Anthocyanins, delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside, inhibit immune checkpoints in human colorectal cancer cells *in vitro* and *in silico*

Candice Mazewski: cmazews2@illlinois.edu

Sanha Kim: msk2@illinois.edu

Elvira Gonzalez de Mejia: edemejia@illinois.edu

Supplementary Figure Legends

Supplementary Figure 1. Summary of the method used for co-culture of peripheral blood mononuclear cells (PBMC) and colorectal cancer cells for viability and supernatant collection for programmed cell death protein 1 (PD-1) ELISA analysis. HCT 116 cells are shown as an example of colorectal cancer cells in the figure; the same procedure was used for HT-29 cells. UNT, untreated; C3G, cyanidin-3-O-glucoside; D3G, delphinidin-3-O-glucoside; PMB, pembrolizumab; LDH, lactate dehydrogenase.

Supplementary Figure 2. High-performance liquid chromatography profiles and picture of a well from a 96-well plate of phenolics in media at 520 and 280 nm initially and after incubation with HCT 116 human colon cancer cells at 37 ˚C in 5% CO2 and 95% air. a. Delphinidin-3-O-glucoside (D3G). b. Cyanidin3-O-glucoside (C3G). c. Malvidin3-O-glucoside (M3G). Two independent experiments were run.

Supplementary Figure 3. Flow cytometry cell count versus apoptosis staining pictures to show where quadrants were place in Figure 3. Red line indicates the quadrant placement which is positioned just before the second cluster of cells to include the higher intensity stained cells for Annexin V fluorescein isothiocyanate (FITC) and propidium iodide (PI). a. Untreated b. Delphinidin-3-O-glucoside (D3G) c. Delphinidin chloride (DC) d. Gallic Acid.

Supplementary Figure 4. Lactate dehydrogenase (LDH) activity of co-cultures with untreated and anthocyanin pre-treated peripheral blood mononuclear cells (PBMC) and colon cancer cells compared to the untreated monoculture of colon cancer cells. a. HCT 116 cells. b. HT-29 cells. UNT, untreated; C3G, cyanidin-3-O-glucoside; D3G, delphinidin-3-O-glucoside; PMB, pembrolizumab.

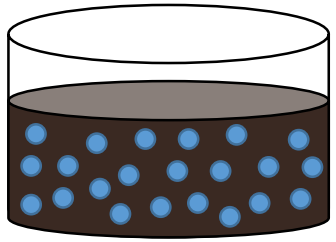
Supplementary Figure 5. Three-dimensional figures and binding free energy between a. Cyanidin-3-O-glucoside (C3G) and programmed cell death protein 1 (PD-1) at the programmed death-ligand 1 (PD-L1) binding site. b. Delphinidin-3-O-glucoside (D3G) and PD-1 at the nivolumab binding site. c. Delphinidin (DC) and programmed death-ligand 1 (PD-L1) at the 8J8 small molecule inhibitor site. d. Malvidin-3-O-glucoside (M3G) and PD-L1 at the Atezolizumab binding site. e. Procyanidin B1 (PB1) and vascular endothelial growth factor (VEGF) at the VEGFR2 binding site. f. Gallic acid (GA) and VEGF at the VEGFR1 binding site.

Supplementary Figure 6. Full-length uncropped western blot images depicting a. programmed death-ligand 1 PD-L1 for HCT 116 cells b. glyceraldehyde 3-phosphate (GAPDH) for HCT 116 cells for the PD-L1 membrane c. vascular endothelial growth factor (VEGF) for HCT 116 cells d. GAPDH for HCT 116 cells for the VEGF membrane for the cropped figures in Fig. 4a. e. VEGF for HT-29 cells f. GAPDH for HT-29 cells for VEGF membrane for cropped figures in Fig. 4b. MCF7 whole cell lysate (sc-2206) was used in lane 10 as a control for the VEGF membranes as recommended by the manufacturer. Recombinant human PD-L1 protein fragment (ab167713) was used in lane 10 of the PD-L1 membrane. BLE, black lentil extract; D3G, delphinidin-3-O-glucoside; DC, delphinidin chloride; GA, gallic acid; RGC, red grape combination; RGE, red grape extract; UNT, untreated; UNTD, untreated DMSO.

Supplementary Figure 7. Full-length uncropped western blot images depicting additional exposures as adjusted in Carestream for Supplementary Fig. 6a for programmed death-ligand 1 PD-L1 for HCT 116 cells a. increased exposure b. decreased exposure. For Supplementary Fig. 6c for vascular endothelial growth factor (VEGF) for HCT 116 cells c. increased exposure d. decreased exposure. For Supplementary Fig. 6e for VEGF for HT-29 cells e. increased exposure f. decreased exposure. MCF7 whole cell lysate (sc-2206) was used in lane 10 as a control for the VEGF membranes as recommended by the manufacturer. Recombinant human PD-L1 protein fragment (ab167713) was used in lane 10 of the PD-L1 membrane. BLE, black lentil extract; D3G, delphinidin-3-O-glucoside; DC, delphinidin chloride; GA, gallic acid; RGC, red grape combination; RGE, red grape extract; UNT, untreated; UNTD, untreated DMSO.

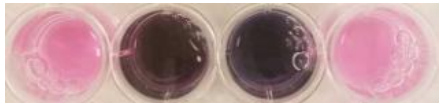
Supplementary Figure 1

Day 1 -PBMC Monoculture

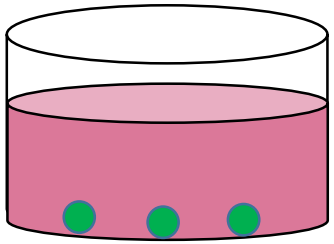


PBMC in 24 well plate 0.5-1x10^6 cells per well with treatments:

