A network pharmacology approach to evaluate the synergistic effect of dihydromyricetin and myricitrin in vine tea on the proliferation of B16F10 cells

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Aim of the study: Although vine tea has demonstrated broad-spectrum anti-cancer properties, its main active compounds, dihydromyricetin (DMY) and myricitrin (MYT), exert weaker effects than the tea extracts. This study aimed to investigate the synergistic inhibitory effects of DMY and MYT on B16F10 cell proliferation and their synergistic inhibitory effects.

Methods: The effect of vine tea extracts (VTEs) and their active compounds on B16F10 cells was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, fluorescence staining, and flow cytometry. The synergistic effects were calculated by the combination index (CI), and its mechanism was discussed by network pharmacology.

Results: Different VTEs varied in their inhibition of B16F10 cell growth, with IC50 values ranging from 4.45 to 12.95 µg/mL. Among these, Guangzhou Qingyuan (Level 2), appeared to have the most potent inhibitory effect. The IC50 value of mix-use of DMY and MYT was 19.94∼64.4 µM, of which DMY:MYT = 8:1 had the minimum IC50 value of 19.94 µM. Combinations in the 1:1∼8:1 range had stronger effects than the isolated active compound. When they were mixed at the ratio of 1:4∼8:1, CI < 1, showing a synergistic effect. The combination of DMY and MYT also significantly inhibited the tyrosinase activity in B16F10 cells, consistent with its impact on cell proliferation. The eight potential targets were identified by network pharmacology regulating melanin metabolism, tyrosine metabolism, and melanogenesis signaling. According to the analysis of protein-protein interactions, TP53, TNF, and TYR might be critical targets for preventing and treating melanoma.
**Conclusion:** We found that DMY and MYT induced apoptosis of B16F10 cells, and their combined application had a significant synergistic effect. The present findings indicated that vine tea had a multi-pathway and multi-target impact on the prevention and treatment of melanoma.

**KEYWORDS**

vine tea, dihydromyricetin, myricitrin, proliferation inhibition, network pharmacology, synergistic effect, cell cycle

**Introduction**

Melanoma originates from melanocytes (1, 2). In addition to genetic and other endogenous risk factors, ultraviolet radiation is the most critical exogenous risk factor for melanoma (3). Early detection and surgical resection are the best choices to cure melanoma. At the same time, radiotherapy and chemotherapy are also commonly used treatments (4). However, surgical resection is limited and cannot effectively treat metastatic tumors. Radiotherapy and chemotherapy have drug toxicity and a high cost (5, 6). Therefore, it is important to prevent melanoma through lifestyle. Phytochemicals as supplements have attracted wide attention because of their low cost and toxicity. Many active ingredients have been reported with anti-cancer, anti-metastatic, and pro-apoptotic effects (7–10). Daphnetin inhibits α-MSH-induced melanogenesis via PKA and ERK signaling pathways in B16F10 cells and inhibits melanin synthesis in UVB-irradiated HaCaT conditioned medium (11). Paclitaxel in combination with a C-C chemokine receptor type 7 monoclonal antibody can both delay B16F10 cell growth and reduce lymphatic metastasis (12). Luteolin inhibits melanoma growth by regulating cell-cell interaction and oncogenic pathways (13). The purified extract of *Nymphaea hybrid* also has a specific inhibitory effect on melanogenesis in B16F10 cells (14).

Vine tea (*Ampelopsis grossedentata*) has more than 600 years of use in China, is widely distributed in the mountainous areas of southern China, and has been used as a new food resource in recent years. It has hypoglycemic (15), antioxidant (16, 17), antibacterial (18), and anti-inflammatory (19, 20) properties. Several bioactive components have been isolated from vine tea, such as DMY, MYT, and myricetin (21). As the most abundant flavonoid in vine tea, the content of DMY can reach as much as 30% in the leaves (15). Several studies have shown it to be anti-tumor in human lung adenocarcinoma cell lines (22), human glioma (23), and cholangiocarcinoma (24).

Natural products have gained popularity due to their low toxicity and low cost. However, due to their complex composition, the use of natural products is sometimes restricted. Network pharmacology can provide insight into natural products. In network pharmacology, multiple targets of a specific molecule are analyzed through network analysis, emphasizing multi-way regulation of signaling pathways, which can help understand the mechanism of prevention and treatment (25).

The present study evaluated the synergistic effect of the main active components in vine tea, DMY, and MYT, on the proliferation of B16F10 cells. The network pharmacological model was used to explain the underlying mechanisms of vine tea in preventing and treating melanoma.

**Materials and methods**

**Materials**

B16F10 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, CAS; MYT standard (CAS: 17912-87-7; ≥ 98%, purity), DMY standard (CAS: 27200-12-0; ≥ 98%, purity), and MTT were purchased from Beijing Solarbio Science and Technology Co., Ltd.; Fetal bovine serum was purchased from Beijing TransGen Biotech Co., Ltd.; RPMI medium and trypsin were purchased from Hyclone from Thermo Fisher Scientific; Hoechst 33342 was purchased from Shanghai Beyotime Biotechnology Co., Ltd.; PI/Rnase staining solution was purchased from Beijing BD Biosciences Co., Ltd., other reagents were commercially available and analytically pure. Six vine tea varieties selected for use in the experiment are all commercially available: A, Wild vine tea in Enshi, Hubei; B, Hubei Enshi selenium-rich vine tea; C, Guangzhou Qingyuan (Level 1); D, Guangzhou Qingyuan (Level 2); E, Wild vine tea in Shiqian, Guizhou; F, Wild vine tea in Zhangjiajie, Hunan.

