



Antiproliferative activity of aqueous and polyphenol-rich extracts of *Larrea divaricata* Cav. upon a melanoma cell line

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Keywords:	<i>Larrea divaricata</i> Cav, melanoma B16F10 cell line, antiproliferative activity, STAT3, apoptosis

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Larrea divaricata Cav.



AQUEOUS EXTRACT



POLYPHENOL-RICH FRACTION

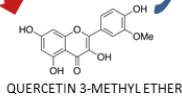


. p-STAT3 inhibition
. induction of early and late apoptosis

↓ PROLIFERATION



B16F10 MELANOMA CELLS



254x190mm (96 x 96 DPI)

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Antiproliferative activity of aqueous and polyphenol-rich extracts of *Larrea divaricata* Cav. upon a melanoma cell line

Abstract

Most of the deaths from skin cancer are caused by melanoma, a malignancy in which STAT3 plays a crucial role. The inhibition of STAT3 is considered a potential target to induce cell death, tumor regression and metastasis inhibition. The objective of this work was to evaluate the activity of the aqueous extract of *Larrea divaricata* (Aq), a fraction rich in polyphenols (EA), and the isolated compound quercetin-3-methyl ether (Q3ME) on B16F10 melanoma cells. The effects of Aq, EA and Q3ME were assessed on B16F10 cells by determining the proliferation, viability, apoptosis induction and the expression and phosphorylation of STAT3. The phytochemical composition of the extracts was determined by High Performance Liquid Chromatography. Aq, EA and Q3ME presented antiproliferative activity on B6F10 cells through p-STAT3 inhibition and early and late apoptosis induction (EC_{50} EA = ≤ 0.1 $\mu\text{g/ml}$; Aq = 316 ± 30 $\mu\text{g/ml}$; Q3ME = < 0.1 $\mu\text{g/ml}$). *L. divaricata* could be considered for the development of adjuvant phytotherapies in melanoma treatment.

Keywords: *Larrea divaricata* Cav., melanoma B16F10 cell line, antiproliferative activity, STAT3, apoptosis.

1. Introduction

According to the World Health Organization (WHO), 80% skin cancer deaths are due to melanoma. In 50-90% of melanomas, the Janus kinase signal transducer and activator of transcription, STAT3, is constitutively activated and is considered a potential treatment target to induce cell death, tumor regression and inhibition of metastasis (Kortylewski et al. 2005; Kamran et al. 2013).

Polyphenol-rich natural products have been reported to exert an antiproliferative activity on melanoma cells (Stevanato et al. 2014; Tong and Young 2014). Since the synthetic agents used for melanoma treatment can cause severe adverse effects, natural safe agents obtained from plant extracts should be considered. *Larrea divaricata* (Zygophyllaceae) is an autochthonous South American plant widely distributed in Argentina and used in popular medicine for the treatment of inflammatory diseases and cancer. This species produces polyphenol compounds such as hydroxycinnamic acid, flavonoids and lignans (Lorenzo et al. 2020). It has previously been demonstrated that an aqueous extract (Aq) of the leaves and a polyphenol-rich sub-fraction obtained by extraction with ethyl acetate (EA) exert antiproliferative effects on the EL-4 lymphoma cell line (Martino et al. 2013; Martino et al. 2016). However, the effect of these extracts has not been studied

on melanoma cells. This work aimed at elucidating the anti-proliferative mechanism of action of Aq and EA on melanoma cells. The results obtained would be useful to develop natural novel phytomedicines to be used along with the adjuvant therapy for melanoma.

2. Results and Discussion

In this work, an aqueous extract and a polyphenol-rich extract from *L. divaricata* exerted antiproliferative effects on a melanoma cell line by inhibiting the phosphorylation of STAT3, and inducing early and late apoptosis.

