

Synergistic Effects of Alpha-Pinene and D-Limonene on Negative Triple Breast Cancer Cell Death Through Enhanced ROS Level

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Received: 23 February 2026, Revised: 21 April 2026, Accepted: 28 April 2026, Published: 5 June 2026

Abstract

Triple-negative breast cancer (TNBC) accounts for approximately 10% - 15% of all breast cancers and is associated with high recurrence rates and poor prognosis, contributing disproportionately to breast cancer mortality worldwide. Its limited therapeutic options and aggressive behavior underscore the need for novel combinatorial strategies. α -Pinene and d-limonene have demonstrated anticancer activity in several models; however, their combined effects on TNBC remain unclear. This study aimed to evaluate the synergistic cytotoxic effects of α -pinene and d-limonene and predict their potential molecular targets in TNBC. Cytotoxicity of single and combined treatments was assessed in MDA-MB-231 cells using an MTT assay across defined concentration ranges, followed by combination analysis using the Chou-Talalay method. Intracellular reactive oxygen species (ROS) levels were measured using the DCFDA assay after specified treatment durations. Target prediction was conducted using SMILES-based similarity across STITCH, SEA, and SwissTargetPrediction, while TNBC-associated genes were curated from TCGA (via UALCAN) and literature sources. Overlapping genes were analyzed using WebGestalt for GO and KEGG enrichment, STRING for protein-protein interaction networks, and Cytoscape-CytoHubba for hub gene identification, with survival analysis performed using TCGA datasets. α -Pinene and d-limonene exhibited cytotoxic effects with IC₅₀ values of 0.84 and 1.20 μ L/mL, respectively. Combination treatment demonstrated synergism (CI < 1) and significantly increased ROS levels compared to single treatments ($p < 0.05$), indicating enhanced oxidative stress. Bioinformatics analysis identified the PPAR signaling pathway as enriched among predicted targets. Five hub genes (PPARG, NOS3, ESRI, PPARG, and CYP19A1) were associated with TNBC-related pathways and patient survival. These findings suggest that the combination of α -pinene and d-limonene may exert synergistic cytotoxic effects in TNBC cells, potentially through modulation of ROS and PPAR-related pathways. However, these mechanisms remain predictive and require further experimental validation.

Keywords: α -pinene, Cell survival, d-limonene, Reactive oxygen species, Triple-negative breast cancer cells

Introduction

Triple-negative breast cancer (TNBC) represents approximately 10% - 15% of all breast cancer cases worldwide and is associated with disproportionately

high mortality due to its aggressive phenotype and limited treatment options [1]. Recent global estimates indicate that breast cancer accounts for over 2.3 million new cases annually, with TNBC contributing

significantly to recurrence and metastasis-related deaths [2,3]. TNBC is characterized by high heterogeneity, strong metastatic potential, and a recurrence rate of up to 47.2%, leading to poor clinical outcomes. Unlike other breast cancer subtypes, TNBC lacks estrogen receptor (ER), progesterone receptor (PR), and HER2 expression, rendering endocrine and HER2-targeted therapies ineffective [4]. Consequently, current treatment strategies rely heavily on chemotherapy, which is often limited by drug resistance and high relapse rates [5,6].

Current chemotherapy approaches primarily rely on apoptotic mechanisms [5,6]. Apoptosis in cancer cells occurs by inducing cell aging and can also be triggered by increased reactive oxygen species (ROS) and oxidative metabolites [7,8]. At moderate levels, ROS promotes tumor progression by enhancing proliferation, survival signaling, and metastasis. In contrast, excessive ROS accumulation can induce oxidative stress, leading to cellular damage, apoptosis, or necrosis [9,10]. This duality makes ROS a critical yet challenging therapeutic target. While increased ROS has been implicated in promoting TNBC progression and metastasis, supra-physiological ROS levels may instead suppress tumor growth and inhibit migration [11-13]. However, the precise mechanisms by which ROS modulation contributes to TNBC cell death and therapeutic response remain insufficiently understood.

Emerging evidence suggests that oxidative stress may interact with key metabolic and transcriptional regulators, including the peroxisome proliferator-activated receptor (PPAR) signaling pathway [14]. PPARs are nuclear receptors involved in lipid metabolism, inflammation, and cancer cell survival [15]. Studies have shown that ROS can modulate PPAR activity either directly through oxidative modification or indirectly via redox-sensitive signaling pathways, thereby influencing cancer cell fate [16,17]. In TNBC, dysregulation of PPAR signaling has been associated with altered proliferation and survival, suggesting a potential link between ROS-mediated stress and PPAR-dependent mechanisms [16,18,19]. Despite this, the integration of ROS modulation and PPAR signaling in TNBC therapy remains underexplored.

Natural compounds such as α -pinene and d-limonene have gained attention as potential anticancer agents due to their multi-target properties and relatively

low toxicity [20-23]. Active compounds in turpentine oil containing α -pinene were shown to be cytotoxic to several cancer types by triggering apoptosis and altering cell cycle arrest at the G2/M phase [6,20,24]. Likewise, citrus oil containing d-limonene as the main component (74.6%) exerted cytotoxicity against A549 lung cancer cells by increasing ROS levels and inhibiting migration [25,26]. Based on the two findings above, it can be inferred that two compounds could synergistically inhibit TNBC growth through distinct targets. However, most studies have evaluated these compounds individually, and their combined effects, particularly in TNBC, remain poorly characterized. Furthermore, limited research has integrated experimental cytotoxicity, ROS modulation, and *in silico* target prediction to elucidate potential molecular mechanisms underlying their combined activity. Based on these considerations, we hypothesize that the combination of α -pinene and d-limonene exerts synergistic cytotoxic effects in TNBC cells by enhancing ROS accumulation and modulating key signaling pathways, including PPAR-related mechanisms. Therefore, this study aimed to examine the combined effect of α -pinene and d-limonene on inducing higher ROS levels of MDA-MB-231 cells. This study also compared the therapeutic effects of pure α -pinene, d-limonene, and their combined formulation on TNBC regulatory genes using bioinformatic analyses.

