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Evidences that *Annona cherimola* leaves extracts suppress the malignant melanoma cells growth and migration

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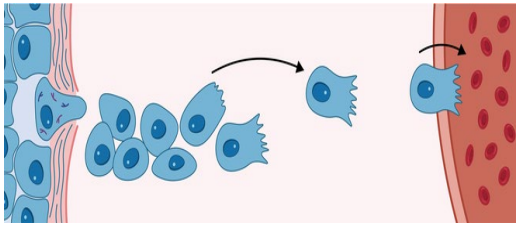
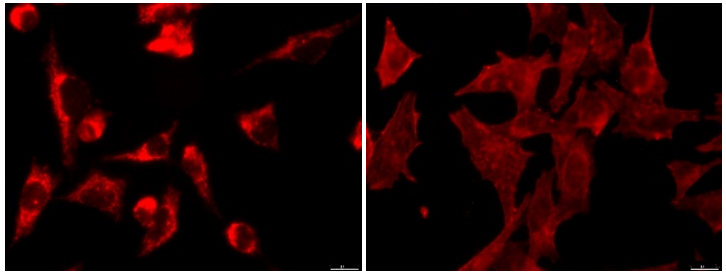
Evidences that *Annona cherimola* leaves extracts suppress the malignant melanoma cells growth and migration

Annona cherimola leaves



Inhibition of malignant melanoma cell migration

Tubulin *Actin*



Cytoskeleton disorganization

Abstract: Cancer represents one of the major causes of mortality worldwide, indeed 19.3 million new cases and almost 10.0 million deaths have been estimated last year. Among the different type of cancers, malignant melanoma represents the most aggressive and deadly skin cancers. Unfortunately, the long-term efficacy of melanoma treatments is limited by the lack of clinical effects, together with the onset of side effects and resistance. The latter is a major obstacle for the success of the melanoma therapy, thus the exploration of new potent and safer anticancer agents is of great importance. Recently, numerous plant species, used for therapeutic purposes and containing various non-toxic nutraceuticals, have been widely studied. In particular, we investigated the antioxidant and anticancer properties on melanoma cells of three *Annona cherimola* leaves extracts (ethanolic, methanolic and aqueous). The ethanolic extract showed the most promising and interesting anticancer activity, mostly on the malignant A2058 melanoma cell line (IC_{50} = 5.6±0.8 ng/mL), without exerting cytotoxicity on the normal cells. It was also able to block the melanoma cells migration process, modulating the expression levels of some involved proteins. In addition, the A2058 cells treated with the ethanolic extract showed a clear disorganization of cytoskeleton dynamic, inducing cell apoptosis. Finally, numerous bioactive compounds, responsible of the antioxidant and antitumoral properties, were identified in the studied extracts.

Keywords: *Annona cherimola*; natural compounds; melanoma; tubulin; actin; epithelial-mesenchymal transition.

Annona cherimola Mill.

Family: Annonaceae
Clade: Angiospermae

An exotic Calabrian fruit with numerous beneficial properties for human health

[1] Albuquerque, T.G. et al. Nutritional and phytochemical composition of *Annona cherimola Mill.* fruits and by-products: Potential health benefits. *Food Chem* **2016**, *193*, 187-195.
[2] Jamkhande, P.G. et al. *Annona cherimola Mill*. (Custard apple): a review on its plant profile, nutritional values, traditional claims and ethnomedicinal properties. Oriental Pharmacy and Experimental Medicine 2017, 17, 189–201.

Extraction yield

Extracts	%
EtOH	11.2
MeOH	16.9
H_20	17.0

*Total Polyphenols Content (**TPC**)*
*Total Flavonoids Content (**TFC**)*

Extracts	TPC (μg GAE g^{-1})	TFC (μg QE g^{-1})
EtOH	7.1 ± 0.1	6.4 ± 0.1
MeOH	6.2 ± 0.2	5.8 ± 0.1
H_20	2.9 ± 0.2	0.9 ± 0.2

