component, the mixture of them in EOs and a combination of additive and/or synergistic effects of components (Jamali et al., 2018).

## 5. CONCLUSIONS

Characterization of commercial essential oils, isolation of EO from dried thyme herb as well as cytotoxicity studies based on two chosen cell lines (MCF-7 and L929) and a comparison with thymol effect on cells may help to determine the crucial components of thyme EOs that might be irritating or potentially useful in therapies. The dose-dependent action of bioactive compounds in *T. vulgaris* L. EOs was observed in CCK-8 and NRU assays. Our study showed that the thymol alone and EO of *T. vulgaris* L. freshly distilled had the strongest cytotoxic activity against L929 and MCF-7 cell lines. Based on obtained results it can be suggested that the thymol/thyme essential oil cytotoxicity is cell dependent and the various mechanisms involved in the cellular response to thyme oil compounds. Thus, individual analysis of the main ingredients of thyme EOs may give a more accurate answer about the effect and mechanism of thyme EO.

## REFERENCES


Aleksandrzak M., Jedrzejczak-Silicka M., Sielicki K., Piotrowska K., Mijowska E., 2019. Size-dependent *in vitro* biocompatibility and uptake process of polymeric carbon nitride. *ACS Appl. Mater. Interfaces*, 11, 47739–47749. DOI: 10.1021/acsami.9b17427.

Anžlovar S., Baričevič D., Ambrožič Avguštin J., Koce J.D., 2014. Essential oil of common thyme as a natural antimicrobial food additive. *Food Technol. Biotechnol.*, 52, 263–268.

Arunasree K.M., 2010. Anti-proliferative effects of carvacrol on a human metastatic breast cancer cell line, MDA-MB 231. *Phytomedicine*, 17, 581–588. DOI: 10.1016/j.phymed.2009.12.008.

Attoub S., Sperandio O., Raza H., Arafat K., Al-Salam S., Al Sultan M.A., Al Safi M., Takahashi T., Adem A., 2012. Thymoquinone as an anticancer agent: evidence from inhibition of cancer cells viability and invasion *in vitro* and tumor growth *in vivo*. *Fundam. Clin. Pharmacol.,* 27, 98-102. DOI: 10.1111/j.1472-8206.2012.01056.x.

Babushok V.I., Linstrom P.J., Zenkevich I.G., 2011. Retention indices for frequently reported compounds of plant essential oils. *J. Phys. Chem. Ref. Data.*, 40, 043101. DOI: 10.1063/1.3653552.

Balladin D.A., Headley O., 1999. Evaluation of solar dried thyme (*Thymus vulgaris* Linné) herbs. *Renewable Energy*, 17, 523–531. DOI: 10.1016/S0960-1481(98)00757-5.

Basch E., Ulbricht C., Hammerness P., Blevins A., Sollars D., 2004. Thyme (*Thymus vulgaris* L.), thymol. *J. Herb. Pharmacother.*, 4, 49–67. DOI: 10.1080/J157v04n01_07.

Blowman K., Magalhães M., Lemons M.F.L, Cabral C., Pires I.M., 2018. Anticancer properties of essential oils and natural products. *Evidence-Based Complementary Altern. Med.*, 2018, 3149362. DOI: 10.1155/2018/3149362.

Chamchoy K., Pakotiprapha D., Pumirat P., Leartsakulpanich U., Boonyuen U., 2019. Application of WST-8 based colorimetric NAD(P)H detection for quantitative dehydrogenase assays. *BMC Biochem.*, 20, 4. DOI: 10.1186/s12858-019-0108-1.

Cicvarek Z., 1964. On the mechanism of a positive thymol turbidity reaction in waldenstroem's macroglobulinemia. *Bratisl. Lek. Listy*, 44, 385–390.

Dapkevicius A., van Beek T.A., Lelyveld G.P., van Veldhuizen A., de Groot A., Linssen J.P.H., Venskutonis R., 2002. Isolation and structure elucidation of radical scavengers from *Thymus vulgaris* leaves. *J. Nat. Prod.*, 65, 892–896. DOI: 10.1021/np010636j.

Deering K., Nethery W., Averill C., Esposito E., 2017. Effects of thyme essential oil chemotypes on breast and cervical cancer cell lineages. *Ann. Pharmacol. Pharm.*, 2, 1008.

Duke J.A., 1992. *Handbook of phytochemical constituents of GRAS herbs and other economic plants*. 1$^{st}$ edition, CRC Press Inc., Boca Raton, 593-597.

European Directorate for the Quality of Medicines & Healthcare., European Pharmacopoeia Commission., Council of Europe, 2010. *European Pharmacopoeia*. 7th edition, Strasbourg, Council of Europe, 1252–1254.

European Medicines Agency (EMA), 2010. Assessment report on *Thymus vulgaris* L. *Thymus zygis* Loefl. ex. L. aetheroleum. EMA/HMPC/52980/2017. Committee on Herbal Medicinal

Products (HMPC). Available at: https://www.fitoterapia.net/archivos/201910/draft-assessment-report-thymus-vulgaris-l-thymus-zygis-l-aetheroleum-revision-1_en.pdf.

Farahbakhsh J., Najafian S., Hosseinifarahi M., Gholipour S., 2021. Essential oil storage conditions affect the chemical composition in cultivated *Mentha spicata*. *Iran. J. Plant Physiol.*, 11, 3617–3624.

Ferreira J.V., Capello T.M, Siqueira L.J.A., Lago J.H.G., Caseli L., 2016. Mechanism of action of thymol on cell membranes investigated through lipid langmuir monolayers at the air-water interface and molecular simulation. *Langmuir,* 32, 3234–3241. DOI: 10.1021/acs.langmuir.6b00600.

Gali-Muhtasib H., Kuester D., Marwin C., Bajbouj K., Diestel A., Ocker M., Habold C., Foltzer-Jourdainne C., Schoenfeld P., Peters B., Dia*b*–Assaf M., Pommrich U., Itani W., Lippert H., Roessner A., Schneider-Stock R., 2008. Thymoquinone triggers inactivation of the stress response pathway sensor *CHEK 1* and contributes to apoptosis in colorectal cancer cells. *Cancer Res.*, 68, 5609–5618. DOI: 10.1158/0008-5472.CAN-08-0884.

Gautam N., Mantha A.K., Mittal S., 2014. Essential oils and their constituents as anticancer agents: a mechanistic view. *BioMed Res. Int.*, 2014, 154106. DOI: 10.1155/2014/154106.

Granger R., Passet J., 1973. *Thymus vulgaris* spontane de France: races chimiques et chemotaxonomie. *Phytochem.*, 12, 1683–1691. DOI: 10.1016/0031-9422(73)80388-7.

Granger R., Passet J., Arbousset G., 1973. L'essence de Rosmarinus officinalis L. II. Influence des facteurs ecologiques et individuals. *Parfum. Cosmet. Savons France*, 3, 307–312.

Habtemariam S., Lentini G., 2018. Plant-derived anticancer agents: lessons from the pharmacology of geniposide and its aglycone, genipin. *Biomedicines*, 6, 39. DOI: 10.3390/biomedicines6020039.

