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Evaluation of cytotoxic, membrane, and DNA damaging effects of *Thymus revolutus* Célak essential oil on different cancer cells

Aysun ÖZKAN*, Ayşe ERDOĞAN
Department of Biology, Faculty of Science, Akdeniz University, Antalya, Turkey

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Background/aim: In this study, we evaluated *Thymus revolutus* Célak essential oil and its two main constituents, γ-terpinene and p-cymene, as potential oxidative agents against lung cancer and epidermoid carcinoma cells.

Materials and methods: Cell viability assessment was made by CellTiter-Blue® cell viability and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after treatment with 5–600 μg/mL concentrations of essential oil, γ-terpinene, and p-cymene. Malondialdehyde and 8-hydroxy-2′-deoxyguanosine levels in parental H1299, epirubicin-resistant (drug-resistant) H1299, A549, and A431 cells were also assayed after essential oil, γ-terpinene, and p-cymene had been administered for 24 h. Glutathione S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GRx), and glucose 6-phosphate dehydrogenase (G6PD) activities were determined.

Results: Parental H1299 cells were found to be more sensitive to the cytotoxic effects of all compounds. While A431 cells had the highest membrane damage, which was caused by essential oil (IC_{50} and IC_{70}), A549 cells had the highest DNA damage at IC_{50} and IC_{70} p-cymene concentrations. G6PD, GST, GRx, and GPx enzyme activities of cells, which increased against these compounds, depended on concentrations, incubation times, and antioxidant capacities of the cells.

Conclusion: This study suggests that different cancer cells showed different cellular responses against potential antitumor and prooxidative effects of the essential oil and its two main constituents.

Key words: *Thymus revolutus* Célak, essential oil, anticancer

1. Introduction

Thymus species are well known as medicinal plants because of their biological and pharmacological properties. Members of this genus are called "kekik" in Turkish and are used as herbal teas and condiments. *Thymus revolutus* Célak is an endemic species in Turkey. *Thymus* oils and their main constituents are widely used in pharmaceutical, cosmetics, and perfume industries and also for flavoring and preservation of several food products (1). Essential oils have many constituents. Some constituents in essential oils are seen in trace amounts. As typical lipophiles, they pass through the cell wall and cytoplasmic membrane; disrupt the structures of their different layers of polysaccharides, fatty acids, and phospholipids; and permeabilize them. Because of the great number of constituents, essential oils seem to have no specific cellular targets (2,3). Phenolic constituents in plant essential oils showed antioxidant activity in many studies. This activity is due to their ability to scavenge free radicals (4–7). In addition, complex factors such as the nature of the antioxidants, the condition of oxidation, and the properties of the substrate are related to the activity of antioxidants. Phenolic antioxidants lose their antioxidant effects at higher concentrations and gain a prooxidant structure. They can cause DNA and membrane damage as prooxidants at higher concentrations. Concentrations of these molecules and types of cells and organisms affect the anti-/prooxidant and toxic properties of those molecules (8). The intracellular redox potential, which is determined by the level of oxidants and reductants, has been shown to play an important role in the regulation of cell growth (9). The principal intracellular reductant is nicotinamide adenine dinucleotide phosphate (NADPH), which is mainly produced by the pentose phosphate pathway through the actions of glucose-6-phosphate dehydrogenase (G6PD) (10). Another important reductant is glutathione (GSH). Oxidative stress generated by oxidants leads to cell death. Antioxidant enzymes such as glutathione reductase

* Correspondence: aozkan@akdeniz.edu.tr

(GRx) and glutathione peroxidase (GPx) can protect the cells from the effects of oxidative stress. When H1299 cancer cells were preincubated with superoxide dismutase (SOD) and catalase (CAT) before oxidant exposure, the GPx activity and GSH amount were found different than in the control cells (11).

Tumors are heterogeneous in many respects, including chemotherapeutic susceptibility and expression of epidermal growth factor (EGF) receptors (12,13). Acquired multidrug resistance is the main obstacle for the cure for lung cancer. A group of drug resistance cells can occur in tumors during chemotherapy. Overexpression of epidermal growth factor receptor (EGFR) is also associated with resistance to hormonotherapy, cytotoxic drugs, or radiotherapy (14–16). In this respect, it has been postulated that EGFR overexpression and multidrug resistance would serve as cell survival responses to counteract apoptotic signaling in cancer cells exposed to a cytotoxic damage.

We observed *Thymus revolutus* Célak essential oil components with GC and GC/MS analysis and found that cymene (32.57%) and γ-terpinene (17.18%) were the major components of the oil in our previous study (6). We therefore used cymene and γ-terpinene as the major components of the oil in the present study.

The purpose of this study was to compare the cellular responses of different types of lung cancer cells, H1299 (parental nonsmall-cell lung cancer cells), drug-resistant H1299 (epirubicin-resistant nonsmall-cell lung cancer cells), A549 (EGFR-positive alveolar epithelial cells derived from human lung carcinoma), and A431 (EGFR-positive human epidermoid carcinoma), against DNA and membrane injury induced by *Thymus revolutus* Célak essential oil and its two main constituents (p-cymene and γ-terpinene) after the cells were treated with these solutions.

2. Materials and methods

2.1. Collection of plant material

Thymus revolutus Célak was collected from the south of the Agricultural Faculty of Akdeniz University, Antalya (50–60 m), Turkey, in June 2014. The taxonomic identification of plant materials was confirmed by a plant taxonomist, Professor Dr Hüseyin Sümbül from the Department of Biology, Akdeniz University, Antalya, Turkey (Voucher No: TR 1011).

2.2. Isolation of the essential oil

The dried aerial parts of the collected plants (100 g) were submitted to water distillation for 3 h using a Clevenger-type apparatus (İldam Ltd., Ankara, Turkey) at the Molecular Biology Department of Akdeniz University. The obtained essential oil was dried over anhydrous sodium sulfate and, after filtration, stored at 4 °C until tested and analyzed. Cymene (purity: 99%) was purchased from Aldrich and γ-terpinene (purity: 99%) was purchased from Sigma.

2.3. Cell culture

H1299 (human nonsmall-cell lung cancer cells), A549 (alveolar epithelial cell line derived from human lung carcinoma), and A431 (human epidermoid carcinoma cells) cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). These cell lines were grown and maintained in a humidified incubator at 37 °C and in 5% CO_2 atmosphere. Cells were grown in Roswell Park Memorial Institute 1640 medium (RPMI 1640) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution in a humidified atmosphere containing 5% CO_2 at 37°C. For subculturing, cells were harvested after trypsin/ethylenediaminetetraacetic acid (EDTA) treatment at 37 °C. Cells were used when monolayer confluence reached 75%. Epirubicin-resistant (drug-resistant) H1299 tumor cells were derived from the parental line by stepwise selection in increasing concentrations of epirubicin until the cells were capable of propagating in 220 ng/mL drug, as described previously (17,18).