The lignan nor-dihydroguaiaretic acid (NDGA) (retention time: 47 min), 4-hydroxybenzoic acid (4-HBA) (retention time: 12 min), together with other flavonoids (retention times between 24-25 min) were identified and quantified by HPLC-UV in Aq and EA. Quercetin-3-methyl ether (Q3ME) was only detected in EA (chromatograms not shown). As it can be seen in Table S1, the amount of polyphenols, Q3ME, NDGA and the 4-HBA, were greater in EA than in Aq.

Aq and EA inhibited B16F10 cell proliferation in a concentration–response manner and decreased cell viability (Figures S1A and B), but a dependency of the latter effect with the extract concentration could be established neither with Aq nor with EA. The highest inhibitory capacity was observed with EA (Table S2). All compounds exerted antiproliferative activity ($p < 0.001$ – $p < 0.0001$). 4-HBA was the least active compound and Q3ME, which also decreased cell viability ($p < 0.0001$), was found to be the most potent (Figure S1C, D and E, Table S2). Taking into account these results, Q3ME was used for further studies.

The flow cytometry analysis demonstrated that Aq, EA and Q3ME at 10 $\mu\text{g/ml}$ were able to induce a significant decrease of cell viability, an increase in the number of cells in early apoptosis ($p < 0.0001$), late apoptosis ($p < 0.01$) as well as necrotic ones ($p < 0.05$, $p < 0.001$) (Figure S1E). It had been previously demonstrated that Aq inhibited the proliferation of the BW 5147 lymphoma cell line and that EA inhibits EL-4 lymphoma cell line by inducing apoptosis mediated by ROS and NO (Davicino et al. 2010; Martino et al. 2013; Martino et al. 2016). Q3ME, 4-HBA and NDGA had also shown antiproliferative effects on dermal tumor cells and breast cancer cells (Kubow et al. 2000; Li et al. 2012; Wang et al. 2018).

It is known that the activation of STAT3 leads to the synthesis of molecules involved in malignant transformation and since several clinical trials are being undertaken to study the effectiveness of STAT3 inhibitors, including natural molecules (Siveen et al. 2014), the interaction of the most active drugs, EA and Q3ME, with STAT3 was analyzed.

The STAT3 proliferative dependency of the melanoma cell line was confirmed, since the STAT3 inhibitor 5,15 DPP decreased cell proliferation and STAT3 phosphorylation, while the pro-inflammatory cytokine IL-6 exerted opposite effects (Figure S2 A, C and D). The anti-proliferative effect of EA and Q3ME was mediated by the inhibition of STAT3 phosphorylation (Figure S2 C and D). The latter finding was confirmed by the fact that IL-6 was able to revert the antiproliferative effect induced by EA and Q3ME (Figure S2B). The inhibition of STAT3 phosphorylation could be related to the induction of apoptosis. It has previously been reported that quercetin regulates the STAT3 pathway in melanoma cells by inhibiting its phosphorylation/activation (Cao et al. 2014). Plant extracts containing quercetin and kaempferol exert antiproliferative effects on B16F10 melanoma cells by inducing late apoptosis and inhibiting the phosphorylation of STAT3. These effects were observed with the herbal formula “Huai-Hua-San”, which contains *Sophorae flos* and *Gardeniae fructus* ethanolic extracts, which were found to decrease viability with an EC₅₀ value of around 400 µg/ml, a value that was higher than that found for *L. divaricata* extracts (Li et al. 2021).

3. Experimental

See supplementary material.

4. Conclusions

The results presented in this work serve as the basis for future *in vivo* studies in induced melanoma models to determine the effect of aqueous or polyphenol-rich extracts from *L. divaricata* as coadjutant phytotherapies for the treatment of this disease.

Competing interests

The authors declare no competing interests.