**Extraction of vine tea**

The preparation of vine tea extracts (VTEs) was as follows: 70% ethanol was added to dried vine tea (tea: solvent = 1:10), and the suspension was incubated in a water bath at 40°C for 30 min. Then, the extracts were filtered and concentrated in a rotary evaporator to eliminate the solvent. Finally, the concentrate was lyophilized and stored at 4°C.
High-performance liquid chromatography analysis of vine tea extracts from different regions

VTEs were detected using a high-performance liquid chromatography (HPLC) system (Waters, Shanghai, China) with a C18 (5 μm, 4.6 × 250 mm) reverse-phase column, and the flow rate was 1.0 mL/min. The mobile phases were 0.1% acetic acid acetonitrile solution (A) and 0.1% acetic acid aqueous solution (B). Under the following gradient profile: 0–22 min 10–30% A, followed by washing and recombinating the column (3 min). The detection wavelength was 254 nm (200–400 nm full-band scanning).

The standard curve equation of DMY was \( y = 9656905.4141x + 39250.3632 \), \( r^2 = 0.9995 \) (0.1677 ~ 0.8 mg/mL), and MYT was \( y = 33785171.6504x + 446.4681 \), \( r^2 = 0.9998 \) (0.0030 ~ 0.035 mg/mL). The VTEs were prepared at a 1 mg/mL concentration, the sample injection volume was 20 μL and repeated three times as parallels.

Cell culture

B16F10 cells were inoculated in RPIM-1640 medium containing 10% FBS at 37°C and cultured in an incubator with 5% CO2 and saturated humidity (MCO-15AC, Sanyo, Japan), and the medium was changed every other day. When cells reached about 80% confluence, they were digested and subcultured with 0.25% trypsin and allowed to continue to develop (26).

The proliferation of B16F10 cells by MTT assay

B16F10 cell suspension was inoculated in a 96-well plate at 3,000 cells/well for 24 h. VTEs were dissolved in dimethyl sulfoxide (DMSO) to 100 mg/mL and diluted with a culture medium to 10–30 μg/mL. For the administration of DMY and MYT, the compounds were diluted in DMSO to 100 mM and then diluted with a culture medium to get the needed concentration. The final concentration of the DMSO was less than 0.5% in the medium.

To evaluate the synergistic effect of DMY and MYT, cells were treated with DMY, MYT, or mix-use. In the mixture, the compounds were present in ratios of 1:4, 1:2, 1:1, 2:1, 4:1, and 8:1. After treatment for 72 h, 10 μL of 5 mg/mL MTT was added to each well. The media containing MTT was removed after 4 h, and 100 μL DMSO was added to each well to dissolve the formazan crystals (27). The plate was shaken for 1 min, the absorbance at 490 nm was measured, the inhibition rate of the drug on cell growth was calculated, and the general equation for the dose-effect relationship was obtained according to the following formula. The combination index (CI) was calculated after 72 h incubation, and the CI value represented the combined effect of two drugs; CI <1 showed synergism, CI = 1 additive effects, and CI > 1 showed antagonism. The CI values of different intervals had strong and weak differences. CI value was calculated by the equation.

\[
\log \left( \frac{fa}{fu} \right) = \log (D/D_0)^m = m \log(D) - m \log(D_m)
\]

\( D_0 \): the dose of the drug
\( D_m \): the median-effect amount is signifying the potency.
\( fa \): the fraction affected by the dose
\( fu \): the fraction unaffected, \( fu = 1 - fa \)

If \( b = m, a = -m \log D_m \), \( Y = \log (fa/fu), X = \log D, Y = bX + a \).

\[
CI = \frac{(D_1)}{(D_0)}^{1} + \frac{(D_2)}{(D_0)}^{2}
\]

In the above formula, \( (D_1) \) and \( (D_2) \) represent the combined inhibition rate X% of drug 1 and drug 2 in the experiment, and \( (D_0) \) and \( (D_0) \) represent their respective inhibition rates X% (28).

Cell fluorescence staining

B16F10 cells were inoculated in 24-well plates at 3 × 10^4 cells/well. DMY, MYT, or mixed-use were applied the next day at a concentration of 75 μM. The cells were stained for 72 h. The original medium was aspirated and discarded, and the well was washed with PBS. Each was stained with 250 μL Hoechst 33342 solution. After 15 min, the dye solution was discarded. After washing 3 times with PBS, fluorescence photomicrographs were obtained by an inverted fluorescence microscope (IX73, Olympus, Japan) (29) and quantitatively analyzed using ImageJ software.

\[
\text{Mean} = \frac{\text{IntDen}}{\text{Area}}
\]

\( \text{IntDen} \): Integrated Density.

Effects of dihydromyricetin and myricitrin on tyrosinase activity in B16F10 cells

B16F10 cells were inoculated into 96-wells at 3,000 cells/well. The cells were cultured for 24 h before administering the test compounds. DMY and MYT were in DMSO to 100 mM and diluted with a culture medium to a concentration gradient of 20–100 μM, either alone or in combination.

After B16F10 cells were treated for 72 h, 90 μL 1% TritonX-100 was added to each well, and then 10 μL 1.0 mg/mL L-DOPA was added. The absorbance at 490 nm was measured after 5 min of ultrasound and treatment at 30°C for 30 min. The enzyme activity was calculated using the following formula: tyrosinase activity = OD_{sample} / OD_{control} × 100% (31).
**Determination of cell cycle**

The digested B16F10 cells were inoculated into three 12-well plates as three parallel groups at $5 \times 10^4$ cells/well. After 24 h, the cells were adherent to the wall before treatment. $75 \mu M$ DMY, MYT, or the combined compounds were added to each well, and then the cells were incubated for 24 h. The digested cells were collected and centrifuged at 1,000 rpm for 5 min; then, the supernatant was discarded. After washing the cells with PBS twice, 4°C 75% ethanol was added slowly, and the cells were kept in darkness at 4°C overnight. The cells were centrifuged at 1,000 rpm for 5 min before analysis, the supernatant was discarded, and the cells were washed with PBS to remove all ethanol and resuspended in 0.5 mL PI/Rnase staining solution, incubated in the dark at room temperature for 15 min, and then analyzed by flow cytometry (FACSVers, BD, America) (32).