Antioxidant activity

		Extracts		
	Trolox	EtOH	MeOH	H_2O
DPPH EC_{50}	10.2 ± 0.4	11.2 ± 0.1	8.2 ± 0.2	88.9 ± 0. 2
ABTS EC_{50}	5.6 ± 0.3	9.8 ± 0.2	6.9 ± 0.3	22.7 ± 0.9

Quantitative profile (HPLC)

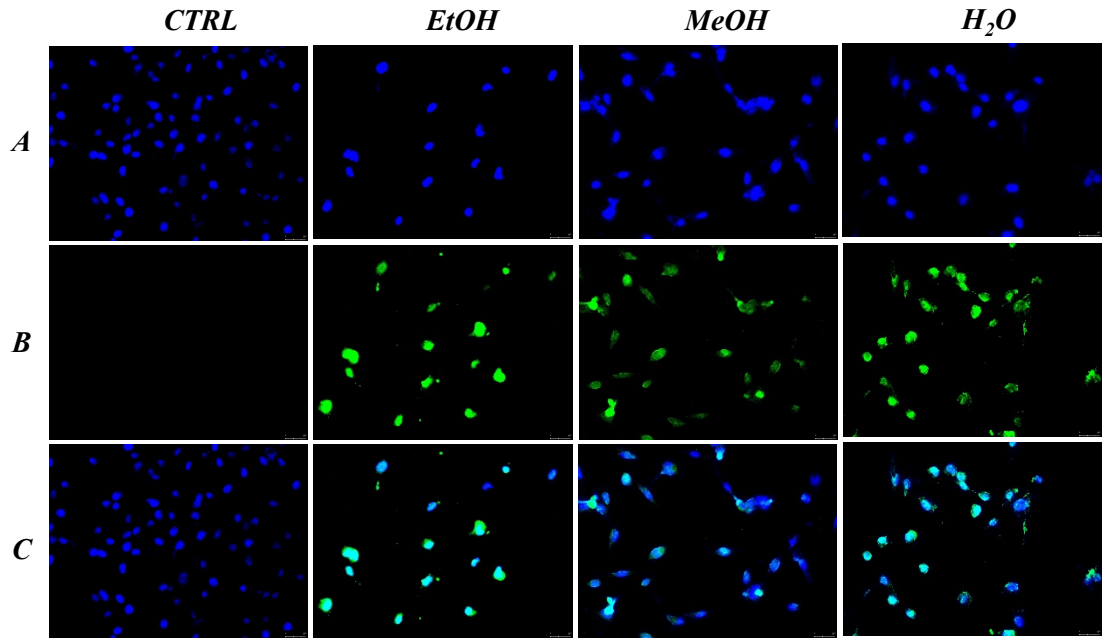
	Extracts (mg 100 g^{-1})		
Standard	*MeOH*	*EtOH*	*H_2O*
Gallic acid	75.7	60.0	51.3
Chlorogenic acid	38.1	34.3	33.7
p-Cumaric acid	14.7	10.3	/
Quercetin	27.2	27.3	/
Ferulic acid	30.8	30.8	/

Anticancer activity: MTT assay

IC_{50} values of the ethanol (EtOH), methanol (MeOH) e aqueous (H_2O) of *Annona cherimola leaves extracts*, expressed in µM.