2.4. Cell viability assays

The cancer cells (10,000 cells/well, monolayer) were plated in a 96-well plate. The next day the cells were treated with different concentrations of essential oil (5–600 µg/mL) and its main components (γ-terpinene and p-cymene) (5–600 µg/mL) for 24, 48, and 72 h. At the end of the incubation period, the cytotoxicity of this solution on cancer cells was determined by the CellTiter-Blue cell viability assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The CellTiter-Blue cell viability assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Nonviable cells rapidly lose metabolic capacity and thus do not generate a fluorescent signal (19). Following cellular reduction, fluorescence was recorded at 560 nm (excitation) and 590 nm (emission) spectrofluorometrically (PerkinElmer LS 55). In the MTT assay, tetrazolium salts such as MTT are metabolized by mitochondrial dehydrogenases to form a blue formazan dye, useful for the measurement of cytotoxicity. Test reagents were added to the culture medium. Briefly, 15% volumes of dye solutions were added to each well after the appropriate incubation time. After 2 h of incubation at 37 °C, an equal volume of solubilization/stop solutions (dimethyl sulfoxide) was added to each well for an additional 1 h of incubation. The absorbance of the reaction solution at 490 nm was recorded (20). Every experiment also included one set of positive controls (paclitaxel). The data were expressed as average values obtained from eight wells for each concentration. IC_{10}, IC_{50}, and IC_{70} concentrations were calculated. For the calculation of these values, Microsoft Excel software

was used. The reading taken from the wells with the cells cultured with the control medium was used as a 100% viability value.

The cells were plated at a density of (5–10) × 10^5 cells/dish (100 mm) and incubated with different concentrations (IC_{50} and IC_{70}) of essential oil, γ-terpinene, and p-cymene for 24 h. Cells were scraped off the culture plates with culture medium and were centrifuged at 400 × *g* for 10 min. The cell pellets were washed with phosphate-buffered saline (PBS) and then sonicated (3 × 15 s) in 50 mM potassium phosphate, pH 7.2, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) and 1 μg/mL leupeptin (Sigma), and centrifuged at 150,000 × *g* for 45 min. The supernatant was used for the determination of biochemical parameters.

2.5. Determination of malondialdehyde level

Malondialdehyde (MDA) levels in the cells were assayed as described by Wasowicz et al. (21). This fluorometric method for measuring thiobarbituric acid-reactive substances (TBARS) in supernatant is based on the reaction between MDA and thiobarbituric acid. The product of this reaction was extracted into butanol and measured by fluorescence spectrometer (525 nm excitation, 547 nm emission).

The concentration of proteins was determined by the Bradford method (22) with bovine serum albumin as a standard.

2.6. Determination of 8-hydroxy-2′-deoxyguanosine level

The cells were plated at a density of (5–10) × 10^5 cells/dish (100 mm) and incubated with different concentrations (IC_{50} and IC_{70}) of essential oil, γ-terpinene, and p-cymene for 24 h. After DNA purification (Genomic DNA Mini Kit, Invitrogen) (23) from the cultured cells, the genomic DNA samples were used to determine the amount of 8-hydroxy-2′-deoxyguanosine (8-OHdG) with a competitive ELISA kit (8-OHdG Check New (High Sensitivity), Japan Institute for Control of Aging, Fukuroi, Japan). Microtiter ELISA plates were precoated with 8-OHdG. Fifty microliters of the sample and the primary antibody were added to each well and they were incubated at 4 °C overnight. The wells were washed three times, and then 100 μL of secondary antibodies was added to each well and wells were incubated for 1 h at room temperature. The wells were again washed three times. After that, enzyme substrate solutions were added and the wells were incubated at room temperature for 15 min. The reaction was stopped by adding the terminating solution. The absorbance was read at a wavelength of 450 nm (24).

2.7. Determination of enzymes activity

Glutathione S-transferase (GST) was determined according to Habig and Jakoby (25) using 1-chloro-2,4-dinitrophenol as substrate. One unit of enzyme activity results in the binding of one mmol GSH/min.

GPx was determined according to Flohe and Gunzler (26) with t-butyl hydroperoxide as a substrate. The assay was based on determination of NADPH at 340 nm. One unit of enzyme activity results in the oxidation of 1 mmol GSH/min.

GRx was assayed spectrophotometrically by following NADPH oxidation at 340 nm (27). The reaction mixture contained 100 mM potassium phosphate (pH 7.4), 0.1 mM NADPH (Sigma), oxidized glutathione (GSSG) (Sigma), and 1 mM EDTA. One unit of enzyme activity represents the reduction of 1 μmol GSSG/min.

G6PD activities were determined by following the reduction of NADP at 340 nm (28).

The concentration of proteins was determined by the Bradford method (22) with bovine serum albumin as a standard.

2.8. Data analysis

The results of the replicates were pooled and expressed as mean ± standard error. Analysis of variance (ANOVA) was carried out. ANOVA was used to determine whether there were any significant differences between the means of three or more independent (unrelated) groups on some variable. Tukey multiple comparisons tests were used. Statistical differences were considered significant at $P < 0.05$ (29). Statistical analyses were performed using the Minitab program (http://www.minitab.com/products/minitab/), release 13.0. Data marked with different letters in tables indicate significant differences between the control and treatments (essential oil, γ-terpinene, and p-cymene) ($P < 0.05$).

3. Results

The antiproliferative activities of *T. revolutus* Célak essential oil and its two main constituents, γ-terpinene and p-cymene, were investigated on the parental H1299 (P-H1299), drug-resistant H1299 (R-H1299), A549, and A431 cell lines using MTT and the CellTiter-Blue cell viability assay. Results were expressed as 70% inhibitory concentration (IC_{70}) and 50% inhibitory concentration (IC_{50}) (Table 1). The CellTiter-Blue cell viability assay was found to be more sensitive than the MTT assay, so we studied other parameters according to CellTiter-Blue assay results. The viability of the cells decreased when the cells were exposed to the essential oil and its constituents at increasing concentrations and incubation times (Figure 1–3). Among the essential oil from *T. revolutus*, γ-terpinene, and p-cymene, the essential oil exhibited the best antiproliferative activity with the lowest IC_{50} values of 108 μg/mL, 62 μg/mL, and 50 μg/mL on parental H1299 cells for 24, 48, and 72 h, respectively. The tested cell sensitivities to essential oil, γ-terpinene, and p-cymene were found to follow an order of P-H1299 > R-H1299 > A431 > A549, P-H1299 > A549 > R-H1299 > A431, and

Table 1. Summary of the cytotoxic effects of *T. revolutus* essential oil, γ-terpinene, and p-cymene on parental H1299, drug-resistant H1299, A549, and A431 cells.