UNT C3G D3G PMB

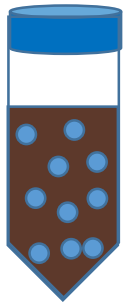


Day 2 - HCT 116 Monoculture

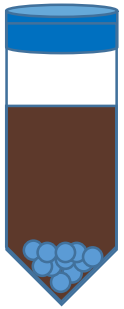


Seed cells at 1x10^4 per well in a 96 well plate

Day 3 – Co-culture

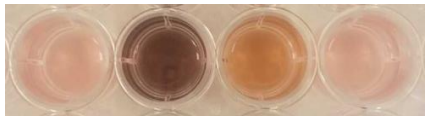


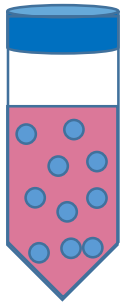
Collect cells from each treatment



Centrifuge at 200g for 15 min

UNT C3G D3G PMB

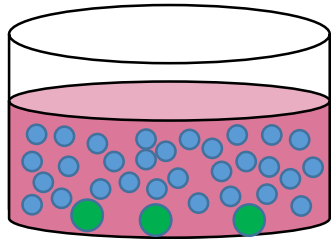




Collect media and add fresh untreated media so each 200 µL has 2x10^6 PBMC cells

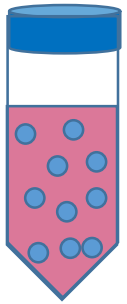
HCT 116

PBMC

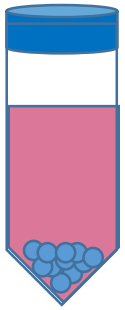


Aspirate media from HCT 116 cells in the 96 well plate and add PBMC at a 10:1 ratio to HCT 116 cells

Day 4 - viability and supernatant collection



Collect media from each 96 well plate well



Centrifuge at 200g for 15 min

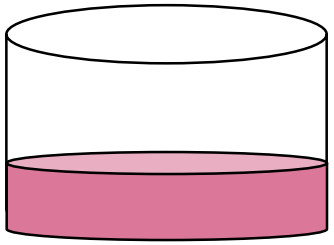
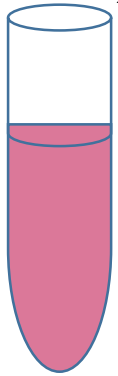
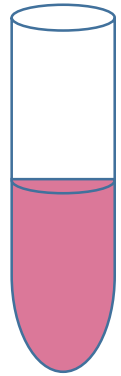


Plate 50 µL of supernatant into a clean 96 well plate for each replicate for Pierce LDH Cytotoxicity Assay

Collected media from monoculture

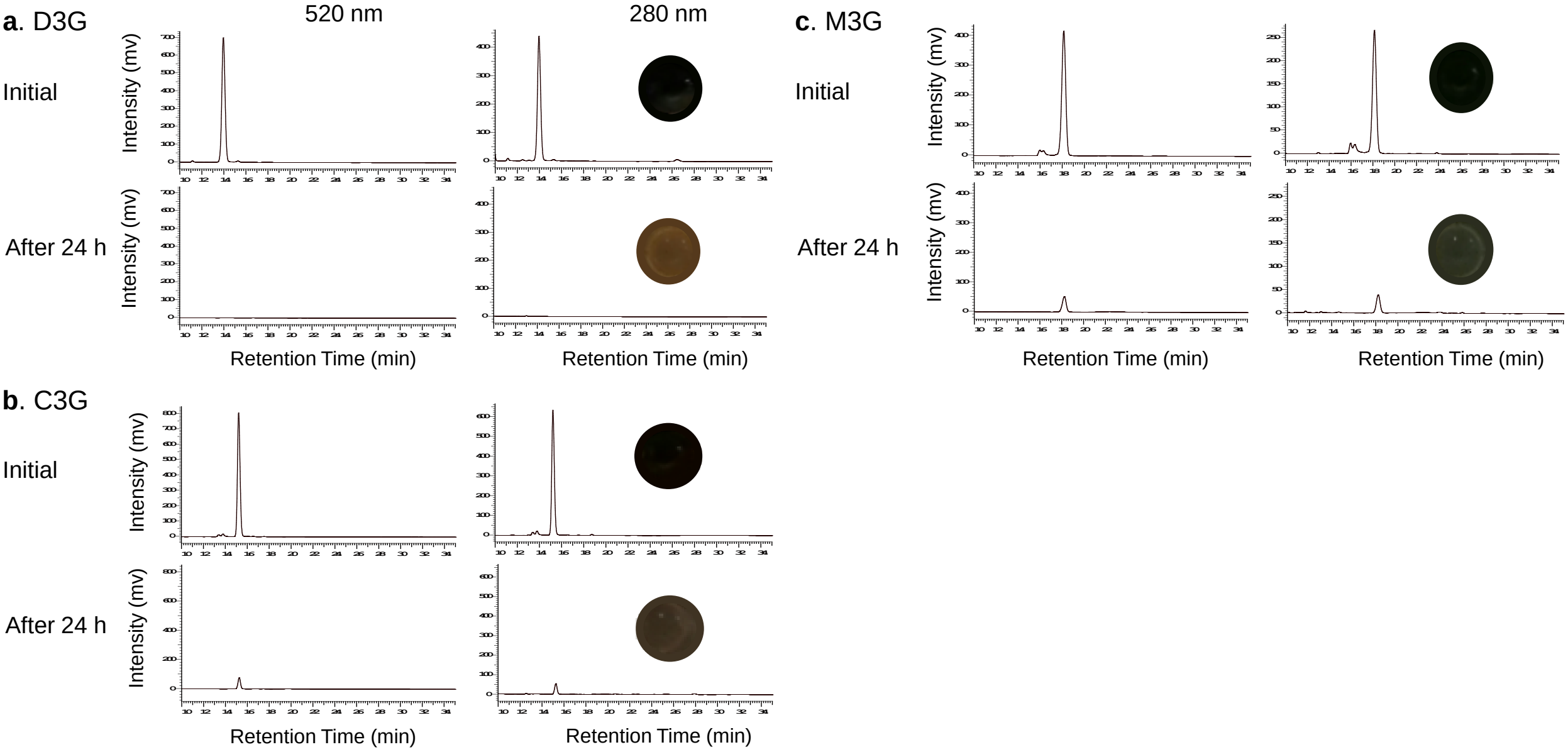


Combine remaining media in each replicate for each treatment (not disturbing the pellet) centrifuge at 4°C at 14,000 rpm for 10 min