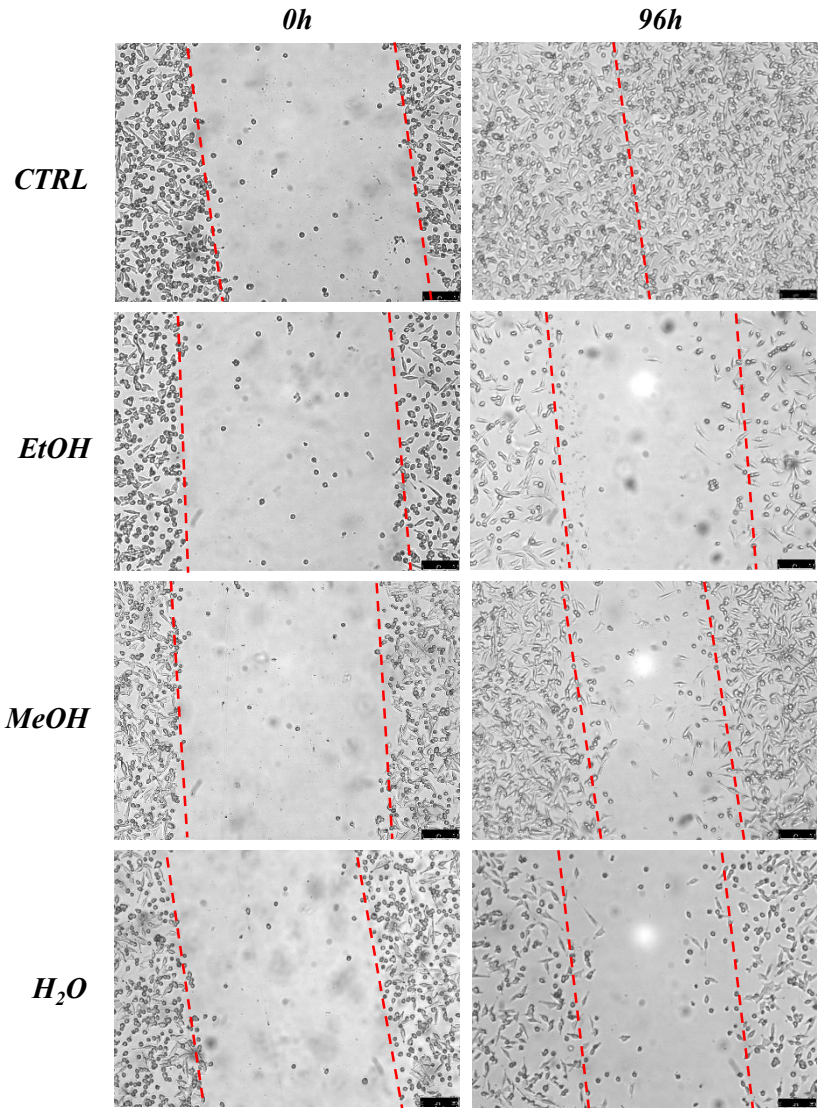
	IC_{50} (µg/mL)			
	EtOH	***MeOH***	***H_2O***	***Vinblastine***
A2058	$5.6x10^{-3}$±0.8	$1.3x10^{-2}$±0.6	2.4±0.9	$1.8x10^{-3}$±0.8
Sk-Mel28	0.12±1.0	0.19±0.5	23.3±0.8	15.3±0.7
3T3-L1	29.7±0.6	3.6±1.0	60.9±1.1	$1.6x10^{-4}$±0.7

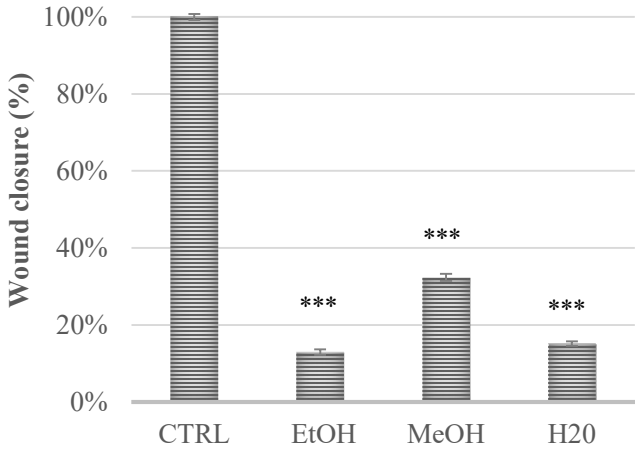
Cell death by apoptosis: TUNEL assay



TUNEL assay. A2058 cells were treated for 24 h with *Annona cherimola* leaves extracts *(*EtOH, MeOH and H_2O) or with vehicle (CTRL). The green nuclear fluorescence the fluorescence indicates that the cells undergone to apoptosis. *Panels **A***: DAPI. *Panels **B***: CF™488A. *Panels **C***: overlay.

