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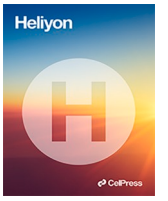

Research article

The therapeutic potential and molecular mechanism of *Alpha-pinene, Gamma-terpinene*, and *P-cymene* against melanoma cells

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ABSTRACT

The purpose of this study is to investigate the potential anticarcinogenic effects of three phytochemicals, namely *Alpha-pinene* (AP), *Gamma-terpinene* (GT), and *P-cymene* (PC), on melanoma cells (A2058 cell line). Additionally, the study aims to explore the synergistic activities of these phytochemicals with Dacarbazine, a chemotherapy drug. To understand the molecular mechanism involved in apoptosis induction in the A-2058 cell line, it was used AO/EB staining for apoptosis detection and cell cycle analysis, monitored through flow cytometry. It also determined the mRNA expression levels of different apoptosis-regulatory genes, including *p53, Bax, NF-kB, Bcl-2, Bcl-xl*, and caspase-3. The antitumor activities of these phytochemicals and their combinations were investigated in a subcutaneous mouse tumor model. The tumor diameter was 21.4 ± 1.1 mm in the Dacarbazine treatment group and 42.4 ± 3.1 mm in the control group. The antitumoral activities of AP and PC in the tumor model were similar to those of Dacarbazine. On the other hand, GT exhibited remarkable antitumoral activity, with a 1.75-fold reduction in tumor diameter compared to the Dacarbazine group. When different combinations of phytochemicals and Dacarbazine were used, the GT plus Dacarbazine treatment group was found to have a 3.5-fold reduction in tumor diameter compared to the Dacarbazine group. The tumor diameters in the Dacarbazine, AP plus GT, GT plus Dacarbazine, and AP plus Dacarbazine treatment groups were 21.4 ± 1.1, 7.6 ± 2.2, 8.6 ± 0.5, and 6.2 ± 1.9 mm, respectively.

1. Introduction

Melanoma is the third most common type of skin cancer after basal cell carcinoma and squamous cell carcinoma. Melanoma is one of the most common types of malignancies in both men and women. Aging, mitochondrial degradation, and impaired telomere function are important risk factors for developing skin cancer. Therefore, older adults have a higher risk of skin cancer due to

Abbreviations: DAC, Dacarbazine; AP, *Alpha-pinene*; GT, *Gamma-terpinene*; PC, *P-cymene*; A-2058, Human Melanoma Cell Line; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, Dimethyl sulfoxide; PCR, Polymerase chain reaction; RT-PCR, Real-Time Polymerase Chain Reaction; qPCR, Quantitative Polymerase Chain Reaction.

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Table 1
Sequences of the oligonucleotide primers used in this study and their expected fragment sizes.

Primers	Sequences	Size	Reference
Bax	5′-TGGCAGCTGACATGTTTTCTGAC-3′ 5′-TCACCCAACCACCCTGGTCTT-3′	195	[20]
Bcl-xl	5′-TCCTTGTCTACGCTTTCCACG-3′ 5′-GGTCGCATT GTGGCCTTT-3′	62	[21]
Bcl-2	5′-CATGTGTGTGGAGAGCGTCAA-3′ 5′-GCCGGTTCAGGTACTCAGTCA-3′	83	[22]
Casp-3	5′-ACAAATGGACCTGTTGACCTGA-3′ 5′-ACACCACTGTCTGTCTCAATGC-3′	146	[23]
NF-kB	5′-AAAGACACATCCGGACCTCG-3′ 5′-TGTAAGAGTTCCCCTCCGGT-3′	118	Designed by authors
p53	5′-CCCATCCTCACCATCATCACAC-3′ 5′-GCACAAACACGCACCTCAAAG-3′	80	[24]
GAPDH	5′-TGCACCACCAACTGCTTAGC-3′ 5′-GGCATGGACTGTGGTCATGAG-3′	87	[25]

weakened immunity. Thousands of melanoma patients die every year due to the lack of appropriate treatment methods [1–3]. Exposure to UV rays is also a significant risk factor for developing skin cancer. UV rays can cause mutations in DNA and lead to melanoma. Other factors such as skin hair color, freckles, hereditary factors, and the number of moles on the body may also play a role in melanoma formation. Melanoma is associated with a devastating pathophysiology, mainly due to the uncontrolled proliferation of melanocytes. However, if not treated early, it can spread to different organs through metastasis. Treatment inadequacy or failure in melanoma leads to higher mortality rates worldwide. Among skin cancers, cutaneous metastatic melanoma is the most devastating type of cancer, with a five-year overall survival rate [2]. Although treatment methods such as surgery, chemotherapy, and radiotherapy are used, the success of the treatment is low. Although various monoclonal antibody treatments have been focused on in recent years, their success rates are debated due to the chemoresistance of melanoma cells [1,4]. Treatment methods against cancers, including melanomas, cause non-selective toxicity to many tissues or organs [1]. Due to the low effectiveness of existing chemotherapeutics, research on new effective drugs in melanoma is carried out intensively. Most of the studies conducted in this field include herbal and natural products (see Table 1).

Phytochemicals are compounds found in plants that show promise in cancer therapy. They can counteract the adverse effects of chemotherapy, target metastasis, and inhibit tumor formation. Phytochemicals have various inhibitory effects on cancer cells, such as cell proliferation, tumor growth, angiogenesis, metastasis, and inflammation, and can also induce cellular apoptosis. Additionally, phytochemicals can modulate immunity and protect normal cells from free radical damage. Most research on the anti-cancer mechanisms of phytochemicals has focused on individual compounds. However, some studies have shown that combining phytochemicals with chemically similar or different compounds can enhance their anti-cancer efficacy and broaden their scope of action [5,6].

The combined use of plant-based compounds and chemotherapeutic drugs in non-toxic doses can lead to increased cytotoxicity, induction of apoptosis, and enhanced anti-proliferative potential. Combinations of these compounds have shown potential in improving cancer treatment. The chemical compositions of many plant essential oils contain high levels of monoterpenes, which have significant pharmacological activities, particularly antitumoral efficiency [5]. Dacarbazine is a type of chemotherapy drug that damages the DNA of cancer cells, thereby preventing them from growing and dividing. It is mainly used to treat metastatic malignant melanoma. Still, it can also be used in combination with other drugs to treat soft tissue sarcoma, neuroblastoma, Kaposi's sarcoma, and different types of tumors. However, the drug's high toxicity and low specific efficacy can lead to significant side effects, which limit its use for some patients [7].

Numerous studies suggest that phytochemicals or natural compounds possess anticarcinogenic properties and can effectively combat various cancer cells [8–11]. Combining phytochemicals and cancer drugs has created significant synergistic activity, which can prove crucial in developing new treatment protocols for cancer chemotherapy. Limited clinical studies have reported that phytochemicals exhibit synergistic effects with conventional chemotherapeutic agents and radiotherapeutic applications and have even shown positive results in humans [12,13].

Combined therapies can be a practical approach for treating cancer cells or harmful pathogens, boosting the host's immune system, or activating repair mechanisms. In modern medicine, using a single-drug treatment is no longer preferred. Researchers have been exploring the potential benefits of combining chemotherapeutic drugs with natural products, intending to improve the treatment's efficacy while reducing possible side effects. In recent years, various studies have been conducted worldwide to investigate the effectiveness of phytotherapeutic agents [14–17].

New research studies on drugs are of utmost importance because of the side effects that come with conventional cancer treatment and the growing resistance to such treatment. Several studies have focused on the anticarcinogenic activity of natural products or herbal extracts. However, no comprehensive research has looked into the in vitro activities of AP, GT, and PC against melanoma, lung, and breast cancer cells and their in vivo activities in a subcutaneous mouse tumor model.

This study aimed to investigate the anti-cancer effects of AP, GT, and PC alone and combined with Dacarbazine against melanoma cells.

The study has the following objectives.

- To investigate the cytotoxic effects of AP, GT, and PC on melanoma cells.
- To evaluate the effects of phytochemicals or combinations found to be effective on cancer cells on the expression of some apoptotic and anti-apoptotic genes (*p53, Bax, NF-kB, Bcl-2, Bcl-xl*, and *caspase-3*).
- To evaluate the antitumor activities of these phytochemicals and their combinations found to be effective on melanoma cells in a subcutaneous mouse tumor model.

2. Experimental

2.1. Cell culture

Studies performed on cancer before clinical applications are called in vivo cancer studies. These studies are more realistic models that contain most cancer features. Although xenotransplantation models are relevant in vivo for studying tumor effects, they cannot substitute for in vivo studies. Therefore, further activity studies were conducted in a subcutaneous tumor model created with the B16F-10 cell line. The Vero cell line was selected, for which non-toxic concentrations were determined and applied in the study. Vero (African green monkey kidney cells) (ATCC CCL-81) was used as a healthy cell line, and A-2058 (human melanoma cells; ATCC-CRL-11147) cells were used as a cancer cell line.

Non-toxic concentrations of the phytochemicals were determined in the Vero cell line. RPMI-1640 containing 10 % fetal bovine serum (FBS), ten mM HEPES, 100 IU/ml penicillin/streptomycin with four mM glutamine was used as cell culture medium. Cell lines were cultivated in a humid incubator at 37 °C, with 5 % CO_2. Cell density in proliferation and activity experiments adjusted to 1×10^6 cells/ml. Cells were harvested at appropriate times for the planned tests and used for ELISA, flow cytometry, and immunofluorescence assay.

2.2. Phytochemicals

AP, GT, and PC (Sigma-Aldrich, USA) were commercially available. Dimethyl sulfoxide (DMSO, Merck) was used as the solvent to dissolve the phytochemicals in the medium. Dacarbazine (Kocak, Farma, TR) was chosen as the standard drug in the experiments. Activation of dacarbazine was performed as reported in the literature [18]. DMSO was used as a solvent to dissolve phytochemicals and Dacarbazine. A 1 % concentration of DMSO was selected for this purpose.

2.2.1. Cytotoxicity assay

Phytochemical toxicity tests were performed using the MTT method in the Vero cell line [19]. The Vero cell line, a reliable indicator, was used to determine non-toxic phytochemical concentrations. Vero cells (1×10^6 cells/well) were treated by adding different concentrations of phytochemicals (0.018, 0.035, 0.07, 0.14, 0.28, 0.56, 1.125, 2.25, 4.5, 9, and 18 mM) to the cell growth medium (RPMI-1640 with 5 % FBS).

The wells containing no phytochemicals were selected as the control group. Cells were incubated at 37 °C in a humidified 5 % CO_2 incubator for 24 h. After incubation, MTT (20 μL, 5 mg/ml) was added to each well, and the cells were incubated for 2 h. Then, the plate wells were aspirated with MTT. DMSO (100 μL) was added to the wells, and the plates were shaken for 5 min. Finally, the absorbance of each well was measured at 540 nm in a microtiter plate reader. Experiments were repeated three times for each sample.

2.2.2. Determination of non-toxic concentration of DMSO

In the experiments, DMSO was used to dissolve the phytochemicals in the medium. To determine the non-toxic concentration of DMSO in the cells, Vero cells were treated with different concentrations of DMSO in the medium at 1×10^5 cells/ml. The experiments unequivocally demonstrated that DMSO did not have any toxicity to cell reproduction or viability at concentrations of $\leq$2 % (w/v). The 1 % DMSO concentrations were selected as a solvent to dissolve phytochemicals in the medium.

2.3. RNA isolation

Cells were harvested from the cell cultures before and after activity experiments. Then, total RNA extractions were performed using highly pure RNA Tissue Kits (Thermo Fisher Scientific, ABD). The commercial company protocol used the deoxyribonuclease I enzyme to prevent DNA contamination. RNA isolation was checked by running agarose gel electrophoresis. Then, to synthetic cDNA, each sample was reverse transcribed into complementary DNA (cDNA) using the Thermo Scientific cDNA Synthesis Kit (Thermo Fisher Scientific, ABD). The resulting cDNA was stored at −20 °C until use.

2.4. RT-qPCR application

After cDNA synthesis, the expression levels of the *Bax, Bcl-xl, Bcl-2, Caps-3*, *NF-kB*, and *p53* genes were determined. The target gene was amplified using 10 μl of cDNA sample from each sample. For real-time PCR experiments, SYBR Green dye was used according to the manufacturer's instructions. Real-time PCR reactions were performed by a real-time PCR system (Rotor-Gene Q-QIAGEN) with the following conditions: one denaturing step at 95 °C for 10 min, followed by 35 cycles consisting of denaturing at 95 °C for 15 s and annealing and elongation at 60 °C for 60 s, followed by an inactivation step of 10 min at 99.9 °C. Each sample was carried out in triplicate, and the *GAPDH* gene was used as a reference gene. The oligonucleotide sequences of the primers used are given in Table 1

[20–25].

2.5. Determination of Caspase-3 (Casp-3) concentration

The caspase three activity test, a crucial step in our experiment, involves binding the colored p-NA molecule released from the cell lysate to the Ac-DEVD-p-NA substrate. Caspase-3 activity was calculated using the caspase-3 colorimetric assay kit (Human Caspase 3 ELISA Kit (Bioassay Technology Laboratory, China).

The effects of *Alpha-pinene, Gamma-terpinene*, and *P-cymene* on caspase-3 activity on A-2058 cells were evaluated and compared with Dacarbazine (10 μM). A-2058 cells and control group cells were collected after being treated with phytochemicals, and the cells were washed with PBS and then resuspended with lysis buffer (50 mM HEPES pH7.4, five mM CHAPS, five mM DTT) for 15 min. The concentration of the lysis buffer was adjusted to 1×10^7 cells per 100 μl. Lysed cells were centrifuged at 16,000×*g* for 15 min at +4 °C. Lysate protein concentration was determined using the Bradford method. Equal amounts of protein (approximately 20 μg) from each sample were added to the wells containing test buffer (20 mM HEPES, pH 7.4, 0.1 % CHAPS, five mM DTT, two mM EDTA) and 10 μl Ac-DEVD-p-NA (20 mM) added. The total volume was completed to 100 μl in each well. Caspase-3 activity measurements were calculated by reading at a wavelength of 405 nm on a spectrophotometer.

2.6. Cell cycle analysis

For DNA content analysis, A2058 cells were treated with these phytochemicals. At the end of incubation, cells were collected, washed, and resuspended 48 h later. Cells were treated with ethanol (75 %) overnight for fixation. Cells were centrifuged and exposed to RNase (100 μl) for 30 min at 37 °C. Cells were incubated with propidium iodide (PI) (400 μl) for 30 min in the dark to stain their DNA. DNA contents were analyzed using flow cytometry and appropriate software. DNA histograms in the G0-G1, S, and G2-M phases were identified, and each phase's ratio was determined.

