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Evaluation of antitumor and immunomodulatory properties of Indicaxanthin from *Opuntia Ficus Indica* (L. Mill) fruit

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CICLO XXXII

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~ DEDICATION ~

I wish to dedicate this thesis to my late grandmother.
She was constant source of inspiration to my life. Although she is not here
to give me strength and support I always feel her presence
that used to urge me to strive to achieve
my goals in life.

Table of contents

ABSTRACT

Cancer is a growing health problem around the world and according to estimates from the International Agency for Research on Cancer (IARC), 14.1 million new cancer cases and 8.2 million cancer deaths worldwide have been reported in 2012 (Ferlay *et al.,* 2015). By 2030, the global burden is expected to grow to 21.7 million new cancer cases and 13 million cancer deaths simply due to the growth and aging of the population.

Indicaxanthin ((2S)-2,3-dihydro-4-[2-[(2S)-2a-carboxypyrrolidin-1- yl]ethenyl]pyridine-2a,6-dicarboxylic acid), a betalain pigment from cactus pear fruit, has been the object of sound experimental work over the latest years. As many phytochemicals, indicaxanthin is a redox-active compound and has been shown to act as antioxidant in a number of *in vitro* studies (Allegra *et al*., 2005; Turco Liveri *et al.,* 2009). Interestingly, thanks to its charged portions, ionizable groups and lipophilic moieties, it is amphiphilic at physiological pH (Turco Liveri *et al*., 2009) and has been demonstrated to interact with cell membranes (Tesoriere *et al*., 2006; Turco Liveri *et al*., 2009). This feature is critical to allow bioactive compounds to interact with cells and to initiate signaling events. In this regard, indicaxanthin has been showed to modulate specific redox-dependent signaling pathways involved in macrophage activation and apoptosis, epithelial and endothelial dysfunction in vitro (Allegra *et al.*, 2014; Tesoriere *et al*., 2015). Remarkably, and in contrast with the majority of dietary phytochemicals, indicaxanthin is highly bioavailable (Tesoriere *et al*., 2004). The molecule has been shown to cross unaltered intestinal epithelial cell *in vitro* being absorbed through paracellular junctions (Tesoriere *et al*., 2013). In line with that, indicaxanthin has been found in human plasma at a 7 μM peak concentration 3 h after the ingestion of four cactus pear fruits containing 28 mg of the

pigment (Tesoriere *et al*., 2004). Moreover, its amphiphilicity allows it to cross the blood-brain-barrier and localize within the CNS (Allegra *et al*., 2015). Finally, thanks to its bioavailability and redox-modulating properties, indicaxanthin exerts significant pharmacological effects *in vivo*. Indeed, oral administration of the PhC at nutritionally-relevant doses (2 μmol/kg) generates, in rats, a plasma peak concentration of 0.2 μM able to exert strong anti-inflammatory effects in an *in vivo* model of acute inflammation (Allegra *et al*., 2014). The causative link between inflammation and melanoma has accurately been explored in the recent years (Bald *et al*., 2014; Meyer *et al*., 2011; Reinhardt *et al*., 2017; Soudja *et al*., 2010). Experiments in mice revealed that UV-induced skin inflammatory responses can cause the reactive proliferation and migration of melanocytes (Zaidi *et al*., 2011). More recently, it has been shown that reciprocal interactions between melanoma and immune cells in a pro-inflammatory microenvironment provide a source of phenotypic heterogeneity that drives therapy resistance and metastasis (Bald *et al*., 2014; Landsberg *et al*., 2012). In keeping this perspective, we decided to investigate the effects of Indicaxanthin against human melanoma cell proliferation and in a model of cutaneous melanoma. We here demonstrate that indicaxanthin induces apoptosis of human melanoma cells through the inhibition of the NF-κB pathway and the downstream anti-apoptotic signaling events *in vitro* and these effects were paralleled *in vivo* in a murine model of melanoma. Finally, preliminary data on six healthy volunteers, showed that indicaxanthin is able to modulate TNF-α and Il-6 production in a whole blood *ex vivo* model. Furthermore, the phytochemical induces an increase in the phagocytosis of 5 different Gram-negative pathogens on whole blood assay, without exerting antimicrobial effects on them. Interestingly, preliminary data on 4 of the 6 volunteers showed that the observed effects maybe attributed to the modulation of LTB4 levels, strictly correlated to the activation of immune cells.

CHAPTER 1

1.1 Melanoma

Melanoma is the most dangerous type of skin cancer because it grows quickly and develops over weeks to months invading nearby tissues and spreading to other parts of the body, such as the lung, liver, bone or brain. Melanoma appears as a new spot or as an existing spot, freckle or mole that changes color, size or shape. As shown in **Figure 1.1**, it usually has an irregular, smudgy outline and is often more than one color and it can occur anywhere on the skin. If caught early, it is usually curable, but if it spreads to other parts of the body, it can be very difficult to cure (Koch *et al.,*2004).

Melanoma is a type of skin cancer that could begin in any melanocytes, so it can originate in any part of the body that contains melanocytes: in the eye, respiratory system, nervous system, even on the cortex of the brain and mucous membranes (e.g. lining of the mouth and nasal passages) and become cancerous.

Skin is the largest organ of our body, it protects the internal organs from heat, sunlight, injury and infection; prevents the loss of too much water and other fluids; helps to control body temperature and produces vitamin D.

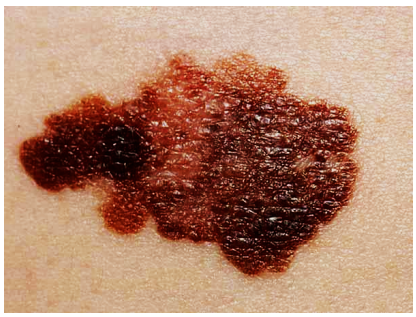


Figure 1.1 Melanoma (Source: National cancer Institute https://www.cancer.gov/types/skin/moles-fact-sheet

The skin consists of three layers: epidermis, dermis and subcutaneous tissue (**Figure 1.2**). Between the epidermis and the dermis layers, there are cells called melanocytes, which

become cancerous in melanoma. Melanocytes produce a brown pigment called melanin which gives the skin its tan or brown color, melanin also protects the deeper layers of the skin from harmful ultraviolet radiation (UVR) and red pheomelanin that contributes to melanoma genesis through a mechanism involving ROS (Brenner *et al.,* 2008).

The melanin display two important biological functions, related to the capacity to act both as an oxidant scavenger and as a system absorbing UV and protecting neighboring cells from DNA damage induced by DNA irradiation.

When there is DNA damage, it could cause the cell to grow out of control, leading to a tumor. Indeed, melanoma is usually caused by damage from UV light from the sun, but UV light from sunbeds can also contribute to the disease. A number of rare mutations, which often run in families, are known to greatly increase one's susceptibility to melanoma. Several different genes have been identified as increasing the risk of developing melanoma. Some rare genes have a relatively high risk of causing melanoma (Gies *et al.,* 2004).

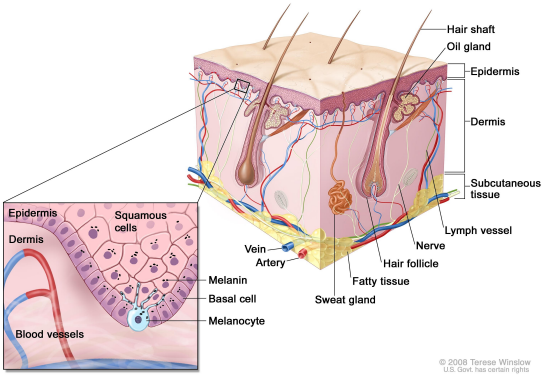


Figure 1.2 Anatomy of the skin (Source: The National Cancer Institute, available at [http://www.cancer.gov/cancertopics/pdq/treatment/melanoma/patient/]

Researchers have found some gene changes inside mole cells that may cause them to become melanoma cells. But it is still not known exactly why some moles become cancerous while most do not. Melanoma is a phenotypically and molecularly

heterogeneous disease: cutaneous, uveal, acral, and mucosal melanomas have different clinical courses, are associated with different mutational profiles, and possess distinct risk factors (Testa *et al.,* 2017). Melanoma is the most aggressive type of skin tumor and extremely difficult to treat in the metastatic stage of the disease. Its incidence rises fast with an increasing global rate per year at 2–7% annually (Gladfelter *et al*., 2017). It represents only 4% of the skin cancer cases, but it accounts for 65% of all skin cancer-related deaths (Cummins *et al.,* 2006). Classification schema for melanoma use Clark model, it describes the histopathological changes that occur in the progression of melanoma, from normal melanocytes to malignant. In Clark Level I, the cancer is in the epidermis only and the nevi are benign. So, normal melanocytes progressively develop a malignant phenotype through the acquisition of various phenotypic features. The second step, Clark level II, some benign nevi became dysplastic nevi, which are not really invasive malignancies but the cancer has begun to spread into the papillary dermis (upper layer of the dermis). In Clark Level III, cancer has spread through the papillary dermis into the papillary-reticular dermal interface but not into the reticular dermis (lower layer of the dermis) and its growth is radial. In Clark Level IV, namely vertical-growth phase, appear the invasive characteristics of the melanoma cells and the cancer has spread into the reticular dermis. Finally, In the last one level V, the cancer has spread into the subcutaneous tissue, it became a metastatic melanoma and the tumor cells dissociate from the primary lesion, migrate through the surrounding stroma, and invade blood vessels and lymphatics to form a tumor at a distant site (Beddingfield *et al.,* 2002).

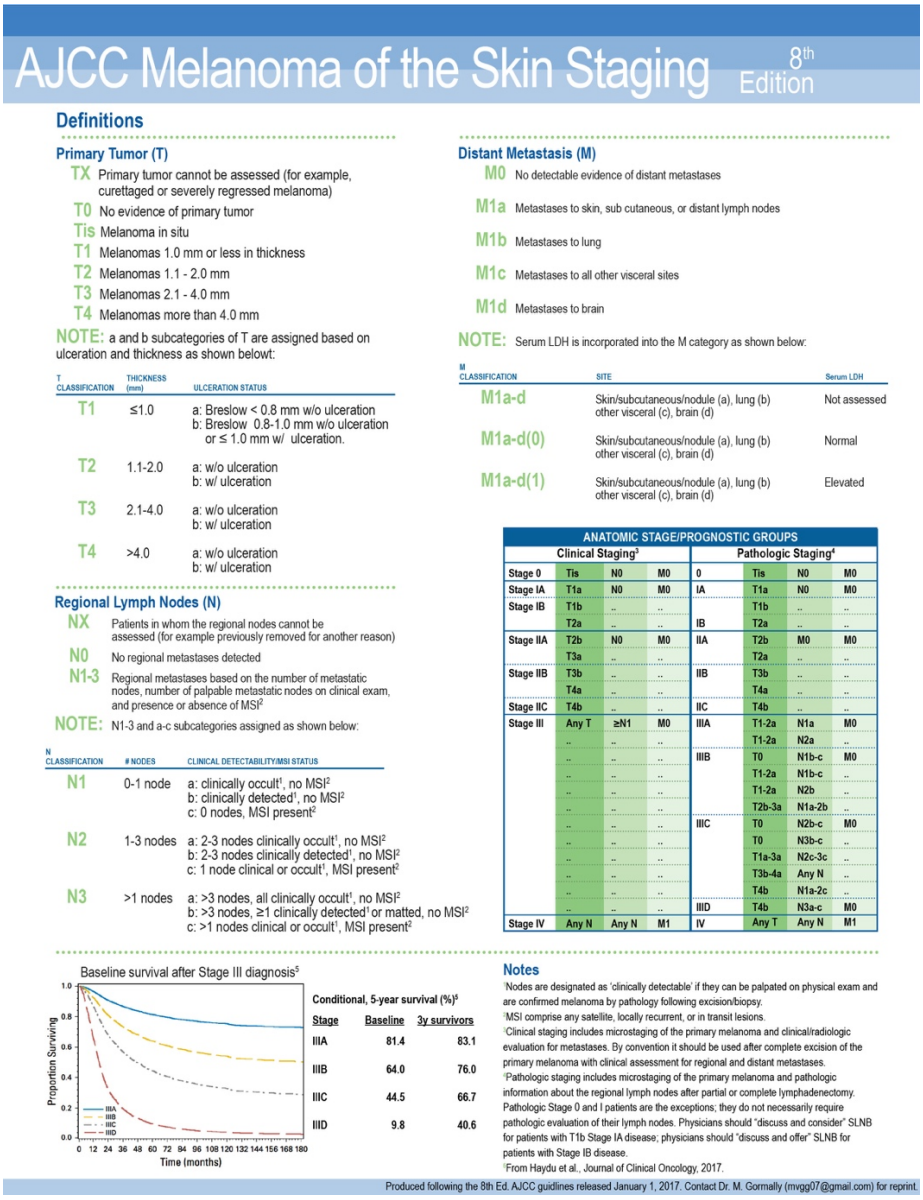
Clinically, the Breslow index is considered one of the most significant factors in predicting the progression of the disease and the primary criterion in melanoma staging (**Figure 1.3**) (Miller *et al.,* 2006).

Fig. 1.3 Classifying melanoma: Both Clark's level and Breslow measurement are often used for staging [https://www.cancer.gov/publications/dictionaries/cancer-terms/def/clark-level-v-skin-cancer]

Instead, **Breslow's thickness** is a measurement of lesion depth in millimeters and it measures the distance between the upper layer of the epidermis and the deepest point of tumor penetration. Tumors are classified into 4 categories based on the depth vertically from the top of the granular layer (or base of superficial ulceration) to the deepest point of tumor involvement.

The AJCC introduced a more precise staging system in 1998, this new system referred to as the TNM staging system uses three parameters, local advancement (T), a combination of Breslow depth and ulceration, lymph node status (N) and distant metastasis (M).

The three parameters (T, N and M) are then combined into four categories (**Figure 1.4**). Stage I represents limited local disease, stage II locally advanced disease, stage III regionally advanced disease and stage IV distant metastasis (Balch *et al.,* 2009).



AJCC Melanoma of the Skin Staging 8th Edition

Definitions

Primary Tumor (T)

TX	Primary tumor cannot be assessed (for example, curettaged or severely regressed melanoma)
T0	No evidence of primary tumor
Tis	Melanoma in situ
T1	Melanomas 1.0 mm or less in thickness
T2	Melanomas 1.1 - 2.0 mm
T3	Melanomas 2.1 - 4.0 mm
T4	Melanomas more than 4.0 mm

NOTE: a and b subcategories of T are assigned based on ulceration and thickness as shown belowt:

T CLASSIFICATION	THICKNESS (mm)	ULCERATION STATUS
T1	≤1.0	a: Breslow < 0.8 mm w/o ulceration b: Breslow 0.8-1.0 mm w/o ulceration or ≤ 1.0 mm w/ ulceration.
T2	1.1-2.0	a: w/o ulceration b: w/ ulceration
T3	2.1-4.0	a: w/o ulceration b: w/ ulceration
T4	>4.0	a: w/o ulceration b: w/ ulceration

Regional Lymph Nodes (N)

NX	Patients in whom the regional nodes cannot be assessed (for example previously removed for another reason)
N0	No regional metastases detected
N1-3	Regional metastases based on the number of metastatic nodes, number of palpable metastatic nodes on clinical exam, and presence or absence of MSI[2]

NOTE: N1-3 and a-c subcategories assigned as shown below:

N CLASSIFICATION	# NODES	CLINICAL DETECTABILITY/MSI STATUS
N1	0-1 node	a: clinically occult[1], no MSI[2] b: clinically detected[1], no MSI[2] c: 0 nodes, MSI present[2]
N2	1-3 nodes	a: 2-3 nodes clinically occult[1], no MSI[2] b: 2-3 nodes clinically detected[1], no MSI[2] c: 1 node clinical or occult[1], MSI present[2]
N3	>1 nodes	a: >3 nodes, all clinically occult[1], no MSI[2] b: >3 nodes, ≥1 clinically detected[1] or matted, no MSI[2] c: >1 nodes clinical or occult[1], MSI present[2]

Distant Metastasis (M)

M0	No detectable evidence of distant metastases
M1a	Metastases to skin, sub cutaneous, or distant lymph nodes
M1b	Metastases to lung
M1c	Metastases to all other visceral sites
M1d	Metastases to brain

NOTE: Serum LDH is incorporated into the M category as shown below:

M CLASSIFICATION	SITE	Serum LDH
M1a-d	Skin/subcutaneous/nodule (a), lung (b) other visceral (c), brain (d)	Not assessed
M1a-d(0)	Skin/subcutaneous/nodule (a), lung (b) other visceral (c), brain (d)	Normal
M1a-d(1)	Skin/subcutaneous/nodule (a), lung (b) other visceral (c), brain (d)	Elevated

ANATOMIC STAGE/PROGNOSTIC GROUPS

Clinical Staging[3]				Pathologic Staging[4]			
Stage 0	Tis	N0	M0	0	Tis	N0	M0
Stage IA	T1a	N0	M0	IA	T1a	N0	M0
Stage IB	T1b	..	..		T1b	..	..
	T2a	..	..	IB	T2a	..	..
Stage IIA	T2b	N0	M0	IIA	T2b	M0	M0
	T3a	..	..		T2a	..	..
Stage IIB	T3b	..	..	IIB	T3b	..	..
	T4a	..	..		T4a	..	..
Stage IIC	T4b	..	..	IIC	T4b	..	..
Stage III	Any T	≥N1	M0	IIIA	T1-2a	N1a	M0
	..	..	..		T1-2a	N2a	..
	..	..	..	IIIB	T0	N1b-c	M0
	..	..	..		T1-2a	N1b-c	..
	..	..	..		T1-2a	N2b	..
	..	..	..		T2b-3a	N1a-2b	..
	..	..	..	IIIC	T0	N2b-c	M0
	..	..	..		T0	N3b-c	..
	..	..	..		T1a-3a	N2c-3c	..
	..	..	..		T3b-4a	Any N	..
	..	..	..		T4b	N1a-2c	..
	..	..	..	IIID	T4b	N3a-c	M0
Stage IV	Any N	Any N	M1	IV	Any T	Any N	M1

Baseline survival after Stage III diagnosis[5]

Conditional, 5-year survival (%)[5]

Stage	Baseline	3y survivors
IIIA	81.4	83.1
IIIB	64.0	76.0
IIIC	44.5	66.7
IIID	9.8	40.6

Notes

[1]Nodes are designated as 'clinically detectable' if they can be palpated on physical exam and are confirmed melanoma by pathology following excision/biopsy.
[2]MSI comprise any satellite, locally recurrent, or in transit lesions.
[3]Clinical staging includes microstaging of the primary melanoma and clinical/radiologic evaluation for metastases. By convention it should be used after complete excision of the primary melanoma with clinical assessment for regional and distant metastases.
[4]Pathologic staging includes microstaging of the primary melanoma and pathologic information about the regional lymph nodes after partial or complete lymphadenectomy. Pathologic Stage 0 and I patients are the exceptions; they do not necessarily require pathologic evaluation of their lymph nodes. Physicians should "discuss and consider" SLNB for patients with T1b Stage IA disease; physicians should "discuss and offer" SLNB for patients with Stage IB disease.
[5]From Haydu et al., Journal of Clinical Oncology, 2017.

Produced following the 8th Ed. AJCC guidlines released January 1, 2017. Contact Dr. M. Gormally (mvgg07@gmail.com) for reprint.

Fig. 1.4 AJCC staging system for melanoma
https://imgur.com/gallery/U2mNP

1.2 Epidemiology of melanoma

Melanoma shows geographical and ethnic variations, the incidence rates of melanoma in Caucasian are 5 times higher than in Hispanics and 20 times higher in African Americans (Rouhani *et al.,* 2008). The incidence and mortality rates of melanoma have increased worldwide in the last 40 years and in USA the incidence has increased 15-fold over the

past (DeSantis *et al*., 2014). Currently, the annual increase in incidence is 3-7% per year within the Caucasian population (Hardy *et al.,* 2008).

In 2019, the American Academy of Dermatology and the National Cancer Institute estimate that there are 1.3 million skin melanoma survivors living in the United States, and 96,480 people will be newly diagnosed (5.6% higher than in 2018). Almost one-half (47%) of melanoma survivors (626,960 men and women) are aged younger than 65 years, including 207,750 survivors who are aged younger than 50 years. Cutaneous melanoma is now the third most common cancer in men and the fifth most common in women in US, with almost 4000 new cases per year (**Figure 1.5**).

Women tend to be diagnosed at a younger age than men (age 60 vs 66 years, respectively), reflecting differences in occupational and recreational exposure to ultraviolet radiation by sex and age. Although melanoma represents less of 5% of cases of skin cancer it counts more of 75% of skin-related deaths (Miller *et al.,* 2019).

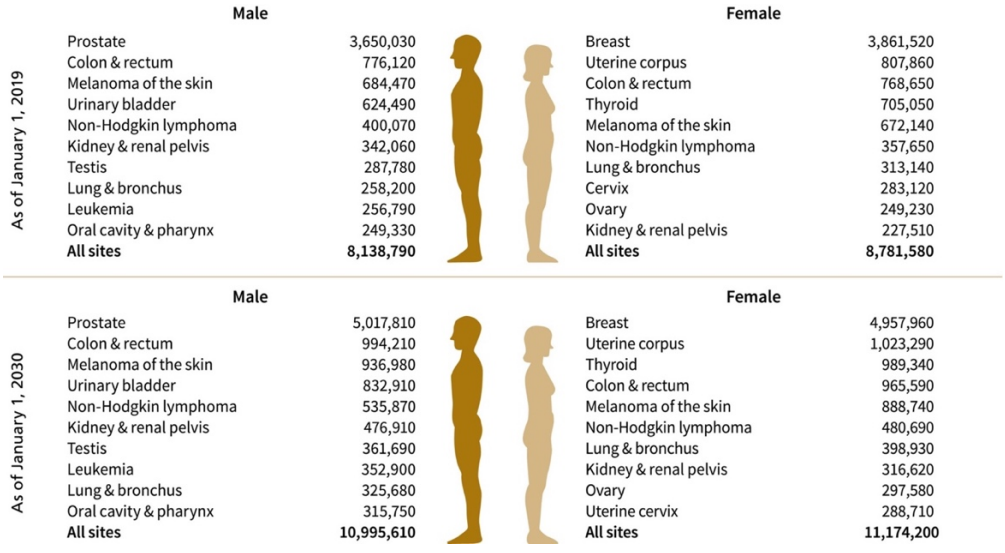


Fig. 1.5 Estimated Number of US Cancer Survivors by Site.
[https://onlinelibrary.wiley.com/doi/full/10.3322/caac.21565]

As a proportion of all cancers, the incidence of melanoma peaks between 20 and 40 years of age and then decreases. It accounted for 11% of all malignant neoplasms in individuals 15 to 29 years of age in the time period 1975 to 2000 and was the second most common type of cancer in this age group (Bleyer *et al.,* 2006). Despite this increase and an overall rise in mortality due to melanoma, the survival rate has improved substantially. As a matter of fact, if the melanoma is diagnostic in the earliest stages, it is treatable and the 5-year and 10-year survival rates for patients are 91.3% and 89.3%, respectively (DeSantis *et al.,* 2014). Roughly 60% of those diagnosed with melanoma in the 1960s died of the disease, compared with just 11% more recently, an improvement attributed mainly to early detection and the improvement of treatments (Marquette *et al.,* 2007).