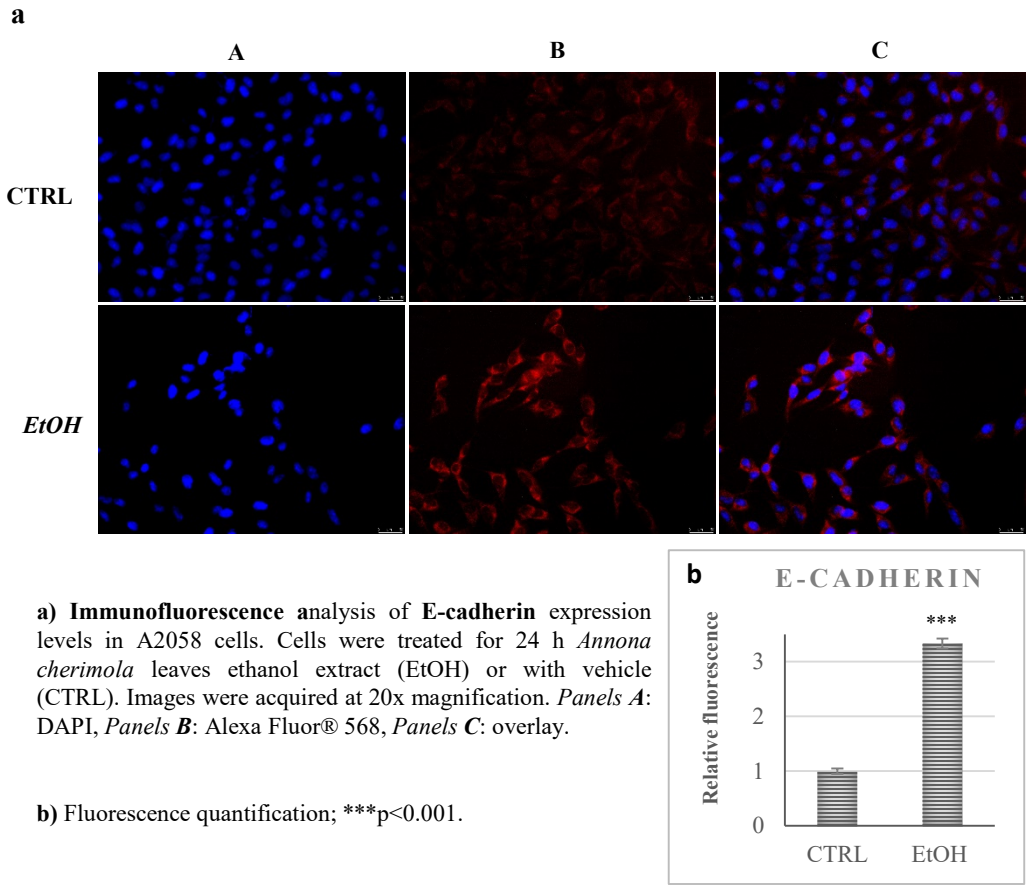
In vitro inhibition of cell migration: *Scratch assay*





Scratch assay on A2058 cells treated with *Annona cherimola* leaves extracts (EtOH, MeOH and H_2O) or with vehicle (CTRL). Wound closure was monitored at 0 and 96 h using an inverted microscope.
Wound closure was graphically reported.