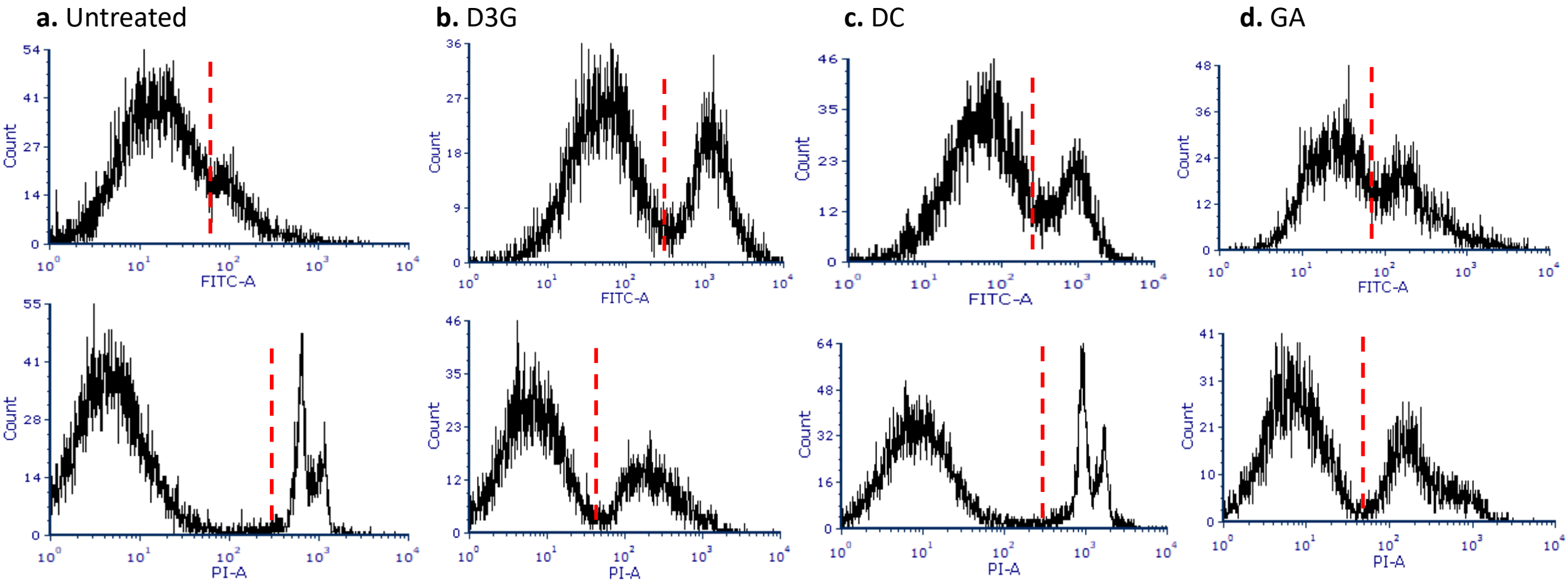


Collect supernatant to be used for the PD-1 ELISA (both from monoculture and co-culture), store at -80°C

