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Evaluation of anti-proliferative and antioxidant potential of tomato extract against melanoma

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Abstract

Melanoma is a form of skin cancer occurring prevalently, in fair-skinned and light-haired person; its incidence is constantly increasing and in Italy is the second most frequent cancer males under 50 and third most frequent in female under 50 years. Increasing evidences are demonstrating that antioxidants from vegetables have both antriproliferative activity and ratio-sensiting property on cancer cell. Here, we will investigate the anti-proliferative and ratio-sensitizing potential of polyphenolic fraction from different tomato cultivars in melanoma cells lines. Aim of this work is to expand the existing knowledge on the effects of anti-oxidants and antiproliferative on melanoma cancer cells. The melanoma cancer cell line we used for this study is M14 with mutation in the BRAF gene which is involved in cancer drug resistance. Polyphenolic fraction obtained from our tomatoes extracts has been examined for identification of polyphenols by High Performance Liquid Chromatography technology, and we will study the effects of these compounds on the main pathways to be deregulated in cancer (Rb2, p21/^{Cip1} and p27/^{Kip1}), according with our published results. Cytotoxicity assay, western blot, qRT-PCR, cell cycle analysis and will be performed to study the impact of these compouds on melanoma cells biology. In the second part of this work we evaluated also the capacity to inhibition of UV-A induced ROS generation in fibroblast cell by tomato extract and the ability to screen UV light and to reduce the harm to DNA caused by free oxygen radicals.

Abbreviations

AIOM: Italian Association of Medical Oncology

CDKN2A: Cyclin-dependent Kinase Inhibitor 2A

CDK4: Cyclin-dependent Kinease Inhibitor 4

TERT: Telomerase Reverse Transcriptase

TBP: Total Body Photography

AJCC: American Joint Committee on Cancer

RGP: Radial Growth Phase

VGP: Vertical Growth Phase

SSM: Superficial Spreading Melanoma

NMM: Nodular Malignant Melanoma

LMM: Lentiginous Malignant Melanoma

ALM: Acral Lentiginous Melanoma

MD: Desmoplastic Melanoma

MM: Mucosal Melanoma

CTLA-4: Cytotoxic T-Lymphocyte Antigen 4

PD-1: Programmed Cell Death Protein 1

PD-L1: Programmed Death-Ligand 1

MD: Mediterranean Diet

HDL: High-Density Lipoprotein

NF-kB: Nuclear Factor Kappa-Light-Chain enhancer of activities B cells

MAPK: Mitogen-Activated Protein Kinase

PGE2: Prostaglandine 2

NO: Nitric Oxide

EM: Effective Microorganism

B: Brandywine

N: New Yellow

P: Cherry Purple

TSS: Total Soluble Solid

TA: Titratable Acidity

HPLC: High Performance Liquid Chromatography

DPPH: 2,2-diphenyl-1-picrylhydrazyl

HSFs: Primary Human Skin Fibroblast

SDS-PAGE: Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis

TBS-T: Tris-Buffered Saline

ROS: Reactive Oxygen Species

RPMI: Roswell Park Memorial Institute

FACS: Fluorescence-Activate Cell Sorting

RTq-PCR: Reverse Transcriptase-Polymerase Chain Reaction

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Chapter 1

Malignant Melanoma

1.1 Introduction

Skin cancer is one of the most common tumors in the world. The impact of this cancer has increased in recent years and this growth can be explained by different factors, such as climatic changes, intense intermittent exposure to the sun, depletion of the ozone and variation of lifestyle. Skin cancers can be classed in two types: Melanoma and Non-Melanoma.

1.2 Epidemiology

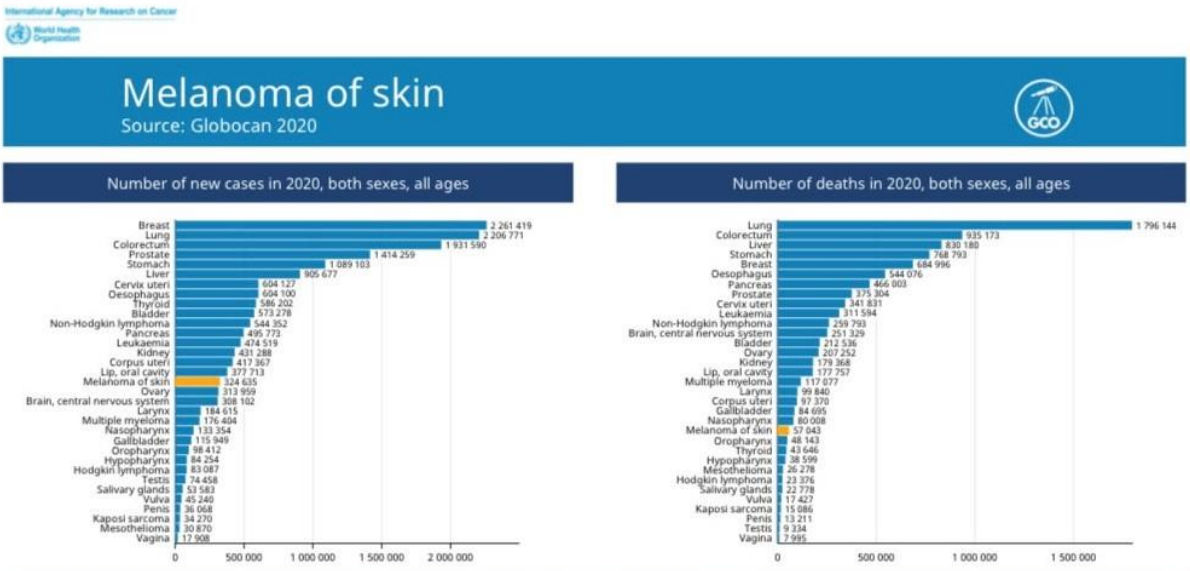
According to GLOBOCAN 2020 (https://gco.iarc.fr/) there were nearly 300,000 new case of malignant melanoma every year, making melanoma the 17th most common cancer occurring in men and women. Non-Melanoma skin cancer, instead, is the 5th most prevalent tumor in men and women, reaching in 2020 about 1 million of diagnosis in the world. Melanoma cancer represents only 1% of all skin tumors but causes approximately 60% of all deaths for skin cancer all over the world . Over the last forty years the incidence of melanoma is considerably increased throughout the world with the highest incidence recorded in Australia with 40 new cases per 100000 population.

Figure 1.1 shows the distribution of incidence and mortality of melanoma with the highest incidence of melanoma cancers in Australia and New Zealand (35.8 per 100000). Even in Australia and New Zealand (2.7 per 100000) there is the highest mortality rate followed by Western Europe (1.5 per 100000).

In Figure 1.2 we can see the distribution of melanoma cases and deaths divided by gender and age. Males seem to be more affected than females; moreover it is one of the major tumors that arise at a young age.

In Italy (Figure 1.3) melanoma constitutes the second most frequent cancer in males under 50 and the third most frequent in females under 50 years. Almost 12,300 new cases are expected in 2019 with a slight preponderance in males. The incidence trend

appears to be on a statistically significant increase both in males (+ 4.4% per year) and in women (+ 3.1% per year). Generally the risk of developing melanoma is 1:66 in males and 1:85 in women but there is considerable geographical variability in the incidence of cutaneous melanoma in our country with an evident decreasing North-South trend: incidence rate is about twice lower in Southern Italy compared to those in the areas of Central and Northern Italy [Italian Association of Medical Oncology-AIOM, https://www.aiom.it/] .



Cancer incidence and mortality statistics worldwide and by region

	Incidence						Mortality					
	Both sexes		Males		Females		Both sexes		Males		Females	
	New cases	Cum. risk 0-74 (%)	New cases	Cum. risk 0-74 (%)	New cases	Cum. risk 0-74 (%)	Deaths	Cum. risk 0-74 (%)	Deaths	Cum. risk 0-74 (%)	Deaths	Cum. risk 0-74 (%)
Eastern Africa	2 056	0.10	735	0.07	1 321	0.12	856	0.04	305	0.03	551	0.05
Middle Africa	1 099	0.19	593	0.23	506	0.15	469	0.08	255	0.10	214	0.07
Northern Africa	968	0.05	478	0.05	490	0.05	429	0.02	205	0.02	224	0.02
Southern Africa	1 885	0.36	968	0.45	917	0.29	517	0.10	288	0.13	229	0.08
Western Africa	955	0.06	337	0.04	618	0.07	408	0.02	140	0.02	268	0.03
Caribbean	414	0.07	232	0.09	182	0.06	179	0.03	105	0.04	74	0.02
Central America	2 452	0.15	1 199	0.16	1 253	0.13	959	0.05	564	0.07	395	0.04
South America	16 015	0.29	7 802	0.32	8 213	0.27	4 519	0.08	2 546	0.10	1 973	0.06
Northern America	105 172	1.76	61 675	2.06	43 497	1.50	8 412	0.12	5 443	0.16	2 969	0.08
Eastern Asia	10 427	0.04	5 414	0.05	5 013	0.04	5 436	0.02	2 917	0.02	2 519	0.02
South-Eastern Asia	3 275	0.05	1 742	0.06	1 533	0.05	1 616	0.03	848	0.03	768	0.02
South-Central Asia	6 232	0.04	3 312	0.04	2 920	0.03	3 579	0.02	2 004	0.02	1 575	0.02
Western Asia	3 819	0.17	1 922	0.18	1 897	0.16	1 355	0.05	807	0.07	548	0.04
Central and Eastern Europe	27 993	0.62	12 191	0.65	15 802	0.60	9 272	0.18	4 614	0.23	4 658	0.15
Western Europe	65 168	1.99	34 745	2.11	30 423	1.89	7 415	0.16	4 300	0.20	3 115	0.12
Southern Europe	23 915	0.93	12 436	0.99	11 479	0.88	4 926	0.15	2 977	0.20	1 949	0.10
Northern Europe	33 551	1.91	16 937	1.92	16 614	1.91	4 747	0.20	2 814	0.26	1 933	0.15
Australia and New Zealand	18 972	3.96	11 003	4.67	7 969	3.29	1 880	0.27	1 212	0.36	668	0.18
Melanesia	240	0.33	109	0.28	131	0.38	64	0.08	37	0.08	27	0.08
Polynesia	27	0.42	14	0.47	13	0.38	5	0.10	4	0.14	1	0.05
Micronesia	0	0	0	0	0	0	0	0	0	0	0	0
Low HDI	3 728	0.09	1 538	0.08	2 190	0.09	1 647	0.04	686	0.04	961	0.04
Medium HDI	7 121	0.04	3 629	0.04	3 492	0.04	3 863	0.02	2 085	0.02	1 778	0.02
High HDI	35 597	0.10	17 517	0.10	18 080	0.09	13 167	0.04	7 255	0.04	5 912	0.03
Very high HDI	277 993	1.12	151 055	1.25	126 938	1.02	38 352	0.13	22 351	0.16	16 001	0.09
World	324 635	0.37	173 844	0.42	150 791	0.33	57 043	0.06	32 385	0.07	24 658	0.05

Figure 1.1 Estimated number of new case, number of deaths, cancer incidence and mortality in 2020 caused by melanoma (Globocan 2020 - IARC)

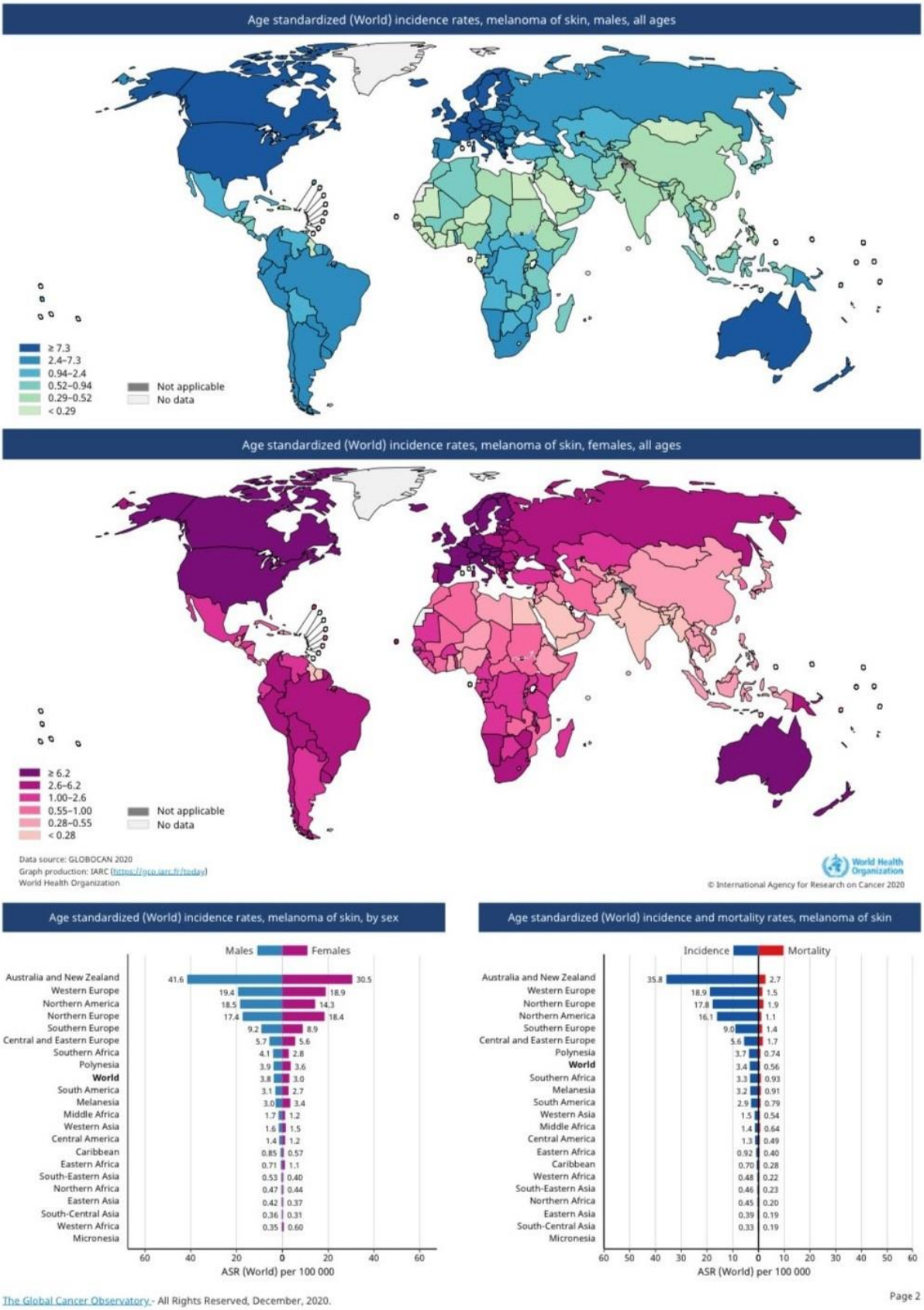


Figure 1.2 Melanoma estimated age-standardized rates (World) per 100,000 (Globocan 2020 – IARC)

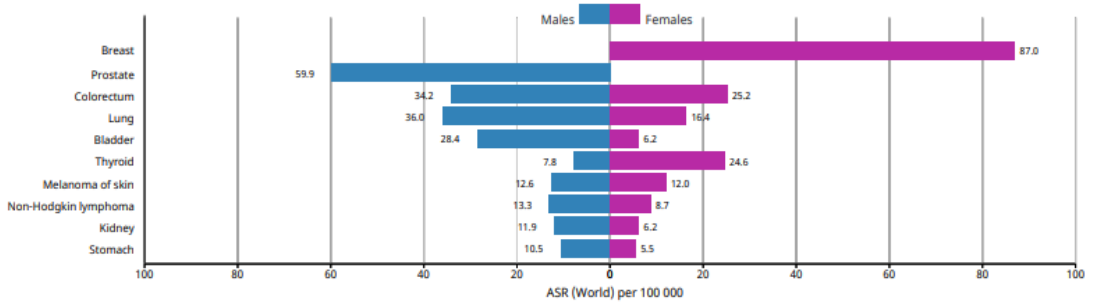
Italy

Source: Globocan

Incidence, Mortality and Prevalence by cancer site

	New cases				Deaths				5-year prevalence (all ages)	
Cancer	Number	Rank	(%)	Cum.risk	Number	Rank	(%)	Cum.risk	Number	Prop.
Breast	55 133	1	13.3	9.27	12 633	4	7.2	1.43	223 090	0
Lung	41 953	2	10.1	3.12	33 602	1	19.2	2.15	49 423	0
Prostate	39 317	3	9.5	7.66	6 902	8	3.9	0.43	147 452	0
Colon	33 957	4	8.2	2.34	16 629	2	9.5	0.78	97 550	0
Bladder	28 336	5	6.8	1.94	7 108	7	4.1	0.27	90 710	0
Stomach	14 372	6	3.5	0.88	8 853	6	5.1	0.46	22 054	0
Pancreas	14 155	7	3.4	0.84	12 917	3	7.4	0.77	9 386	0
Non-Hodgkin lymphoma	14 032	8	3.4	1.19	5 175	10	3.0	0.29	43 960	0
Rectum	13 326	9	3.2	1.01	4 812	11	2.8	0.25	41 829	0
Melanoma of skin	12 515	10	3.0	1.23	2 224	16	1.3	0.16	42 364	0
Kidney	12 306	11	3.0	1.04	4 280	13	2.4	0.25	36 112	0
Thyroid	12 288	12	3.0	1.45	526	26	0.30	0.03	46 013	0
Liver	11 739	13	2.8	0.90	9 798	5	5.6	0.61	11 165	0
Corpus uteri	10 013	14	2.4	1.71	2 152	17	1.2	0.25	37 836	0
Leukaemia	9 352	15	2.3	0.73	6 324	9	3.6	0.35	26 829	0
Multiple myeloma	5 772	16	1.4	0.41	3 775	14	2.2	0.20	15 290	0
Brain, central nervous system	5 732	17	1.4	0.55	4 568	12	2.6	0.39	16 920	0
Ovary	5 370	18	1.3	0.88	3 285	15	1.9	0.45	15 119	0
Lip, oral cavity	4 037	19	0.97	0.32	1 585	20	0.91	0.10	12 519	0
Larynx	3 229	20	0.78	0.29	1 459	21	0.83	0.10	10 740	0
Cervix uteri	3 152	21	0.76	0.68	1 011	22	0.58	0.17	9 806	0
Testis	2 382	22	0.57	0.64	104	35	0.06	0.02	10 460	0
Oesophagus	2 164	23	0.52	0.18	1 975	18	1.1	0.13	2 543	0
Hodgkin lymphoma	2 120	24	0.51	0.27	416	28	0.24	0.03	8 379	0
Mesothelioma	1 973	25	0.48	0.14	1 774	19	1.0	0.11	2 265	0
Oropharynx	1 494	26	0.36	0.15	762	24	0.44	0.07	4 385	0
Anus	1 293	27	0.31	0.10	348	29	0.20	0.02	4 091	0
Vulva	1 204	28	0.29	0.13	544	25	0.31	0.04	3 599	0
Kaposi sarcoma	1 120	29	0.27	0.08	150	32	0.09	0.00	3 504	0
Gallbladder	1 071	30	0.26	0.07	780	23	0.45	0.04	1 148	0
Salivary glands	1 034	31	0.25	0.08	430	27	0.25	0.02	3 393	0
Hypopharynx	772	32	0.19	0.08	319	30	0.18	0.03	1 463	0
Nasopharynx	629	33	0.15	0.07	273	31	0.16	0.03	2 125	0
Penis	540	34	0.13	0.09	144	33	0.08	0.02	1 805	0
Vagina	236	35	0.06	0.03	110	34	0.06	0.01	670	0
All cancer sites	415 269	-	-	28.05	174 759	-	-	9.50	1 230 693	0

Age-standardized (World) incidence rates per sex, top 10 cancers



Age-standardized (World) incidence and mortality rates, top 10 cancers

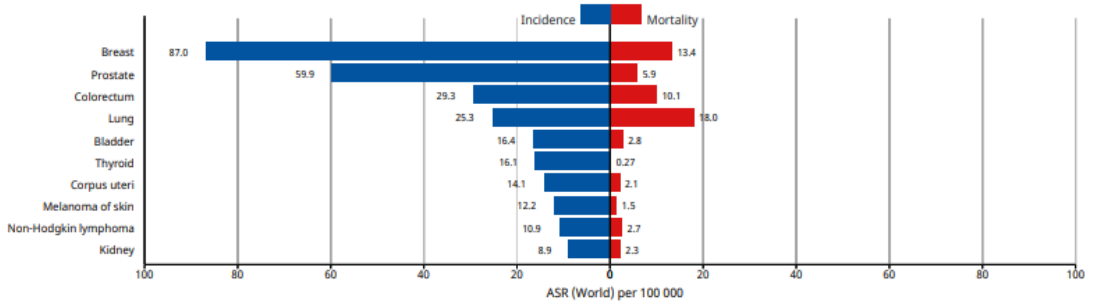


Figure 1.3 Melanoma data in Italy (Globocan 2020 – IARC)

1.3 Risk Factor and Prevention

Nowadays cancer, including melanoma, is considered a multifactorial disease because born from interaction between environmental causes and genetic factors. The most important and editable risk factor for the development of malignant melanoma is the direct exposure to UV rays, considered mutagenic and genotoxic for human healthy [Pennello G et al 2000; Gilchrest BA et al 1999]. A greater effect is recognized for intermittent exposure to sunlight compared to chronic exposure and for sunburn occurring in childhood/adolescence compared with those in mature age [Elwood JM, et al 1997; White E et al 1994]. The protective role of sunscreen creams is still under debate either for the long follow-up that would be necessary to study their effects both for the tendency to increase sun exposure in those who use them. Among the exogenous risk factors associated with the development of melanoma it is worth mentioning the use of sunbeds. It is known that the amount of UVA light that reaches the surface of the skin is greater in a single session of tanning bed in comparison to the normal exposure during ordinary outdoor activities or even during sunbathing [International Agency for Research on Cancer Working Group on artificial ultraviolet (UV) light and skin cancer 2007]. Recent studies have shown a significant increase in the risk of development melanoma at the young age especially in the region head – neck related to the use of sunbeds.[Lazovich D. et al 2010; Wehner MR. et al 2014; Le Clair MZ. et al 2016].