1.3 Risk factor and prevention of melanoma

Genetic predisposition or an environmental stressor contributes to the genesis of melanoma. The strongest melanoma risk factors are a family history of melanoma, atypical moles or dysplastic nevi (Miller *et al.,* 2006).

Sun sensitivity, immunosuppression, and exposure to ultraviolet radiation are additional risk factors (Miller *et al.,* 2006). In particular, the exposure to ultraviolet light induces mutations in particular genes that affect both the defensive response of the skin to ultraviolet light and the risk of melanoma. (Thompson *et al.,* 2005). Risk factors and relative risk for melanoma were shown in **Table 1** (Rubin *et al.,* 2009).

Ultraviolet radiation has multiple effects on the skin: causes genetic changes, reduces cutaneous immune function, increases the local production of growth factors, and induces the formation of DNA-damaging reactive oxygen species that affect keratinocytes and melanocytes (Gilcherest *et al.,* 1999).

If DNA damage remains unrepaired, the cell undergoes irreversible permanent genetic mutations, enabling the cell with the ability for autonomous growth and acquiring a tumor phenotype (Chau *et al.,* 2018).

Table 1: Risk Factors for Melanoma
○ **Uv exposure**
○ **Number of moles (both normal and atypical**
○ **Fair skin, freckling, light hair**
○ **Family history of melanoma**
○ **Personal history of melanoma**
○ **Immunosuppression**
○ **Age: Risk increases with age**
○ **Gender: Males > femals**
○ **Xeroderma pigmentosum**

Information from the American Cancer Society, 2009

Table. 1 Risk factors for melanoma
https://www.cancernetwork.com/sites/default/files/cn_import/1435084.png

Clinically, variations in pigmentation and the protective response to ultraviolet light are associated with variations in susceptibility to melanoma. Endogenous factors, like specific phenotypic characteristics have been shown to correlate with increased risk of melanoma such as green/blue eye color, red/blond hair color, fair skin, the presence of freckles and inability to tan. (Pho *et al.,* 2006).

Exogenous factors are exposing to natural sunlight or artificial sunlight and having a history of many blistering sunburns, especially as a child or teenager. Risk factors and relative risk for melanoma were shown in **Table 2** (Thompson *et al.,* 2005).

Table 2: Phenotype/Genetic Risk Factors	Relative Risk for Melanoma
Genetic	
Strong family history (≥3 first degree relatives affected)	35-70
Weak family history	3
Naevi	
Multiple benign naevi (>100)	11
Multiple atypical naevi	11
Previous skin cancer	
Previous melanoma	8.5
Previous non-melanoma skin cancer	2.9
Immunosuppression	
Transplant recipients	3
Patients with AIDS	1.5
Surrogates of sun sensitivity	
Type I skin (burns without tanning)	1.7
Freckling	2.5
Blue eyes	1.6
Red hair	2.4
UV exposure	
History of blistering sunburn	2.5

Table. 2 Phenotype/Genetic Risk Factors (Thompson, *et al*., 2005)

The most effective method to prevent melanoma is avoiding too much sun exposure. Besides, it is very important for the people has moles, watch them closely with regular exams and remove some of them if they have certain features that suggest they might change into a melanoma (Weyers *et al.,* 2018).

1.4 Current therapeutic approaches

Currently, there is not a "gold therapy" for melanoma and the treatment options vary, depending on the extent of disease and risk of recurrence, as well as, patient characteristics such as age, comorbidity, and personal preferences.

Actually, there are mainly five therapeutic options for melanoma: surgical treatments, radiation therapy, chemotherapy, molecular targeted treatments, and biological therapy, but the surgery is the primary treatment for most melanomas. Melanoma remains unique among solid tumors in that its treatment primarily is surgical. Radiation is only of limited benefit, and chemotherapy has been disappointing in both the adjuvant and metastatic settings (Siegel *et al.,* 2019).

Early treatment of melanoma is essential; removal of the non-disseminated primary lesion is usually curative, whereas the median survival rate for patients with disseminated disease is 8.1 months, with only approximately 2% surviving for 5 years (Lee *et al.,* 2000).

Radiation therapy is using high energy X-rays or other types of radiation to kill cancer cells and reduces tumors, it can be used to help the decreasing of big tumors and reducing symptoms. Radiation therapy is used in 1–6% of patients with melanoma in the US and usually use it on inoperable disease, that is when the tumor is localized in the head and neck region. It is commonly used as adjuvant therapy or as palliative therapy to improve local control or in patients with desmoplastic neurotropic melanoma (Pirard *et al.,* 2004).

In the last 30 years, many drugs and combinations of drugs have introduced in melanoma therapy to improve patient survival (**Figure 1.6**). Between 1976 and 2011, dacarbazine chemotherapy (1976) and high-dose IL-2 (1998) with or without cisplatin or its derivate, were the only approved agents for treatment of metastatic melanoma, but these therapies have shown modest response rates of less than 20%. The number of approved agents more

than tripled in the last 3 years with the approvals of ipilimumab (antibody to CTLA-4) and vemurafenib (BRAF inhibitor) in 2011, dabrafenib (BRAF inhibitor) and trametinib (MEK inhibitor) in 2013, and pembrolizumab (antibody to PD-1) in 2014 (Li *et al.,* 2017). These new therapies constitute the most advanced forms of immuno and target therapy.

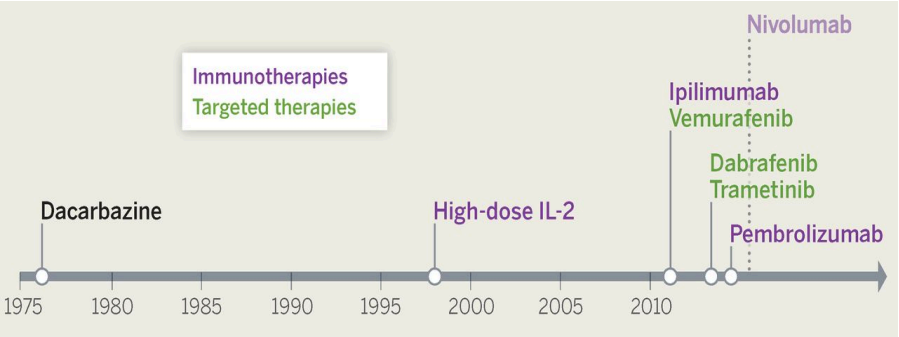


Fig. 1.6 Timeline of FDA regulatory approval for metastatic melanoma.
Targeted therapies are labeled in green; immunotherapies are labeled in purple.
https://science.sciencemag.org/content/346/6212/945/F3

The importance of immune responses in melanoma has long been appreciated, with reports of spontaneous melanoma regressions published more than 50 years ago (Cole *at al*., 1956). The first immune-based therapy FDA-approved for advanced melanoma in 1998 was IL-2, an immune cytokine that activates cytotoxic T cell activation and promotes their proliferation. The use of high-dose IL-2 showing long-term, durable complete responses in previously treated patients with metastatic melanoma (Li *et al.,* 2017).

However, its use is limited by high toxicity due to anaphylactic shock and development of infectious granulomas at sites of injection and low response rates. To reduce systemic toxicity and increase local therapeutic effects, it was introduced intra-lesional interleukin-2 for the treatment of cutaneous melanoma (Oberle *et al.,* 2014).

The most successful immunotherapy approach has been "immune checkpoints" inhibition. This therapy is based on the fact that T lymphocytes are critical to antitumor immunity. Two of the best-studied checkpoints involve cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed cell death-1 (PD-1), two co-inhibitory T cell receptors that mediate immune tolerance (Sharpe *et al.,* 2009).

Ipilimumab, CTLA-4 blocking monoclonal antibody, was the first treatment to increase survival in advanced melanoma and was approved by the FDA in 2011 (**Figure 1.7**). Although ipilimumab responses were limited to 11% of patients in the phase III trial, many of these responses were durable (Hodi *et al.,* 2010).

Monoclonal antibodies that antagonize PD-1 or PD-L1 have been even more impressive in clinical studies, with higher response rates and fewer autoimmune toxicities (Topalian *et al.,* 2012, Hamid *et al.,* 2013).

The PD-1–blocking antibody pembrolizumab won FDA approval in 2014, and approval of another PD-1 antibody, nivolumab, is expected in the near future. With the successes of these new checkpoint inhibitors, in the last years, the scientists have accelerated and expanded their study on other hypotheticals immunological targets inducing progress in immunotherapy (Li *et al.,* 2017).

Concurrent advances in targeted molecular therapy have also improved the treatment and prognosis of a subset of advanced melanoma patients. Approval of single substances directed against mutated proteins has dramatically changed the options available in melanoma therapy.

About half of all melanomas have mutations in BRAF gene. Melanoma cells with these changes make an altered BRAF protein that helps them grow, for this reason, some drugs target BRAF and related proteins, such as the MEK proteins (Manzano *et al.,* 2016).

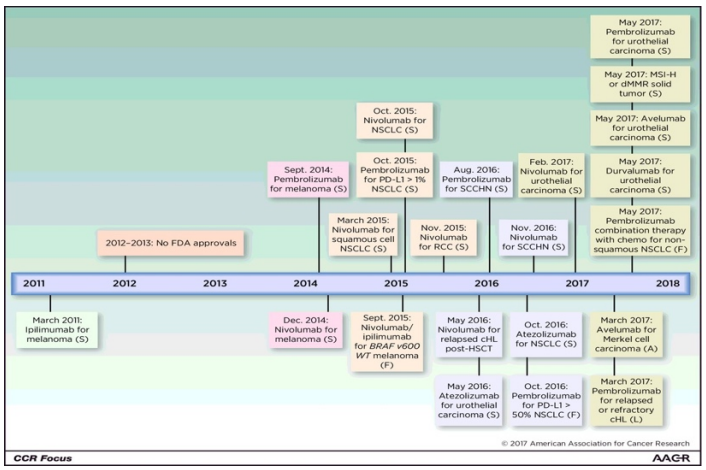


Fig. 1.7 FDA approval timeline of immune checkpoint inhibitors in advanced/metastatic malignancies - https://www.fda.gov/drugs

Vemurafenib (Zelboraf), dabrafenib (Tafinlar), and encorafenib (Braftovi) are drugs attack the BRAF protein directly. These drugs reduce or slow the growth of tumors in some people with metastatic melanoma have a BRAF gene change. They can also help some patients live longer. (Manzano *et al.,* 2016).

The MEK gene works together with the BRAF gene, so drugs that block MEK proteins can also help treat melanomas with BRAF gene changes. MEK inhibitors include trametinib (Mekinist), cobimetinib (Cotellic), and binimetinib (Mektovi**)** (Morris *et al.,* 2013).

Trametinib/Mekinist (GSK1120212) is a non-competitive MEK1/2 inhibitor and it was FDA-approved in 2013 for the treatment of adult patients with the BRAF V600E/K mutation and unresectable or metastatic melanoma (Wright *et al.,* 2013).

Others targets are ERK1/2, they are the kinases below MEK in the MAPK cascade. Preclinical evidence suggests that ERK inhibition is effective in BRAF inhibitor-resistant melanoma with common MAPK-reactivating resistance drivers (Carlino *et al.,* 2013). Several other targets exist in BRAF-mutant melanoma, ranging from growth factors in

the tumor microenvironment, receptor tyrosine kinases at the cell surface, epigenetic regulators, and apoptosis regulators. It has been found that in 10–20% of cases that develop early resistance, or are intrinsically resistant to the MAPK inhibition, there is a loss of PTEN, or mutations in PI3K or AKT (Manzano *et al*., 2016).

Drugs that target the BRAF protein, with the name of BRAF inhibitors, or the MEK proteins, with the name MEK inhibitors, aren't likely to work on melanomas that have a normal BRAF gene.

CHAPTER 2

2.1 Molecular pathway activated in melanoma

Cell signaling pathways regulate cell growth and death, cell metabolism, migration and angiogenesis. In cancer progression, there is a loss of control of signaling events that allows cancer cells to acquire specific phenotypes, such as the ability to resist to apoptosis, abnormal proliferation, angiogenesis, and invasion.

Recent studies have focused on various dysfunctional signaling pathways, to identify key factors involved in the induction and progression of melanoma. Indeed, melanoma is commonly divided into three mutually exclusive genetic subsets: BRAF mutant melanoma, NRAS mutant melanoma and melanoma of wild type at both loci; over 50% of melanomas have mutations in the BRAF gene (Gordon *et al*., 2005). These mutations cause the gene to make an altered BRAF protein that signals the melanoma cells to grow and divide quickly. The most common BRAF mutation (nearly, 90% of cases) is the T1799A point mutation in exon 15 within the kinase domain, in which a T-A transversion converts glutamic acid for valine at the 600 position of the amino acid sequence (BRAFV600E) and constitutively activates the protein. Oncogenic activation of BRAF act in the RAS–RAF–MEK–ERK mitogen-activated protein kinase (MAPK) pathway stimulating cyclin D1-mediated cell cycle progression, (Palmieri *et al.,* 2009).

The mitogen-activated protein kinase (MAPK) pathway regulates many physiological processes, including mitosis, gene expression, metabolism and apoptosis. Under normal physiological conditions, growth factors bound their tyrosine kinases receptors and activate a small GTP binding protein RAS that existing in three isoforms: ARAF, BRAF and CRAF in humans on cell membranes, which triggers intracellular signalling inside the cell. Mutational activation of BRAF, common in human melanomas, has been also associated with an enhanced IKK activity and the activation of NF-κB transcriptional

activity resulting in increased survival of melanoma cells. Moreover, constitutively active ERK may indirectly activate NF-κB through the upregulation of inflammatory cytokines such as TNF-α and IL-1α/β, as well as chemokines, that activate NF-κB (Li *et al.,* 2002, Castelli *et al.,* 1994). However, activating mutations in BRAF alone is not sufficient to cause melanoma. Indeed, additional genetic alterations in BRAF-mutant cells are required to elicit a fully cancerous phenotype. (Shtivelman *et al.,* 2014). Interestingly, melanomas that possess wild-type BRAF typically have mutations in key genes encoding upstream proteins of the MAPK pathway, including NRAS, KIT, GNAQ, and GNA11 (Abildgaard *et al.,* 2015).

Mutations on NRAS is present in 15% across all types of melanoma, other mutation could be on MEK1, MEK2 as well as in c-KIT. The latter has been shown to be amplified or mutated in some cases of melanomas and in particular, the prevalence is closer to 1% in the Caucasian population. Activation of this tyrosine kinase results in the stimulation of the MAPK and PI3K-AKT pathways, producing both proliferative and survival advantages (Madonna *et al.,* 2012).

One genetic lesion that cooperates with activating BRAF mutations and represents another high-penetrance genetic alteration in melanoma, is the loss or inactivating mutation of the cyclin-dependent kinase inhibitor 2 A (CDKN2A) locus. This locus is located on chromosome 9p21 and encodes two tumor suppressor proteins, $p16^{INK4A}$ and $p14^{ARF}$ (differing in post-translational modifications), which both function to arrest the cell cycle (Liu *et al.,* 2014).

Another genetic lesion associated with melanoma is amplification or mutations in CDK4, which is associated with a small number of cases of familial melanoma (Soufir *et al.,* 1998).

CDK4 is located on chromosome 12q13 and is the binding partner of $p16^{INK4a}$. Mutations of CDK4 are often found in its binding domain, making this protein incapable of binding to functional $p16^{INK4A}$. Indeed, the CDK4 pathway is dysregulated in most melanomas as a result of hyper-activation of ERK or loss of $p16^{INK4A}$. Both high-penetrance genes, CDKN2A and CDK4, are involved in cell cycle control, as both mutations, which affect $p16^{INK4A}$ and CDK4, respectively, disturb the G_1/S-phase checkpoint (**Figure 2.1**) (Paluncic *et al.,* 2016).

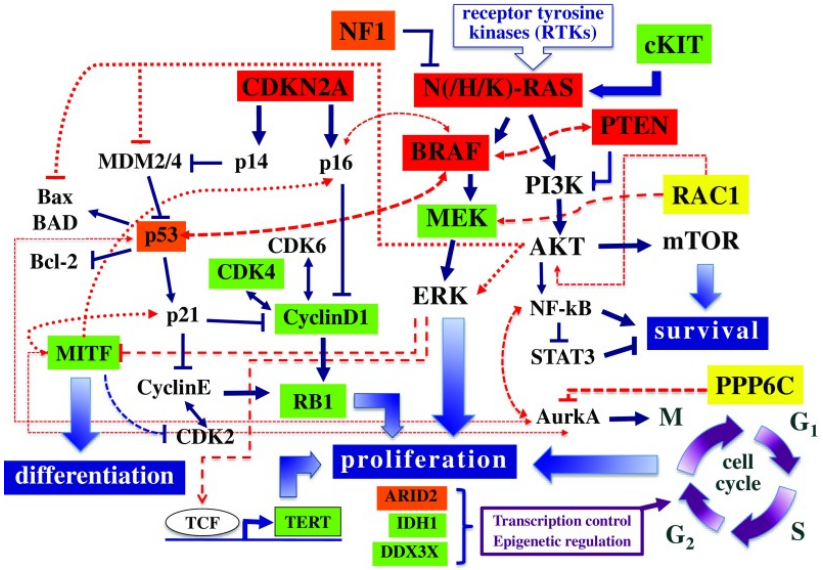


Fig. 2.1 Signal transduction pathways involved in melanomagenesis.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6153571/figure/Fig1/

2.2 NF-κB pathway

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) represents a group of homo- or heterodimeric transcription factors that regulate the transcription of several genes involved in various critical pathways. It was identified more than 20 years ago as a regulator of B cell-specific gene expression. Recently, studies have highlighted that it is a member of the structurally-related eukaryotic transcription factors family NF-κB/Rel that regulates the expression of many inducible genes involved in the control of a large number of normal cellular and organismal processes (Tian *et al.,* 2003).

NF-κB protein usually is a dimer consisting of P50-P65 dimer (NF-κB1/RelA). The dimer formation is necessary for DNA binding, two NF-κB monomers bind to DNA as a dimer. The N-terminal regions of dimer are responsible for specific DNA contact. The C-terminal regions are usually highly conserved, they are responsible for dimerization and nonspecific DNA phosphate contact. (**Figure 2.2**).

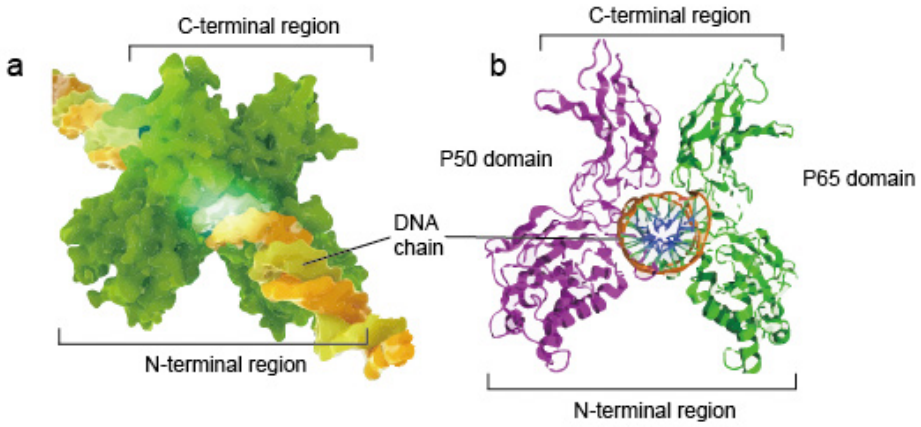


Figure 2.2 The structure of NF-κB protein dimer binding with DNA chain.

In mammals NF-κB transcription factor family consists of 5 proteins: p65/RelA, RelB, c-Rel, p105/p50 (NF-κB1), and p100/p52 (NF-κB2), that associated together forming distinct transcriptionally active homo/heterodimeric complexes (**Figure 2.3**). NF-κB proteins share an approximately 300 amino acid N-terminal domain called Rel homology (RH) domain containing important sequences for binding DNA or inhibitor of NF-κB (Iκ B) as well as sites of dimerization. However, they differ in their C-terminal domain in that RelA, RelB and c-Rel exhibit transactivating functions, while p100 and p105 contain inhibitory domains. In fact, NF-κB1/p105 and NF-κB2/p100 are the inactive precursors of the p50 and p52 proteins, respectively.

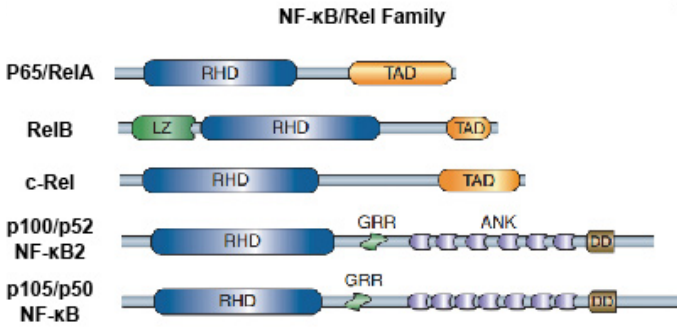


Figure 2.3. The 5 proteins and their homo/heterodimeric complexes of NF-κB family.