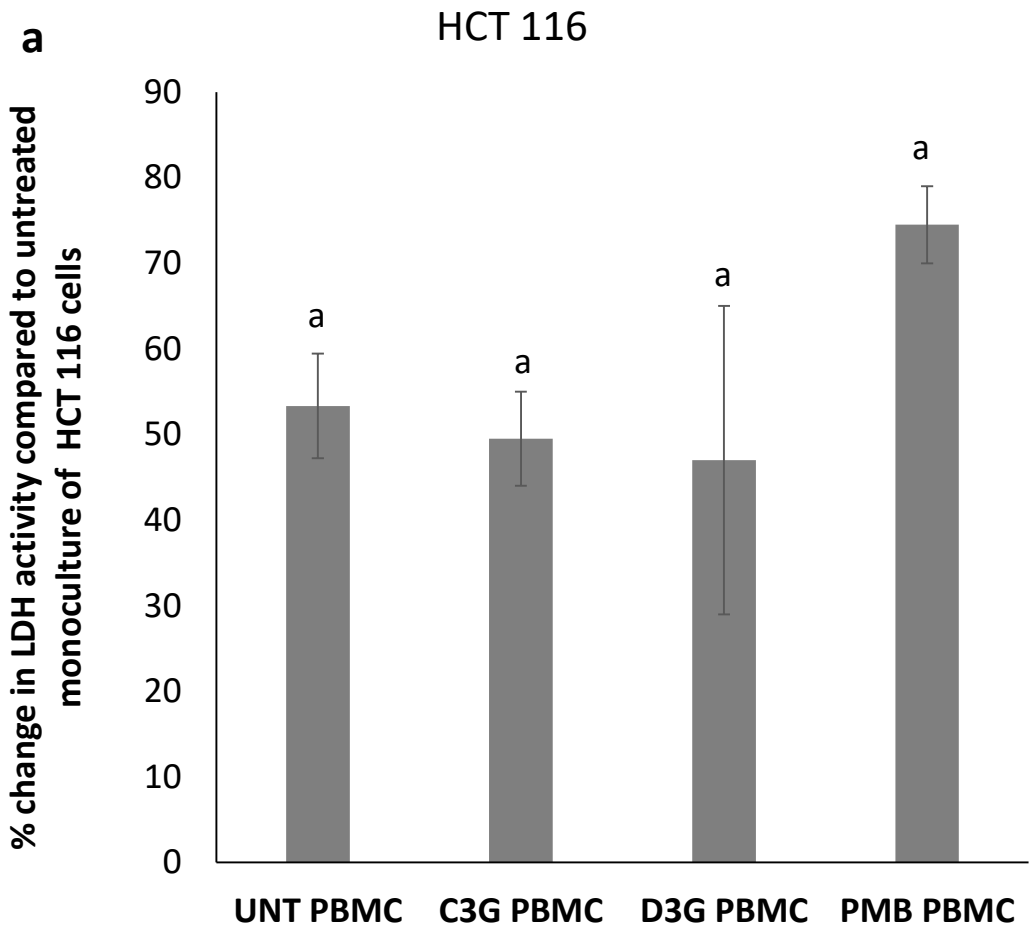
Supplementary Figure 2

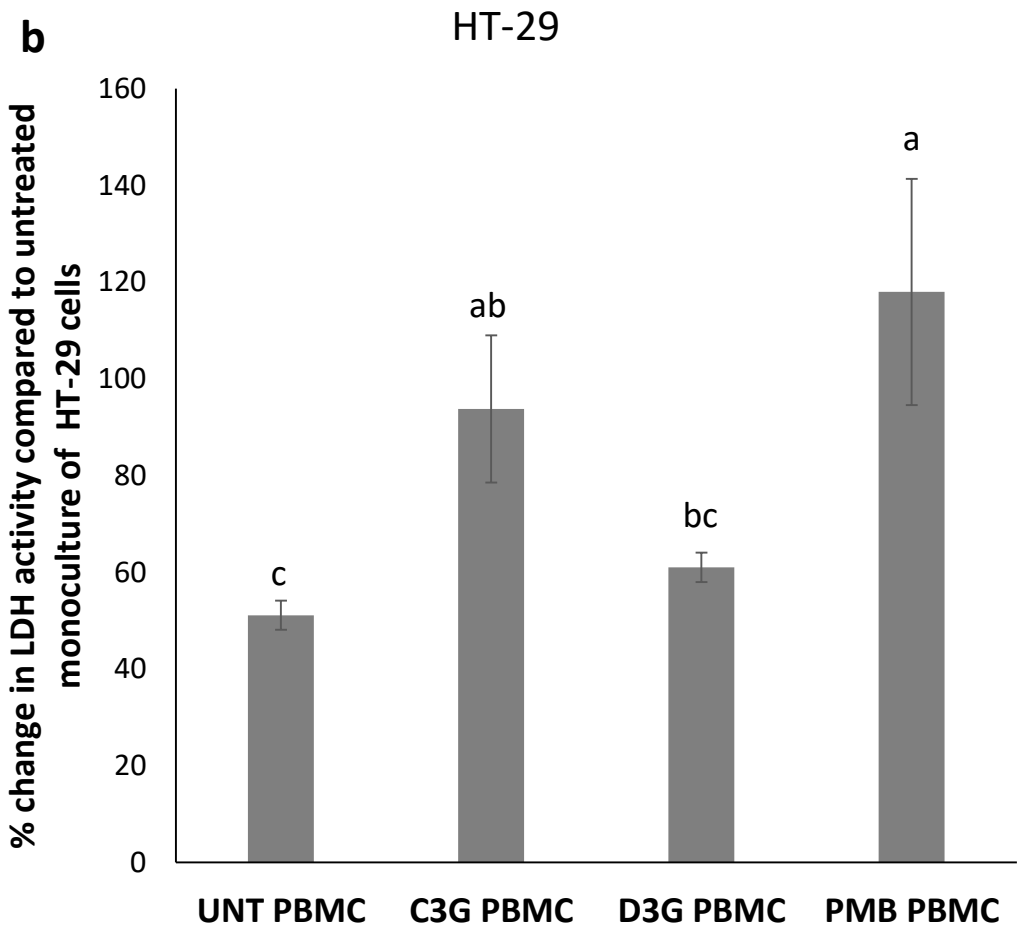


Supplementary Figure 3



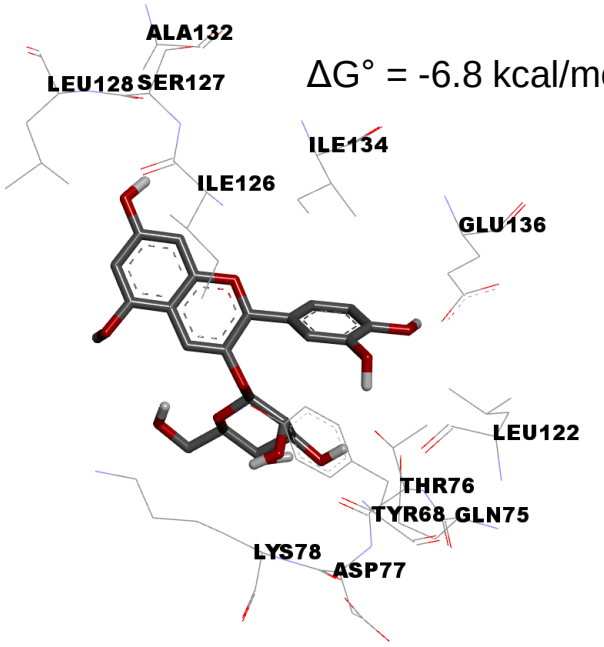
Supplementary Figure 4





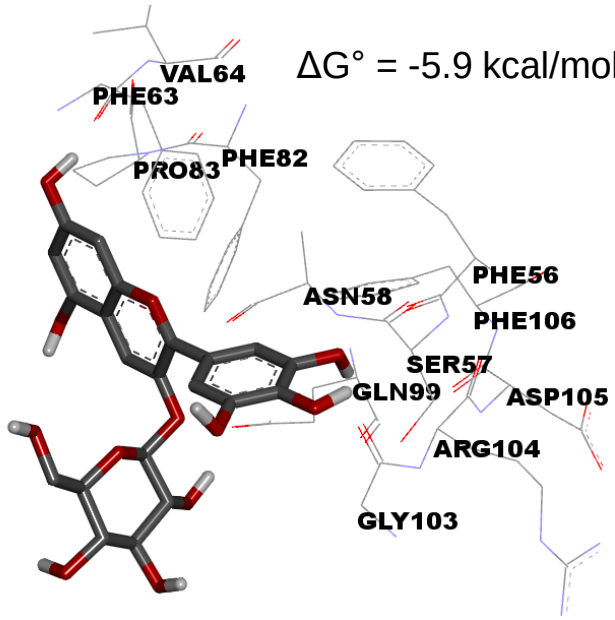
Supplementary Figure 5

a. C3G and PD-1



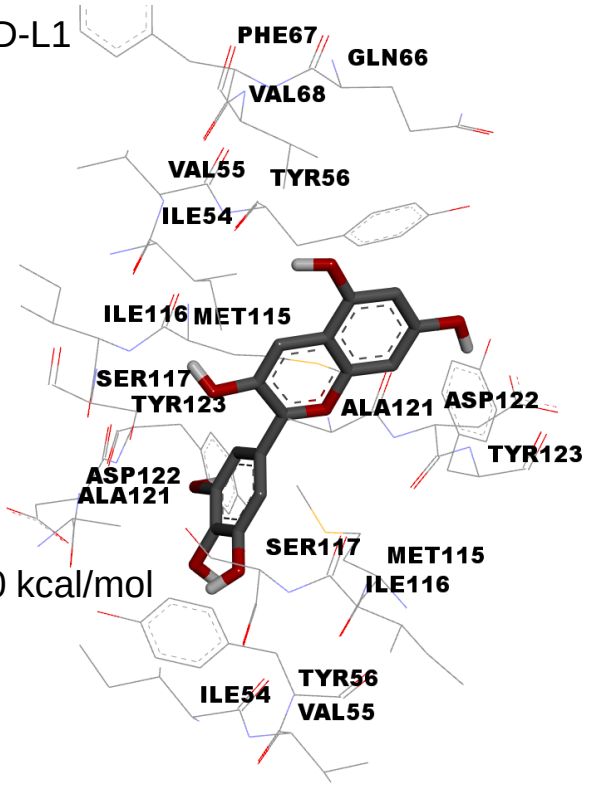