2.7. AO/EB staining

Alpha-pinene (300 μM), Gamma-terpinene (300 μM), and P-cymene (200 μM) were added to the pre-prepared cell cultures for the detection of morphological changes, and incubated at 37 °C. At the end of the incubation, the cells were washed three times for 2 min with PBS solution. Then, 1 ml of 70 % ethanol was added to the cells and kept at room temperature for 5 min. After this procedure, cells were incubated for 5 min at room temperature with a mixture of 200 μl of acridine orange (100 μg/ml) and ethidium bromide (100 μg/ml) (Sigma) prepared in PBS. Cells were examined under an immunofluorescent microscope (Nikon, Japan), and the number of viable, apoptotic, or necrotic cells was calculated. Three hundred cells per sample were counted, reporting evaluations as viable, apoptotic, or necrotic [26].

Then, viable cells (green-stained chromatin), apoptotic cells (condensed and fragmented green-stained chromatin), normal nucleated dead cells (necrotic cells homogeneously stained orange chromatin), and dead cells with apoptotic nuclei (condensed and fragmented orange-stained chromatin) were counted in the preparation. The apoptotic index was calculated according to the formula below [26].

Apoptotic index = (Number of Apoptotic Cells + Number of Dead Cells with Apoptotic Nuclei)/Number of Viable Cells + Number of Apoptotic Cells + Count of Normal Nuclear + Dead Cells with Apoptotic Nuclei) × 100.

A2058 cells were inoculated in a 24-well plate exposed to various concentrations of the phytochemicals (AP, GT, and PC) for 24 h. At the end of the incubation, the cells were washed 3 times for 2 min with PBS solution. Then, 1 ml of 70 % ethanol was added to the cells and kept at room temperature for 5 min. After this procedure, cells were incubated for 5 min at room temperature with a mixture of 200 μl of acridine orange (100 μg/ml) and ethidium bromide (100 μg/ml) (Sigma) prepared in PBS. Cells were examined under an immunofluorescent microscope (Nikon, Japan), and the number of viable, apoptotic, or necrotic cells was calculated [26].

2.8. In vivo tests for tumor growth

This study has the ethics committee approval of Hatay Mustafa Kemal University Animal Experiments Local Ethics Committee dated December 27, 2018 and numbered 2018/11-9. Animal experiments were conducted at Hatay Mustafa Kemal University Experimental Research and Application Center. BALB-c female mice were used in the experiments. Mice were randomly divided into groups. All mice in the study were fed a standard diet and water ad libitum throughout the experiments. According to international animal experimentation and care guidelines, mice were monitored in standard laboratory conditions (temperature 23 ± 2 °C, 12 h day/night cycle) in cages with free access to standard pellet feeds and water. The efficacy of the phytochemicals and their different combinations was investigated in a subcutaneous mouse tumor model to determine whether the phytochemicals regulate tumor growth. B16-F10 cells (2×10^5/mouse) were injected into the left and right dorsal flank of BALB/c mice, which were 6–8 weeks of age.

When the mice exhibited a palpable tumor (approximately one week after tumor cell injection), they were randomly divided into ten groups (n = 5 animals/group): 1) Healthy control group: Mice in this group were not injected with B16-F10 cells. 2) Cell control group: Mice were injected with B16F10 cells but not treated. 3) DMSO group: Mice in this group were injected with DMSO (at the ratio chosen as solvent). 4) Standard drug group: Mice were treated with Dacarbazine (10 μM). 5) PC treatment group: Treatment was administered with PC (60 mM). 6) GT treatment group: Treatment was administered with GT (60 mM). 7) AP treatment group 3: Treatment was with AP (20 mM). 8) AP plus Dacarbazine treatment group: "AP (20 mM) plus Dacarbazine (10 μM)" combination was

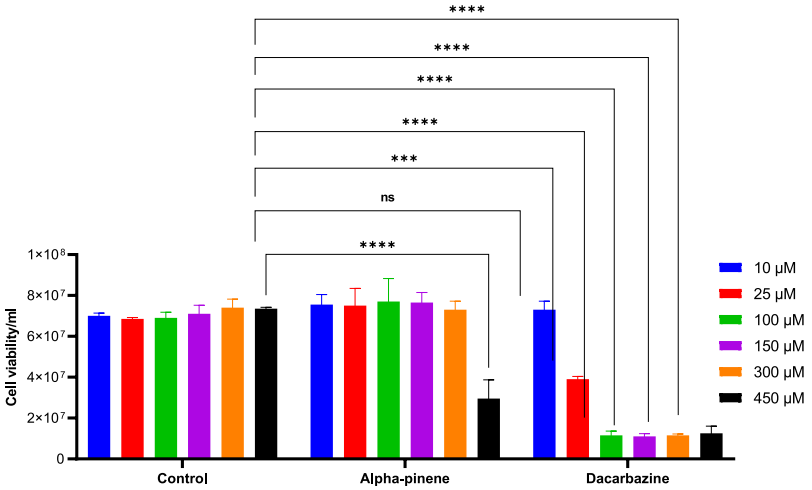

Fig. 1a. Non-toxic concentrations of *Alpha-pinene* on Vero cells compared to Dacarbazine.

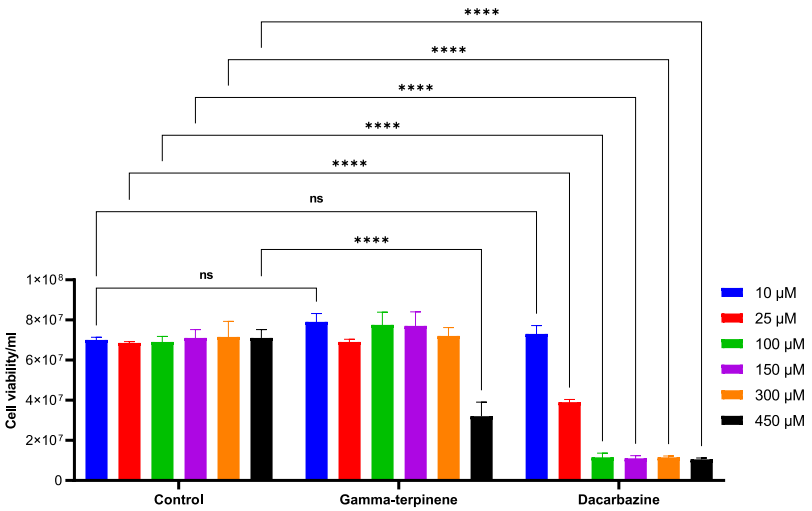

Fig. 1b. Non-toxic concentrations of *Gamma-terpinene* on Vero cells compared to Dacarbazine.

applied for treatment. 9) GT plus Dacarbazine treatment group: "GT (60 mM) plus Dacarbazine (10 µM)" combination was applied for treatment. 10) AP plus GT plus PC plus Dacarbazine treatment group: "The combination of "AP (20 mM) plus GT (60 mM) plus PC (60 mM) plus Dacarbazine (10 µM)" was administered for treatment.

2.9. Dissection and histological-pathological evaluations

At the end of the treatment protocols, mice were euthanized with 100 µL of a 10:1 mixture of ketamine (100 mg/mL) and xylazine (100 mg/mL) by injection into the lateral tail vein. Histological-pathological evaluations were carried out in the Department of Pathology. The animals were gently dissected, and the subcutaneous mass, brain, kidney, spleen, heart, lung, liver, and large intestines were removed. Tissue samples were placed in a 10 % formaldehyde solution.

2.10. Preparation of tissue samples for pathological evaluations

The mouse tissue samples taken were fixed in 10 % buffered formalin. The fixed tissues were washed overnight to remove formalin under tap water. After this process, the tissues were dehydrated by immersion in 70, 80, 96, and 100 % alcohol. Tissues were cleared with xylene before embedding in paraffin. Then, tissues were embedded in paraffin, sectioned at five µm thickness from each block,

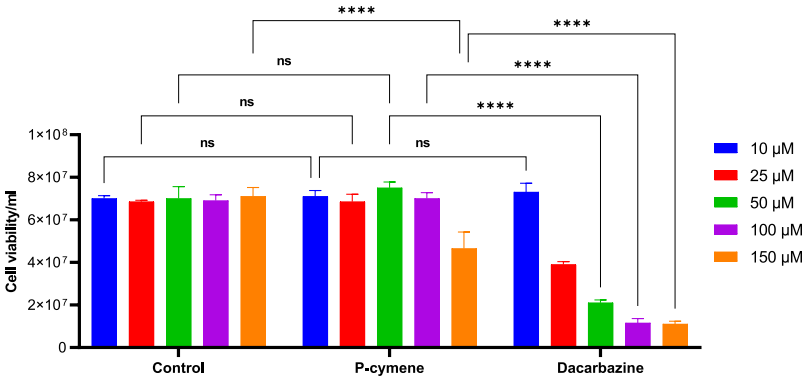

Fig. 1c. Non-toxic concentrations of *P-cymene* on Vero cells compared to Dacarbazine.

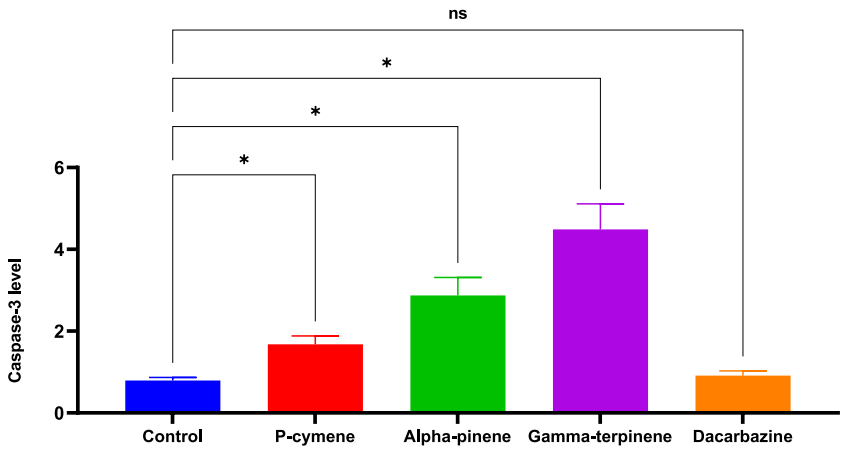

Fig. 2. Comparison of Caspase-3 activities of *Alpha-pinene* (300 µM), *Gamma-terpinene* (300 µM), and *P-cymene* (200 µM) on A-2058 cells with the control and Dacarbazine (10 µM) groups. ns: not significant.

Groups	Summary	P value
Control vs. *P-cymene*	*	0,0158
Control vs. *Alpha-pinene*	*	0,0436
Control vs. *Gamma-terpinene*	*	0,0162
Control vs. Dacarbazine	ns	0,6994

ns: not significant

deparaffinized in xylol, then passed through a series of 100 %, 96 %, 80 %, and 70 % alcohol, respectively. After the tissues were stained with Hematoxylin and Eosin (H&E), microphotographs (Olympus DP12) of the tissues were obtained under a light microscope (Olympus CX31).

2.11. Statistical analysis

GraphPad Prism software (version 10) was used for statistical analysis. The mean and standard deviation of at least three experiments were calculated. Comparisons between each group and between groups were performed with one-way and two-way ANOVA tests. Only findings with a p-value less than 0.05 were considered significant. Experiments with two subgroups were analyzed using a two-tailed unpaired *t*-test. Experiments with three or more univariate subgroups were analyzed using one-way ANOVA. Post hoc analysis was performed using Tukey's multiple comparison tests between two groups. A two-way ANOVA was performed for multivariate experiments. Post hoc analysis was performed using Tukey or Sidak's multiple comparison tests. Each statistical test is explained in figure or table descriptions. Only data containing $p < 0.05$ were considered statistically significant. Symbols for different test significance levels are assigned as follows: $p > 0.05$, *$p < 0.05$, **$p < 0.001$, *** not significant for $p < 0.0001$, and ****$p < 0.00001$ (ns). Data were expressed as mean ± SD. The sample size was $n \geq 3$ and contained biological duplicates.

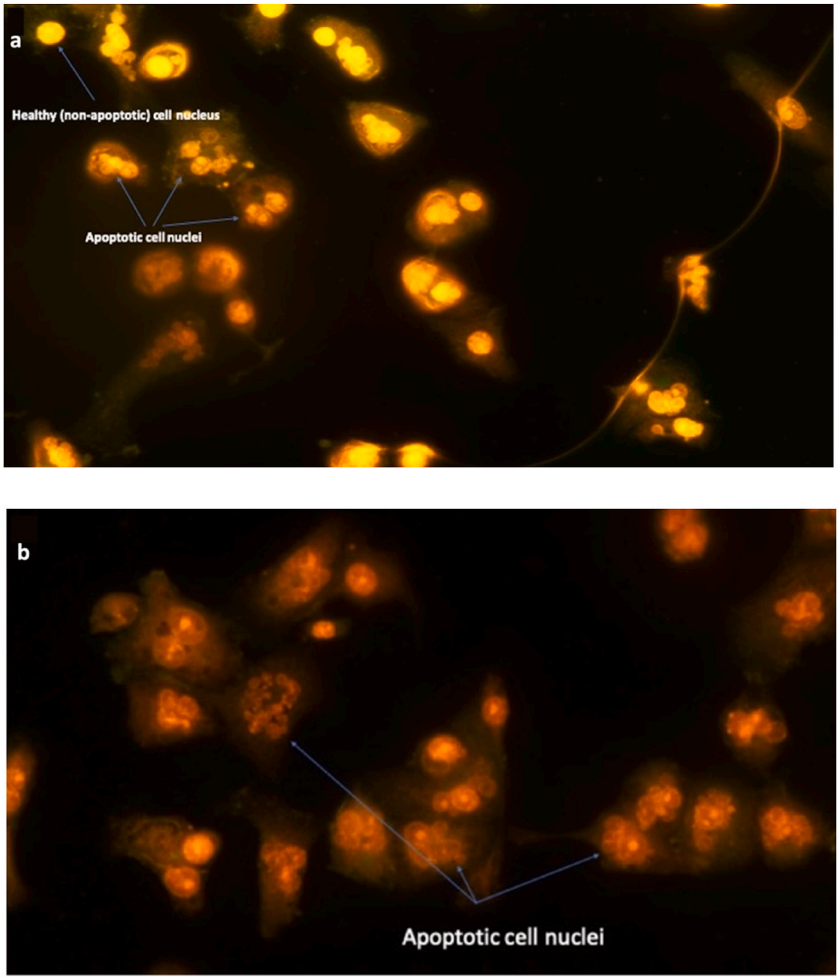

Fig. 3. a–b. Morphological changes in cells detected by Acridine-Orange staining method. A. Appearance of healthy and apoptotic cell structures. B. Apoptotic cell nuclei. Cells undergoing apoptosis were demonstrated in comparison with healthy cells using the acridine orange staining method. A crucial aspect of our study was comparing cells undergoing apoptosis to healthy cells, demonstrated using the acridine orange staining method.