**Network analysis of component-disease interactions**

The genes of targets associated with “melanoma” disease names were collected through CTD,1 and the genes of targets with “DMY” and “MYT” as chemical names were similarly searched. Target genes were screened for overlapping with DMY, MYT, and melanoma. Then, they were uploaded to Cytoscape 3.8.2 to generate a network map of component-gene-disease interactions, and the protein interaction diagrams, gene ontology (GO), and kyoto encyclopedia of genes and genomes (KEGG) pathway diagrams were obtained for further analysis.

**Data analysis and processing**

Calcusyn 2.0 software was used to calculate the CI of DMY and MYT for further analysis. The data were visualized using by Origin 9.0 and were statistically analyzed by SPSS. For statistical analysis, one-way ANOVA was used. Results are presented as means and error bars represent standard deviation (SD). $p < 0.05$ was considered statistically significant.

**Results**

**High-performance liquid chromatography analysis of vine tea extracts**

VTEs were analyzed by HPLC (Figure 1), and three peaks revealed the main components of vine tea were DMY, MYT, and myricetin. The range of DMY was 53.36 $\sim$ 67.09%, of which the wild vine tea in Zhangjiajie, Hunan province, was the highest, with a content of 67.09% (Figure 2).

**Inhibitory effects of vine tea extracts on the proliferation of B16F10 cells**

B16F10 cells were treated with 10 $\mu g/mL$ VTEs, and the inhibition rate of cell proliferation increased gradually within 48 h (Figure 3). Within 24 h, the inhibition rate of VTEs on B16F10 cells was 9.87 $\sim$ 53.62%. The inhibition rate was 14.87 $\sim$ 75.95% within 48 h; the results showed that vine tea had an inhibitory effect on B16F10 cells. Among these, sample D had the best inhibitory effect.

**Correlation analysis**

As shown in Table 1, Quantitative Composition-Activity Relationship analysis showed a very significant correlation between DMY and the proliferation inhibition rate of B16F10 cells, which indicated that DMY in vine tea played an essential role in inhibiting the proliferation of B16F10 cells.

Previous experiments showed that the IC$_{50}$ value of DMY on B16F10 cells was 14.73 $\mu g/mL$. IC$_{50}$ of VTEs on B16F10 cells was 4.45$\sim$12.95 $\mu g/mL$, in which sample D showed the best inhibitory effect (Figure 3). The IC$_{50}$ values of six VTEs were lower than DMY, indicating that VTEs had a better inhibitory effect on B16F10 cells. Therefore, we inferred that MYT in vine tea had no significant correlation with inhibiting B16F10 cell proliferation, but MYT may have a synergistic effect.

**Inhibition of B16F10 cell proliferation by dihydromyricetin and myricitrin**

Single or combined administration of DMY and MYT could inhibit the proliferation of B16F10 cells. The rate of cell proliferation inhibition increased gradually in a dose-dependent manner as drug concentration increased (Figure 4). When the content of DMY in the mixed drug increased, the IC$_{50}$ decreased gradually. When the DMY: MYT was more significant than 1: 1, the IC$_{50}$ of the mix-use group was lower than that of the single-drug group, and the lowest IC$_{50}$ was 19.94 $\mu M$ when DMY: MYT = 8:1 (Table 2). The CI value was used to analyze the experimental results further. DMY and MYT had a synergistic effect on inhibiting the proliferation of B16F10 cells. When they were mixed in the ratio of 1:4–8:1, the CI at IC$_{25}$, IC$_{50}$, and IC$_{75}$ was less than 1, showing a synergistic effect. Among them, the CI of IC$_{25}$ was the lowest, and DMY: MYT = 8:1 showed strong synergism, indicating that the synergistic effect of DMY and MYT was more apparent when the cell inhibition rate was low. Among different proportions, when the ratio of them was 8:1, the CI value was the lowest,
indicating that the synergistic effect of the ratio was the best (Figure 5).

**Effects of combination of dihydromyricetin and myricitrin on tyrosinase activity in B16F10 cells**

DMY and MYT inhibited tyrosinase activity in B16F10 cells when applied alone or in combination. With the increase in drug concentration, the inhibition of tyrosinase activity increased gradually, showing a dose-dependent relationship in which the activity of DMY was more substantial than MYT’s (Figure 6). The IC\(_{50}\) of the combined drug group was lower than that of the single-drug group. The lowest IC\(_{50}\) of DMY: MYT = 2:1 was 62.59 µM (Table 3). When they were mixed in the ratio of 1:4–8:1, the CI of IC\(_{25}\), IC\(_{50}\), and IC\(_{75}\) was less than 1, showing a synergistic effect. Among them, the CI of IC\(_{25}\) was the smallest, and DMY: MYT = 8:1 showed strong synergism, indicating that the synergistic effect was higher when DMY and MYT were used together. Among the different ratio concentrations, the CI value of DMY: MYT = 8:1 was the lowest at IC\(_{50}\) and IC\(_{75}\), while the lowest CI at IC\(_{25}\) was the ratio of 2:1 (Figure 7).

**Inhibition of B16F10 cell proliferation by dihydromyricetin and myricitrin—Fluorescence staining**

Hoechst's staining results showed that DMY and MYT alone or in combination could effectively inhibit the proliferation
FIGURE 2
Contents of DMY and MYT in the extract of different vine teas. Values are means ± SD (n = 3). Different groups of vine tea were prepared at 1 mg/mL, DMY and MYT standards were prepared at different concentrations with methanol solution and were determined by HPLC. Different letters and capitalization indicate significant differences between means at \( P < 0.05 \) by one-way ANOVA followed by Duncan comparison test. DMY, dihydromyricetin; MYT, myricitrin.

