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Postprint of Preliminary Study on the Anti-melanoma Material Basis of Veronica didyma

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Abstract

To identify the anti-melanoma active fraction and material basis of Veronica didyma Tenore, this study employed the CCK8 assay to evaluate the proliferation inhibitory effects of the four extraction fractions of the ethanol extract of V. didyma (petroleum ether layer, ethyl acetate layer, n-butanol layer, water layer), the ethanol extract, and monomeric compounds on melanoma cell lines (B16 and A375); phytochemical techniques and methods were utilized to conduct systematic isolation and purification studies on the chemical constituents of the active fraction. The results demonstrated: (1) The ethyl acetate extraction fraction (PPNE) exhibited superior inhibitory effects on B16 and A375 cell proliferation compared to other samples, with IC50 values of 0.177 mg·mL-1 (B16) and 2.826 mg·mL-1 (A375), respectively. (2) Seven monomeric compounds were isolated from the active fraction PPNE, namely p-hydroxybenzaldehyde (1), picroside II (2), isoscutellarein 7 - O - (6’’’ - Oacetyl) - β - allopyranosyl (1’’ ‘$→$2’ ‘) - β - glucopyranoside (3), 3’ - hydroxyl - 4’ - O - methylisoscutellarein 7 - O - [6’’’ - O - acetyl - β - D - allopyranosyl - (1$→$2) - β - D - Glucopyranoside (4), 6 - O - veratroylcatalposide (5), Veronicoside (6), and isoscutellarein 4’ - methyl ether 7 - O - (6’’’ - O - acetyl) - ballopyranosyl (1’’ ‘$→$2’’) - β - glucopyranoside (7). All seven compounds were isolated from this plant for the first time, and HPLC analysis revealed that these seven compounds are relatively abundant constituents of PPNE. Except for compound 1, the other six monomeric compounds demonstrated favorable inhibitory effects on melanoma cell proliferation, with the anti-melanoma activity of compounds 3, 4, and 7 being reported for the first time in this study. The ethyl acetate extraction fraction of Veronica didyma (PPNE) represents its anti-melanoma active fraction, with iridoid compounds 2, 5, 6 and flavonoid compounds 3, 4, 7 potentially constituting the material basis for the anti-melanoma activity of PPNE. These findings provide a scientific foundation for the rational utilization of this resource.

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Full Text

Preliminary Study on the Material Basis of Anti-Melanoma Activity of *Veronica didyma* Tenore

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Abstract

To identify the anti-melanoma active fraction and material basis of *Veronica didyma* Tenore, we evaluated the proliferation inhibitory effects of four extraction fractions (petroleum ether, ethyl acetate, n-butanol, and water layers) derived from the ethanol extract, as well as the ethanol extract itself and monomeric compounds, on melanoma cell lines (B16 and A375) using the CCK8 assay. Phytochemical techniques and methods were employed to systematically separate and purify the chemical constituents of the active fraction. The results demonstrated that: (1) The ethyl acetate extract (PPNE) exhibited superior inhibitory activity against B16 and A375 cell proliferation compared to other samples, with IC_{50} values of 0.177 $mg \cdot mL^{-1}$ (B16) and 2.826 $mg \cdot mL^{-1}$ (A375), respectively. (2) Seven monomeric compounds were isolated from the active PPNE fraction: p-hydroxybenzaldehyde (1), Picroside II (2), isoscutellarein 7-O-(6"'-O-acetyl)-β-allopyranosyl(1"'$\rightarrow$2")$-$ $-glucopyranoside(3), 3'-hydroxy-4'-O-methylisoscutellarein7-O-[6'''-O-acetyl- -D-allopyranosyl-(1\rightarrow2)- -D-glucopyranoside(4), 6-O-veratroylcatalposide(5), Veronicoside(6), andisoscutellarein4'-methylether7 - O - (6''' - O - acetyl) - ballopyranosyl(1'''\rightarrow2'')-$-glucopyranoside (7). All seven compounds were isolated from this plant for the first time, and HPLC analysis revealed that these compounds are major constituents of PPNE. Except for compound 1, the other six monomeric compounds demonstrated significant inhibitory effects on melanoma cell proliferation. This study reports for the first time the anti-melanoma activity of compounds 3, 4, and 7. The ethyl acetate extract (PPNE) of *Veronica didyma* represents the anti-melanoma active fraction, with iridoid compounds 2, 5, 6 and flavonoid compounds 3, 4, 7 likely constituting the material basis for its anti-melanoma activity. These findings provide a scientific foundation for the rational utilization of this resource.