Materials and methods

Cell culture

MDAMB-231 (ECACC#92020424), representing TNBC cells, is cultured in high glucose Dulbecco's modified Eagle's Medium (DMEM) (Gibco, USA). The media were enriched with fetal bovine serum 10% v/v (Gibco, USA), amphotericin B 12.5 μ g/mL (Gibco, USA), streptomycin 150 μ g/mL, and penicillin 150 IU/mL (Gibco, USA), and cultured at 5% CO₂ and 37 °C. The medium is replaced every two to three days, and the cells are subcultured when they reach a confluence rate of 80% - 90%. The experiment employed only cells with >90% viability, passage number <10, and in the logarithmic growth phase.

Cell viability assay

The MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to

evaluate the antiproliferative effect of α -pinene and d-limonene on MDA MB-231. Briefly, cells with a density of 5×10^3 cells/well were seeded into a 96-well plate and incubated at 37 °C under 5% CO₂ for 24 h. The cells underwent triplicate exposure to concentration ranges of 62.5 - 12,500 μ M for α -pinene (density 0.858 g/mL, MW 136.23 g/mol) and 100 - 15,000 μ M for d-limonene (density 0.841 g/mL, MW 136.23 g/mol), equivalent to 0.25 - 20 μ L/mL for 24 h, with untreated cells as the negative control. Next, the cells were treated with 0.5 mg/mL MTT (Biovision) and further incubated for 4 h. DMSO (100 μ L) was used to dissolve MTT formazan, which was incubated for 15 min before absorbance was measured at 595 nm using an ELISA reader (Bio-Rad iMark™ Microplate Reader). The absorbance was converted to a percentage of cell viability by comparing the treated and untreated groups at a specified time point. The IC₅₀ value was calculated by linear regression of the concentration (x) and % cell viability (y) to obtain the equation $y = Bx + A$. The concentration that prevents a 50% cell proliferation was determined from the linear graph at y-axis = 50 to obtain the IC₅₀ value on the x-axis.

Data represent mean $\pm$ SD of $n = 3$ independent experiments [27].

Combination experiments by MTT assay

Synergy was assessed using the Chou-Talalay method via CompuSyn software (CompuSyn Inc., Paramus, New Jersey). Full dose-effect curves were generated for single agents (as above), and the cells were treated for 24 h with a half-dose combination of α -pinene and d-limonene, corresponding to $\frac{1}{4}$ of the IC₅₀ value. The dose level was used to spanning ED₂₀-ED₈₀ (effective dose for 20% - 80% effect), ensuring fractional effects 0.1 - 0.9 for accurate combination index (CI) calculation ($CI < 1$ = synergy). Treatments followed 24 h protocol above, with isobologram analysis confirming interactions [28,29].

Measurement of ROS levels

Intracellular ROS levels were analyzed by flow cytometry using dichlorodihydrofluorescein diacetate (DCFH-DA). Briefly, cells were plated at 1×10^5 density in 6-well plates for 24 h, then harvested. Then, the cells were stained with DCFH-DA (10 μ M) at 37 °C for 30

min in the dark, treated with pure α -pinene, d-limonene, or their mixture for 4 h (to capture early oxidative burst, prior to 24 h cytotoxicity peak that consistent with rapid ROS dynamics in cancer cells), and then treated with doxorubicin (10 nM) as the positive control. Cells were collected, and the fluorescence of dihydrodichlorofluorescein (DCF) was measured using an Accuri C6 flow cytometer (BD Bioscience). The percent fluorescence intensity (%) = the mean fluorescence of each group divided by the mean fluorescence of the negative control group $\times 100$ [30,31].

Data acquisition from genes expressed in TNBC

TNBC-associated genes were identified via systematic PubMed search (January 2015 - 2025): (“triple-negative breast cancer” OR TNBC) AND (gene OR target OR biomarker) AND (“differentially expressed” OR upregulated OR downregulated); $n = 1,248$ hits. Inclusion: Human studies, TCGA/GEO validation, fold-change > 1.5 , $p < 0.05$, yielding ~ 200 genes (e.g., EGFR, FOXC1). Curated with UALCAN (TCGA-BRCA, TNBC subtype $n = 115$ tumors vs. $n = 124$ normals).

The identification of α -pinene and d-limonene as direct protein targets

We identified the direct protein target genes for α -pinene and d-limonene using a variety of online tools, including SEA-Predict (<https://sea.bkslab.org/>), SwissTargetPrediction (<http://www.swisstargetprediction.ch/>), and STITCH (<https://stitch.org/>). The SMILES code from PubChem was used to store the default setting in each database. We next utilized Venny 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/>) to find the overlapping genes implicated in TNBC as well as α -pinene and d-limonene target genes (CPTG). Each gene was classified using PANTHER v.16 (<http://www.pantherdb.org/>) based on protein class [32].

Analysis of pathway enrichment and functional annotation chart

Gene Ontology (GO) databases are useful for analyzing gene enrichment in overlapping genes. The GO analysis was done using Overrepresentation Enrichment Analysis (ORA) via WebGestalt 2019

(<http://www.webgestalt.org/>). The false discovery rate (FDR) of less than 0.05 was chosen as the cut-off value. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was analyzed using STRING-DB [27].