On the contrary, there are also protective factors; for example some foods seem useful for prevention, reducing risk of developing melanoma, such as carotenoids and vitamins from tomatoes, sulforaphane from broccoli flowers and polyphenols by green tea extracts [Ombra MN. et al 2019].

The most important endogenous risk factors are the number of nevi, familiar history and genetic susceptibility. Currently it is known that 25% of melanoma cases occurs on a pre-existing nevus [Bevona C. et al. 2003]. The presence of a large number of common, atypical or multiple nevi also increases the tendency to develop this pathology especially in the trunk; moreover the results of a recent meta-analysis pointed out that patients with a more 100 nevi have a 7 times higher risk of developing cancer [Rastrelli M. et al., 2014]. Among the phenotypic factors, subjects with light skin, eyes, hair and photo-type I/II have a double risk of development melanoma compared to subjects with dark-olive skin, dark–black eyes and hair photo-type IV. To those patients who fall into

that lower photo-types often develop amelatonic melanomas (are an atypical presentation of cutaneous melanoma) that are very difficult to diagnose, due to their lack of pigment, such lesion may be misdiagnosed as different pathologys [Titus-Ernstoff L et al 2005] .

≈7-15 % of malignant melanoma cases are diagnosed in patients with a family history of melanoma [Dessinioti C et al 2011]. However, true hereditary melanoma (i.e., multi-generational, unilateral lineage, multiple primary lesions and early onset of the disease) are infrequent; the familial clustering of the disease is considered to be responsible for the presence of a transmitted genetic mutation. [G. Leonardi et al., 2018]. In recent years it was found that melanomas that arise in families are linked to specific cancer patterns, such as Familial Atypical Multiple Male Melanoma Syndrome (FAMMM Syndrome) and the Melanoma-Astrocytoma syndrome (MAS) . The most important and frequent genetic abnormalities recognized in these families are the hereditary mutation in the tumor suppressor gene p16 or *CDKN2A*, inhibitor cyclin-dependent kinase 2A, less frequently are found mutations in cyclin dependent Kinase 4 *(CDK4)* or a decreased activity of tumor suppressor factors linked to Rb gene [Zuo L et al 1996 ; Soura E et al 2016]. Also patients with other cancer syndrome such as familiar retinoblastoma, Li-Fraumeni cancer syndrome and Lynch Syndrome type II have a higher risk of developing melanoma cancer [Goldstein AM et al 2001].

1.4 The Genesis of Malignant Melanoma

Melanocytes developed from neuronal crest cells [Kanitakis J 2002] and in the human melanocytes are present in different parts as basal epidermis and hair follicle but also along mucosal surfaces, meninges and in the choroidal of the eye. In order to respond to UV induced damage, the skin's keratinocytes produce melanocyte stimulating hormone (MSH) that binds to the melanocortin receptor 1 (MC1R) on the melanocytes inducing the release of melanin [Leonardi GC et al., 2018]. The melanin behaves like a protective screen for UV radiation to prevent DNA damage [Lin JY et al 2007]. In the general Caucasian population, malignant melanoma is classified on the basis of type of sun-exposure (chronic or intermittent), that caused different sites of origin, a degree of cumulative UV exposure, age at diagnosis, types of oncogenic drivers and mutational load [Leonardi GC et al., 2018]. Indeed, malignant melanoma diagnosed in old age (>

55 years) is typical of chronic skin sun exposure where are involved the areas more exposed to sun such as the head and neck at dorsal of the upper extremities alteration. The genetic mutations, associated with melanoma connected to chronically sun-exposed, are: B-Raf proto-oncogene (*BRAF*) present in about half cases, neurofibromin (*NFI*) and *NRAS* mutation [Leonardi GC et al., 2018]. B-Raf proto-oncogene codes for B-raf kinase involved in a complex signal transmission, resulting in the central of mitotis, organization of the cytoskeleton, metabolic regulation and apoptosis [Curtin JA et al 2005; Bastian BC 2014]. Conversely, melanoma associated with intermittent sun-exposed skin cases developed in youngers (<55 years), on less sun-exposed areas, such as the trunk and proximal extremities, and is usually associated with a specific BRAF mutation ($BRAF^{V600E}$) [Bastian BC 2014].

In the last decade, significant improvements have been made in order to understand why there is a conversion from normal melanocytes to melanoma cancer cell and it is totally clear that for a fully-evolved melanoma many genes mutated are necessary. It is very interesting that in 80 % of benign nevi the gene BRAF turn out to be mutated [Pollock PM et al 2003; Leonardi GC et al., 2018]. This data suggest that oncogenic BRAF alone is not sufficient for melanoma development; rarely benign nevi progress to melanoma. For cancer progression others mutation have to occur, such as telomerase reverse transcriptase (TERT) gene encodes catalytic reverse transcriptase subunit of telomerase, the enzyme that mantains telomere lenght or CDKN2A [Horn S et al 2013;Tsao H et al 2004]. Different is melanomas associated with chronic sun-exposed skin because do not arise form pre-existing nevi, but from melanomas *in situ* or dysplastic lesions and carry a different set of mutations [Shain AH et al 2016].

1.5 Diagnosis of Melanoma

Because of the aggressive biological behavior and poor prognosis of melanoma, early diagnosis is essential. Diagnosis is based on the histopathological examination of lesion, that shall be previously removed [Garbe C et al 2011]. For this reason it is necessary to perform an accurate analysis of the skin surface via a good lighting system and a dermoscopic examination, using the ABCDE rule (Asymmetry, Irregular border, Color variation, Diameter> 6 mm, Evolution), for defining the characteristics of the lesion [Abbasi NR et al 2004., Rigel DS et al 2010]. Another useful clinical indicator to

identify melanomas is the “ugly duckling” sign, a concept that is based on the idea that nevi in the same individual normally have common characteristics and the presence of a lesion that looks different strongly increase the suspicion of a malignant melanoma [Grobe JJ et al 1998; Scope A et al 2008].

Dermoscopy is a useful tool to support the visual inspection of a suspicious skin lesion for the analysis of both melanoma and atypical melanocytic lesions; this analysis must be performed by experienced personnel [Rigel DS et al 2010]. For avoid false negative the clinical examination and general medical history must be integrated with. dermoscopy. Fortunately, in recent years new techniques of diagnosis are still emerging, among these there are computerized approaches and non invasive assessment of genetic markers that could reduce melanoma mortality. Total Body Photography (TBP) is used to create a complete archive of the skin surface of the patient for the evaluation of new lesions or to identify changes in a pre-existing lesion [Davis L et al., 2019]. This multimodal application should be applied especially to patients with numerous melanocyte nevi that are at high risk of developing melanoma. In the context of non-invasive skin imaging, confocal microscopy, defined as a virtual optical biopsy, could be used as a diagnostic level after evaluation clinical-dermoscopic in selected cases. Confocal microscopy can have a potential role in clinical practice particularly for the identification of lesions difficult to diagnose, in particular for facial lesions, because seems to be more sensitive and specific than dermoscopy [M. Rastrelli et al., 2014].

The presence of a common international staging system is central to cancer management particularly for the development of guidelines for surveillance and treatment of melanoma. The actual cutaneous melanoma staging system used is the one introduced in 2018 by the American Joint Committee on Cancer (AJCC), the eighth edition of Cancer Staging Manual and is based on the evaluation of primary tumor (T), regional (N) and distant lymph nodes metastasis (M), TNM stage system [Keung EZ et al., 2018]. Tumor (T) describes the thickness of the melanoma. There are 6 main stage of tumor thickness in melanoma (Tis to T4). Tis means that the cancer cells are only growing in the layer of cells where they started, without growing into deeper layers. This may also be called *in situ* cancer or pre-cancer. The number after T (such as T1, T2, T3, T4) describes the tumor size and/or amount of spread into nearby structures. Higher is the number, larger is the tumor and/or the more it has grown into nearby

tissues. The node stage (N) describes whether cancer cells are in the nearby lymph node. The (N) category can be assigned a letter or a number. NX means there is not information about the nearby lymph node, N0 indicates instead nearby lymph nodes do not contain cancer. The number after N (N1, N2 and N3) might describe the size, location, and/or the number of near lymph nodes affected by cancer. Higher is N number, greater is the cancer spread to nearby lymph nodes. Metastasis (M) describes whether the cancer has spread to different part of the body. There are 2 stages of metastasis (M0 and M1), M0 means the cancer has not spread to another part of the body. M1 means that the cancer has been found to have spread to distant organs or tissues.

1.5.a Growth phase

The growth phase of melanoma determines the prognosis. Primary melanoma may progress through three recognizable steps: *in situ* radial growth phase (RGP), invasive RGP and the vertical growth phase (VGP) also known as ‘‘tumorigenic melanoma’’ [Guerry D et al 1993]. The vertical growth phase represents the tumorigenic phase in which melanoma acquires the ability to metastasize. This lesional step is characterized by the appearance of a new population of cells within the melanoma, not an expansion of the cells forming the preexisting radial growth phase [Clark WH et al 1984].
The histopathology report should contain this information, namely the vertical growth except for nodular melanoma which by definition presents the vertical growth pattern.

1.5.b Breslow thickness

Breslow thickness is the measurement of the depth of the melanoma from the surface of your skin down through to the deepest point of the tumor. It is considered the most important prognostic and it must always be reported in the histopathological reference of any invasive melanoma. Breslow thickness is measured from the granular layer or, if the lesion is ulcerated, from the base ulceration, up to the point of maximum infiltration and it is measured in millimetres (mm) with a small ruler, called a micrometer. [Breslow A et al 1970]. The Breslow thickness in another melanoma staging system:

- Stage I: less than or equal to 0.75 mm
- Stage II: 0.76 − 1.5 mm

- Stage III: 1.51–2.25 mm
- Stage IV: 2.26–3.0 mm
- Stage V: greater than 3.0 mm

1.5.c Clark level

As well as Breslow thickness, also Clark scale look at the depth of melanoma cells in the skin. The Clark scale is a way of measuring how deeply the melanoma has grown into the skin and which levels of the skin are affected and, although the parameter must be reported in the histopathology report, this staging system is not included in the current AJCC staging system (8th Ed.) [Clark WH, et al 1969]. The parameter is considered poorly reproducible due expecially to the difficulty to recognize the interface between the dermis papillary and reticular dermis in the presence of marked solar dermal elastosis. The Clark scale is composed of 5 levels [Rebecca VW et al 2013; Scolyer RA et al 2011]:

- Level 1: melanoma cells are confined to the epidermis (melanoma *in situ*)
- Level 2: invasion of single cells or very small nests of melanoma into the papillary dermis
- Level 3: melanoma cells “fill and expand” the papillary dermis
- Level 4: invasion into the reticular dermis
- Level 5: invasion into the subcutaneous fat

The Clark level was inserted together with the mitotic rate for the sub classification of T1 tumors. The third factor (the first factor is Breslow thickness and the Clark level is the second) used for classification in localized melanoma is the presence of ulceration of the primary tumor. Based on these three factors, invasive melanomas are classified from T1a to T4b, with 10-year survival rates between 93% and 39% [Balch CM et al 2009]. The search for the sentinel lymph node is a fundamental moment for the surgical staging of melanoma. The risk of lymph node involvement is directly proportional to the thickness of the melanoma or to the presence of mitosis; in a melanoma with a thickness of <1 mm, secondary tumors at this level are rare, while for melanomas with a thickness between 1.5 and 4 mm this involvement occurred in 25% of cases and increases up to 60% for melanomas with a thickness greater than 4 mm. The sentinel lymph node technique is a procedure considered to be minimally invasive and allows

for assess the state of lymph node involvement and is better to identify patients with metastatic lymph nodes, clinically not palpable or candidates for complete lymph node dissection. The method requires the pre-operative execution of a dynamic lymphoscintigraphy, all in order to correctly identify the lymph node to be removed. If the sentinel node contains even as little as a single melanoma cell, it confers a stage III diagnosis. Melanoma is divided into stages using five roman numerals (0 through IV) and up to four letters (A through D) that indicate a higher risk within each stage. The stage is determined mostly by specific datails about the tumor and its growth that are tallied in a system called TNM, as described above.

1.6 Biopsy and Histological Classification of Melanoma

Biopsy can be of two types: excisional or incisional (Figure 1.4). The first choice method is excisional biopsy that removes the entire tumor (along with a 2 mm small margin of normal skin around it and subcutaneos fat), in order to be able to make an accurate histopathological and molecular diagnosis. Indeed the incisional biopsy remove only a small portion of the melanoma with the disadvantage to have less accuracy in determining lymphatic infiltration and regression. The punch biopsy is a technique based on the use of a skin punch instrument with a circular blade (Figure 1.4 b), looks like a tiny round cookie cutter, that remove a deeper sample of skin well into the subcutaneous fat. Punches are used both for excisional and incisional biopsies. Punch biopsies, with rare exception, are closed with simple interrupted or vertical mattress stitches for wound edge eversion to obtain the best cosmetic result. [Swanson NA et al 2002]. The shave biopsy consist in shaves off the top layers of the skin with a small surgical blade (Figure1.4 c) . A shave biopsy is useful in diagnosing many types of skin diseases and in sampling moles when the risk of melanoma is very low, because it does not allow a correct evaluation of Breslow thickness.

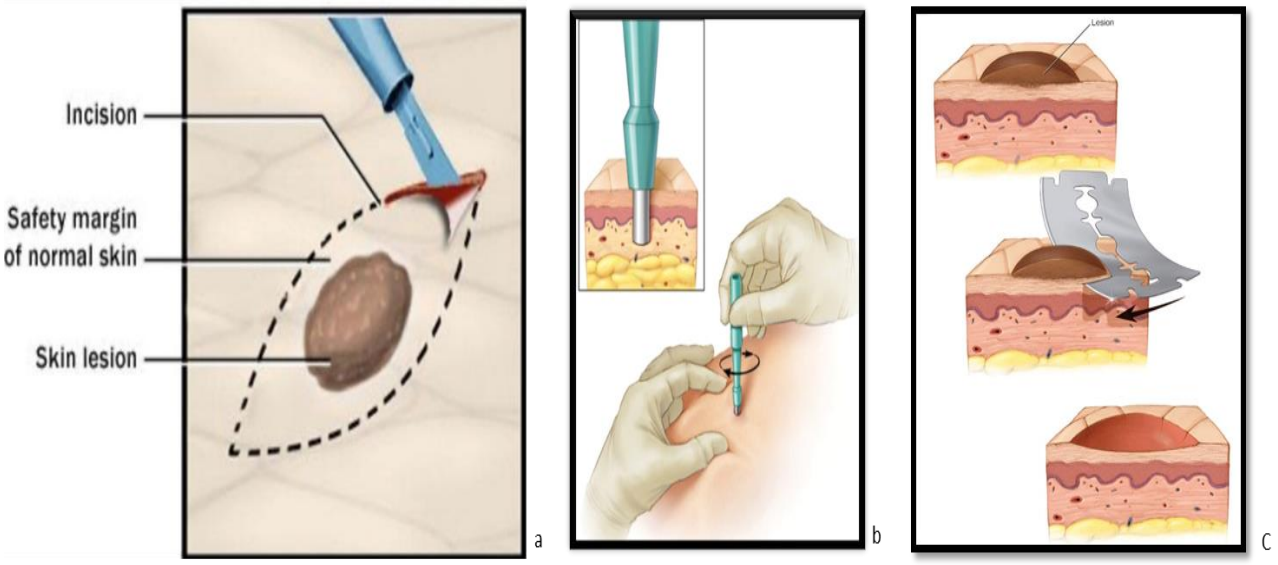


Figure 1.4 Types of Biopsy used for melanoma diagnosis a) Excisional, b) Punch and c) Shave (mayoclinic.org)

Once obtained the tissue, this should be supported to macroscopic analysis, in order to assess the size of lesion, the characteristics of the lesion and the minimum margin. This is followed by microscopic analysis based on the evaluation of multiple architectural and cytological parameters that include the asymmetry in the distribution of melanocytes, the circumscription, the single or theca mode of melanocyte proliferation, the asymmetry in the pigmentation, the formation of melanocyte aggregates, the infiltrating profile of the deep growth front, the ascent of the cells above the dermo-epidermal junction, the atrophy of the epidermis, the cytology of melanocytes, the absence of maturation, the presence of a strong mitotic activity in the deep portions [M. Rastrelli et al., 2014].

On the basis of clinical and histological criteria, malignant melanoma can be classified into 3 main types: 1) superficial spreading melanoma, 2) nodular melanoma 3) lentigo maligna melanoma plus some rare forms.

Superficial Spreading Melanoma (SSM). SSM is the most frequent type of melanoma, including approximately 70% of melanoma cases. Its development is correlated to intermittent sun exposure and the areas of body mostly involved are the legs for women

and the backs for men. Generally SSM is characterized by a pigmented, asymmetrical lesion with irregular edges often with areas of different pigmentation [Markovic SN et al 2007] (Figure 1.5 a). This type of tumor can have different variety of colors including brown, gray, black, violaceus and pink, rarely blue or white. Change of color often occurs over a period of time ranging from a few months to years.

Nodular Malignant Melanoma (NMM). NMM is the second in order of incidence representing the 15-30% of melanoma cases. It is characterized by a rapid growing pigmented lesion (even a few weeks), often accompanied by ulceration and bleeding [Bergman R et al 2006] (Figure 1.5 b). This cancer is easy to find on the limbs and trunk of patients >50 years old. It has only a fast and vertical growth and higher rate of metastasis. Nodular Melanoma have a variety of colors including brown, black, or blue-black. It occurs in the form of nodule, ulcerated polyp or as a plaque with irregular edges [Duncan LM et al 2009].

Lentiginous Malignant Melanoma (LMM). LMM represents the 4-15 % of all melanoma cases (Figure 1.5 c). The lesion appears aspigmented lesion with irregular edges of flat form. LMM generally is located in the photo-exposed areas of the face and neck and is characterized by a slow growth with a long phase of melanoma *in situ* [Markovic SN et al 2007; Cohen LM et al 1995], but we can also check her on the trunk in men or limb extremities in women. Clinically, it shows a variety of colors black, brown or brown on a tan background. [Duncan LM et al 2009].

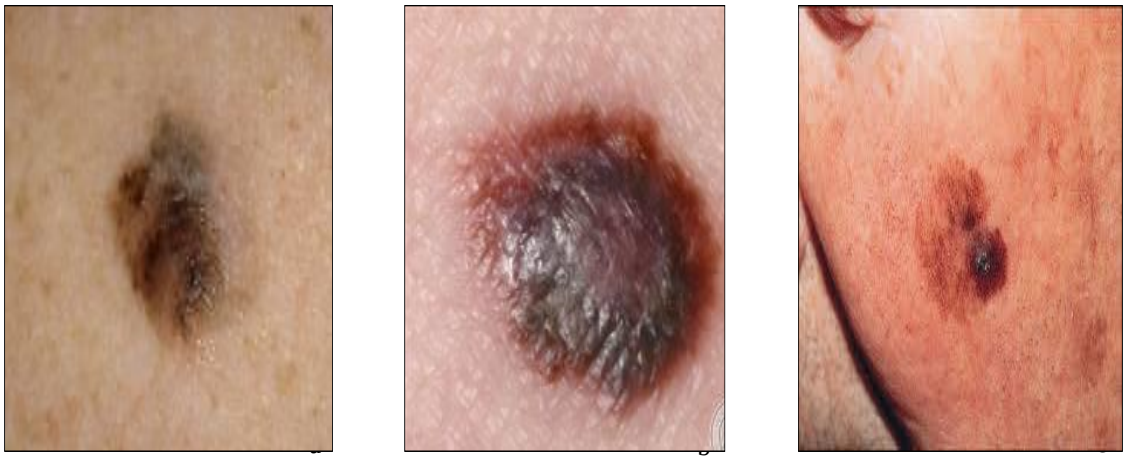


Figure 1.5 Types of Melanoma a) Superficial Spreading , b) Nodular Malignant and c) Lentiginous Malignant

The rare forms are acral lentiginous melanoma, desmoplastic melanoma and mucosal melanoma.

Acral Lentiginous Melanoma (ALM). This melanoma is a rare pigmented lesion that occurs at the level of the palm of the hands, soles of the feet or in the nail bed (Figure 1.6 a); accounted for 5% of melanoma in white people while it is a more common subtype among Asian, Latin America and African patients [Bradford PT et al. 2005].

Desmoplastic Melanoma (MD). Desmoplastic Melanoma is a rare form of invasive cancer representing less than the 4-5% of all melanomas (Figure 1.6 c). This cancer is histologically characterized by fusiform cells distributed within the stroma. MD often affects male people 60-70 years old, especially on the head and neck, but may appear on adifferent cutaneous and mucosal areas. MD can develop *ex novo* or be associated with different types of pre-existing melanoma, usually to LMM [Markovic SN et al 2007].

Mucosal Melanoma (MM). MM is a rare tumor of mucous membrane that contain melanocytes, such as uvea, mucosa of the eye, oral, gastrointestinal, respiratory and genitourinary mucosae (Figure 1.6 b). This cancer represents about 1,4 % of all melanoma cases and ≈40% of these cases are located in the head and neck region [Chang AE et al.1998; McLaughlin CC et al. 2005] . Unlike the other subtype of melanoma, etiopathogenesis it's not linked to sun exposure. Data show that most of these cancer are due to environmental pollution such as tobacco smoke and formaldehyde [Axéll T et al 1982]. This tumor are found in highly vascularized and hidden cavities of human body and for this reason often the lesions are diagnosed late becoming fatal.