ΔG° = -6.8 kcal/mol

b. D3G and PD-1



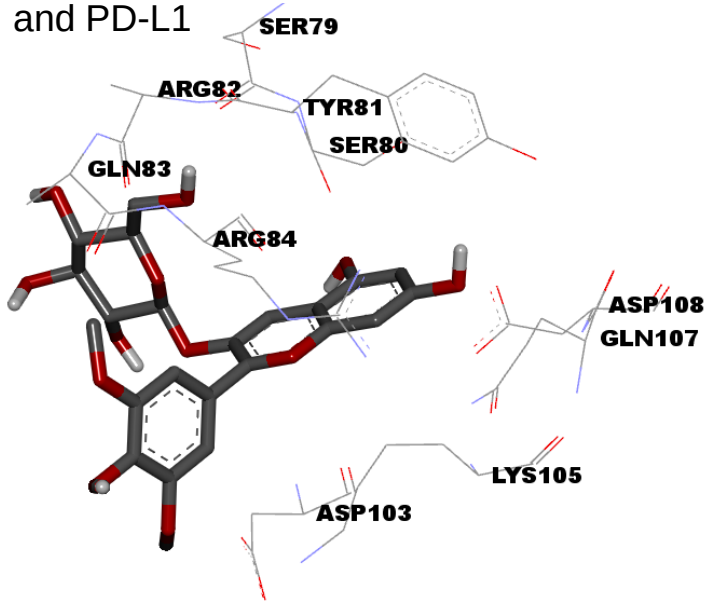
ΔG° = -5.9 kcal/mol

c. DC and PD-L1



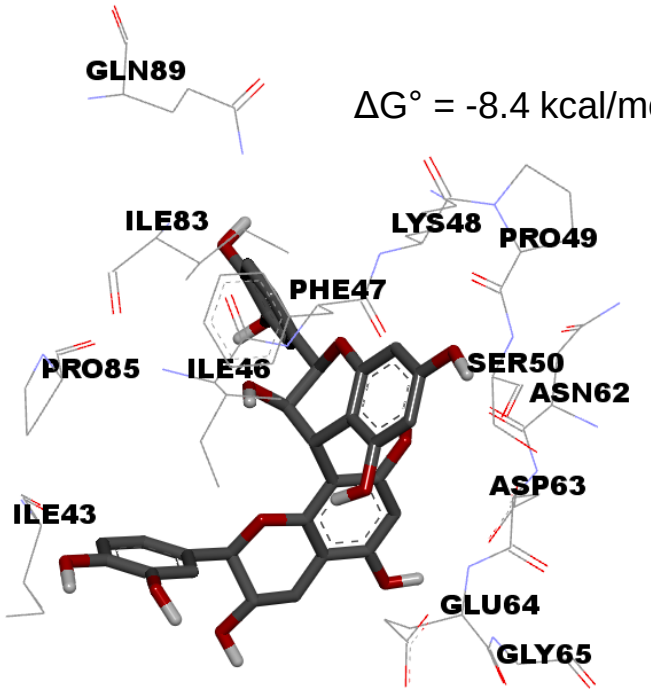
ΔG° = -8.0 kcal/mol

d. M3G and PD-L1



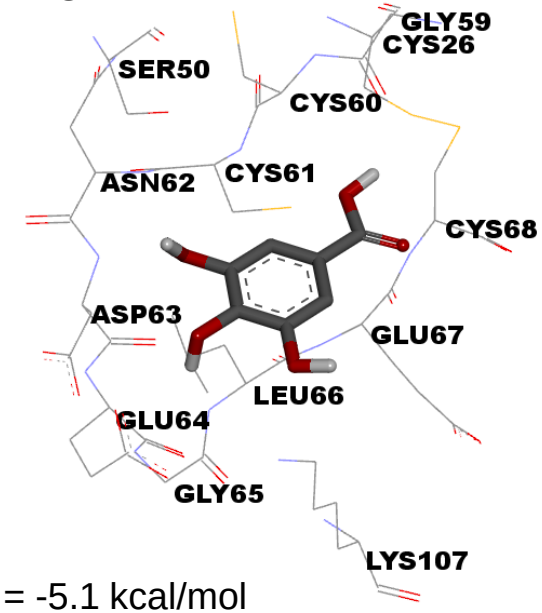
ΔG° = -5.5 kcal/mol

e. PB1 and VEGF



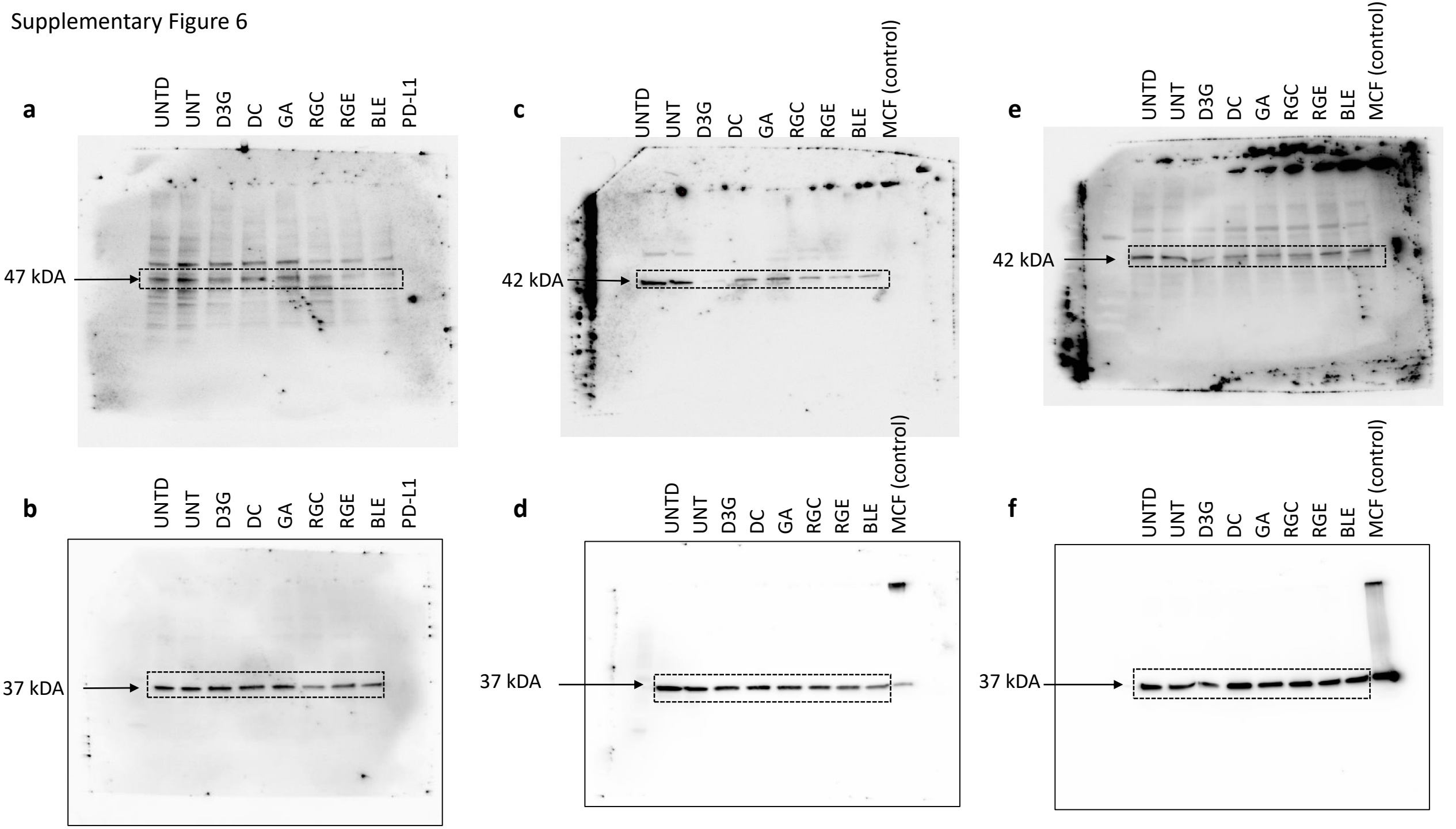