In an unstimulated state, these proteins are localized into the cytoplasm. Proteolytic processing removes the C-terminal inhibitory domains, allowing the resulting proteins to enter the nucleus. p50 and p52 usually form homodimers or heterodimers with one of the three proteins that has a transactivation domain. NF-κB proteins are sequestered in the cytoplasmic compartment with inhibitory proteins called IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha).

NF-κB activation is the result of a variety of different stimuli, primarily inflammatory stimuli, as well as proinflammatory cytokines (IL-1 and TNF-α) and several extracellular stimuli (viral and bacterial infections, oxidative and DNA-damaging agents, UV light and osmotic shock) (Ghosh *et al.,* 2002).

This activation leads to activation of the IκB kinase (IKK) complex. IKK activated phosphorylates IκB proteins that are recognized by the ubiquitin ligase machinery, leading to their poly-ubiquitination and subsequent degradation by the proteasome 26S. During this process, also p65 is phosphorylated and p100 and p105 are cleaved into active forms p52 and p50 respectively. These events generate active NF-κB complex that is free to translocate into the nucleus where it binds to specific sequences in the promoters of target genes, inducing transcription (Senftleben *et al.,* 2001).

In melanoma cells, some components of NF-κB family, such as p50 and p65/RelA proteins, are overexpressed in nuclei of dysplastic nevi and melanoma cells compared to those of normal nevi and healthy melanocytes, respectively (McNulty *et al.,* 2004).

These phenomena are more closely associated with an increased activity of IKK, resulting in more rapid degradation of IκB, nuclear localization of NF- κB, and enhanced trans-activating capacity of the NF-κB complex. In fact, the equilibrium between Iκ-Bα degradation and re-synthesis has been found altered in Hs294T melanoma cells, with a shift of the process toward the first event (degradation) leading to constitutive nuclear translocation and activation of NF-κB (Shattuck-Brandt *et al.,* 1997).

The translocation of NF-κB into the nuclei start the transcription of immunoregulatory and inflammatory genes for cytokines and chemokines, acute phase proteins, cell adhesion proteins, inducible nitric oxide synthase (iNOS), immunoglobulins, viral and anti-apoptotic proteins, tumor necrosis factor receptor-associated factor 1 and 2 (TRAF1 and TRAF2), inhibitor-of-apoptosis protein 1 and 2 (c-IAP1 and c-IAP2), caspase8/FADD, c-FLIP, members of BCL2 family, proteins that positively regulate cell cycle progression (cyclin D1, c-myc, MMP9) and negative regulators of NF-κB (Hayden *et al.,* 2004). NF-κB proteins are, therefore, key regulators, directly and indirectly, of innate and adaptive immune responses, able to controls inflammation, cancer cell proliferation and survival, accelerating cell proliferation, inhibiting apoptosis, promoting cell migration and invasion, and stimulating angiogenesis and metastasis (**Figure 2.4**) (Karin *et al.*, 2015).

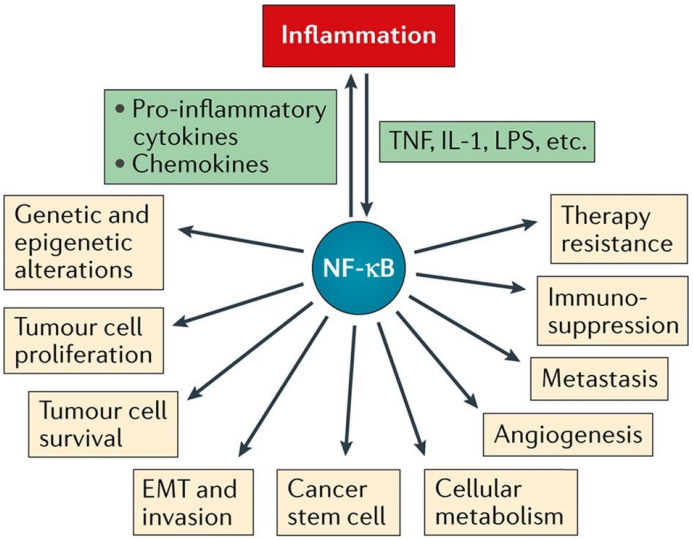


Fig 2.4 Roles of NF-κB in cancer
https://www.nature.com/articles/nri.2017.142

Furthermore, it able to promote epithelial-to-mesenchymal transition (EMT), genetic and epigenetic alterations, cancer stem cell formation, and therapy resistance (Tang and Wang 2016).

Many news findings suggest that inhibition of NF-κB can be used as a strategy to effectively interfere with the pathogenesis and/or progression of melanoma. However, the multiplicity of actions of NF-κB could have the drawback that this type of therapy might entail disadvantageous effects: a reduction of the NF-κB activation could lead to an impairment of either the migration of natural killer (NK) cells or the concentration of the tumor-infiltrating lymphocytes (TILS) and dendritic cells into the developing tumor. All these effects might reduce or abolish the immune response against the tumor.

Recent studies with substances that inhibit the activity of NF-κB have hypothesized that these unwanted biological effects in tumor tissues may be apparently overcome by a much stronger enhancement of apoptosis into the tumor cells (Baldwin *et al.*, 2001).

2.3 Oxidative stress and cancer

Life is unimaginable without oxygen and the production of reactive oxygen species (ROS) is a continual process in the human body. ROS are the byproducts generated from the metabolism of molecular O_2, and include superoxide (O_2^-) and hydroxyl radical (HO^-) and non-radical hydrogen peroxide (H_2O_2). The organelle where most of ROS is generated in mitochondria, during ATP synthesis through oxidative phosphorylation. One or two, instead of four, electron reductions lead towards the formation of O_2^- or H_2O_2 which later transformed into others ROS (Mao *et al*., 2014, Kumar *et al.,* 2017). In the mitochondria, cytochrome P450 and peroxisomes are the major endogenous factors leading to reactive oxygen species (ROS) and reactive nitrogen species (RNS) formation. Other exogenous factors, such as radiation, tobacco smoking, chemotherapy, and diet, are also important inductors of free radical production (Bhattacharyya *et al.,* 2014). Noteworthy it is now clear that intermediate reactive species, that are naturally produced under physiological conditions, have a crucial role in metabolic regulation, the cell cycle and intracellular signaling pathways (**Figure 2.5**) (Nathan *et al.,* 2003).

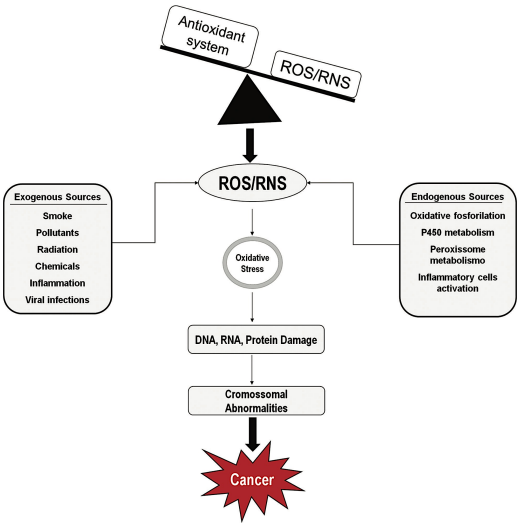


Fig 2.5 Redox system is involved in the cancer progression
http://www.scielo.br/img/revistas/clin/v73s1//1807-5932-clin-73-e548s-gf01.jpg

However, an increased level of ROS is harmful to the cells and can initiate various disease processes. A homeostasis of ROS in the human body is maintained by several antioxidant systems working simultaneously (Rahal *et al.,*2014).

There are major antioxidant systems working in the human body such as catalases, thioredoxin system, glutathione peroxidases, peroxiredoxins, eosinophil peroxidases, and myeloperoxidases. An imbalance in this *in vivo* homeostasis is defined as 'oxidative stress'. The generation of oxidative stress is dependent on the available molecular oxygen in the cell, physical or chemical external stimuli and type of the cell/tissue or organ (Sies *et al.,* 2017). Oxidative stress can initiate and maintain the progression of cancer in multiple ways, causing DNA damage and increasing the mutagenicity (Gupta *et al.,* 2014), promoting cell survival and proliferation by increasing transcriptional activity (Sies *et al.,* 2017), exerting pro-survival functions by activating ERK/MEK and PI3K/AKT signal transduction pathways (Oh *et al.,* 2017), and enhancing invasiveness and metastasis (Auyeung *et al.,* 2017).

Master regulators of oxidative stress are responsible for the control stimulation/inhibition of redox signaling cycles and for the modulation of redox sensing systems. Molecular redox switches, which act as master regulators and control diverse biological activities, involve mainly NF-κB pathway.

2.4 Inflammation and carcinogenesis

Inflammation is a primary defense process against various extracellular stimuli, such as viruses, pathogens, foods and environmental pollutants. When cells respond to stimuli for short periods of time, it results in acute or physiological inflammation. On the other hand, if the stimulation is prolonged or if a pathological state occurs, it is known as chronic or

pathological inflammation (Hong *et al.,* 2010). Under persistent infection or injury, chronic inflammation drives the transformation of cancer-originating cells by producing reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) that are capable of inducing DNA damage and genomic instability (Khansari *et al.,* 2009, Reuter *et al.,* 2010). In addition, tumor-infiltrating myeloid and lymphoid cells produce cytokines that signal to transformed cells and support their growth and survival. These pro-tumorigenic cytokines include IL-6, IL-11, IL-21 and IL-22 that activate the STAT3 transcription factor; TNFα, IL-1 and IL-18 that activate NF-κB; and the IL-23 to IL-17 axis of inflammation that activates both STAT3 and NF-κB in tumor cells (Wang *et al.,* 2015, Elinav *et al.,* 2013) (**Figure 2.6**).

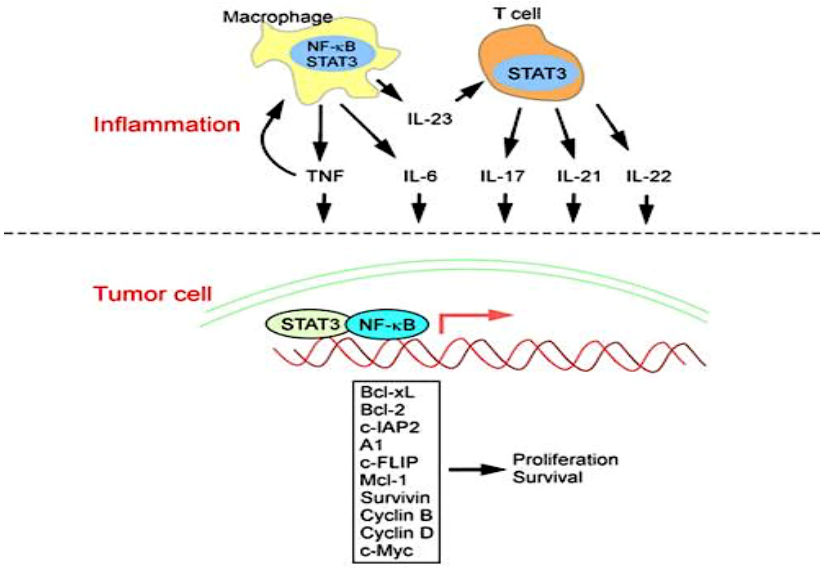


Fig 2.6 Inflammation promotes tumor growth and survival
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4911907/

The idea that inflammation and cancer may be linked is not entirely new, in 1863 Rudolf Virchow understood that chronic irritation and inflammation cause cancer (Balkwill *et al.,* 2001, Coussens *et al.,* 2002).

The importance of inflammation in the onset of cancer and the mechanisms through which it exerts its pro-tumorigenic effects were not fully appreciated and understood until the 1990s, when many studies, using molecular biology techniques and genetically modified mice, revealed the importance of inflammatory cells, cytokines, chemokines and growth factors in cancer-related inflammation (Balkwill *et al.,* 2001, Coussens *et al.,* 2002). Persistent infections and chronic inflammation are estimated to be associated with at least 15–20% of cancer deaths worldwide (Plummer *et al.,* 2016, Grivennikov *et al.,* 2010), and obesity-associated inflammation may be responsible for another 15% of the death toll of cancer (Thun *et al.,* 2003).

Inflammation can also be induced after tumor initiation, owing to the necrotic death of cancer cells subject to an insufficient blood supply or microbial invasion into the tumor bed caused by barrier deterioration. Such 'tumor-elicited inflammation' appears to be a key driver of malignant progression in most solid malignancies. Chronic inflammation can also augment tumor development and progression by triggering immunosuppression and compromising anticancer immunity (Shalapour *et al.,* 2015).

Correspondingly, the molecular mechanisms that connect inflammation to tumorigenesis have become a major branch of cancer research (Hanahan *et al.,* 2011). It has also become apparent that the tumorigenic function of inflammation is a grotesque manifestation of its essential role in tissue regeneration and repair (Karin *et al.,* 2016).

CHAPTER 3

3.1 Phytochemicals, redox modulation and inflammation

Phytochemicals (from a Greek *phyto*, meaning plant) are secondary metabolites, produced by plants and herbal products. They are produced as a response to external stimuli and play an important role in plant growth or defense against competitors, pathogens, or predators. They include a diverse range of entities such as alkaloids, flavonoids, coumarins, glycosides, gums, polysaccharides, phenols, tannins, terpenes, terpenoids and vitamins (**Figure 3.1**) (Venkatalakshmi *et al.,* 2016).

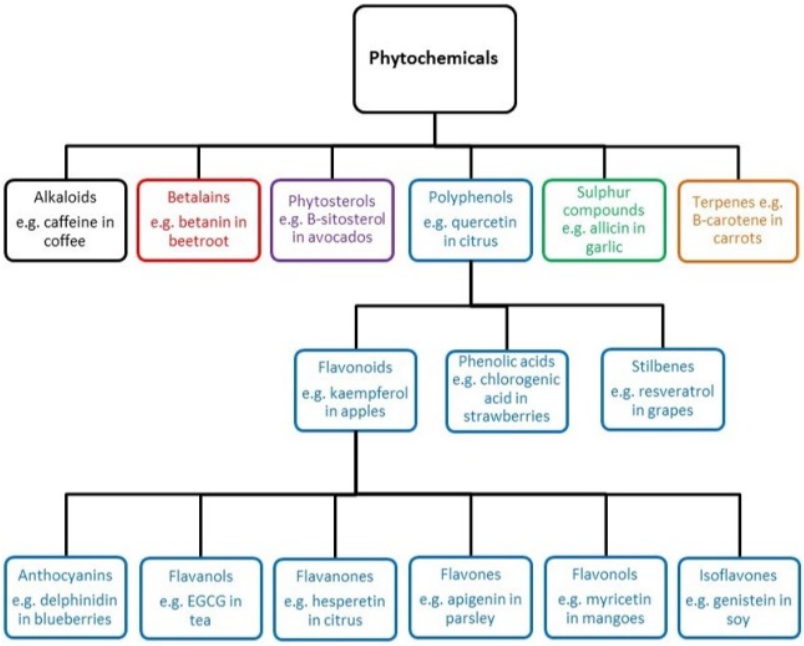


Fig 3.1 Classification of Phytochemicals

Dietary phytochemicals often reported in literature as "nutraceutical", have been studied for their health benefits for a long time, and for the prevention and treatment of pathologies like metabolic syndrome, obesity, cardiovascular diseases, cancer, and neurodegenerative diseases (Winter *et al*., 2017). They exhibit a wide range of protective roles and in particular, a large number of studies emphasize their antioxidant and anti-inflammatory properties, able to function as redox modulators, protecting the cells from

stress oxidative (Alrawaiq *et al*., 2014). They show a dual behavior both anti-oxidant that pro-oxidant, depending upon microenvironment and concentration of ROS already present within cells. Indeed, in normal cells that have a nominal quantity of ROS, the phytochemicals exert antioxidant activity, but when the imbalance between ROS production and antioxidant defenses brings to oxidative stress conditions (accumulation of ROS), the phytochemicals acts as pro-oxidants, increasing the amount of ROS to lethal levels. (Arulselvan *et al*., 2016). In cancer cells, already there are high levels of ROS (particularly H_2O_2), so these cells are already adapted to live under these conditions, but the phytochemicals act on this balance further increasing the levels of stress oxidative and pushing the cells to death. This dual role of plant constituents reflects their utilization in cancer chemoprevention. Chemoprevention is defined as the use of natural or synthetic compounds able to inhibit, suppress, reverse, or delay the development and progression of cancer (Tong *et al.,* 2014). It has been estimated that more than two-thirds of human cancers could be prevented through modification of the lifestyle, with special attention to diet. As a matter of fact, high consumption of fruits and vegetables is associated with a reduced risk of colon cancer (Liang *et al.,* 2009), prostate cancer (Kirsh *et al.,* 2007), and esophageal cancer (Terry *et al*., 2001). The chemopreventive action of the phytochemicals can be exerted at different stages of carcinogenesis, dividing it in primary prevention, referring to inhibiting the formation or facilitating the repair of mutagenic molecular species in normal tissue, secondary where it intervenes in the progression of premalignant cells by slowing, blocking, or reversing their conversion to melanoma, last but not least, tertiary prevention refers to preventing tumorigenic recurrence in patients with treated disease (Keith *et al*., 2018, Surh *et al*., 2003). It is well known that ROS represents physiologic activators of transcription factors, such as Activator Protein-1 (AP-1) or NF-κB, which in turn are able to modulate the transcription of pro-

inflammatory cytokines such as TNF-α, IL-6, IL-8, and IL-1 (Nordberg *et al*., 2001). In fact, ROS, acting as an intracellular signaling component, is associated with inflammatory response and with autoimmune diseases (**Figure 3.2**) (Phull *et al*., 2018). Therefore, the use of natural products with antioxidant and anti-inflammatory activity represents an intriguing strategy for future clinical applications.

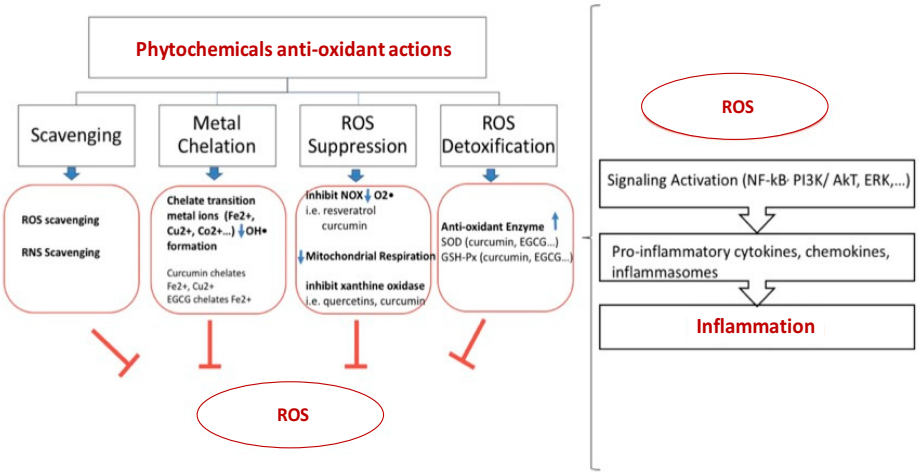


Figure 3.2 Key Phytochemivals anti-oxidant actions in relation to anti-inflammation https://www.mdpi.com/2072-6643/10/11/1618#

The use of natural products with antioxidant and anti-inflammatory activity represents, then, an intriguing strategy for future clinical applications.

In particular, phytochemicals showed to able to chelate metal ions, like Fe^{2+} and Cu^{2+}, (Heim *et al*., 2002) and free radicals, leading to a reduction of them and inhibiting multiple enzymes responsible of ROS generation (Mishra *et al.*, 2012). Phytochemicals can also attenuate the mitochondrial ATP synthesis by blocking the mitochondrial respiratory chain and ATPase, affect the activity of cyclooxygenase, lipoxygenase, and NOS (nitric oxide synthase), can also restrain LPS-induced iNOS gene expression in cultured macrophages. They can decrease oxidative damage and finally, they may act by upregulating endogenous antioxidant enzymes (Sarkar *et al*., 2001).

In the last years many phytochemicals were identified to have these properties, for example, curcumin is able to chelate transition metal (Cu^{2+} and Fe^{2+}) ions, EGCG and quercetin chelate Fe^{2+} (Heim *et al*., 2002), while other phytochemicals, like apocynin, resveratrol, and curcumin can inhibit NOX (NADPH oxidase) causing a reduction in the generation of O_2 during infections consecutively in endothelial cells in THP1-monocytes (Deby-Dupont *et al*., 2005; Petrônio *et al*., 2013).

Additionally, curcumin (Shen *et al*., 2009), phenolic acids (Schmidt *et al*., 2009), capsaicin (Nguyen *et al*., 2012) quercetins (Bräunlich *et al*., 2013), anthocyanins (Bräunlich *et al*., 2013), and resveratrol analogs (Huanget *et al.*, 2008) inhibit xanthine oxidase, a type of enzyme that generates reactive oxygen species.

3.2 Natural phytochemicals and Melanoma

Skin cancer is a devastating disease; therefore, novel protective and adjuvant treatment methods are needed to improve its prognosis. The current therapies, especially for malignant melanoma, have relatively low success rates. Therefore, there is an urgent need to develop new remedies that are both safe and effective (Furman-Toczek *et al.,* 2016)

In the last years, medicinal plants and their bioactive compounds have been tested on many skin cancer cell lines and animal models and have shown promising anti-skin cancer results by inhibiting angiogenesis, metastasis, proliferation, induces apoptosis and arrest cell cycle (**Figure 3.3**) (Javed *et al.,* 2019).

Numerous diet-derived phytochemicals such as epigallocatechin-3-gallate, resveratrol, curcumin, proanthocyanidins, silymarin, apigenin, capsaicin, genistein, indole-3-carbinol, and luteolin have shown promising anti-inflammatory and/or anti-cancer effects in melanoma due to their antioxidant property (Ombra *et al.,* 2019). Also, most of these

molecules are safe, widely available, highly-tolerable, and cost-effective which makes them ideal agents to improve cancer chemoprevention and treatment.