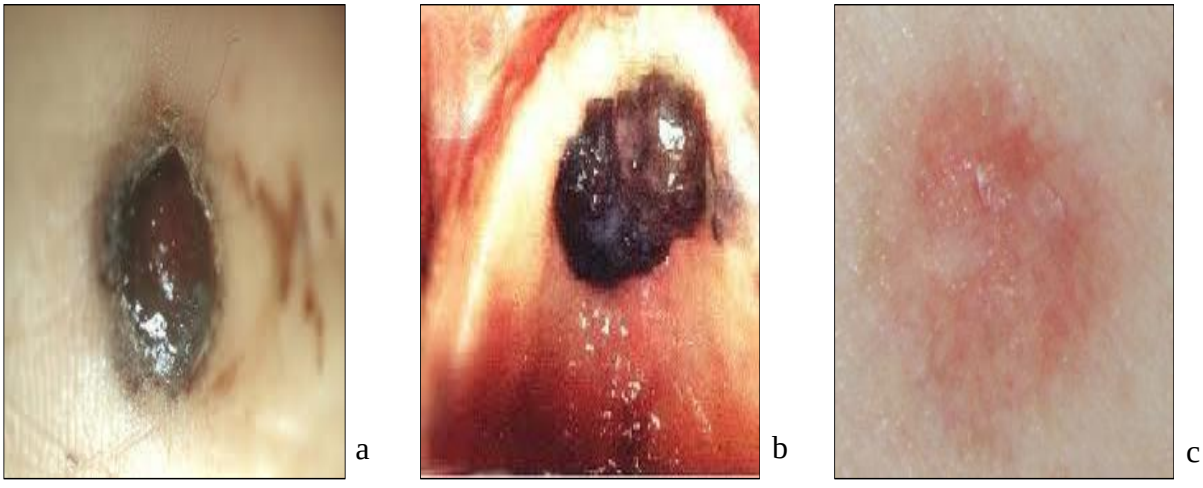


Figure 1.6 Type of Melanoma a) Acral Lentiginous, b) Mucosal and c) Desmoplastic

1.7 Melanoma: types of Treatment

1.7.a Surgical Treatment

In the case of localized primary melanoma, surgical treatment is completed, if necessary, with the enlargement of the excision based on the histological thickness of the lesion [Lee C et al 2013]. An excision with a 5 mm margin is considered mandatory and sufficient for melanoma *in situ*. A crucial step, both for staging and prognosis, is the search for the sentinel lymph node. It is recommended for all patients with stage > TIa. If the sentinel lymph node is histologically positive or there is the presence of clinically evident lymph node metastases, complete lymph node dissection is the standard treatment. Patients with metastases in transit (classified as stage IIIB or IIIC) or dermal metastasis (metastasis that spread through the lymphatic system and that are localized between the site of the primary lesion and the lymphatic drainage station for that site) have as therapeutic choice the surgical resection with histologically free margins, with subsequent adoption of the sentinel lymph node technique. In some situation, metastatic tumors can be surgically removed as well, but surgical treatment alone is not curative and will require other treatment options.

1.7.b Chemoterapy

As mentioned above, for patients with metastatic disease, surgical treatment alone is not curative and drug therapies are the next line of treatment. Different chemotherapeutic agents have demonstrated activity when used in mono-chemotherapy including decarbazine, fotemustine, temozolomide [Serrone L et al 2000]. For a long time drug treatment with decarbazine alone was considered the standard treatment for metastatic melanoma, even if its activity is quite limited, with global responses not exceeding15%. Fotemustine and temozolomide are more recent drugs that act with the same mechanism of action and with a demonstrated non-inferiority compared to the decarbazine [Avril MF et al 2004; Patel PM et al 2011]. The ability to cross the blood-brain barrier makes them suitable for the treatment of patients with brain metastases. Multi-chemotherapy treatment did not demonstrate a median survival benefit compared to single-agent chemotherapy [Legha SS et al 1996; Chapman PB et al 1999]. The addition of an immunotherapy treatment with interferon alpha and interleukin 2 to the polychemotherapy regimen did not lead to improvements in terms of average overall

survival but only to a greater frequency of side effects [Chapman PB et al 1999; Bajetta E et al 2006]. In recent years the treatment with conventional chemotherapy is considered second-line drug therapy; treatment with targeted therapies and immunotherapy is preferred because showed a significant improvement of prognosis especially for the patients affected by metastatic melanoma.

1.7.c Targeted Therapy

Until a few years ago, the treatment of inoperable metastatic disease was considered almost exclusively palliative, since the available chemotherapeutic agents showed a limited and poorly curative effect. Since 2011 for the treatment of metastatic melanoma the use of the BRAF inhibitors was evaluated to challenge molecular defects present in melanoma. The most important drugs belonging to this class of drugs are vemurafenib and dabrafenib; these can be used alone or in combination with Mek inhibitors (cobimetinib and trametinib) [Hodi FS et al 2010; Ascierto PA et al 2016]. Selective inhibitors of mutated B-Raf kinase showed efficacy in melanoma with B-Raf^{V600E} mutation and no effect on non-mutated lines. Several studies, conducted in patients with V600E mutation, have shown a 38% reduction in the risk of death and a 66% reduction in the risk of progression compared to dacarbazine [McArthur GA et al 2014]. Although these medications may have significant action in countering the progression of the disease, a large number of patients tend to develop secondary resistance in a short time.

1.7.d Immunotherapies

Among the older generation immunotherapeutic agents, interleukin-2, aMDinistered intravenously to high doses, is able to induce an objective response rate of 16% in selected patients and a 6-7% of complete responses [Atkins MB et al 2000]. Follow-up data indicates that approximately half of these complete responses are long-lasting, with some patients that are disease free 15 years after therapy. In general, the median duration of response in patients with complete response was beyond 59 months. Since activity (not high) do not justify the toxicity associated with such treatment, represented by the capillary hyper-permeability syndrome (increased risk of pulmonary edema, renal insufficiency, hypotension and cardiac dysfunction), this approach did not receive authorization in Italy and Europe for the treatment of metastatic melanoma. In recent

years, the introduction of immunological checkpoint inhibitor drugs represented one of most important breakthrough in melanoma immunotherapy. Ipilimumab is a monoclonal antibody directed to CTLA4, a membrane protein receptor expressed by cytotoxic T lymphocytes, through which some ligands (expressed by other immune cells) inhibit the lymphocytes themselves. Ipilimumab, by binding to the receptor, prevents this inhibition and allows T lymphocytes to proliferate, infiltrate and attack cancer cells [Lipson EJ et al 2011]. Treatment with this drug demonstrated significant survival (10 years in 20% of cases); the information is significant when compared to one-year survival in stage IV melanoma patients [Wolchok JD et al 2010; Sharma P et al 2015; Topalian SC et al 2015]. The tolerability profile of this drug is affected by the immune-related side effects due to its mechanism of action: the most frequent adverse events are diarrhea and dermatitis. More recently, antibodies directed against the PD-1 inhibitory checkpoint have been introduced into the clinic (nivolumab and pembrolizumab) for treatment of advanced melanoma (unrespectable stage IV or III). These immunomodulating antibodies seems to be superior in efficacy and tolerability compared to ipilimumab. In particular nivolumab is a human IgG4 antibody that binds to PD-1 thus blocking the interaction between PD-1 and its ligands PD-L1 and PD-L2 [Larkin J et al 2015].

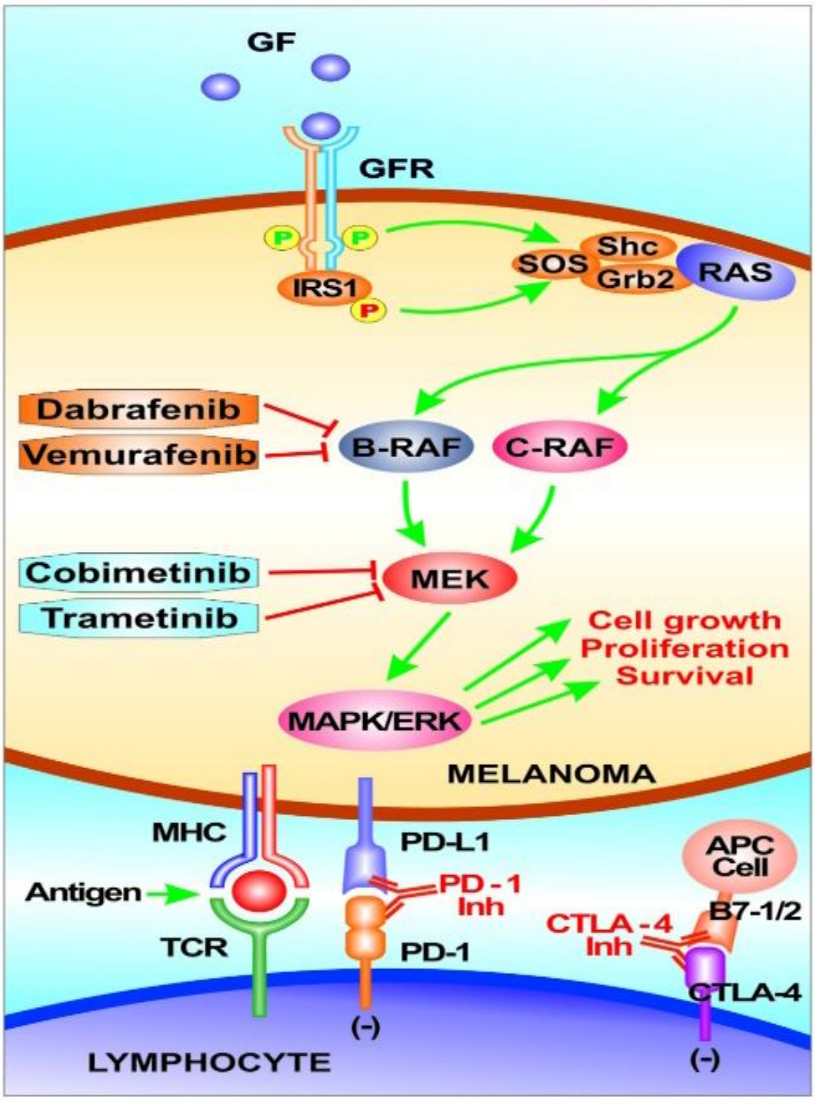


Figure 1.7 Medical treatment of melanoma. The therapeutic approaches for the melanoma treatment are based on drugs of last generation. (Cutaneos Melanoma: from pathogenesis to therapy-Review, Int J Oncology 2018)

Chapter 2

Tomato

2.1 Mediterranean Diet

Several studies demostrated how the adoption of the Mediterranean Diet represents a protective factor against the development of some common chronic diseases [Hoffman R et al 2013]. In the studies conducted, the concept of the Mediterranean diet (MD) has been concretely translated into a diet characterized by a high intake of vegetables, fruit, legumes, nuts, olive oil and cereals (whole meal), a moderate consumption of fish dairy products (especially cheese and yogurt), a reduced consumption of red wine with meals and a low consumption of red meat, white meat and saturated fatty acids (Figure 2.1) [Castro—Quezada I et al 2014]. The increase in the level of adherence to the MD was significantly associated with reduced cases of hypertension, hypercholesterolemia, diabetes and obesity in adults. Moreover it is emerged that MD represents a protective factor against all causes of mortality and, specifically, those related to cardiovascular diseases and cancer, but also Parkinson's and Alzheimer's disease [Esposito K et al 2013; Sofi F et al 2008; Román GC et al 2019].Unfortunately, the younger generations seem to gradually and steadily abandon the MD, in favour of new food trends characterized by high-fat foods [Baldini M et al 2009; Martinez-Lacoba R et al 2020].

The MD is known to protect against weight gain preventing obesity [Funtikova AN et al 2014; Bonaccio M et al 2013]. A possible explanation is that MD is rich in plant foods that provide a large amount of dietary fiber which increases satiety and secretion of cholecystokinin [Schroder H et al 2007]. Moreover MD foods have a low energy density, a low glycemic index and high water content, these characteristics lead to an increase in satiety and an intake lower calorie, helping to prevent weight gain. The MD has a positive influence on cancer prevention; many foods typical of MD (legumes, fresh fruit or nuts, vegetables, fish, and olive oil, especially extra-virgin olive oil) are rich in bioactive compounds (carotenoids and vitamins, as vitamin C and E, folates and

flavonoids) that have a protective effect, preventing cell degeneration and proliferation of cancer cells [Ciancarelli MG et al 2017].

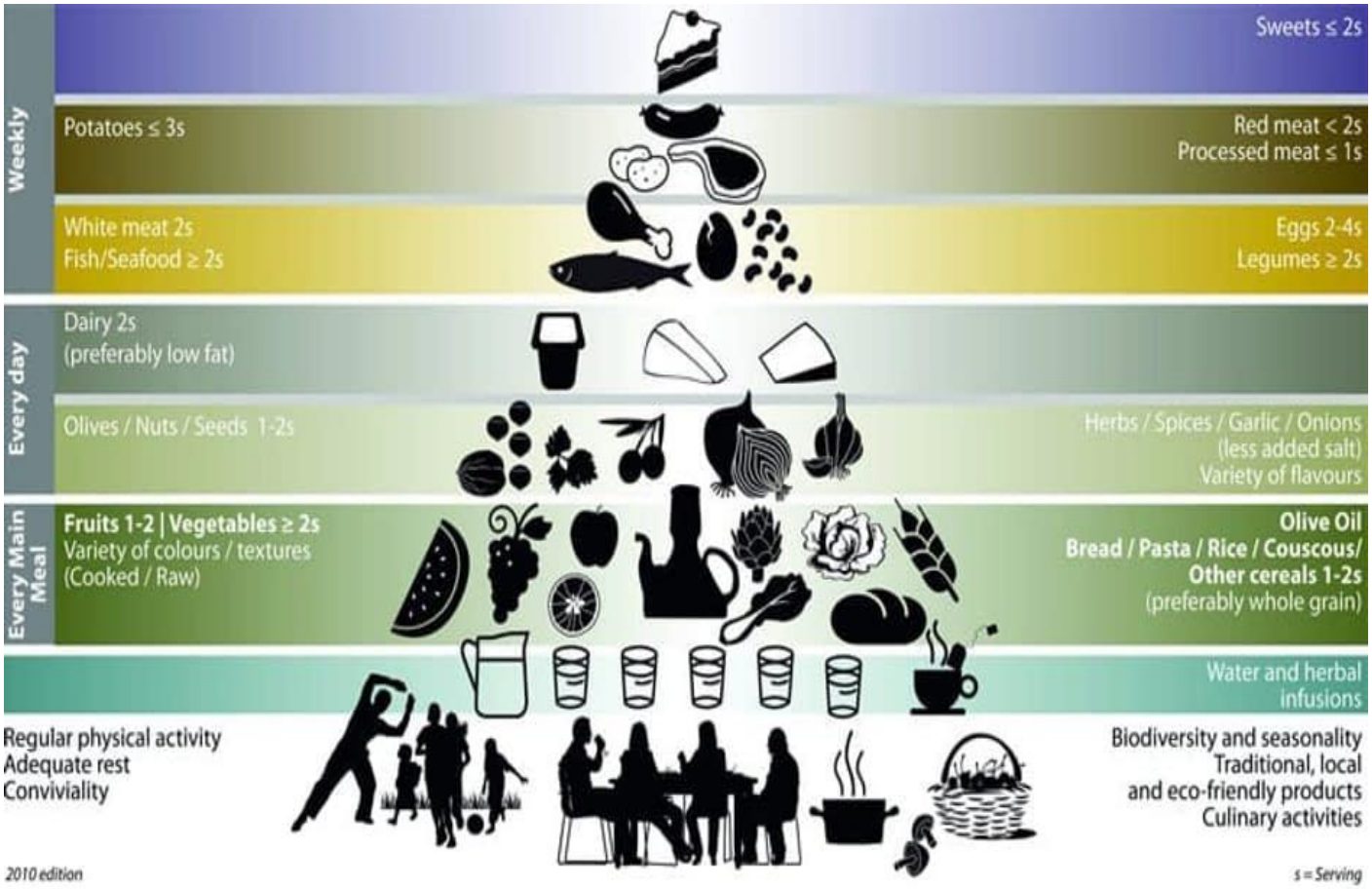


Figure 2.1 The new MD pyramid provides key elements for selection of foods, both quantitative and qualitative, indicating the relative proportions and consumption frequency of the main food servings groups plus suggestions regarding cooking, socializing, use of seasonal and local foods, activity, moderation and adequate rest. (Mediterranean diet pyramid today. Science and cultural updates. Public Health Nutr. 2011)

2.2 Nutraceutical

The neologism “Nutraceutical” originates from the fusion of the words “nutrition” and “pharmaceutical” and it was coined in 1989 by Sthephen De Felice, an American nutritionist and biochemist, member of Foundation for innovation in Medicine (New Jersey, USA). This definition was used to state the discipline that study the food components which, through specific physiological action–interaction, have a positive role on the health contributing to the prevention from chronic illnesses as cardiovascular diseases, osteoporosis, arthritis, II type diabetes and neurodegenerative pathologies.

Therefore, with the word “nutraceutics”, specific components of vegetal or animal food are identified and, thanks to their functional features, can be used alone or in mixture to produce supplements.

2.3 Functional Food

Although there is no universal definition of functional food, a simple description is “processed foods having disease-preventing and/or health-promoting benefits in addition to their nutritive value.” Functional foods overlap with nutraceuticals, medical foods, probiotics, designer foods, pharmafoods, and vitafoods [Arihara K et al 2014]. Functional food can be divided in two categories [Aschwell M et al 2001]:

- Type A: food that improves a specific physiological function beyond their specific role in the body growth and development. This kind of food does not have functions related to diseases or pathological conditions. For example, coffee could increase the cognitive capabilities because of the caffeine contained in it.
- Type B: food that reduce the risk of developed disease, for example, tomato could reduce the risk of pathologies connected to oxidative stress (atheriosclerosis, myocardium heart attack, cataract, cancer etc.), thanks to its lycopene content.

It needs to do a distinction between “neutracetical” and “functional food”: the first one refers to individual substance with medicinal features contained in the food, the second one try to identify the entire food that presents healthy features. The nutraceutic substances normally derive from plants, food and/or microbial sources. Examples are probiotics, antioxidants, polyunsaturate fat acid (omega-3 omega–6), vitamins and enzymatic complexes. They are usually used to prevent chronic diseases, to improve health conditions, to hold over the ageing process and to increase life expectations. [Aschwell M et al 2001]. Therefore the nutraceutics can be taken introducing in the diet functional food, or as simple food or as as food enriched with a specific active ingredient (for example, milk with D vitamin or acids omega-3). They can also be intoduced as liquid supplement, pills or capsules [Roberfroid M B 2002]. The better knowledge of consumers about the possibility to protect the health by an appropriate

diet has lead to the development of a flourishing market of nutraceutic products that, despit the economic crisis, records a constant increase.

2.4 Tomato: the Superfood

2.4.a Botanical description

Tomato (*Lycopersicon esculentum*) is an herbaceous plant that belongs to magnoliopside class (dicotyledon); firstly it was classified by Linneo as belonging to the solanacee family and gathered in the type Solanum. In 1768 Miller described in a specic way the type Solanum, introducing *Lycopersicon genus* which includes two sub–categories *Eulycopersicon C. H. Muller* characterized by red and smooth skinned fruits and *Eriopersicon C. H. Mull* with green and tomentose fruits.

Tomato is a seasonal plant, up to 2 meters high, when is young it is upright but then warped because of the weight of its fruits; it has branching growth (every segment of the plant ends with an inflorescence) and shows buns at the angle of every leaf . Leaves are big, separated, irregularly formed by little unequal leaves. Flowers take shape on raceme inflorescences that arise at leaves angle and are in number from 4 to 12. The fruit, the only edible part, is a berry with changeable shapes and sizes (globular, flatten, lengthened, navel shaped, smooth or ribbed), with a different number of locules and generally red colored when it is ripe (Figure 2.2).

Figure 2.2 Different type of tomatoes.

From a morphological point of view (Figure 2.3) berry can be divided in:

- Epicarp (peal): yellow colored film
- Mesocarp (flesh of the fruit): composed of rounded–ovoid cells which contains a straw-colored liquid in which are dissolved several components (sugars, salts, acids, nitric substances etc.); besides lycopene grains, mesocarp contains thin vessels composed by lignin, cellulose, hemicelluloses and pectic substances.
- Endocarp: is composed by seminal locules and stylus axis which brings seeds.

Figure 2.3 Tomato Sections

The berry composition depends on the variety and climate conditions of cultivation areas. In fruit pulp there are several smashed disc-shaped seeds and when they are dried, they are tomentose and yellow-greysh coloured.

Tomato plant presents a radical taproot apparatus that reaches a depth of 2 m even though about the 60% of active layer is absorbed in the first 0.30 m of ground and the 25% in the first 0.50 m. The fruit adapts itself well to different temperate; thermal limits for the crop are: 0-2°C as lethal temperature, 8-10° C for vegetation, 13-16° C as night optimum thermal values and 22-26° C as daily optimum values. Tomato plant adapt itself to several kinds of ground, with variations which depend on the choice variety even if some basic nutritive conditions that must be guaranteed, for example it needs a bigger quantity of potassium followed by nitrogen, phosphorus and calcium (order of importance). Moreover it prefers sub-acid reaction grounds and it is particularly sensitive to salinity. Potassium plays an important role expecially for the berries quality

influencing the sugars contents, dried residue and colour. Nitrogen enhances the vegetative vigour therefore it is an important component to obtain high yields, even if its surplus could excessively facilitate the flourishing of the vegetation to the detriment of fruits development.