3. Results

3.1. Non-toxic concentrations of phytochemicals (AP, GT, and PC) and dacarbazine

Non-toxic concentrations of AP, GT, PC, and Dacarbazine were determined on Vero cells by calculating cell viability. Cell toxicity was performed by comparing cell viability in control groups containing the same PBS concentrations. The study involved testing the effects of different concentrations of phytochemicals and Dacarbazine (10, 25, 50, 100, 150, 300, and 450 μM) on the cells. Non-toxic concentrations were determined as 300 μM for AP and GT, 200 μM for PC, and 10 μM for Dacarbazine. These findings are summarized in Fig. 1(a–c).

3.2. Effect of phytochemicals on Caspase-3 activity

Caspase-3 is a well-defined protease with multiple roles that impact a cell's destiny. During apoptosis, it functions as an executioner caspase and plays crucial proteolytic roles, ultimately leading to the final phases of programmed cell death. Because of its pivotal role, caspase-3 is utilized as an intracellular control target of apoptosis for therapeutic purposes.

These three phytochemicals significantly increased Caspase-3 activity on A-2058 cells compared to the control and Dacarbazine groups. In particular, GT triggered Caspase-3 activity very strongly in melanoma cells. Caspase-3 activity in the dacarbazine group was 1.15 times (0.91/0.79) higher than the control group. There was no significant difference between these two groups. Whereas, P-cymene, Alpha-pinene, and Gamma-terpinene increased Caspase-3 release 1.85 (1.68/0.91)-fold, 3.14 (2.86/0.91) and 4.91 (4.47/0.91)-fold, respectively, when compared to the Dacarbazine group (Fig. 2).

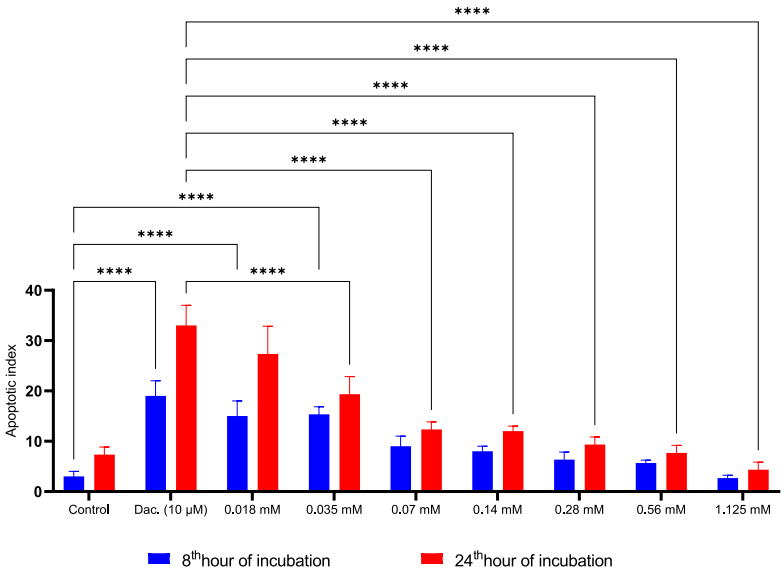

Fig. 4a. Apoptotic cell index of *Alpha-pinene* at the 8th and 24th hours on A-2058 cells.

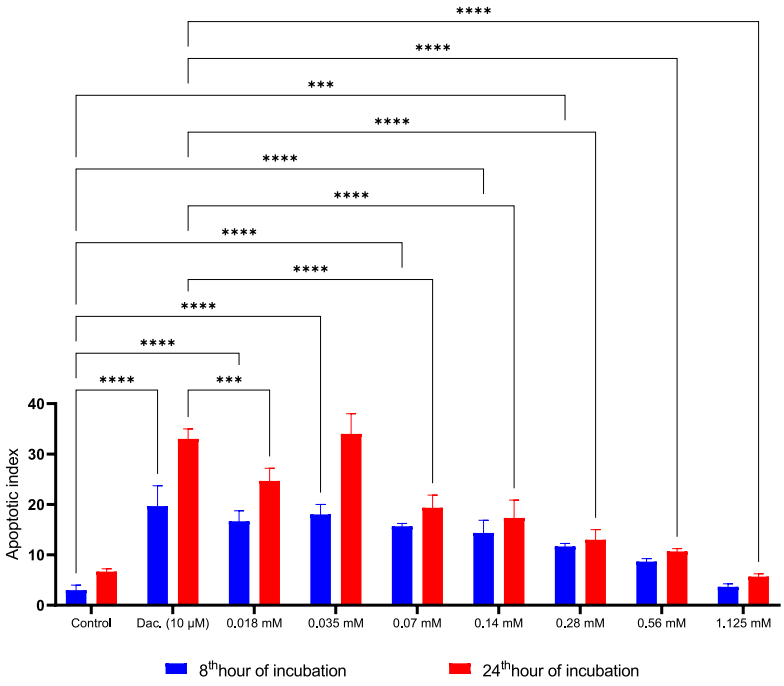

Fig. 4b. Apoptotic cell index of *Gamma-terpinene* at the 8th and 24th hours on A-2058 cells.

3.3. Determination of apoptotic effects of phytochemicals

3.3.1. Acridine orange-ethidium bromide staining method

Morphological changes in cells were analyzed using the Acridine-Orange staining method. The number of cells undergoing apoptosis increased as the exposure to phytochemicals continued. After 8 h of exposure, the cells showed morphological changes, and apoptotic structures began to develop around the cell membrane, causing atypical cell appearances (Fig. 3a and b). The apoptotic indexes of GT, AP, and PC cells ranged from 3 to 34, and the apoptotic index of phytochemicals by cell type is presented in Fig. 4a–c.

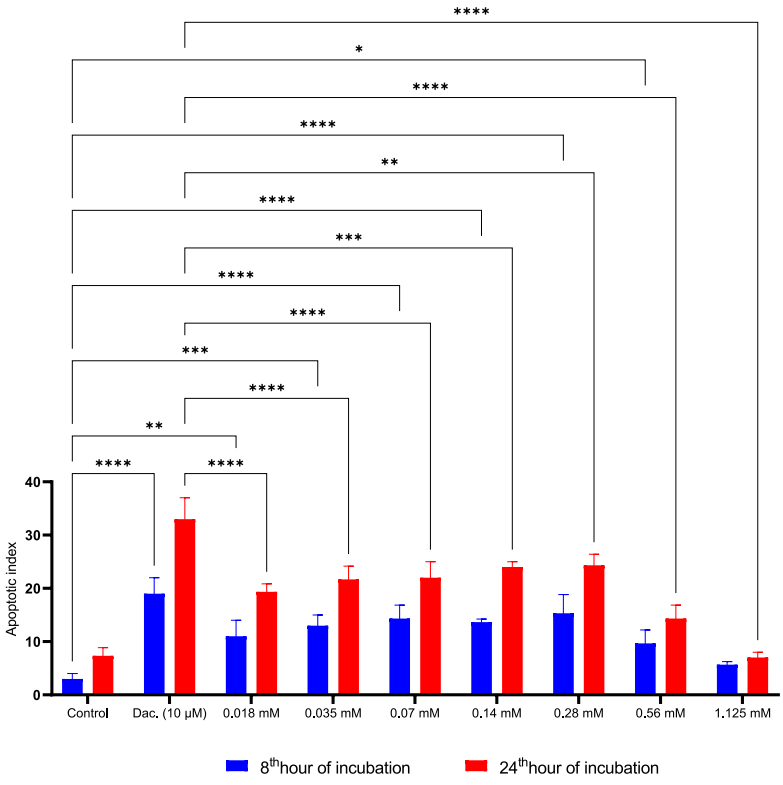

Fig. 4c. Apoptotic cell index of *P-cymene* at the 8th and 24th hours on A-2058 cells.

Although the activity of AP on A-2058 cells was lower than that of Dacarbazine, the activities of AP at concentrations of 00.19 and 0.039 µg/ml were significant. The activity of GT on melanoma cells was quite high, and the efficacy of GT at a concentration of 0.039 µg/ml was not significantly different compared to the standard drugs ($p < 0.05$; Fig. 4a–b).

Compared to Dacarbazine on A-2058 cells, the effectivity of PC was lower than that of other phytochemicals. However, at a concentration of 0.312 µg/ml of PC, melanoma cells showed a significant increase in apoptosis ($p < 0.05$; Fig. 4c).

3.4. Flow cytometric analysis

The cell cycle is a series of events that occur in a cell and lead to cell division. In eukaryotes, the cell cycle typically consists of four phases: G1, S, G2, and M phases, and each stage of cell cycle progression depends on the proper completion of the previous phases. Analyzing the cell cycle in cancer cell lines is crucial for cancer diagnosis. In this regard, cell cycle analyses were performed on cells treated with the phytochemicals AP, GT, and PC, as well as the standard Dacarbazine, and compared to a control group, which was free of any phytochemicals or drugs.

After cell passage, the cycle stages of synchronized cells were analyzed. The results showed that significant inhibition of DNA synthesis occurred in synchronized cells (document number 1) 12 h after treatment. Gamma-terpinene activity in these cells was similar to that of Dacarbazine. Most cells treated with these two molecules were in the G0/1 phase (74 % and 72 %, respectively) (Fig. 5a–b). When the cell cycle of the control group cells, which do not contain any phytochemicals or drugs, is examined, it is seen that the cycle continues the mitotic cycle by passing to the next phase, the S phase, to a large extent (43.2 %), without being inhibited in the G0/1 phase (Fig. 5c). Most cells in the control group were in the S phase, whereas those treated with Dacarbazine were in the G0/1 phase. Cell cycles of the cells treated with AP, GT, and PC were mainly inhibited at the G0/1 stage.

3.5. Gene expression results

The study evaluated the effects of AP, GT, PC, and standard cancer drugs on gene expression in experiments. Specifically, the study analyzed the impact of these phytochemicals and drugs on proapoptotic (p53, Bax, and NF-kB) and anti-apoptotic (Bcl-2 and Bcl-xl) gene expressions and compared their results.

The results showed that PC significantly inhibited the expression of the Bcl-2 gene on A-2058 cells, while AP, GT, and Dacarbazine did not affect its expression. Compared to Dacarbazine and AP, PC significantly inhibited the expression of the Bcl-xl gene. On the other hand, GT significantly increased the expression of this gene. The study also found that the combination of GT plus AP did not

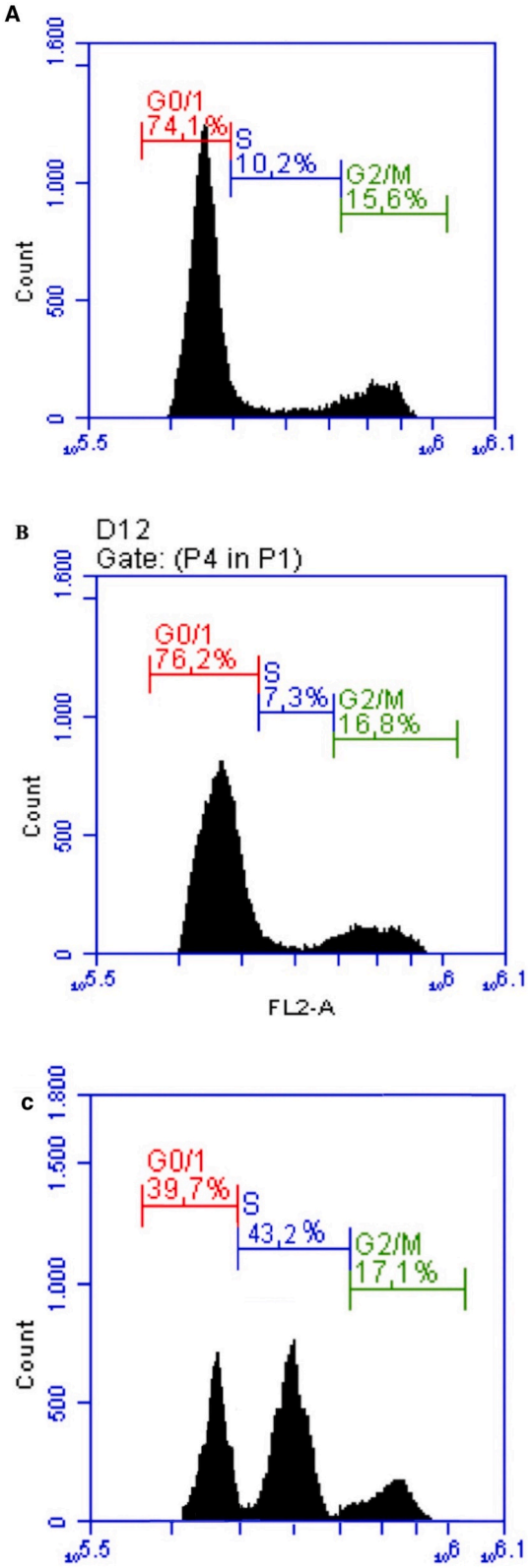

Fig. 5. a–c. Effects of the phytochemicals on cell cycles of the cancer cells. Effects of *Gamma-terpinene* on A-2058 cells (A), Dacarbazine on A-2058 cells (B), and the control group (C).