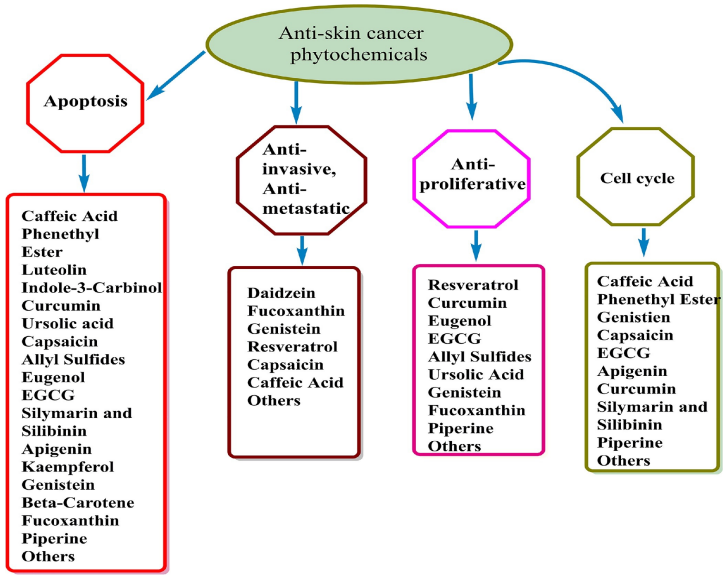


Fig 3.3 Chemotherapeutic effects of natural phytochemicals on different molecular processes. https://www.sciencedirect.com/science/article/pii/S0753332218338538#fig0010

Detailed information about these phytochemicals, their sources and molecular processes that are regulated by these phytochemicals are given in **Figure 3.4**, where it is possible to see phytochemicals that down-regulate or inhibit the expression of different proteins, enzymes and signaling pathways to either prevent or treat skin cancer (Soldati *et al.*, 2018)

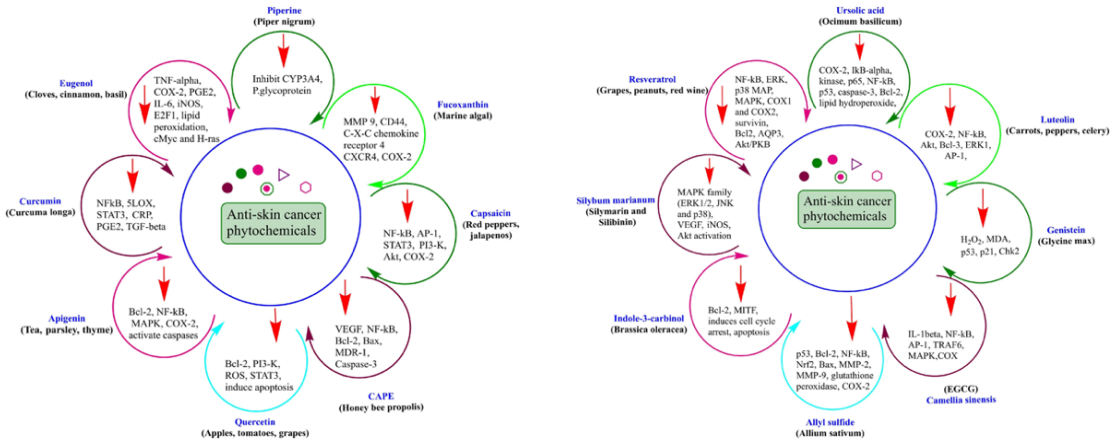


Fig 3.4 chemotherapeutic of different phytochemicals against skin cancer. https://www.sciencedirect.com/science/article/pii/S0753332218338538#fig0010

Evidence has indicated that the anti-carcinogenic properties of phytochemicals are due to their anti-oxidative, anti-inflammatory, anti-proliferative, and anti-angiogenic effects (Chau *et al.,* 2018).

Moreover, they can regulate various transcription factors including nuclear factor erythroid 2-related factor 2 (Nrf2), NF-κB, β catenin and TGF-β (Russo *et al.,* 2007).

3.4 *Opuntia Ficus Indica* (L.) Miller

Plants with beneficial properties are known in traditional medicine. Nowadays, in spite of the widespread availability of synthetic compounds, the search goes towards natural compounds to lower cost and few side effects. The increasing interest in preventive medicine encourages the use of nutraceuticals, bioactive compounds of vegetable origin with important nutritional values.

In recent years various studies have been published on the beneficial properties of prickly pear cactus, (*Opuntia ficus indica)*. The plant is characterized by its remarkable adaptation to arid and semi-arid climates in tropical and subtropical regions of the globe like as North Africa, America, Asia, Oceania and the entire Mediterranean basin, including Sicily (Aragona *et al.,* 2018).

Opuntia ficus indica, commonly referred to as prickly pear, is a dicotyledonous angiosperm plant and belonging to the Cactaceae family (Family Cactaceae, subfamily Opuntiodeae, Genus Opuntia, subgenus Platyopuntia, species *Opuntia ficus-indica* (L.) Miller). Main varieties of *Opuntia ficus -indica* (L.) Miller are: Gialla di Sarroch, Gialla di Bonacardo, Bianca di Macomer, Gialla di Ozieri, Bianca di Bronte, Rossa di Castelsardo, Hybrid Gialla x Rossa, Nopalea cochenillifera Salm-Dick (Beccaro *et al.,*. 2015). Different parts of *Opuntia ficus -indica* (L.) Miller are fruit, cladodes and flowers

which have been historically used as food for human and farm animals and in folk medicine, thanks to their nutritional properties and beneficial activities (Pèrez-Torrero *et al.,* 2017.) The plants can grow up to 3–5 m height, with thick, succulent and oblong to spatulate stems called cladodes with a size of 30-40 cm of length per 15-25 cm of width and 1,5-3,0 cm of thickness.

The cladodes ensure the chlorophyll photosynthesis and replace the functions normally ascribable to the leaves, but only in the first years of life: in fact, from the fourth year onwards, the basal cladodes lignify, losing photosynthetic activity and acquiring a purely structural role (**Figure 3.5**).

Fig. 3.5 *Opuntia ficus indica* plant

Cladodes are covered with a waxy cuticle that limits transpiration and it is a barrier against predators. The real leaves have a conical shape and are just a few millimeters long. They appear on young cladodes and are ephemeral. The leaves, before falling, are gathered in "tufts", at the base of which there are particular structures, called areoles, which are nothing but modified leaf axils.

Two types of spines develop from the tissue that makes up the areolas: the glochids and the actual spines. The glochids are thin thorns some millimeters long, of brownish color,

which are easily detached, and being equipped with tiny hook-shaped scales, they are solidly implanted in the skin and are very difficult to extract, as they break easily when trying to take them off. The thorns in the strict sense are whitish, sclerified, solidly implanted, 1 to 2 cm long.

Each cladode contains on average a hundred areolas, whose fabric, in addition to generating thorns, can also be differentiated in shoots and leaves (or form new blades), in adventitious roots and even in flowers. The root system is superficial, generally does not exceed 30 cm deep in the soil, but on the contrary, it is very extensive (Aragona *et al.,* 2017). The flowers are hermaphrodite and the color of petals is generally yellow, but sometimes also pink or orange. They have a diameter of about 5 cm and numerous and well-developed stamens (**Figure 3.6**) (Miller *et al.,* 2015).

Fig 3.6 *Opuntia ficus indica*, yellow flower

The flowers first appear in early May through the early summer in the Northern Hemisphere, and the fruits ripen from August through October and they generally grow on cladodes over a year old more, often on the areoles located on the top of the cladode or on the surface most exposed to the sun.

The fruit with the name of "prickly pear fruit", (**Figure. 3.7**) is a fleshy berry, unilocular, with numerous seeds (up to 300) with a weight of 150 to 400 g.

Fig 3.7 *Opuntia ficus indica*, fruits

The yellow cultivar (sulfarin) is the main cultivar in Sicily and represents 90% of the total plantations, while the red (sanguine) and white (muscaredda) cultivars represent 10% and 2% respectively. According to spectrophotometric analysis, the yellow cultivar has a higher content of betalains (**Figure 3.8**) compared to red and white, and is particularly rich in indicaxantina (89%) (Butera *et al.,* 2002). *Opuntia ficus indica* has a rich composition in polyphenols, vitamins, polyunsaturated fatty acids and amino acids has been highlighted through the use of a large panel of extraction methods.

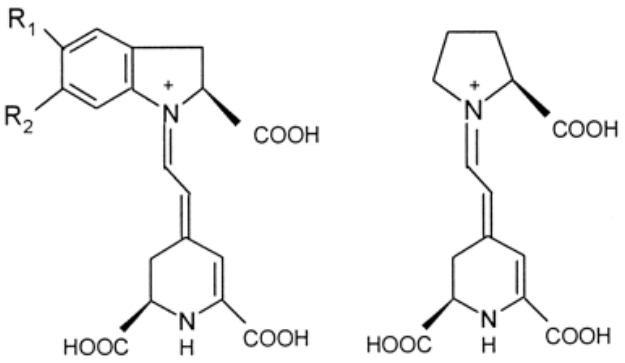


Fig 3.8 structures of betalains pigments

The identified natural cactus compounds and derivatives were shown to be endowed with biologically relevant activities including anti-inflammatory, antioxidant, hypoglycemic,

antimicrobial and neuroprotective properties (Morales *et al.,* 2012; Valente *et al.,* 2010; Butera *et al.,* 2002).

3.5 Indicaxanthin

Indicaxanthin ((2S)-2,3-dihydro-4-[2-[(2S)-2a-carboxypyrrolidin-1- yl]ethenyl]pyridine-2a,6-dicarboxylic acid, **Figure 3.9**), a betalain pigment from cactus pear fruit, has been the object of sound experimental work over the latest years. Indicaxanthin is the adduct of betalamic acid with proline and has a typical yellow - orange color due to the presence of a wide resonance system (Allegra *et al.,* 2014).

It is a reducing molecule with two redox potentials at 414 and 683 mV (Butera *et al.,* 2002) and it is also able to fit into the phospholipid bilayer of multilamellar liposomes like as an amphipathic molecule (Liveri *et al.,* 2007). The reducing capacities combined with amphiphilicity make the indicaxanthin able to behave as a reductant (scavenger) against numerous free radicals, both hydrophilic and lipophilic nature, generated in chemical solution (Tesoriere *et al.,* 2007).

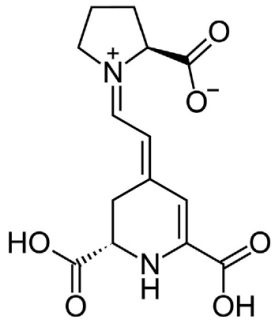


Fig 3.9 Indicaxanthin chemical structure

As many phytochemicals, indicaxanthin is a redox-active compound and has been shown to act as antioxidant in several of *in vitro* studies (Allegra *et al.,* 2005; Turco Liveri *et al.,* 2009).

In this regard, indicaxanthin has been showed to modulate specific redox-dependent signaling pathways involved in macrophage activation and apoptosis, epithelial and endothelial dysfunction in vitro (Allegra *et al.,* 2014; Tesoriere *et al.,* 2013, 2014, 2015). Interestingly, thanks to its charged portions, ionizable groups and lipophilic moieties, it is amphiphilic at physiological pH (Turco Liveri *et al.,* 2009) and has been demonstrated to interact with cell membranes (Tesoriere *et al.,* 2006, 2013; Turco Liveri *et al.,*2009). This feature is critical to allow bioactive compounds to interact with cells and to initiate signalling events. Indicaxanthin can modulate the activity and/or redox-dependent expression of key enzymes in the inflammatory process, such as myeloperoxidase and NADPH oxidase (NOX) (Tesoriere *et al.,* 2013; Allegra *et al.,* 2015).

Remarkably, and in contrast with the majority of dietary phytochemicals, indicaxanthin is highly bioavailable (Tesoriere *et al.,* 2004). The molecule has been shown to cross unaltered intestinal epithelial cell in vitro being absorbed through paracellular junctions and does not appear to be metabolized either during digestion or in the liver (Tesoriere *et al.,* 2013). Investigation from our group provided evidence that the ingestion of a dietary-consistent amount of cactus pear fruit pulp (4 fruits, containing 28 mg of indicaxanthin) generates, in humans, a plasma concentration of 7 mmol/L, quite a high amount compared with most of the dietary polyphenol phytochemicals, such as flavonoids (Allegra *et al.,* 2015; Tesoriere *et al.,* 2004). Moreover, its amphiphilicity allows it to cross the blood-brain-barrier and localize within the CNS (Allegra *et al.,* 2015). Finally, thanks to its bioavailability and redox-modulating properties, indicaxanthin exerts significant pharmacological effects in vivo. Indeed, oral administration of the PhC at nutritionally-relevant doses (2 μmol/kg) generates, in rats, a plasma peak concentration of 0.2 μM able to exert strong anti-inflammatory effects in an in vivo model of acute inflammation (Allegra *et al.,* 2014).

Indicaxanthin has been demonstrated to protect endothelial cells from both TNF-α and oxidized LDL (oxLDL)-induced dysfunction. In these systems, the phytochemical modulated specific redox-dependent signaling pathways responsible for endothelial cells activation. Indeed, when stimulated with 1 ng/ml TNF-α, HUVEC pre-treatedwith indicaxanthin at 5mM showed a reduction of cytokine-induced ICAM-1 over-expression (15%) (Allegra *et al.,* 2019). Similarly, when stimulated with 100mg/ml oxLDL, EC pre-treated with indicaxanthin in the range between 5 and 20mM, showed a concentration-dependent reduction of oxLDL-induced ICAM-1, VCAM-1 and ELAM-1 over-expression (both at mRNA and protein levels). At the same time, oxLDL-induced ABC-A1 down-regulation was pre-vented. From a mechanistic perspective, these effects were correlated with a reduction of endocellular RONS levels that in turn counteracted the redox-dependent NF-κB activation (Attanzio *et al.,* 2019). Within, the innate immunity, macrophages are crucial effectors cells able to synthesize relatively high amounts of RONS as a part of their molecular defense machinery against pathogens and tumor cells (Hamidzadeh, *et al.,* 2017). On the other hand, the same cells finely modulate their redox milieu to control metabolic responses during inflammation, in order to foster the switching from the acute phase to its resolution, thus avoiding chronicity. The redox-dependent metabolism of arachidonic acid (AA), with expression of inducible cyclo-oxygenase-2 (COX-2), its downstream enzymes PGE2 synthase-1(PGES-1) and PGD2 synthase (PGDS), the release of the pro-inflammatory prostaglandin E2 (PGE2), the anti-inflammatory PGD2 and its derivative 15-deoxy PGJ2 (15D-PGJ2), are central in the inflammatory process (Allegra *et al.,* 2014). In contrast to the majority of drugs and nutraceuticals that commonly exert anti-inflammatory effects by down-regulating COX-2 expression that in turn shut down the biosynthesis of all PG, indicaxanthin counteracts macrophages activation and elicits anti-inflammatory effect in a completely different

fashion. The molecule has been demonstrated to divert the metabolic axis of PG biosynthesis from PGE2 to PGD2. Indeed, LPS-activated RAW 264.7macrophages pre-incubated with indicaxanthin in a range between 50 and 100mM, showed a significant COX-2 overexpression and at the same time a PGES-1 down-regulation and PGDS up-regulation both at protein and mRNA level (Allegra *et al.,* 2014). As a consequence, COX-2 reaction product (PGH2) cannot react with PGES-1 and conversely becomes substrate for PGDS; this leads to a decrease of PGE2 release and an increase of PGD2 synthesis that is subsequently metabolized to PGJ2. The mechanism for such a metabolic rewiring is NF-κB-dependent and passes through the modulation of macrophages redox-state i.e. a pro-oxidant activity of indicaxanthin through lipid peroxide production. Rather than simply arresting the inflammatory reaction, the molecule appears to induce a cellular reprogramming towards the anti-inflammatory state and these effects could be of interest to project a novel class of anti-inflammatory compounds (Nathan *et al.,* 2002). Interestingly enough, not only modulates indicaxanthin the activation of LPS-stimulated macrophages, but also protects these cells from 7-ketocholesterol (7-KC)-induced apoptosis, a key event in the development of atherosclerosis (Tesoriere *et al.,* 2013). 7-KC-stimulated macrophages pre-treated with indicaxanthin in the range between 0.1 and 2.5mM, showed a significant reduction of 7-KC-inducedapoptosis. The mechanism for these protective effects where mediated by: the modulation of endocellular macrophage redox state and Ca^{++} homeostasis; the inhibition of NF-κB activation and NOX-4 activity and expression; the protection of mitochondria from oxidative damage.

Noteworthy, the anti-inflammatory potential of indicaxanthin is not confined to EC and macrophages but it is also evident in other cellular districts such as epithelial cells.

(Ligumsky *et al.,* 1990). Pre-incubation of IL-1β activated Caco-2 cells with indicaxanthin, in the range between 1 and 25mM, prevented the release of the pro-

inflammatory cytokines IL-6 and IL-8, PGE2 and NO, the endocellular production of RONS and the decrease of thiols in a concentration-dependent manner. Moreover, co-incubation of the cells with indicaxanthin and IL-1β also prevented the IL-1β induced increase of epithelial permeability. These anti-inflammatory effects were mediated by the inhibition of NOX-1, COX-2, iNOS over-expression and NF-κB activation (Tesoriere *et al.,* 2014). Finally, the above-reported anti-inflammatory effects, exerted *in vitro* by indicaxanthin, were paralleled *in vivo* in a reproducible model of acute inflammation, i.e. the carrageenin pleurisy, widely considered a gold standard tool to evaluate anti-inflammatory drugs (Allegra *et al.,* 2014). Administration of indicaxanthin (0.5, 1, 2mmol/kg) per os in rat challenged with λ-carrageenin, time- and dose-dependently, reduced the exudate volume and the number of leukocytes recruited in the pleural cavity. Pre-treatment with indicaxanthin at 2mmol/kg inhibited the carrageenin-induced release of PGE2, NO, IL-1β, TNF-α in the exudate and caused a decrease of IL-1β, TNF-α, iNOS, and COX-2 mRNA as well as iNOS and COX-2 protein expression in the recruited leukocytes. These effects were mediated by the time- and dose-dependent inhibition of NF-κB activation.

Tutone *et al*., in their recent paper using a Reverse Docking method, have identified potential targets for indicaxanthin, shown in **Table 3.1**.

Target protein	[a]ΔG_{bind}	Pathway
ITP3K	−50.460	Decrease of the release of Neurotransmitters (Synaptic plasticity)
GCPII	−100.903	Glutamate release (Glutamatergic transmission)
LTA4H	−48.457	Inflammation associated carcinogenesis
HPSP	−52.715	Increase of synthesis of D−serine, co-agonist for NMDA receptor in the glycine binding site (Glutamatergic transmission)
PDE4D	−43.116	cAMP Hydrolysis, PKA Activator (Mnemonic processes and intestine contractility)
GluA3	−55.390	Glutamatergic transmission
GluA2	−55.262	Glutamatergic transmission
GluK1	−59.335	Glutamatergic and GABAergic transmission

[a] Free energies (kcal/mol) (best value predicted by MM-GBSA calculations).

Table 3.1 Target identified by Reverse Docking methodology, with related biological pathways, and calculated binding data.

They include Inositol Trisphosphate 3-Kinase (ITP3K), Glutamatecarboxypeptidase II (GCPII), Leukotriene-A4 hydrolase (LTA4H), Phosphoserine phosphatase (HPSP), Phosphodiesterase 4D(PDE4D), AMPA receptor (GluA3 and GluA2 sub-units) and Kainate receptor (GluK1 isoform). These targets are implicated in neuromodulation, and inflammatory regulation, normally expressed mostly in the CNS, and expressed (or overexpressed) in cancer tissues (i.e. breast, thyroid, and prostate cancer cells) (Tutone *et al.,* 2018).

CHAPTER 4

Hypothesis and Aims

Malignant melanoma is one of the most common causes of cancer deaths among young adults. Increased incidence of melanoma is a result from the co-occurrence of careless sun exposure and the ageing society. Unfortunately, current therapeutic options are still limited. Melanoma is known to be a tumor with a high mutational load and should thus be recognized by the immune system as foreign. The fact that the tumor is able to evade the immune system opens up treatment possibilities in priming the immune system. Although treatment for melanoma has improved in recent years, most patients do not benefit from the treatment, which also often causes side effects. For this reason, the combination of drugs is often applied to reduce adverse effects and the emergence of drug resistance. This pushed clinicians and researchers to test combinatorial therapies in order to obtain significant clinical benefits. The aim of this study was to investigate novel therapeutic adjuvants for the treatment of melanoma. The specific aims for this project were to:

- Investigate the effects of indicaxanthin on human melanoma cell proliferation and explain its possible mechanism of action, *in vitro*;
- Evaluate, *in vivo,* the antitumor activity of indicaxanthin in melanoma development and progression;
- Determine the effects of indicaxanthin in more complex and multicellular systems, using one of the most useful translation models, the whole blood *ex vivo* assay.

This work was developed in collaboration with the University of Naples, Federico II, under the supervision of Prof. Angela Ianaro (Department of Pharmacology), and with the University of Roehampton, London, under the supervision of Prof. Fulvio D'Acquisto (Department of Life Sciences).

CHAPTER 5

Methods

5.1 Reagents

Unless stated otherwise, all reagents were from Sigma (Milan, Italy) and of the highest grade commercially available.

5.2 Extraction and purification of Indicaxanthin

Indicaxanthin was isolated from cactus pear (*Opuntia Ficus-Indica*) fruits (yellow cultivar) as previously described (Allegra *et al*., 2014). Briefly, the phytochemical was separated from a methanol extract of the pulp by liquid chromatography on Sephadex G-25. Fractions containing the pigment were submitted to cryodesiccation and purity of indicaxanthin assessed by HPLC on a Varian Microsorb C-18 column (4.6 Å~ 250 mm; Varian, Palo Alto, CA) eluted with a 20min linear gradient elution from solvent A (1% acetic acid in water) to 20% solvent B (1% acetic acid in acetonitrile) with a flow rate 1.5 ml/min. Spectrophotometric revelation was at 482nm. Under these conditions, indicaxanthin eluted after 8.15 min and was quantified by reference to standard curves constructed with 5−100 ng of purified compounds and by relating its amount to the peak area.