Harbeck N., Penault-Llorca F., Cortes J., Gnant M., Houssami N., Poortmans P., Ruddy K., Tsang J., Cardoso F., 2019. Breast cancer. *Nat. Rev. Dis. Primers.*, 5, 66. DOI: 10.1038/s41572-019-0111-2.

Hudaib M., Aburjai T., 2007. Volatile components of *Thymus vulgaris* L. from wild-growing and cultivated plants in Jordan. *Flavour Fragr. J.*, 22, 322–327. DOI: 10.1002/ffj.1800.

Islam M.T., 2019. Anticancer activity of thymol: A literature-based review and docking study with Emphasis on its anticancer mechanisms. *IUBMB Life*, 71, 9–19. DOI: 10.1002/iub.1935.

Jamali C.A., Kasrati A., Fadli M., Hassani L., Leach D., Abbad A., 2018. Synergistic effects of three moroccan thyme essential oils with antibiotic cefixime. *Phytothérapie,* 16, S149–S154. DOI: 10.3166/phyto-2019-0143.

Jedrzejczak-Silicka M., 2017. Cytotoxicity and genotoxicity of GO-$Fe_3O_4$ hybrid in cultured mammalian cells. *Pol. J. Chem. Technol.*, 19, 27–33. DOI: 10.1515/pjct-2017-0004.

Jedrzejczak-Silicka M., Kordas M., Konopacki M., Rakoczy R., 2020. The cell type-dependent response to the rotating magnetic field (RMF) – An in vitro wound healing study, In: Sosnowski T.R., Szwast M. (Eds.), *Chemical and Process Engineering for Environment and Health*. Publishing House of Łukasiewicz – Institute for Sustainable Technologies, Radom, 122–133.

Jedrzejczak-Silicka M., Kordas M., Konopacki M., Rakoczy R., 2021. Modulation of cellular response to different parameters of the rotating magnetic field (RMF) – an in vitro wound healing study. *Int. J. Mol. Sci.*, 22, 5785. DOI: 10.3390/ijms22115785.

Jedrzejczak-Silicka M., Urbas K., Mijowska E., Rakoczy R., 2017. The covalent and non-covalent conjugation of graphene oxide with hydroxycamptothecin in hyperthermia for its anticancer activity. *J. Alloys Compd.*, 709, 112–124. DOI: 10.1016/j.jallcom.2017.03.146.

Kang S.-H., Kim Y.-S., Kim E.-K., Hwang J.-W., Jeong J.-H., Dong X., Lee J.-W., Moon S.-H., Jeon B.-T., Park P-J., 2016. Anticancer effect of thymol on AGS human gastric carcinoma cell. *J. Microbiol. Biotechnol.*, 26, 28–37. DOI: 10.4014/jmb.1506.06073.

Kokkini S., Vokou D., 1989. *Mentha spicata* (Lamiaceae) chemotypes growing wild in Greece. *Econ. Bot.*, 43, 192–202. DOI: 10.1007/BF02859860.

Kubatka P., Uramova S., Kello M., Kajo K., Samec M., Jasek K., Vybohova D., Liskova A., Mojzis J., Adamkov M., Zubor P., Smejkal K., Svajdlenka E., Solar P., Samuel S.M., Zulli A., Kassayova M., Lasabova Z., Kwon, T.K., Pec M., Danko J., Büsselberg D., 2019. Anticancer activities of *Thymus vulgaris* L. in experimental breast carcinoma in vivo and in vitro. *Int. J. Mol. Sci.*, 20, 1749. DOI: 10.3390/ijms20071749.

Lesgards J.-F., Baldovini N., Vidal N., Pietri S., 2014. Anticancer activities of essential oils constituents and synergy with conventional therapies: A review. *Phytother. Res.*, 28, 1423–1446. DOI: 10.1002/ptr.5165.

Maclagan N.F., Pradhan K., Sule U., Wallsgrove R., Cooke K.B., Lee J., 1974. A modi?ed thymol turbidity test in hyperglobulinaemia. *Clin. Chim. Acta*, 53, 339–349. DOI: 10.1016/0009-8981(74)90273-3.

Manikandan P., Murugan R.S., Priyadarsini R.V., Vinothini G., Nagini S., 2010. Eugenol induces apoptosis and inhibits invasion and angiogenesis in a rat model of gastric carcinogenesis induced by MNNG. *Life Sci.*, 86, 936–941. DOI: 10.1016/j.lfs.2010.04.010.

Marzec M., Polakowski C., Chilczuk R., Kołodziej B., 2010. Evaluation of essential oil content, its chemical composition and price of thyme (*Thymus vulgaris* L.) raw material available in Poland. *Herba Polonica*, 56, 37–52.

Nagoor Meeran M.F., Javed H., Al Taee H., Azimullah S., Ojha S.K., 2017. Pharmacological properties and molecular mechanisms of thymol: prospects for its therapeutic potential and pharmaceutical development. *Front. Pharmacol.*, 8, 380. DOI: 10.3389/fphar.2017.00380.

Niksic H., Becic F., Koric E., Gusic I., Omeragic E., Muratovic S., Miladinovic B., Duric K., 2021. Cytotoxicity screening of *Thymus vulgaris* L. essential oil in brine shrimp nauplii and cancer cell lines. *Sci. Rep.*, 11, 13178. DOI: 10.1038/s41598-021-92679-x.

Oliviero M., Romilde I., Beatrice M.M., Matteo V., Giovanna N., Consuelo A., Claudio C., Giorgio S., Filippo M., Massimo N., 2016. Evaluations of thyme extract effects in human normal bronchial and tracheal epithelial cell lines and in human lung cancer cell line. *Chem. Biol. Interact.*, 256, 125–133. DOI: 10.1016/j.cbi.2016.06.024.

Polo M.P., Crespo R., de Bravo M.G., 2011. Geraniol and simavastatin show a synergistic effect on a human hepatocarcinoma cell line. *Cell Biochem. Funct.*, 29, 452–458. DOI: 10.1002/cbf.1772.

Rabi T., Bishayee A., 2009. *d*-Limonene sensitizes decetaxel-induced cytotoxity in human prostate cancer cells: generation of reactive oxygen species and induction of apoptosis. *J. Carcinog.* 8, 9. DOI: 10.4103/1477-3163.51368.

Rahman F.-U., Bhatti M.Z., Ali A., Duong H.-Q., Zhang Y., Ji X., Lin Y., Wang H., Li Z.-T., Zhang D.-W., 2018. Dimetallic Ru(II) arene complexes appended on bis-salicylaldimine induce cancer cell death and suppress invasion via p53-dependent signaling. *Eur. J. Med. Chem.*, 157, 1480–1490. DOI: 10.1016/j.ejmech.2018.08.054.

Rios-Estepa R., Turner G.W., Lee J.M., Croteau R.B., Lange B.M., 2008. A systems biology approach identifies the biochemical mechanisms regulating monoterpenoid essential oil composition in peppermint. *Proc. Natl. Acad. Sci. U.S.A.*, 105, 2818–2823. DOI: 10.1073/pnas.0712314105.