Cells treatments	Es. oil (μg/mL) (MTT) X ± S.E.	Es. oil (μg/mL) (Cell. titer.) X ± S.E.	γ-Terpinene (μg/mL) (MTT) X ± S.E.	γ-Terpinene (μg/mL) (Cell. titer) X ± S.E.	p-Cymene (μg/mL) (MTT) X ± S.E.	p-Cymene (μg/mL) (Cell titer.) X ± S.E.
P, 24 h, IC_{50}	138 ± 3.11	108 ± 2.99	139 ± 2.11	138 ± 2.11	170 ± 2.22	159 ± 2.11
P, 24 h, IC_{70}	181 ± 2.71	151 ± 2.45	222 ± 3.71	221 ± 3.43	242 ± 2.98	231 ± 3.71
P, 48 h, IC_{50}	119 ± 3.81	62 ± 1.71	92 ± 1.72	90 ± 1.22	100 ± 1.78	99 ± 1.87
P, 48 h, IC_{70}	151 ± 3.71	94 ± 1.66	172 ± 2.73	160 ± 2.43	170 ± 2.01	169 ± 2.02
P, 72 h, IC_{50}	92 ± 171	50 ± 1.09	72 ± 1.61	71 ± 1.32	70 ± 1.04	56 ± 1.00
P, 72 h, IC_{70}	121 ± 1.33	79 ± 1.02	159 ± 2.66	158 ± 2.03	125 ± 2.71	111 ± 2.41
R, 24 h, IC_{50}	144 ± 2.82	128 ± 2.45	164 ±1.99	154 ± 2.11	195 ± 2.88	171 ± 2.70
R, 24 h, IC_{70}	191 ± 3.50	175 ± 2.33	245 ± 2.99	235 ± 3.67	272 ± 3.44	248 ± 3.79
R, 48 h, IC_{50}	135 ± 2.71	96 ± 1.22	133 ± 2.01	126 ± 2.06	131 ± 2.34	127 ± 1.89
R, 48 h, IC_{70}	187 ± 2.85	148 ± 2.11	218 ± 3.45	211 ± 3.44	211 ± 3.05	207 ± 2.56
R, 72 h, IC_{50}	109 ± 1.99	88 ± 1.23	92 ± 2.61	90 ± 1.76	96 ± 1.23	73 ± 0.98
R, 72 h, IC_{70}	167 ± 2.21	146 ± 3.00	175 ± 2.66	173 ± 3.03	145 ± 2.14	122 ± 1.01
A5, 24 h, IC_{50}	195 ± 3.66	193 ± 2.38	176 ± 1.99	145 ± 3.00	182 ± 2.01	181 ± 1.56
A5, 24 h, IC_{70}	274 ± 4.11	272 ± 4.90	260 ± 2.34	229 ± 3.71	262 ± 2.88	261 ± 3.65
A5, 48 h, IC_{50}	160 ± 2.88	150 ± 2.98	128 ± 2.86	88 ± 1.63	167 ± 2.03	166 ± 2.45
A5, 48 h, IC_{70}	249 ± 5.22	239 ± 2.35	184 ± 3.03	175 ± 2.65	275 ± 2.66	274 ± 3.71
A5, 72 h, IC_{50}	140 ± 2.88	134 ± 2.66	86 ± 1.65	79 ± 1.43	86 ± 1.21	75 ± 0.71
A5, 72 h, IC_{70}	218 ± 4.05	212 ± 3.11	178 ± 2.77	171 ± 3.24	143 ± 2.33	132 ± 1.81
A4, 24 h, IC_{50}	150 ± 2.66	146 ± 2.34	189 ± 2.34	185 ± 2.77	199 ± 3.00	180 ± 3.71
A4, 24 h, IC_{70}	202 ± 4.00	198 ± 2.76	266 ± 3.66	262 ± 2.32	279 ± 3.66	260 ± 3.72
A4, 48 h, IC_{50}	125 ± 2.33	123 ± 1.71	147 ± 2.35	146 ± 1.88	123 ± 2.71	122 ± 3.43
A4, 48 h, IC_{70}	167 ± 1.99	165 ± 2.77	226 ± 3.03	225 ± 2.78	206 ± 2.31	205 ± 3.71
A4, 72 h, IC_{50}	89 ± 1.33	87 ± 1.75	120 ± 3.00	119 ± 1.99	73 ± 1.56	72 ± 1.01
A4, 72 h, IC_{70}	137 ± 2.89	135 ± 3.71	198 ± 2.89	197 ± 2.43	129 ± 1.89	128 ± 2.71

X is an average of five repetitions. S.E., standard error. P, parental H1299 cells. R, resistant H1299 cells. A5, A549 cells. A4, A431 cells.

P-H1299 > R-H1299 > A431 > A549 depending on IC concentrations, respectively. p-Cymene and essential oil were found more effective on A431 cells (EGFR-positive control cells) than γ-terpinene. The 50% inhibition concentrations (IC_{50}) of paclitaxel for 24, 48, and 72 h were calculated by using the CellTiter-Blue cell viability assay. IC_{50} values were calculated, respectively, for paclitaxel on parental H1299 cells as 6.2×10^{-3}, 4.7×10^{-3}, and 3.5×10^{-3} μg/mL; on drug-resistant cells as 7.6×10^{-3}, 6.8×10^{-3}, and 5.4×10^{-3} μg/mL; on A431 cells as 5.5×10^{-2}, 4.3×10^{-2}, and 3.1×10^{-2} μg/mL; and on A549 cells as 4.4×10^{-3}, 3.1×10^{-3}, and 1.8×10^{-3} μg/mL.

The induction of cytotoxic cell death can be accompanied by membrane and DNA damage. Essential oil, γ-terpinene, and p-cymene solutions induced membrane and DNA damage at IC_{50} and IC_{70} concentrations (Table 2) that mediated their anticancer activities. Essential oil, γ-terpinene, and p-cymene caused increasing MDA levels, an end product of lipid peroxidation of membrane, and 8-OHdG formation, a product of oxidative DNA damage following specific enzymatic cleavage after 8-hydroxylation of guanine bases. Generally, the MDA and 8-OHdG amounts in cells exposed to IC_{50} and IC_{70} essential oil, γ-terpinene, and p-cymene concentrations were found to be statistically different from the control cells ($P \leq 0.05$). The membrane and DNA damage increased when the cells were exposed to the essential oil, γ-terpinene, and p-cymene at increasing concentrations