Funding

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Abstract

Most of the deaths from skin cancer are caused by melanoma, a malignancy in which STAT3 plays a crucial role. The inhibition of STAT3 is considered a potential target to induce cell death, tumor regression and metastasis inhibition. The objective of this work was to evaluate the activity of the aqueous extract of *Larrea divaricata* (Aq), a fraction rich in polyphenols (EA), and the isolated compound quercetin-3-methyl ether (Q3ME) on B16F10 melanoma cells. The effects of Aq, EA and Q3ME were assessed on B16F10 cells by determining the proliferation, viability, apoptosis induction and the expression and phosphorylation of STAT3. The phytochemical composition of the extracts was determined by High Performance Liquid Chromatography. Aq, EA and Q3ME presented antiproliferative activity on B6F10 cells through p-STAT3 inhibition and early and late apoptosis induction (EC_{50} EA = ≤ 0.1 $\mu\text{g/ml}$; Aq = 316 ± 30 $\mu\text{g/ml}$; Q3ME = < 0.1 $\mu\text{g/ml}$). *L. divaricata* could be considered for the development of adjuvant phytotherapies in melanoma treatment.

Experimental

1. Plant material and preparation of extracts

Larrea divaricata Cav. (Zygophyllaceae) leaves were obtained from the wild by Dr Ignacio Peralta, during January 2017, in Córdoba, Argentina, Rio Dolores district, quarter Aguas Azules (Sector B), Capilla del Monte, Department of Punilla (land register nomenclature: Dep. 23- Ped. 01- Pueblo 06- Circ. 05- Secc. 02- Manzana 054- Parcela 003) and identified by Dr Gustavo Giberti† and Hernán Gerónimo Bach. A voucher specimen (BAFC no. 38) was deposited at the Museum of Pharmacobotany, School of Pharmacy and Biochemistry, University of Buenos Aires. No state permissions were necessary to collect the sample. The plant material was air-dried at 25°C. A 7.5% aqueous extract was prepared by boiling the leaves in distilled water for 20 min. The extract was then filtered using a Buchner funnel and a 125 mm no. 42 Whatman paper and lyophilized. The final yield was 26.6 g/100 g of plant material. A fraction enriched in flavonoids was obtained by liquid-liquid partition of Aq with dichloromethane (DM) and ethyl acetate (Davicino et al. 2011). Aq and EA (obtained from ethyl acetate) were used for subsequent studies.

2. HPLC analysis

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3 The HPLC analysis was performed in a Varian Pro Star instrument equipped with a Rheodyne
4 injection valve (20 μ l) and a photodiode array detector set at 280 nm (NDGA) and 260 nm
5 quercetin 3-methyl ether (Q3ME). A Phenomenex-Kinetex (no. 00G-4605-EO, 5 μ m XB-C18
6 100A, size: 250 mmx 4.6 mm, surface area: 200 m²/g) was used. For the identification and
7 quantification of NDGA, samples were eluted with a gradient of solution A (water and acetic
8 acid, 98:2), and solution B (methanol and acetic acid, 98:2). The gradient was from 15% B to
9 40% B in 30 min, 40% B to 75 % B in 10 min, 75% B to 85% B in 5 min and 100% B in 5 min.
10 The flow rate was 1.2 ml/min. A calibration curve of NDGA was obtained by injecting 20 μ l of
11 solutions ranging from 2 μ g/ml to 200 μ g/ml. (Pearson's correlation coefficient: $r^2= 0.9988$).
12

13 For the identification and quantification of Q3ME and 4-hydroxybenzoic acid (4-HBA), three
14 chromatographic systems were used: System 1 was the same as that described for the
15 quantification of NDGA. In system 2, a mixture of water/methanol/phosphoric acid (100:100:1)
16 was used as mobile phase. In system 3, two mobile phases were used: solution A was a water-
17 phosphoric acid (0.5%) mixture and solution B was a methanol-phosphoric acid (0.5%) mixture.
18 The elution profile was 100% A to 25% A in 30 min, 25% A to 0% A in 2 min, and back to the
19 initial conditions.
20

21 NDGA, 4-HBA and Q3ME pure standards (Sigma, USA) were used for identification and
22 quantification by comparing retention times and by plotting peak areas, respectively. Aq and EA
23 at 10 mg/ml and the pure standard were dissolved in methanol:water (70:30). Chromatograms
24 are not shown.
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3. Quantification of total polyphenols