ΔG° = -8.4 kcal/mol

f. GA and VEGF

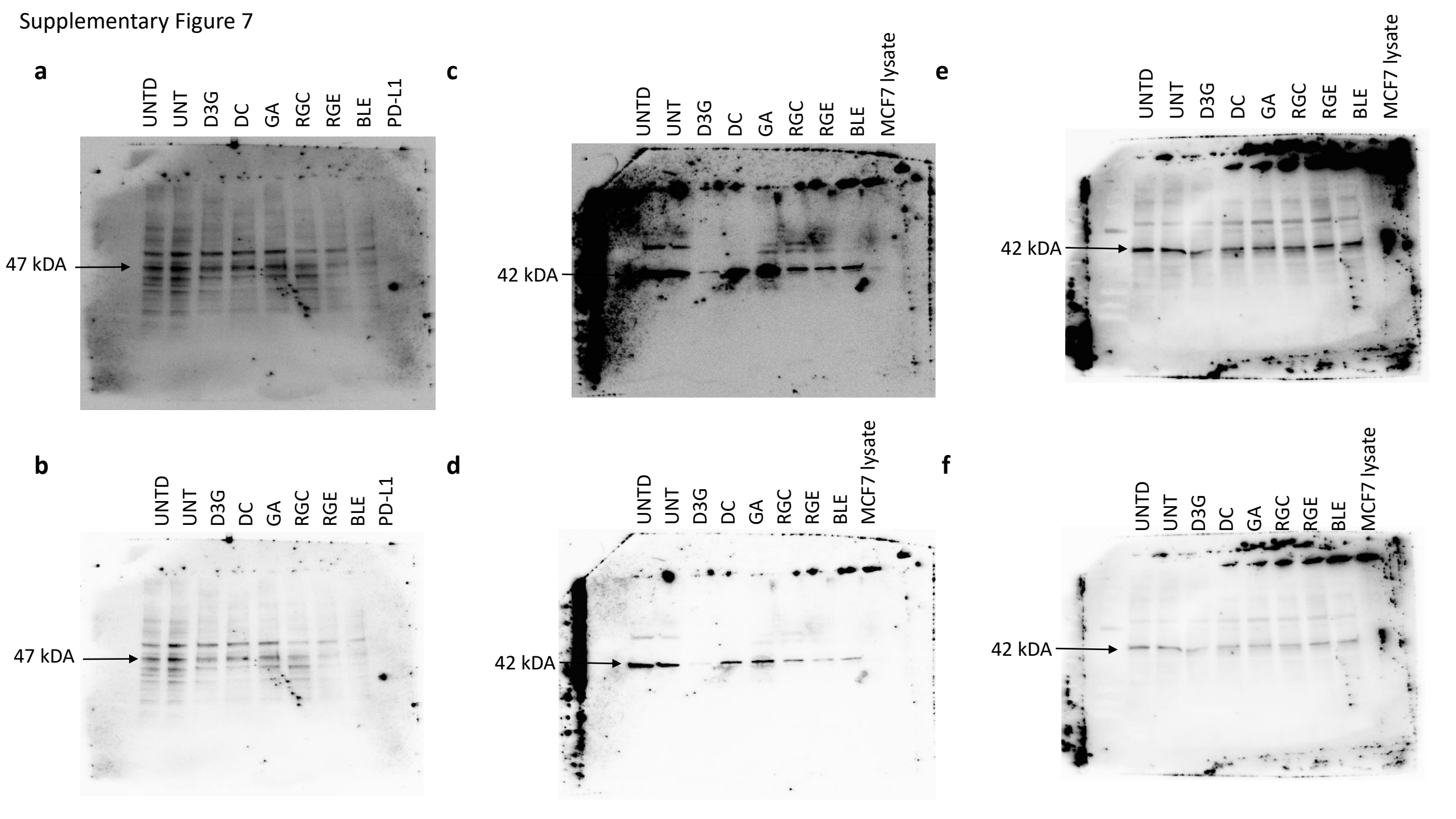


ΔG° = -5.1 kcal/mol

Supplementary Figure 6



Supplementary Figure 7



Supplementary Table 1: Comparison of color parameters of anthocyanins in the media initially and after incubation with HCT 116 cells for 24 h at 37˚C.