Generating protein-protein interaction (PPI) networks containing overlapping genes

The STRINGDB 11.0b was used to create the PPI network analysis, which had confidence scores higher than 0.7. The network analysis was then constructed using Cytoscape-CytoHubba, and only the top 5 genes were selected for further analysis.

The survival analysis of associated genes in TNBC patients

We curated the survival analysis for each gene using UALCAN (<http://ualcan.path.uab.edu/>), TCGA-BRCA, TNBC $n = 115$; median split cut-off for high/low expression. Kaplan–Meier survival curves (log-rank test, $p < 0.05$); overall and disease-free survival.

Statistical analysis

Data from $n = 3$ independent experiments (mean $\pm$ SD). One-way ANOVA (95% CI) followed by Tukey's post-hoc test for multiple comparisons ($p < 0.05$ significant; GraphPad Prism). Nonlinear regression for dose-responses/IC₅₀ (GraphPad Prism).

Results and discussion

Natural compounds, particularly essential oil constituents, have attracted attention as potential anticancer agents because they may interfere with proliferation, survival, and migration pathways in cancer cells. In this study, we evaluated the effects of α -pinene, d-limonene, and their combination on MDA-MB-231 TNBC cells and explored predicted molecular targets using bioinformatic analyses.

Cytotoxic effect of α -pinene and d-limonene on MDA-MB-231 cells

The cytotoxic activities of various concentrations of α -pinene and d-limonene were assessed by MTT assay, which measures mitochondrial dehydrogenase activity. The α -pinene and d-limonene inhibited cell proliferation in a dose-dependent manner, with IC₅₀ values of 0.84 and 1.20 $\mu\text{L/mL}$, respectively (**Figure 1**). The dose-response analysis showed that α -pinene produced slightly greater potency than d-limonene under the conditions tested. A previous study reported that α -pinene inhibits cancer invasion by downregulating nuclear factor kappa B (NF- κ B) in MDAMB-231 breast cancer cells [33]. D-limonene in *Citrus sinensis* extract also inhibits MDAMB-231 cells by downregulating matrix metalloproteinases-9 [34]. The previous study found that 77% of HCT116 viable cells were treated with a combination of d-limonene and hispolon [35], indicating the effectiveness of combination therapy.

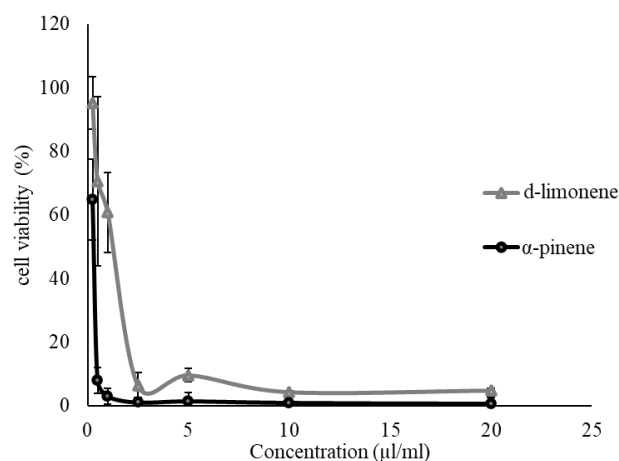


Figure 1 Percentage cell viability in MDA-MB-231 cells after treatment with various concentrations of α -pinene and d-limonene. Data are presented as mean $\pm$ SD, $n = 3$.

Combination effect of α -pinene and d-limonene on MDA-MB-231 cells

Combination treatment reduced cell viability more than the single-compound treatments, with the strongest effect observed at the pairwise concentration corresponding to the single-agent IC₅₀ range. A total of 16 combination conditions were tested over 24 h, and the combination of α -pinene and d-limonene produced the greatest suppression of viability at 0.84 μ L/mL α -pinene + 1.2 μ L/mL d-limonene (**Figure 2(A)**). The Chou-Talalay analysis indicated synergistic interaction

across most tested combinations, with the strongest synergy at the IC₅₀-equivalent pair (CI = 0.01), while other combinations showed strong to moderate synergy depending on the dose pair (**Figures 2(B)** and **2(C)**). The low-dose increase in viability suggests a possible biphasic or hormetic response, where lower exposure may transiently stimulate survival pathways before higher doses become cytotoxic. This pattern has been reported for several natural compounds and may reflect dose-dependent stress adaptation in cancer cells, but it requires direct experimental confirmation in this model.