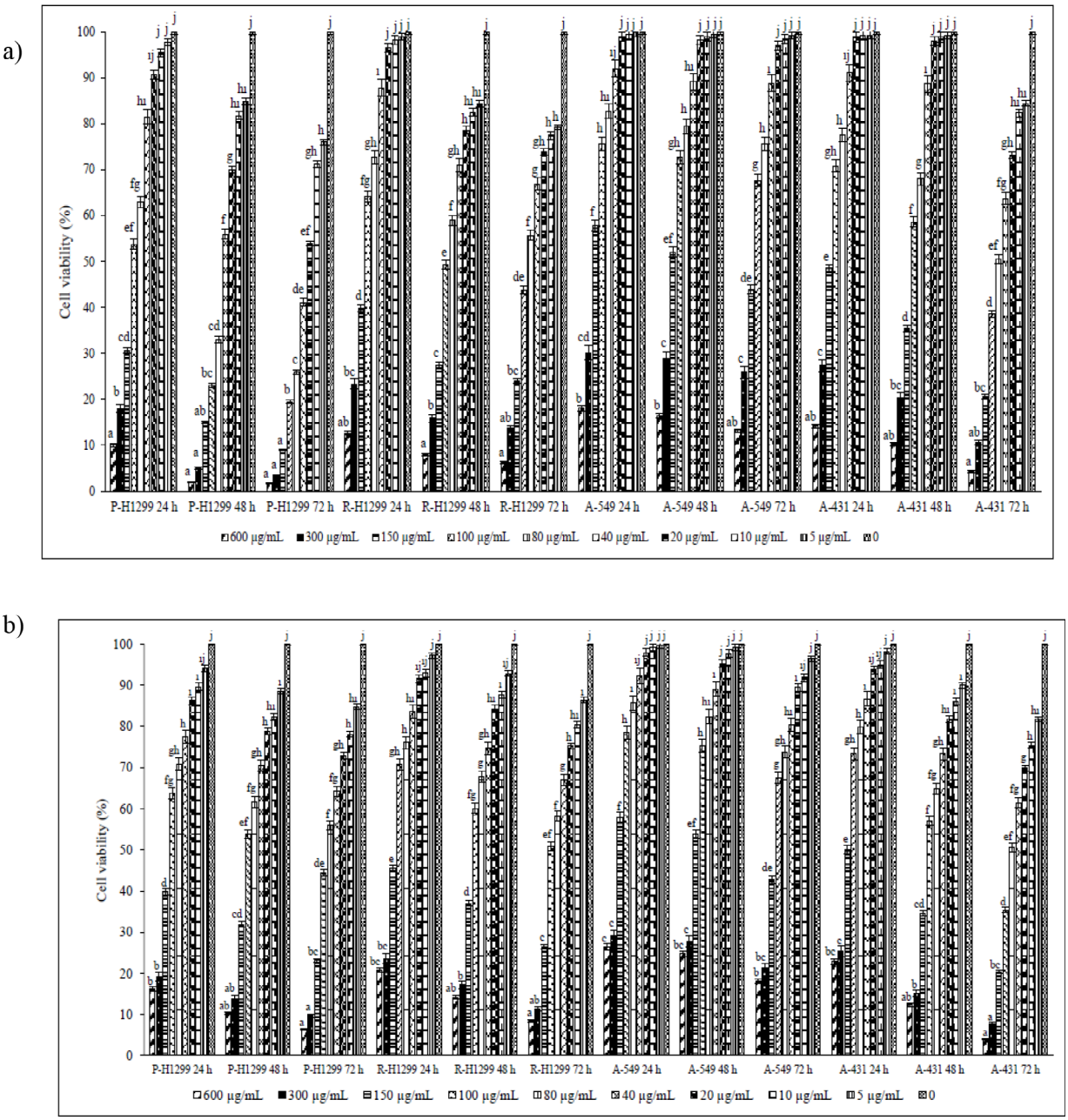
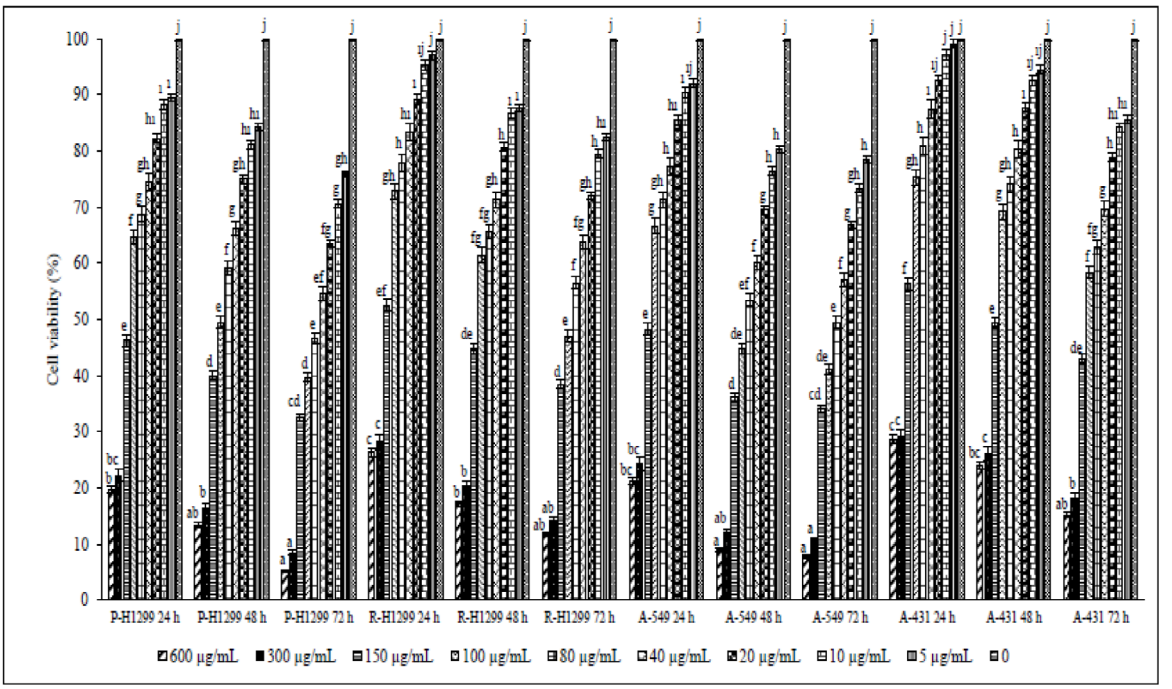


Figure 1. The cytotoxic effects of *T. revolutus* essential oil on parental H1299, drug-resistant H1299, A549, and A431 cells as measured by a) CellTiter-Blue cell viability assay and b) MTT assay. Results are presented as viability ratio compared with the control group (treated with only the medium-untreated cells). Values are expressed as the mean of three separate experiments ± S.E. Error bars represent standard error of the mean from three replications, and bars with the same letter indicate no significant difference (ANOVA with Tukey's test, $P < 0.05$). Different letters represent significant differences among treatments with the essential oil ($P < 0.05$; treatment groups after 24 h, 48 h, and 72 h) in P-H1299, R-H1299, A549, and A-431 cells. P, parental cells. R, resistant cells.