FIGURE 3
Inhibitory effect of different kinds of vine tea extracts on the proliferation of B16F10 cells. Values are means ± SD (n = 3). B16F10 cells were treated for 24 h with inhibitor (10 \( \mu \)g/mL VTEs of different varieties) and medium (control), and cell viability was determined using the MTT assay. Different letters and capitalization indicate significant differences between means at \( P < 0.05 \) by one-way ANOVA followed by Duncan comparison test. (A) Wild vine tea in Enshi, Hubei. (B) Hubei Enshi selenium-rich vine tea. (C) Guangzhou Qingyuan (Level 1). (D) Guangzhou Qingyuan (Level 2). (E) Wild vine tea in Shiqian, Guizhou. (F) Wild vine tea in Zhangjiajie, Hunan.

TABLE 1 Correlation analysis.

| Administration time | 24 h | 48 h |
|---------------------|------|------|
| Single use          | R    | P    | R    | P    |
| DMY                 | -0.882 | <0.001 | 0.713 | 0.009 |
| MYT                 | 0.443  | 0.150  | 0.423 | 0.170 |
| Myricetin           | -0.562 | 0.057  | 0.540 | 0.070 |

FIGURE 4
The effects of DMY and MYT on B16F10 cell proliferation. Values are means ± SD (n = 3). B16F10 cells were treated with DMY, MYT, or mix-use (1:4 ~ 8:1) for 72 h, before assessment of viability by MTT assay. The dose-response relationship was obtained by transformation according to the equation. DMY, dihydromyricetin; MYT, myricitrin.

TABLE 2 Half-inhibitory concentration of drugs on B16F10 cell proliferation.

| Sample          | DMY | MYT | DMY: MYT |
|-----------------|-----|-----|----------|
|                 | 1:4 | 1:2 | 1:1      | 2:1  | 4:1  | 8:1  |
| IC50 (\( \mu \)M)| 45.98 | 109.1 | 64.64 | 43.65 | 41.87 | 27.41 | 19.94 |
| r                | 0.9  | 0.95 | 0.99    | 0.99 | 0.99 | 0.98 | 0.98 |

MYT. The best effect was at 8:1 in the combined drug group (Figure 8A). The quantitative fluorescence results showed that the 8:1 group had the lowest mean fluorescence intensity, which was consistent with the image results (Figure 8B).

Effects of B16F10 cells on cell cycle in combination with dihydromyricetin and myricitrin

The effects of DMY and MYT on the cell cycle of B16F10 cells were analyzed by flow cytometry. As shown in Table 4, the results of DMY, MYT, and the combination group on the cell cycle of B16F10 cells were mainly characterized by reducing...
FIGURE 5
Analysis of the combined inhibitory effects of DMY and MYT on B16F10 cell proliferation. Values are means ± SD (n = 3). B16F10 cells were treated with DMY, MYT, or mix-use (1:4 ~ 8:1) for 72 h, before assessment of viability by MTT assay. The CI value was calculated by the equation. DMY, dihydromyricetin; MYT, myricitrin.

FIGURE 6
Tyrosinase inhibition by DMY and MYT in B16F10 cells. Values are means ± SD (n = 3). After B16F10 cells were treated with DMY, MYT, or mix-use (1:4 ~ 8:1) for 72 h, 90 μL of 1% TritonX-100 was added to each well, and then 10 μL of 1.0 mg/mL L-DOPA was added. The absorbance at 490 nm was measured after 5 min of ultrasound and treatment at 30°C for 30 min. The dose-response relationship was obtained by transformation according to the equation. DMY, dihydromyricetin; MYT, myricitrin.

TABLE 3 Half-inhibitory concentrations of drugs on tyrosinase in B16F10 cells.

| Sample | DMY    | MYT    | DMY: MYT |
|--------|--------|--------|----------|
|        | IC₅₀ (μM) |        |          |
| 1:4    | 107.7  | 233.95 | 98.68    |
| 1:2    | 99.42  | 54.07  | 82.34    |
| 1:1    | 75.67  | 32.34  | 62.59    |
| 2:1    | 73.07  | 62.59  | 78.91    |
| 4:1    | 73.07  | 78.91  | 85.42    |
| 8:1    |        |        | 73.07    |

The mechanism of action of vine tea in the prevention and treatment of melanoma was studied. The targets of DMY and MYT on melanoma were shown in Figure 9A. 8 targets of DMY and MYT related to melanoma, among which DMY acted on TYRP1, PARP1, MC1R, and TYR, while MYT acted on TP53, TNF, PPARG, and PTGS2.

Two components-disease cross targets were uploaded to the String database to construct a protein-protein interaction network (PPI). In this network, eight targets of dihydromyricetin and myricitrin on melanoma were studied.
FIGURE 7
Analysis of the combined effect of DMY and MYT on inhibiting tyrosinase in B16F10 cells. Values are means ± SD (n = 3). After B16F10 cells were treated with DMY, MYT, or mix-use (1:4 ∼ 8:1) for 72 h, 90 µL of 1% TritonX-100 was added to each well, and then 10 µL of 1.0 mg/mL L-DOPA was added. The absorbance at 490 nm was measured after 5 min of ultrasound and treatment at 30°C for 30 min. The dose-response relationship was obtained by transformation according to the equation. The CI value was calculated by the equation. DMY, dihydromyricetin; MYT, myricitrin.

could interact with proteins, and 14 edges represent the interactions between proteins. The average degree of freedom of each node in the network was 3.5, the average betweenness centrality was 0.101190, and the average closeness centrality was 0.645117 (Figure 9B). There were three targets above the average, speculating that TP53, TNF, and TYR might be the critical targets of vine tea in preventing and treating melanoma (Table 5). Interleukin-4 and 13 signaling, thyroid cancer, pathways in cancer, signaling by interleukin, interleukin-10 signaling, melanogenesis, melanin biosynthesis, and tyrosine metabolism might be the key to treating melanoma.