5.3 Reagents and cell culture

NHEM (Normal Human Epidermal Melanocytes) were purchased from Lonza (Walkersville, MD, USA) and grown in melanocyte growth medium 2 (Lonza). The melanoma cell lines B16/F10 and Sk-Mel-28 were purchased from IRCCS AOU San Martino – IST (Genua, Italy),

A375 from Sigma–Aldrich (Milan, Italy), MALME from American Type Culture Collection (ATCC). B16/F10, A375 and Sk-Mel-28 were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mmol/l L-glutamine, 100 µlmol/l non-essential amino acids, penicillin (100 U/ml), streptomycin (100 µg/ml) and 1 mmol/l sodium pyruvate (all from Sigma–Aldrich). MALME was cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing 20% fetal bovine serum, 2 mmol/l L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) and 1 mmol/l sodium pyruvate. Cells were grown at 37 °C in a humidified incubator under 5% CO_2. All cell lines used in this study were characterized by the cell bank of the supplier.

5.4 Proliferation assay

Cell proliferation was measured by the 3-[4,5-dimethyltiazol-2-yl]- 2,5-diphenyl tetrazolium bromide (MTT). Briefly, the human melanoma cells and the NHEM cells were seeded on 96-well plates (2Å~103 cells/ well) and treated with indicaxanthin (50-100-200 µM) for 72 h before adding 200 µl of MTT (Sigma, Milan, Italy) (0,25 mg/ml in medium).

Cells were thus incubated for an additional 3 h at 37 °C. After this time interval, cells were lysed, and dark blue crystals were solubilized with 100 µl of dimethyl sulfoxide (DMSO). The optical density of each well was measured with a microplate spectrophotometer (TitertekMultiskan MCC/340), equipped with a 490nm filter.

5.5 Apoptosis assay

The externalization of phosphatidylserine to the cell surface was detected by flow cytometry using a double-staining with Annexin V Fluorescein Isothiocyanate (AxV-

FITC) and Propidium Iodide (PI) as previously reported (Tesoriere *et al*., 2013). Briefly, cells were seeded in triplicate in 24-well plates at a density of 2.0 x 10^5 cells/cm2. After an overnight incubation, they were washed with fresh medium and incubated with indicaxanthin at 100 µM. After 48 h, cells were harvested by trypsinization and adjusted at 2.0 x 105 cells/ml with combining buffer. One hundred microliter of cell suspension was then incubated with 5 µl of a 5 µM AxV-FITC solution and 10 µl of a 20µg/ml PI solution, at room temperature in the dark for 15 min. Samples of at least 1.0 x 104 cells were then analyzed by Epics XL flow cytometer using Expo32 software (Beckman Coulter, Fullerton, CA) and an appropriate bi-dimensional gating method.

5.6 Preparation of cellular extracts

A375 cells (1 x10^6) were seeded in 6-well plates and treated with indicaxanthin 100 µM for 15-30- 60 min or 6-24-48 h. To obtain cytosolic or nuclear extracts the cell pellet was re-suspended in 100 µL of ice-cold hypotonic lysis buffer (10 mmol/L Hepes, 10 mmol/L KCl, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1.5 µg/mL soybean trypsin inhibitor, 7 µg/mL pepstatin A, 5 µg/mL leupeptin, 0.1 mmol/L benzamidine, 0.5 mmol/L dithiothreitol) and incubated on ice for 15 minutes. The cells were lysed by rapid passage through a syringe needle five times and centrifuged for 10 minutes at 13 000g. The supernatant containing the cytosolic fraction was removed and stored at −80°C. The nuclear pellet was re-suspended in 30 µL of high-salt extraction buffer (20 mmol/L Hepes pH 7.9, 10 mmol/L NaCl, 0.2 mmol/L EDTA, 25% v/v glycerol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1.5 µg/mL soybean trypsin inhibitor, 7 µg/mL pepstatin A, 5 µg/mL leupeptin, 0.1 mmol/L benzamidine, 0.5 mmol/L dithiothreitol) and incubated at 4°C for 30 minutes with constant agitation. The nuclear extract was then centrifuged for 10 minutes at 6000g, and the supernatant was aliquoted and stored at −80°C.

Whole-cell extracts of A375 cells were prepared after lysis in extraction buffer (50 mM Tris [tris(hydroxymethyl)aminomethane]/HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 5 mM EDTA [ethylenediaminetetraacetic acid], 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor cocktail tablets, Roche). The protein concentration was measured by the Bradford method (Bio-Rad, Milan, Italy).

5.7 Western blot analysis

The protein concentration was measured by the Bradford method (Bio-Rad, Milan, Italy). Equal amounts of protein (40 μg/sample) from whole or nuclear cell extracts were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Trans-Blot Turbo Transfer Starter System, Biorad). The membranes were blocked for 2 h in 5% low-fat milk in PBS with 0.1% Tween 20 (PBST) at room temperature. Then the filters were incubated with the following primary antibodies: Caspase-3 and NF-κB p65 (Cell Signaling, USA; diluted 1:1000), IκBα (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:200); LC3B (Novusbio, USA; diluted 1:1000); Bcl-2 (Cell Signaling, USA; diluted 1:1000), c-FLIP (Millipore; diluted 1 μg/ ml), β-actin (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:1000), GAPDH (Cell Signaling, USA; diluted 1:1000), α-Tubulin (Cell Signaling, USA; diluted 1:1000) overnight at 4 °C. The membranes were washed 3 times with PBST and then incubated with horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:2000) for 2 h at room temperature. The immune complexes were visualized by the ECL chemiluminescence method and acquired by the Image Quant 400 system (GE Healthcare).

5.8 Invasion assay

The assay was performed using chambers with polycarbonate filters with 8 μM nominal pore size (Millipore, USA) coated on the upper side with Matrigel (Becton Dickinson Labware, USA) as previously described (Panza *et al*., 2016). Briefly, the chambers were placed into a 24-well plate and melanoma cells (2.5×10^5/ml) were plated in the upper chamber, with or without indicaxanthin 100 μM, in serum-free DMEM. After the incubation period (16 h), the filter was removed, and nonmigrant cells on the upper side of the filter were detached with the use of a cotton swab. Filters were fixed with 4% formaldehyde for 15 min, and cells located in the lower filter were stained with 0.1% crystal violet for 20 min and then washed with PBS. The filters were examined microscopically and cellular invasion was determined by counting the number of stained cells on each filter in at least 4–5 randomly selected fields. Data are presented as a mean of invaded cells ±SD /microscopic field of three independent experiments.

5.9 Animals

Animal care was in accordance with Italian and European regulations on the protection of animals used for experimental and other scientific purposes. Mice were observed daily and humanely euthanized by CO_2 inhalation if a solitary subcutaneous tumor exceeded 1.5 cm in diameter or mice showed signs referable to metastatic cancer. All efforts were made to minimize suffering. Male C57BL/6 mice (age 6–7 weeks, 18–20 g) were from Charles River Laboratories, Inc. Mice were housed at the Animal Research Facility of the Department of Pharmacy of the University of Naples Federico II.

5.10 Induction of subcutaneous B16 lesions

Mice were subcutaneously (s.c.) injected in the right flank with B16/F10 cells (1×10^5/0.1 ml) and divided in two groups. The treated group received indicaxanthin (3.2 mg/kg) orally three time a day for 14 days. Control mice received only vehicle. Tumor size was measured using a digital caliper, and tumor volume was calculated using the following equation: tumor volume=π/6(D1xD2xD3) where D1=length; D2=width; D3=height and expressed as cm^3.

5.11 Blood Donors

Human blood donors were 20- to 50-year-old healthy men and women who were tested to be negative for HIV, hepatitis B virus and hepatitis C virus. Further exclusion criteria were manifest infections during the last 4 weeks, fever, symptomatic allergies, abnormal blood cell counts or increased liver enzymes. All healthy volunteers did not receive anti-platelet or anticoagulant therapy and gave oral and written consent. Cell collection and separation was covered by ethical approval 05/Q0603/34 (East London and The City Research Ethics Committee).

5.12 Blood Collection

Whole blood assay was conducted on 12-h fasting blood samples collected through finger prick in sterile Microvette 200 K3E (Catalog No. 20.1278, Sarstedt) tubes containing K_3EDTA. The blood was held at room temperature (20–22°C) until processed. The processing of whole blood started no later than 3 hours after collection.

5.13 *Ex vivo* whole blood Assay

Whole blood (20µl) was diluted 1:4 with RPMI 1640 medium (Catalog No. 2187503; Gibco) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere.

Samples were transferred to 1,5 ml tube and pre-incubated for 1 hour with indicaxanthin 0.001-10µM prior to the stimulation with LPS 100 ng/mL (*Escherichia coli* O127:B8 Catalog No. L3129; Sigma-Aldrich).

To obtain a time-dependent analysis of the production of inflammatory cytokines, 100 µL of sample were collected at 4-8-24 hours after the stimulation with LPS. These were transferred in a new tube and centrifuged at 500 *g* for 5 minutes. The supernatants were collected and stored at -80 °C for further analysis.

5.14 ELISA

The levels of CXCL1/KC, Tumor Necrosis Factor Alpha (TNF- α) and Interleukin (IL-6) in the above-mentioned supernatants were measured by standard ELISA kits and according to the manufacturer's instruction (CXCL1/KC; DuoSet ELISA, R&D Systems, Minneapolis, MN, USA; TNF- α and IL-6; Uncoated Elisa Assay Catalog No. 88-7346 Invitrogen). For the measurement of TNF-α and IL-6 the samples were diluted 1:10 and 1:20 respectively, with assay diluent before loading them onto the plates.

For the measurement of Leukotriene B4 we used the Elisa Assay Kit from Cayman Chemical (LTB4 ELISA KIT, Catalog No. 520111). As for the cytokines, samples were diluted 1:10 before loading onto the pre-coated plates.

5.15 Bacterial strains and culture conditions

Escherichia coli (K12 NCM3722), *Pseudomonas aeruginosa* (PAO1), *Staphylococcus aureus* (USA300), *Klebsiella pneumonia* (ATCC 43816) and *Acinetobacter baumannii* (ATCC 19606) (kindly supplied by A. Edwards lab, Imperial College) were cultured in Luria Bertani (LB) Broth Miller (Catalog No. L3522, Sigma Aldrich). Stocks of colonies were prepared by growing cells to an optical density of 1 at 600 nm (OD600) and by and re-suspending them in 1.5 ml of PBS supplemented with 20% glycerol. Aliquots of this suspension (100 µl) were stored at −80°C and used for future experiments.

5.16 Ex vivo Phagocytosis assay in whole blood

The day before the test, aliquots of the above-mentioned bacteria were defrosted and grown overnight in 5 ml of LB at 37 °C with shaking (300 rpm; Incu-shake MIDI 4020 incubator, SciQuip). The concentration of each strain was determined by measuring the optical density of the cells suspension at 600 nm.

Aliquots of bacterial suspensions at MOI 100 (100 bacterial for each cell) in 10 µl of PBS were added to samples of diluted blood treated or not with indicaxanthin as described before (see paragraph 5.13). After 2 and 4 hours, the phagocytosis was stopped by adding 10µl of a solution of 1 mg/ml gentamicin (Catalog No. G1397, Sigma) for 1 additional hour. During this time, all the extracellular non-phagocyted bacteria were eliminated.

To measure the number of alive phagocyted bacteria samples were centrifuged at 500 × g for 5 min and cell pellets were lysed with 50 µl lysis buffer (PBS + 0,1 % Triton X 100) for 10 minutes at room temperature. Intact and alive intracellular bacteria were counted

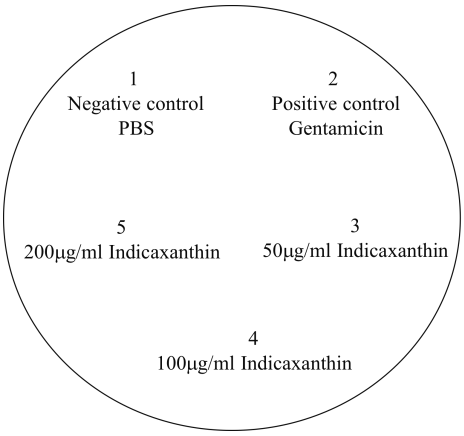
by serial dilutions and plating on agar plates (Freebern *et al.,* 2013; Gupta et al., 2017). The results were expressed as colony-forming units (CFU):

AVGdil x dilution / 0.05 (the inoculation volume) = CFU/ml

5.17 Antimicrobial susceptibility testing

To determine if indicaxanthin had any antimicrobial activity *per se*, we used the classical paper disc plate method (Giardina *et al.,* 2010; Lo Grasso *et al.,* 2015). Briefly, 100 µl of a suspension of approximately 10^8 cells/ml was spread onto LB-agar plates to obtain a homogenous layer of cells.

Thereafter, we soaked sterile paper discs (1 cm diameter) of Whatman filter paper No. 42 into solutions of indicaxanthin (50-100-200µg/ml) or Gentamicin 1 mg/ml (positive control) (Catalog No. G1397, Sigma) or PBS (negative control). Soaked discs were placed on agar plate at a distance of 1cm from each other and in a circular way as shown in the figure below.



Plates with discs were then incubated overnight at 37º C. The minimal inhibitory concentration (MIC) was calculated as the lowest concentration of the compound that was

able to produce a transparent halo of growth inhibition larger than 1 cm (size of the disk paper) in a plate containing 20 ml of LB-agar.

5.18 Statistical analysis

Data from all experiments are reported as mean ±SEM unless otherwise noted. Data were analyzed and presented using GRAPHPAD PRISM software (GraphPad). Significance was determined using Student's 2-tailed t-test and the multiple comparisons were determined using One-way ANOVA followed by Bonferroni's post-hoc. Results were considered significant at P values less than 0.05 and are labeled with a single asterisk. Besides, P values less than 0.01 and 0.001 are designated with double and triple asterisks, respectively.

CHAPTER 6

Results and discussions

6.1 Indicaxanthin suppresses human melanoma cell proliferation

Indicaxanthin was purified as above reported and its HPLC profile along with the chemical structure are shown in **Figure. 6.1**. To explain the role of indicaxanthin in human and murine melanoma, we assessed the effect on different cells line. The antiproliferative effect of indicaxanthin on human (A375, SKMel- 28, MALME) or murine (B16F10) melanoma cells was evaluated by MTT assay.

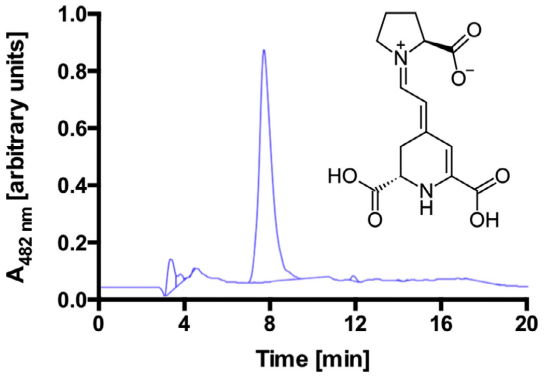


Fig 6.1 Representative chromatographic profile of indicaxanthin used in the present study; in the inset the chemical structure of the phytochemical.

Incubation of cells with indicaxanthin (50-100-200 μM) for 72 h, caused a concentration-dependent inhibition of all cell used. The most sensitive cell lines to the inhibitory effect of indicaxanthin were A375 and the B16F10, whose proliferation was inhibited by 21%, 36%, 56% and 34%, 46%, 69%, respectively (**Table 6.1**).

On the other hand, NHEM growth was not affected by any of the concentration of indicaxanthin tested at the time point considered (data not shown), thus indicating a selective cytotoxic activity toward cancer cell lines. Curcumin was used as positive control (**Table 6.1**).

	IND 50 µm	IND 100 µm	IND 200 µm	CUR 100 µm
A375	20.7***	35.7***	56***	73.3***
Sk-Mel-28	4.2	4.7	24.4 ***	65.2***
MALME	4.9	12.9	30.8 ***	69.4***
B16/F10	33.9***	46.4***	69.2***	70.2***

Table 6.1 Effect of indicaxanthin (IND) on A375, Sk-Mel-28, MALME and B16/F10 melanoma cells proliferation. Growth inhibition was measured by MTT assay and is expressed as percent of inhibition at 72 h. Curcumin (CUR) was used as positive control. Experiments were run in triplicate, each performed in quadruplicate (***P<0.001 vs. untreated cells).

6.2 Indicaxanthin induce apoptosis of human melanoma A375 cell

To gain insights into the mechanisms of indicaxanthin-induced cell growth inhibition (necrosis or apoptosis), externalization of plasma membrane phosphatidylserine, a reliable marker of cell apoptosis, was evaluated for A375 cell line at 48 h. The choice of this cell line was prompted by its high sensitivity to indicaxanthin effect (see **Table 6.1**). The concentration of the phytochemical chosen for this study (100 µM), was selected taking into account the value of IC_{50} measured for this cell line.

As shown in **Figure 6.2 A** and **B**, the treatment of A375 cells with indicaxanthin induced a significant increase of the percentage of apoptotic/necroptotic cells (AxV-FITC+/PI+, [31.5 ± 0.2] % (P<0.01) as compared to untreated cells. On the other hand, indicaxanthin treatment determined only neglectable necrotic events as evaluated by the percentage of AxV-FITC−/PI+ cells ([1.3 ± 0.01] %, **Figure 6.2 A**). As caspase-3 is the final stage of apoptosis initiation, we also examined its activity. Results obtained showed that treatment with indicaxanthin markedly promoted cleavage and subsequent activation of caspase-3 (**Figure 6.2 C**). However, melanoma is one type of cancer that constantly develops drug resistance due to dysregulation of apoptosis (Grossman and Altieri, 2001). Therefore, the

induction of other forms of cell death, especially autophagy, is necessary and fundamental to conquer this resistance.

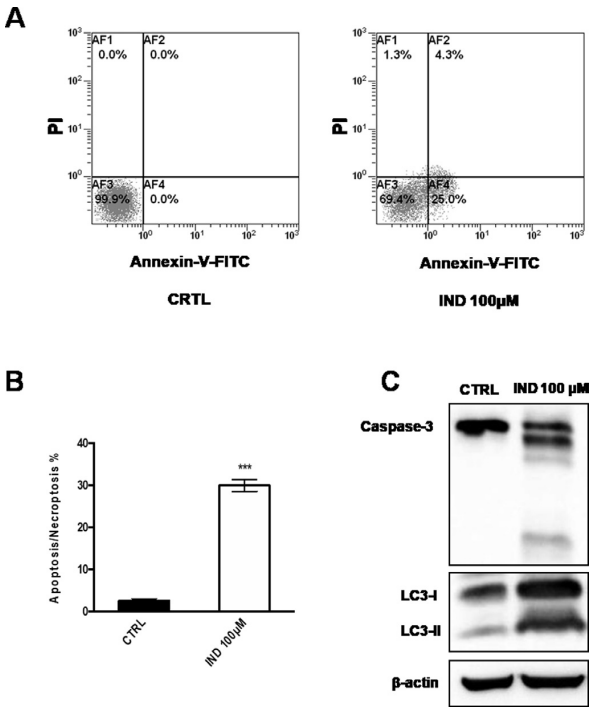


Fig. 6.2 Effect of indicaxanthin on A375 melanoma cells apoptosis. (A) Cells were incubated for 48 h in the absence (CTRL) or presence of indicaxanthin (IND) 100 µM and apoptosis was determined by Annexin V-FITC/Propidium Iodide (PI) staining. AF3, viable cells (Annexin V-FITC−/PI−); AF4, early apoptotic cells (Annexin V-FITC+/PI−); AF2, tardive apoptotic/necroptotic cells (Annexin V-FITC+/PI+); AF1, necrotic cells (Annexin V-FITC−/PI+). Images are representative of three experiments with comparable results. (B) Quantitative analysis of indicaxanthin-induced A375 cell apoptosis at 48 h. Experiments were run in triplicate, each performed in quadruplicate (**P<0.01; ***P<0.001 vs. CTRL). (C) Analysis of caspase 3 activation and LC3II/I conversion in A375 cells treated with IND 100 µM by immunoblotting. β-actin was used as a loading control.

To gain further insights into the mechanisms of indicaxanthin-induced cell growth inhibition A375 cells were treated with indicaxanthin (100 µM) and autophagy was evaluated by measuring the conversion of LC3-I to LC3-II, a sign of autophagic activity (Mizushima *et al*., 2010). As shown in **Figure 6.2 C**, 100 µM indicaxanthin treatment caused a marked increase of LC3-II level in A375 cells as compared to control. These results revealed that indicaxanthin could potently induce both apoptosis and autophagy

in A375 cells. Caspase-8 and autophagy are apparently gatekeepers preventing necroptosis (Kaczmarek *et al*., 2013). Since caspase-3 is activated downstream of caspase-8 in the death-receptor pathway we can exclude an involvement of necroptosis in indicaxanthin induced cell death.

6.3 Indicaxanthin inhibits NF-κB activation and down-regulate NF-κB-dependent anti-apoptotic proteins

Nuclear factor kappa B (NF-κB) signaling pathway is a complex network linking extracellular stimuli to cell survival and proliferation. NF-κB is, indeed, one of the major transcription factors associated with the development and progression of both hematologic and solid tumors, including melanoma, breast, prostate, ovarian, pancreatic, colon, lung, and thyroid cancers. Aberrantly activated NF-κB plays a fundamental role in cell survival, resistance to apoptosis (Madonna *et al*., 2012), angiogenesis, tumor cell invasion, metastasis (Basseres and Baldwin, 2006) and chemoresistance (Fujioka *et al*., 2012). Along these lines, NF-κB is currently considered an ideal target for cancer therapy. NF-κB is normally retained in an inactive form in the cytoplasm tightly associated with the inhibitory protein IκB-α.

In order to investigate indicaxanthin effects on NF-κB activity, A375 cells were treated with indicaxanthin (100 μM) and western blot analysis was carried out on cellular extracts obtained at different time points (15-30-60 min and 3-6-24-48 h). As shown in **Figure 6.3 A** indicaxanthin inhibited IκBα degradation in A375 cells as demonstrated by an increase in band intensity at 30 min. This effect was paralleled by a reduction in the nuclear levels of the NF-κB active subunit p65, at 3 and 6 h after incubation with indicaxanthin (**Figure 6.3 B**).