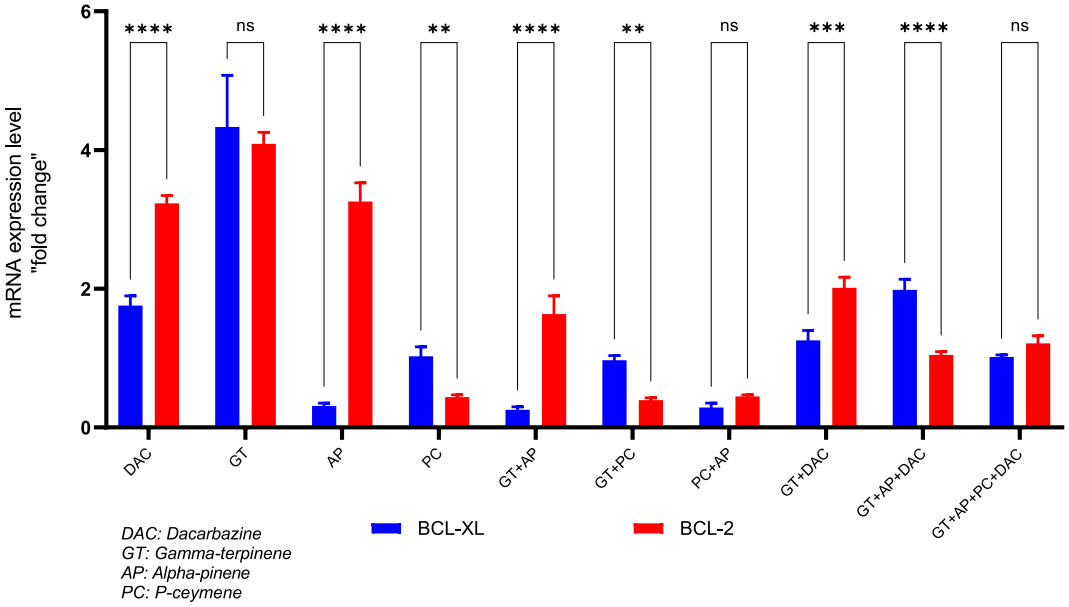

Fig. 6. Effects of the phytochemicals and their combinations on the expression levels of Bcl-2 and Bc-xl genes on A-2058 cells.

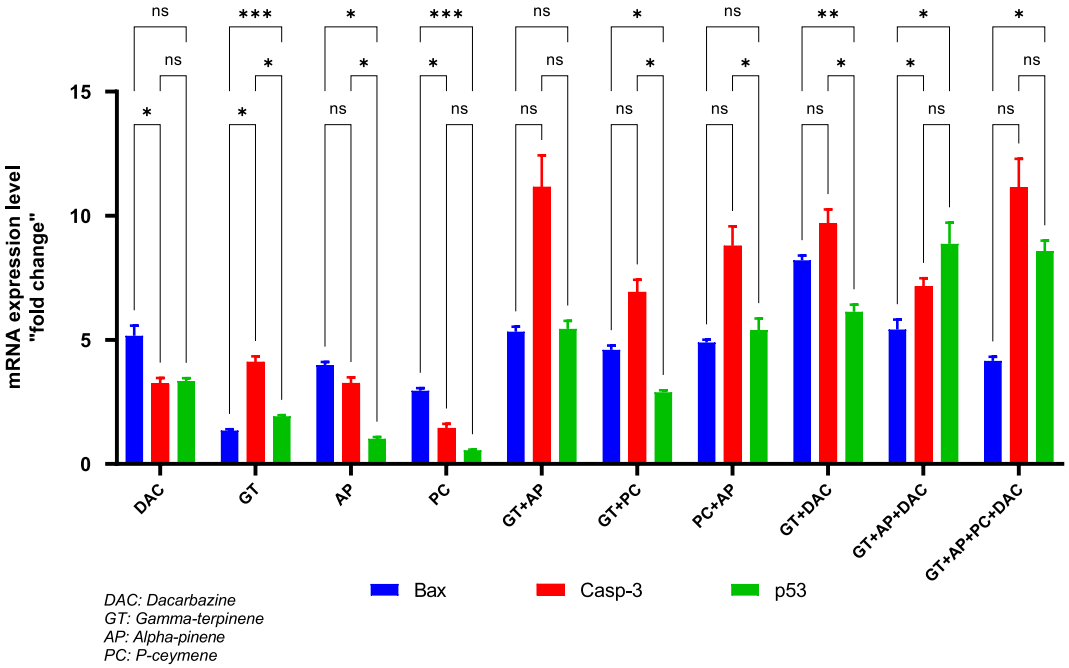

Fig. 7. Effects of phytochemicals and their combinations on the expression levels of Bax, Caspase-3, and p53 genes on A-2058 cells.

significantly inhibit the expression levels of both Bcl-xl and Bcl-2 genes compared to Dacarbazine. However, combining GT plus PC and AP notably inhibited Bcl-xl and Bcl-2 gene expressions. In the PC plus AP treatment group in A2058 cells, expressions of the Bcl-xl and Bcl-2 genes were inhibited by 6.01-fold (1.75/0.29) and 7.2-fold (3.24/0.45), respectively, compared to Dacarbazine. The study also found that the expressions of the Bcl-xl and Bcl-2 genes were significantly inhibited in the GT plus Dacarbazine treatment group compared to Dacarbazine [1.38-fold (1.75/1.27) and 1.61-fold (3.24/2.01), respectively)] (Fig. 6).

The study found that PC and AP did not affect the expression of the *p53* gene in A-2058 cells. However, GT significantly increased the expression of the *p53* gene. Although GT's efficacy was lower than that of Dacarbazine, it significantly impacted the expression level of the *p53* gene.

Regarding the Caspase-3 gene, both AP and GT significantly increased their expression level, while PC did not cause a significant

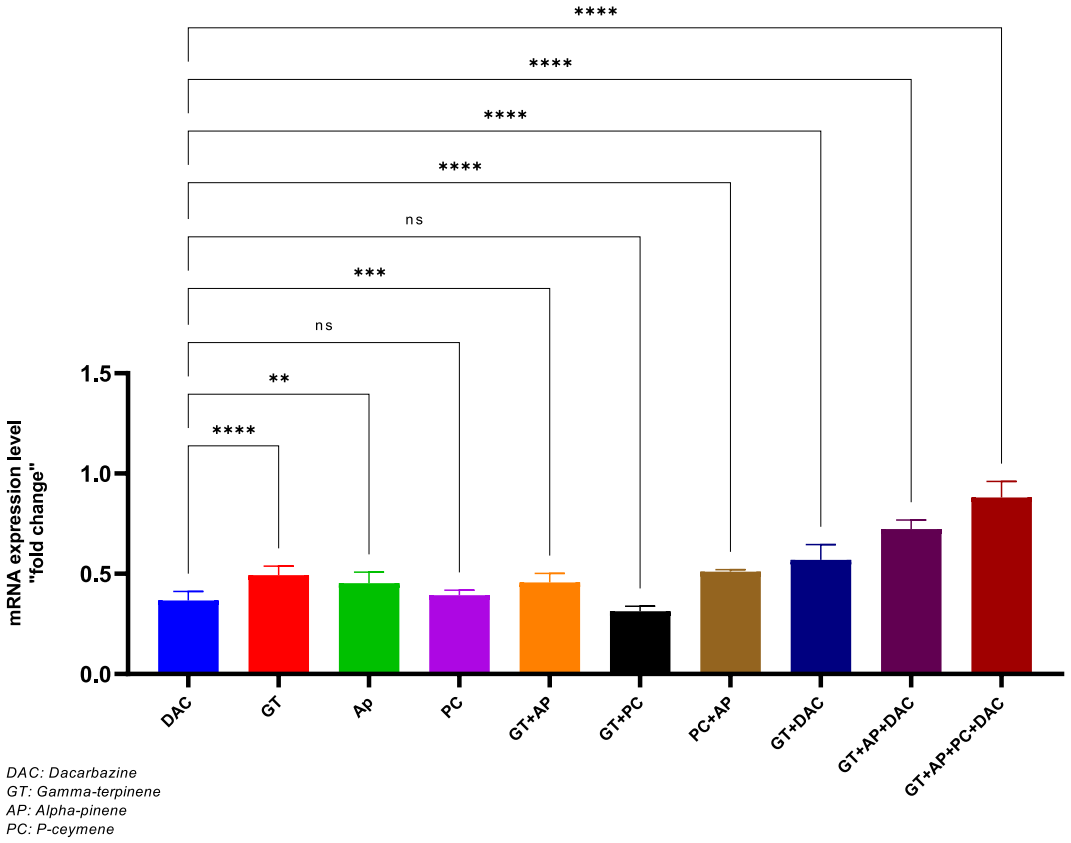

Fig. 8. Effects of phytochemicals and their combinations on the expression of *NF-kB* gene on A-2058 cells.

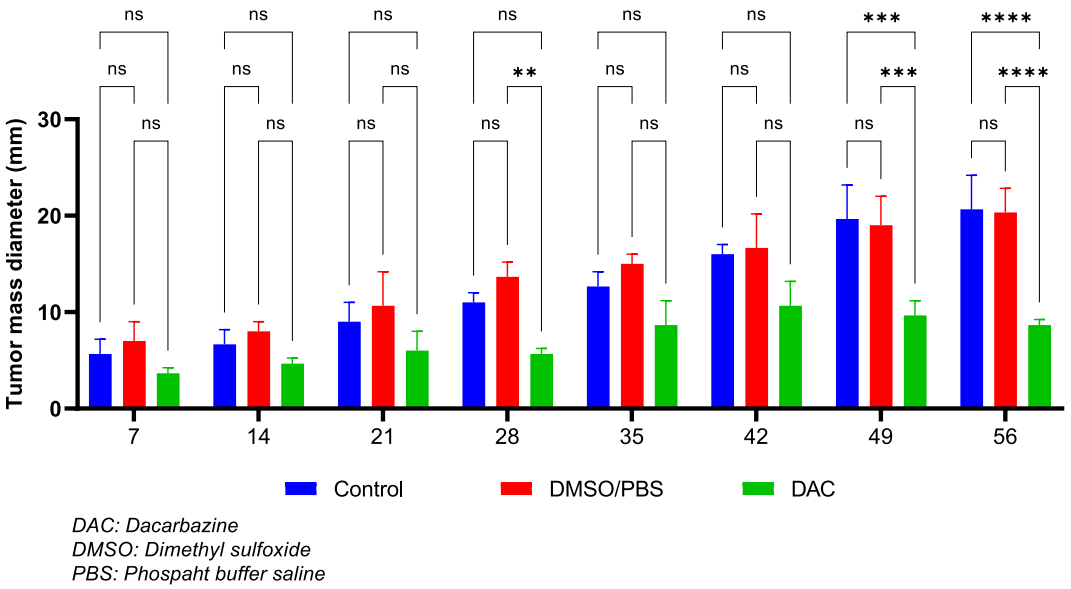

Fig. 9. Efficacy of Dacarbazine on tumor growth in the subcutaneous tumor model.

increase. The Bax gene's expression level significantly increased when treated with PC and AP, but GT increased it non-significantly. Combining GT with AP caused a statistical increase in the expression levels of the Caspase-3 and p53 genes. Meanwhile, combining GT with PC increased the expression of the Caspase-3 gene but did not affect the expression of the p53 gene. Combining GT with

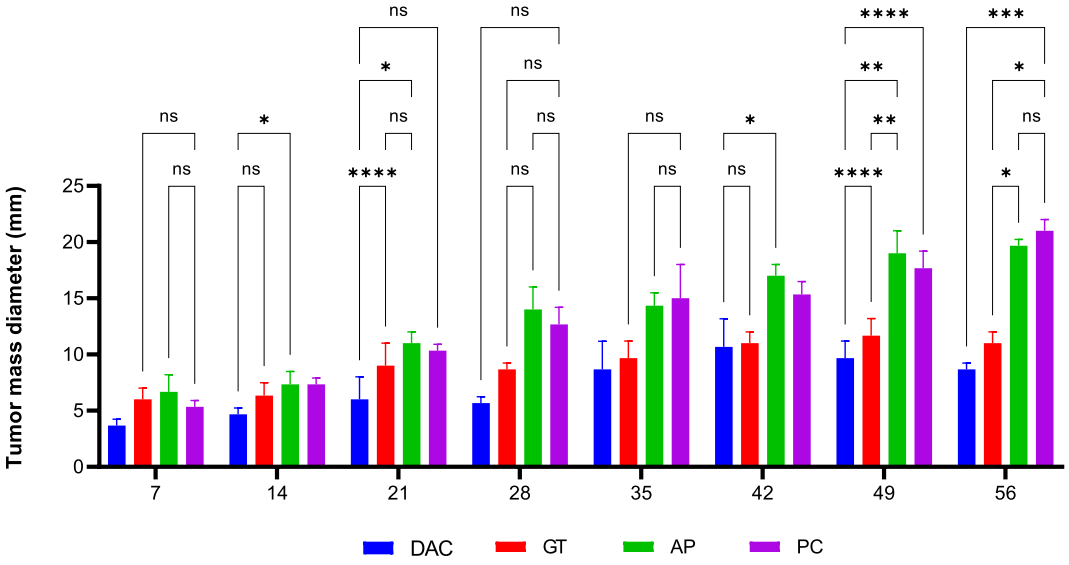

Fig. 10. Efficacy of *Alpha-pinene*, *Gamma-terpinene*, and *P-cymene* in the subcutaneous tumor model.

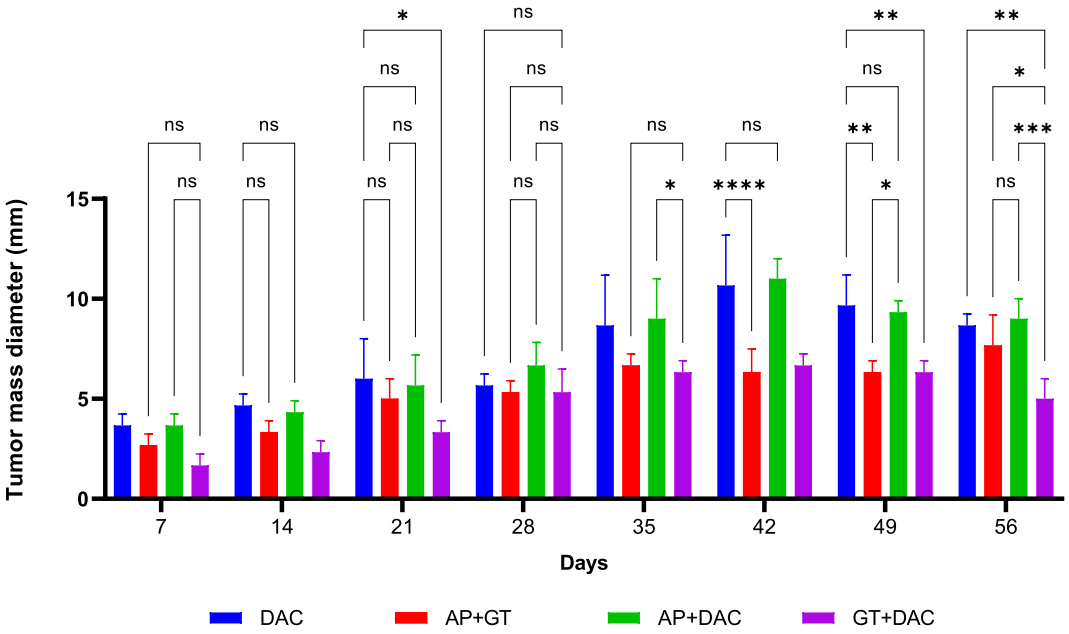

Fig. 11. Combined efficacy of *Alpha-pinene* plus *Gamma-terpinene*, *Alpha-pinene* plus Dacarbazine, and *Gamma-terpinene* plus Dacarbazine in the subcutaneous tumor model.