GO pathway enrichment analysis

The functional enrichment analysis of GO terms was carried out. A total of 474 enrichment results were obtained in the biological process (P < 0.01), and 10 categories with the highest functional values were selected, mainly related to the metabolic synthesis of melanin, including the corresponding response to light stimulation and the negative regulation of gene silencing. Twenty six enrichment results were obtained by molecular function, mainly related to oxidoreductase activity, and 12 enrichment results were obtained by cell composition, including melanosome membrane, and complex transcription mechanism (Figure 10A).

KEGG pathway enrichment analysis

The selected targets were analyzed by KEGG pathway enrichment analysis, and 6 enrichment results were obtained (P < 0.01). It included the melanogenesis signaling pathway, NF-κB signaling pathway, tyrosine metabolism signaling pathway, thyroid cancer signaling pathway, and apoptosis signaling pathway, suggesting that DMY and MYT play a role in preventing and treating melanoma by acting on the multiple pathways (Figure 10B).

Discussion

Melanoma is challenging to treat because of its ability to metastasize at early stages and its resistance to conventional cancer treatments (33, 34). The use of molecular targeted drugs and immunotherapy for melanoma is limited by the high cost and significant side effects (2). Therefore, safer and more effective treatments are necessary. Natural products have few side effects and do not contain any drug residues. The compatibility of active components can improve disease prevention and treatment efficacy and has been widely used in anti-tumor. For example, Cuphea aequipetala extracts can induce cell accumulation in the G1 phase of the cell cycle, induce apoptosis, and thus exhibit inhibitory activity (35). Menke et al. reported that dandelion extract and mistletoe
FIGURE 8
Analysis of the synergistic inhibitory effect of DMY and MYT on cell proliferation by fluorescence staining. Cell viability was determined by Hoechst 33342 fluorescent staining and pictures were quantified with ImageJ. Values are means ± SD (n = 3). Different letters indicate significant differences between means at P < 0.05 by one-way ANOVA followed by Duncan comparison test. (A) Fluorescence photography. (B) Fluorescence quantitative analysis. (A) Control. (B) DMY. (C) MYT. (D) DMY: MYT = 1:4. (E) DMY: MYT = 1:2. (F) DMY: MYT = 1:1. (G) DMY: MYT = 2:1. (H) DMY: MYT = 4:1. (I) DMY: MYT = 8:1. DMY, dihydromyricetin; MYT, myricitrin; Mean, mean gray value.

extract could promote neuroblastoma cell apoptosis (36). Sturza et al. found that quercetin could simultaneously regulate the pathway of glycolysis and mitochondria to produce ATP to kill cancer cells (37). Some studies have shown that flavonoids can inhibit mTOR and RAS carcinogenic pathways, activate apoptosis, and lead to cell cycle stagnation. The vine tea contains many flavonoids, which have certain biological activities in cells and have anti-tumor effects (38). Huang et al. found that DMY inhibits melanin synthesis through its antioxidant properties and down-regulation of protein kinase A, protein kinase C, and mitogen-activated protein kinase signal pathways (39). Our data showed that the VTEs could inhibit B16F10 cells, and the inhibitory effect on tyrosinase was consistent with cell inhibition experiments. In organisms, tyrosinase is the key enzyme involved in melanin biosynthesis (31). It has been reported that individual flavonoids are potential melanin
synthesis inhibitors in mammalian melanocytes (40, 41). For melanoma diseases, only flavonoids with an IC$_{50}$ value of less than 50 $\mu$M can inhibit mammalian tyrosinase, thus reducing the melanin synthesis of B16F10 (31).

Some studies have shown that natural products target pathogens through a combination of different structures and functions (42). The active components in vine tea are present as mixtures. The activity of purified extracts of single compounds is weaker than that of crude extracts, suggesting that maximum bioactivity is obtained through the interaction of different functional factors (43). Previous studies have compared the theoretical value (T-EM) with the actual experimental value (EM). If the EM is higher than the T-EM, the two compounds are considered to have a synergistic effect. The EM value is the sum of the effect values of each combination. For example, it has been found that the synergistic effect of EGCG and metformin can inhibit cancer cell proliferation and promoting cell apoptosis (44). These flavonoids have also been shown to inhibit melanoma (37). This inhibitory effect has two main aspects: inhibiting cancer cell proliferation and promoting cancer cell apoptosis (45–47). Recent studies have shown inhibitory effects on the expansion of human acute promyelocytic leukemia cells and K562 cells (48), Bel-7402 cells (49), human breast cancer cells (50, 51), and nasopharyngeal carcinoma HK –1 cells (27). We expected that both DMY and MYT would have inhibitory effects on B16F10 cells. However, the IC$_{50}$ value of DMY on B16F10 cells was 14.73 $\mu$g/mL, and the IC$_{50}$ of MYT on B16F10 cells was 50.66 $\mu$g/mL, and the IC$_{50}$ of VTEs on B16F10 cells was 4.45–12.95 $\mu$g/mL, which showed that the effect was not as significant as a crude extract. We hypothesized that the combination of active compounds in the extracts could enhance the inhibitory effect on melanoma disease. Therefore, the correlation between DMY, MYT, and myricetin in vine tea was analyzed, and DMY had a very significant correlation. Further inhibition experiments showed that the IC$_{50}$ of VTEs was lower than that of DMY. There might be a synergistic effect between the active compounds. We selected DMY and MYT for further study since our previous experimental results indicated that DMY and myricetin had no synergistic effect, whereas DMY and MYT did show an effect. B16F10 cells were treated with different ratios of DMY and MYT, ranging from 1:4 to 8:1; it was found that DMY:MYT = 8:1 was the most effective in inhibiting B16F10 and tyrosinase activity. Fluorescence staining showed that the number of cells decreased with the increase of DMY concentration, and the inhibitory effect of DMY was more substantial than MYT’s. Compared with the control group, the proportion of cells in S phase, or G2 phase was increased, but G1 phase was decreased. The effects of DMY and MYT on B16F10 cells were analyzed by flow cytometry, which showed that the cell cycle was arrested in the S phase and G2 phase. The combined treatment could effectively reduce the number of cells entering the G1 phase. The inhibition of DMY on melanoma cells was higher than that of MYT. We speculated that the proportion of DMY had an important effect on the synergism. It was observed that the IC$_{50}$ values of the mix-drugs decreased with increased DMY content. Especially in the range of 1:1~8:1, they showed lower IC$_{50}$ than single-use. Based on CI and isobole methods, the 8:1 group showed a strong synergistic effect. However, only a narrow range of ratios were tested in this experiment; perhaps increasing the ratios will produce stronger effects, which will be tested in the follow-up experiments.