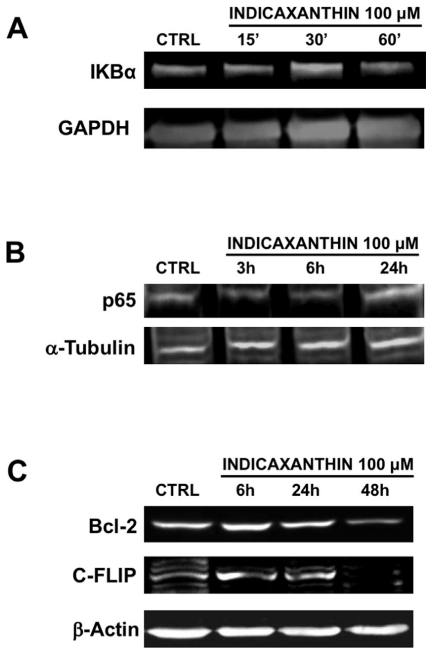


Fig. 6.3. Effect of indicaxanthin on NF-κB activation and NF-κB-dependent antiapoptotic proteins in A375 human melanoma cells. (A) Western blot analysis of IκBα carried out on the cytosolic extracts obtained from A375 cells incubated in the absence or in the presence of indicaxanthin 100 μM for 15, 30, or 60 min; (B) western blot analysis of p65 carried out on nuclear extracts obtained from A375 cells incubated in the absence or in the presence of indicaxanthin 100 μM for 6–24 h; (C) western blot analysis of Bcl-2 and C-FLIP carried out the cytosolic extracts obtained from A375 cells incubated in the absence or in the presence of indicaxanthin 100 μM for 6–48 h. GADPH, α-tubulin and β-actin were detected as a loading control. Images are representative of three experiments with comparable results.

To further confirm that indicaxanthin-induced apoptosis of melanoma cells was strictly related to NF-κB-inhibition western blot experiments were carried out to evaluate the effect on the expressions of two anti-apoptotic proteins, B cell lymphoma gene-2 (Bcl-2) and FLICE inhibitory protein (c-FLIP) whose transcription is modulated by NF-κB.

As shown in **Figure 6.3 C**, A375 cells incubated with indicaxanthin exhibited a marked reduction in both proteins band intensity.

Our data showing the ability of indicaxanthin to inhibit NF-κB activation provide a molecular insight into the mechanism through which the PhC could impair cell proliferation and stimulate apoptosis.

Moreover, it gains a remarkable interest in the light of the current great interest of researchers and pharmaceutical companies in the identification of new molecules able to modulate NF-κB pathway. Over 750 inhibitors of the NF-κB pathway have been identified and a lot of them are natural compounds (Gilmore and Herscovitch, 2006). Therefore, novel dietary supplements to inhibit NF-κB activation may offer a very promising option in melanoma treatment.

6.4 Effect of indicaxanthin on cells invasion

To determine whether indicaxanthin affected the invasive ability of the metastatic melanoma cells A375, we performed a cell invasion assay using a transwell system. As shown in **Figure 6.4**, indicaxanthin 100 μM (a concentration not affecting cell proliferation at 16 h) significantly inhibited the invasiveness of A375 cell line by 71% ($P<0.001$ vs control; n=3).

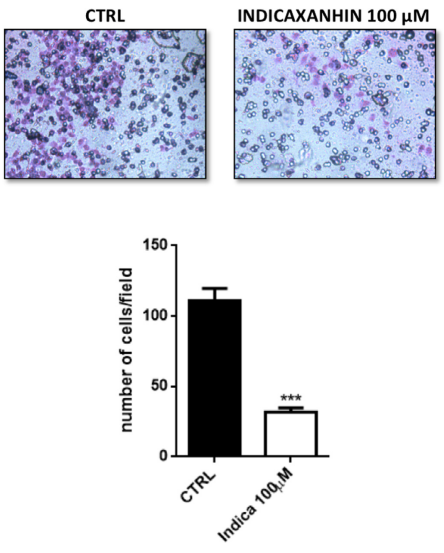


Fig. 6.4. Effect of indicaxanthin on the invasiveness of A375 cells. Indicaxanthin (100 μM) suppresses A375 cell invasion as measured by a transwell cell invasion assay. Representative field of invasive cells A375 on the membrane and relative average number of invasive cells from triplicate measurements. (***P<0.001 vs. CTRL).

6.5 Indicaxanthin inhibits growth of melanoma tumors *in vivo* by reducing plasma levels of melanoma-associated chemokines

Finally, to confirm the antitumor capacity of indicaxanthin we examined its effects on tumor growth in vivo. To this end, we used a murine model of cutaneous melanoma obtained by the subcutaneous injection of B16F10 murine melanoma cells in C57BL/6 mice. Tumor-bearing mice were treated with indicaxanthin (3.2 mg/kg) or vehicle (saline) by oral gavage twice daily. This dose was 5-times higher than that employed in previous in vivo experiments investigating the anti-inflammatory effects of the PhC (0.65 mg/kg) (Allegra *et al*., 2014). At day 14 after tumor implantation, a reduction in tumor volume (86%) and weight (83%) was observed in indicaxanthin-treated mice (0.035 ± 0.01 cm3 mean tumor volume vs. control mice 0.260 ± 0.04 cm3 mean tumor volume, P<0.001) (**Figure 6.5 A** and **B**). Moreover, in the plasma of indicaxanthin-treated mice, we found a significant reduction of CXCL1 levels by 42% (140 ± 17 pg/ml; P<0.05) as compared to control mice (240 ± 28) (**Figure 6.5 C**). CXCL1 is a chemokine belonging to the CXC chemokine subfamily and it has been associated with metastatic melanoma since it facilitates the recruitment of tumor-promoting myeloid cells into the tumor and enhances angiogenesis (Richmond *et al*., 2010).

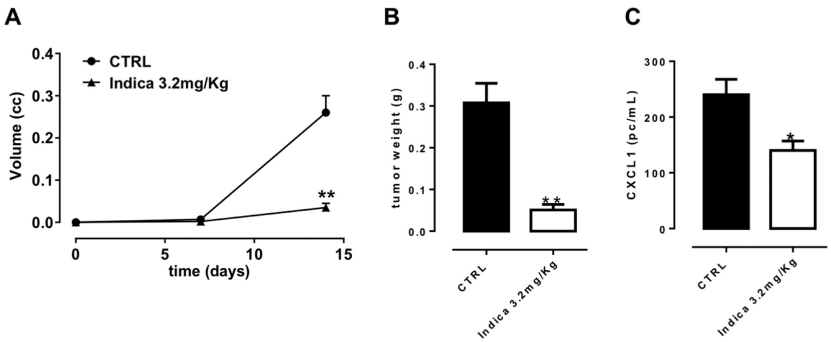


Fig. 6.5. Effect of indicaxanthin on tumor growth in vivo and plasma levels of the chemokine CXCL1. Indicaxanthin (3.2 mg/kg) was given orally to mice, while control mice received vehicle only. (A) the average tumor volume with standard error is plotted against the days after tumor implant; (B) tumor weight and (C) CXCL1 levels in control and indicaxanthin-treated mice. (*P<0.05 vs. CTRL; **P<0.01 vs. CTRL).

As above stated, indicaxanthin is a bioavailable PhC in rats where it reaches a therapeutical plasma concentration of 0.22 μM after an oral administration of 0.64 mg/kg (Allegra *et al*., 2014). Taking into account that we here administered a 5-time higher amount of the PhC in mice, we estimate that the plasma concentration of indicaxanthin in this study may well be close to this value.

Indicaxanthin is, however, a thermo-unstable compound: 50% of the molecule is lost after a 24 incubation at 37 °C (not shown). Taking all this into account, we then believe that the antiproliferative effects observed in the present study should be induced by a concentration of the molecule in a range much lower than above-mentioned IC_{50} (100 μM) and more likely between 12.5-50 μM.

6.6 Whole blood (WB) *ex vivo* stimulation to predict the systemic cytokine response

The whole blood assay (WB) is a convenient and widely used experimental system to investigate the production of inflammatory mediators in response to stimulation with a variety of inflamogens, including bacterial lipopolysaccharide (LPS), antigens and allergens (Abbasi *et al*., 2014).

The majority of the results we have described so far were obtained by testing a single cell line or the whole animal (data on mice shown in paragraph **6.5**). As we wanted to determine the effects of indicaxanthin in more complex and multicellular systems were cell-to-cell communication and crosstalk might take place, we sought to use the whole blood as this has been repeatedly reported as one of the most useful translational *in vitro* models. We used blood of six healthy volunteers of different ages and gender (male and female) to assess possible differences in response between male and female and age range. This is an underpowered pilot study that we carried out mainly to assess feasibility

and explore unexpected effects that have not emerged in the other experimental systems such as in cell culture. Lipopolysaccharide (LPS) is a wall component of gram-negative bacteria that has been widely used as powerful stimulants of both monocytes and lymphocytes (Abbasi *et al*., 2018). LPS binds to and activates toll-like receptor (TLR)-4 in mammals, resulting in the release of TNF-α, IL1, IL-6 and an array of regulatory cytokines like IL-10 and TGF-β. Before starting the testing of indicaxanthin, we run some setting up tests to optimize our system and experimental conditions. As we aimed to ultimately expand the study to a wide range of volunteers, we wanted these tests to be simple and effective. For instance, we wanted to collect the minimal amount of blood to perform a screening using the most simple and fast method such as the finger prick. With this idea in mind, we first investigated what would be the minimal amount of blood that would provide us with some readings for inflammatory cytokines. We used 10-25-50 µl of whole blood in a final volume of 100µl, so we carried out 1:10, 1:4 and 1:2 dilution factors with RMPI medium complete as described in Materials and Methods.

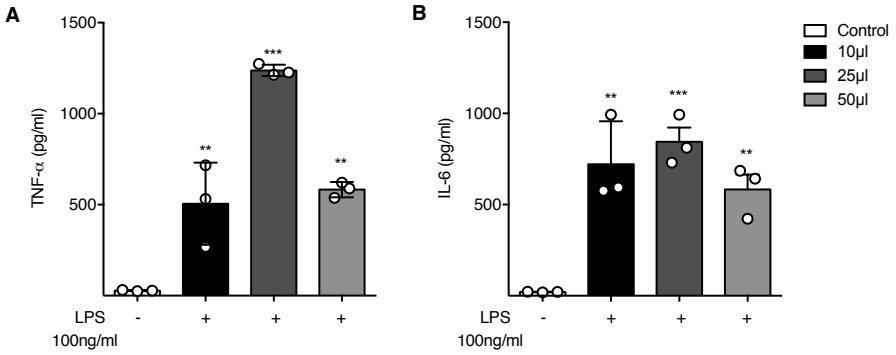


Fig 6.6 Production of TNF-a and IL-6 in LPS-stimulated whole blood assay. Different volume (10µl, 25µl and 50µl) of whole blood from healthy human volunteers was diluted with RPMI medium to obtain a final volume of 100µl. Samples were stimulated with LPS 100ng/ml for 8 hours and the supernatants used to measure TNF-α (A) and IL-6 (B) as reported in Material and Methods. The graphs are representative of three experiments with comparable results. Values are the mean ±SEM of three separate experiments carried out in triplicate. Significance calculated by One-way ANOVA multiple comparisons (**P<0,01vs. control and ***P<0,001 vs. control)

As shown in **Figure 6.6**, the production of TNF-α was the highest using the 1:4 dilutions while it was almost the same in the 1:2 or in the 1:10. Similar observations could be made for the IL-6 production although here the differences are not as clear as for the TNF-α.

As next step, determined the best time of incubation with LPS as studies have previously shown that the production of TNF-α and IL-6 in time *in vivo* different significantly with TNF-α showing a peak first and IL-6 after. Consistent with this literature, our results showed that the levels of TNF-α increased by about 30 folds and peaked at 8 hours while they raised by 8-fold at 4 and 8 hrs. Conversely, the levels of IL-6 increased by about the same (30 folds) at 8 hours and remained the same at 24 hours (**Figure 6.7**).

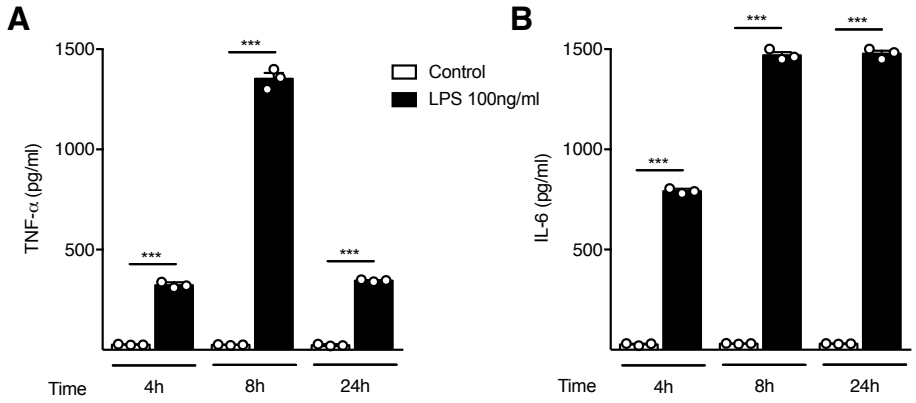


Fig 6.7 The kinetic of TNF-α and IL-6 production by LPS-stimulated whole blood assay. The whole blood from healthy human volunteers was diluted 1:5 with RPMI medium, it was stimulated with LPS 100ng/ml for 4-8-24 hours and the supernatants used to measure TNF-α (A) and IL-6 (B) as reported in Material and Methods. The graphs are representative of three experiments with comparable results. Values are the mean ±SEM of three separate experiments carried out in triplicate. Significance calculated by One-way ANOVA multiple comparisons (***P<0,001 vs Control).

6.7 Indicaxanthin modulates cytokine production *in the whole blood assay*

Having set up the best condition to measure cytokine production using the smallest amount of blood, we next tested the effect of indicaxanthin in this system using

concentrations that correspond to what is possible to find in blood after the assumption of 4 prickly pear fruits. These concentrations range from 0,001μM to 10μM.

We first tested the effects of indicaxanthin alone, without LPS stimulation. As shown in **Figure 6.8** below, there were no significant effects on both TNF-α and IL-6 although the samples showed a high variability of responses. More specifically, the blood of two of the volunteers seem to show detectable amounts of these cytokines following incubation with both high and lower amount of the compound.

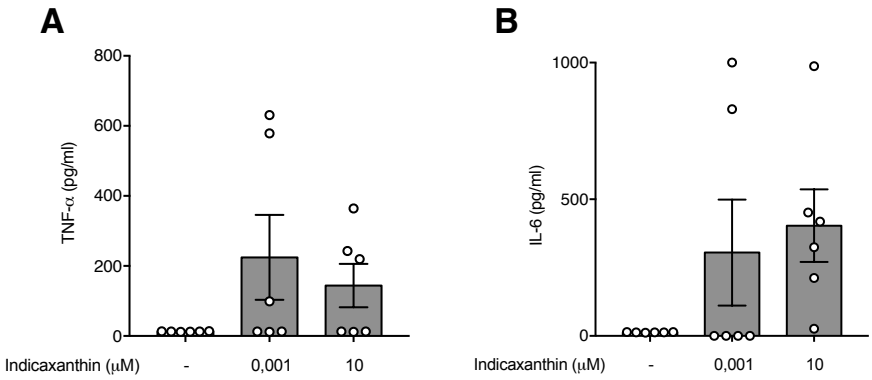


Fig 6.8 Effect of indicaxanthin on production of TNF-α and IL-6 in whole blood assay. The whole blood from healthy human volunteers was diluted 1:5 with RPMI medium, it was treated with different concentrations of Indicaxanthin (0,001-10μM) for 4 hours and the supernatants used to measure TNF-α (A) and IL-6 (B) as reported in Material and Methods. The graphs are representative of three experiments with comparable results. Values are the mean ±SEM of 6 volunteers. Significance calculated (none found) by One-way ANOVA.

We next investigated if indicaxanthin had any effect on LPS-stimulated cytokine production. **Figure 6.9 A** shows that indicaxanthin at both concentrations, 0.001 and 10μM caused a slight and non-significant reduction of TNF-α production (-12.5% at 0,001μM and -8.7% at 10μM), after 4 hours of stimulation with LPS 100 ng/ml. Once again, there was a large degree of variation in the effects that depended on the volunteer.

This is clearly 'visible' in the graph in **Figure 6.9 B** where the single value of each volunteer has been plotted.

Interestingly, indicaxanthin seems to increase the production of IL-6 (6.46% at 0,001μM and 17.8% at 10μM) induced by LPS (**Figure 6.9 C-D**) but as in the case of TNF-α, these differences were highly variable and non-significant.

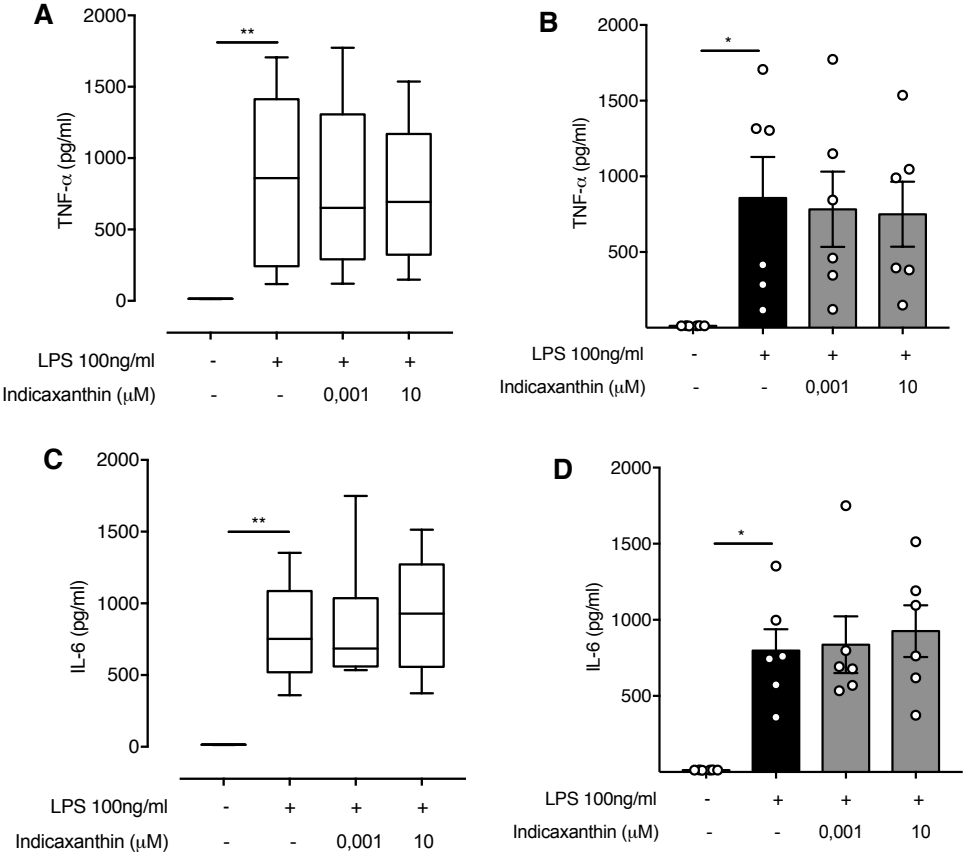


Fig 6.9 Effect of indicaxanthin on LPS-stimulated whole blood assay. The whole blood from healthy human volunteers was diluted 1:5 with RPMI medium, it was pre-treated with different concentrations of Indicaxanthin (0,001-10μM) and stimulated with LPS 100ng/ml for 4 hours and the supernatants used to measure TNF-α (A) and IL-6 (B) as reported in Material and Methods. The graphs are representative of three experiments with comparable results. Values are the mean ±SEM of 6 volunteers. Significance calculated (none found) by One-way ANOVA.

6.8 Indicaxanthin induces an increase in phagocytosis on WBA

The results of the previous tests showed some inconsistent and conflicting results on cytokine production. We reasoned that this could be to a number of reasons and variables but we also wanted to make sure that these puzzling data could also be obtained in another experimental system that is relevant to inflammation – namely the phagocytosis of pathogens. Therefore, we incubated blood pretreated or not with indicaxanthin with 5 different Gram-negative bacterial strains: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Acinetobacter baumannii*.

As shown in **Figure 7.0**, the results were very similar to those obtained with the cytokines and showed great variability together with a clear trend towards a positive increase in phagocytosis.

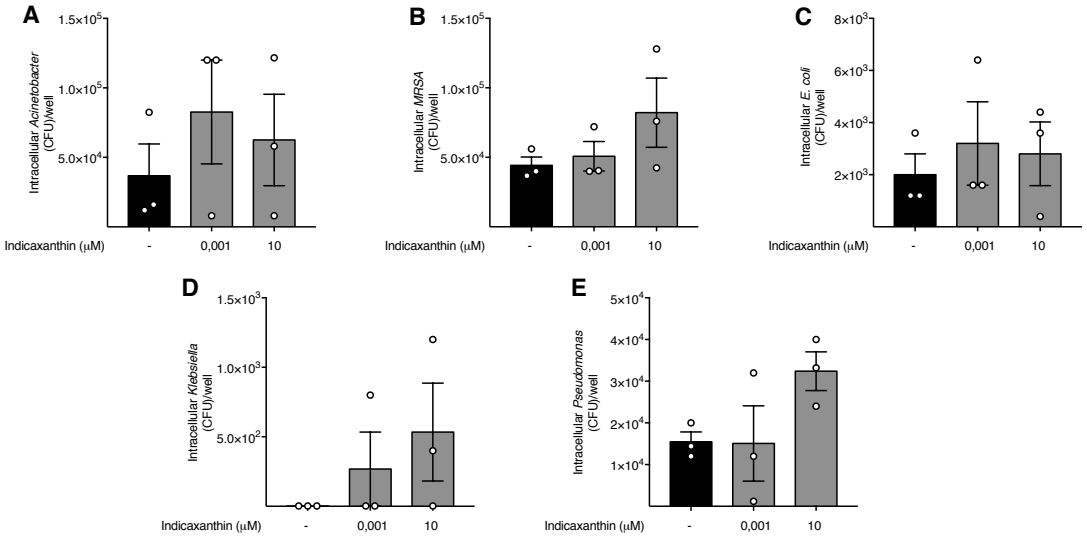


Fig 7.0 Effect of indicaxanthin on the phagocytosis in whole blood assay. The whole blood from healthy human volunteers (*n=3)* was diluted 1:5 with RPMI medium, it was pre-treated with different concentrations of Indicaxanthin (0,001-10μM). After one hour of pre-treatment were added the following bacterial strains: (A) *Acinetobacter baumannii vivo*, (B) *Staphylococcus aureus*, (C) *Escherichia coli,* (D) *Klebsiella pneumonia* and (E) *Pseudomonas aeruginosa*. At each time point the number of phagocyte bacteria was determinates as reported in Material and Methods. The graphs are representative of three experiments with comparable results. Values are the mean ±SEM of three separate experiments carried out in triplicate. Significance calculated (none found) by One-way ANOVA.