Keywords: *Veronica didyma*, chemical constituents, melanoma, isolation and purification

Introduction

The genus *Veronica* L. (Scrophulariaceae) comprises approximately 250 species distributed worldwide, primarily across Europe and Asia. To date, over 60

species have been identified in China, mainly in the southwestern region, most of which are traditional medicinal plants used to treat various ailments including colds, hemoptysis, hernias, hemorrhage, blood stasis, detoxification, tissue regeneration, and rheumatism. Recent studies have increasingly focused on the biological activities of *Veronica* species due to their remarkable traditional efficacy, revealing promising antioxidant, antiviral, antitumor, anti-inflammatory, hepatoprotective, and antimicrobial properties. Notably, these plants exhibit significant activity against malignant tumors. For instance, methanol extracts of *Veronica anagallis-aquatica* and *Veronica hederifolia* have demonstrated pronounced inhibitory effects on oral epidermoid carcinoma (KB) and B16 melanoma cells, highlighting the genus' s potential as a source of anticancer agents, though the underlying active constituents remain unclear.

Veronica didyma, a representative traditional medicinal species within the genus, is used as a whole herb for detoxification, swelling reduction, blood cooling, hemostasis, and pain relief in the treatment of carbuncles, hemoptysis, hernias, orchitis, and leukorrhea. The plant is widely distributed across China with abundant resources. Our previous research revealed that the ethanol extract of *V. didyma* effectively inhibited B16 cell proliferation with an IC_{50} of 1.955 mg $\cdot$ mL^{-1}. However, to our knowledge, no studies have investigated the chemical constituents of this plant, and its active material basis remains unknown.

Building upon these reports and our preliminary findings, this study aims to elucidate the anti-melanoma active fraction and constituents of *V. didyma* to facilitate better resource utilization. Using phytochemical separation and purification techniques guided by inhibitory activity against B16 and A375 melanoma cell lines, we addressed three key questions: (1) identification of the anti-melanoma active fraction from the ethanol extract; (2) characterization of the material basis in the active fraction; and (3) evaluation of the anti-melanoma activity of individual chemical constituents. The results provide a scientific basis for the rational development and utilization of *V. didyma* resources.

1. Instruments and Materials

Nuclear magnetic resonance spectrometers (Bruker AM-400 and AM-500, Germany), high-performance liquid chromatography system (Shimadzu LC-20A series), preparative chromatography system (Shimadzu Essentia Prep LC-16P series), multi-functional microplate reader (Cytation™ 3), CO_2 incubator (Thermo Fisher Scientific Form 311), biological safety cabinet (Thermo Fisher Scientific MSC-Advantage 1.8), dextran gel (Sephadex LH-20, Pharmacia), pre-coated silica gel plates (Qingdao Marine Chemical, GF254), preparative chromatography column (C18 column, YMC-Pack ODS-A, 250$×$20 mm, 5 m, YMC Co., Ltd., Kyoto, Japan), methanol (chromatographic grade, Macklin), and other conventional reagents (analytical grade, Sinopharm Group) were used. Mouse melanoma cell line B16 and human melanoma cell line A375 were obtained from Wuhan Boster Biological Technology. The CCK8 assay kit was purchased from Shanghai Sangon Biotech. DMSO was from Biosharp, fetal

bovine serum from Gibco, RPMI-1640 medium from HyClone, and trypsin from Gibco.

Plant material was collected in April 2020 from Suxian District, Chenzhou City, Hunan Province, and identified as the whole herb of *Veronica didyma* Tenore (Scrophulariaceae) by Professor Liu Peng of Xiangnan University. A voucher specimen (PPN202005CZ) is deposited at the Key Laboratory of Natural Products for Cardiovascular and Cerebrovascular Diseases, Hunan Provincial Department of Education, Xiangnan University.