Furthermore, network pharmacology was used to create a network map of the “active ingredient-acting gene-disease” of vine tea on melanoma disease. It was found that DMY and MYT have the same target and act on eight different targets of melanoma disease, which might be the reason for the synergism between DMY and MYT. Based on the interaction analysis of these targets, TP53, TFE, and TYR might be the key targets for the prevention and treatment of melanoma diseases. P53 protein promoted cancer cell apoptosis by responding to the anti-proliferation effects of various physiological processes such as aging (52). GO, and KEGG enrichment analysis found that the prevention and treatment of melanoma were related to ultraviolet reaction, melanosome membrane, chitosan, receptor binding, NF-$\kappa$B signal pathway, and apoptosis. Abnormal expression of NF-$\kappa$B has been associated with various cancers (53). Exposure to ultraviolet (UV) radiation, that is, UVA (315–400 nm), and UVB (280–315 nm), is considered to be a significant risk factor for melanoma (54). Melanosomes could synthesize and organize melanin. Melanin synthesis and melanosome transport disorders are associated with pigmented diseases (55). The crosstalk between protease-activated receptor 1 and platelet-activating factor receptor has been demonstrated.

### Table 4 Effects of the drugs on the cell cycle of B16F10 cells.

| Sample | Control | DMY | MYT | DMY: MYT |
|--------|---------|-----|-----|----------|
|        |         |     |     |          |
| G1/%   | 75.9 ± 0.58a | 59.11 ± 2.00d | 67.81 ± 5.3b | 63.5 ± 0.75c |
| S/%    | 22.05 ± 1.39d | 35.32 ± 1.10a | 27.25 ± 4.97c | 28.51 ± 0.75bc |
| G2/%   | 2.05 ± 0.82d | 5.57 ± 0.94bc | 4.96 ± 0.33c | 7.98 ± 0.03a |

The mean value of different letters in the same row is significantly different from the mean value of control ($P < 0.05$).
FIGURE 9
Analysis of action targets of DMY and MYT on melanoma. (A) The targets of dihydromyricetin and myricitrin on melanoma. (B) The protein-protein interaction network of DMY, MYT, and melanoma disease.
TABLE 5 Basic information of crucial gene in the control of melanoma by vine tea.

| Gene | Pathway                                                                 | Degree | Betweenness | Closeness |
|------|-------------------------------------------------------------------------|--------|-------------|-----------|
| TP53 | Interleukin-4 and 13 signaling, thyroid cancer, pathways in cancer, signaling by Interleukins | 6      | 0.381       | 0.875     |
| TNF  | Interleukin-4 and 13 signaling, signaling by interleukins, interleukin-10 signaling | 5      | 0.167       | 0.778     |
| TFR  | Melanogenesis, melanin biosynthesis, tyrosine metabolism                | 4      | 0.190       | 0.700     |

FIGURE 10 Enrichment analysis of key genes. (A) Results of GO enrichment analysis. (B) Results of KEGG enrichment analysis. BP, biological process; CC, cellular component; MF, molecular function.
to regulate the expression of melanoma cell adhesion molecule (MCAM/MUC18) metastasis of melanoma (56).

Conclusion

The combination of DMY and MYT in vine tea could synergistically inhibit the proliferation of B16F10 cells, and they have a synergistic effect on different targets. Through network pharmacology, it is concluded that TP53, TNF, and TYR are the main targets of DMY and MYT in melanoma disease and regulate signaling pathways such as melanogenesis, NF-κB, and apoptosis. Regular application of low-toxicity and vine tea extract can contribute to the prevention and treatment of melanoma.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

Q C and X Q designed the study, NZ and HK performed the experiments, sorted out the data, and drew the drawings. HL and QS provided the experimental guidance. NZ participated in manuscript writing. QC revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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References