Salehi B., Mishra A.P., Shukla I., Sharifi-Rad M., del Mar Contreras M., Segura–Carretero A., Fathi H., Nasrabadi N.N., Kobarfard F., Sharifi-Rad J., 2018a. Thymol, thyme, and other plant sources: health and potential uses. *Phytother. Res.*, 32, 1688–1706. DOI: 10.1002/ptr.6109.

Salehi B., Zucca P., Sharifi-Rad M., Pezzani R., Rajabi S., Setzer W.N., Varoni E.M., Iriti M., Kobarfard F., Sharifi-Rad J., 2018b. Phytotherapeutics in cancer invasion and metastasis. *Phytother. Res.*, 32, 1425–1449. DOI: 10.1002/ptr.6087.

Satooka H., Kubo I., 2012. Effects of thymol on B16-F10 melanoma cells. *J. Agric. Food Chem.*, 60, 2746–2752. DOI: 10.1021/jf204525b.

Satyal P., Murray B.L., McFeeters R.L., Setzer W.N., 2016. Essential oil characterization of *Thymus vulgaris* from various geographical locations. *Foods*, 5, 70. DOI: 10.3390/foods5040070.

Sebaugh J.L., 2011. Guidelines for accurate EC50/IC50 estimation. *Pharm. Stat.*, 10, 128–134. DOI: 10.1002/pst.426.

Sertel S., Eichhorn T., Plinkert P.K., Efferth T., 2011. Cytotoxity of *Thymus vulgaris* essential oil towards human oral cavity squamous cell carcinoma. *Anticancer Res.*, 31, 81–87.

Shin S.-H., Park J.-H., Kim G.-C., Park B.-S., Gil Y.-S., Kim C.-H., 2007. The mechanism of apoptosis induced by eugenol in human osteosarcoma cells. *J. Kor. Assoc. Oral Maxillofacial Surg.*, 33, 20–27.

Sikkema J., de Bont J.A., Poolman B., 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Mol. Biol. Rev.*, 59, 201–222. DOI: 10.1128/mr.59.2.201-222.1995.

Slamenová D., Horváthová E., Sramková M., Marsálková L., 2007. DNA-protective effects of two components of essential plant oils carvacrol and thymol on mammalian cells cultured in vitro. *Neoplasma*, 54, 108–112.

Stahl-Biskup E., Saez F., 2002. *Thyme. The Genus Thymus*. 1$^{st}$ edition, CRC Press Inc., London, 1–20. DOI: 10.4324/9780203216859.

Thompson J.D., Chalchat J.-C., Michet A., Linhart Y.B., Ehlers B., 2003. Qualitative and quantitative variation in monoterpene co-occurrence and composition in the essential oil of *Thymus vulgaris* chemotypes. *J. Chem. Ecol.*, 29, 859–880. DOI: 10.1023/a:1022927615442.

Torras J., Grau M.D., Lopez J.F., de las Heras F.X., 2007. Analysis of essential oils from chemotypes of *Thymus vulgaris* in Catalonia. *J. Sci. Food Agric.*, 87, 2327–2333. DOI: 10.1002/jsfa.2995.

Uribe S., Alvarez R., Peña A., 1984. Effects of $\beta$-pinene, a non-substituted monoterpene, on rat liver mitochondria. *Pestic. Biochem. Physiol.*, 22, 43–50. DOI: 10.1016/0048-3575(84)90008-7.

Uribe S., Ramirez J., Peña A., 1985. Effects of beta–pinene on yeast membrane functions. *J. Bacteriol.*, 161, 1195–1200. DOI: 10.1128/jb.161.3.1195-1200.1985.

Venskutonis P.R., 1997. Effect of drying on the volatile constituents of thyme (*Thymus vulgaris* L.) and sage (*Salvia officinals* L.). *Food Chem.*, 59, 219–227. DOI: 10.1016/S0308-8146(96)00242-7.

Verrax J., Calderon P.B., 2011. Comparison between the TC10$^{TM}$automated cell counter and the lactate dehydrogenase (LDH) assay to assess cellular toxicity in vitro. *Bioradiations. A Resource for Life Science Research*. Available at: https://www.bioradiations.com/comparison-between-the-tc10-automated-cell-counter-and-the-lactate-dehydrogenase-ldh-assay-to-assess-cellular-toxicity-in-vitro/.

Vidhya N., Devaraj S.N., 2011. Induction of apoptosis by eugenol in human breast cancer cells. *Indian J. Exp. Biol.*, 49, 871–878.

Vokou D., Kokkini S., Bessiere J.-M., 1993. Geographic variation of Greek oregano (*Origanum vulgare* ssp. *hirtum*) essential oils. *Biochem. Syst. Ecol.*, 21, 287–295. DOI: 10.1016/0305-1978(93)90047-U.

Wesołowska A., Jadczak D., Grzeszczuk M., 2012. Influence of distillation time on the content and composition of essential oil isolated from wild thyme (*Thymus serpyllum* L.). *Herba Polonica*, 58, 40–50.

Yoo C.-B., Han K.-T., Cho K.-S., Ha J., Park H.-J., Nam J.-H., Kil U.-K., Lee K.-T., 2005. Eugenol isolated from the essential oil of *Eugenia caryophyllata* induces a reactive oxygen species-mediated apoptosis in HL-60 human promyelocytic leukemia cells. *Cancer Lett.*, 225, 41–52. DOI: 10.1016/j.canlet.2004.11.018.

Zu Y., Yu H., Liang L., Fu Y., Efferth T., Liu X., Wu N., 2010. Activities of ten essential oils towards *Propionibacterium acnes* and PC-3, A-549 and MCF-7 cancer cells. *Molecules*, 15, 3200–3210. DOI: 10.3390/molecules15053200.