2.4.b Historical information

Tomato plant seems to originate from South America, in particular from Chile and Ecuador, where it lives as wild plant; thanks to the tropical climate it is able to give fruits for the whole year, whereas in European regions, when grown outdoors, it has a seasonal cycle limited to summer (Figure 2.4) [Bergougnoux V 2014]. Later from South America, it spreads in central America and in the XVI Spanish spread out it around in Europe. Tomato plant was already famous in pre-Colombian age where was used as ornamental plant bacause it was considered poisonous due to its high content of solanine, substance considered harmful, so tomato was unused for cooking.

Figure 2.4 Tomato – San Marzano, a typical variety cultivates in Southern Italy (agro Nocerino-Sarnese)

In the XVII century, tomato begin to be eaten as fresh food and used to prepare sauces before in Southern Europe but also in Bohemia and England. In 1820, Livingstone was the first person to set up the basics of the tomato varieties selection. After the creation of tin box, Bryan Donkin and Woodbull Crosby in 1847 commercialized the first preserved tomato boxes. While probably the first tomatoes industrial processing is owed to the

Italian Francesco Cirio at the end of XIX century. From this moment on, the first derivatives start to come up. In 1888, the knight Brandino Vignali started the manufacturing of tomato stock. Simultaneously, in Salerno area, a technique to produce peeled tomatoe was studied and developed; this technique is used with oblongata tomatoes which grown at hillside of Vesuvio.

2.5 Chemical composition and nutritional aspects

Nutritional features of tomato considerably change in relation to several environmental causes as the light, temperature, topdressing, transplanting time and crop soil composition.

Tomato fruit includes an edible part composed by 98% (fruit flesh and juice) and 2% non edible part (peel, stem and seeds). In 100g of the product the percentage composition is 93% water, 0.2% fats, 1% proteins and 1.8% fibres, with energizing value around 73 KJ (17Kcal). The importance of the tomato fruit, from a nutritional point of view, is due to its high contents of vitamins and minerals, carotinoids, folic acids and potassium. Tomato is one of the main food in the Mediterranean diet, it is usually eaten both raw or processed, contributing with its compounds to healthy effects of MD [Lenucci et al 2006]. Tomato can be eaten directly or as an ingredient in many food recipes; both fresh and in sauce form, it contains a high number of antioxidant substances, about 40 different phenolics compounds and a high content of carotenoids. [Rinaldi de Alvarenga et al 2019]. Correlation between ingestion of tomato and human health has been aim of many studies mostly for the presence of these componds expecially lycopene, the most abundant carotenoid present in this fruit. Moreover tomato represents an important source of phenol compounds in the human diet followed by maize and beans; main phenols presents are flavonoyds which are present from 12 to 49% according to different varieties. Even vitamins (A, B group vitamins and mostly C vitamin) contribute to increasing the nutritional value of this fruits. Among mineral elements, potassium is the most important (200-300mg /100g juice) followed by chlorine (30-60 mg/100g juice), iron, zinc, calcium and selenium. A negative aspect of some Solanaceae as the tomato is the presence of a toxin called α-tomatine that is a steroid glucose- alkaloid; it is toxic for the human being in doses higher than 2-5 mg/kg corporeal weigh [Kuete V, 2014]. This substance is more concentrated when the fruit is

green and it disappears after the maturation (for example small immature green fruit we have a content of α-tomatine of 465 mg/kg fw) [Schrenk DJK et al. 2020].

2.6 Main functional composites of the tomato fruit

Functional compounds represent important molecules in the metabolism of the plant and represent a source of healthy molecules that can be taken by nutrition. Antioxidants play an essential role among functional composites. Antioxidants are a family of chemical species, natural or synthetic, that are able to neutralize, handing over electrons, free radicals avoiding damages on cells. Antioxidants can be divided in three categories: 1) true antioxidants, 2) reducing agents and 3) synergist antioxidants. True antioxidants (also known as anti-oxygen) are compounds that inhibit the oxidation reacting to free radicals. These substances are effective against auto-oxidation and belong to this category for example tocopherols and butylatedhydroxyanisole. Reducing agents are substances with a smaller redox potential if compare to the compound to protect and therefore they are oxidized more easily in place of it. These substances include ascorbic acid and sodium and potassium salts of sulphurous acid (used in wine production). In the end the synergist antioxidants have few antioxidant power, but they strengthen the effect of the molecules that belong to first group. An example is tartaric acid or lecithin that make stronger the antioxidant action of citric acid. Tomato fruit is a concentrate of antioxidants, both lipophilic (carotenoides, E vitamin and some flavonoids) and hydrophilic (ascorbic acid, phenol acids and glycosylate flavonoids); the mostly representative are polyphenols and carotenoids, which makes tomato a unique food for its nutritional properties.

2.6a Polyphenols

Polyphenols represent a very wide category of substances, all characterized by the presence of more condensed phenolic cycles. Phenol composites are secondary products and play different roles within the plant. Some are useful against herbivorous animals and pathogens, others are useful as mechanical support, for attract pollinators and for fruit dispersion or as growth inhibitors of plants in competition.

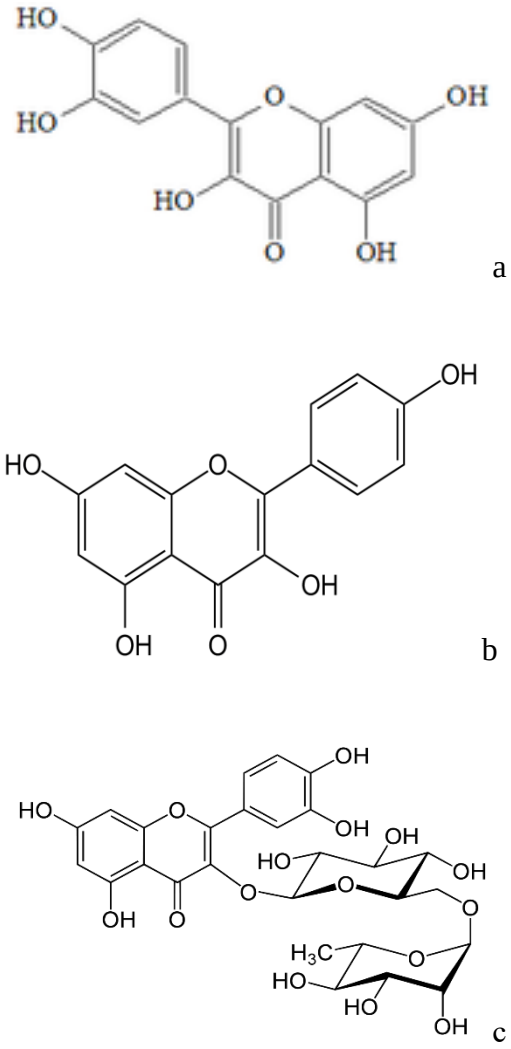


Figure 2.5 Structure of the most important flavonoids introduced with diet a) Quercetin, b) Kaempferol and c) Rutin

Polyphenols are widely distributed in nature, quercetin, kaempferol and rutin (Figure 2.5) are the most abundant flavonoids in the diet intoduced with fruits and vegetables expecially with garlic, onions, lettuce and tomato. A diet abundant of fresh vegetables gives a daily supply from 200 mg to 1 g of these substances, ensuring a sufficient amount of antioxidants. Antioxidant action is not the only activity attributed to these compounds; studies, perfermod both *in vivo* and *in vitro*, showed that polyphenols are anti-carcinogenic, anti-atherogenic, anti-inflammatory, antibacterial and antiviral [Havsteen BH 2002]. of phenols has been related to the inhibition of. Flavonoids and polyphenols generally have an anti-carcinogenic effect on the first steps of cancer development, protecting cells against the direct attack of carcinogens or altering their

activation mechanism; this has been studied in different cancer types (colon, oesophagus, lung, liver, breast and skin cancer) [Batra P et al 2013; Briguglio G et al 2020]. Flavonoids have anticancer and preventive effects because induce apoptosis and cell cycle arrest at G_1 or G_2/M phase by inhibiting key cell cycle regulators such as cyclin-dependent kinases (CDKs) [Abotaleb M et al 2018] . Regarding anti-atherogenic action, the main mechanisms involved are the reduction of platelets coagulation and produce an increace of HDL[Millar CL et al 2017]. Other mechanisms are the inhibition of lypoproteins oxidation and increased antioxidant capacity (free radical scavenging and metal chelating activities) [Amarowicz R et al 2017; Bravo L 1998]. Anti-inflammatory action is the first effect known, exerted mainly by anthocyanins. These last seem to inhibit NF-κB activity via mitogen-activated protein kinase (MAPK) pathways [Garcìa-Barrado M. et al 2020].

Tomato is a source of polyphenols targeted to different disease prevention, as mentioned above. Major tomato polyphenols are hydroxycinnamic acids, flavanones, flavonols, and anthocyanins [Martí R et al 2016]. Naringenin chalcone (Figure 2.6) is the principal polyphenol contained in the tomato with a maximum concentrations of 18.2 mg 100 g^{-1} fw [Slimestad R et al 2008], quercetin is the main flavonol and one of the most important flavonoids from tomato. Its content varies from 0.7 to 4.4 mg 100 g^{-1} fw [Martínez-Valverde I et al 2002], depending on the type of tomato. The accumulation of this class of compounds in tomato takes place during the last stages of ripening and is also almost restricted to the peel [Martí R et al 2016].

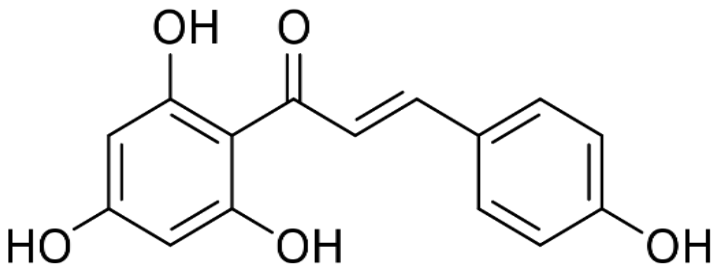


Figure 2.6 Naringenine Chalcone structure

2.6b Carotenoids

Carotenoids take their name from carotene, a yellow-orange substance, found for the first time (1831) in the Daucus carota (common carrot). Carotenoids are a group of pigments from yellow to violet, widespread in nature and soluble in the lipids. The intensity of their colour is due to the chromophore system constituted by an high number of double combined connections, even responsible of the large carotenoids instability, which easily oxidize in contact with air and are considerably modified by the presence of mineral acids. Because of light exposure or other agents, carotenoids, that are always in trans configuration, can transform themselves in a mixture of stereo-isomers, in which one or more double connections moved to cis position. From a chemical point of view, carotenoids are terpenes, usually at 40 carbon atoms, made of eight isoprene unit [Rodriguez-Concepcion M et al 2018]. The skeleton of their molecule generally consists of a central share, with 22 carbon atoms and two terminal shares made of 9 carbon atoms each. Terminal units can be no-cyclical like in the lycopene or both cyclical like in α and β carotene, or one cyclical and the other no-cyclical like in γ-carotene (Figure 2.7). Moreover, terminal cyclical units can tie a wide variety of groups (alcoholics, ketones, epoxies, benzenics, etc). Two families of carotenoids are known: carotenes with a hydrocarbon diene structure and xanthophylls, that is oxygenate derivates, plus carotene acids [Maoka T 2020] .

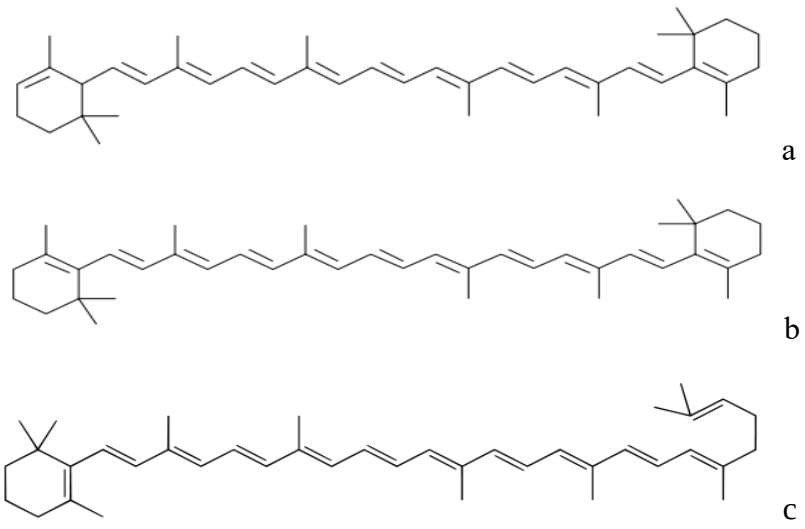


Figure 2.7 a) α-Carotene, b) β-carotene and c) γ-Carotene Structures

The most famouse carotene are lycopene, β-carotene and α- carotene, while among the xanthophylls the main are lutein, zeaxanthin, violaxanthin and auroxanthin (Figure 2.8), bixin and crocetin belong to carotenoid acids (Figure 2.9).

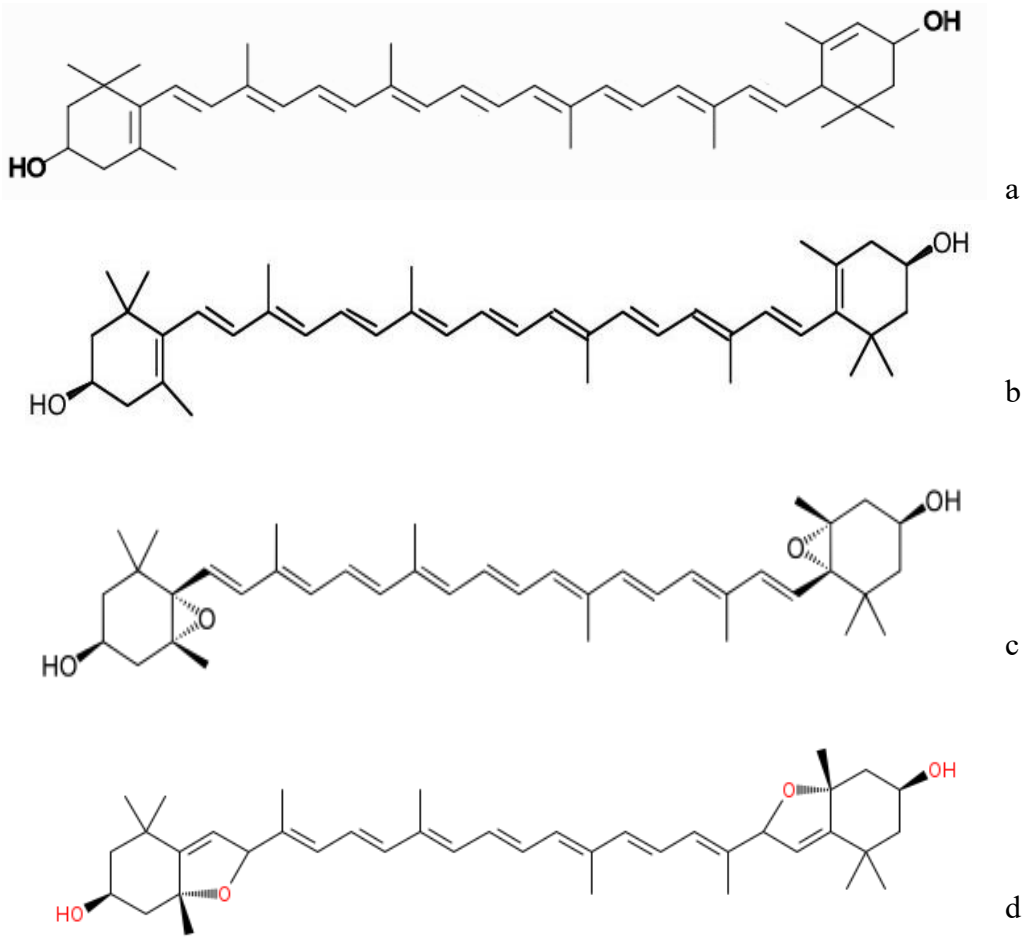


Figure 2.8 The main xantophylls a) Lutei, b) Zeaxanthin, c) Violaxanthin and d) Auroxanthin

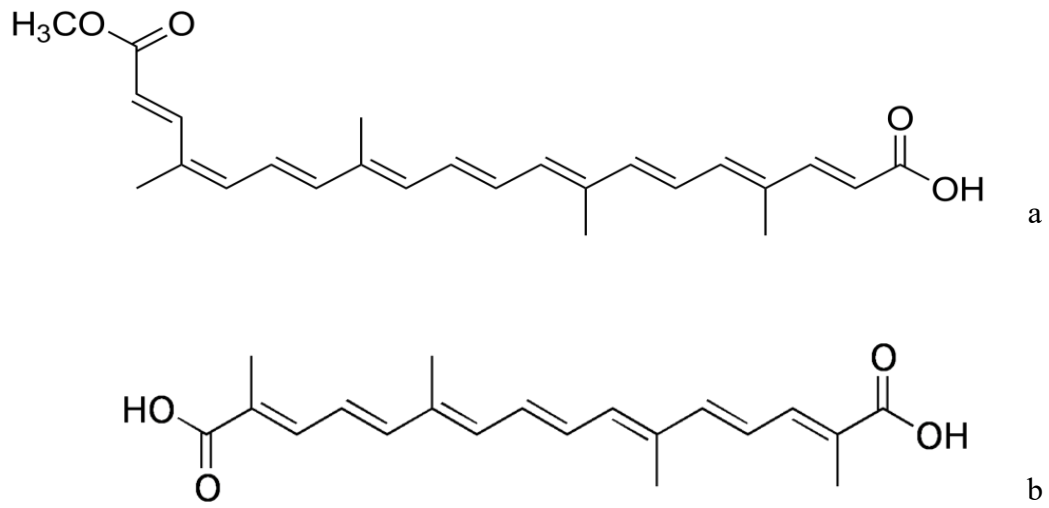


Figure 2.9 a) Biaxin Structure and b) Crocetin Structure

Carotenoids has been isolated from plants, seaweeds, bacteria and from animal kingdom and are responsible for different coloration, for example red of tomato (lycopene), orange of carrots (β-carotene) and corn (zeaxanthin), yellow of marigolds (violatxanthin and auroxanthin), crocein for saffron and lutein for autumn leaves. In unripe fruits they are not visible because are produced during ripening process in presence of oxygen, displaying the typical colourings. Carotenoids, not only take part in photosynthesis of plants but also are essential in the protection against free radicals formed during the photosynthesic process. Animals, included men, are not able to synthesize these substances; therefore they take them from vegetative kingdom through the nutrition and then they modify them; particularly important to man, is the conversion of α –β- y carotenes to A vitamin (Figure 2.10), in the intestinal mucous membrane, that allows us to obtain this essential molecule for our health. A vitamin have a prominent role in biosynthetic processes of many glycol-proteins, which regulate cellular differentiation and genes expression; for this reason it is an important factor for the development and growth of body. Lack of A vitamin and carotene strikes first tissues with a fast cells turover as the epithelial tissue, which tend to iperkeratinize.

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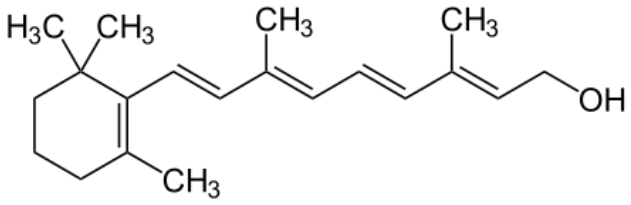


Figure 2.10 Vitamin A Structure

Fruits and vegetables red-purple coloured, as tomato fruit, red cabbage and berries, in addition to carotenoids contain also other pigments such as flavonoids, wich have not A pro vitamin activity. Different studies [Murillo AG et al 2016; Lee Y et al 2019] have highlighted that carotenoids are not only an essential A vitamin source but have many other protective actions for our body; licopene, zeaxanthin and lutein, demonstrated to have an high antioxidant activity. In tomato, the carotenoids are among the main bioactive compounds and they are present in leaves, flowers and fruits. The main carotenoids present in tomato is lycopene with concentration up to 7.8 to 18.1 mg 100 g^{-1} fresh weight (fw), β-carotene is the second main carotenoid found in tomato with concentration up to 1.2 mg 100 g^{-1} fw; it is also responsible for the orange color of certain types of tomato. Other carotenoids can be found at a lower concentration in the tomato, among them are γ-carotene, δ-carotene, lutein, neurosporene, α-carotene.

- **Lycopene**

Lycopene is a carotenoid pigment, mostly present in tomato fruit, which give it the typical red colouring [Trombly HH et al 1953]. During tomato riping, lycopene is the last carotenoid that is formed; its biosynthesis considerably increased when chloroplasts differentiated in chromoplasts. [Kirk JTO el al 1978]. Tomato skin and pericarp are particularly rich in lycopene (Figure 2.11). The skin, alone, contains 12 mg of lycopene for 100 g (damp weight), while ripe whole fruit contains only 3,4 mg for 100 g (damp weight) [Al-Wandawi H. et al 1985].

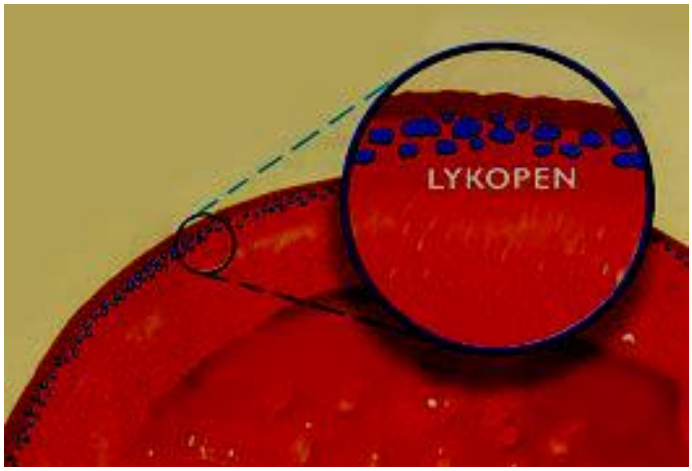


Figure 2.11 Distribution of lycopene in mesocarp

Lycopene contained in tomato varies considerably because reflects the influence of tomato variety (genetic factors) but also maturation degree and conditions both agricultural and environmental during its growth [Periago MJ et al 2004].