1. Quintanilla-Diez MJ, Bichakjian CK. Management of early-stage melanoma. *Facial Plast Surg Clin North Am.* (2019) 27:35–42. doi: 10.1016/j.fsc.2018.08.003
2. Namikawa K, Yamazaki N. Targeted therapy and immunotherapy for melanoma in Japan. *Curr Treat Options Oncol.* (2019) 20.7. doi: 10.1007/s11864-019-00607-8
3. Lodde G, Zimmer L, Livingstone E, Schadendorf D, Ugurel S. [Malignant Melanoma]. *Haustartz.* (2020) 71:63–77. doi: 10.1007/s00105-019-04514-0
4. Puza C, Bressler ES, Terando AM, Howard JH, Brown MC, Hanks B, et al. The emerging role of surgery for patients with advanced melanoma treated with immunotherapy. *J Surg Res.* (2019) 236:209–15. doi: 10.1016/j.jss.2018.11.045
5. Austin E, Mamalis A, Ho D, Jagdeo J. Laser and light-based therapy for cutaneous and soft-tissue metastases of malignant melanoma: a systematic review. *Arch Dermatol Res.* (2017) 309:229–42. doi: 10.1007/s00105-017-1720-9
6. Zhang Q, Jin L, Jin Q, Wei Q, Sun M, Yue Q, et al. Inhibitory effect of Dihydroartemisinin on the proliferation and migration of melanoma cells and induced melanogenesis via Pka and Erk signaling pathways in B16f10 melanoma cells. *Front Pharmacol.* (2021) 12:727275. doi: 10.3368/fphar.2021.727275
7. Zhang Q, Jin L, Jin Q, Wei Q, Sun M, Yue Q, et al. Inhibitory effect of dihydroartemisinin on the proliferation and migration of melanoma cells and experimental lung metastasis from melanoma in mice. *Front Pharmacol.* (2021) 12:727275. doi: 10.3389/fphar.2021.727275
8. Ghazizadeh F, Shafiei M, Falak R, Panahi M, Rakhshani N, Ebrahimi SA, et al. Xanthomicrol exerts antiangiogenic and antitumor effects in a mouse melanoma (B16F10) allograft model. *Evid Based Complement Alternat Med.* (2020) 1914. doi: 10.1155/2020/8543872
9. Guo L, Kang JS, Kang NJ, Choi YWS. Petasin induces apoptosis and inhibits cell migration through activation of P53 pathway signaling in melanoma B16F10 Cells and A375 Cells. *Arch Biochem Biophys.* (2020) 692:108519. doi: 10.1016/j.abb.2020.108519
10. Huang HC, Wang SS, Tsai TC, Ko WP, Chang TM. Phoenix Dactylifera L. Seed extract exhibits antioxidant effects and attenuates melanogenesis in B16F10 murine melanoma cells by downregulating Pka signaling. *Antioxidants.* (2020) 9:1270. doi: 10.3390/antioxid9121270
11. Nam G, An SK, Park IC, Bae S, Lee JH. Daphnetin inhibits alpha-Msh-induced melanogenesis via Pka and Erk signaling pathways in B16F10 melanoma cells. *Biosci Biotechnol Biochem.* (2022) 86:596-609. doi: 10.1093/bbb/aclc016
12. Zhang L, Zhu L, Yao X, Lou X, Wan J, Duan X, et al. Paclitaxel treatment enhances-lymphatic metastasis of B16F10 melanoma cells via Cdx2/Cxcl7 Axis. *Int J Biol Sci.* (2022) 18:1476–90. doi: 10.7150/ijbs.67138
13. Schomberg J, Wang Z, Farhat A, Guo KL, Xie J, Zhou Z, et al. Luteolin inhibits melanoma growth in vitro and in vivo via regulating Ecm and oncogenic pathways but not Ros. *Biochim Biophys Acta Mol Cell Biol.* (2020) 177:114025. doi: 10.1016/j.bmcb.2020.114025
14. Liu HM, Lei SN, Tang W, Xun MH, Zhao ZW, Cheng MY, et al. Optimization of ultrasound-assisted cellulase extraction from nymphaea hybrid flower and biological activities: antioxidant activity, protective effect against Ros oxidative damage in hacat cells and inhibition of melanin production in B16 cells. *Molecules.* (2022) 27:1914. doi: 10.3390/molecules27061914
Phytother Res. peel suppress the metastatic potential of murine melanoma b16f10 cells in vitro and
Cytometry. et al. Best practices in cell culture: an overview. J Cancer. tumor growth and epithelial-mesenchymal transition through regulating Mir-455-
Sci Rep. potential targets of astragalus membranaceous-angelica sinensis compound acting
glioma through inducing apoptosis and autophagy dependent on ros generation
Molecules. tyrosinase inhibitory activity and melanin synthesis in melanocytes.
Food Chem. exclusive and nonexclusive inhibitors.
Nat Prod Res. In vitro and myricetin as major bioactive compounds.
Ther. Research on vine tea, a potential and functional herbal tea with dihydromyricetin
Brain Res Bull. the oxidative stress response induced by methylglyoxal via the Ampk/424
Mol Cell Biochem. mode of action of dihydromyricetin from ampelopsis grossedentata leaves against
Food Chem. hypericum perforatum extracts in the forced swimming test. Planta Med. (1999) 179:297–304. doi: 10.1002/(SICI)1097-4652(199906)179:3<297::