Dacarbazine and three phytochemicals plus Dacarbazine significantly increased the expression levels of the Caspase-3 and p53 genes.

The study also found that in A2058 cells, the combination of GT and DAC (Dacarbazine) treatment increased the expression of the *Bax* gene by 1.56 times compared to Dacarbazine treatment alone. Similarly, the *p53* and Caspase-3 gene expression increased 1.84-fold and 3.03-fold in the GT plus DAC treatment group (Fig. 7).

Three phytochemicals were found to significantly increase the expression of the NF-kB gene in A-2058 cells in comparison to Dacarbazine. The expression of the NF-kB gene was further increased in the groups that received combinations of GT plus AP plus Dacarbazine and GT plus AP plus PC plus Dacarbazine. In the quadruple combined treatment group of GT + AP + PC + DAC on A2058 cells, the expression level of the NF-kB gene was increased 2.38 times (0.88/2.38) compared to Dacarbazine (Fig. 8).

Additionally, the efficacy of Dacarbazine was compared to that of the control group and the DMSO control group in vivo. In the subcutaneous tumor model, the tumor diameter was 21 mm in the Dacarbazine treatment group at the end of the 56th day, compared to 42 mm in the control group. The dacarbazine treatment group showed a 0.5-fold reduction in tumor diameter on the 56th day of

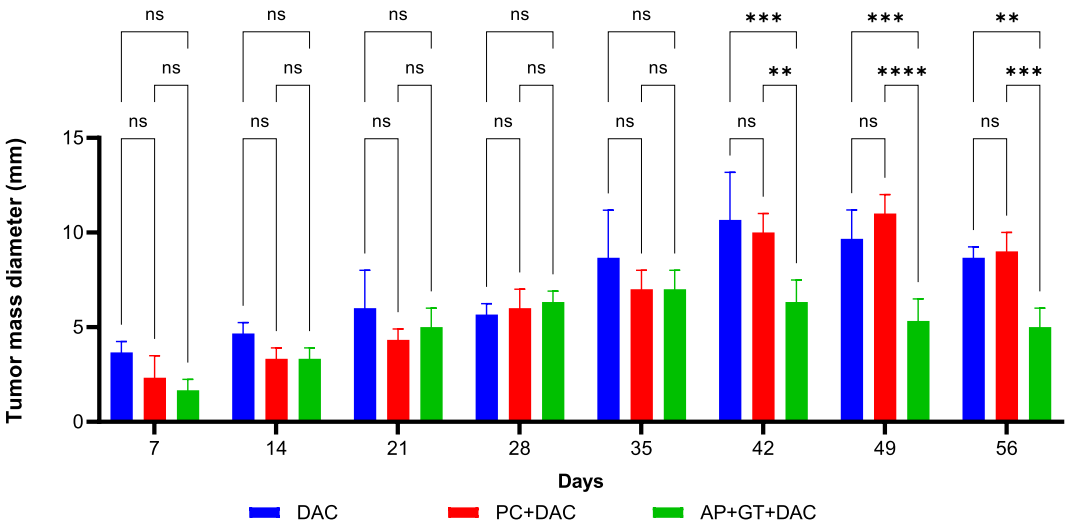

Fig. 12. Efficacy of the phytochemicals and their different combinations with Dacarbazine in the subcutaneous tumor model.

treatment compared to the control group (Fig. 9).

The study observed that tumor growth continued to increase in the control and DMSO groups in a time-dependent manner. However, significant shrinkages in the size of tumors were observed in mice treated with Dacarbazine. The anti-tumor activities of AP, GT, and PC were compared to those of Dacarbazine. The results showed that the antitumoral activities of AP and PC in the tumor model were similar to those of Dacarbazine. In contrast, the GT treatment group had a 1.75-fold (12/21) reduction in tumor diameter compared to the Dacarbazine group. GT exhibited remarkable antitumoral activity, and the activities of AP and PC were similar to those of Dacarbazine (Fig. 10). The phytochemicals showed remarkable anti-tumor activity on melanocytes when compared to Dacarbazine. Interestingly, the antitumoral activity of GT was significantly higher than the activities of both AP and PC, as well as Dacarbazine ($p < 0.05$).

The combination of Dacarbazine with both AP and GT significantly increased antitumoral efficacy. Compared to the Dacarbazine group, the tumor diameters were lower in the AP plus GT treatment group, AP plus Dacarbazine, and GT plus Dacarbazine treatment groups from day 28 of treatment. At the end of treatment, the tumor diameters in the Dacarbazine, AP plus GT, GT plus Dacarbazine, and AP plus Dacarbazine treatment groups were 21.4 ± 1.1, 7.6 ± 2.2, 8.6 ± 0.5, and 6.2 ± 1.9 mm, respectively. A 3.5-fold reduction in tumor diameter was detected in the GT plus Dacarbazine treatment group compared to the Dacarbazine group (Fig. 11). The efficacy of the combination of PC plus Dacarbazine was higher than the efficacy of Dacarbazine alone. Besides, GT plus AP plus Dacarbazine had remarkable antitumoral activity. On the 56th day of treatment, the PC plus Dacarbazine and AP plus GT plus Dacarbazine treatment group had a 0.47-fold and 0.29-fold reduction in tumor diameter, respectively, compared to Dacarbazine (Fig. 12). The effectiveness of GT was compared to that of Dacarbazine. There was no significant difference in tumor mass diameters from the first week to the seventh week of treatment. However, efficacy increased significantly in the following weeks. The effectiveness of the combination of GT plus Dacarbazine was found to be significant from the first week. The antitumoral effect of GT was 1.75 times more potent compared to Dacarbazine. The antitumoral activity of Dacarbazine plus GT was 3.5 times higher than that of GT. The efficacy of GT compared with the GT plus Dacarbazine was two times higher in the combined treatment group (Fig. 13a).

The combination of AP plus Dacarbazine was found to have potent activity on melanocytes, similar to the combination of GT plus Dacarbazine. The efficacy of AP plus Dacarbazine was significantly higher than that of Dacarbazine alone. However, the antitumoral activity of AP was not statistically significant compared to Dacarbazine alone. Nevertheless, the tumor diameter in the AP plus Dacarbazine treatment group decreased 2.33-fold more than the Dacarbazine group and 2.22-fold more than the AP group ($p < 0.05$, Fig. 13b).

While PC activity against melanoma cells was lower than AP and GT activities, PC plus Dacarbazine activity was higher than Dacarbazine's efficacy alone. The activity of PC was not different from the activity of Dacarbazine alone. However, the antitumoral action detected in the PC plus Dacarbazine treatment group was significantly higher. Tumor diameter was 2.1-fold decreased than the PC plus Dacarbazine treatment group ($p < 0.05$, Fig. 13c).

3.6. Histopathological findings

This study performed histopathological evaluations on tissue samples from treatment groups to analyze the presence of normal tissue, mononuclear cell infiltration, and melanocytes. Sample pictures of the histopathological findings of the tissues prepared from the treatment groups are given in Fig. 14a–m. Additionally, photos of the appearance of tumor diameters in rats treated with different options are shown in Fig. 15.

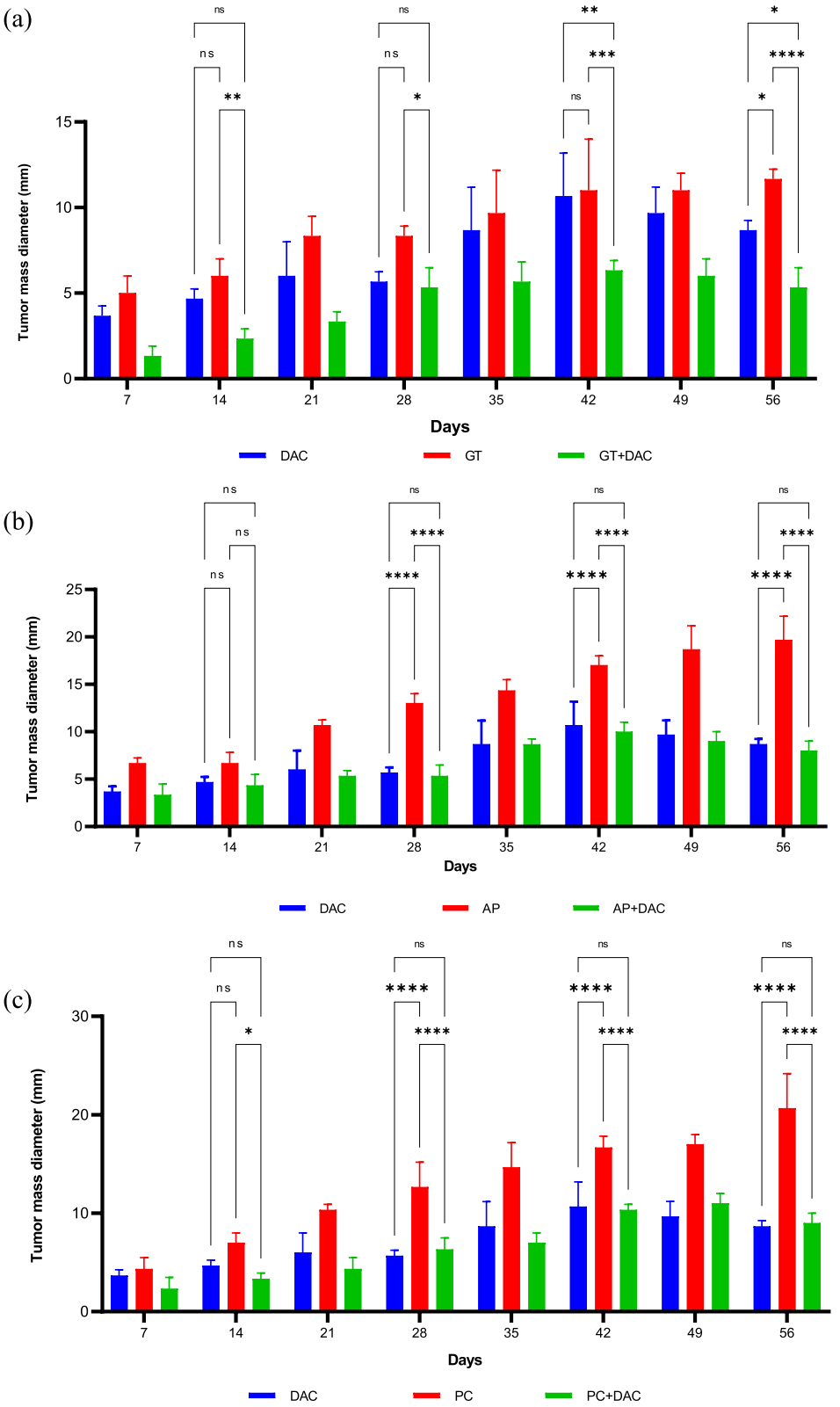

Fig. 13. a–c. Synergistic effects of Dacarbazine combinations of the phytochemicals on melanoma cells in a subcutaneous tumor model. A: *Gamma-terpinene*, B: *Alpha-pinene*, C: *P-cymene*.

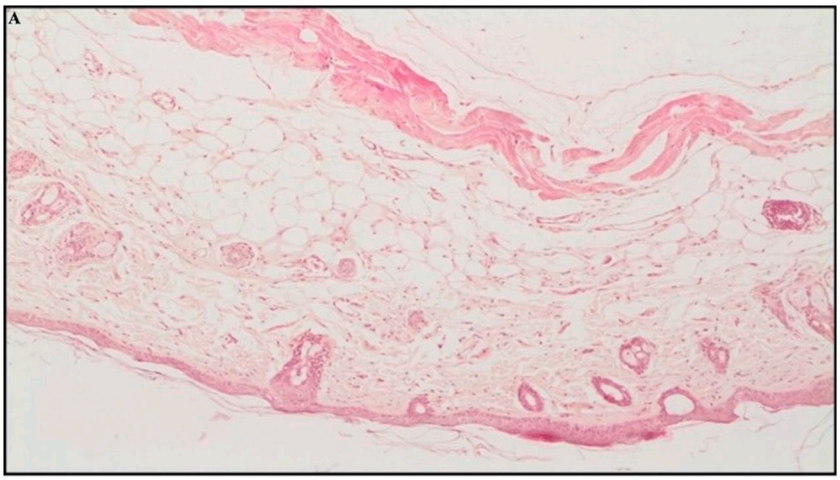

Fig. 14a. Healthy control group. The skin tissue has a standard histological structure. No inoculation was performed, and no treatment was given. 40x, HE.

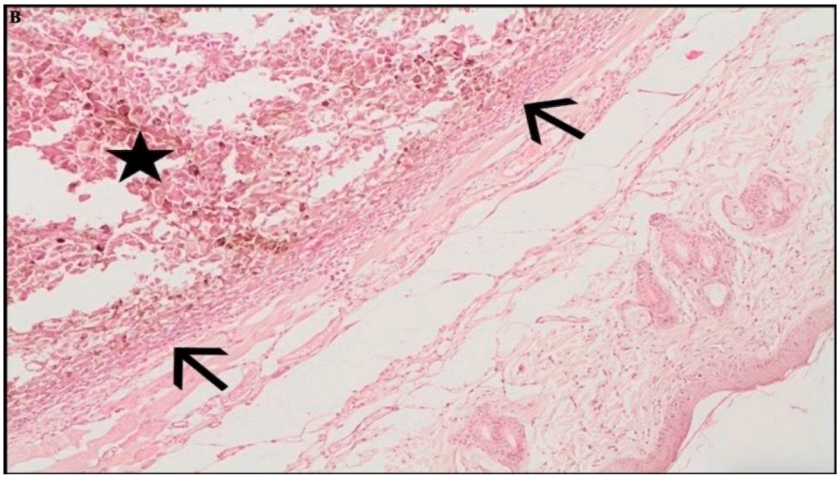

Fig. 14b. Tumor control group. Mice in this group were inoculated with A-2058 cells but received no treatment. Atypical melanotic melanocytes are delimited by connective tissue (arrows) under the skin (star). 100x, HE.