## Appendix

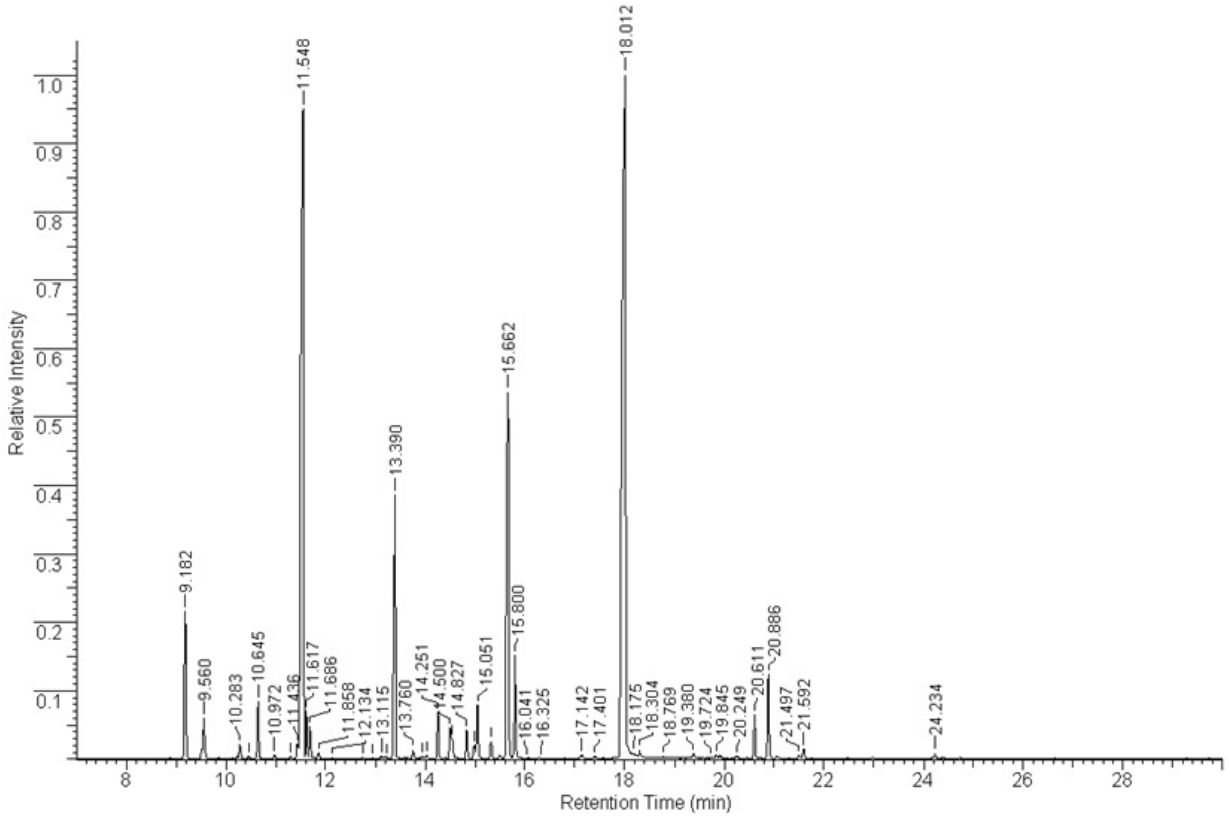


Figure A1. GC-MS chromatogram of commercial thyme essential oil produced by Bamer company.

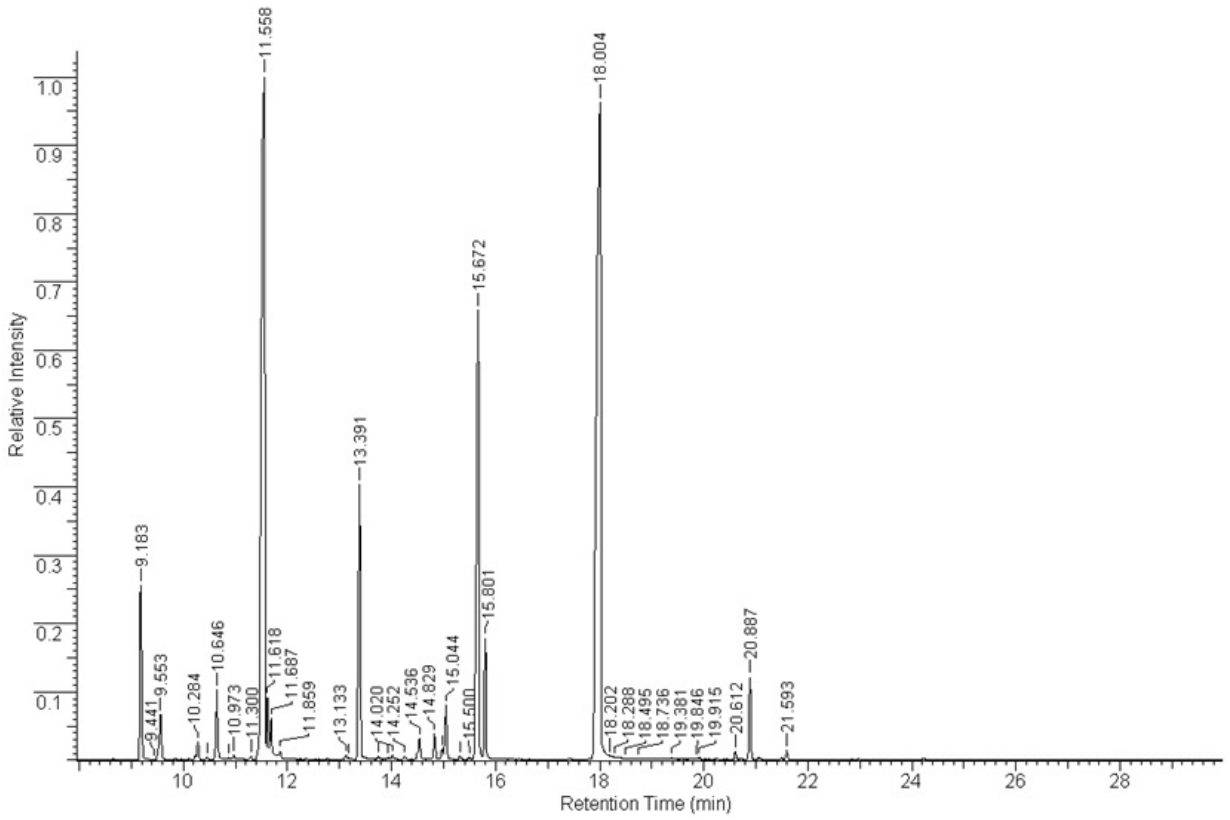


Figure A2. GC-MS chromatogram of commercial thyme essential oil produced by Ecospa company.