28 Polyphenols were determined spectrophotometrically by the Folin-Ciocalteu's method using
29 gallic acid as standard (Hosseinzadeh et al. 2013). Aq and EA were weighed and dissolved in
30 distilled water. One ml of each extract was transferred to separate tubes containing 7.0 ml of
31 distilled water, 0.5 ml of Folin-Ciocalteu's reagent, and 1.5 ml of a 20% anhydrous sodium
32 carbonate solution. Mixtures were left at room temperature for 60 min and then the absorbance
33 was measured at 765 nm. The concentration of polyphenols in samples was calculated from a
34 standard curve of gallic acid ranging from 10 to 50 μ g/ml (Pearson's correlation coefficient: r^2
35 = 0.9996). Results were expressed as mg GAE/g extract.
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4. Cells and assays

4.1 Cell culture conditions

38 The melanoma ATCC cell line B16F10 was cultured at 3×10^5 cells/ml in RPMI 1640 medium
39 supplemented with 10% of fetal calf serum (Sigma, St. Louis, MO, USA), 2 nM of L-glutamine,
40 100 IU/ml penicillin and 100 μ g/ml streptomycin (Sigma, St. Louis, MO, USA).
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4.2 Proliferation assays

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3 The effect of the extracts and isolated compounds on cell proliferation was evaluated by the
4 tritiated thymidine [^3H]TdR uptake assay. Cells were pulsed with [^3H]TdR (20 Ci/mmol) for
5 the last 6 h of incubation as previously described (Anesini et al. 1996).

6
7 B16F10 cells were incubated alone or in the presence of different concentrations of either EA,
8 Aq, Q3ME, 4-HBA or NDGA. B16F10 cells were also incubated in the presence of either 5,15
9 diphenylporphirin (5,15-DPP) (Sigma, St. Louis, MO, USA), a STAT3 inhibitor, and IL-6 (10
10 ng/ml, Sigma, St. Louis, MO, USA), a STAT3 activator, and 10 $\mu\text{g/ml}$ Aq, 10 $\mu\text{g/ml}$ EA or 10
11 $\mu\text{g/ml}$ Q3ME. The optimal incubation time (24 h) was previously determined by kinetics
12 studies. Results were expressed as cell proliferation inhibition (%) and cell proliferation (%).
13 Data represent the mean \pm SEM of three experiments performed in triplicate.

14
15 The EC_{50} values for the antiproliferative activity were calculated from concentration-response
16 curves employing a mathematical method based on the right-angled triangle principle according
17 to the following formula:

$$\text{EC}_{50} = \text{anti log} [D - [(A - 50\% \text{ max response}) \cdot (D - C)] / (A - B)]$$

18
19 where A is the response just above the 50% maximum response; B is the response just below
20 the 50% maximum response; D=log concentration corresponding to A; C=log concentration
21 corresponding to B (Alexander et al. 1999).

22 23 24 25 26 27 28 29 30 31 32 **4.3 Viability assays**

33 The effect of Aq, EA and Q3ME at 0.1 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ on cell
34 viability for 24 h was determined by the Trypan blue exclusion method. Briefly, the cell
35 suspension was mixed with 0.4% Trypan blue in a 1: 9 ratio and the mixture was transferred to a
36 Neubauer hemocytometer to determine the number of viable and non-viable cells. Viability was
37 expressed as %Viable cells = [number of viable cells /number of total cells] x 100