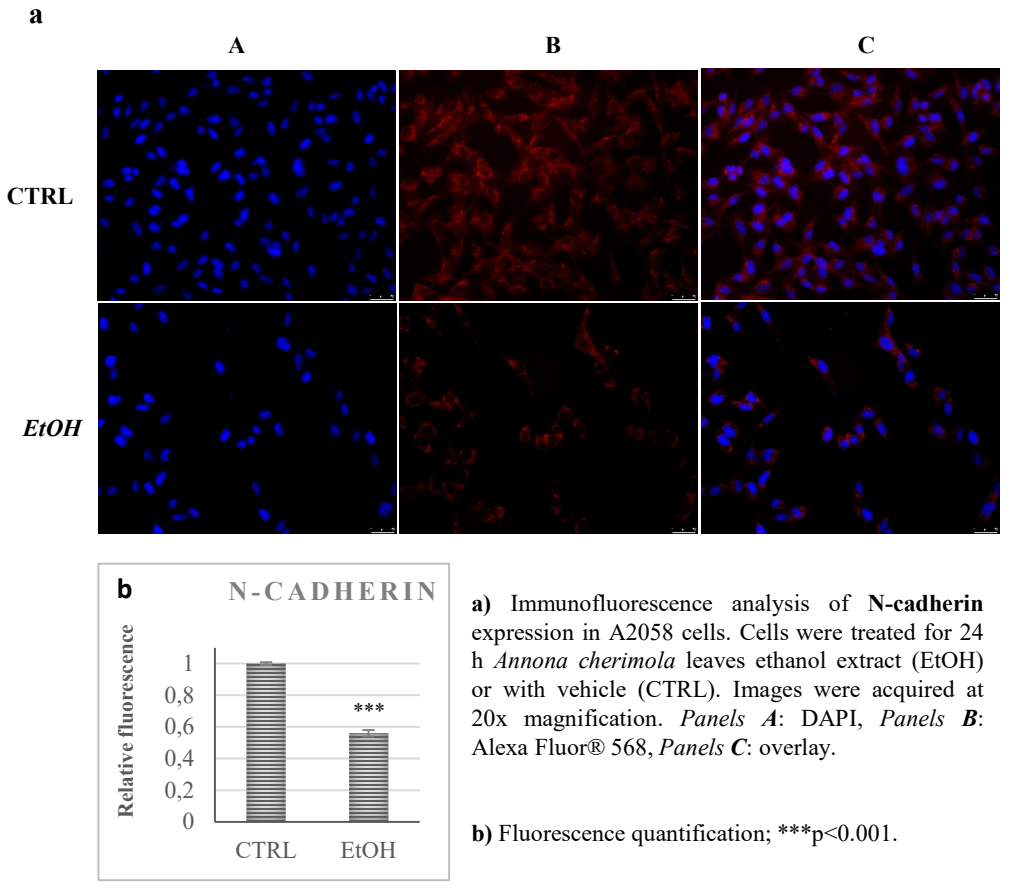
Increase in the E-CADHERIN expression levels



a) Immunofluorescence analysis of **E-cadherin** expression levels in A2058 cells. Cells were treated for 24 h *Annona cherimola* leaves ethanol extract (EtOH) or with vehicle (CTRL). Images were acquired at 20x magnification. *Panels **A***: DAPI, *Panels **B***: Alexa Fluor® 568, *Panels **C***: overlay.

b) Fluorescence quantification; ***p<0.001.

Decrease in the N-CADHERIN expression levels

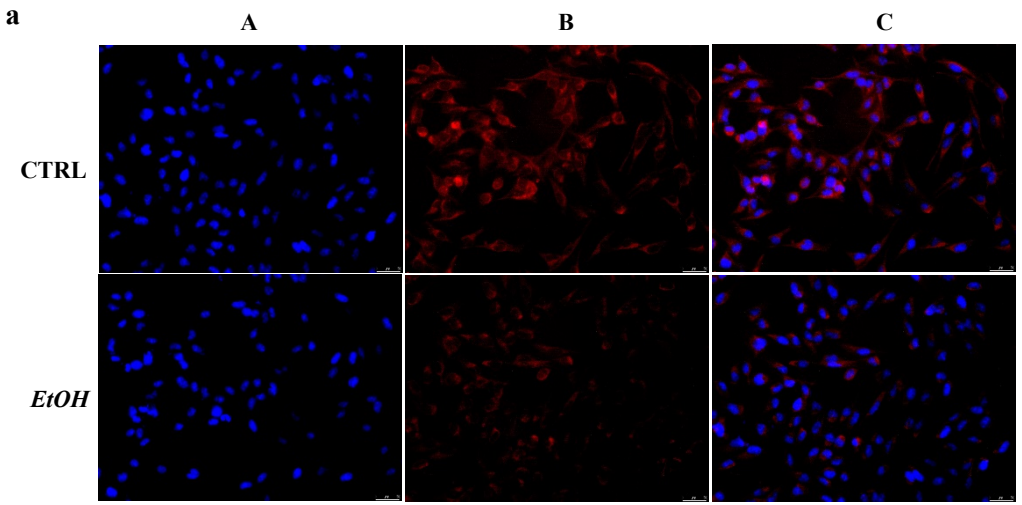


a) Immunofluorescence analysis of **N-cadherin** expression in A2058 cells. Cells were treated for 24 h *Annona cherimola* leaves ethanol extract (EtOH) or with vehicle (CTRL). Images were acquired at 20x magnification. *Panels **A***: DAPI, *Panels **B***: Alexa Fluor® 568, *Panels **C***: overlay.

b) Fluorescence quantification; ***p<0.001.