2. Methods

2.1 Extraction and Separation

Fresh whole herb of *V. didyma* was air-dried, and 1 kg of the dried material was pulverized and extracted three times with 90% ethanol at room temperature for 72 hours each. The extracts were combined and concentrated under reduced pressure until ethanol-free, then diluted with pure water to a final volume of 1 L. The aqueous dispersion was sequentially extracted with petroleum ether, ethyl acetate, and n-butanol in small portions until the extract color became faint, yielding 7.7 g of petroleum ether fraction, 13.30 g of ethyl acetate fraction, 9.30 g of n-butanol fraction, and 25.46 g of water fraction after solvent recovery.

Bioactivity evaluation revealed that the ethyl acetate fraction (PPNE) exhibited prominent inhibitory activity against B16 and A375 melanoma cells compared to other fractions. Therefore, PPNE was subjected to systematic separation and purification. The PPNE sample (12.56 g) was dissolved in methanol, filtered through a 0.45 m microporous membrane, mixed with reversed-phase silica (ODS), and subjected to ODS column chromatography using methanol:water (1:1 to 1:0) as eluent. Fractions were monitored by HPLC and TLC, combined to yield five fractions (A-E). Fraction A was repeatedly chromatographed on a dextran gel column (Sephadex LH-20, methanol:water system) to obtain compound 1 (45 mg) and compound 2 (39 mg). Fraction B was similarly processed on Sephadex LH-20 to yield compound 5 (19 mg). Fraction C was subjected to repeated Sephadex LH-20 chromatography to obtain compound 6 (15 mg). Fractions containing compounds 3 and 4 were combined and purified by semi-preparative HPLC (detection wavelength 254 nm, methanol:water 55:45) followed by gel column chromatography to yield compound 3 (21 mg) and compound 4 (19 mg). Fraction E was processed by Sephadex LH-20 chromatography followed by semi-preparative HPLC (detection wavelength 254 nm, methanol:water 65:35) and gel column chromatography purification to obtain compound 7 (35 mg).

[Figure 1: see original paper] Extraction and isolation procedure of *Veronica didyma* Tenore

2.2 HPLC Analysis

HPLC analysis of the active ethyl acetate fraction (PPNE) was performed on a Kromasil 100-5-C18 column (4.6$\times$250mm)at a detection wavelength of 276nm. The mobile phase consisted of 0.1^ 1}$ with an injection volume of 5 L. Gradient elution was programmed as follows: 15% B for column equilibration; 0-5 min, 30% B; 5-10 min, 30-40% B; 10-20 min, 40% B; 20-30 min, 40-50% B; 40-45 min, 50-60% B; 45-50 min, 60-98% B; 50-55 min, 98% B; 55-60 min, 15% B. The HPLC chromatogram of PPNE is shown in Figure 2.

[Figure 2: see original paper] HPLC chromatogram of PPNE

2.3 Cell Culture

Cells were seeded in 6 cm culture dishes and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a 5% CO_2 incubator. Upon reaching approximately 85% confluence, cells were subcultured following trypsin digestion.

2.4 CCK8 Assay for Evaluating Inhibitory Effects on B16 and A375 Cells

Logarithmic-phase cells were trypsinized, centrifuged, and resuspended in culture medium to prepare a single-cell suspension at 1$\times$10^{4}$ cells $\cdot$ mL^{-1}. The suspension was seeded in 96-well plates (100 L per well) and incubated at 37 °C with 5% CO_2 for 12 hours. The medium was then replaced with fresh medium containing *V. didyma* extracts or monomeric compounds (100 L per well) and incubated for 24 hours. After replacing with fresh medium, 10 L of CCK8 reagent was added to each well, mixed thoroughly, and incubated for an additional 4 hours. Absorbance was measured at 450 nm using a multifunctional microplate reader. Experimental wells (A_1), cell control wells (A_2), and blank wells without cells (A_0) were prepared with five replicates each. The inhibition rate of cell proliferation was calculated as: Inhibition % = {[(A_2-A_0)-(A_1-A_0)]/(A_2-A_0)} $\times$ 100%.