38 39 40 41 42 43 **5 Flow cytometry analysis**

44 The induction of apoptosis in the tumor cell line was quantified by flow cytometry using FITC-
45 conjugated annexin V/propidium iodide (PI) (SIGMA San Diego USA). After the incubation of
46 cells with either 10 $\mu\text{g/ml}$ Aq, 10 $\mu\text{g/ml}$ EA or Q3ME 10 $\mu\text{g/ml}$ for 24 h, cells were washed
47 twice with PBS, resuspended in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl_2 ,
48 pH 7.4) at a concentration of 1×10^6 cells/ml Aliquots of 1×10^5 treated cells were incubated
49 with annexin-FITC and propidium iodide for 15 min at room temperature in the dark. Samples
50 were shaken gently, diluted in binding buffer and analyzed by flow cytometry within 1 h. Cells
51 treated with culture medium alone (basal conditions) and fixed with 2.3% ethanol were used as
52 controls. At least 10,000 events per tube were acquired in a Becton-Dickinson
53 FACSCaliburflow cytometer (Becton Dickinson Biosciences, San José, CA.) The data analysis
54 was performed on the FCS Express V3 platform, plotting FL1 (annexin-V-FITC) against FL2

(IP). The results were expressed as percentage of cells \pm standard deviation of the total of cells acquired (Davicino et al. 2010).

6 Determination of the expression of STAT 3 by Western blot

B16F10 cells were cultured in either the absence or the presence of either EA (100 μ g/ml), Q3ME (10 μ g/ml), DPP (10 μ M) or IL-6 (10 ng/ml) for 24 h. Cells were then lysed for 30 min at 4 °C with lysis buffer (50 mmol/l Tris-HCl, 150 mmol/l NaCl, 1 mmol/l EGTA, 1% NP-40, 1 mmol/l NaF, 1 mmol/l Na_3VO_4 , 0.25% sodium deoxycholate, 1 μ mol/l phenylmethylsulfonylfluoride, 10 μ g/ml aprotinin, 10 μ mol/l pepstatin and 10 μ mol/l leupeptin). After centrifugation (14,000 xg , 15 min, 4 °C), whole-cell protein extracts (50 μ g) were mixed with sample buffer (2% SDS, 10% v/v glycerol, 62.5 mM Tris-HCl, pH 6.8, 0.2% bromophenol blue and 1% v/v 2-mercaptoethanol). Equal amounts of protein were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to a PVDF membrane. Non-specific binding sites were blocked with blocking buffer (5% non-fat dried milk containing 0.1% Tween 20 in 100 mM Tris-HCl, pH 7.5 and 0.9% NaCl) for 1 h. Membranes were then incubated overnight with a mouse antibody anti-active phospho-STAT3 and antibody anti-STAT3 (Santa Cruz Biotech). After washing with PBS-Tween, membranes were sequentially incubated with a horseradish peroxidase-conjugated anti-mouse antibody (1:2500) for 1 h. After washing with PBS-Tween, the immunological reaction was developed with an enhanced chemiluminescent system. Band intensities were normalized to those of total STAT3. The densitometric analysis of bands was performed using the Image J software (version 5.1, Silk Scientific Corporation, NIH, Bethesda, MA, US), and expressed as p-STAT3/ total STAT3 (arbitrary units) (Park et al. 2014).

7 Statistical analysis

The Student's *t* test for unpaired measures was used to determine the level of significance of results. When multiple comparisons were necessary, one way ANOVA plus the Dunnett or Tukey's test was applied. Differences between means were considered significant when $p < 0.05$.

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Table S1. Content of polyphenols, Q3ME, NDGA and 4-HBA in Aq and EA

Extracts	Yield (%)	Polyphenols g%*	NDGA g%	Q3ME g%	4-HBA g%
Aq	26.6	11.7 ± 1.6 ^a	0.80±0.05 ^a	----	0.0343 ±0.003 ^a
EA	3.83	50.24 ± 2.13 ^b	3.22±0.40 ^b	0.52 ± 0.03	1.3 ± 0.10 ^b

Polyphenols were determined by spectroscopic methods, NDGA, 4-HBA and Q3ME were determined by HPLC-UV. Values are expressed as mean ± SEM of three independent measures.

* Gallic acid equivalent. a and b significant differences ($p < 0.001$, Student's *t* test.)