Since is a lypophilic sustance, it disperses itself in edible oils and it is soluble in apolar organics. Conversely, in aqueous systems it tends to aggregates and precipitate in crystals. This characteristic is considered the cause of low lycopene bioavailability within human system [Zumbrunn A et al 1985]. Structurally, lycopene is an unsaturated aliphatic hydrocarbon (Figure 2.12); its chain contains 13 double connections carbon–carbon, 11 of these are combined and put in linear order. This structure is the key of its biological activity, which includes its susceptibility to oxidative degradation. Contrary to other carotenoids, lycopene is not a pro-vitamin A [Hill HM et al 1969].

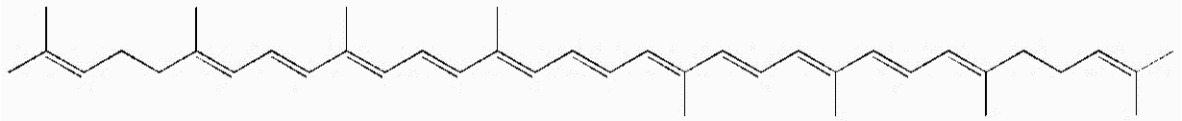


Figure 2.12 *Trans*-lycopene Structure

The degradation of lycopene not only has negative effects on quality of final processed product, but also has a loss of benefits. Main causes of degradation during the processing are isomerisation and oxidation [Shi J et al 2000]. Low temperature of stocking, low levels of oxygen and the reduced light exposure are factors that limits the degradation of lycopene due to the oxidation [Granado F et al 1992; Clinton SK et al 1996; Porrini M et al 1998]. Concerning isomerisation, lycopene within fresh tomato is mainly in all-trans configuration [Shi J et al 2000] even if cis isomers are better

absorbed. This could be due to higher solubility of such isomers in micelles that allow the incorporation in chylomicrons favoring intestinal absorption [Sakamoto H et al 1994; Britton G 1995; Stahl W et al 1996; Boileau AC et al 1999]. Also composition and structure of food matrix influence lycopene bioavailability; cooking or grinding can increase the bioavailability by destructing or weakening cellular membranes, breaking lycopene-proteins complexes [Hussein L et al 1990]. According to recent studies, eating tomato and its derived has been associated with the decreased risk of developed chronic diseases as cancer and cardiovascular problems [Stice CP et al 2018; Cheng HM et al 2017]. Antioxidant properties of lycopene are believed to be responsible for such healthy effects [Blum A et al 2005]. *In vitro* and *in vivo* studies have highlighted that the high usage of tomatoes decrease the risk of developed diseases caused by oxidative stress thanks to lycopene antioxidant capabilities [Fang Y et al 2020; Qu M et al 2016]. Indeed lycopene is considered the most steady and powerful agent of extinction of singlet oxygen ($_1O^2$), that is notoriously harmful, and an efficient "scavenger" of a wide spectrum of free radicals [Pennathur S et al 2010; Bhuvaneswari V et al 2005; Di Mascio P et al 1989; Conn PF et al 1991]. Human body system is not able to synthesize lycopene, therefore is necessary take it by diet. Once taken it, we can find it in higher concentration within the liver, testicles, adrenal glands, prostate and blood.

Chronic inflammation (oxidave stress play an important role in its onset) is known as one of the most important milestones in cancer. Since lycopene is one of the most important antioxidant in nature, many *in vitro* studies analyzed its anti-inflammatory effect; data show that levels of prostaglandin E2 (PGE2) and NO decreased after administration of lycopene [Imran M et al 2020]. Increasing evidences, about the usage of products made with tomato, revealed promising regarding prostate cancer; moreover high concentrations of lycopene in the blood are associated with a decrease the risk for different cancers including gastric and colorectal cancer [Tsugane S et al 1992; Farinetti A et al 2017].

2.7 EM® TECHNONOLOGY

EM® technology (Effective Microorganisms) has been developed for the first time in 1970 by the professor Teruo Higa University of Ryukyus in Okinawa . The EM® consists of *in vivo* micro-organisms crops selection, isolated in nature from the soils

[Mohan B 2008]. EM® micro organisms include lactic bacteria as *Lactobacillus plantarum, L. Casei, Streptococcuslactis,* photosynthetic bacteria as *Rhodopseudomonas lalustris* and *Rhodobacter speroides,* yeasts as *Saccharomyces cerevisiae* and *Candida utilis,* actinium-fungus as *Streptomycesalbus and S.griseus,* fermenting fungus as *Aspergillu oryzae Penicillum sp, Mucorhiemalis, Trichoderma harzianum* and *Trichoderma viride*.

The main activity of EM is to increase soil biodiversity. Photosynthetic bacteria, that are part of EM in synergy with other microorganisms, increase energetic substances available for plants and reduce the stress [Condor AF et al 2007]. EM microorganisms interact with plant–soil system abolishing plants pathogens and disease agents, solubilising mineral substances of the soil, increasing energy, keeping the balance of microbial flora of the soil, increasing photosynthetic efficiency and fixing nitrogen. Data from different analysis showed significant results regarding seeds germination and tomato plants vigour [Siqueria MFB et al 2013]. Moreover many authors underlined an increase of tomato growth in open field and a better resistance for several infections as *Phytophora* and *Downy mildew* [XU HL et al 2001]. Electrophoresis analysis performed on tomato plants grown using Effective Microorganisms demonstrated a decreasing of nitrogen compounds and nitrates (highly produced in stress conditions by plants) [XU HL et al 2001]. Integrated results suggest that the increase of resistance to infections were strictly connected to nitrogen metabolism.

Inoculation by EM establishes an increase of photosynthetic levels of plants with a resulting early fructification [Nceube L et al 2011] and plants height [Idris II et al 2008]. Usage of EM in cultivation increase the C vitamin concentration within fruits [Xu HL et al 2001] and contents of sugars, proteins and amino acids. More important advantages following roots colonization by EM microorganisms are:

- Growth promotion of vegetables and better tolerance toward water stresses.
- Better mobilization and absorption of nutrients, especially phosphorus.
- Availability of minerals, usually insoluble.
- Modification of plant-pathogen interaction (the root colonization by pathogen as nematodes, fungus and phito-pathogen bacteria is negatively influenced).

This healthy effect of protection against terricoulus pathogen can be a consequence of radical exudates alteration or through the creation of a real physical barrier around the root made by microrganisms.

Chapter 3

Purpose

Bioactive natural compounds have been long used for healing purposes. Although many *in vivo* and *in vitro* studies focus on the possible anti-carcinogenic properties of food, they usually investigate the action of pure phytochemicals in order to identify novel drugs for cancer chemoprevention and/or therapy. Far fewer studies in fact focus upon interventions with whole foods or whole food fractions. Here, we aimed to evaluate the effects of treatment with total polyphenolic (methanolic) extracts from three tomato cultivars, Brandywine (B), New Yellow (N) and Cherry Purple (P) using as biological system an *in vitro* model of melanoma cancer. All the experiments are performed, used the polyphenolic extracts obtained from tomatos cultivated with EM technology to improve quality.

Chapter 4

MATERIALS and METHODS

4.1 Tomato Plants and EM® Technology

Tomato Plants of Brandywine (B), New Yellow (N) and Cherry Purple (P) have been cultivated in an expanded and experimental field named "Giordano", located in Corbara (Salerno, Italy). Seed of tomato were germinated at the final of february 2017, after this, the tomato plants are ready for planting in the "Giordano" field. The "Giordano" field cultivated with EM® technology was treated, at first, with *Bokashi* product, bran enriched with EM® liquid solution [a mix of isolated soil microorganisms, such as lactic (*Lactobacillus plantarum*, *L. casei*, *Streptococcus lactis*) and photosynthetic bacteria (*Rhodopseudomonas lalustris* and *Rhodobacter speroides*), yeasts (*Saccharomyces cerevisiae* and *Candida utilis*), actinomicetes (*Streptomyces albus* and *S. griseus*) and fungi (*Aspergillus oryzae*, *Penicillum* sp., *Mucor hiemalis*, *Trichoderma harzianum* and *Trichoderma viride*]. Two weeks before placing the tomato seedlings, *bokashi* was dispersed on the ground, by mixing the first 6 cm of soil. The amount of *bokashi* used was 100 kg/500 m^2 of field. Next, EM® (liquid solution) was used on tomato plants starting from their transfer in open field. Before the use EM® was diluted 1:500 (v/v) in water and sprayed on the plants once every 5-7 days at sunset.

4.2 Extraction Process

Samples were homogenized in a blender and next centrifuged at 9,500 rpm for 20 min at 4°C to obtain surnatant (hydrophilic fraction) and a solid part (the pellet). These different fraction were collected separately and kept for analysis. Polyphenolic (methanol) extracts were obtained from pellets through three cycle of overnight stirring in the dark with methanol (1:2 wt/vol). Extracts were then filtered, concentrated in a rotary evaporator under vacuum (temperature <35°C), and dried under N_2 [Tommonaro G et al 2012].

4.3 Analysis of chemical-physical parameters

The total soluble solids (TSS) content of samples, expressed as °Brix, was estimated by using refractometer and saccharose as standard. The total titratable acidity (TA) of samples was evaluated by means of 0.1 N NaOH titration up to get to pH 8.1 value. TA was expressed as grams of acid citric equivalent for 100 g of fresh product (g CA/100 g FW).

4.4 Bioactive Compound Content

The total polyphenols content was estimated by using the Folin-Ciocalteau method [Singleton and Rossi 1965]. Briefly, 50 μL of Folin-Ciocalteau's phenol reagent, a volume of samples ranging from 10 to 50 μL and 800 μL of deionized water were accurately mixed. A final volume of 1 mL was reached by adding deionized water. Quercitin was used as standard. Samples were kept ar room temperature for 2 h and next the total phenol content was estimated by reading at 765 nm (DU-Beckman, USA). All determinations were petrformed in triplicates.

4.5 High-Performance-Liquid-Chromatography (HPLC)

The polyphenolic extract obtained from each sample was analyzed by reversed-phase High-Performance-Liquid-Chromatography (HPLC) in order to evaluate polyphenol (Rutin and Naringenin Chalcone) content. For polyphenol analysis, the system used was an Agilent 1200 Infinity II LC with a Kinetex 100A C18 column, 2.6 μm, 100 × 3 mm (Phenomenex, Torrance, CA) with DAD detector 1260 DAD WR and Agilent OpenLAB CDS (EZChrom Edition) software version A. 04. 07. HPLC analysis was performed by using the following chromatographic conditions: gradient elution with water (A) and acetonitrile (B), 85%/15% v/v, A/B for 10 min, gradually changing to 75%/25% A/B for 10 min, gradually changing to 10%/90% A/B for 3 min, 10%/90% A/B for 5 min, gradually changing to 85%/15% A/B for 2 min and holding 85%/15% A/B for 5 min to return to the starting condition before next injection; flow rate 1.0 ml/min; injection volume 5 μl; UV detector λ = 365 nm. Polyphenolic extracts were analyzed compararing with Rutin and Naringenin Chalcone standards (range from 5.00 mg to 0.312 mg) Stock solutions of each standard were prepared at concentration of

50mg/5 ml of Dimethyl Sulfoxide (DMSO). Retention times of each carotenoid were found as follow: Rutin 2.45 min and Naringenin Chalcone 19.15 min

Time / min	% A	% B
0	85	15
10	85	15
20	75	15
23	10	90
28	10	90
30	85	15
35	85	15

4.6 Antioxidant Activity

The antioxidant activity was evaluated by using the DPPH decolouration methods which are based on the capacity of different components to inhibit the DPPH radical [Blois MS 1958]. The antioxidant capacity was expressed as radical percentage inhibition.

4.7 Cell Culture

Human M14 melanoma cell lines and a primary human skin fibroblasts (HSFs) cell lines were used. The human melanoma M14 cell lines were kindly provided by Doctor Paolo Antonio Ascierto from National Tumor Institute of Naples “G. Pascale”, M14 cell lines harbor BRAF-V600E mutations. Primary human skin fibroblasts were kindly provided by Michele Fimiani, Giancarlo Mariotti and Stefania Mei from University of Siena (IT) [Pianigiani et al., 2010]. Cells were cultured and maintained in RPMI supplemented with 10% fetal bovine serum, 100U/mL of penicillin and 0.1mg/mL of streptomycin. All the cell lines were incubated at 37°C in a 5% CO2 incubator until they reached 70% confluency (48 h) for M14 and 70% confluency (72 h) for HSFs. Before being used for the experiments, cells were routinely maintained under exponential-proliferation conditions.

4.8 Trypan Blue Assay

Trypan blue assay was used to determine the number of viable cells present in a cell suspension (live cells, excluding trypan blue are not coulored, whereas dead cells let pass dye and appeare blue stained). 1.0×10^5 cells were plated in tissue culture dishes (60 mm) containing RPMI with 10% FBS. Cells were treated with 25, 50 and 100 μg/ml of methanolic extracts of Brandywine (B), New Yellow (N) and Cherry Purple (P) and DimethylSulfoxide (DMSO) as control. After 72 hours (h) culture medium was removed, adherent cells were trypsinized and collected. Trypan Blues was added to cells suspension (1:1) the number of live/died cells in each well was quantified using a Burker chamber. Data obtained from three independent experiment were used to calculate cell survival rate.

4.9 Cell Viability Analysis by XTT Assay

XTT assay was used to assess cell viability after treatment with polyphenolic extracts and to evaluate the effect of different tomato extracts. Briefly, M14 cells were seeded at the density of 7×10^3 cells well in 96 well microplates. After 24h of incubation, the medium was changed with 200 μl of 10 % FBS RPMI culture medium plus polyphenolic extracts at three concentration (25, 50, 100 μg/mL). After 72h 50 μl of XTT was added and cells incubated for 6h. The absorbance values was measured at 450 nm by an automatic multifunctional microplate reader (FLUOstar Omega, BMG Labtech). Cell viability was calculated with the following formula:

$$\text{Cell Viability (\%)} = (\text{A sample} - \text{A blank}) / (\text{A control} - \text{A blank}) \times 100\ \%$$

4.10 Cell Colony Formation Assay

A total of 100 cells/well were seeded in multiwells plates (6 well) and treated with our tomato extracts and DMSO as control. After 7 day of incubation, cells were stained with Crystal Violet plus methanol for 20 minutes, PBS (Phosphate-buffered saline) 1x

washed and air-dried. Cell colonies formed were counted and then photographed. The cell colony formation assay was performed in triplicate (mean±standard deviation [SD]).

4.11 Cell Cycle Analysis

Cells, after 72h of incubation with 25, 50, 100 μg/mL of tomatoes extracts or DMSO, were trypsinized and $1x10^6$ cells were fixed with 70% ethanol at −20°C overnight. Cells were washed with and stained with 5 μg/mL propidium iodide and 20 μg/mL RNAase at 4°C overnight. DNA content and cell cycle distribution were analyzed by FACS (CytoFLEX, Beckman Coulter).

4.12 Protein Extraction and Western Blotting

For total protein extraction, cells were lysed on ice for 30 min in lysis buffer containing 250 mM NaCl, 0.5% NP-40, 50 mM Hepes pH 7.5 supplemented with protease and phosphatase inhibitor cocktails (Roche Applied Science). Protein concentration was quantified using the Bradford protein assay. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate cell lysates. Proteins were transferred to nitrocellulose membranes and blocked with tris-buffered saline and 0.1% Tween 20 (TBS-T 1X) containing 5% not fat dry milk (blotto A), and then incubated with the following primary antibodies at 4°C overnight: rabbit anti-pRb2/p130 (Abcam, Cat #Ab 76234) (1:250), rabbit anti-$p21^{Cip1}$ (Cell Signaling, Cat # 2947) (1:100), rabbit anti-p27Kip1(Abcam, Cat #Ab 32034) (1:250), rabbit anti-GAPDH (Abcam, Cat # 9485) (1:1000), and mouse anti- Lamin (Cell Signaling, Cat #4777) (1:250). Membranes were washed three times with TBS-T 1X and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies (1:3000) for 1 h at room temperature. Protein bands were visualized using ECL detection reagents. Protein expression levels were normalized to GAPDH or Lamin.

4.13 Real-time Reverse Transcriptase-Quantitative PCR (RTq-PCR)

Total RNA was isolated from cell lines using the RNeasy Mini kit (Qiagen, Hilden, Germany Cat #74106). RNA concentration was determined using a NanoDrop™ ND-1000 (Thermo Fisher). Complementary DNA (cDNA) was synthesized from 500 ng of RNA using the iScript cDNA Synthesis Kit (Bio-rad, Hercules, Cat #1708891BUN) and amplified in the LightCycler™ instrument (Roche Applied Sciences) using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-rad, Cat #1725274) according to the manufacturer's instruction. The primers used were from Bio-Rad and SinoBiological: *Cyclin A2* (*CCNA2),* Assay ID qHsaCID0017452; *Cyclin-dependent kinase 2* (*CDK2),* Assay ID qHsaCEP0051246; *P27 Kip 1* Assay ID HP101024 ; *GAPDH* and Assay ID qHsaCED0038674. The housekeeping glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used to normalize the expression of genes of interest. Gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Fold Change).

4.14 Quantification of UV-A-induced intracellular ROS

Intracellular ROS formation was measured using an oxidative stress reagent (CellROX deep red reagent, Life Technologies Corporation, Carsband, CA, USA) using the manufacturer's recommended protocol. Fibroblasts were suspended in RPMI (2×10^4). After 1-day incubation, a stepwise dilution series of polyphenols, described above and 100% DMSO without polyphenols, were diluted 500 fold with RPMI. 50 µl of the dilution with or without polyphenols were added to each well after removing the growth medium (50µl in each well). The final concentration of each polyphenols extracts was in the range 25 to 100 µg/ml. The polyphenols extract/cell mixtures were incubated for 3 h at 37°C, since it was previously shown that a 2 h pre-incubation of flavonoids with cells ensured their permeation membrane into the plasma membrane. UV-A irradiation was performed in a box controlled at 30 °C. The culture plate was irradiated with a total fluence dose of 3 J/cm³ using the UV-A LED device position 65 mm over the plate. The cells were stained with 40 uM CellROX reagent during the last 30 min of the irradiation period. Next, the fluorescence of each well was measured with Tecan, (lex = 640 nm, l em = 665 nm).

4.15 Statistical Analysis

Statistical analyses were performed using t Microsoft Excel Software, version 2019 for Windows. Statistically significant differences between the means of groups were evaluated using paired Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference (*significant $p < 0.05$ and **very significant $p<0.01$).

Chapter 5

RESULTS

5.1 Tomatoes Chemical-Physical Parameters are comparable between three tomatoes varieties

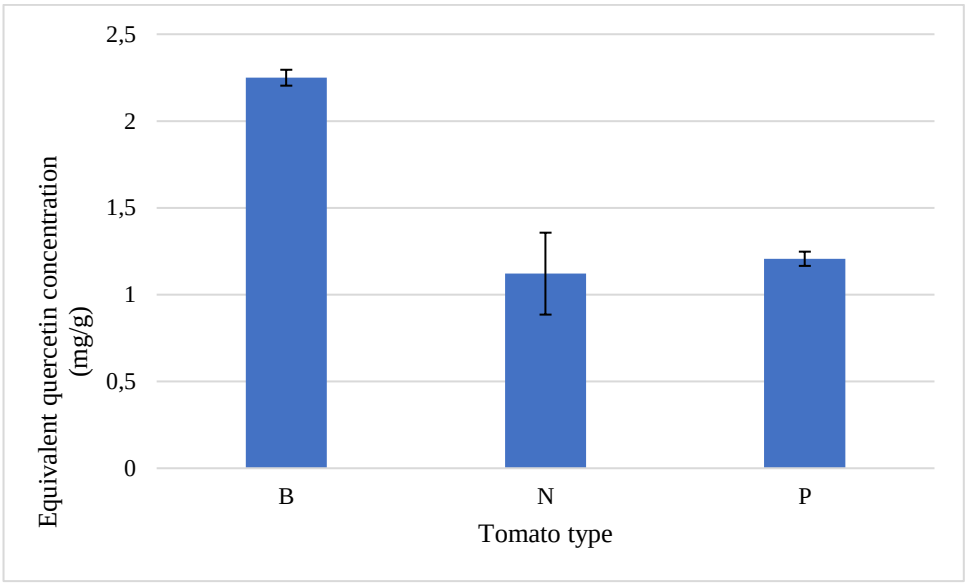
Fruits of three different tomato cultivars were investigated for their chemical-physical parameters: the total soluble solids (TSS) expressed as °Brix, the total titratable acidity (TA) expressed as g CA/100g FW and pH (Caliman F.R.B. 2010). For all tomato varieties no relevant differences were detected (Table 5.1).

Table 5.1 Total soluble solids (TSS) content, pH and total titratable acidity (TA) of B, N and N tomato varieties. Values are given as mean ± SD

Tomato	pH	TSS (°BRIX)	TA (g CA/100g FW)	Maturity Index (TSS/TA)
Brandywine (B)	4,40	5,20±0,28	0,32±0,05	15,63±2,00
New Yellow (N)	4,30	5,15±0,29	0,27±0,04	15,27±2,35
Cherry Purple (P)	4,44	5,55±0,29	0,25±0,02	15,64±2,06

5.2 Rutin is the most abundant polyphenols in tomato extract

The polyphenolic content of all methanolic tomato extracts was estimated by using the Folin-Ciocalteau method. The tomato extract which contains the most amount of polyphenols is Brandywine (2,24 mg quercetin equivalent/g extract), followed by Cherry Purple and New Yellow (Figure 5.2). Data obtained from Folin-Ciocalteau experiment is in accordance with results coming HPLC and confirming the greater content of polyphenolic in B extract.