Mediators implicated in migration and angiogenesis processes

Decrease in VIMENTIN expression levels



a) Immunofluorescence analysis of **vimentin** expression in A2058 cells. Cells were treated for 24 h *Annona cherimola* leaves ethanol extract (EtOH) or with vehicle (CTRL). Images were acquired at 20x magnification. *Panels **A***: DAPI, *Panels **B***: Alexa Fluor® 568, *Panels **C***: overlay.

b) Fluorescence quantification; ***p<0.001.

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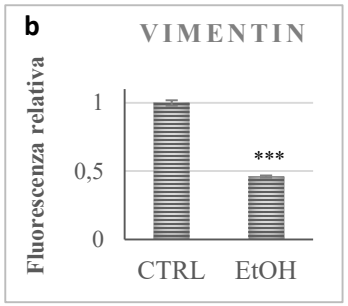
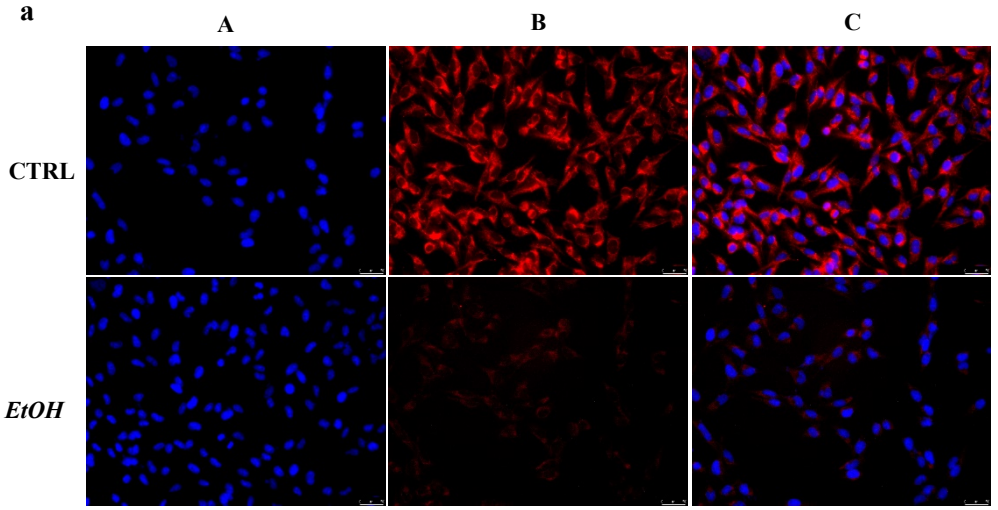
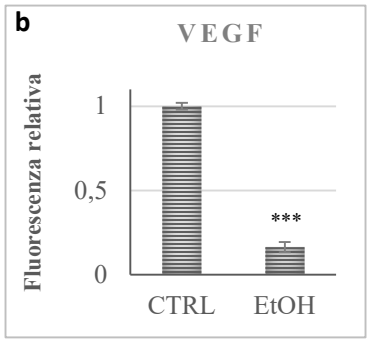