(Table 2). The highest membrane damage was caused by essential oil at IC_{50} and IC_{70} concentrations in A431 cells. The cell membrane damage increased 85% at IC_{50} and 94% at IC_{70} concentration in A431 cells compared to control cells. A549 cells treated with IC_{50} p-cymene and R-H1299 treated with IC_{70} *γ*-terpinene had the lowest membrane damages, but A549 cells had the highest DNA damages at IC_{50} and IC_{70} p-cymene concentrations. DNA damage increased 254% at IC_{50} and 378% at IC_{70} concentrations in A549 cells compared to control cells. The responses of cells

a)



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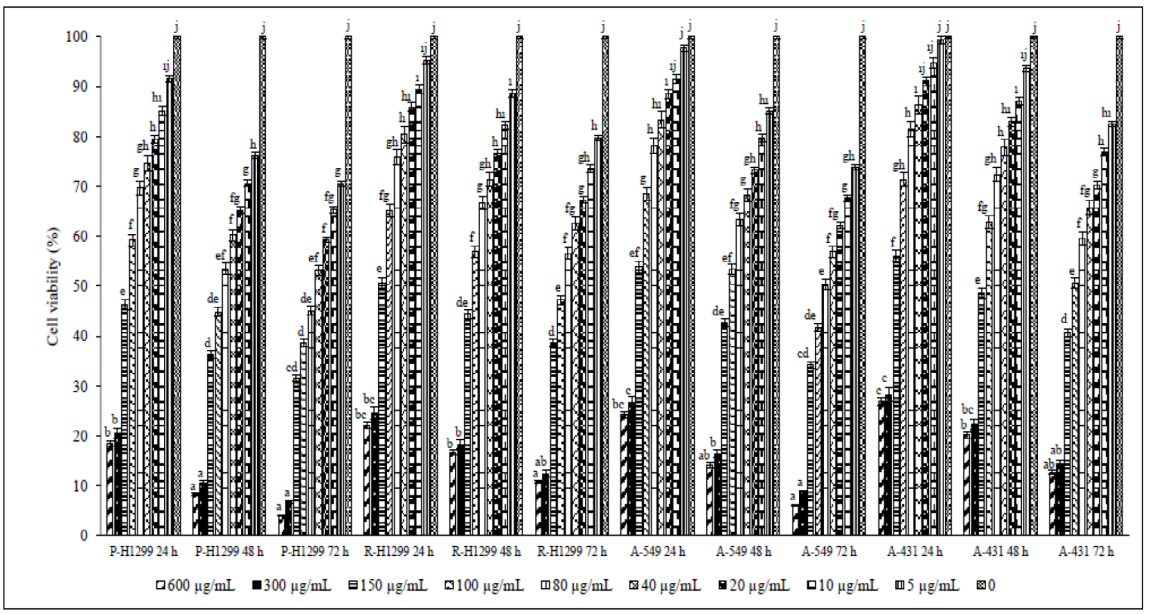

Figure 2. The cytotoxic effects of *γ*-terpinene on parental H1299, drug-resistant H1299, A549, and A431 cells as measured by a) CellTiter-Blue cell viability assay and b) MTT assay. Results are presented as viability ratio compared with the control group (treated with only the medium-untreated cells). Values are expressed as the mean of three separate experiments ± S.E. Error bars represent standard error of the mean from three replications, and bars with the same letter indicate no significant difference (ANOVA with Tukey's test, $P < 0.05$). Different letters represent significant differences among treatments with *γ*-terpinene ($P < 0.05$; treatment groups after 24 h, 48 h, and 72 h) in P-H1299, R-H1299, A549, and A-431 cells. P, parental cells. R, resistant cells.

were found different when comparing the membrane and DNA damaging effects of essential oil, *γ*-terpinene, and p-cymene.

The cellular redox status was determined from the reduced glutathione to oxidized glutathione ratio (GSH/GSSG) and the enzymes involved in glutathione