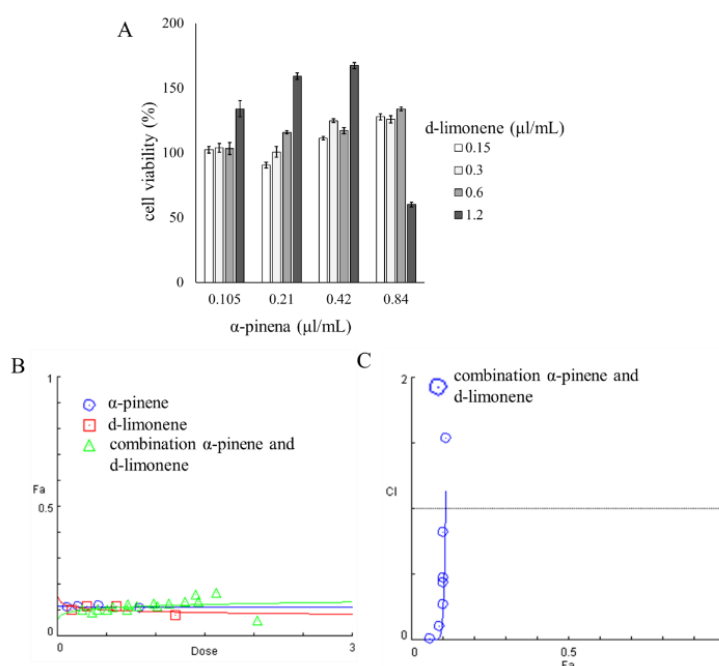


Figure 2 Synergistic cytotoxicity of α -pinene and d-limonene on MDAMB-231 cells. (A) Combination of α -pinene and d-limonene treatment for 24 h. Cell viability profiles were expressed as mean $\pm$ SD from three independent experiments. (B) Dose-effect curves of α -pinene, d-limonene, and their combination. The CompuSyn algorithm generated dose-effect curves, and the values represent the mean of three experiments. (C) Combination index plot among 16 combinations, 15 data points on the synergism side (CI < 1) and 1 data point on the antagonism side (CI > 1).

ROS induction by α -pinene and d-limonene

Given that the combination of α -pinene and d-limonene had been confirmed to induce cytotoxicity in MDAMB-231 cancer cell lines, we next explored the role of α -pinene in combination with d-limonene-induced ROS levels on MDAMB-231 cells. The ROS level measured by the DCFDA assay was significantly higher in the combination treatment; α -pinene and d-limonene led to elevated ROS levels in a dose-dependent manner (**Figures 3(A)** and **3(B)**). Compared with d-limonene alone, α -pinene produced a stronger

increase in ROS under the conditions tested, suggesting that α -pinene may contribute more strongly to the oxidative response observed here. In addition, Doxorubicin was used as a positive control to confirm assay responsiveness. Doxorubicin increases ROS levels, pushing already elevated cancer cells over a threshold that triggers cell death [36]. A previous study confirmed that α -pinene induces apoptotic cell death by increasing ROS levels in human ovarian cancer cells [37]. The results suggest that increased ROS levels may induce cell death.

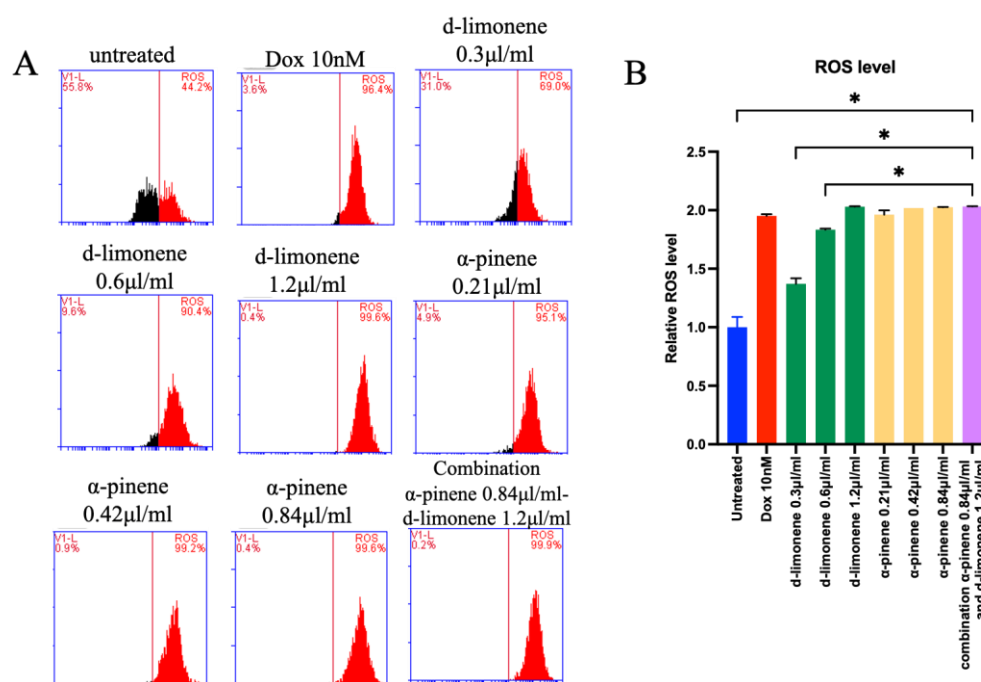


Figure 3 ROS level enhanced by α -pinene and d-limonene. (A) ROS level morphology under flow cytometry in treatment with single α -pinene and d-limonene. (B) interpretation levels of ROS. Data are represented as mean $\pm$ SD using the one way-ANOVA test (* indicates $p < 0.05$)

Data collection and processing of regulated genes in TNBC and α -pinene and d-limonene direct protein target

Based on preliminary *in vitro* data, we investigated target genes associated with the combination of α -pinene and d-limonene as a potential anticancer agent against TNBC. To identify the potential therapeutic target of combined α -pinene and d-limonene in TNBC, we collected 1061 TNBC-related genes from the PubMed-NCBI database. Then, we identified the direct protein targets (DTPs) of α -pinene and d-limonene using three online open-source databases to obtain more comprehensive data. It was shown that 17 and 11 genes (excluding duplicates) in the database were encoded for α -pinene and d-limonene DTP, respectively. The genes from α -pinene and d-limonene contained 26.7% and 33.3% of nuclear receptor groups (**Figure 4(A)**).