6.9 The Kirby-Bauer disk diffusion

To further assess a possible effect of indicaxanthin as antibacterial agent, we next testes the direct effects of the compound on the bacterial growth of *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumonia* and *Acinetobacter baumannii.*

After seeding the bacteria on LB agar plates, 3 paper disks containing 3 concentrations of indicaxanthin 50-100-200 µg/ml, a positive control containing 1mg ml of gentamicin, and a negative control containing PBS were added. The inhibition halos were evaluated after 24 hours of incubation. As it is possible to see in **Figure 7.1**, none bacterial strain was found to be sensitive to the action of indicaxanthin under the conditions examined. Putting everything together, it is possible to conclude that indicaxanthin does not show any direct effects on bacterial growth while it might have some indirect host-directed antibacterial function but this needs to be further investigated and tested.

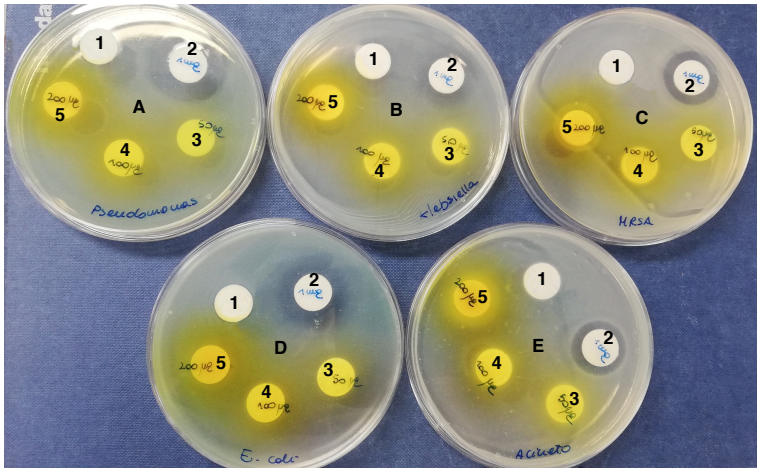


Fig 7.1 Antimicrobial activity of indicaxanthin against different Gram-negative bacteria. The potential antimicrobial activity of indicaxanthin was assessed by Kirby-Bauer plate diffusion assay on the following Gram-negative strains: (A) *Pseudomonas aeruginosa* (B) *Klebsiella pneumonia* and (C) *Staphylococcus aureus,* (D) *Escherichia coli* (E) *Acinetobacter baumannii*. The conditions tested on each bacterial strain are: (1) Negative Control (PBS), (2) Positive Control (1mg/ml Gentamicin), (3) (4) (5) 50-100-200µg/ml indicaxanthin respectively.

6.10 Indicaxanthin modules the production of LTB4

A recent molecular docking study on the mechanism of action of indicaxanthin (Tutone *et al*., 2018) has suggested that this molecule might bind and modulate the activity of several molecules news hypothetical molecular targets of indicaxanthin including the A4 hydrolase leukotriene (LTA4H), an enzyme that catalyzes the hydrolysis of A4 leukotriene into B4 leukotriene. This last lipid derivative works mainly as a chemotactic and activator of immune cells (Primiano *et al*., 1999). We, therefore, sought to investigate if, following treatment of whole blood with indicaxanthin, there was a change in LTB4 compared to control. Results obtained on the blood of 4 volunteers showed that after 4 hours of incubation with indicaxanthin there was an almost doubling of the level of LTB4 compared to control but once going, their response was very variable and some samples also showed an inhibition rather than an increase (**Figure 7.2**).

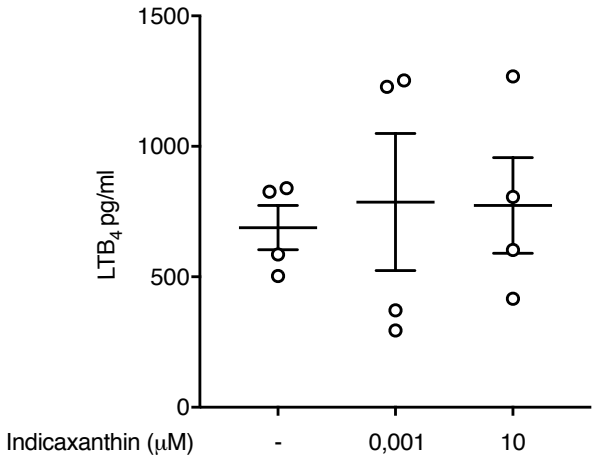


Fig 7.2 Effect of indicaxanthin on production of LTB4 in whole blood assay. The whole blood from healthy human volunteers was diluted 1:5 with RPMI medium, it was treated with different concentrations of Indicaxanthin (0,001-10μM) for 4 hours and the supernatants used to measure LTB4 as reported in Material and Methods. Values are the mean ±SEM of 4 volunteers. Significance calculated (none found) by One-way ANOVA.

CHAPTER 7

Conclusions

In conclusion, we demonstrated that indicaxanthin represents a novel phytochemical able to significantly inhibit human melanoma cells proliferation *in vitro* and markedly impair tumor progression *in vivo*. The retention of the antitumoral effects of indicaxanthin *in vivo*, its ability to interact with a key molecular target in the ethiopathogenesis of melanoma, i.e. NF-κB, and the ability to modulate the secretion of proinflammatory cytokines, appear of interest taking into account the limited and inadequate number of pharmacological agents for melanoma treatment. In this regard, it is important to underlie how the complexity and aggressiveness of melanoma made it hardly to be controlled with just one pharmacological/phyto-therapeutical agent. Along these lines, we propose indicaxanthin as a novel therapeutical agent to be further explored in more complex studies in combo therapy, i.e. with other therapeutical agents targeting different checkpoints of melanoma development.

Pilot human *ex vivo* data also showed that indicaxanthin is able to modulate TNF-α and Il-6 production in a whole blood *ex vivo* model. Furthermore, the phytochemical showed an increase in phagocytosis of 5 different Gram-negative pathogens on whole blood assay, without exerting antimicrobial activity on them. Preliminary studies aimed to understanding the hypothetical mechanism of action, showed that probably the observed effect could be attributed to the modulation of the LTB4, a chemotactic and activator of immune cells.

CHAPTER 8

Inhibitory effects of cynaropicrin on human melanoma progression

The promising results obtained of indicaxanthin' s experimentation, pushed us to evaluate the activity of another phytochemical, the cynaropicrin, so during my period at the Department of Pharmacy of University of Naples, I studied its effect in different *in vitro* model.

8.1 Inhibitory effects of cynaropicrin on human melanoma progression

As already stated in the previous chapters of this thesis, consumption of plant-based foods, such as fruits, vegetables and whole grains, rich in diverse phytochemicals, is inversely associated whit cancer incidence (Allen *et al*., 2018). Indeed, during the last decade, the use of phytochemicals as adjuvant chemotherapeutic agents in heterogeneous human carcinomas has shown an upward trend (Sak *et al*., 2012). A growing body of scientific evidence has established the role of dietary factors in the protection of the skin from various diseases such as cancer (Ng *et al*., 2018).

Among plant-derived phytochemicals, sesquiterpene lactones (SQLs) represent one of the largest group (Chadwick *et al.*, 2013). They have received widespread attention because of their potent bioactivities, including antibiotic, anti-tumor, anti-inflammatory, insect-feeding deterrent, phytotoxic, and schistosomicidal effects (Adekenov *et al.,* 2017; Bosco *et a*l., 2017). Cynaropicrin is a sesquiterpene lactone of a guaianolide type that has been firstly found in the edible plant artichoke (*Cynara scolymus L.*), where it is responsible for approximately 80% of the characteristic bitter taste (Eljounaidi *et al*., 2015). To date, cynaropicrin it has also been isolated, from different botanical sources, particularly from several species of the genus Saussurea and Centaurea (Formisano *et al*., 2017; Milosevic *et a*l., 2013; Pandey *et al.,* 2007; Ren *et al.,* 2007; Saito *et al*., 2012). Cynaropicrin has

shown to possess various biological activities and has demonstrated extraordinary pharmacologic properties such as anti-hepatitis C virus, anti-hyperlipidemic, anti-trypanosomal, anti-malarial, antifeedant, antispasmodic, anti-photoaging, activation of bitter sensory receptors, as well as anti-tumor action and anti-inflammatory action (Elsebai et al., 2016).

In our study, cynaropicrin was obtained from the aerial parts of *Centaurea drabifolia* subsp. detonsa (Bornm.) Wagenitz, a plant endemic of Turkey, very popular to prepare infusions to be used both as refreshing drink and as traditional remedy, with anti-inflammatory applications (Honda *et al*., 1996). In the present study, we explored for the first time the protective and therapeutic potential of the phytochemical cynaropicrin in human melanoma cells. We selected the highly invasive A375, Sk-Mel-28 and WM983B human melanoma cell lines as clinical model expressing the BRAFV600E oncogenic mutation and the WM3060 cell line as control since they do not express the BRAFV600E mutation.

8.2 Materials and methods

Reagents and Cell Culture

The human melanoma cell lines A375 cells were purchased from Sigma-Aldrich (Milan, Italy), Sk-Mel-28 were purchased from IRCCS AOU San Martino – IST (Genua, Italy), WM3060 and WM983B cells were purchased from Rockland (Limerick, Ireland). A375 and Sk-Mel-28 were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 µmol/L non-essential amino acids, penicillin (100 U/mL), streptomycin (100 µg/mL), and 1 mmol/L sodium pyruvate (all from Sigma-Aldrich, Milan, Italy). WM3060 and WM983B were cultured

in Tumor Specialized Media (1:5 Leibovitz's MCDB153), containing 2% Inactivated FBS and 1.68 mM $CaCl_2$. Cells were grown at 37◦C in a humidified incubator under 5% CO_2. All cell lines used in this study were characterized by the cell bank where they were purchased. Cynaropicrin was diluted in DMSO to produce a stock solution of 10 mM for in vitro experiments.

Proliferation Assay

Cell proliferation was measured by the 3-[4,5-dimethyltiazol2yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. Melanoma cells (A375, Sk-Mel-28, WM3060 and WM983B) were seeded on 96-well plates (2-5×10^3 cells/well) and treated with cynaropicrin (0,1-3-10-30-µM) for 24–48 h before adding 25 µl of MTT (Sigma, Milan, Italy) (5 mg/ml in saline). Cells were incubated for additional 3 h at 37◦C. Thereafter, cells were lysed and dark blue crystals were solubilized with a solution containing 50% (vol/vol) N,Ndimethyl formamide, 20% (wt/vol) sodium dodecylsulfate with an adjusted pH of 4.5. The OD of each well was obtained by measuring the absorbance at 620 nm using Multiskan GO microplate reader (Thermo Fisher Scientific, MA, USA).

Apoptosis assay

A375 were seeded in 35 mm culture dishes and allowed to attach overnight. The cells were treated with cynaropicrin (30 µM) for 24–48 h, collected, and washed twice with PBS. Apoptosis was detected using the Annexin V- FITC apoptosis detection kit (eBioscience. Thermo Fisher Scientific MA, USA) according to the manufacturer's instructions. Following the Annexin V and PI double staining, the cells were subjected to flow cytometric analysis. A minimum of 20,000 events for each sample were collected and data were analyzed using BriCyte E6 (Mindray, P.R. China).

Preparation of Cellular Extracts and Western Blot Analysis

A375 cells were treated with cynaropicrin 30 µM for 24 and 48 h. Whole-cell or nuclear extracts were prepared as previously described (De Cicco *et al*., 2017). The protein concentration was measured by the Bradford method (Bio-Rad, Milan, Italy). Equal amounts of protein (40 µg/sample) from whole or nuclear cell extracts were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a nitrocellulose membranes (Trans-Blot Turbo Transfer Starter System, Biorad). The membranes were blocked for 2 h in 5% low-fat milk in PBS with 0.1% Tween 20 (PBST) at room temperature. Then the filters were incubated with the following primary antibodies: Bcl-2 (#2876; Cell Signaling, USA; diluted 1:1000), caspase 3 (#9662; Cell Signaling, USA; diluted 1:1000), PARP (#9542; Cell Signaling, USA; diluted 1:1000), p44/42 MAPK (Erk1/2) (#9102; Cell Signaling, USA; diluted 1:1000), Phospho-p44/42 Erk MAPK (Erk1/2, Thr202/Tyr204) XP (#4370; Cell Signaling, USA; diluted 1:2000), NF-κB p65 XP (#8242; Cell Signaling, USA; diluted 1:1000), Nrf2 (sc-722; Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:100), β-actin (sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:1000), GAPDH (#2118; Cell Signaling, USA; diluted 1:1000), α-tubulin (#3873; Cell Signaling, USA; diluted 1:1000), overnight at 4 ◦C. The membranes were washed 3 times with PBST and then incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:2000) for 2 h at room temperature. The immune complexes were visualized by the ECL chemiluminescence method and acquired by the Image Quant 400 system (GE Healthcare).

Invasion Assay

The assay was performed using chambers with polycarbonate filters with 8-µm nominal pore size (Millipore, USA) coated on the upper side with Matrigel (Becton Dickinson Labware, USA). Briefly, the chambers were placed into a 24-well plate and A375 cells (2.5×10^5 /mL) were plated in the upper chamber, with or without cynaropicrin (3-10 µM), in serum-free DMEM. After the incubation period (16 h), the filter was removed, and non-migrant cells on the upper side of the filter were detached with the use of a cotton swab. Filters were fixed with 4% formaldehyde for 15 min, and cells located in the lower filter were stained with 0.1% crystal violet for 20 min and then washed with PBS. The filters were examined microscopically and cellular invasion was determined by counting the number of stained cells on each filter in at least 4– 5 randomly selected fields.

Wound healing assay

A375 cells were seeded in 12-well plates ($2x10^5$ cells/well) and grown to full confluence. A 200µL pipette tip was used to create a wound in the monolayer. Cynaropicrin (3-10 µM) was added after imaging of the wells with the microscope at 0 hours (wound induction). Imaging was repeated after 24 and 48 h. An ImageJ macro, the MRI Wound Healing Tool (MRI Redmine) was used for calculating the area of the cell-free gap at 0-24-48 hours after wound induction.

Clonogenic Assay

A375 cells ($1x10^3$ cells/well) were seeded in 6-well plates and treated with cynaropicrin 3 µM for 48 h. After, fresh medium (drug-free) was changed every 2 days. Cells were cultured for 14 days to allow the colonies to form. Formed colonies were washed twice with 1xPBS, fixed by 4% paraformaldehyde, and stained with 0.5% crystal violet and

colonies containing more than 50 cells (established by microscopy) were counted manually. Images of the colonies were obtained using a digital camera.

RNA purification and quantitative real-time PCR (RT-qPCR)

A375 cells were treated with cynaropicrin 30 μM for 24 and 48 h. Total RNA was isolated by using TRI-Reagent (Sigma-Aldrich, Milan, Italy), according to the manufacturer's instructions, followed by spectrophotometric quantization. Final preparation of RNA was considered DNA- and protein-free if the ratio between readings at 260/280 nm was ≥1.7. Isolated mRNA was reverse-transcribed by use of iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Milan, Italy). Then, quantitative real-time-PCR was performed using CFX384 real-time PCR detection system (Bio-Rad, Milan, Italy). Primer sequences were:

HMOX1:

5'GCCGTGTAGATATGGTACAAGGA3';5'AAGCCGAGAATGCTGAGTTCA-3'

GCLM:

5'-AGGAGCTTCGGGACTGTATCC-3'; 5'-GGGACATGGTGCATTCCAAAA-3'

GCLC:

5'GTTGGGGTTTGTCCTCTCCC-3'; 5'-GGGGTGACGAGGTGGAGTA-3'

Samples were amplified in triplicate using SYBR Green master mix kit (Bio- Rad, Milan, Italy). A control blank for each primer pair was used to control for contamination or primer non-template dimers formation, and the Ct value for each experimental group was determined. The S16 housekeeping gene was used as an internal control to normalize the Ct values, using the $2^{-\Delta Ct}$ formula.

Intracellular ROS measurement

The generation of intracellular reactive oxygen species (ROS) was estimated using the fluorescence probe 2′,7′-dichlorfluorescein-diacetate (H2DCF-DA). For the experiments, A375 cells were plated in 96-multiwell black plates (Corning, USA) at the density of 3×10^3 cells per well and incubated with cynaropicrin (3–10-30 µM) for 24 h. After washing, cells were incubated for 1 h with 200 µl of 100 µM H2DCF-DA in HBSS containing 1% FBS. Finally, cells were rinsed and incubated with the Fenton's reagent (H_2O_2/Fe^{2+} 2 mM) for 3 h at 37 °C. The DCF fluorescence intensity was detected using a fluorescent microplate reader (excitation 485 nm and emission 538 nm; GloMax®-Multi Detection System, Promega). The intracellular ROS levels were expressed as fluorescence intensity.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed with a number of $n \geq 3$. Data were analyzed with GrapdPad Prism 6.0 software program (GraphPad Software, Inc., San Diego, CA, United States). Significance was determined using Student's 2-tailed t-test. Results were considered significant at P-values ≤ 0.05 and are labeled with a single asterisk. In addition, P-values ≤ 0.01 and 0.001 are designated with double and triple asterisks, respectively.

8.3 Results

Cynaropicrin inhibits proliferation and induces apoptosis in human melanoma cells

To detect the effect of cynaropicrin (structure shown in **Figure 8.1 A**) on the proliferation of human melanoma cells, an MTT assay was performed. The human melanoma metastatic cells A375, Sk-Mel-28, WM983B and WM3060 were incubate with different

concentrations of cynaropicrin (0.1-3-10-30 μM) and cell viability was determined after 24h and 48h. As shown in **Table 8.I**, cynaropicrin reduced cell proliferation in a time-and concentration-dependent manner. The IC_{50} values for each cell line were respectively 25 μM, 57 μM, 27 μM and 162 μM following 48h of incubation with cynaropicrin. On the basis of the IC50 values we selected the A375 cell line for further experiments. To confirm the anti-proliferative effect of cynaropicrin we performed Annexin V/PI double staining analysis. A375 cells were treated with cynaropicrin (30μM) and the percentage of cells undergoing apoptosis or necrosis was evaluated by flow cytometric analysis at 24h and 48h. Cynaropricrin treatment induced apoptosis in a time–dependent manner (**Figure 8.1 B-C**). The pro-apoptotic effect of cynaropicrin was confirmed by the activation of caspase-3 and the cleavage of its substrate poly (adenosine diphosphate-ribose) polymerase (PARP), evaluated by western blot analysis, in A375 cells following cynaropicrin treatment (30 μM) (**Figure 8.1 D**). We further assessed the expression of three anti-apoptotic proteins, X-chromosome-linked inhibitor of apoptosis protein (XIAP) and B cell lymphoma gene-2 (Bcl-2), whose expression is modulated by the transcriptional activity of NF-κB. Western blot experiments showed that cynaropicrin markedly decreased the expression of all the anti-apoptotic genes considered (**Figure 8.1 E**) in A375 human melanoma cells.

Table 8.1: Effect of cynaropicrin on human melanoma cells proliferation.

(A) 24h

Cell line	CTRL	Cyn 0.1 µM	Cyn 3 µM	Cyn 10 µM	Cyn 30 µM
A375	0,459±0,01	0,450±0,02	0,432±0,02**	0,362±0,005***	0,274±0,02***
SK-Mel-28	0,536±0,008	0,516±0,003	0,500±0,001	0,488±0,003***	0,475±0.003***
WM983B	0,293±0,01	0,285±0,01	0,268±0,004	0,234±0,005***	0,200±0,005***
WM3060	0,556±0,007	0,532±0,009	0,516±0,002	0,475±0,006***	0,431±0,007***

(B) 48h

Cell line	CTRL	Cyn 0.1 µM	Cyn 3 µM	Cyn 10 µM	Cyn 30 µM
A375	0,819±0,03	0,736±0,02	0,689±0,007**	0,578±0,008***	0,356±0,03***
SK-Mel-28	0,758±0,010	0,684±0,020	0,630±0,006***	0,615±0,004***	0,540±0.003***
WM983B	0,280±0,008	0,275±0,008	0,245±0,006	0,169±0,002***	0,141±0,003***
WM3060	0,846±0,01	0,853±0,005	0,819±0,002	0,793±0,005	0,761±0,01***

Table 8.1: Effect of cynaropicrin on human melanoma cell proliferation. Human melanoma cells A375, Sk-Mel-28, WM983B and WM3060 were incubate with different concentrations of cynaropicrin (cyn) (0.1-30 µM) and cell viability was determined after 24 and 48h by using the MTT assay. Cynaropicrin inhibited the growth of all melanoma cells in a time-and concentration-dependent manner. Data are expressed as mean ± SEM of OD values. Results were generated with three biological replicates each of them performed in technical quadruplicate (**P < 0.01; ***P < 0.001 vs CTL).