		L*	a*	b*	Hue Angle	Chroma	Saturation	ΔE	Color
Cyanidin-3-*O*-Glucoside	Initial	1.3 ± 0.1	9.8 ± 0.6	0.9 ± 0.7	5.1 ± 4.5	9.8 ± 0.6	7.6 ± 0.1		
	After 24 h	10.9 ± 1.3	23.7 ± 1.8	13.3 ± 1.3	29.2 ± 1.3	27.1 ± 1.8	2.5 ± 1.3		
	Δ	9.6	13.9	12.4	24.2	17.3	-5.1	20.9	
Delphinidin-3-*O*-Glucoside	Initial	0.3 ± 0.0	1.8 ± 0.2	0.3 ± 0.1	10.4 ± 5.2	1.8 ± 0.2	6.5 ± 0.8		
	After 24 h	28.6 ± 2.5	19.7 ± 0.3	40.0 ± 1.3	63.7 ± 1.0	44.6 ± 1.1	1.6 ± 0.1		
	Δ	**28.4**	**18.0**	**39.7**	**53.3**	**42.9**	-5.0	**52.0**	
Malvidin-3-*O*-Glucoside	Initial	0.3 ± 0.0	1.9 ± 0.0	0.4 ± 0.0	13.2 ± 0.1	1.9 ± 0.0	7.5 ± 0.1		
	After 24 h	8.7 ± 2.1	3.7 ± 1.4	7.9 ± 0.7	64.9 ± 8.0	8.7 ± 0.8	1.0 ± 0.2		
	Δ	8.4	1.8	7.4	51.8	6.8	**-6.5**	11.4	

All parameters were statistically different ($p < 0.05$) between the initial and after 24 h per anthocyanin except for a* for malvidin-3-*O*-glucoside.

Supplementary Table 2: Estimated free energy of binding and interactions between amino acids of the phenolic ligand (or small molecule inhibitor) and protein, programmed cell death protein 1 (PD-1).

Ligand	PD-1 Binding Site	Binding Energy (kcal/mol)	Hydrogen Bonding	van der Waal Forces	π-Interactions	Carbon-Hydrogen Bonds
Cyanidin-3-*O*-glucoside	Nivolumab	-5.9	PHE56, **SER57**, SER62, PHE63	THR59, SER60, **GLU61**, **PHE82**, PRO83, **GLN99**, **ARG104**, PHE106		ASN58, GLY103
	PD-L1	-6.8	TYR68, GLN75, THR76	LYS78, LEU122, LEU128, ALA132	**ILE126**, **ILE134**	
Procyanidin B1	Nivolumab	-6.6	**SER57**, PHE63	PHE56, **ASN58**, **GLU61**, **SER62**, **VAL64**, **PHE82**, PRO83, PHE106	GLN99	
	PD-L1	-6.1	LYS78, GLY124, GLU136	VAL64, ASN66, THR76, LEU122, ALA125, LEU128	**ILE126**, **ILE134**	TYR68
Delphinidin-3-*O*-glucoside	Nivolumab	-5.9	PHE56, **SER57**, PHE63, GLN99, GLY103	**ASN58**, **VAL64**, **ARG104**, PHE106	PHE82, **PRO83**	
	PD-L1	-5.3		ASN66, LYS78, LEU122, GLY124, ALA132, ILE134	**ILE126**	TYR68, THR76
Malvidin-3-*O*-glucoside	Nivolumab	-5.6	GLU61	PHE56, SER57, **ASN58**, SER60, **SER62**, PHE63, **PHE82**, **GLN99**, GLY103, **ARG104**, PHE106		
	PD-L1	-5.5	ASN66, THR76, LYS78	VAL64, GLY124, ALA132, **GLN133**, ILE134, GLU136	**ILE126**	TYR68
Delphinidin chloride	Nivolumab	-5.4	PHE56, **SER57**, PHE63	**ASN58**, **VAL64**, GLY103, **ARG104**, PHE106	**PHE63**, PHE82, **PRO83**	PRO83
	PD-L1	-5.2	THR76, GLU136	LEU122, GLY124, LEU128, ALA132	TYR68, **ILE126**, **ILE134**	
Gallic acid	Nivolumab	-3.9	ASN33, **SER57**, SER60, SER127, GLN133	PHE56, **ASN58**, THR59, LYS135		
	PD-L1	-3.8	GLU136	**TYR68**, THR76, LEU122, GLY124, ILE126	**ILE134**	
8YZ (small molecule inhibitor)	Nivolumab	-7.0	**SER57**	**ASN58**, **GLU61**, **SER62**, **VAL64**, **PHE82**, **GLN99**, PRO101, ASN102, **ARG104**	PHE56, **PHE63**, **PRO83**, GLY103, PHE106	
	PD-L1	-7.0	ILE134	**TYR68**, PRO83, **GLN133**	VAL64, **ILE126**, LEU128, ALA132, **ILE134**	

Bolded interactions are those that are the same between the phenolics and the small molecule inhibitor; Underlined interactions are those that are the same between phenolics.

Supplementary Table 3: Estimated free energy of binding and interactions between amino acids of the phenolic ligand (or small molecule inhibitor) and protein, programmed death-ligand 1(PD-L1).