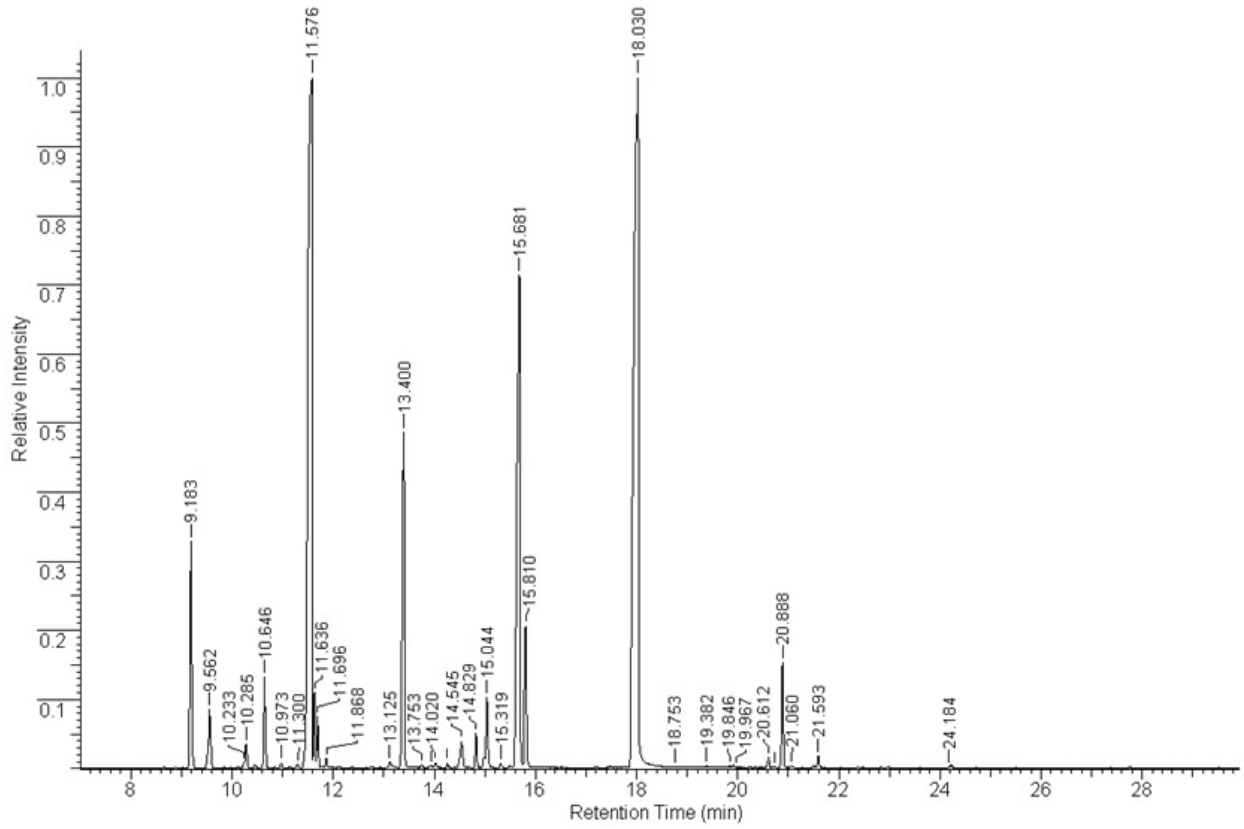


Figure A3. GC-MS chromatogram of commercial thyme essential oil produced by Senti company.

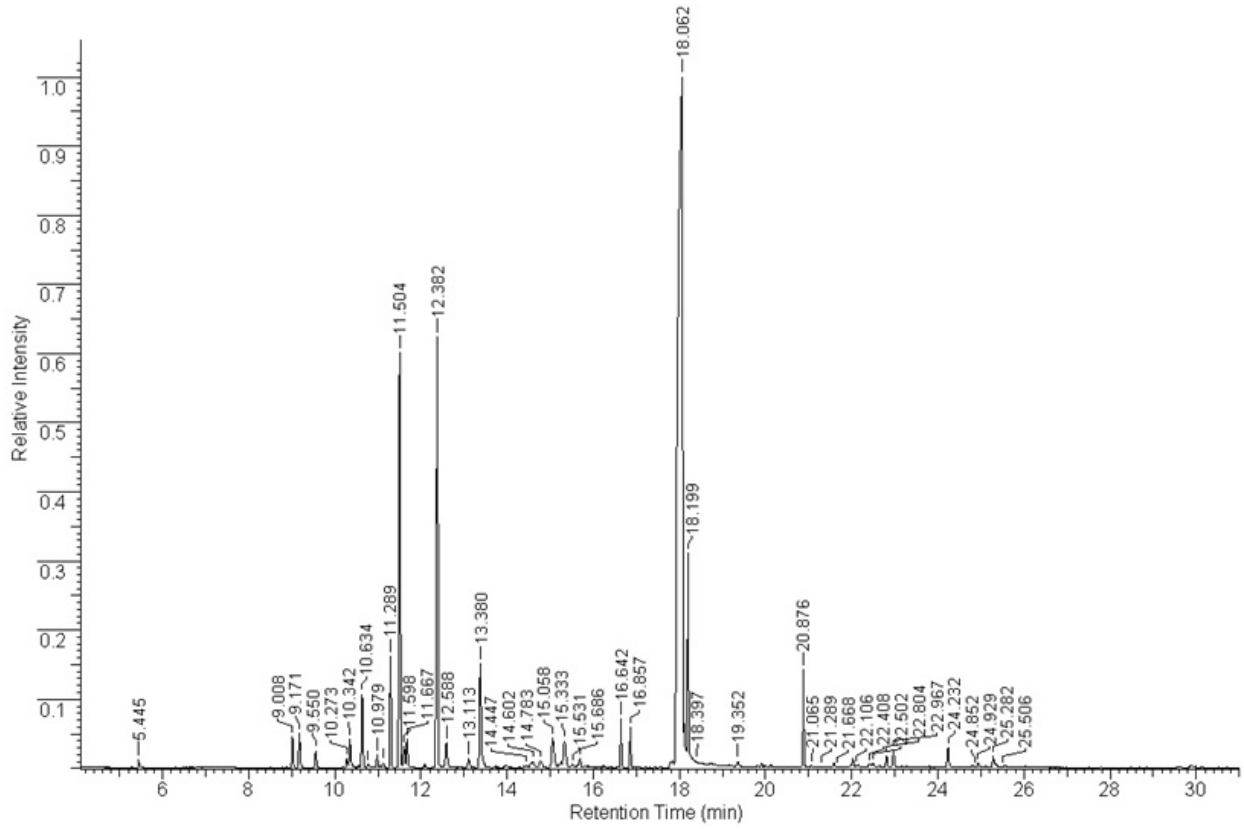


Figure A4. GC-MS chromatogram of essential oil isolated from thyme herb (*Thymus vulgaris* L.) produced by Kawon company.

Table A1. Statistically significant differences between means of L929 cells exposed to commercial BAMER thyme oil obtained from CCK-8, LDH and NR assays (small letters present differences considered as significant at a level of $p < 0.05$).

| | Concentration (μg/mL) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 50 | | | 100 | | | 250 | | | 500 | | | 1000 | | |
| | a | | | b | | | c | | | d | | | e | | | f | | |
| | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU |
| BAMER | e, f | f | f | f | f | f | f | f | f | f | f | f | a, f | f | – | a, b, c, d, e | a, b, c, d, e | a, b, c, d |

Table A2. Statistically significant differences between means of L929 cells exposed to commercial ECOSPA thyme oil obtained from CCK-8, LDH and NR assays (small letters present differences considered as significant at a level of $p < 0.05$).

| | Concentration (μg/mL) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 50 | | | 100 | | | 250 | | | 500 | | | 1000 | | |
| | g | | | h | | | i | | | j | | | k | | | l | | |
| | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU |
| ECOSPA | k, l | – | l | k, l | – | l | k, l | – | l | k, l | – | l | g, h, i, j, l | – | | g, h, i, j, k | – | g, h, i, j |

Table A3. Statistically significant differences between means of L929 cells exposed to commercial SENTI thyme oil obtained from CCK-8, LDH and NR assays (small letters present differences considered as significant at a level of $p < 0.05$).

| | Concentration (μg/mL) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 50 | | | 100 | | | 250 | | | 500 | | | 1000 | | |
| | m | | | n | | | o | | | p | | | r | | | s | | |
| | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU |
| SENTI | n, o, p, r, s | r, s | s | m, s | s | s | m, s | s | s | m, s | s | s | m, s | m, s | s | m, n, o, p, r | m, n, o, p, | m, n, o, p, r |