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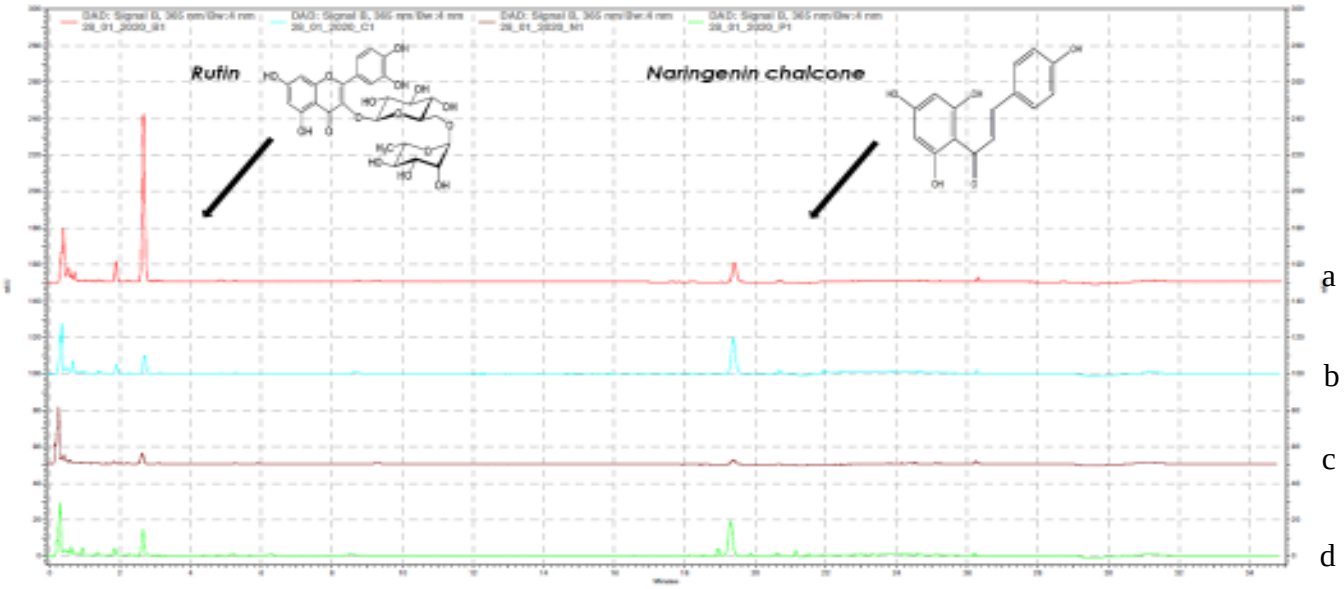
Cultivar	Poliphenols
Brandywine **(B)**	**2,24**± 0,045 mg quercetin/g extract
New Yellow **(N)**	**1,20**± 0,041 mg quercetin/g extract
Cherry Purple **(P)**	**1,70**± 0,106 mg quercetin/g extract

Figure 5.2 Polyphenols content was evaluated by using Folin-Ciocalteau method. B (Brandywine), New Yellow (N), Cherry Purple (P). All values are the means ± SD from three independent experiments.

The Rutin and Naringenine chalcone content was determined by HPLC. As can be seen in the (Figure 5.3 e), among all methanol extracts, the tomato extract Cherry Purple had the most balanced profile for these two major polyphenols, expressed mg kg $^{-1}$ fresh product (13,10 mg kg^{-1} of Rutin and 9,24 mg kg^{-1} of Naringenine chalcone). The amount of these compounds in the other extracts, is not similar, ranging from 80,41 and 4,05 k^{-1} in B and from 3,97 to 0,58 kg^{-1} in N for Rutin and Naringenine chalcone respectively. Although Brandywine extract had a very high content of Rutin and the highest amount of polyphenols overall, its Naringenine chalcone content was

significantly lower than that of the Cherry Purple extract, while the New Yellow variety has significant lower levels of both polyphenols.



Tab. 5.3 Polyphenols concentration for mg/kg^{-1} of fresh sample. All values are the means ± SD from three independent experiments.

Cultivar	Rutin (mg/Kg^{-1}) of fresh tomato	Naringenine Chalcone (mg/Kg^{-1}) of fresh tomato
Brandwine (B)	85,4173±0,32	4,050185±0,14
New Yellow (N)	3,972967±0,65	0,585539±0,78
Cherry Purple (P)	13,10976±0,62	9,244927±0,35

Figure 5.3 HPLC spectra of polyphenols- Rutin and Naringenine Chalcone (a) standard, (b) Brandywine extract, (c) New Yellow extract and (d)Cherry Purple extract.

5.3 Tomato extracts shown antioxidant activity

The antioxidant activity related to different extracts and expressed as equivant of Trolox (index of inhibition of DPPH* radical) is summarized in (Figure 5.4). Data showed that

all varieties have a radical scavenging capacity and the Charry purple extract (at the maximum concentration) exhibited the best DPPH radical scavenger activity.

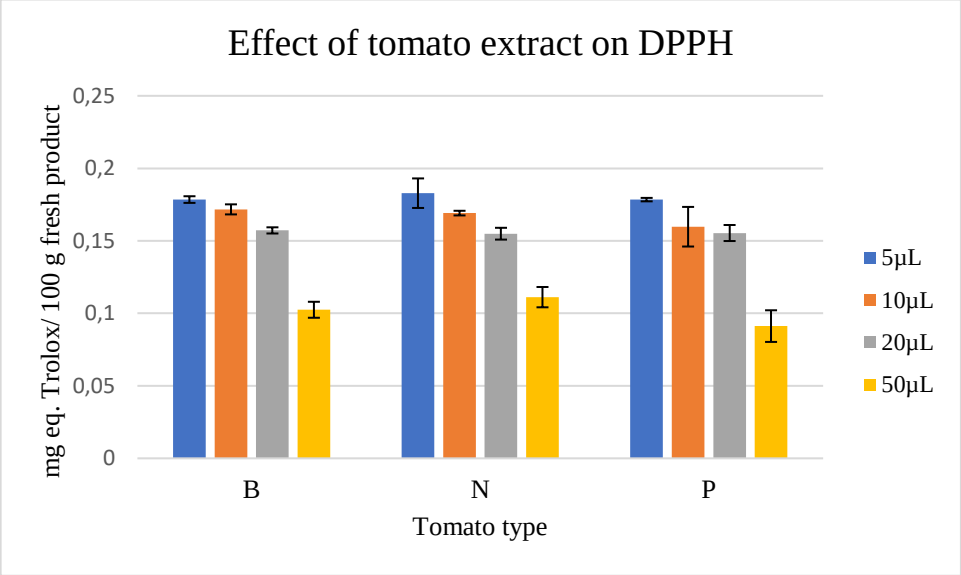
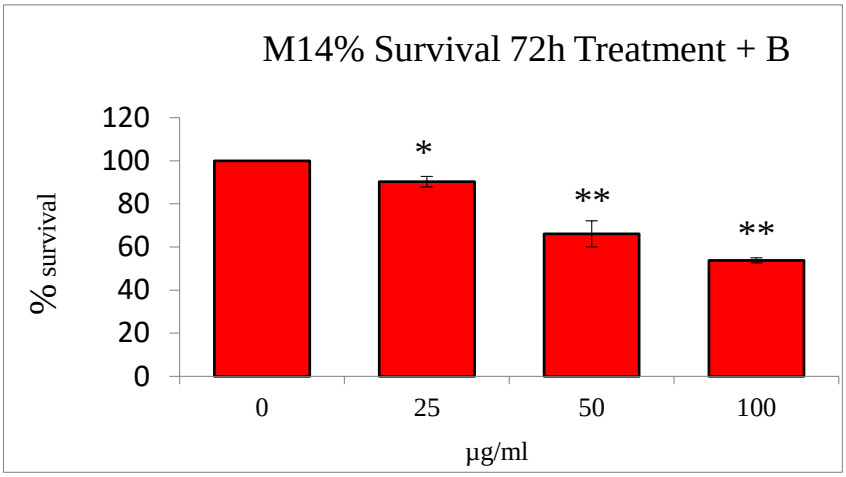


Figure 5.4 Antioxidant activity of polyphenolic extracts evaluated by DPPH method. All values are the means ± SD from three independent experiments.

5.4 Polyphenolic tomato extracts inhibit cancer cells viability in a dose response way

After studying the physical and chemical characteristics of our tomato polyphenolic extracts we tested the effect of various doses of these methanolic extracts on melanoma cells viability. Firstly we treated M14 with 25, 50 and 100 µg/mL of methanolic extracts or DMSO as control, after 72 h of treatment cells were stained with Trypan Blue and counted. Results (Figure 5.5 and 5.6) showed that all doses and all tomato vareties used have a significant inhibitory effect on cancer cells vitality showing a good antiproliferative efficacy on M14 cell lines. Obviously the best result was found in cells treated with the highest dose. To analze if this toxic effect was cancer cells specific, we performed the same assay on HSFs. After 72h of treatment with the three different concentration of B, N and P methanolic extracts we can't find any change in cells vitality, and so for that reason is not reported the significance (Figure 5.7) suggesting that our tomato extracts have effect only on cancer cells and no on normal one.



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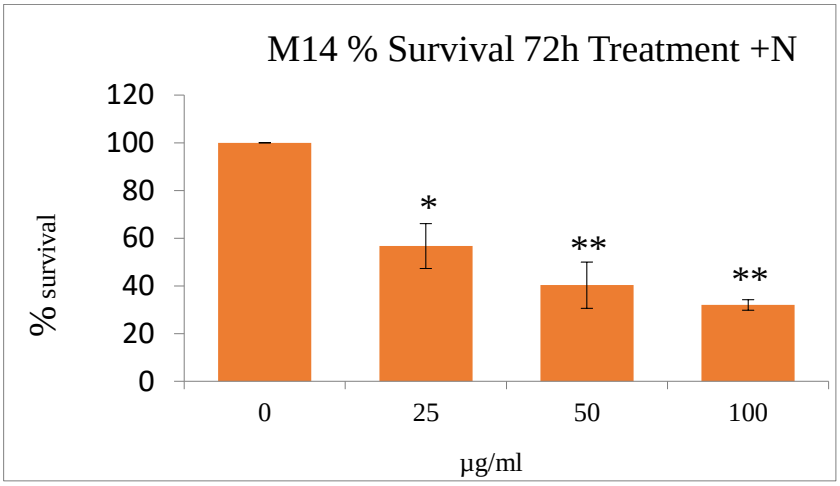

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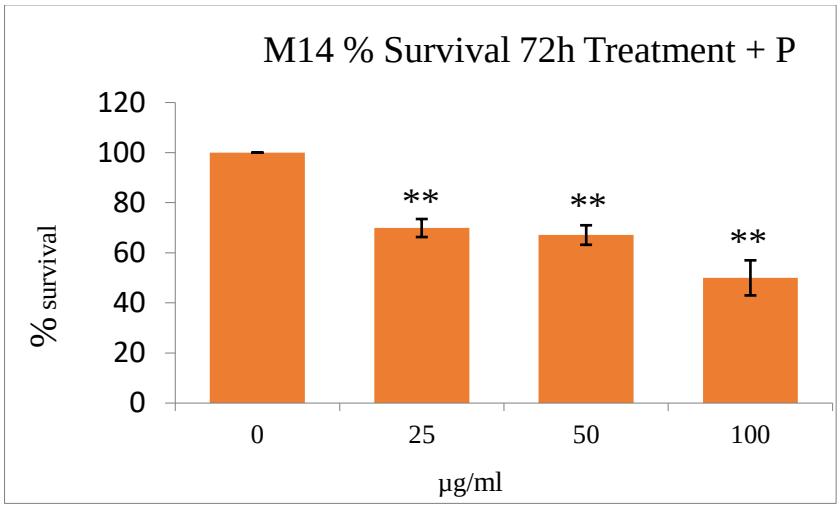


c

Figure 5.5 M14 dose response survival histograms. We treated with 25, 50 and 100 of methanolic B (a), N (b) and P (c) tomato extracts versus DMSO and counted cells at 72h. All values are the means ± SD from three independent experiments. Statistical analysis was performed by subjecting total number of trated and control cells to Student's *t*-test. Statistically significant differences are indicated with: *significant $p<0.05$ and ** very significant $p<0.01$

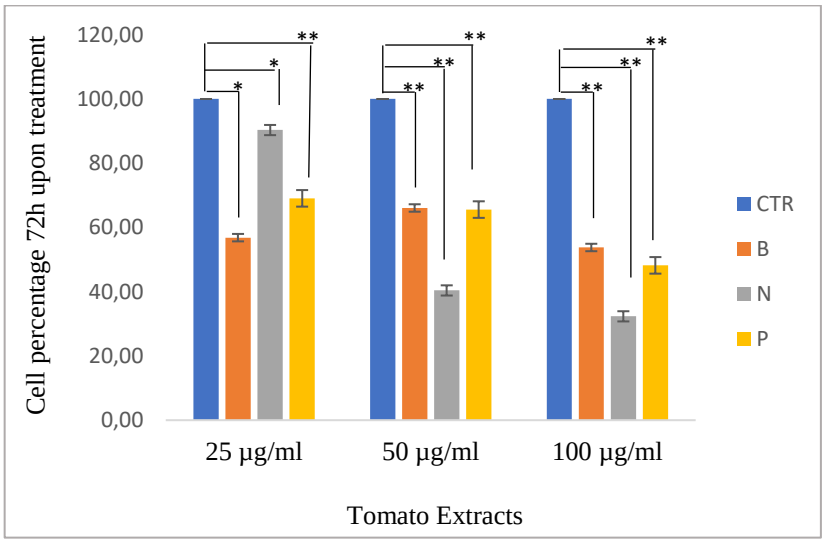
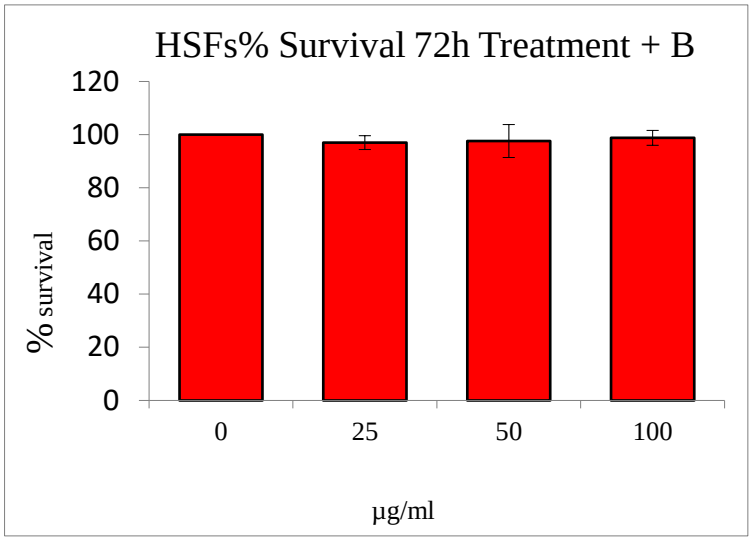


Figure 5.6 Comparison of dose-responce effect of B, N, P polyphenolic extracts. All values are the means ± SD from three independent experiments. Statistical analysis was performed by subjecting total number of trated and control cells to Student's *t*-test. Statistically significant differences are indicated with: *significant $p<0.05$ and ** very significant $p<0.01$.



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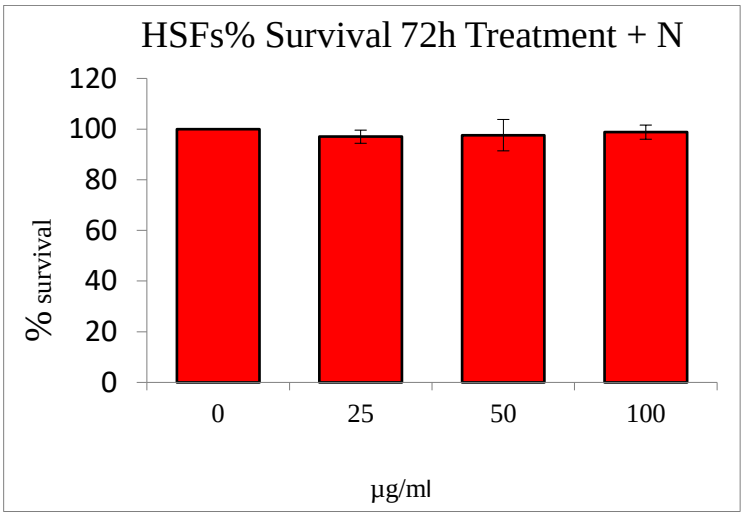

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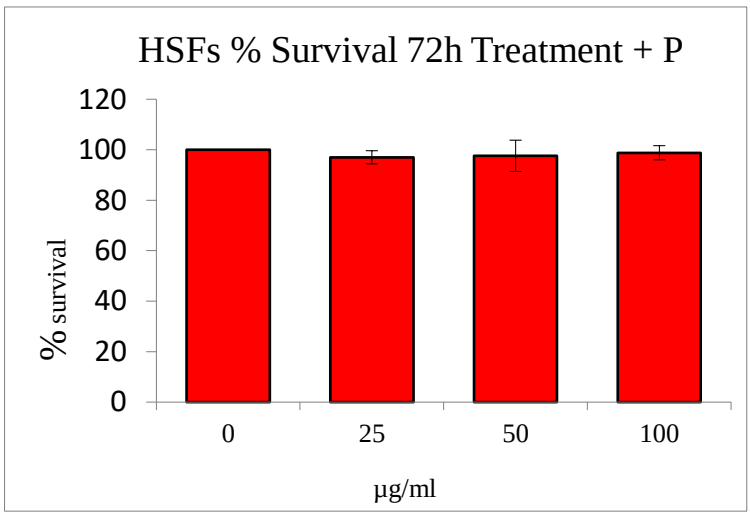


c

Figure 5.7 HSFs dose response survival histograms. We treated with 25, 50 and 100 µg/ml of methanolic B (a), N (b) and P (c) tomato extracts versus DMSO for 72h. All values are the means ± SD from three independent experiments

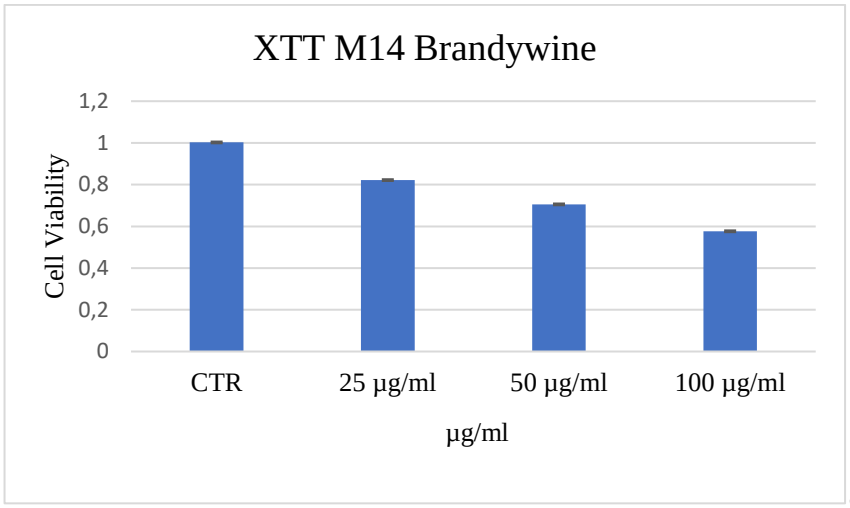
XTT assay was performed to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. Data obtained from XTT assay confirmed that the treatment with B, N and P phenolic extracts reduce cancer cell viability in a dose-response way (Figure 5.8). Even with this assay no effect is found in normal cell line (HSFs) (Figure 5.9).

5.5 Cell Colony Formation Assay

We investigated the effects of our tomato extracts on the colony formation ability of M14 cancer cells. After 7 days of treatment data showed a reduction in cell colonies number ad size. We can see a dose-dependent loss of the ability of individual cells to proliferate into viable colonies (at the maximum concentration the colonies colonies are minor in number and size). The number and size of colonies decreases after the treatment with all three polyphenolic extracts used (Figure 5.10, 5.11, 5.12). This assay essentially tests cancer cell ability to undergo “unlimited” division, so data obtained suggested that polyphenols in tomatoes can block cell divion and proliferation.

5.6 B, N and P methanolic extract effects on cell cycle.

To investigate whether the inhibitory effects of tomato extracts were due to an arrest of cell cycle or to an increased apoptosis, we performed the analysis of the cell cycle by staining with PI and reading by FACS. We found that all tomato extracts were able to induce a slight increase in the G0/G1 phase with a decrease in S phase in M14 cell line (Figure 5.13, 5.14, 5.15). Also in this experiment it is interesting to note that the blocking of cell cycle is closely related the tomato extract dose used (dose-dependent response); the best effect occurs following treatment with the maximum dose. Even these data are compatible with the inhibitory effects observed in the previous assays, suggesting that polyphenols present in tomatoes could regulate cancer cell proliferation influencing cell cycle.