Next, we used a Venn diagram to identify genes shared among the DTPs of α -pinene, d-limonene, and TNBC. We identified 7 overlapping genes for further analysis as potential target genes (CPTG) for α -pinene and d-limonene (**Figure 4(B)**). Based on 7 CPTG, we found 2 downregulated genes and 5 overexpressed genes in TNBC (**Figure 4(C)**). Next, we used the

PANTHER web tools to categorize the overlapping genes according to protein. Most of the listed genes were classified as metabolite interconversion enzymes (57.14%), including NOS3, AR, and CYP2C19, which convert one small molecule into another (**Figure 4(D)**). A previous study reported that NOS3 enhances the migration and invasive capacity of breast cancer cells by activating the ERK1/2/MAPK pathway, thereby increasing ROS levels [21,38-40]. Furthermore, the listed genes are classified into gene-specific transcriptional regulators (42.86%), including NR1H3, PPARG, and PPARG. PPARG overexpression was shown to improve breast cancer cell survival by multiple mechanisms, including increased antioxidant signaling and AKT/protein kinase B activation [41]. Based on this information, α -pinene and d-limonene have the potential to target 7 genes involved in preventing cancer cell migration and survival via ROS and multiple signaling pathways. Based on the obtained data, we decided to focus on overlapping genes overexpressed in TNBC for further investigation. Overlapping genes indicate that these genes may be directly targeted by α -pinene and d-limonene against TNBC.

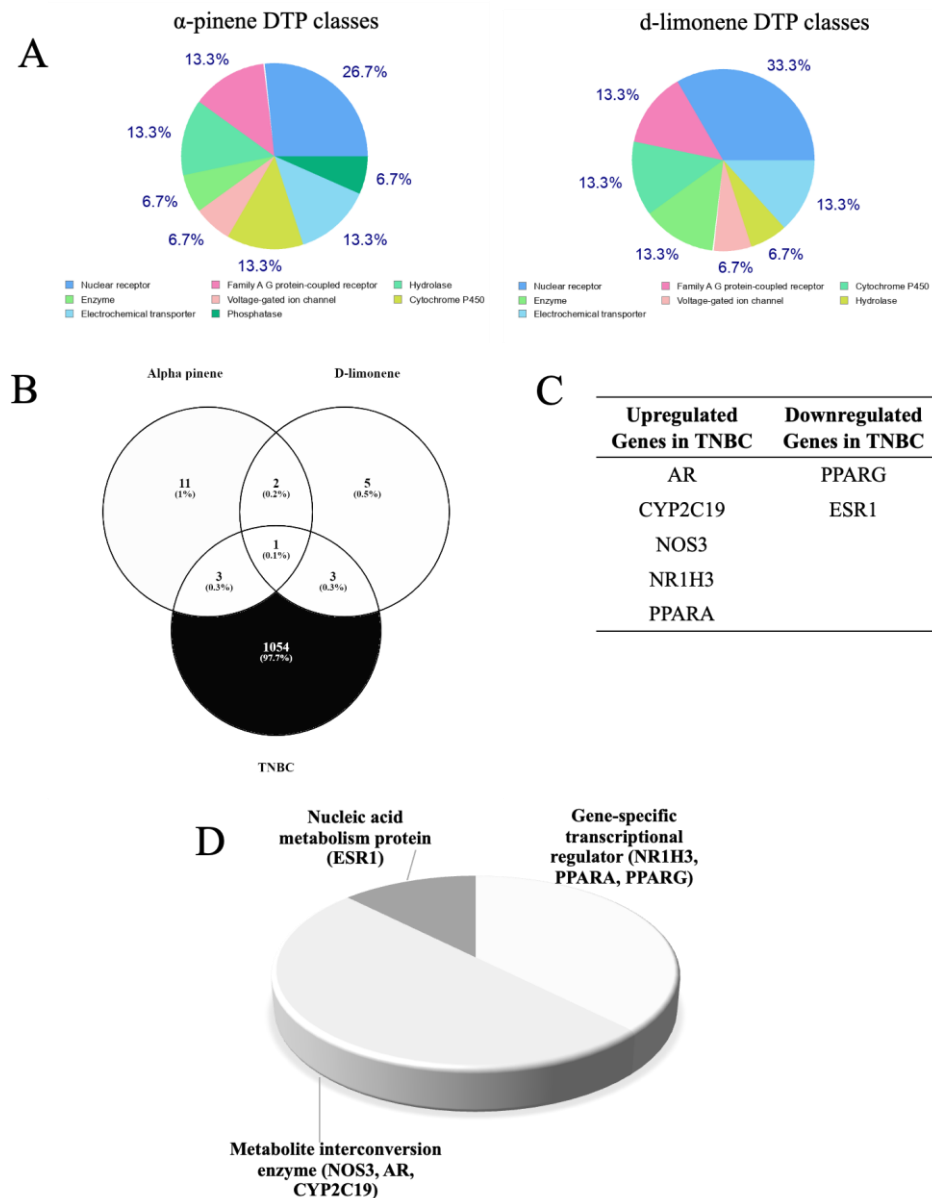


Figure 4 Direct protein target of α -pinene and d-limonene. (A) Several classes of DTP α -pinene and d-limonene. (B) According to the Venn diagram (C), DTP overlapped with TNBC biomarkers; (C) resulted in a total of 7 potential therapeutic targets of α -pinene and d-limonene (CPTG) as listed in the table. (D). PANTHER is used to classify genes by protein class.