Table A4. Statistically significant differences between means of L929 cells exposed to hydrodistilled thyme oil (KAWON) obtained from CCK-8, LDH and NR assays (small letters present differences considered as significant at a level of $p < 0.05$).

| | Concentration (μg/mL) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 50 | | | 100 | | | 250 | | | 500 | | | 1000 | | |
| | t | | | u | | | w | | | x | | | y | | | z | | |
| | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU |
| KAWON | u, w, x, y, z | z | u, w, x, y, z | t, y, z | z | t, z | t, y, z | z | t, z | t, y, z | z | t, z | t, u, w, x, z | z | t, z | t, u, w, x, y | t, u, w, x, y | t, u, w, x, y |

Table A5. Statistically significant differences between means of L929 cells exposed to thymol obtained from CCK-8, LDH and NR assays (small letters present differences considered as significant at a level of $p < 0.05$).

| | Concentration (μg/mL) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 50 | | | 100 | | | 250 | | | 500 | | | 1000 | | |
| | a1 | | | b1 | | | c1 | | | d1 | | | e1 | | | f1 | | |
| | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU |
| THYMOL | b1, c1, d1, e1, f1 | b1, e1, f1 | b1, c1, d1, e1, f1 | a1, d1, e1, f1 | a1, e1, f1 | a1, e1, f1 | a1, d1, e1, f1 | e1, f1 | a1, e1, f1 | a1, b1, c1, e1, f1 | e1, f1 | a1, e1, f1 | a1, b1, c1, d1, f1 | a1, b1, c1, d1, f1 | a1, b1, c1, d1 | a1, b1, c1, d1 | a1, b1, c1, d1, e1 | a1, b1, c1, d1, e1 |

Table A6. Statistically significant differences between means of MCF-7 cells exposed to commercial BAMER thyme oils obtained from CCK-8, LDH and NR assays (small letters present differences considered as significant at a level of $p < 0.05$).

| | Concentration (μg/mL) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 50 | | | 100 | | | 250 | | | 500 | | | 1000 | | |
| | a | | | b | | | c | | | d | | | e | | | f | | |
| | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU |
| BAMER | b, d, e, f | b, c, d, e, f | d | a | a | – | – | a | – | a | a | – | a | a | – | a | a | a |

Table A7. Statistically significant differences between means of MCF-7 cells exposed to commercial ECOSPA thyme oil obtained from CCK-8, LDH and NR assays (small letters present differences considered as significant at a level of $p < 0.05$).

| | Concentration (μg/mL) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 50 | | | 100 | | | 250 | | | 500 | | | 1000 | | |
| | g | | | h | | | i | | | j | | | k | | | l | | |
| | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU |
| ECOSPA | j, l | – | - | – | – | – | – | k | k | g | – | – | – | i | i | g | – | – |

Table A8. Statistically significant differences between means of MCF-7 cells exposed to commercial SENTI thyme oil obtained from CCK-8, LDH and NR assays (small letters present differences considered as significant at a level of $p < 0.05$).

| | Concentration (μg/mL) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 50 | | | 100 | | | 250 | | | 500 | | | 1000 | | |
| | m | | | n | | | o | | | p | | | r | | | s | | |
| | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU |
| SENTI | n, o, p, r, s | – | – | m, s | – | – | m, s | – | – | m, s | – | – | m, s | – | – | m, n, o, p, r | – | – |

Table A9. Statistically significant differences between means of MCF-7 cells exposed to hydrodistilled thyme oil (KAWON) obtained from CCK-8, LDH and NR assays (small letters present differences considered as significant at a level of $p < 0.05$).

| | Concentration (μg/mL) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 50 | | | 100 | | | 250 | | | 500 | | | 1000 | | |
| | t | | | u | | | w | | | x | | | y | | | z | | |
| | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU |
| KAWON | x, y, z | z | x, y, z | x, y, z | z | x, y, z | y, z | z | x, y, z | t, u, y, z | z | t, u, w, y, z | t, u, w, x | z | t, u, w, x | t, u, w, x | t, u, w, x, y | t, u, w, x |

Table A10. Statistically significant differences between means of MCF-7 cells exposed to thymol obtained from CCK-8, LDH and NR assays (small letters present differences considered as significant at a level of $p < 0.05$).

| | Concentration (μg/mL) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 50 | | | 100 | | | 250 | | | 500 | | | 1000 | | |
| | a1 | | | b1 | | | c1 | | | d1 | | | e1 | | | f1 | | |
| | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU | CCK-8 | LDH | NRU |
| THYMOL | b1, c1, d1, e1, f1 | e1, f1 | c1, d1, e1, f1 | a1, e1, f1 | e1, f1 | e1, f1 | a1, e1, f1 | e1, f1 | 1a | a1, e1, f1 | e1, f1 | a1 | a1, b1, c1, d1 | a1, b1, c1, d1, f1 | a1, b1 | a1, b1, c1, d1 | a1, b1, c1, d1, e1 | a1, b1 |

Table A11. Main point of difference between tested EOs and thymol.

| Feature of EO | Bamer | Ecospa | Senti | Kawon |
|---|---|---|---|---|
| Total number of compounds | 47 | 42 | 40 | 54 |
| Main differences in compounds concentration | The highest concentration of (Z)-*p*–2,8-Menthadien-1-ol; camphor | The highest concentration of D–limonene; Lack of γ–terpinene | Lack of γ–terpinene | The highest concentration of thymol; α–terpinene; γ–terpinene; terpinen-4-ol; carvacrol; The lowest concentration of α–pinene; camphene; *p*–cymene; linalool; α–terpineol; γ–terpineol Lack of (Z)-*p*–2,8-Menthadien-1-ol |
| Cell morphology of L929 cells | Typical morphology even at the highest concentrations (500 μg·mL$^{-1}$ and 1000 μg·mL$^{-1}$) | | | Changes in morphology – as shrinkage/cell spherical shapes at concentrations of 500 μg·mL$^{-1}$; Cells death at concentration 1000 μg·mL$^{-1}$ |

Table A11 *continued on the next page*

Table A11 *continued from previous page*

| Feature of EO | Bamer | Ecospa | Senti | Kawon |
|---|---|---|---|---|
| Total number of compounds | 47 | 42 | 40 | 54 |
| Cell morphology of MCF-7 cells | Typical morphology even at concentration 500 $\mu g \cdot mL^{-1}$; changes in morphology – spherical shapes of cells at concentration of 1000 $\mu g \cdot mL^{-1}$ | | Typical morphology even at concentration 500 $\mu g \cdot mL^{-1}$; major part of cell population display untypical morphology at concentration of 1000 $\mu g \cdot mL^{-1}$ | Cells demonstrate shrinkage, spherical shapes at concentration of 500 $\mu g \cdot mL^{-1}$; Cells death at concentration of 1000 $\mu g \cdot mL^{-1}$ |
| L929 cell viability (CCK-8 assay) | The relative cell viability reduced to 17% at concentration of 1000 $\mu g \cdot mL^{-1}$ | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 61% | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 25% | Full reduction of cell viability of 1000 $\mu g \cdot mL^{-1}$ |
| MCF-7 cell viability (CCK-8 assay) | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 53% | The relative cell viability reduced to 53% at concentration of 1000 $\mu g \cdot mL^{-1}$ | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 29% | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 6% |
| Membrane integrity of L929 cells (LDH assay) | The relative cell viability reduced to 60% at concentration of 1000 $\mu g \cdot mL^{-1}$ | The relative cell viability reduced to 91% at concentration of 1000 $\mu g \cdot mL^{-1}$ | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 84% | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 66% |
| Membrane integrity of MCF-7 cells (LDH assay) | The relative cell viability reduced to 86% at concentration of 1000 $\mu g \cdot mL^{-1}$ | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 92% | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 91% | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 64% |
| Cell viability of L929 cells (NRU assay) | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 41% | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 34% | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 25% | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 10% |
| Cell viability of MCF-7 cells (NRU assay) | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 51% | The relative cell viability reduced to 54% at concentration of 1000 $\mu g \cdot mL^{-1}$ | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 64% | The reduction of cell viability at concentration of 1000 $\mu g \cdot mL^{-1}$ to 14% |