3. Results

3.1 Anti-Melanoma Activity Evaluation of Ethanol Extract and Different Fractions

The inhibitory effects of different solvent fractions from the ethanol extract on B16 and A375 melanoma cell proliferation are summarized in Table 1. Overall, *V. didyma* samples exhibited stronger inhibitory activity against B16 cells than A375 cells. The ethyl acetate fraction displayed the most potent anti-melanoma activity, followed by the petroleum ether fraction. The ethanol extract showed inhibitory effects, particularly against B16 cells, while the water and n-butanol fractions demonstrated weak or negligible activity. These results indicate that the ethyl acetate fraction contains the primary anti-melanoma constituents of

V. didyma, with the active components predominantly distributed in the ethyl acetate and petroleum ether fractions, especially the former.

Inhibitory effects of different samples of *Veronica didyma* on melanoma cells

3.2 Chemical Constituents of the Ethyl Acetate Fraction (PPNE)

Systematic separation and purification of the ethyl acetate layer yielded seven compounds identified as: compound 1, p-hydroxybenzaldehyde; compound 2, Picroside II; compound 3, isoscutellarein 7-O-(6' ' '-O-acetyl)-β-allopyranosyl(1' ' ' $\rightarrow$2'')- -*glucopyranoside*; *compound* 4, $3' - hydroxy - 4' - O - methylisoscutellarein 7 - O - [6''' - O - acetyl - -D - allopyranosyl - (1\rightarrow2) - -D - glucopyranoside$; *compound* $5, 6 - O - veratroylcatalposide$; *compound* 6, *Veronicoside*; *andcompound* 7, $isoscutellarein 4' - methylether 7 - O - (6''' - O - acetyl) - ballopyranosyl(1'''\rightarrow2'')$- $-glucopyranoside. Their chemical structures are shown in Figure 3. Compounds 2, 5, and 6 are iridoid glycosides, while compounds 3, 4, and 7 are flavonoid glycosides.

[Figure 3: see original paper] Chemical structures of compounds from PPNE

Compound 1 was obtained as a white powder, readily soluble in methanol. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 10.94 (1H, s, -CHO), 7.78 (2H, d, J=8.5 Hz, H-2,6), 6.81 (2H, d, J=8.5 Hz, H-3,5); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 131.99 (C-1), 131.95 (C-2,6), 115.53 (C-3,5), 161.98 (C-4), 189.29 (-CHO). These data are consistent with literature reports (Huang et al., 2020), identifying compound 1 as p-hydroxybenzaldehyde.

Compound 2 was obtained as colorless square crystals, soluble in methanol. Co-injection with a Picroside II standard on HPLC showed identical retention times and DAD spectra, with a single peak observed at the Picroside II standard position in the mixed sample, confirming compound 2 as Picroside II.

Compound 3 was obtained as a yellow powder, soluble in methanol. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 6.85 (1H, s, H-3), 6.70 (1H, s, H-6), 7.98 (1H, d, J=8.5 Hz, H-2'), 7.98 (1H, d, J=8.5 Hz, H-6'), 6.95 (1H, d, J=8.5 Hz, H-3'), 6.95 (1H, d, J=8.5 Hz, H-5'), 12.37 (1H, s, 5-OH), 5.08 (1H, d, J=6.5 Hz, H-Glu-1), 3.60 (1H, m, H-Glu-2), 3.52 (1H, m, H-Glu-3), 3.26 (1H, m, H-Glu-4), 3.46 (1H, m, H-Glu-5), 3.75 (1H, m, H-Glu-6a), 3.52 (1H, m, H-Glu-6b), 4.93 (1H, d, J=8.0 Hz, H-All-1), 3.26 (1H, m, H-All-2), 3.93 (1H, m, H-All-3), 3.43 (1H, m, H-All-4), 3.88 (1H, m, H-All-5), 4.03 (1H, m, H-All-6a), 4.02 (1H, m, H-All-6b), 1.88 (3H, s, -OAc); ^{13}C-NMR (125 MHz, DMSO-d_6): see Table 2. These data match literature values (Albach et al., 2003), identifying compound 3 as isoscutellarein 7-O-(6'' '-O-acetyl)-β-allopyranosyl(1''' $\rightarrow$2'')- $-glucopyranoside.