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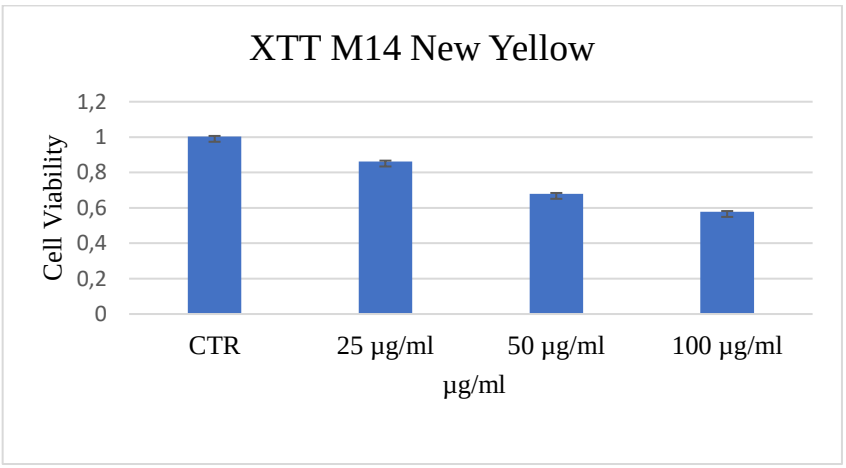

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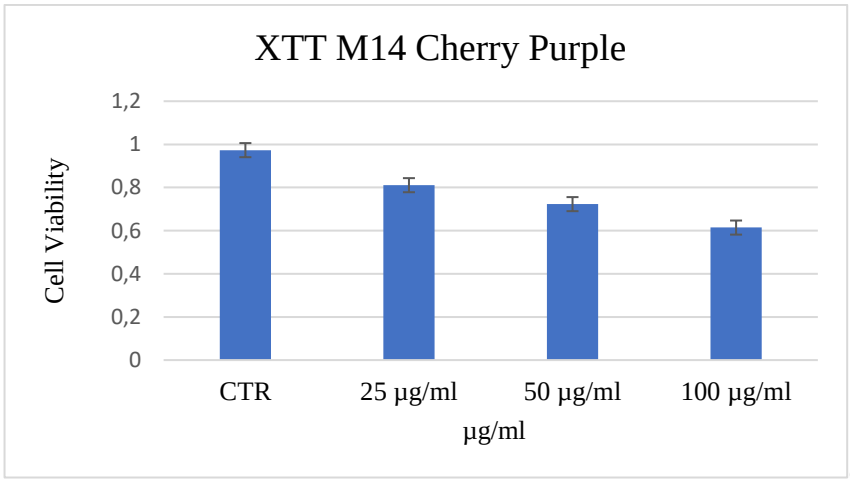
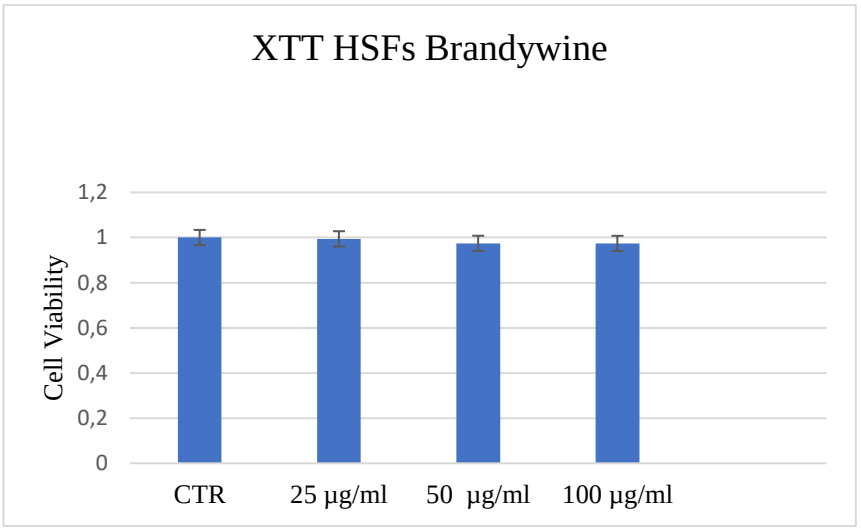


c

Figure 5.8 (a) B, (b) N, (c) P Polyphenolic extracts effect on cell viability of the M14 cells tested using XTT assay. All values are the means ± SD from three independent experiments.



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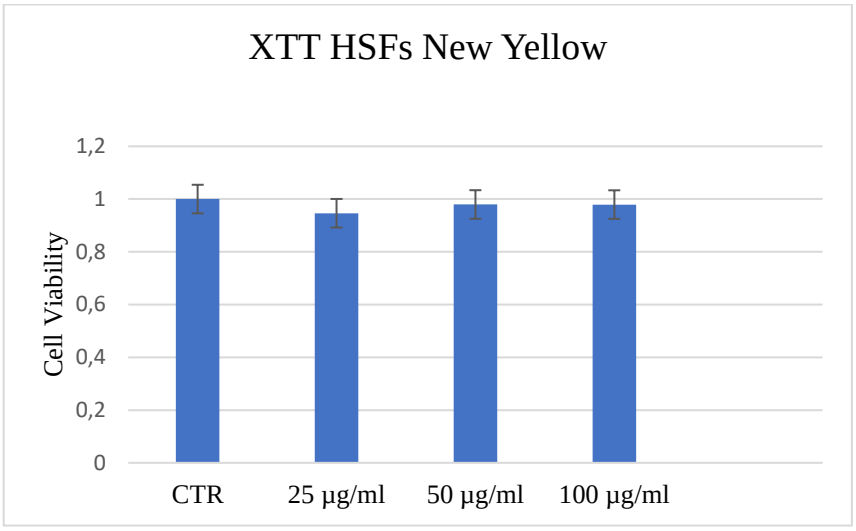

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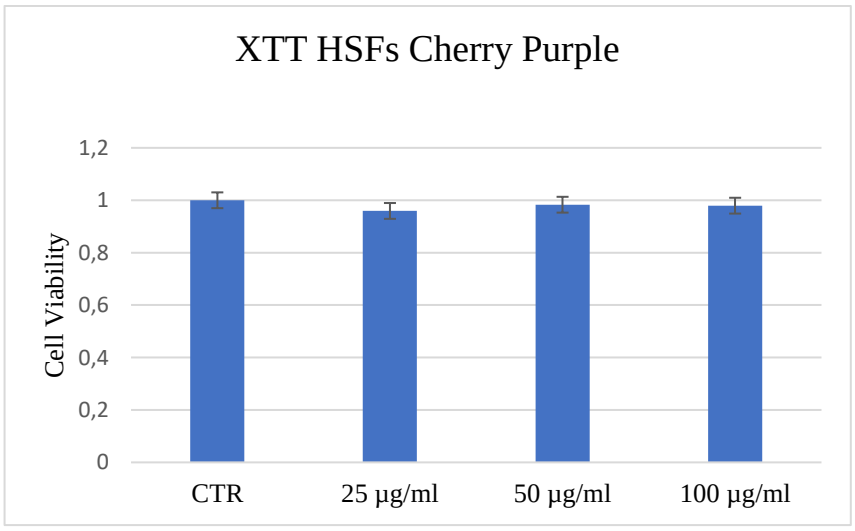

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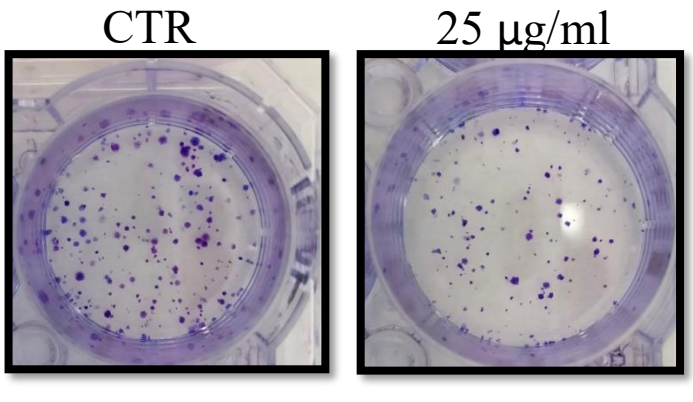
Figure 5.9 (a) B, (b) N, (c) P polyphenolic extracts effect on cell viability of the HSFs cells tested using XTT assay. All values are the means ± SD from three independent experiments .



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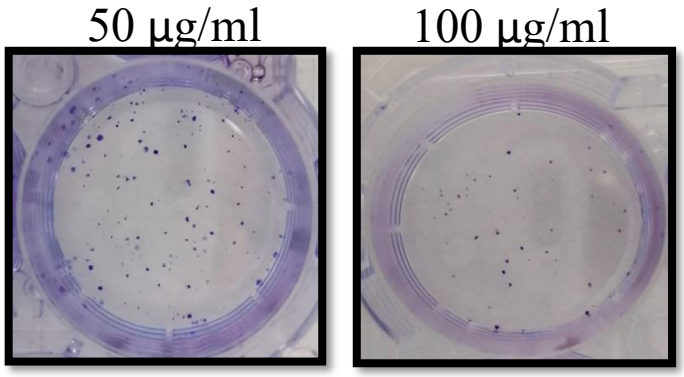
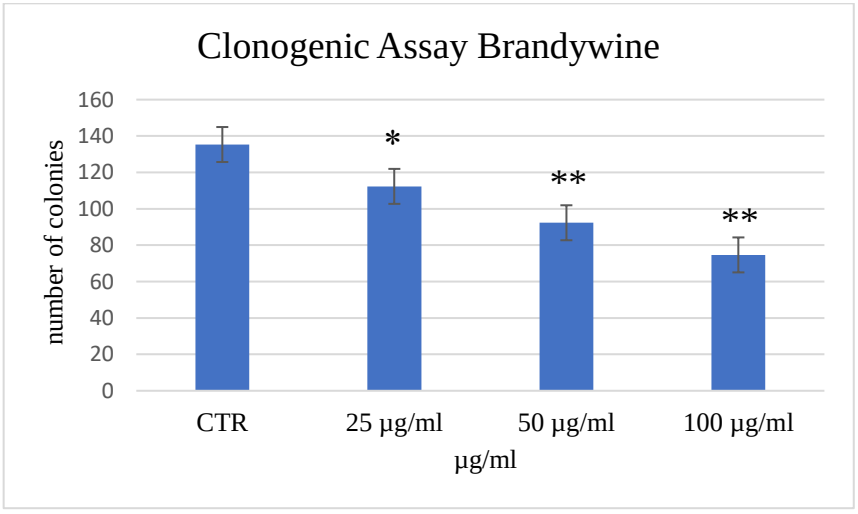
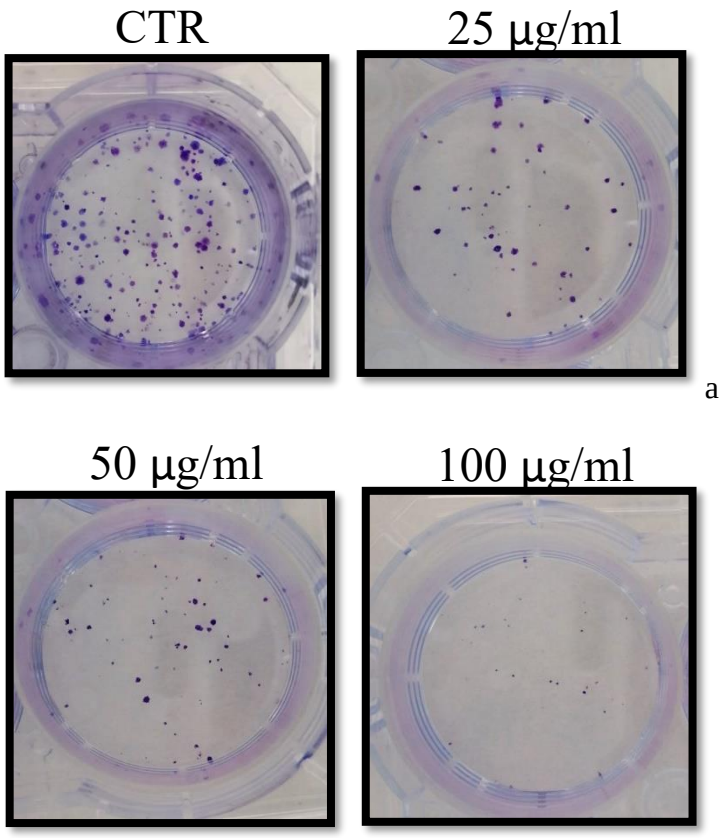




b

Figure 5.10 (a) Rapresentative Colony formation of M14 after 7 days of treatment with B polyphenolic extract and DMSO as a control (CTR). (b) Histogram rapresenting the mean numbers of colonies with standard deviation obtained from three independent experiments. Statistical analysis was performed by subjecting total number of trated and control cells to Student's *t*-test. Statistically significant differences are indicated with: *significant $p<0.05$ and ** very significant $p<0.01$.



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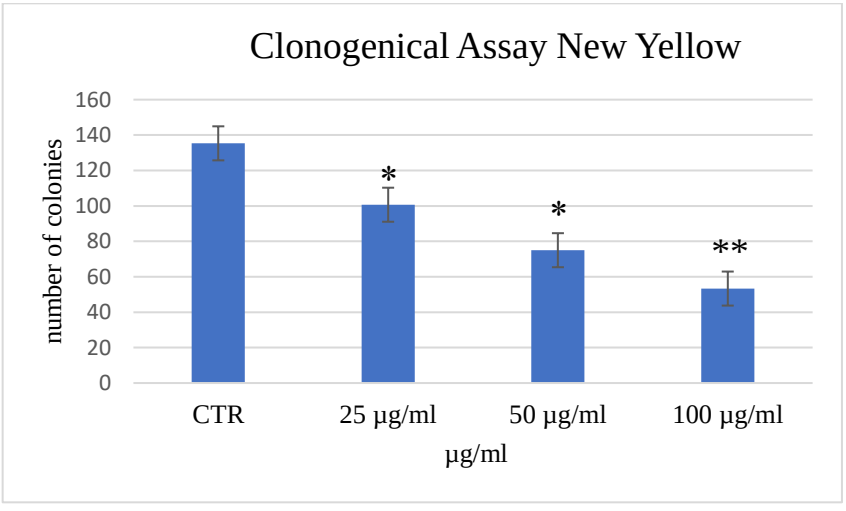
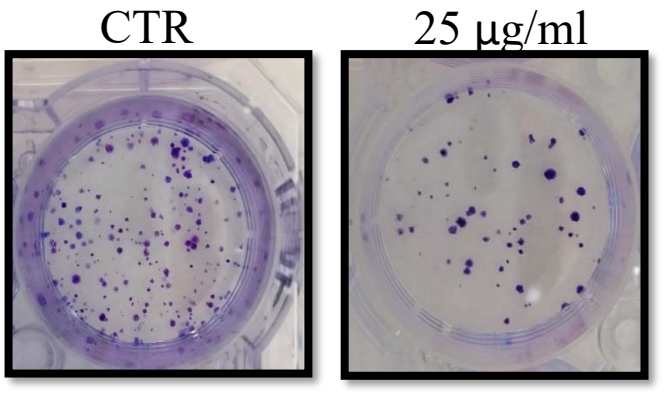
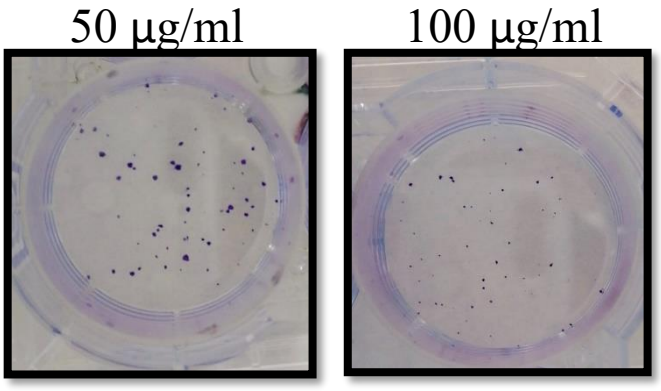


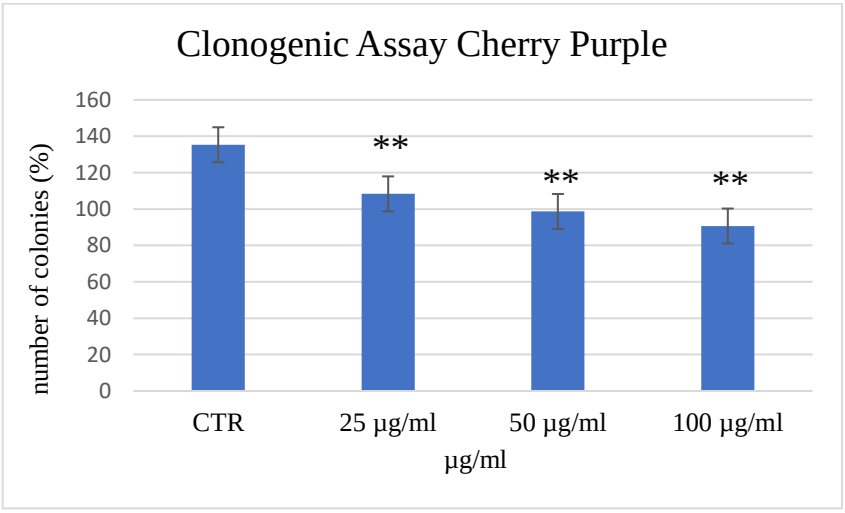
b

Figure 5.11 (a) Rapresentative Colony formation of M14 after 7 days of treatment with N polyphenolic extract and DMSO as a control (CTR). (b) Histogram rapresenting the mean numbers of colonies with standard deviation obtained from three independent experiments. Statistical analysis was performed by subjecting total number of trated and control cells to Student's *t*-test. Statistically significant differences are indicated with: *significant $p<0.05$ and ** very significant $p<0.01$.



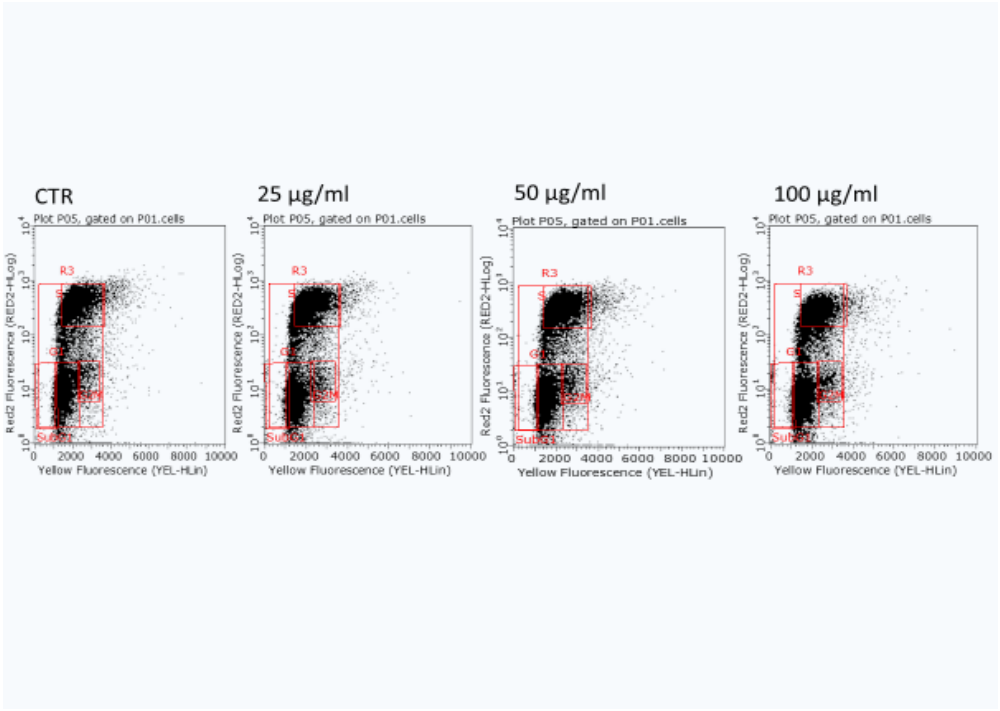
a





b

Figure 5.12 (a) Rapresentative Colony formation of M14 after 7 days of treatment with P polyphenolic extract and DMSO as a control (CTR). (b) Histogram rapresenting the mean numbers of colonies with standard deviation obtained from three independent experiments. Statistical analysis was performed by subjecting total number of trated and control cells to Student's *t*-test. Statistically significant differences are indicated with: *significant $p<0.05$ and ** very significant $p<0.01$.