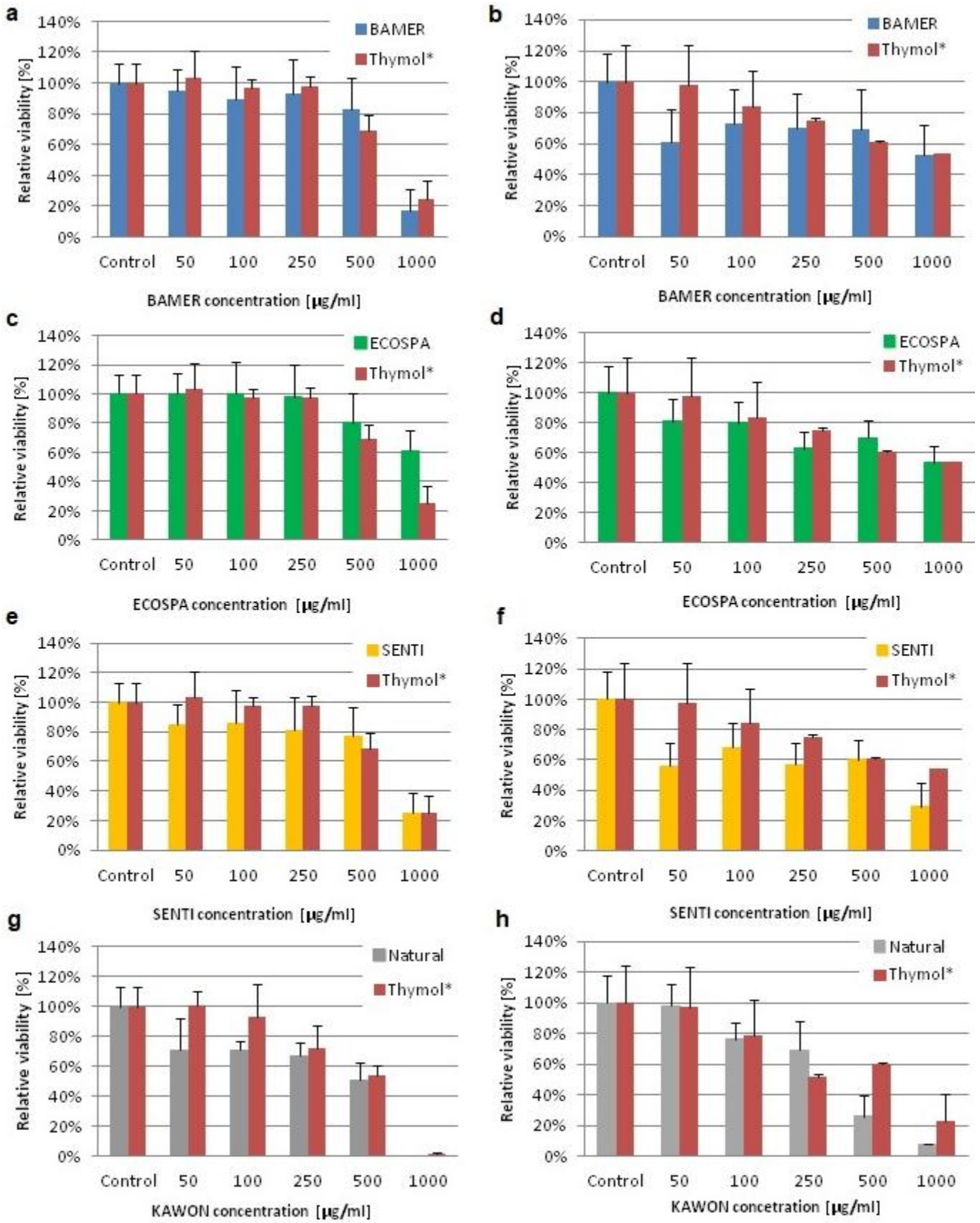


Figure A5. The effect of essential oils versus thymol (*at the concentration corresponding to the content in individual oils) on cell lines - CCK-8 assay results; L929 cell viability incubated with BAMER EO (a); MCF-7 cell viability incubated with BAMER EO (b); L929 cell viability incubated with ECOSPA EO (c); MCF-7 cell viability incubated with ECOSPA EO (d); L929 cell viability incubated with SENTI EO (e); MCF-7 cell viability incubated with SENTI EO (f); L929 cell viability incubated with KAWON EO (g); MCF-7 cell viability incubated with KAWON EO (h).