GO and KEGG pathway enrichment of CPTG

WebGestalt web tools were used to demonstrate the functional annotations of CPTG using GO functional annotations and KEGG pathway enrichment analysis. We also classified GO functions using over-representation analysis (ORA). As shown, the CPTG is involved in several biological processes, including metabolism, response to stimuli, and cell communication (**Figure 5(A)**). Besides, CPTG was associated with cellular components in the nucleus and membrane (**Figure 5(B)**) and had a molecular function

in ion- and protein-binding (**Figure 5(C)**). Furthermore, KEGG pathway analysis revealed that the CPTG were enriched in the PPAR signaling pathway (**Figure 5(D)**). High expression of PPAR isoforms, including PPARG, PPARA, and PPAR γ , is associated with cancer progression [42]. A previous study reported that PPAR γ ligands had a stronger antiproliferative effect in TNBC cells than in ER α -positive breast cancer cells [39], suggesting that PPAR agonists such as α -pinene and d-limonene might be a useful therapeutic strategy for the treatment of more aggressive breast cancer subtypes.

Ligand-activated PPAR also influences cell cycle in breast cancer cells through repressing cyclin D1, leading to cell cycle arrest [43]. PPAR activation transactivates the p53 promoter and increases p53 protein expression, as well as that of its target gene p21, thereby activating

the intrinsic apoptotic pathways [23,44-46]. Based on these data, suggest that TNBC cell death is probably triggered by α -pinene and d-limonene via the PPAR pathway associated with elevated ROS levels.

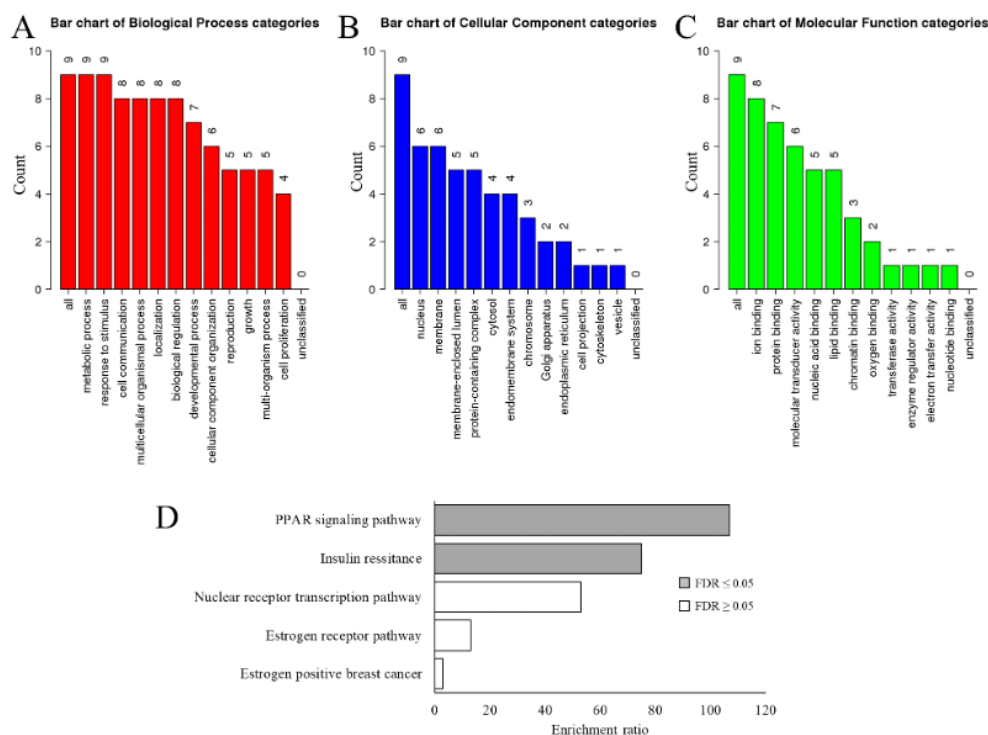


Figure 5 The involvement of CPTG in (A) biological process, (B) cellular component, (C) function categories, and (D) the KEGG pathway of CPTG using gene enrichment analysis under Webgestalt.

PPI Network and Hub-genes analysis of CPTG

Furthermore, we curated the CPTG to determine each gene's interactions and represented the network using the STRING web tools. In the PPI network, we found 7 nodes with 7 edges and an average node degree of 2 (**Figure 6(A)**). We ranked the nodes by degree and identified the top five genes with the highest scores (**Figures 6(B)** and **6(C)**). Moreover, five genes with the highest scores are involved in the progression of apoptosis. PPARG is another PPAR subfamily shown to induce cancer cell apoptosis by destabilizing Bcl2 and stabilizing endogenous Bcl2 protein regulated by PPARG, leading to reduced cancer cell chemoresistance

[47]. Activation of PPARG may induce apoptosis in several cancer cells by inducing DR5 and/or downregulating c-FLIP and/or survivin [48]. On the other hand, a previous study suggested that knockdown of ESR1 induces autophagy, leading to promoting ROS-induced apoptosis in breast cancer cells [49]. NOS3, a redox regulator enzyme, initiates ROS production, which, in turn, promotes apoptosis by upregulating caspase-8 and disrupting the mitochondrial membrane [50]. We presume that α -pinene and d-limonene are involved in those proteins that cause TNBC cancer cell death.