4. Discussion

Cancer is one of the leading causes of death globally and is often associated with uncontrolled cell growth, metastasis, and frequent recurrence. Traditional cancer treatments can have harmful side effects, which has led to the exploration of alternative therapeutic options. Natural plant compounds or phytochemicals are effective against cancer and other diseases or increase the activity of existing chemotherapeutics. Phytochemicals can be co-administered with chemically similar groups or traditional chemotherapeutics. This combination therapy approach can be a promising strategy for cancer treatment [5,27].

In addition to investigating the therapeutic effectiveness of three important phytochemicals, the study considered using these products alone or combined with existing chemotherapeutic agents to minimize the adverse effects of chemotherapy.

Combination therapy, where a synergistic effect on multiple targets is possible, has gained importance because it is more valuable than a single-target therapeutic effect with a single drug. Combinations of plant compounds with active pharmaceutical ingredients may be helpful in cancer treatment. Phytochemicals abundant in plants exhibit many anticarcinogenic properties, including inhibitory effects on cancer cell proliferation, tumor growth, angiogenesis, metastasis, inflammation, and inducing apoptosis [28]. In addition, these phytochemicals can modulate the immune system response and protect normal cells from free radical damage. Most research on the anti-cancer mechanisms of phytochemicals has been applied to individual compounds. However, many studies have shown that anti-cancer efficacy and scope of action can be further enhanced by synergistically combining them with chemically similar or different compounds [6]. While most studies investigated the anti-cancer effects of combinations of the two compounds, our study aimed to further strengthen the anticarcinogenic activity with combinations of three phytochemicals and one chemotherapeutic agent.

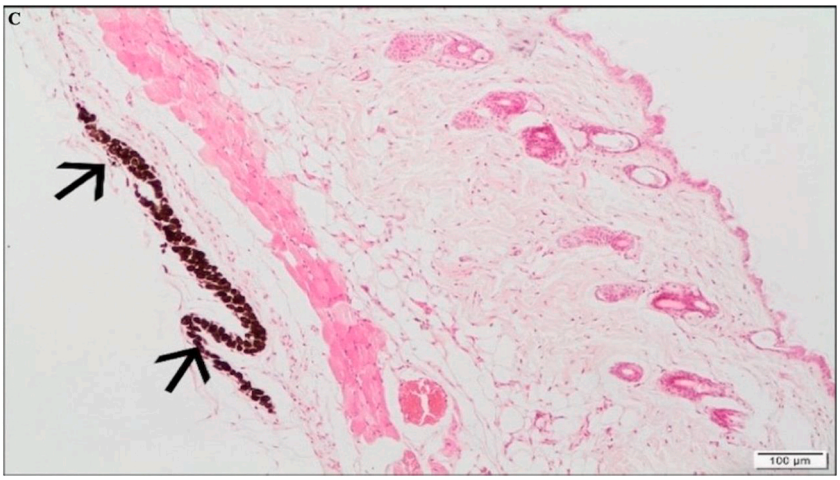

Fig. 14c. *Gamma-terpinene* treatment group. Localized subcutaneous atypical melanotic melanocytes. 100x, HE.

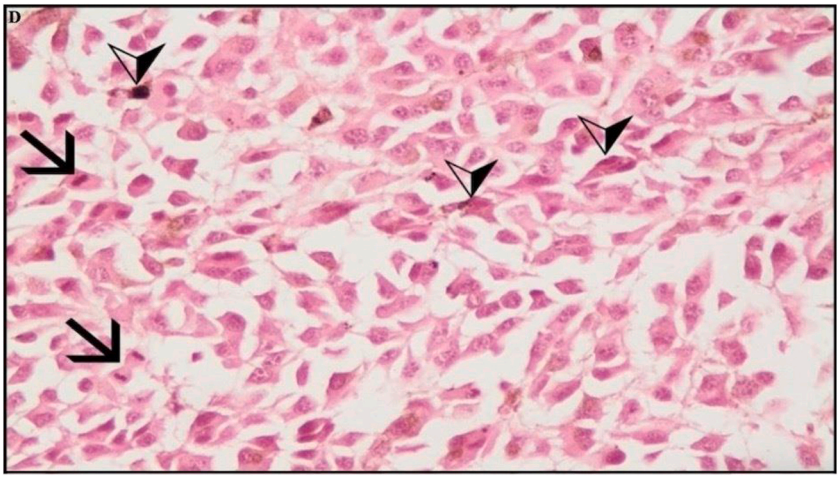

Fig. 14d. Atypical melanotic melanocytes (arrowheads) and mitotic figures (arrows) under the skin (arrows). 400x, HE. Mice were injected with A-2058 cells, but no treatment was administered. Diffuse melanocytes were detected in the subcutaneous tissue.

Because they can modulate the activity of many carcinogenic targets, phytochemicals can inhibit the proliferation of cancer cells. However, despite the promising results of in vitro studies, the use of phytochemicals as the sole anticancer agent in clinical practices is limited. This is mainly due to their low bioavailability in the human body. New strategies are needed to increase the efficacy of phytochemicals as anticarcinogenic agents [29–32].

Conventional methods such as chemotherapy and radiotherapy are the gold standard methods for cancer treatment. Unfortunately, tumor cells become resistant to drugs by removing chemotherapeutic agents from the cell or changing the gene expression involved in carcinogenesis, altering their drug targets. They do this by increasing the expression of proapoptotic genes such as *p53* and Bax or by decreasing the expression of anti-apoptotic genes such as *Bcl-2* and *Bcl-xl* [31–34].

Various studies have reported that phytochemicals increase the anticarcinogenic effects of drugs used for cancer chemotherapy, modulate different signal transduction pathways that play a role in carcinogenesis, and inhibit tumor growth. It has been reported that combining phytochemicals with drugs used for conventional cancer therapy may help overcome drug resistance and reduce the side effects of standard anticancer treatments [23,35,36]. In vitro and in vivo studies have shown that combining phytochemicals inhibits cancer cell growth more strongly than a single phytochemical [37–40].

The most significant problem in using phytochemicals is their poor bioavailability in the human body. Poor bioavailability may prevent or reduce the in vivo efficacy of phytochemicals in their single use. The most important approach to overcome this problem is using more than one phytochemical or combining phytochemicals and drugs [41–43].

Our study showed that the phytochemicals' combined effectiveness was more significant than their effectiveness. The combined use of phytochemicals significantly increased pro-apoptotic gene expression and decreased anti-apoptotic gene expression. Combined with Dacarbazine, phytochemicals showed a strong synergistic effect on A-2058 cells. However, further studies are needed to support

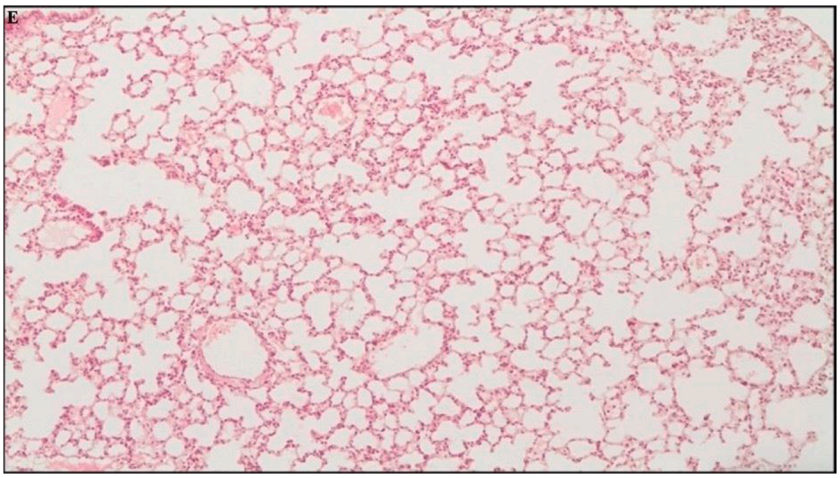

Fig. 14e. Lung tissue, normal histological structure. 100x, HE. The *Gamma-terpinene* plus Dacarbazine treatment group. No melanocytes were detected in the lung tissue.

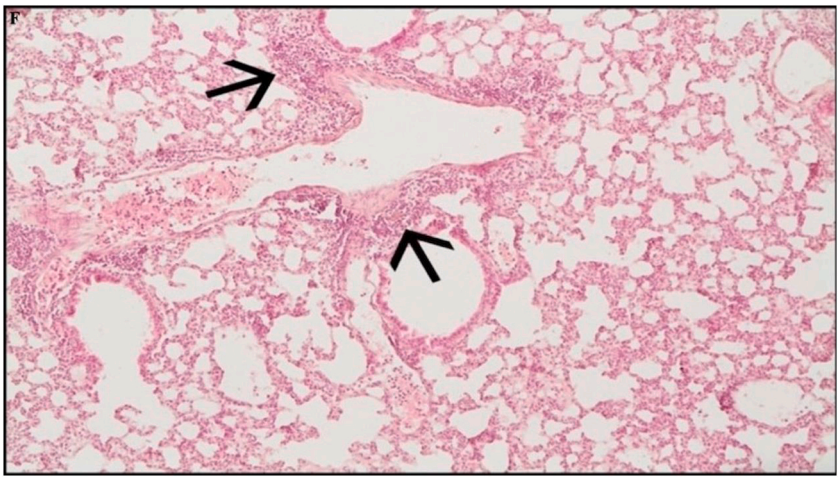

Fig. 14f. Peribronchial mononuclear cell infiltration in the lung (arrows), 100x, HE. *Gamma-terpinene* treatment group. There was no melanocyte in the lung tissue, and mononuclear cell infiltration was detected.

our apoptosis findings.

The efficacy of phytochemicals and their combinations, which had striking activity against the cancer cells in vitro, was evaluated in the experimental subcutaneous tumor model. Tumor growth was monitored by measuring tumor mass diameters weekly for two months following treatment. The mice were sacrificed at the end of the treatment, and tissue samples taken from the skin, lung, liver, kidney, spleen, brain, heart, and intestines were evaluated histopathologically for metastasis.

In the group that received GT treatment, a significant reduction in tumor mass diameters was observed from the 21st day of treatment. Furthermore, substantial reductions in the size of the tumor mass were detected from the first week of treatment, particularly in the combined treatment groups as compared to the group treated with Dacarbazine. This effect has been evaluated as having potent in-vivo synergistic effects in the combined treatments with phytochemicals. The tumor mass diameters were reduced to a level that could not be detected by palpation, particularly in the combined treatment groups of AP plus GT, AP plus Dacarbazine, and GT plus Dacarbazine. The antitumoral efficacy became more pronounced after the 15th day of treatment. The combination that exhibited the most striking antitumoral activity in animal experiments was the GT plus Dacarbazine combination. In this treatment group, antitumoral efficacy was observed from the first week of treatment, unlike in the other treatment groups.

Histopathological evaluations were performed on tissue samples of sacrificed animals at the end of the treatment to examine for metastasis. The subcutaneous mouse tumor model was used to compare the anti-tumoral efficacy of phytochemicals or their combinations with the highest efficacy in vitro experiments. Following the injection of B16-F10 cells, cell metastases were found in all tissue samples from untreated mice. Tumor shrinkage was observed in all mice treated with Dacarbazine, but melanoma cells were still present in skin samples. Although Dacarbazine reduced the rates of lung, liver, and kidney metastases by 20 %, 20 %, and 50 %,

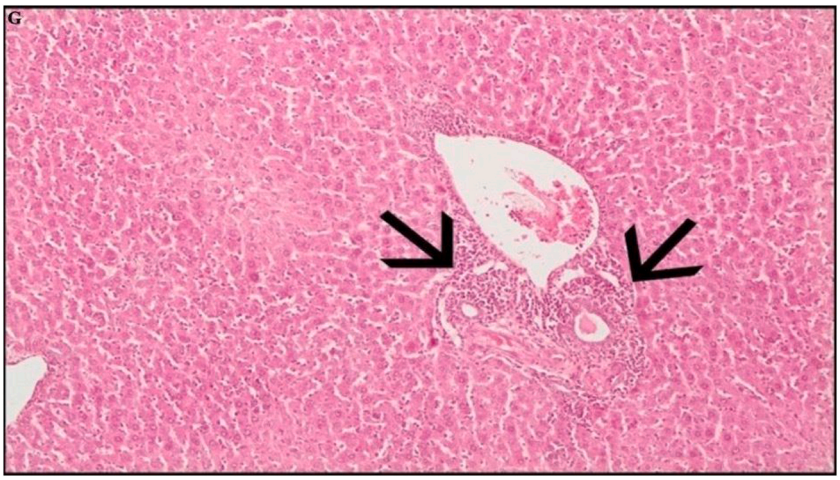

Fig. 14g. Perivascular mononuclear cell infiltration in the liver (arrows), 100x, HE. *Gamma-terpinene* treatment group. There was no liver metastasis; only mononuclear cell infiltration was detected.

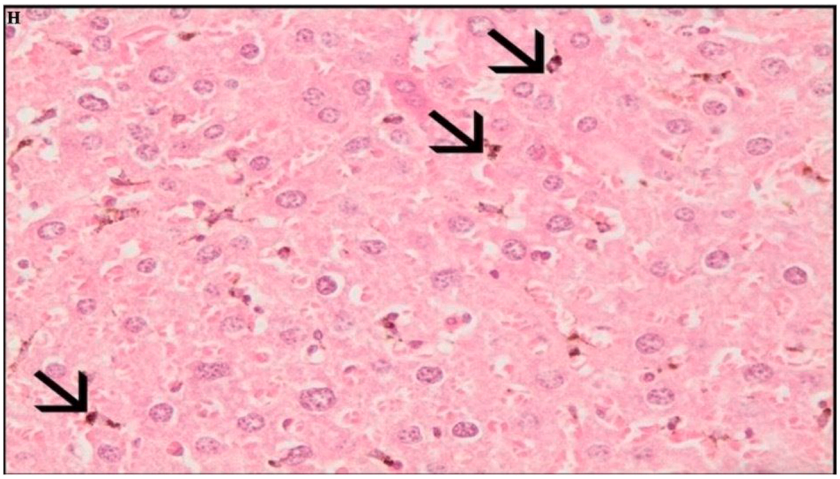

Fig. 14h. Metastasis and melanotic melanocytes in the liver (arrows), 400x, HE. Dacarbazine treatment group. Melanocytes were detected in liver tissue after treatment with Dacarbazine.

respectively, it could not eliminate cell metastasis.