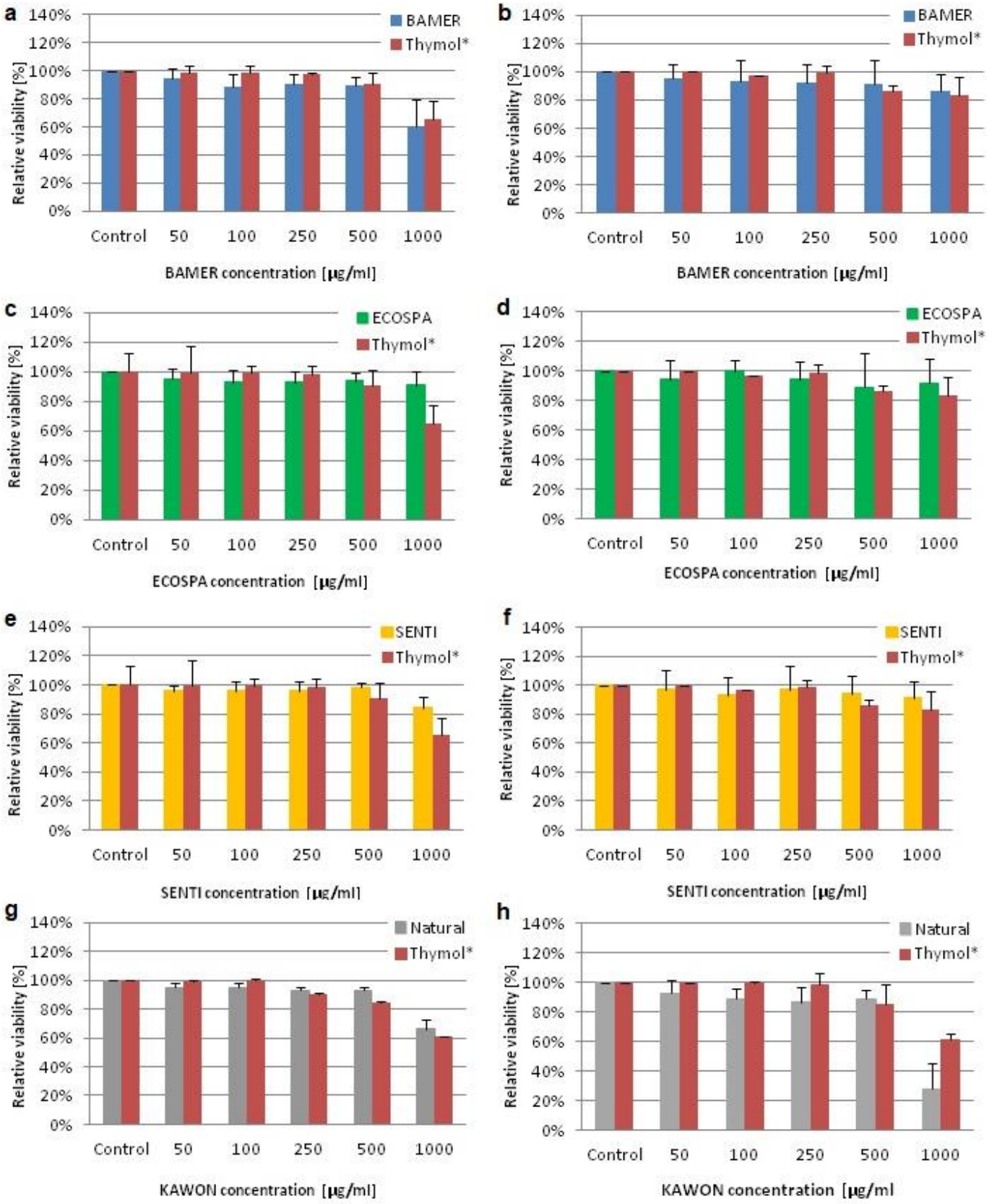


Figure A6. The effect of essential oils versus thymol (*at the concentration corresponding to the content in individual oils) on cell lines – LDH assay results; L929 cells incubated with BAMER EO (a); MCF-7 cells incubated with BAMER EO (b); L929 cells incubated with ECOSPA EO (c); MCF-7 cells incubated with ECOSPA EO (d); L929 cells incubated with SENTI EO (e); MCF-7 cells incubated with SENTI EO (f); L929 cells incubated with KAWON EO (g); MCF-7 cells incubated with KAWON EO (h).

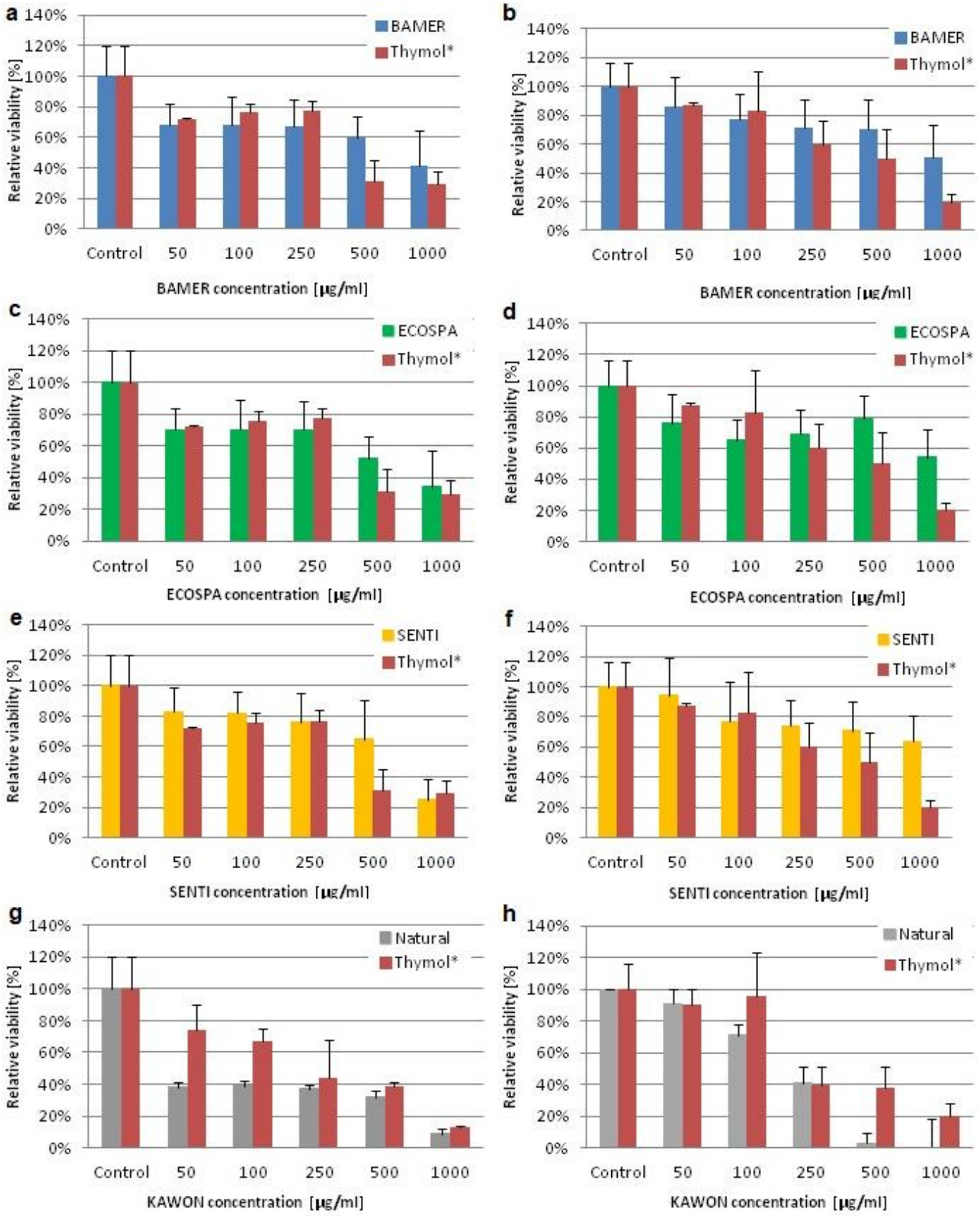


Figure A7. The effect of essential oils versus thymol (*at the concentration corresponding to the content in individual oils) on cell lines – NRU assay results; L929 cell viability incubated with BAMER EO (a); MCF-7 cell viability incubated with BAMER EO (b); L929 cell viability incubated with ECOSPA EO (c); MCF-7 cell viability incubated with ECOSPA EO (d); L929 cell viability incubated with SENTI EO (e); MCF-7 cell viability incubated with SENTI EO (f); L929 cell viability incubated with KAWON EO (g); MCF-7 cell viability incubated with KAWON EO (h).