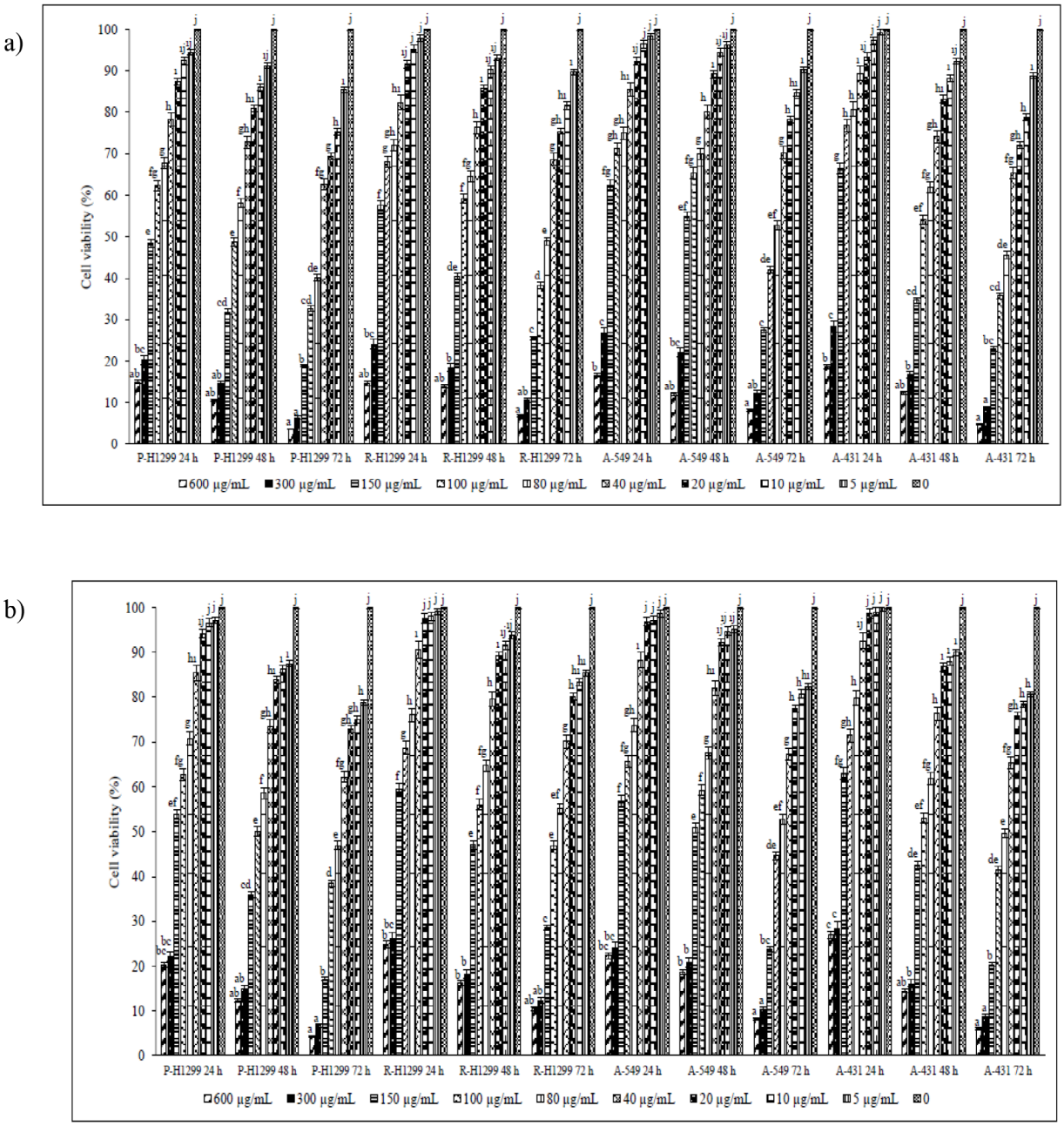

Figure 3. The cytotoxic effects of p-cymene on parental H1299, drug-resistant H1299, A549, and A431 cells as measured by a) CellTiter-Blue cell viability assay and b) MTT assay. Results are presented as viability ratio compared with the control group (treated with only the medium-untreated cells). Values are expressed as the mean of three separate experiments ± S.E. Error bars represent standard error of the mean from three replications, and bars with the same letter indicate no significant difference (ANOVA with Tukey's test, $P < 0.05$). Different letters represent significant differences among treatments with p-cymene ($P < 0.05$; treatment groups after 24 h, 48 h, and 72 h) in P-H1299, R-H1299, A549, and A-431 cells. P, parental cells. R, resistant cells.

metabolism, including GRx, GPx, and GST. When the cells were challenged with IC_{50} and IC_{70} concentrations of essential oil, γ-terpinene, and p-cymene, clear signs of oxidative stress, i.e. increased GRx in all cells due to decreasing reduced glutathione amount in the cells, were observed. Increased GPx and GST activities, especially after IC_{70} treatment of cells, as well as increased G6PD levels were seen. Changes in the levels of enzyme activities in all cell types were found statistically significant at IC_{70} concentrations (Table 3). GST activity increased in all

Table 2. Membrane and DNA damaging effects of *T. revolutus* essential oil, γ-terpinene, and p-cymene on parental H1299, drug-resistant H1299, A549, and A431 cells.

Concentrations	MDA (nmol/mg protein) X ± S.E.	8-OHdG (ng/mL) X ± S.E.
IC_{50} Es. oil (P)	6.20 ± 0.30 c	0.54 ± 0.03 c
IC_{70} Es. oil (P)	7.20 ± 0.23 d	0.73 ± 0.04 e
IC_{50} Es. oil (R)	7.50 ± 0.11 de	0.58 ± 0.02 cd
IC_{70} Es. oil (R)	7.71 ± 0.16 de	0.82 ± 0.07 f
IC_{50} Es. oil (A549)	7.72 ± 0.32 de	0.50 ± 0.03 c
IC_{70} Es. oil (A549)	8.11 ± 0.29 e	0.7 ± 0.04 e
IC_{50} Es. oil (A431)	8.90 ± 0.35 ef	0.55 ± 0.03 c
IC_{70} Es. oil (A431)	9.32 ± 0.43 fg	0.72 ± 0.01 e
IC_{50} p-Cymene (P)	6.40 ± 0.20 c	0.51 ± 0.02 c
IC_{70} p-Cymene (P)	7.11 ± 0.13 d	0.63 ± 0.04 d
IC_{50} p-Cymene (R)	7.12 ± 0.22 d	0.55 ± 0.03 c
IC_{70} p-Cymene (R)	8.41 ± 0.26 e	0.70 ± 0.05 e
IC_{50} p-Cymene (A549)	5.00 ± 0.34 b	0.94 ± 0.06 g
IC_{70} p-Cymene (A549)	7.50 ± 0.14 de	1.40 ± 0.07 h
IC_{50} p-Cymene (A431)	8.00 ± 0.19 e	0.50 ± 0.01 c
IC_{70} p-Cymene (A431)	8.60 ± 0.27 ef	0.55 ± 0.02 c
IC_{50} γ-Terpinene (P)	8.40 ± 0.38 e	0.53 ± 0.02 c
IC_{70} γ-Terpinene (P)	8.80 ± 0.40 ef	0.70 ± 0.04 e
IC_{50} γ-Terpinene (R)	5.20 ± 0.10 b	0.50 ± 0.02 c
IC_{70} γ-Terpinene (R)	5.70 ± 0.18 bc	0.73 ± 0.03 e
IC_{50} γ-Terpinene (A549)	5.50 ± 0.31 bc	0.50 ± 0.02 c
IC_{70} γ-Terpinene (A549)	6.00 ± 0.21 c	0.6 ± 0.01 d
IC_{50} γ-Terpinene (A431)	7.80 ± 0.43 de	0.52 ± 0.03 c
IC_{70} γ-Terpinene (A431)	8.90 ± 0.25 ef	0.65 ± 0.02 de
Control (P)	4.10 ± 0.12	0.30 ± 0.01
Control (R)	4.30 ± 0.37	0.40 ± 0.02
Control (A549)	4.70 ± 0.19	0.37 ± 0.04
Control (A431)	4.80 ± 0.25	0.40 ± 0.01
0.5% DMSO (P)	4.20 ± 0. 14 a	0.30 ± 0.02 a
0.5% DMSO (R)	4.50 ± 0.28 ab	0.40 ± 0.05 b
0.5% DMSO (A549)	4.90 ± 0.13 ab	0.39 ± 0.02ab
0.5% DMSO (A431)	5.20 ± 0.17 b	0.41 ± 0.03 b

Values that are followed by different letters within each column are significantly different ($P < 0.05$). S.E., standard error. P, parental H1299 cells. R, resistant H1299 cells. MDA, malondialdehyde. 8-OHdG, 8-hydroxy-2′-deoxyguanosine. Es. oil, essential oil. X, each value is an average of five repetitions of MDA and 8-OHdG amounts. The letter labels in the table indicate statistically significant differences between controls and essential oil, γ-terpinene, and p-cymene treatments ($P < 0.05$) based on ANOVA with Tukey's test.

Table 3. Effects of *T. revolutus* essential oil, *γ*-terpinene, and p-cymene on enzyme activities of parental H1299, drug-resistant H1299, A549, and A431 cells.