Compound 4 was obtained as a yellow powder, soluble in methanol. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 6.81 (1H, s, H-3), 6.69 (1H, s, H-6), 7.51 (1H, d, J=1.5 Hz, H-2'), 7.62 (1H, dd, J=8.5, 2.0 Hz, H-6'), 7.12 (1H, d, J=8.5 Hz, H-5'), 12.35 (1H, s, 5-OH), 3.88 (1H, s, -OCH_3), 5.10 (1H, d, J=2.5 Hz, H-Glu-1), 3.60

(1H, m, H-Glu-2), 3.50 (1H, m, H-Glu-3), 3.25 (1H, m, H-Glu-4), 3.46 (1H, m, H-Glu-5), 3.74 (1H, m, H-Glu-6a), 3.52 (1H, m, H-Glu-6b), 4.92 (1H, d, J=8.0 Hz, H-All-1), 3.25 (1H, m, H-All-2), 3.92 (1H, m, H-All-3), 3.43 (1H, m, H-All-4), 3.88 (1H, m, H-All-5), 4.03 (1H, m, H-All-6a), 4.03 (1H, m, H-All-6b), 1.878 (3H, s, OAc); ^{13}C-NMR (125 MHz, DMSO-d_6): see Table 2. These data are consistent with literature values (Lenherr et al., 1987), identifying compound 4 as 3' -hydroxy-4' -O-methylisoscutellarein 7-O-[6''' -O-acetyl-β-D-allopyranosyl-(1$→2)− $-D-glucopyranoside.

Compound 5 was obtained as a white powder. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 7.65 (1H, dd, J=8.0, 1.5 Hz, H-6"), 7.47 (1H, d, J=1.5 Hz, H-2"), 7.10 (1H, d, J=9.0 Hz, H-5"), 6.43 (1H, dd, J=10.0, 1.0 Hz, H-3), 5.14 (1H, m, H-1), 5.11 (1H, m, H-6), 5.01 (1H, m, H-4), 4.63 (1H, d, J=8.0 Hz, H-1'), 3.94 (1H, dd, J=13.5, 5.0 Hz, H-10a), 3.74 (1H, m, H-10b), 3.74 (1H, m, H-6' a), 3.74 (1H, m, H-7), 3.43 (1H, m, H-6' b), 3.03-3.20 (4H, m, H-2' ,3' ,4' ,5'), 2.60 (1H, m, H-5), 2.50 (1H, dd, J=9.0, 7.0 Hz, H-9); ^{13}C-NMR (125 MHz, DMSO-d_6): see Table 2. These data match literature values (Gao et al., 2003), identifying compound 5 as 6-O-veratroylcatalposide.

Compound 6 was obtained as a light brown powder. ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 8.02 (2H, d, J=7.5 Hz, H-2" ,6"), 7.70 (1H, t, J=7.5 Hz, H-4"), 7.56 (2H, t, J=8.0 Hz, H-3" ,5"), 6.43 (1H, dd, J=6.0, 1.5 Hz, H-3), 5.14 (1H, H-1), 5.12 (1H, m, H-6), 5.01 (1H, m, H-4), 4.63 (1H, d, J=8.0 Hz, H-1'), 3.94 (1H, dd, J=13.3, 4.8 Hz, H-10a), 3.74 (1H, m, H-10b), 3.74 (1H, m, H-6' a), 3.74 (1H, brs, H-7), 3.44 (1H, m, H-6' b), 3.03-3.20 (4H, m, H-2' ,3' ,4' ,5'), 2.61 (1H, m, H-5), 2.50 (1H, H-9); ^{13}C-NMR (125 MHz, DMSO-d_6): see Table 2. These data are consistent with literature values (Kwak et al., 2009), identifying compound 6 as veronicoside.