Decrease in VEGF expression levels





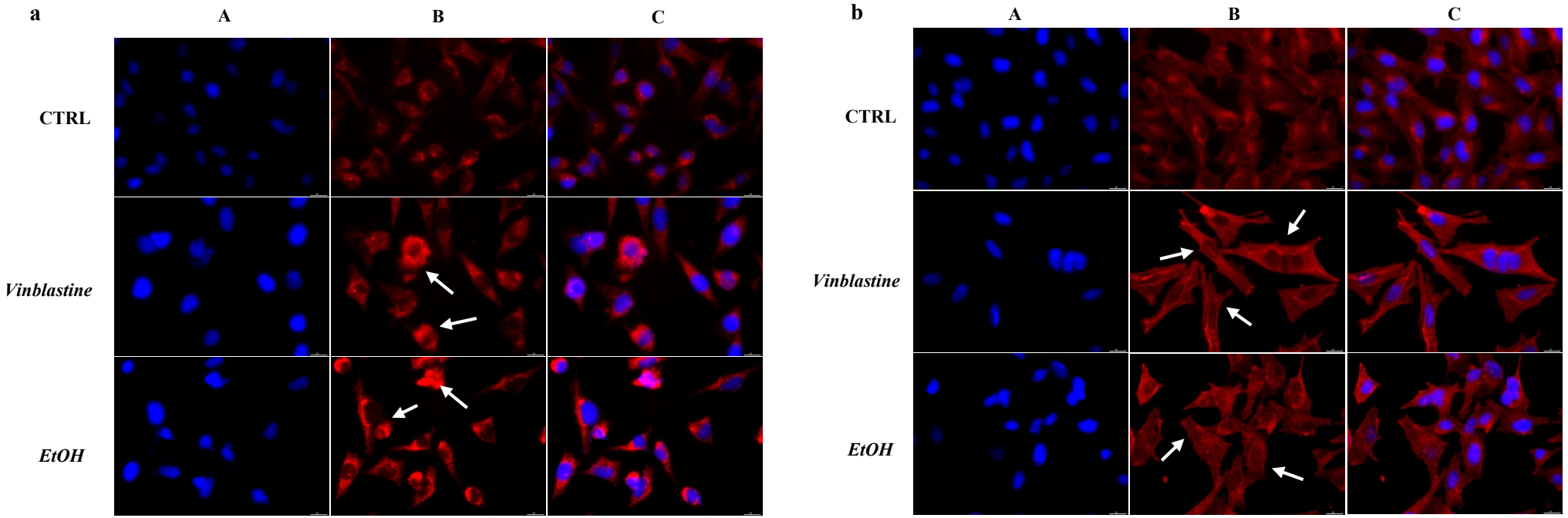
a) Immunofluorescence analysis of **VEGF** expression in A2058 cells. Cells were treated for 24 h *Annona cherimola* leaves ethanol extract (EtOH) or with vehicle (CTRL). Images were acquired at 20x magnification. *Panels **A***: DAPI, *Panels **B***: Alexa Fluor® 568, *Panels **C***: overlay.

b) Fluorescence quantification; ***p<0.001.

Perturbation of cytoskeleton dynamics

TUBULIN accumulation around the cell nucleus

Formation of abnormal **ACTIN** microfilaments prominent at the cell periphery



Fluorescence analysis of **tubulin (a)** and **actin (b)** expression in A2058 cells. Cells were treated for 24 h *Annona cherimola* leaves ethanol extract (EtOH) or with vehicle (CTRL). Images were acquired at 20x magnification. *Panels **A***: DAPI, *Panels **B***: Alexa Fluor® 568, *Panels **C***: overlay.

Conclusions

- The ethanolic extract of *Annona cherimola* leaves showed an interesting antioxidant and antitumor activity, especially on the A2058 melanoma line with IC_{50} equal to 5.6 ± 0.8 ng/mL, without showing cytotoxicity on embryonic murine fibroblasts (3T3- L1).

- All the extracts were able to trigger cell death by apoptosis in A2058 melanoma cells and to interfere with the *in vitro* cell migration process.

- The ethanolic extract was also able to modulate the expression levels of some proteins involved in this process: E-cadherin, N-cadherin, vimentin and VEGF.

- The ethanolic extract caused a clear disorganization of the cytoskeleton with an accumulation of tubulin around the cell nucleus and with the formation of abnormal actin microfilaments on the cell periphery.

Considering these promising data, *Annona cherimola* leaves could represent an innovative nutraceutical for cancer treatment, used as an alternative or an adjuvant therapy to the traditional drugs, unfortunately often affected by high systemic toxicity.

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Thank you for your kind attention