Concentrations	G6PD X ± S.E.	GRx X ± S.E.	GPx X ± S.E.	GST X ± S.E.
IC_{50} Es. oil (P)	33 ± 1.01 c	2.85 ± 0.11 b	4.50 ± 1.20 a	97 ± 3.10 a
IC_{70} Es. oil (P)	24 ± 2.03 b	3.44 ± 0.31 bc	6.00 ± 2.00bc	106 ± 5.22 b
IC_{50} Es. oil (R)	32 ± 1.82 c	2.75 ± 0.21 b	4.40 ± 1.90 a	98 ± 4.31 a
IC_{70} Es. oil (R)	27 ± 3.03 cb	3.30 ± 0.29 bc	6.32 ± 1.76 c	106 ± 3.20 b
IC_{50} Es. oil (A549)	29 ± 2.02 cb	2.60 ± 0.21 b	5.00 ± 1.99 ab	99 ± 3.71 a
IC_{70} Es. oil (A549)	23 ± 1.42 b	3.32 ± 0.29 bc	6.00 ± 2.00 bc	109 ± 6.11 b
IC_{50} Es. oil (A431)	27 ± 2.03 cb	2.51 ± 0.11 b	4.81 ± 2.44 ab	96 ± 5.22 a
IC_{70} Es. oil (A431)	22 ± 2.04 ab	3.21 ± 0.31 bc	6.42 ± 2.01 c	108 ± 3.71 b
IC_{50} p-Cymene (P)	26 ± 1.03 cb	3.10 ± 0.29 bc	6.49 ± 1.22 c	96 ± 2.91 a
IC_{70} p-Cymene (P)	19 ± 1.33 ab	4.50 ± 0.31 cd	7.51 ± 1.55 de	110 ± 4.20 b
IC_{50} p-Cymene (R)	27 ± 2.02 cb	3.20 ± 0.22 bc	4.00 ± 1.69 a	97 ± 3.20 a
IC_{70} p-Cymene (R)	18 ± 1.04 ab	4.50 ± 0.33 cd	6.73 ± 2.11 cd	109 ± 4.11 b
IC_{50} p-Cymene (A549)	26 ± 3.03 cb	2.52 ± 0.19 b	4.11 ± 1.22 a	99 ± 3.52 a
IC_{70} p-Cymene (A549)	11 ± 1.13 a	3.50 ± 0.13 bc	4.50 ± 1.89 a	106 ± 5.23 b
IC_{50} p-Cymene (A431)	25 ± 2.02 b	2.55 ± 0.23 b	5.26 ± 2.12 b	97 ± 3.44 a
IC_{70} p-Cymene (A431)	11 ± 1.02 a	3.44 ± 0.31 bc	5.64 ± 2.09 bc	107 ± 3.75 b
IC_{50} *γ*-Terpinene (P)	27 ± 3.03 cb	2.65 ± 0.22 b	4.64 ± 1.67 ab	97 ± 4.06 a
IC_{70} *γ*-Terpinene (P)	20 ± 2.12 ab	3.20 ± 0.20 bc	6.24 ± 2.01 c	109 ± 5.66 a
IC_{50} *γ*-Terpinene (R)	31 ± 3.03 c	2.61 ± 0.11 b	4.25 ± 2.33 a	96 ± 4.96 a
IC_{70} *γ*-Terpinene (R)	25 ± 2.04 b	3.20 ± 0.20 bc	6.26 ± 2.89 c	99 ± 5.17 a
IC_{50} *γ*-Terpinene (A549)	28 ± 2.13 cb	2.60 ± 0.18 b	5.32 ± 3.00 b	96 ± 4.23 a
IC_{70} *γ*-Terpinene (A549)	22 ± 1.31 b	3.24 ± 0.24 bc	7.34 ± 3.02 d	105 ± 6.09 ab
IC_{50} *γ*-Terpinene (A431)	33 ± 2.01 c	2.63 ± 0.17 b	4.46 ± 1.20 a	96 ± 4.30 a
IC_{70} *γ*-Terpinene (A431)	26 ± 2.61 b	3.20 ± 0.31 bc	4.80 ± 1.66 ab	104 ± 4.09 ab
Control (P)	34 ± 3.03	1.91 ± 0.11	4.40 ± 1.56	97 ± 3.54
Control (R)	34 ± 3.01	1.94 ± 0.13	4.50 ± 1.34	98 ± 2.94
Control (A549)	33 ± 2.24	1.95 ± 0.12	4.43 ± 1.54	97 ± 3.52
Control (A431)	34 ± 2.51	1.93 ± 0.15	4.42 ± 1.23	96 ± 3.79
0.5% DMSO (P)	34 ± 0.01 c	1.95 ± 0.17 a	4.33 ± 1.88 a	99 ± 4.19 a
0.5% DMSO (R)	33 ± 0.01 c	1.91 ± 0.13 a	4.22 ± 0.01 a	96 ± 3.91 a
0.5% DMSO (A549)	34 ± 0.01 c	1.91 ± 0.16 a	4.31 ± 0.01 a	97 ± 3.08 a
0.5% DMSO (A431)	33 ± 0.01 c	1.92 ± 0.12 a	4.12 ± 0.01 a	96 ± 2.98 a

Values that are followed by different letters within each column are significantly different ($P < 0.05$). S.E., standard error. P, parental H1299 cells. R, resistant H1299 cells. Es. oil, essential oil. GST, Glutathione S-transferase. GPx, Glutathione peroxidase. GRx, Glutathione reductase. G6PD, Glucose 6-phosphate dehydrogenase. X, each value is an average of five repetitions of enzymes activity. Enzyme activities (nmol of product formed/mg of protein per min). The letter labels in the table indicate statistically significant differences between controls and essential oil, *γ*-terpinene, and p-cymene treatments ($P < 0.05$) based on ANOVA with Tukey's test.

cells after treatment of IC_{70} essential oil, γ-terpinene, and p-cymene concentrations, except in R-H1299 cells. A549 cells had the lowest GPx activity while P-H1299 cells had the highest after p-cymene treatment. Parental and drug-resistant H1299 cells had the highest GPx activity after p-cymene treatment. Changing GPx activities in all cells were found statistically important ($P \leq 0.05$) compared to control cells. G6PD has an important role in the regeneration of NADPH, which is critical for maintaining GSH in its reduced form. GSH is essential for detoxification of reactive free radicals and lipid hydroperoxides. GSH is also a cofactor for the transferase and peroxidase enzymes. G6PD activities in R-H1299 cells were found less after p-cymene than after essential oil and γ-terpinene treatments.