Compound 7 was obtained as a yellow powder, soluble in methanol. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 6.93 (1H, s, H-3), 6.70 (1H, s, H-6), 8.10 (1H, d, J=9.2 Hz, H-2'), 8.10 (1H, d, J=9.2 Hz, H-6'), 7.13 (1H, d, J=8.5 Hz, H-3'), 7.13 (1H, d, J=8.5 Hz, H-5'), 12.33 (1H, s, 5-OH), 3.87 (1H, s, -OCH_3), 5.08 (1H, d, J=7.6 Hz, H-Glu-1), 3.60 (1H, m, H-Glu-2), 3.52 (1H, m, H-Glu-3), 3.25 (1H, m, H-Glu-4), 3.46 (1H, m, H-Glu-5), 3.74 (1H, m, H-Glu-6a), 3.52 (1H, m, H-Glu-6b), 4.92 (1H, d, J=8.0 Hz, H-All-1), 3.25 (1H, m, H-All-2), 3.92 (1H, m, H-All-3), 3.43 (1H, m, H-All-4), 3.88 (1H, m, H-All-5), 4.03 (1H, m, H-All-6a), 4.03 (1H, m, H-All-6b), 1.88 (3H, s, -OAc); ^{13}C-NMR (100 MHz, DMSO-d_6): see Table 2. These data are consistent with literature values (Nugroho et al., 2008), identifying compound 7 as isoscutellarein 4' -methyl ether 7-O-(6'' '-O-acetyl)-ballopyranosyl(1''' $→2″)− $-glucopyranoside.

^{13}C-NMR spectral data of compounds 3-7

3.3 Anti-Melanoma Activity Evaluation of Monomeric Compounds

The anti-proliferative activities of seven monomeric compounds isolated from PPNE against B16 and A375 cells were evaluated. The results are presented

in Table 3. Except for compound 1, compounds 2-7 demonstrated significant inhibitory effects on both B16 and A375 cell lines, with stronger activity against B16 cells, consistent with the activity pattern observed for the ethanol extract and PPNE. Flavonoid glycosides 3, 4, and 7 showed weaker inhibition than iridoid glycosides 2, 5, and 6. Collectively, these findings suggest that both iridoid and flavonoid compounds constitute the material basis for the anti-melanoma activity of *V. didyma*.

Inhibitory effect of compounds from *Veronica didyma* Tenore on melanoma cells

4. Discussion and Conclusion

Seven major compounds were isolated from the anti-melanoma active PPNE fraction of *V. didyma*, all reported for the first time from this plant. This study provides the first report of the anti-melanoma activity of flavonoids 3, 4, and 7 and iridoid 5. Compound 5 (6-O-veratroylcatalposide) was initially isolated from *Gardenia jasminoides* (Lee et al., 1987), with no prior activity data reported. Compound 4 differs from compound 3 by an additional methoxy group on the C-ring of the flavonoid nucleus. Compounds 3 and 4 were first obtained from *Stachys recta* Labiatae (Lamiaceae) by Lenherr et al. (1984), with compound 3 later found in other plants including *Stachys anisochila* and *Veronica ciliata*, though no activity had been reported for either compound prior to this study. Compound 7 differs from compound 3 by a methoxy group on the flavonoid C-ring and was first isolated from *Veronica filiformis* (Chari et al., 1981), later from *Stachys japonica*, where it exhibited significant acetylcholinesterase (IC_{50}=39.94 $mg \cdot mL^{-1}$) and butyrylcholinesterase inhibition (IC_{50}=86.98 $mg \cdot mL^{-1}$) (Nugroho et al., 2018).

Evaluation of different extraction fractions revealed that anti-melanoma constituents are primarily distributed in the PPNE and petroleum ether fractions, particularly PPNE. Activity assessment of monomeric compounds from PPNE demonstrated that iridoid glycosides 2, 5, and 6 significantly inhibited melanoma cell proliferation. At 50 $g \cdot mL^{-1}$, these compounds exhibited 89.62%, 91.62%, and 93.36% inhibition against B16 cells, and 58.29%, 53.96%, and 53.49% inhibition against A375 cells, respectively. Flavonoid glycosides 3, 4, and 7 also showed good inhibitory activity, though slightly weaker against B16 cells compared to iridoid glycosides, while demonstrating similar activity against A375 cells.

In summary, the PPNE fraction of *V. didyma* ethanol extract represents the active fraction inhibiting melanoma cell proliferation, with both iridoid and flavonoid compounds serving as the material basis, particularly the iridoid constituents. Preliminary mechanistic studies indicate that the active fraction and monomeric compounds can inhibit melanoma cell migration and invasion, though the detailed mechanisms require further investigation.

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