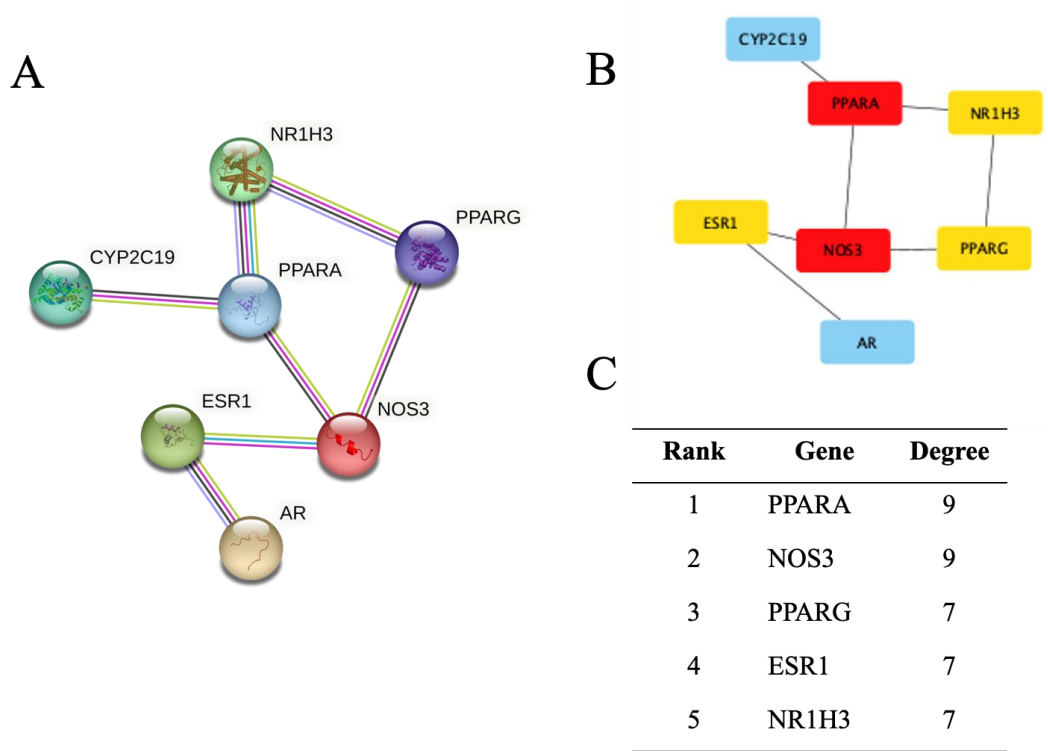


Figure 6 Protein interaction network of α -pinene and d-limonene and TNBC. (A) The protein-protein interactions of CPTG were established in STRING. (B) The top five CPTG genes were selected based on the highest degree score in Cytoscape-cytohubble. (C) The ranking is based on the top five identified CPTG genes.

Survival analysis of CPTG in TNBC patients

Furthermore, using the UALCAN TCGA web tools, we performed the survival analysis using overall survival parameters with a median cut-off. We plotted the PPARA, NOS3, PPARG, ESR1, and NR1H3 genes into a Kaplan-Meier graph. In a total of 115 TNBC patients, elevated expression of these genes is associated with reduced overall survival over a 4,000-day period (**Figure 7**). Low PPARG expression (log-rank test, $p = 0.44$), low PPARA expression (log-rank test, $p = 0.98$), and Low NR1H3 expression (log-rank test, $p = 0.023$) were all significantly associated with poor prognosis for TNBC. However, we also found that high ESR1

expression (log-rank test, $p = 0.096$) and high NOS3 expression (log-rank test, $p = 0.015$) were associated with lower survival. These bioinformatic data provide valuable insights into the specific mechanism of this α -pinene and d-limonene combination, which differs from that of existing chemotherapeutic agents.

We concluded that experimental studies should be conducted to complement these detailed bioinformatic analyses and establish the therapeutic target(s) for the combination of α -pinene and d-limonene in TNBC. Our findings provide valuable insights for further research into the putative biological mechanisms underlying the killing of TNBC cells by α -pinene and d-limonene.