Table S2. EC₅₀ values for the antiproliferative activity of Aq, EA, Q3ME and NDGA

Extracts /Compounds	EC ₅₀ (µg/ml)
Aq	316 ± 30 µg/ml ^a
EA	≤ 0.1 µg/ml ^b
Q3ME	< 0.1 µg/ml ^b
NDGA	2.3± 0.2 µg/ml ^c

EC₅₀ values (µg/ml) were calculated from concentration vs. effect graphs (Figure 1) by the Alexander's method. Results are expressed as mean ± SEM of three experiments made in triplicate. a,b,c significant differences between treatments (One way ANOVA plus Tukey's multiple comparison test).

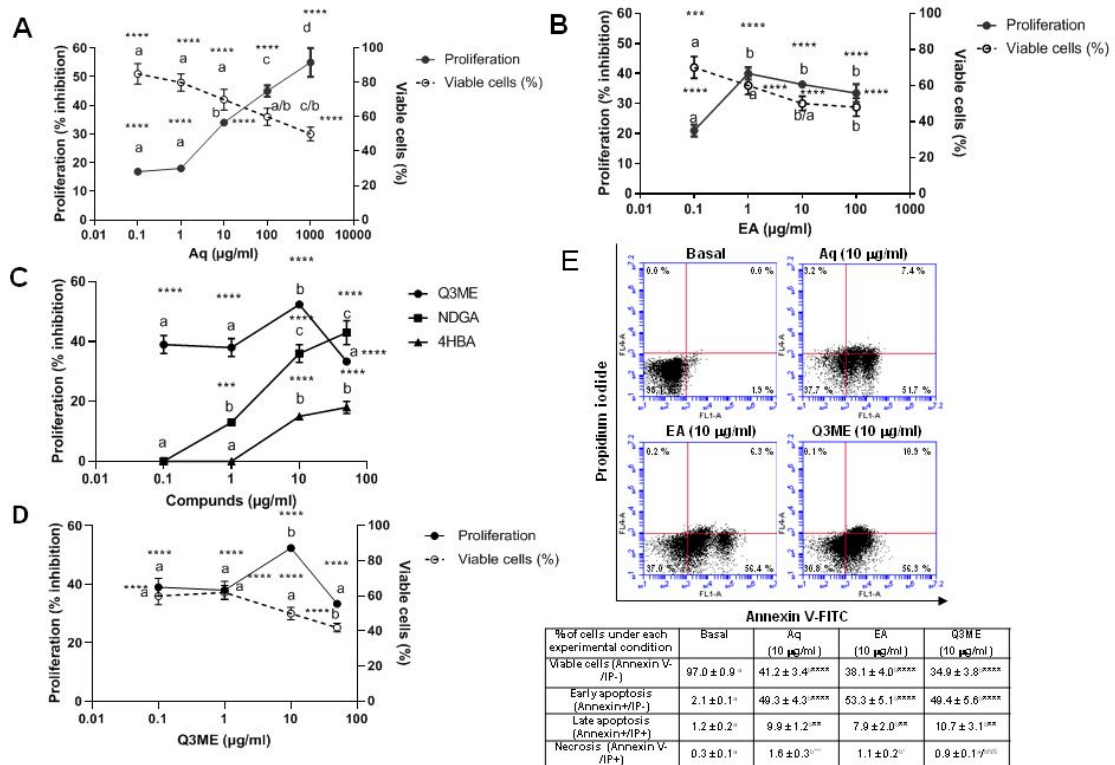


Figure S1. Effect of Aq, EA, Q3ME, 4-HBA and NDGA on the proliferation and viability of B16F10 melanoma cells. Cells were incubated for 24 h in the presence of Aq (A), EA (B), Q3ME, 4-HBA and NDGA (C). D: effect of Q3ME on cell proliferation and viability. E: Representative flow cytometry analysis of three experiments. Results are expressed as mean ± SEM of three experiments made in triplicate. a,b,c significant differences (One way ANOVA plus Tukey's multiple comparison test). Table embedded: Percentage of viable cells and apoptotic cells obtained by flow cytometry. *p<0.05, **p<0.01; ***p<0.001, ****p<0.0001: significant differences with respect to basal values (One way ANOVA plus Dunnett's multiple comparison test), a, b significant differences (One way ANOVA plus Tukey's multiple comparison test).