Ligand	PD-L1 Binding Site	Binding Energy (kcal/mol)	Hydrogen Bonding	van der Waal Forces	π-Interactions	Carbon-Hydrogen Bonds
Cyanidin-3-*O*-glucoside	Atezolizumab	-5.9		SER79, **SER80**, **ASP103**, **GLN107**, ASP108	**ARG82**, **LYS105**	
	8J8 small molecule	-9.6	**TYR56**, ARG125	ALA18, PHE19, **ILE54**, ASP61, ASN63, GLN66, **VAL68**, **VAL76**, MET115, **LYS124**	**TYR56**, **ALA121**, **ASP122**, **TYR123**	THR20, **ASP122**
Procyanidin B1	Atezolizumab	-5.4	ARG82, THR102	SER79, **SER80**, GLN83, **ASP103**, **GLN107**	**ARG82**, **ARG84**, **LYS105**	
	8J8 small molecule	-2.6	ASP61, ASN63, TYR123	THR20, TYR56, LYS62, GLN66, LYS75, HIS78, SER79, **ASP122**	VAL76, **TYR123**, LYS124, ARG125	
Delphinidin-3-*O*-glucoside	Atezolizumab	-5.3	SER79, ASP103	TYR81, ARG82, **GLN107**, ASP108	SER80	ARG84
	8J8 small molecule	-5.9	PHE19, THR20, **TYR56**, ASP61, LYS124, ARG125	ALA18, GLU58, LYS62, ASN63, **TYR123**	VAL76, **ASP122**, LYS124	
Malvidin-3-*O*-glucoside	Atezolizumab	-5.5	SER79, ARG82, ARG84	TYR81, GLN83, **GLN107**, ASP108	SER80, ASP103, **LYS105**	
	8J8 small molecule	-6.8	THR20, GLN66, LYS124	PHE19, VAL21, TYR56, GLU58, LYS62, ASN63, **TYR123**, **ARG125**	**ASP122**, LYS124	
Delphinidin chloride	Atezolizumab	-5.4	ASP103, ASP108	ARG82, GLN83, **GLN107**	SER80, **ARG82**, **LYS105**	
	8J8 small molecule	-8.4	SER117*	**ILE54***, TYR56*, GLN66, **ILE116***, **SER117***, **ASP122***, **TYR123***	**TYR56***, **MET115***, **ALA121***	
Gallic acid	Atezolizumab	-3.9	ARG84	**SER80**, ASP108	**LYS105**	
	8J8 small molecule	-6.7	ILE54, MET115*	VAL55, TYR56, MET115*, **ILE116**, ALA121*, **ASP122***, **TYR123**	**MET115***, **ALA121***	SER117
8YZ (small molecule inhibitor)	Atezolizumab	-5.9	GLN83	LYS62, **SER80**, THR102, **ASP103**, **GLN107**	**ARG82**, **ARG84**, **LYS105**	
8J8 (small molecule inhibitor)	8J8 small molecule	-12.1	**TYR56***	**ILE54***, **VAL68**, **VAL76**, **ILE116***, **SER117*** **ASP122***, **TYR123***, **LYS124**, **ARG125**	**TYR56***, **MET115***, **ALA121***, **ASP122***, **TYR123***	GLN66, **ASP122***, TYR123*

Bolded interactions are those that are the same between the phenolics and the small molecule inhibitor; Underlined interactions are those that are the same between phenolics
*The 5N2D PD-L1 protein structure used is a homodimer and for these ligands identical amino acids from both PD-L1 protein chains participated in binding interactions.

Supplementary Table 4: Estimated free energy of binding and interactions between amino acids of the phenolic ligand (or small molecule inhibitor) and protein, vascular endothelial growth factor (VEGF).

Ligand	VEGF Binding Site	Binding Energy (kcal/mol)	Hydrogen Bonding	van der Waal Forces	π-Interactions	Carbon-Hydrogen Bonds
Cyanidin-3-*O*-glucoside	VEGFR1	-9.4	PHE47, **SER50**, ASN62, **ASP63**, LEU66	**ASP34**, **LYS48**, **CYS60**, **CYS61**, GLU67, LYS107	PHE36, **ILE46**, **GLU64**	
	VEGFR2	-6.4	SER50, GLU64	**PHE47**, PRO49, ASN62, **ASP63**, PRO85, HIS86, **GLN89**	**ILE46**, LYS48, **ILE83**	
Procyanidin B1	VEGFR1	-7.5	GLY59, GLU64, CYS68	CYS26, **ASP34**, **PHE36**, ILE46, PHE47, SER50, **ASN62**, ASP63, LEU66, GLU67	CYS60, CYS61, **GLU64**, LYS107	
	VEGFR2	-8.0	**ASN62**, ASP63	**PHE47**, PRO49, **SER50**, GLY65, **GLN89**	**ILE46**, LYS48, GLU64, **ILE83**, PRO85	
Delphinidin-3-*O*-glucoside	VEGFR1	-7.5	**SER50**, CYS68	**ASP34**, **PHE36**, ILE46, **LYS48**, **GLY59**, **ASN62**, ASP63, LEU66, GLU67, **ILE83**	CYS60	CYS61
	VEGFR2	-6.2	PHE47, GLN89	PRO49, **SER50**, ASN62, **ASP63**, GLU64, PRO85	**ILE46**, LYS48, **ILE83**	
Malvidin-3-*O*-glucoside	VEGFR1	-7.2	ASP34, **ASP63**	PHE47, **LYS48**, SER50, CYS51, **CYS60**, **CYS61**, **ASN62**, LEU66, GLU67, LYS107	PHE36, **ILE46**, **GLU64**	
	VEGFR2	-6.1	**ASN62**, GLN89	**PHE47**, PRO49, **SER50**, **ASP63**, GLU64	**ILE46**, LYS48, **ILE83**	PRO85
Delphinidin chloride	VEGFR1	-6.9	**SER50**, **ASP63**	**ASP34**, **PHE36**, **LYS48**, **CYS60**, LEU66, **CYS68**, **ILE83**	**ILE46**, CYS61, ASP63, **GLU64**	GLU67
	VEGFR2	-5.9	SER50, **ASN62**	**PHE47**, PRO49, **ASP63**, GLU64, PRO85, **GLN89**	**ILE46**, LYS48, **ILE83**	SER50
Gallic acid	VEGFR1	-5.1	GLY59, CYS61, ASN62, **ASP63**, LYS107	CYS26, SER50, **CYS60**, GLU64, LEU66, GLU67, **CYS68**		
	VEGFR2	-4.2	SER50, **ASN62**	**PHE47**, PRO49, **ASP63**, GLU64, ILE83	**ILE46**, LYS48	SER50
Vatalanib	VEGFR1	-7.6	**SER50**, **ASP63**	**ASP34**, **PHE36**, **LYS48**, **GLY59**, **CYS60**, **CYS61**, **ASN62**, **CYS68**, **ILE83**	**ILE46**, **GLU64**	PHE47
PTC-858 (small molecule inhibitor)	VEGFR2	-5.8	**ASN62**	**PHE47**, LYS48, **SER50**, **ASP63**, **GLN89**	**ILE46**, ASN62, **ILE83**	GLU64

Bolded interactions are those that are the same between the phenolics and the small molecule inhibitor; Underlined interactions are those that are the same between phenolics.