4. Discussion

The essential oil from *T. revolutus* and its two main components, cymene (32.57%) and γ-terpinene (17.18%), were found cytotoxic in concentration- and time-dependent manners in Hep G2 and 70.15, 94, and 103.8 μg/mL were calculated as IC_{50} values of essential oil, cymene, and γ-terpinene, respectively, for 24 h (6). The essential oil of *T. caespititius* from Planalto Central, Brazil, used at 0.08 mg/mL on adenocarcinoma gastric cells (ACC201) decreased the viability to 45% in the first 30 min, but at the end of the first hour viability had recovered to 64% and at the end of the assay (8 h) the viability was maintained at 95%. Higher concentrations of the essential oil, 0.50 and 1.00 mg/mL, were detrimental to the viability of the gastric cell line (30). Cytotoxicity of *Thymus vulgaris* L. (thyme) essential oil was investigated on head and neck squamous cell carcinoma cell line UMSCC1. The IC_{50} of thyme essential oil extract was found as 369 μg/mL (31).

T. revolutus essential oil and its two main components increased MDA levels according to controls in Hep G2 cells. On the other hand, Hep G2 cells preincubated with essential oil ($IC_{2.5}$, IC_5, $IC_{7.5}$, and IC_{10}) for 1 h before H_2O_2 treatment (IC_{50} and IC_{70}) for 24 h had lower MDA levels than cells that were not preincubated (IC_{50} and IC_{70} H_2O_2 treatment). Decreasing H_2O_2-induced cytotoxic effects and membrane damage can be accompanied by the antioxidant action of essential oil with lower concentrations (6). *Origanum onites* (Lamiaceae) essential oil and its two phenolic components, thymol and carvacrol, induced membrane damage on Hep G2 cells (32). Carvacrol, thymol eugenol, eucalyptol, terpinen-4-ol, and camphor at higher concentrations increased MDA levels, causing membrane damage, and 8-OHdG levels, causing DNA damage to both parental and epirubicin-resistant H1299 cells (32,33).

Thymus sipyleus methanol extract increased SOD activity, although it did not affect MDA, protein products (AOPP), and GSH levels significantly in liver tissue of Swiss albino mice (34). Pretreatment with *Dracocephalum multicaule* essential oil and *Perilla* aldehyde (71.5%) and limonene (28.1%) as main constituents protected K562 cells 49.5% against H_2O_2-induced oxidative damage by increasing the activities of antioxidant enzymes and glutathione content in K562 cells.

G6PD activity increased parallel to antioxidant enzyme activities (35). The ethyl acetate extract of *C. sativum* roots showed cytotoxicity in MCF-7 cells due to increased SOD activity, whereas decreased GPx and CAT activities arrest the cell cycle at the G2/M phase and induce apoptosis by both extrinsic and intrinsic pathways. *C. sativum* root also exhibited DNA protective effects against H_2O_2 in MCF-7 cells (36). In animal studies, the levels of antioxidant enzymes CAT, SOD, and GPx, as well as glutathione, were found to be increased in groups treated with *Wedelia chinensis* (Osbeck), whereas lipid peroxidation and nitric oxide were reduced (37). In general, the cytotoxic activity of essential oils is mostly due to the presence of phenols, aldehydes, and alcohols such as γ-terpinene and p-cymene (38,39). In this study, we investigated the cellular responses of different cancer cells (P-H1299, R-H1299, A549, and A431) against injury induced by *Thymus revolutus* Célak essential oil, γ-terpinene, and p-cymene after the cells were treated with these solutions.

The responses of cells to chemotherapy are different. These differences play a role in therapy failures in many tumors (12). The differences in the sensitivities of different cell lines can be understood in terms of their natural antioxidant levels, which is a key behind their natural defense mechanisms during oxidative stress. Therefore, this probably governs the sensitivities of the cell lines upon exposure to the same compounds. One study showed that drug-resistant cells could resist oxidants with their higher antioxidant enzymes (33). Our previous study showed that epirubicin-resistant H1299 cells had more GPx and GST activity, but lower GSH amounts, than parental H1299 cells (18). One study suggested that localization of EGFR is related to the sensitivity/resistance of cells to chemotherapy and radiotherapy. Discrepancies were observed between total EGFR and surface EGFR levels in H1299 cells. In A549 cells, total EGFR expression was similar to that of surface EGFR, and A431 cells were used as a positive control for EGFR (40). In this study, R-H1299 cells were found more resistant than P-H1299 cells against cytotoxicity induced by essential oil, γ-terpinene, and p-cymene. P-H1299 cells showed the highest sensitivity to these three compounds among all the cell lines. A549 cells had higher resistance to essential oil and p-cymene cytotoxicity. A431 cells (EGFR positive control) had the highest resistance to γ-terpinene cytotoxicity of all the cells. A431 cell sensitivity to essential oil and p-cymene

was found less than the sensitivity of H1299 cells. When oxidative stress induced by essential oil, γ-terpinene, and p-cymene as a mechanism of toxicity was assessed, depletions of GSH, which caused GRx increases, were seen, which probably resulted in the shift of the overall redox balance towards oxidation, leading to functional damage of cells and enhanced lipid peroxidation and DNA oxidation. The chemical reduction of GSSG and the maintenance of adequate GSH for reactive oxygen species (ROS) detoxification, as well as for the detoxification of free radical intermediates, are ultimately dependent on NADPH supplied by G6PD (41). Results obtained by our study clearly indicate that these compounds could increase GRx activity in the cells at IC_{50} concentration and GPx, GST, and G6PD activity at IC_{70} concentration. Differences of cell responses against prooxidant effects of the compounds at IC_{70} concentrations were found statistically significant ($P \leq 0.05$). Therefore, the observed changes in enzyme activities might be due to the generation of ROS after higher exposure to compounds in order to protect the cells. In one study, sulforaphane induced ROS production and impaired glutathione recycling as evidenced by the change in GR and GPx gene expression and enzyme activity in MG-63 cells (42). Similar observations were made by Tirkey et al. (43), who reported inactivation of antioxidant enzyme activity due to carbon tetrachloride-induced oxidative stress in rat liver and kidney cells. Antioxidant enzyme activity in cancer cells changed depending on plant essential oil components and extract prooxidant/antioxidant effects related to its concentrations (8). The present results also showed that our compounds acted as an oxidizing agent on membranes and DNA, causing elevations of MDA levels as a result of membrane lipid peroxidation and 8-OHdG levels as a result of oxidation of DNA. Some cells had the highest DNA and membrane damages due to their weak antioxidant defense mechanisms. The induction of cytotoxic cell death can be accompanied by membrane and DNA damage (44). Many constituents were already cytotoxic and mutagenic, and caused depleted levels of glutathione. They induced DNA damage in mammalian cells (45,46).

Cellular responses of different lung cancer cells against potential antitumor and prooxidative effects of *Thymus revolutus* Célak essential oil and its two main constituents depended on the application of concentrations, incubation times, and cell properties such as drug resistance and EGFR expression capacity.

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