15. Chen S, Zhao X, Wan J, Ran L, Qin Y, Wang X, et al. Dihydromyricetin improves glucose and lipid metabolism and exerts anti-inflammatory effects in nonalcoholic fatty liver disease: a randomized controlled trial. Pharmacol Res. (2015) 99:74–81. doi: 10.1016/j.phrs.2015.05.009
16. Jiang B, Le I, Pan H, Hu K, Xu X, Xiao P. Dihydromyricetin ameliorates the oxidative stress response induced by methylglyoxal via the Ampk/GL4 signaling pathway in Pc12 Cells. Brain Res Bull. (2014) 109:117–26. doi: 10.1016/j.brainresbull.2014.10.010
17. Ye L, Wang H, Duncan SE, Engel WN, O’Keefe SF. Antioxidant activities of vine tea (Amelopsis Grossedentata) extract and its major component dihydromyricetin in soybean oil and cooked ground beef. Food Chem. (2015) 172:416–22. doi: 10.1016/j.foodchem.2014.09.091
18. Xiao XN, Wang F, Yuan YT, Liu J, Liu YZ, Yi X. Antibacterial activity and mode of action of dihydromyricetin from amelopsis grossedentata leaves against food-borne bacteria. Molecules. (2019) 24:2831. doi: 10.3390/molecules24152831
19. Hou XL, Tong Q, Wang WQ, Shi CY, Xiong W, Chen J, et al. Suppression of inflammatory response by dihydromyricetin, a flavonoid from amelopsis grossedentata, via inhibiting the activation of NF-kappp and mapk signaling pathways. J Nat Prod. (2015) 78:1689–96. doi: 10.1021/acs.jnatprod.5b00275
20. Wu J, Zhao FT, Fan KJ, Zhang J, Xu BX, Wang QS, et al. Dihydromyricetin inhibits inflammation of fibroblast-like synovocytes through regulation of nuclear factor-kappp signaling in rats with collagen-induced arthritis. J Pharmacol Exp Ther. (2019) 368:218–28. doi: 10.1121/jpet.11.253369
21. Zhang Q, Zhao Y, Zhang M, Zhang Y, Ji H, Shen L. Recent advances in research on vine tea, a potential and functional herbal tea with dihydromyricetin and myricetin as major bioactive compounds. J Pharm Anal. (2021) 11:555–63. doi: 10.1007/s12267-020-0216-0
22. Zhu L, Zhang B, Luo J, Dong S, Zhang K, Wu Y. Amelopsis-sodium induces apoptosis in human lung adenocarcinoma cell lines by promoting tubulin polymerization in vitro. Oncol Lett. (2019) 18:1899–96. doi: 10.3892/ol.2019.10288
23. Guo Z, Guozhang H, Wang H, Li Z, Liu N. Amelopsin inhibits human glioma through inducing apoptosis and autophagy dependent on ros generation and Jnk pathway. Biomed Pharmacother. (2019) 116:108524. doi: 10.1016/j.biopharm.2018.12.136
24. Li X, Yang ZS, Cai WW, Deng Y, Chen I, Tan SL. Dihydromyricetin inhibits tumor growth and epithelial-mesenchymal transition through regulating Mr-455-3p in cholangiocarcinoma. J Cancer. (2021) 12:6058–70. doi: 10.7150/jca.61311
25. Dong Y, Zhao Q, Wang Y. Network pharmacology-based investigation of potential targets of astragals membranaceous-angelica sinensis compound acting on diabetic nephropathy. Sci Rep. (2021) 11:19496. doi: 10.1038/s41598-021-10288-5
26. Baust JM, Buehring GC, Campbell L, Elmore E, Harbell JW, Nims RW, et al. Recent advances in development and disease. Pharmacol Ther. (2019) 368:218–28. doi: 10.1016/j.phrs.2019.05.009
27. Nordin ND, Schötz K. Rutin is essential for the antidepressant activity of hypericum perforatum extracts in the forced swimming test. Planta Med. (2002) 68:577–80. doi: 10.1055/s-2002-32908
28. Xu A, Le J, Zhao Y, Wang Y, Li X, Xu P. Potential effect of Egg on the anti-tumor efficacy of metformin in melanoma cells. J Zhejiang Univ Sci B. (2021) 22:548–62. doi: 10.1613/jzus.B200455
29. Yang Y, DeMarco VG, Nicholl MB. Resveratrol enhances radiation sensitivity in prostate cancer by inducing cell proliferation and promoting cell senescence and apoptosis. Cancer Sci. (2012) 103:1990–8. doi: 10.1111/j.1349-7006.2012.02272.x
30. Tan SM, Deng XT, Zhou J, Li QP, Ge XW, Mao L. Pharmacological basis and new insights of queretin action in respect to its anti-cancer effects. Biomed Pharmacother. (2020) 121:109604. doi: 10.1016/j.biopharm.2019.109604
31. Ji X, Donovan MG, Branco GM, Limesand KH, Burd R. Quercetin as an emerging anti-melanoma agent: a four-focus area therapeutic development strategy. Front Nutr. (2021) 18:34. doi: 10.3892/fnut.2016.00488
32. Davenport A, Frezza M, Shen M, Ge Y, Hoo C, Chan TH, et al. Celastrol and an Egg prote-drug exhibit potent chemosensitizing effect in human leukemia cells. Int J Mol Med. (2010) 25:465–70. doi: 10.3892/ijmm_00000366
33. Jia J, Wei X, Qian J, Mo X, Kai G, An F, et al. 2′,4′-Dihydroxy-6′-Methoxy-3′,5′-dimethylchalcone induced apoptosis and G1 cell cycle arrest through Ptk/Akt pathway in bel-7402/5-Fu Cells. Food Cells Toxicol. (2019) 131:110533. doi: 10.1016/j.fct.2019.05.041
34. Lu R, Serrero G. Resveratrol, a natural product derived from grape, exhibits antiangiogenic activity and inhibits the growth of human breast cancer cells. J Cell Physiol. (1999) 179:101–7. doi: 10.1002/(SICI)1097-4652(199906)179:3<101::AID-JCP2>3.0.CO;2-P
35. Kai W, Yating S, Lin M, Kaiyong Y, Baoqin H, Wu Y, et al. Natural product tosoendanin reverses the resistance of human breast cancer cells to adriamycin as a novel Ptk inhibitor. Biochem Pharmacol. (2018) 152:153–64. doi: 10.1016/j.bcp.2018.03.022
36. Kanapathipillai M. Treating P53 mutant aggregation-associated cancer. Cancers. (2018) 10:1514. doi: 10.3390/cancers10061514
37. Prasad S, Ravindran J, Aggarwal BB. NF-kappab and cancer: how intimate in this relationship. Mol Cell Biochem. (2010) 336:25–37. doi: 10.1007/s11010-009-0827-2
38. Sample A, He YY. Mechanisms and prevention of Uv-induced melanoma. Photodermatol Photomed Photobiol. (2018) 34:13–24. doi: 10.1111/jppp.12329
39. Tian X, Cui Z, Liu S, Zhou J, Cui R. Melanosome transport and regulation in development and disease. Pharmacol Ther. (2021) 219:107707. doi: 10.1016/j.pharmthera.2020.107707
40. Melnikova VO, Balasubramanian K, Villares GI, Dobrof AS, Zigler M, Wang H, et al. Crosstalk between protease-activated receptor 1 and platelet-activating factor receptor regulates melanoma cell adhesion molecule (Mcam/Muc18) expression and melanoma metastasis. J Biol Chem. (2009) 284:28845–55. doi: 10.1074/jbc.M109.042150