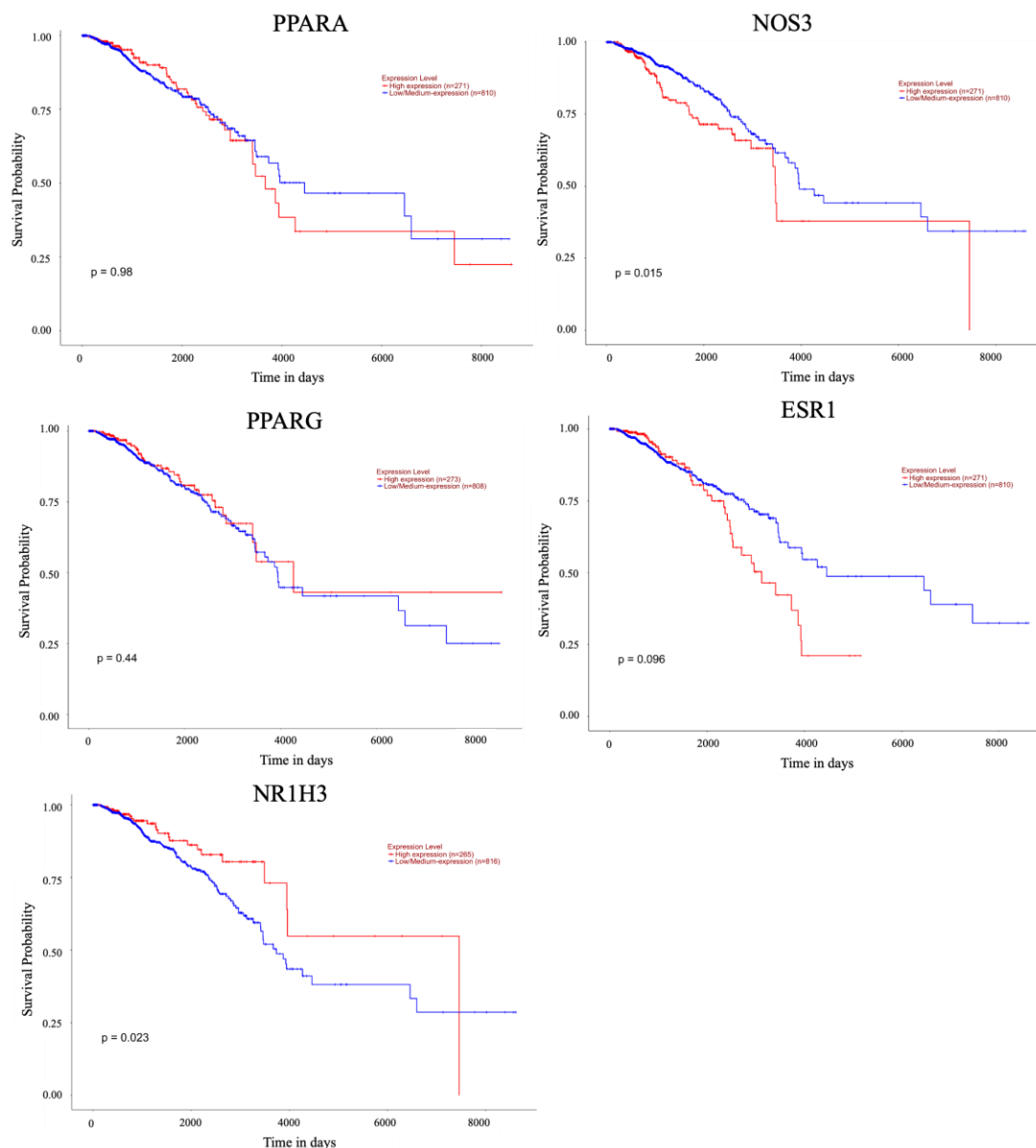


Figure 7 Overall survival analysis of PPARA, NOS3, PPARG, ESR1, and NR1H3 genes in TNBC patients.

Although this study is a step towards understanding the mechanism of the combination of α -pinene and d-limonene in TNBC, it was unavoidably subject to limitations. A limitation of this study is that the combination doses of α -pinene (0.84 $\mu\text{L/mL}$) and d-limonene (1.2 $\mu\text{L/mL}$) were not assessed at higher doses. However, our results are consistent with the highest cell death and ROS induction observed with the combination dose used in this study.

In combination, these findings indicate that both α -pinene and d-limonene have significantly cytotoxic effects separately against MDA-MB-231 TNBC cells (IC_{50} 0.84 and 1.20 $\mu\text{L/mL}$), being synergic in a very high level ($\text{CI} < 0.1$) when jointly applied. This additive

cytotoxicity is associated with marked ROS overproduction compared with single treatments and is even comparable to that of doxorubicin, thereby confirming oxidative stress as a leading cause of cell death. Bioinformatics analyses support these results by identifying 7 overlapping target genes (CPTG) enriched in the PPAR signaling pathway, a previously reported survival mechanism in TNBC. The topmost hub genes (PPARG, NOS3, ESR1, PPARA, and CYP19A1) identified through PPI network analysis demonstrate prognostic relevance in the TCGA data set, with overexpression consistently associated with poor survival. The multitarget mechanism includes ROS-mediated inhibition of the PPAR pathway, hub gene

regulation, and apoptosis induction via caspase activation, Bcl-2 downregulation, and mitochondrial damage. Overall, these convergent *in vitro* and computational findings establish that the α -pinene/d-limonene combination is a potential multitarget therapeutic candidate for TNBC, exploiting oxidative stress vulnerability and disrupting key survival signaling networks. Direct target engagement and *in vivo* efficacy should be validated in follow-up studies to drive clinical translation. This study was limited to one TNBC cell line, and no normal breast epithelial cells were included to assess selectivity. In addition, the proposed ROS-related mechanism was not experimentally validated with a scavenger or downstream molecular assays. The bioinformatics findings should therefore be viewed as predictive, and further *in vivo* and mechanistic studies are needed to confirm the therapeutic potential of the α -pinene and d-limonene combination.

Conclusions

In conclusion, our study found that α -pinene and d-limonene exhibited strong cytotoxicity against TNBC cells by increasing ROS levels. Overall, using a bioinformatics approach, we predict that the genes PPARA, NOS3, PPARG, ESR1, and NR1H3 play a major role in TNBC cell death.

Acknowledgements

The authors appreciate all who have contributed during the research and the making of this manuscript. This research is funded by Universitas Negeri Semarang, Jawa Tengah 50229, Indonesia through the 2021 Dana Penelitian Terapan DIPA grant and LPDP for publication support.

Declaration of Generative AI in Scientific Writing

During the preparation of this work, the author(s) used Grammarly and ChatGPT solely for grammar checking, language polishing, and final proofreading of the manuscript. After using this tool/service, the author(s) carefully reviewed, edited, and verified all content for scientific accuracy, factual correctness, and integrity. The authors take full responsibility and accountability for the final content of the published article, including all interpretations, data presentation, and conclusions.

CRedit Author Statement

Nanik Wijayati and **Nur Dina Amalina:** Conceptualization; Methodology; Software. **Widhi Mahatmanti** and **Nur Dina Amalina:** Data curation; Writing - Original draft preparation. **Retno Ariadi Lusiana:** Visualization; Investigation. **Nanik Wijayati** and **Roswanira Abdul Wahab:** Supervision. **Nur Dina Amalina:** Software; Validation. **Nanik Wijayati** and **Nur Dina Amalina:** Writing - Reviewing and Editing.

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