The study found that all mice in the AP treatment group had melanocytes in their skin tissue. Metastasis rates in the lung, liver, and kidney were 70 %, 80 %, and 60 %, respectively, with no metastasis detected in other organs. In contrast, the GT treatment group showed more potent antitumoral efficacy. Melanocytes were detected in all skin samples in this group, but the metastasis rates in the lung, liver, and kidney were lower at 50 %, 50 %, and 40 %, respectively. Finally, the study found that the antitumoral activity of PC was lower compared to AP and GT.

In the group of mice treated with a combination of AP and Dacarbazine, melanocytes were found in all skin samples. Still, only 30 % of the mice had lung metastases, and 20 % had liver metastases. In the group treated with GT plus Dacarbazine, 50 % of the mice had skin metastases, 30 % had lung metastases, and 20 % had liver metastases. The combination of three phytochemicals along with Dacarbazine showed highly effective anti-tumor efficacy. In the group treated with AP plus GT plus PC plus Dacarbazine, only 10 % of the mice had melanocytes in their skin and lungs.

5. Conclusion

The antitumor activities of the treatment combinations, which are very effective in cell culture experiments, were investigated in a mouse tumor model. The AP treatment groups showed reduced metastasis levels in the lung, liver, and kidney organs. GT exhibited more antitumoral activity than AP, while PC was determined to be the phytochemical with the lowest antitumor activity. The combinations of two or more phytochemicals and combinations of phytochemicals plus Dacarbazine showed more potency than single

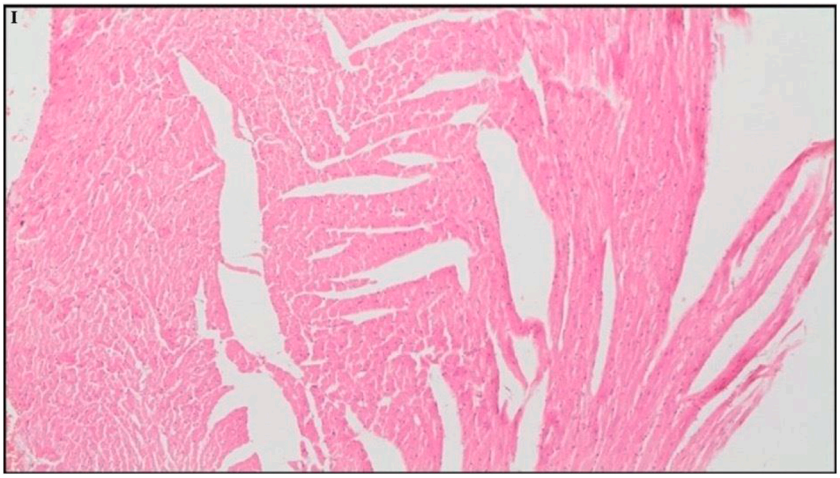

Fig. 14i. The heart tissue has a typical histological structure. 40x, HE. *Gamma-terpinene* plus Dacarbazine treatment group. No melanocytes were detected in the heart tissue.

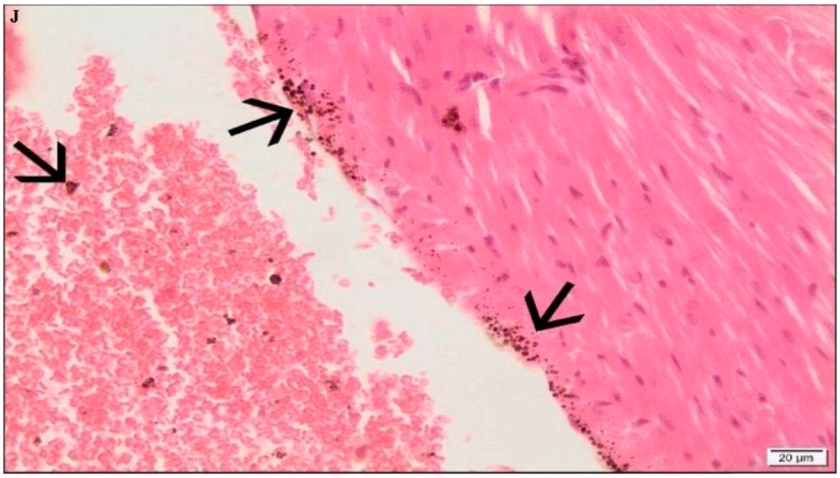

Fig. 14j. Untreated group. Melanotic melanocytes in heart tissue (arrows), 400x, HE.

uses. The lung and liver metastasis rates in the AP plus Dacarbazine combined treatment group were 30 % and 20 %, respectively. The most effective treatment group was the combination of three phytochemicals plus Dacarbazine. Only 10 % of mice treated with this combination had melanocytes in their skin and lungs.

These three phytochemicals (AP, GT, and PC) inhibited A-2058 cell proliferation and anti-apoptotic gene expression and induced pro-apoptotic gene expression. Double and triple combinations of these phytochemicals exhibited much stronger antitumoral activity with different molecular mechanisms.

In addition, these three phytochemicals have increased the efficacy of Dacarbazine, which is used for melanoma treatment and is very promising in reducing its side effects. Different combinations of phytochemicals exhibited synergistic solid activities. These combinations can both increase drug efficacy and decrease side effects, as well as provide a solution to the problem of drug resistance. This study can be a guide for new studies in this field.

The AP, GT, and PC combination has shown significant antiproliferative activities against A-2058 cells. When used together, these phytochemicals' potency was enhanced, resulting in increased effectiveness of Dacarbazine in melanoma cells. These findings are significant as they demonstrate the use of phytochemicals to improve the effectiveness of chemotherapy.

Combining phytochemicals with chemotherapeutic agents may be a safer way to reduce or eliminate side effects on the host while increasing efficacy. Moreover, combination options with phytochemicals have the potential to overcome drug resistance, which is a significant issue that limits the effectiveness of chemotherapeutics. Using multiple combinations of phytochemicals or natural products rather than a single activity can produce more substantial synergistic effects. Therefore, in new drug research, synergistic effect studies should be considered a significant option for achieving strong efficacy.

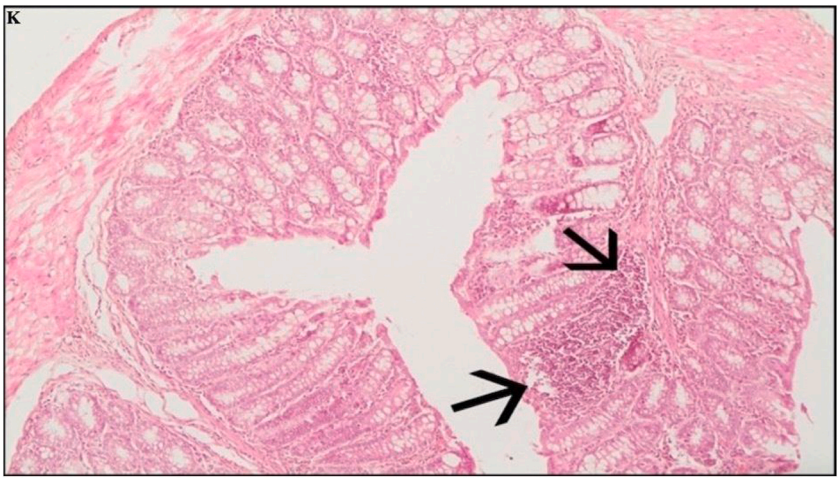

Fig. 14k. *Gamma-terpinene* plus Dacarbazine treatment group. Focal mononuclear cell infiltration in the lamina propria of the intestine (arrows), 100x, HE. No melanocytes were in the intestinal tissue; only mononuclear cell infiltration was detected.

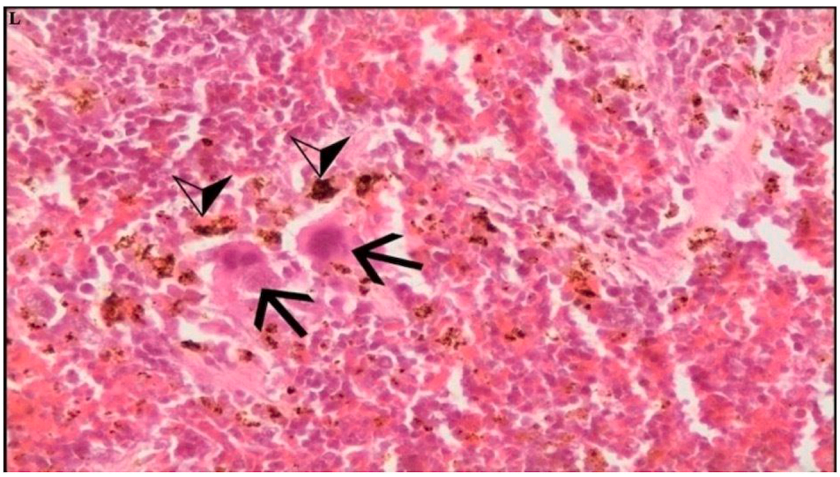

Fig. 14l. The untreated group. Melanotic melanocytes (arrowheads) are the spleen's giant cells (arrows). 400x, HE.

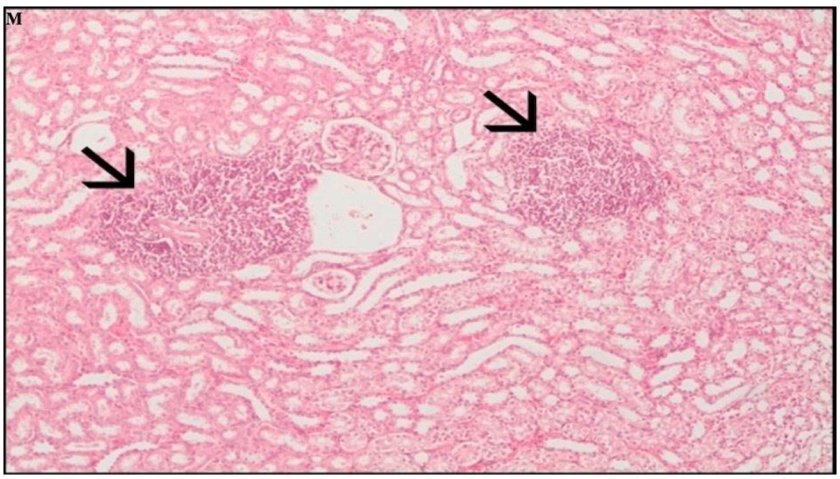

Fig. 14m. *Gamma-terpinene* treatment group. Focal mononuclear cell infiltration in the kidney, (arrows), 100x, HE.

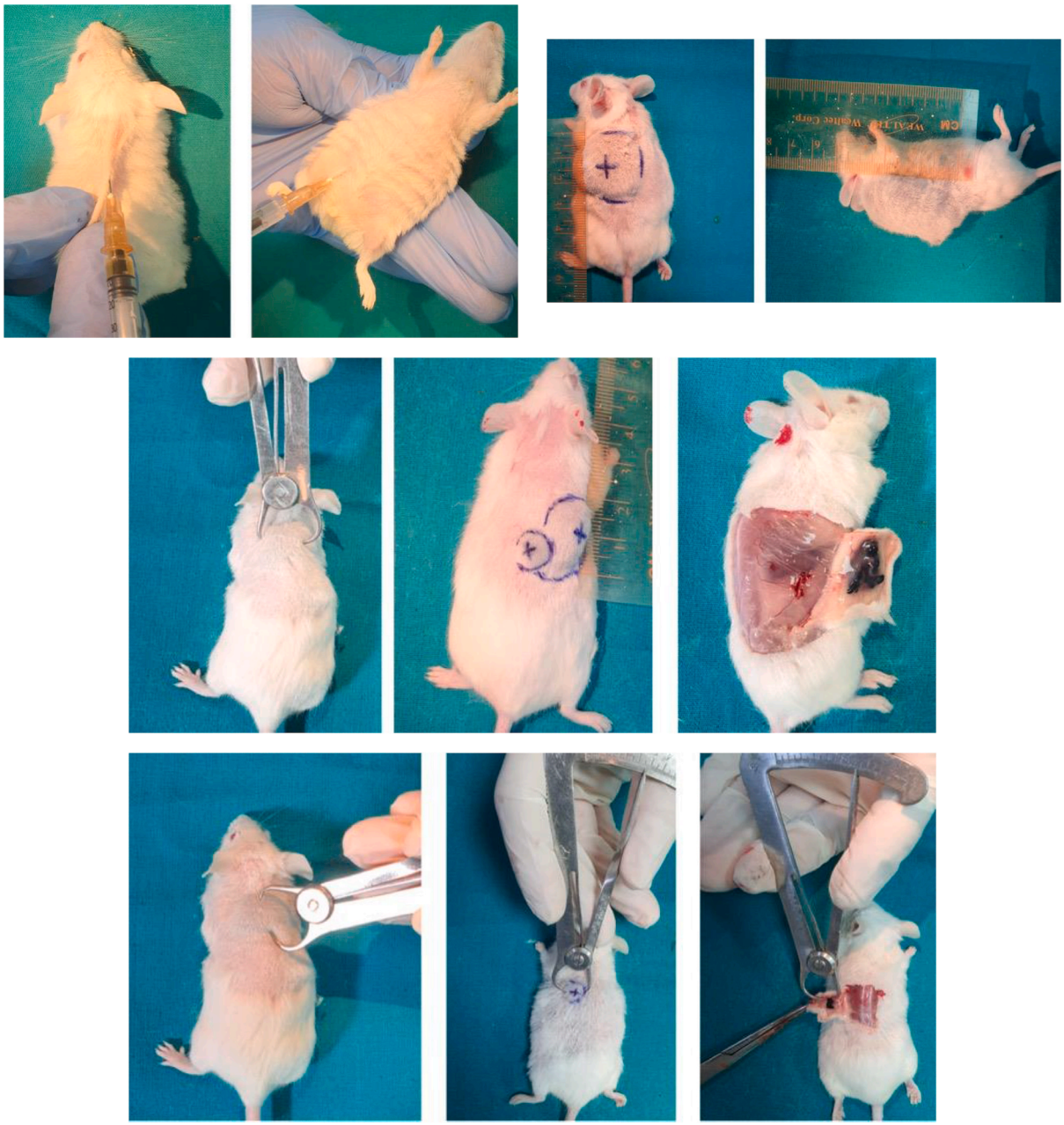

Fig. 15. Sample pictures from the treatment groups. 15.1. Subcutaneous injection of A2058 cells into the dorsal region of mice (left). Intraperitoneal treatment administration to mice (right). 15.2. Inoculation of A 2058 cells and tumor development in the control group (no treatment group). In untreated mice, the tumor diameter increased daily and became a huge mass (right). 15.3. Tumor shrinkage following administration of Dacarbazine therapy. Although the tumor diameter was significantly smaller compared to the control group, the mass remained large. 15.4. Tumor shrinkage following administration of *Gamma-terpinene* plus Dacarbazine therapy. The recovery was very successful, and the tumor shrank greatly. 15.5. Tumor shrinkage following administration of *Alpha-pinene* plus *Gamma-terpinene* therapy. A significant reduction in mass size has been detected. 15.6. *Alpha-pinene* plus Dacarbazine treatment group. The diameter of the mass has significantly reduced; it has almost shrunk to the point that the tumor has disappeared (center and right).