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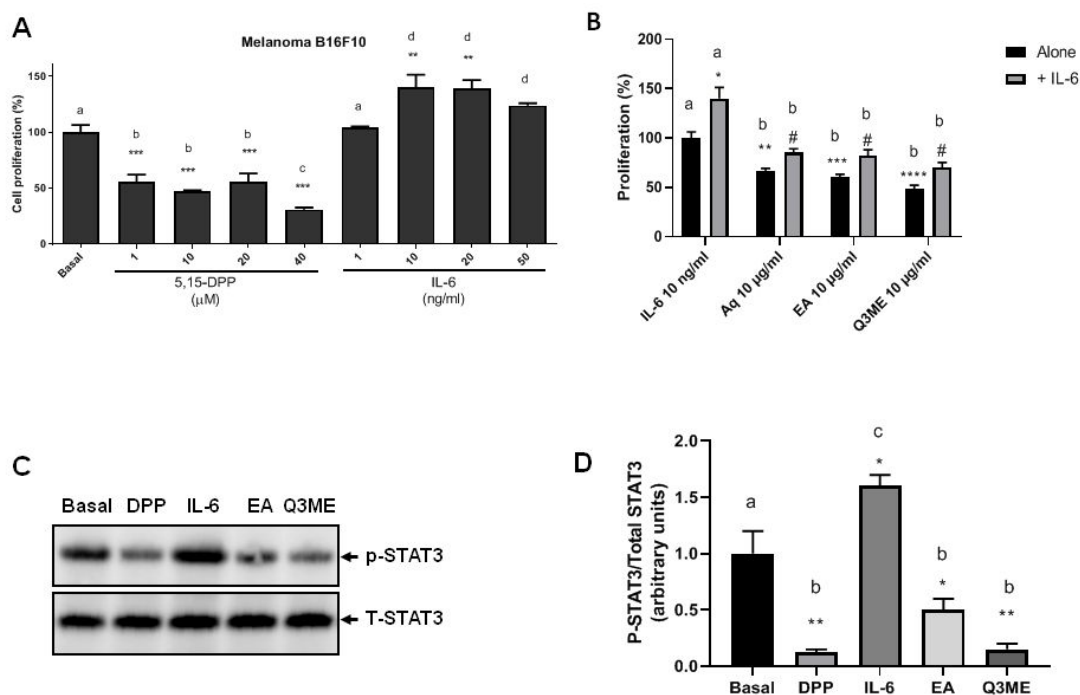


Figure S2. Effect of Aq, EA and Q3ME on p-STAT3 activation and expression in B16F10 melanoma cells. (A) Cells were incubated for 24 h with either IL-6 (1 ng/ml, 10 ng/ml, 20 ng/ml, and 50 ng/ml), a STAT3 activator, or 5,15-DPP (1 μM, 10 μM, 20 μM, 40 μM), a STAT-3 inhibitor. (B) Cells were incubated for 24 h with either Aq, or EA or 10 μg/ml Q3ME alone or in the presence of 10 ng/ml IL-6. (C) Western blot analysis of p-STAT3 and (E) densitometric analysis: T-STAT3 and p-STAT3/T-STAT3 (arbitrary units). B16F10 cells were incubated for 24 h with either EA (100 μg/ml) or Q3ME (10 μg/ml), or 10 μM 5.15 DPP or 10 ng/ml IL-6. p-STAT3: phosphorylated STAT3; T-STAT3: total-STAT3. Results are expressed as mean ± SEM of three experiments performed in triplicate. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$: significant differences between basal and post-treatment values, # $p < 0.05$: significant differences between treatments in the presence of IL-6 and IL-6 alone (ANOVA followed by the Dunnett's multiple comparison test). a, b, c, significant differences (Tukey's multiple comparisons test).