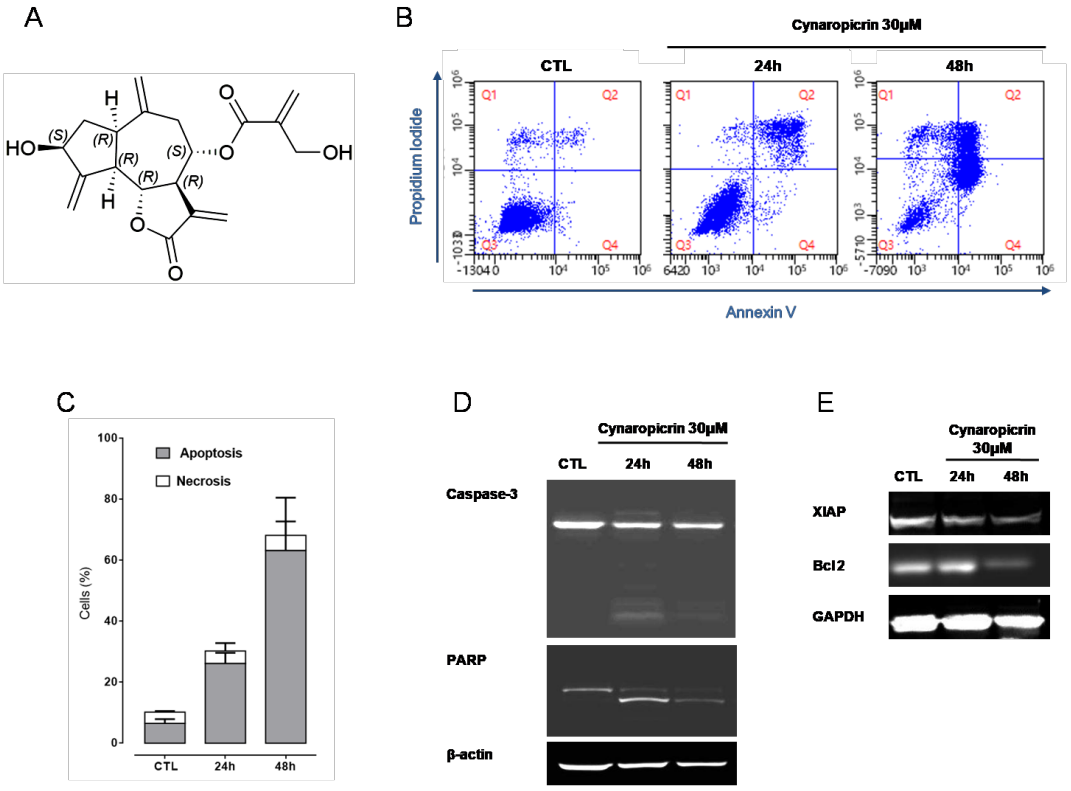


Figure 8.1. Cynaropicrin induces apoptosis in human melanoma cells (A) Chemical structure of cynaropicrin. **(B)** Induction of apoptosis by cynaropicrin in A375 cells was detected by annexin V/propidium iodide staining. A375 cells were treated with cynaropicrin 30 µM and apoptosis was determined at 24 and 48 h by flow cytometric analysis. **(C)** Quantification of cell apoptosis at various time points, showing that 60% of cells treated with cynaropicrin for 48h exhibit markers of apoptosis. Data are expressed as percentage of apoptotic or necrotic cells. Experiments were performed in triplicate (n = 3). **(D)** Western blot analysis of caspase-3 and PARP in A375 whole-cell lysates following the treatment with cynaropicrin (30 µM) for 24 and 48 h. The representative blots show a time-dependent cleavage of caspase-3 and of its substrate PARP.β-actin was detected as a loading control. The data shown are representative of three independent experiments with the similar results. **(E)** Western blot analysis of XIAP and Bcl-2 carried out on A375 whole-cell lysates following the treatment with cynaropicrin (30 µM) for 24 and 48 h. The representative blots show a reduction of the expression of all proteins. GAPDH was detected as a loading control. The data shown are representative of three independent experiments (n = 3) with similar results.

Cynaropicrin inhibits cell motility, invasion and colony formation of human melanoma cells

Anti-metastatic activity of cynaropicrin was further investigated by evaluating its ability to inhibit some of the malignant features of melanoma cells, *in vitro*. Wound healing assay was used to determine whether sub-IC50 concentration of cynaropicrin could inhibit A375 cells motility. Treatment with cynaropicrin (3 and 10 µM) significantly and time-dependently reduced A375 cells migration as compared to untreated cells (**Figure 8.2 A-B**).

A critical event in tumor invasion and metastasis is the ability of tumor cells to invade through the extracellular matrix, allowing tumor cells to move beyond the confines of primary tumor environment (Obenauf *et al.,* 2015). To examine the effect of cynaropicrin on cell invasivity, Boyden chamber invasion assay was carried out. As shown in Fig. 2C and D treatment with cynaropicrin (3 µM) significantly reduced trans-well invasion of A375 cells across biological matrices. Further, we found that cynaropicrin was able to reduce colony formation ability of A375 cells, which correlates with the capacity of tumor cells to produce progeny and form metastasis *in vivo* (Franken *et al*., 2006). As shown in **Figure 8.2 E** and **F** sub-IC_{50} doses of cynaropicrin significantly reduces the number of colonies by 48% (3 µM) and by 84% (10 µM) as well as focus diameter.

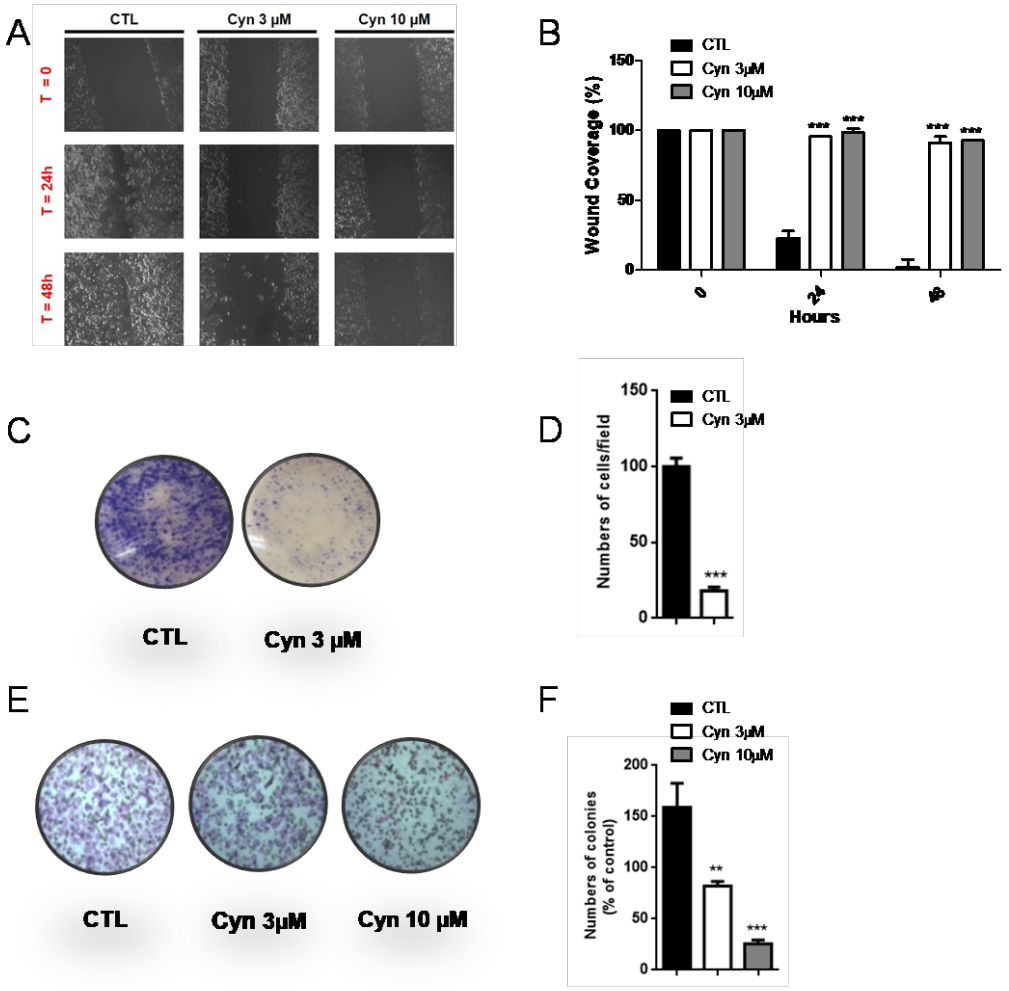


Figure 8.2 Cynaropicrin inhibits motility, invasion and colony formation ability of A375 cells. (A) A375 cells were grown to confluence and scratched with sterile tip; cynaropicrin 3 and 10 µM was added to cultures as indicated and the migration was determined evaluating the rate of cells filling the scratched area. Representative photographs of migratory A375 cells at zero hour and at 24 h and 48h later to assess the degree of wound healing. **(B)** The scratched areas were quantified in three random fields in each treatment. Data are shown as mean ± SEM of three indipendent experiments (n = 3). ***P<0.001 *vs* CTL. **(C)** Cell invasion was determined by using Boyden chamber coated with matrigel. A375 cells were treated with cynaropicrin 3 µM and after 16 h the number of cells passing throughout the matrigel was determined. **(D)** Invasive cells were counted using image software as the number of migrated cells per high-power field (HPF). Five fields were counted in triplicate from each insert. Data are shown as mean ± SEM of three indipendent experiments (n = 3). ***P<0.001 *vs* CTL. A375 cells were treated with cynaropicrin 3 and 10 µM for 48 h and then cultured for 14 days to allow cells to form colonies. Representative photographs **(E)** and average number **(F)** of A375 stained colonies obtained from three independent experiments. Data are shown as mean ± SEM of three independent experiments (n = 3). **P<0.01, ***P<0.001 *vs* CTL.

Cynaropicrin inhibits the MAPK/ERK and NF-κB pathways in human melanoma cells

Among the various intracellular signaling pathways activated in melanoma cells, the NF-κB and the MAPK signaling pathways have been considered the crucial regulators in the initiation and development of cutaneous melanoma. Aberrant activation of the MAPK pathway is responsible for cell proliferation, survival, invasion and motility (Holderfield, *et al*., 2014). Therefore, we examined whether the ability of cynaropicrin to reduce the proliferation, migration and invasion of melanoma cells was associated to the MAPK pathway activity. ERK, a downstream substrate of the MAPK signaling, is hyperactivated in about 90% of melanoma cells following BRAF mutation. Our results demonstrate that phosphorylation of ERK was significantly downregulated in A375 cells after treatment with cynaropicrin (30 μM) for 24h (**Figure 8.3 A**). Mutational activation of BRAF has been also associated with an enhanced activity of NF-κB and increased survival of melanoma cells (Liu *et al*., 2007). NF-κB is the major anti-apoptopic factor, inducing the transcription of several anti-apoptotic proteins. Thus, constitutive induction of NF-κB lead to a consequent increase in proliferation rates and resistance to apoptosis of melanoma cells (Madonna *et al*., 2012). In order to verify the effect of cynaropicrin on NF-κB activity, western blot analysis was carried out on nuclear extracts obtained from A375 cells. The p65/RelA protein, which is a component of NF-κB family normally overexpressed in melanoma cells, was reduced in the nuclei of A375 cells, following treatment with cynaropicrin (30μM) for 24 and 48h (**Figure 8.3 B**).

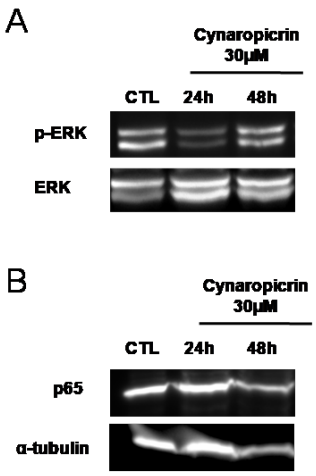


Figure 8.3. Cynaropicrin inhibits ERK and NF-κB activation in A375 cells. (A) Western blot analysis carried out on the whole-cell lysates obtained from A375 cells treated with cynaropicrin (30 µM) for 24 and 48 h. The representative blots show an inhibition of ERK activation as demonstrated by the reduction of p-ERK band intensity at 24h. **(B)** Western blot analysis carried out on the nuclear extracts obtained from A375 cells treated with cynaropicrin (30 µM) for 24 and 48 h. The representative blot shows a time-dependent reduction of p65 nuclear translocation. α-tubulin were detected as a loading control. Experiments were performed in triplicate (n = 3).

Cynaropicrin reduces ROS production and modulates the expression of anti-oxidant enzymes in human melanoma cells throughout the Nrf-2 pathway

Oxidative stress is associated with cancer development and progression by a direct mechanism involving DNA damage or indirectly by modulating cell signal transduction pathways (Toyokuni *et al*., 2006). Therefore, we evaluate the ability of cynaropicrin to reduce oxidative stress in melanoma cells. The exposure of A375 melanoma cells to H_2O_2/Fe^{2+} (2 mM) produced a significant increase in ROS formation. Interestingly, pre-treatment for 24 h with cynaropicrin (10-30 µM) greatly reduced ROS formation as measured by the inhibition of DCF fluorescence intensity (**Figure 8.4 A**).

To investigate the cellular mechanism responsible for the ROS neutralizing activity of cynaropicrin in melanoma cells, we measured the effect of cynaropicrin on the expression of some anti-oxidant enzymes such as glutamate-cysteine ligase (GCL) and heme

oxygenase-1 (HMOX-1). GCL, the rate-limiting enzyme involved in the de novo glutathione (GSH) synthesis, consists of catalytic (GCLC) and modulatory (GCLM) subunits. Treatment with cynaropicrin at 30 μM significantly enhanced the expression of both GCLC and GCLM mRNA in a time dependent manner (**Figure 8.4 B-C**). Similar results were obtained for HMOX-1 mRNA (Figure 4D). Most of the genes encoding phase II detoxifying and antioxidant enzymes are under the control of NF-E2-related factor 2 (Nrf2), the major transcription factor involved in the cellular protection against oxidative stress (Loboda *et al*., 2016). Therefore, we determine whether cynaropicrin could activate Nrf2 in A375 cells. As show in **Figure 8.4 E**, cynaropicrin-treated cells displayed increased Nrf2 accumulation in the nuclear fraction.

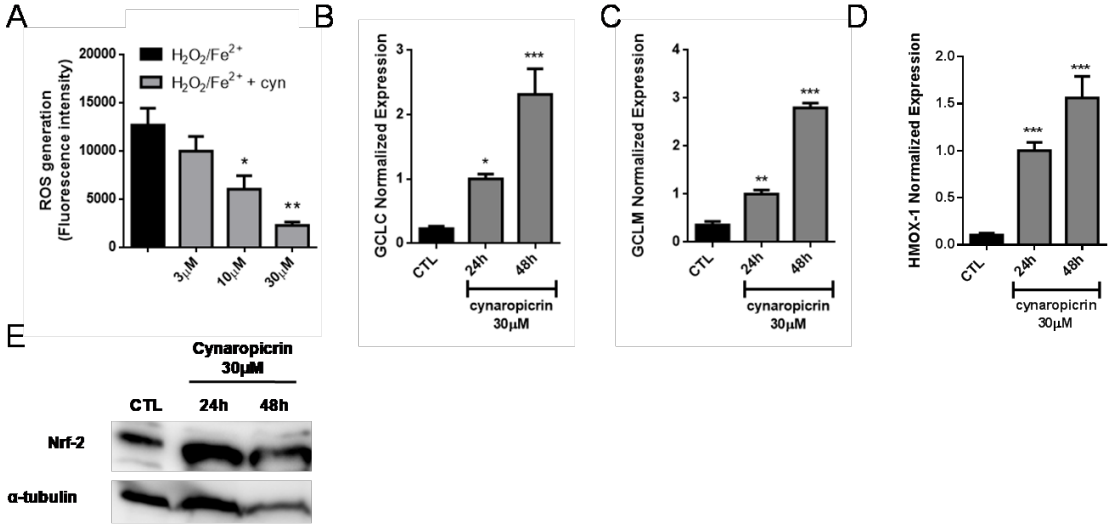


Figure 8.4. Cynaropicrin reduces ROS production and modulates the expression of anti-oxidant enzymes in human melanoma cells throughout the Nrf-2 pathway. (A) Intracellular ROS production in A375 cells was measured using a DCF- DA assay. A375 cells were treated with Fenton's reagent (2 mM H_2O_2/Fe^{2+}) alone or in presence of cynaropicrin (3–30 μM) for 24 h. Pre-treatment with cynaropicrin reduced ROS generation in A375 cells in concentration-dependent manner. Results are show as mean ± SEM of three indipendent experiments (n = 3). *P<0.05, **P<0.01 *vs* H_2O_2/Fe^{2+} alone. mRNA expression levels of GCLC **(B)**, GCLM **(C)** and HMOX-1 **(D)** were increased in A375 cells treated with cynaropicrin (30 μM) for 24 and 48 h. Total RNA was extracted, and the amounts of each gene was quantified with real-time PCR analysis. The results were obtained using the $2^{-\Delta Ct}$ formula and normalized to the corresponding

amounts of S16 mRNA. Data are shown as mean ± SEM of three indipendent experiments (n = 3), each performed in technical quadruplicate. *P<0.05, **P<0.01, ***P<0.001 *vs* CTL. **(E)** Western blot analysis carried out on the nuclear extracts obtained from A375 cells shows a great increase of Nrf-2 nuclear localization following the treatment with cynaropicrin (30 μM) for 24 and 48 h. α-tubulin was detected as a loading control. Experiments (n = 3) were performed in triplicate.

8.4 Discussion

Despite the increased knowledge of the biology and pathogenesis of melanoma reached in the last years, the incidence has grown considerably throughout the world, making the development of preventive measures particularly urgent. A great number of studies have been published recently investigating the beneficial role of natural dietary compounds in the prevention and therapy of malignant melanoma. Due to their safety, low toxicity and antioxidant properties, fruit, vegetables and other dietary elements (phytochemicals and minerals) have been identified as chemopreventive agents (Ombra *et al*., 2019).

In the present study, we explored the anticancer effects of cynaropicrin, an abundant constituent of artichoke and of other numerous plants belonged to the Asteraceae (Compositae) family. In detail, we found that cynaropicrin reduced the proliferation of human metastatic melanoma cells in a time and dose dependent manner. The results of the MTT assay demonstrated that all BRAF mutant melanoma cells (Sk-Mel-28, A375, WM983B) were more sensitive to cynaropicrin treatment, with lower IC50, compared to BRAF wild-type melanoma cells (WM3060). The anti-proliferative effect of cynaropicrin was mediated by the induction of apoptosis in human melanoma cells A375 as demonstrated by the cytofluorimetric studies with Annexin V and PI. Caspase-3 is a frequently activated death protease which cleave PARP, a DNA repair enzyme. Our results clearly demonstrate that cynaropicrin-induced apoptosis was mediated by caspase pathway activation as consequence of caspase-3 and PARP cleavage in cynaropicrin-

treated A375 cells. In addition, the expression of anti-apoptotic proteins such as Bcl-2 and XIAP was also reduced in melanoma cells following the treatment with cynaropicrin. Collectively, these results suggest that cynaropicrin is a cytotoxic and apoptosis inducing natural product derivative. Cutaneous melanoma is an aggressive malignancy due to its high metastatic ability. Patients with metastatic melanoma show poor prognosis with 5-year survival rate less than 15% (Tas *et al.*, 2012). Melanoma metastasis formation is related to the capacity of melanoma cells to acquire distinctive features such as: motility and invasion, plasticity, and ability to colonize secondary tissues (Welch et al., 2019). Thus, chemopreventive agents should be also able to prevent or delay tumor promotion and progression. We found that cynaropicrin significantly reduced migration, invasion and clonogenic ability of human melanoma cells. A growing body of evidence supports that constitutive activation of the Ras/Raf/MEK/ERK (MAPK) signalling pathway in melanoma is associated with increased migratory and metastatic capacity (Lin *et al*., 2010; Testa *et al.,* 2017). In addition, MAPK also promotes hyperactivation of the transcription factor NF-κB that regulates the expression of many anti-apoptotic, pro-proliferative and pro-metastatic genes (Liu *et al*., 2007). In melanoma, the NF-κB signalling pathway has been also found to be hyperactivated and it plays an important role is in cell survival, invasion and metastasis (Ueda & Richmond, 2006). Thus, inhibition of MAPK and NF-κB pathways represent the most promising strategy to prevent melanoma invasion and metastasis. In the present study, we found that cynaropicrin greatly reduced the activation of ERK as well as the translocation of NF-κB p65 in to the nucleus suggesting the potential mechanism of its anti-proliferative, anti-invasive and anti-migratory activity.

Oxidative stress also contributes to the melanoma initiation and progression either by a direct mechanism involving DNA damage or indirectly by activating pro-tumorigenic

pathways (Cannavo *et al*., 2019). The importance of oxidative stress in melanoma is reinforced by the findings that mutations in several melanoma-associated genes, including the common somatic BRAFV600E mutation, result from ROS activity (Landi *et al*., 2006; Meierjohann *et al*., 2014). Secondarily, ROS promotes several signalling pathways, including MAPK, leading to cell proliferation and tumor initiation and promotion (Venza *et al*., 2015). Therefore, reducing oxidative stress might be a protective mechanism of action of many chemopreventive agents in order to stop or delay the occurrence of malignancy. Here, we found that cynaropicrin shows antioxidants properties since it reduces ROS intracellular generation in A375 cells. An important role in the regulation of cellular redox balance in mammals is played by the transcription factor Nrf-2, which is considered as a master regulator of antioxidant response. Nrf-2 regulates the expression of protective antioxidant and phase II detoxificant enzymes including GCL, HMOX1, thioredoxin reductase 1 (Txnrd1), and NAD(P)H-quinone oxidoreductase 1 (NQO1) (Kansanen, Kuosmanen, Leinonen, & Levonen, 2013). These enzymes protect against the development of cancer by catalyzing reactions that convert highly reactive, carcinogenic chemicals to less reactive products (Kensler *et al.,* 2010). Thus, Nrf-2 represents an attractive target for cancer cells and Nrf-2 inducers could be beneficial in cancer treatment approaches. Our results demonstrate that cynaropicrin increase Nrf-2 activity in melanoma cells and the expression of its antioxidant target gene, such as GCL and HMOX-1, suggesting that inactivation of ROS via the Nrf-2 pathway is one of the contributing factors towards the chemopreventive potential of cynaropicrin. Natural products are in most cases the main font of cytotoxic compounds for cancer treatment, while less attention has been paid to natural compounds with antimigratory properties. In this light, the evidences shown in our study for a protective role of cynaropicrin in melanoma carcinogenesis, including cell proliferation inhibition,

apoptosis induction, anti-metastatic and antioxidants potential, modulation of pro-tumorigenic signalling pathways, assume great relevance.

Thus, cynaropicrin may serve as a potential chemopreventive agent in the development of therapeutic strategies for the prevention and/or treatment of metastatic melanoma.

CHAPTER 9

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