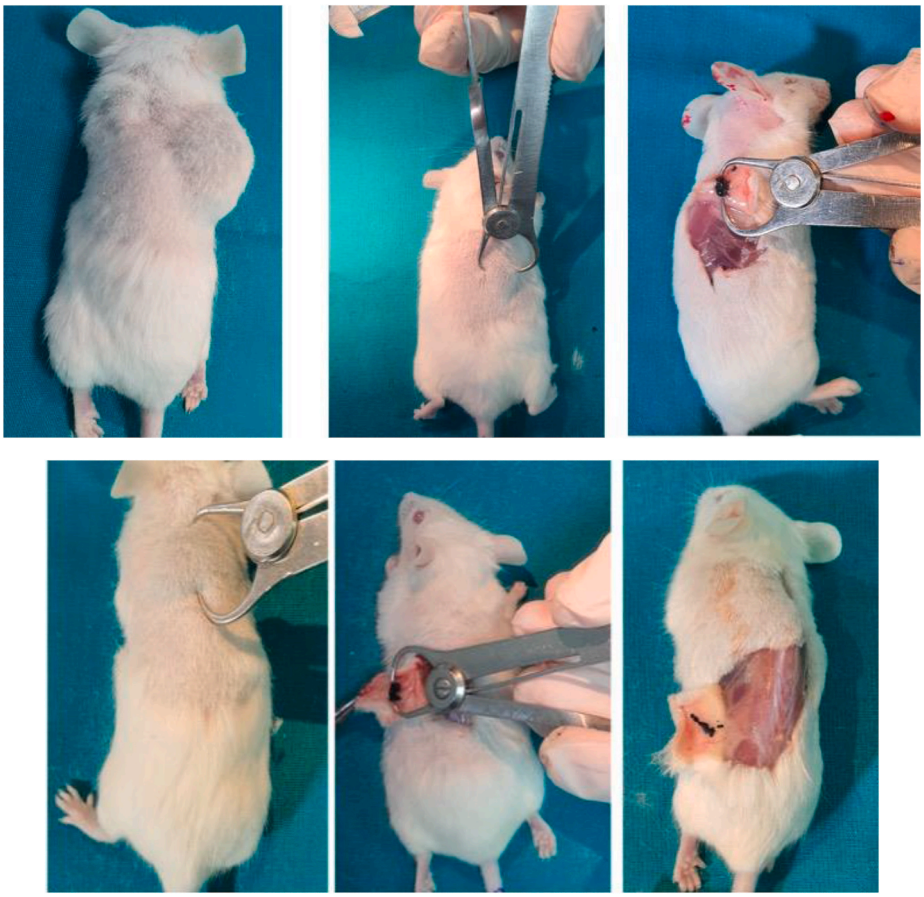

Fig. 15. (*continued*).

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Availability of data and materials

The data associated with this study are included in this published article. Additional files are available from the corresponding author upon reasonable request.

Ethics approval and participation approval

The study protocol was approved by the Local Ethical Committee of Experimental Animal Ethics of Hatay Mustafa Kemal University and was performed entirely according to ethical rules (Approval no: 2018/7-2, July 26, 2018).

Consent for publication

Not applicable.

CRediT authorship contribution statement

Funda Cimen: Methodology, Formal analysis, Data curation, Conceptualization. **Nizami Duran:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Tuncer Kutlu:** Methodology, Formal analysis. **Emrah Ay:** Methodology, Formal analysis, Data curation. **Erhan Tek:** Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Suphi Bayraktar:** Visualization, Methodology, Investigation.

Declaration of competing interest

No conflict interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36223.

References

[1] J. Liu, J. Zhang, N.M. Beeraka, K. Chen, M.Y. Sinelnikov, P. Manogaran, et al., Perspectives on the nanocarriers with miRNAs for targeting melanoma stemness through epigenetic regulation, Pigment Cell Melanoma Res. 36 (3–4) (2023) 268–287.
[2] M. Ciążyńska, G. Kamińska-Winciorek, D. Lange, B. Lewandowski, A. Reich, M. Sławińska, et al., The incidence and clinical analysis of non-melanoma skin cancer, Sci. Rep. 11 (1) (2021) 4337.
[3] H. Cortes, O.D. Reyes-Hernandez, S. Alcala-Alcala, S.A. Bernal-Chávez, I.H. Caballero-Florán, M. González-Torres, et al., Repurposing of drug candidates for treatment of skin cancer, Front. Oncol. 10 (2020) 605714.
[4] C. Desjobert, A. Carrier, A. Delmas, D.M. Marzese, A. Daunay, F. Busato, et al., Demethylation by low-dose 5-aza-2′-deoxycytidine impairs 3D melanoma invasion partially through miR-199a-3p expression revealing the role of this miR in melanoma, Clin. Epigenet. 11 (1) (2019) 9.
[5] R. Dutt, V. Garg, N. Khatri, A.K. Madan, Phytochemicals in anticancer drug development, Anti Cancer Agents Med. Chem. 19 (2) (2019) 172–183.
[6] A. Niedzwiecki, M.W. Roomi, T. Kalinovsky, M. Rath, Anticancer efficacy of polyphenols and their combinations, Nutrients 8 (9) (2016) 552.
[7] P. Torka, E. Przespolewski, A.M. Evens, Treatment strategies for advanced classical hodgkin lymphoma in the times of dacarbazine shortage, J Oncol Pract 18 (7) (2022) 491–497.
[8] M.V. Sobral, A.L. Xavier, T.C. Lima, D.P. de Sousa, Antitumor activity of monoterpenes found in essential oils, Sci. World J. 2014 (2014) 953451.
[9] L. Yeruva, K.J. Pierre, A. Elegbede, R.C. Wang, S.W. Carper, Perillyl alcohol and periodic acid-induced cell cycle arrest and apoptosis in non-small cell lung cancer cells, Cancer Lett. 257 (2) (2007) 216–226.
[10] M.F. Halabi, B.Y. Sheikh, Anti-proliferative effect and phytochemical analysis of Cymbopogon citrus extract, BioMed Res. Int. 2014 (2014) 906239.
[11] A. Ranjan, S. Ramachandran, N. Gupta, I. Kaushik, S. Wright, S. Srivastava, Role of phytochemicals in cancer prevention, Int. J. Mol. Sci. 20 (20) (2019) 4981.
[12] R. Pezzani, B. Salehi, S. Vitalini, M. Iriti, F.A. Zuñiga, J. Sharifi-Rad, Synergistic effects of plant derivatives and conventional chemotherapeutic agents: an update on the cancer perspective, Medicina (Kaunas) 55 (4) (2019) 110.
[13] M.Z. Aumeeruddy, M.F. Mahomoodally, Combating breast cancer using combination therapy with 3 phytochemicals: Piperine, sulforaphane, and thymoquinone, Cancer 125 (10) (2019) 1600–1611.
[14] S. Hemaiswarya, A.K. Kruthiventi, M. Doble, Synergism between natural products and antibiotics against infectious diseases, Phytomed 15 (8) (2008) 639–652.
[15] A. Zhang, H. Sun, Y. Yuan, W. Sun, G. Jiao, X. Wang, An *in vivo* analysis of the therapeutic and synergistic properties of Chinese medicinal formula yin-Chen-hao-tang based on its active constituents, Fitoterapia 82 (8) (2011) 1160–1168.
[16] M. Ayaz, F. Ullah, A. Sadiq, F. Ullah, M. Ovais, J. Ahmed, Synergistic interactions of phytochemicals with antimicrobial agents: potential strategy to counteract drug resistance, Chem. Biol. Interact. 308 (2019) 294–303.
[17] J.Z. Shen, T.L.J. Ng, W.S. Ho, Therapeutic potential of phytochemicals in combination with drugs for cardiovascular disorders, Curr. Pharm. Des. 23 (6) (2017) 961–966.
[18] H.R. Metelmann, D.D. Von Hoff, In vitro activation of dacarbazine (DTIC) for a human tumor cloning system, Int J Cell Cloning 1 (1) (1983) 24–32.
[19] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[20] G.I. Karaliotas, K. Mavridis, A. Scorilas, G.C. Babis, Quantitative analysis of the mRNA expression levels of *BCL2* and *BAX* genes in human osteoarthritis and normal articular cartilage: an investigation into their differential expression, Mol. Med. Rep. 12 (3) (2015) 4514–4521.
[21] R.A. Olie, C. Hafner, R. Küttel, B. Sigrist, J. Willers, R. Dummer, et al., Bcl-2 and Bcl-xL antisense oligonucleotides induce apoptosis in melanoma cells of different clinical stages, J. Invest. Dermatol. 118 (3) (2002) 505–512.
[22] Q. Yang, M. Li, T. Wang, H. Xu, W. Zang, G. Zhao, Effect of STAT5 silenced by siRNA on proliferation apoptosis and invasion of esophageal carcinoma cell line Eca-109, Diagn. Pathol. 8 (2013) 132.
[23] Y. Wang, L. Yan, L. Zhang, H. Xu, T. Chen, Y. Li, et al., NT21MP negatively regulates paclitaxel-resistant cells by targeting miR-155-3p and miR-155-5p *via* the CXCR4 pathway in breast cancer, Int. J. Oncol. 53 (3) (2018) 1043–1054.
[24] S. Hoseinzadeh, E. Raeisi, Y. Lemoigne, E. Heidarian, Effects of combined 5-Fluorouracil and ZnO NPs on human breast cancer MCF-7 Cells: P53 gene expression, Bcl-2 signaling pathway, and invasion activity, Nanomed. J. 6 (2019) 232–240.
[25] T. Hruz, M. Wyss, M. Docquier, M.W. Pfaffl, S. Masanetz, L. Borghi, RefGenes: identification of reliable and condition specific reference genes for RT-qPCR data normalization, BMC Genom. 12 (2011) 156.
[26] C.X. Geng, Z.C. Zeng, J.Y. Wang, Docetaxel inhibits SMMC-7721 human hepatocellular carcinoma cell growth and induces apoptosis, World J. Gastroenterol. 9 (4) (2003) 696–700.
[27] J.J. Wang, K.F. Lei, F. Han, Tumor microenvironment: recent advances in various cancer treatments, Eur. Rev. Med. Pharmacol. Sci. 22 (12) (2018) 3855–3864.
[28] S. Zhu, T. Zhang, L. Zheng, H. Liu, W. Song, D. Liu, et al., Combination strategies to maximize the benefits of cancer immunotherapy, J. Hematol. Oncol. 14 (1) (2021) 156.
[29] M.F. Halabi, B.Y. Sheikh, Anti-proliferative effect and phytochemical analysis of Cymbopogon citrus extract, BioMed Res. Int. 2014 (2014) 906239.
[30] M.M. Chan, D. Fong, Overcoming drug resistance by phytochemicals, in: K. Metha, S.E. Bates, Z.H. Siddik (Eds.), Drug Resistance in Cancer Cells, Springer Science+Business Media, LLC, New York, NY, USA, 2009, pp. 315–342.
[31] M.D. Mund, S. Alam, U.H. Khan, U. Tahir, M.S. Zubair, T. Younas, Phytochemicals as Complementary and alternative therapeutic formulations with potential proapoptotic effects on various cancerous cell lines: a literature survey, Focus Sci 2 (2016) 111–121.
[32] S. D'Aguanno, D. Del Bufalo, Inhibition of anti-apoptotic *Bcl-2* proteins in preclinical and clinical studies: current overview in cancer, Cells 9 (5) (2020) 1287.
[33] F. Pentimalli, S. Grelli, N. Di Daniele, G. Melino, I. Amelio, Cell death pathologies: targeting death pathways and the immune system for cancer therapy, Genes Immun 20 (7) (2019) 539–554.
[34] P. Ferrer, M. Asensi, R. Segarra, A. Ortega, M. Benlloch, E. Obrador, et al., Association between pterostilbene and quercetin inhibits the metastatic activity of B16 melanoma, Neoplasia 7 (1) (2005) 37–47.
[35] B.I.M. Silva, E.A. Nascimento, C.J. Silva, T.G. Silva, J.S. Aguiar, Anticancer activity of monoterpenes: a systematic review, Mol. Biol. Rep. 48 (7) (2021) 5775–5785.
[36] S. Hoseinzadeh, E. Raeisi, Y. Lemoigne, E. Heidarian, Effects of combined 5-Fluorouracil and ZnO NPs on human breast cancer MCF-7 Cells: *P53* gene expression, *Bcl-2* signaling pathway, and invasion activity, Nanomed. J. 6 (2019) 232–240.

[37] P. Ferrer, M. Asensi, R. Segarra, A. Ortega, M. Benlloch, E. Obrador, et al., Association between pterostilbene and quercetin inhibits the metastatic activity of B16 melanoma, Neoplasia 7 (1) (2005) 37–47.
[38] P. Wang, J.V. Vadgama, J.W. Said, C.E. Magyar, N. Doan, D. Heber, S.M. Henning, Enhanced inhibition of prostate cancer xenograft tumor growth by combining quercetin and green tea, J. Nutr. Biochem. 25 (1) (2014) 73–80.
[39] P. Wang, D. Heber, S.M. Henning, Quercetin increased the antiproliferative activity of green tea polyphenol (-)-epigallocatechin gallate in prostate cancer cells, Nutr. Cancer 64 (4) (2012) 580–587.
[40] M.A. Rahman, M.A. Hannan, R. Dash, M.H. Rahman, R. Islam, et al., Phytochemicals as a complement to cancer chemotherapy: pharmacological modulation of the autophagy-apoptosis pathway, Front. Pharmacol. 12 (2021) 639628.
[41] A. Crozier, I.B. Jaganath, M.N. Clifford, Dietary phenolics: chemistry, bioavailability, and effects on health, Nat. Prod. Rep. 26 (8) (2009) 1001–1043.
[42] G. Williamson, C. Manach, Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies, Am. J. Clin. Nutr. 81 (1 Suppl) (2005) 243S–255S.
[43] Vimala Ki, S. Kannan, Phyto-drug conjugated nanomaterials enhance apoptotic activity in cancer, Adv. Protein Chem. Struct. Biol. 125 (2021) 275–305.