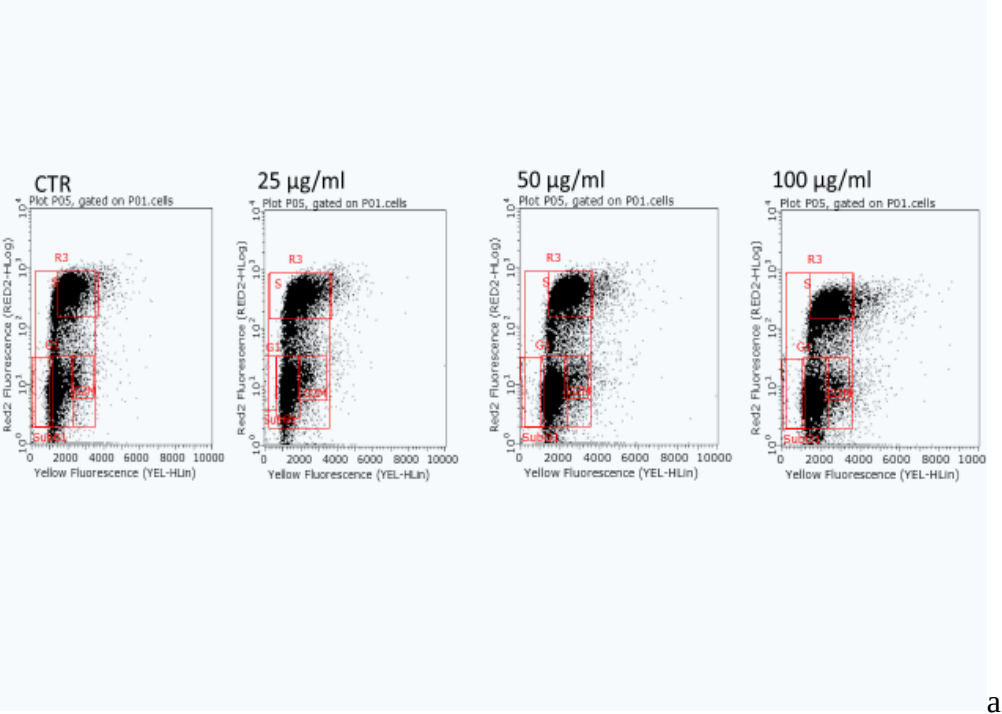


a

Brandywine	G0/G1	S	G2/M
CTR	30,07±0,81	39,51±0,94	5,71±0,61
25 µg/ml	33,50±0,50	33,11±1,00	4,33±0,99
50 µg/ml	36,60±0,62	30,02±0,97	5,18±0,84
100 µg/ml	37,31±0,54	28,10±1,18	5,12±0,35

b

Figure 5.13 B polyphenolic extract effects cell cycle in M14 cell line. FACS analysis data after 72h of treatment. (a), representive plots of a flow cytometry analysis. (b) Results indicated cell cycle distribution determinated by three independent experiments (values are the means ± SD)

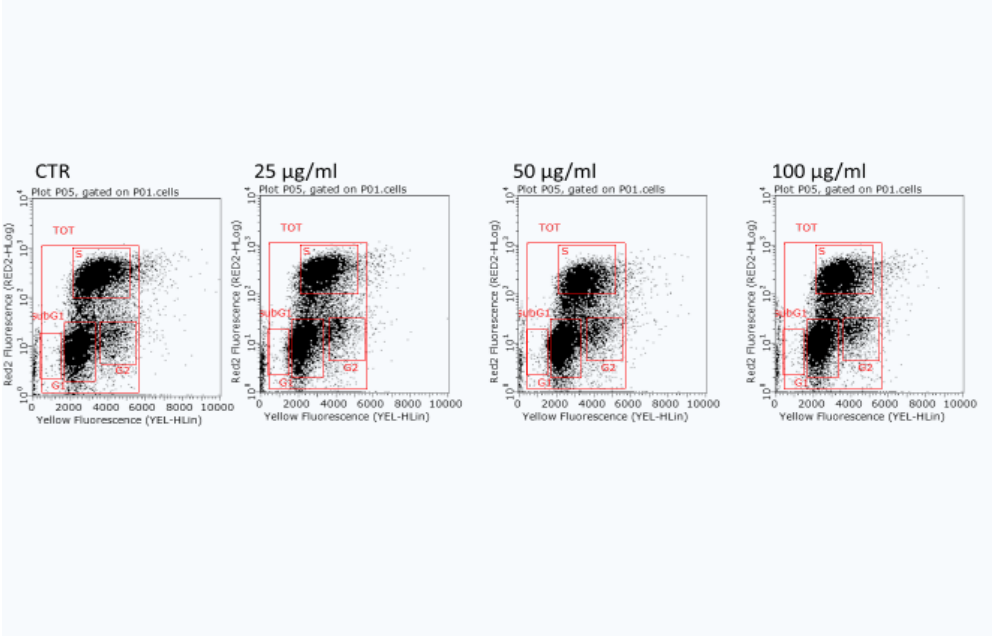


a

New Yellow	G0/G1	S	G2/M
CTR	30,7+1,00	40,20±0,33	5,78±0,86
25 µg/ml	34,81±0,27	37,06±0,53	4,42±0,71
50 µg/ml	37,15±0,97	35,73±0,58	5,37±0,66
100 µg/ml	41,01±0,68	30,15±0,78	5,35±0,39

b

Figure 5.14 N polyphenolic extract effects cell cycle in M14 cell line. FACS analysis data after 72h of treatment. (a), representive plots of a flow cytometry analysis. (b) Results indicated cell cycle distribution determinated by three independent experiments (values are the means ± SD)



a

Cherry Purple	G0/G1	S	G2/M
CTR	29,17±0,63	38,50±0,62	5,77±1,1
25 µg/ml	34,98±0,98	34,11±0,0,27	4,44±0,93
50 µg/ml	36,01±0,61	29,02±0,38	6,37±0,75
100 µg/ml	38,30±1,03	27,04±0,36	6,35±0,85

b

Figure 5.15 P polyphenolic extract effects cell cycle in M14 cell line. FACS analysis data after 72h of treatment. (a), representive plots of a flow cytometry analysis. (b) Results indicated cell cycle distribution determinated by three independent experiments (values are the means ± SD)

5.7 Polyphenolic tomato extracts regulate expression of different proteins involved in cell cycle regulation

Cyclin-dependent Kinase inhibitors p21/^{Cip1} and p27/^{Kip1} fuction as guardian of genoma [Shamloo B et al. 2019] thanks to their different fuctions; probably one of their most important function is the regulation of the cell cycle (tumor-suppressor fuction). After the treatment with our tomato extracts, we can observed in M14 cell line an increased expression level of both p21/^{Cip1} and p27/^{Kip1}. Data could support the cell cycle block observed by FACS. However, also here, raising levels of expression for p21 and p27 are dose-response with the highest concentration (100 µg/ml) of all methanolic extracts that induces the greatest increase. Pocket proteins (pRb/p105, pRb2/p130 and p107) have a central role in proliferation and apoptosis [Paggi MG et al. 2001; Classon M et al. 2001; Rana C et al. 2015; Wang SL et al. 2013], so we thought it necessary to analyze also the expression level of these proteins. Polyphenolic extracts increased the expression levels only of Rb2/p130 protein. Even this result is closely linked to extract dose. Summarazing, these data at the molecular level seem to explain the biological effects observed following treatment with methanolic extracts (Figure 5.16), and support the theory that tomatoes extract can hinder tumor development by the block of cell cycle.

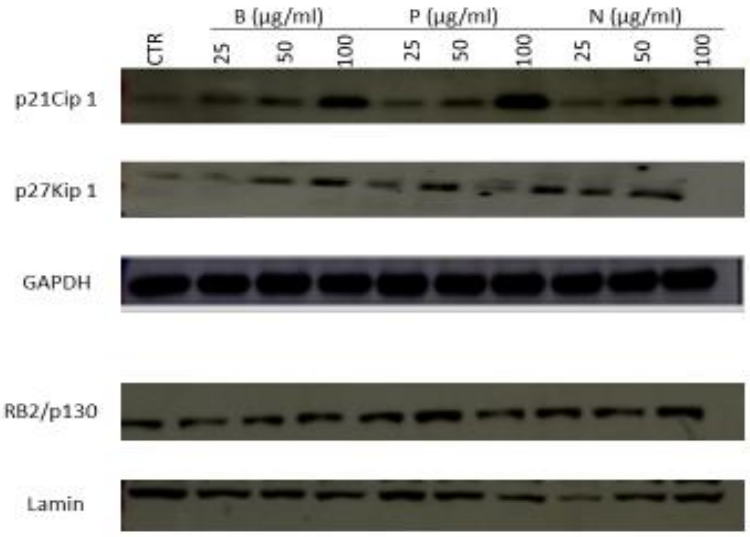
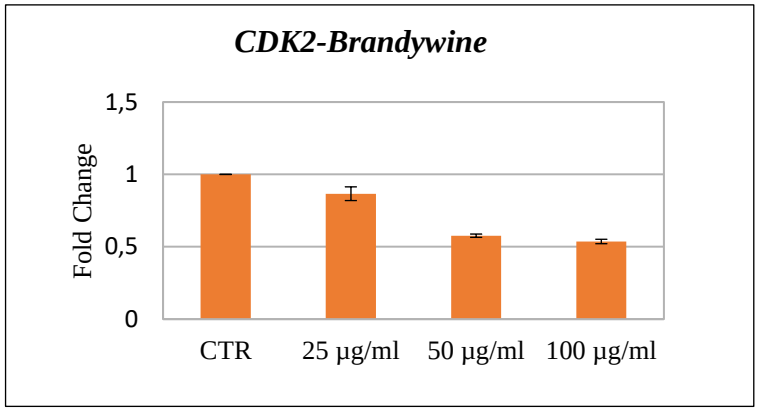


Figure 5.16 Western Blotting analysis showed upregulation of different protein involved in cell cycle regulation.

5.8 RT- qPCR showed that treatment with phenolic tomato extracts regulate mRNA levels of CDK2/cyclin A complex and p27/^{Kip1}

Normal cell proliferation requires a successful transition across cell cycle checkpoints. Access to the mitotic phases of the cell cycle is controlled by growth promotion and inhibition signals; these signaling pathways ensure that cells do not unnecessarily engage in DNA replication and cell division. In eukaryotes, cyclins and cyclin dependent kinases (CDKs) are responsible for controlling cell cycle regulation. When this complex is in an active state, it allows cells to pass cell cycle details checkpoints through phosphorylation of unique protein substrates. Gene expression of CCNA2 and CDK2 was evaluated using RT-qPCR; mRNA expression levels of CCNA2 and CDK2 were consistently decreased in M14 cells already when treated with the lowest dose of B, N and P extracts. Data showed a dose dependent decrease when exposed to the lowest dose (25 µg / ml) of polyphenolic extract for 72 h. Since CDK2 coud be regulate by p27/^{kip1} we also investigated mRNA levels of this inhibitor. Results from RT-qPCR showed an upregulation of this gene (result consistent with data aforementioned). This enhancement in p27 expression associated with data related to CCNA2 and CDK2 lead to cell cycle arrest and tumor suppression (Figure 5.17, 5.18, 5.19).



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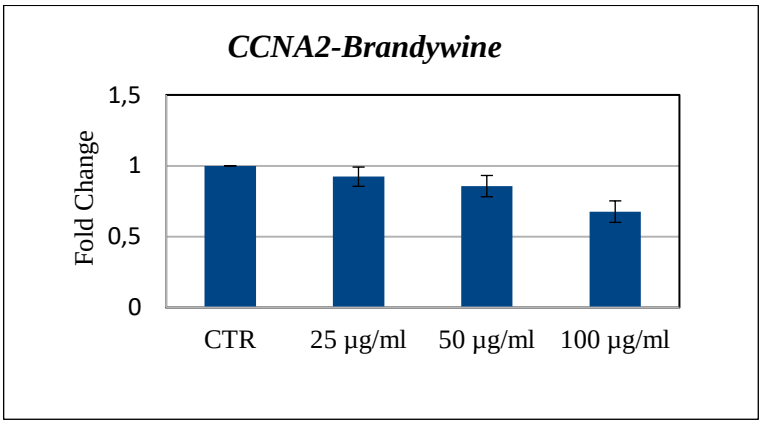

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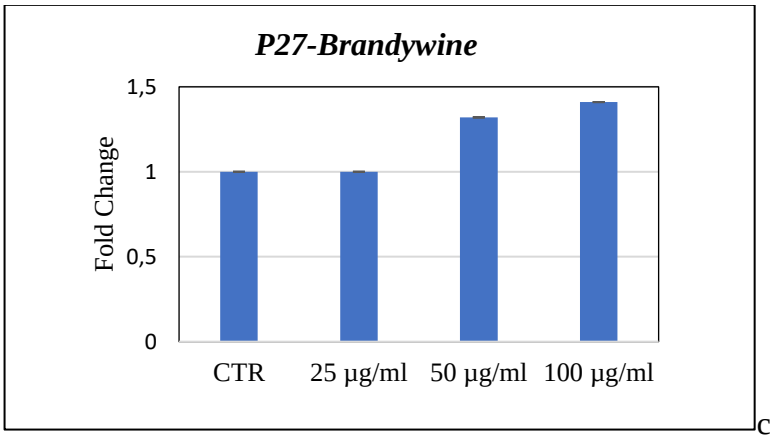
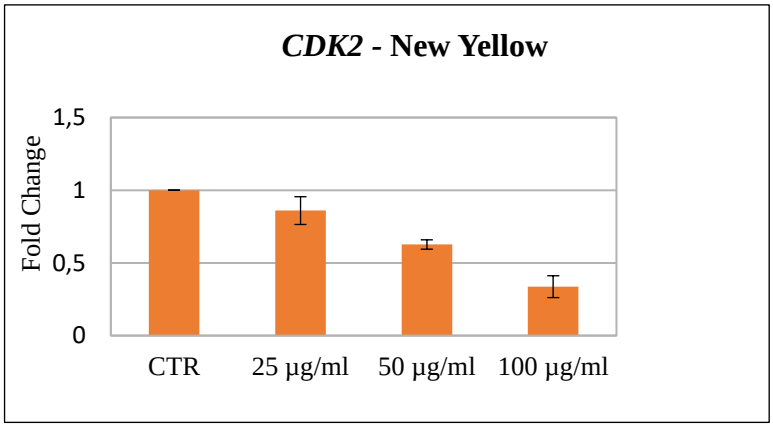


c

Figure 5.17 RT-qPCR analysis of (a) *CDK2*, (b) *CCNA2*, and (c) *P27* expression in M14 after treatment with Brandywine polyphenolic extracts. The amount of mRNA was normalized to GAPDH. The relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method. All values are the means ± SD from three independent experiments.



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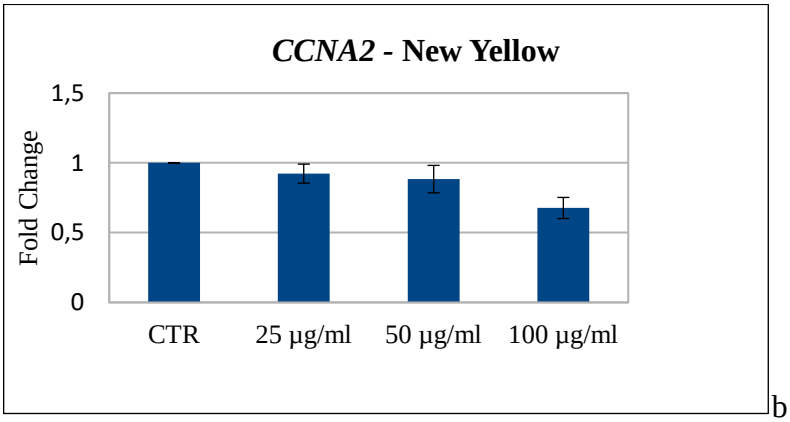

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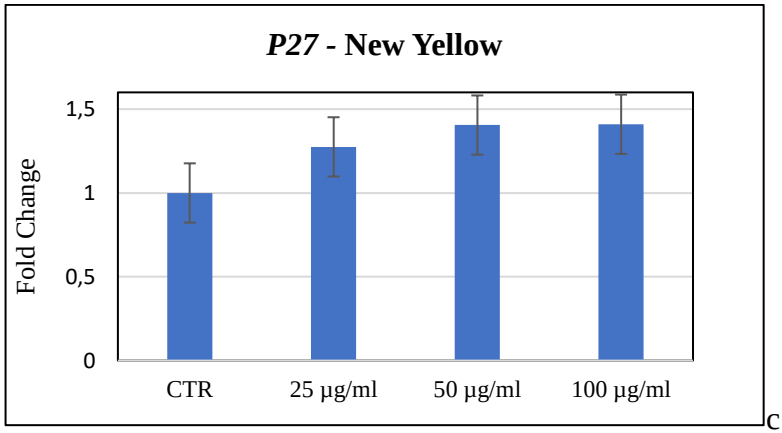
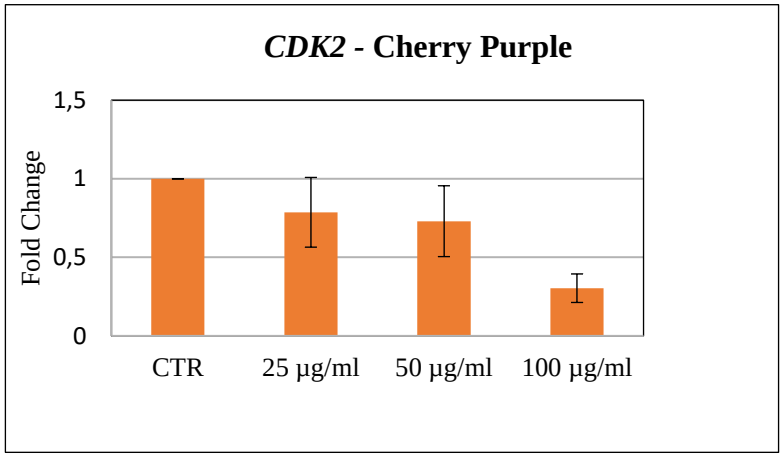


c

Figure 5.18 RT-qPCR analysis of (a) *CDK2*, (b) *CCNA2*, and (c) *P27* expression in M14 after treatment with New Yellow polyphenolic extracts. The amount of mRNA was normalized to GAPDH. The relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method. All values are the means ± SD from three independent experiments.



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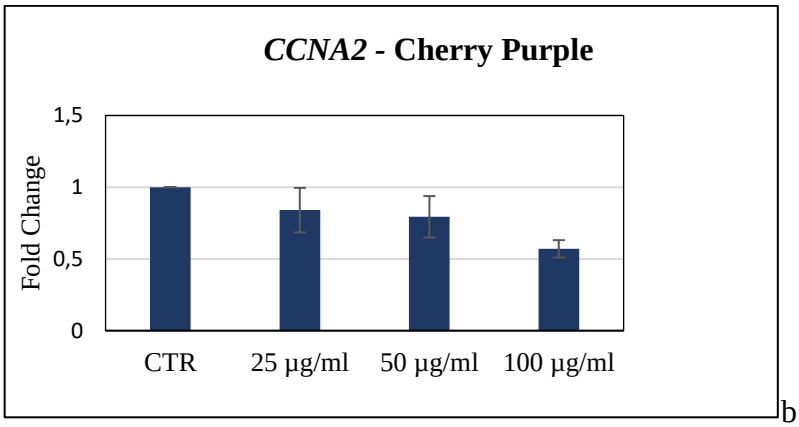

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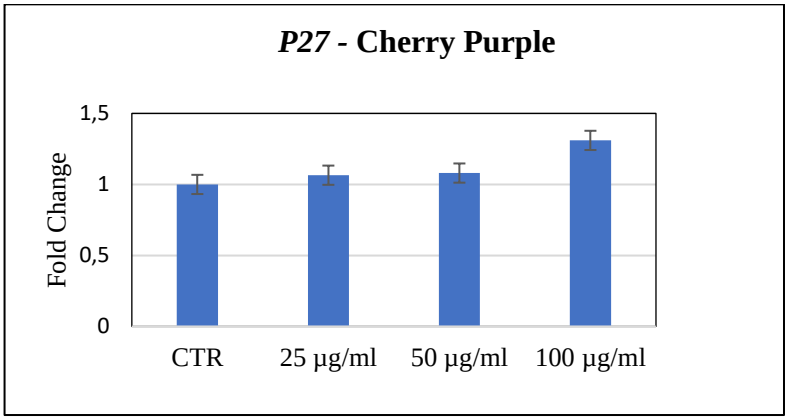
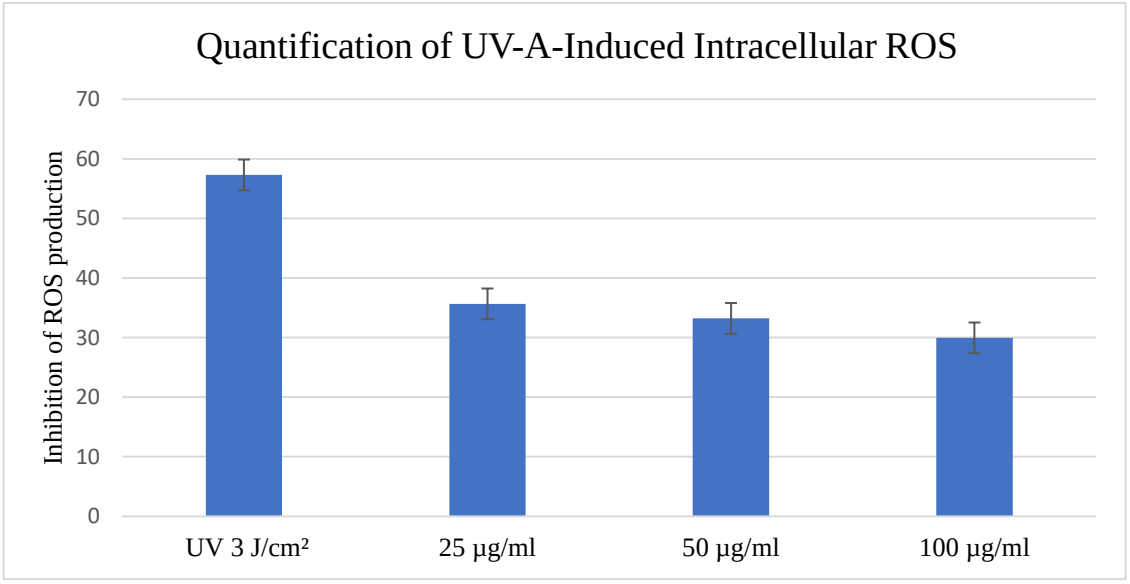


c

Figure 5.19 RT-qPCR analysis of (a) *CDK2*, (b) *CCNA2*, and (c) *P27* expression in M14 after treatment with Cherry Purple polyphenolic extracts. The amount of mRNA was normalized to GAPDH. The relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method. All values are the means ± SD from three independent experiments.

5.9 Polyphenols in tomatoes reduce intracellular ROS UV-A-Induced

In the end the antioxidative potency of polyphenols extract against ROS generation UV-A- induced was investigated. In Figure 5.20 we can see a dose-dependent changes in the inhibition of ROS production by co-incubation with polyphenols after exposure to UV-A light (3 J cm³). The production of ROS was reduced significantly after treatment with all three different polyphenols extracst at concentration between 25 and 100 µg/ml. Data indicated that polyphenols could have a protection on ROS production in normal cell, reducing the harmful effect that ROS have in cells.



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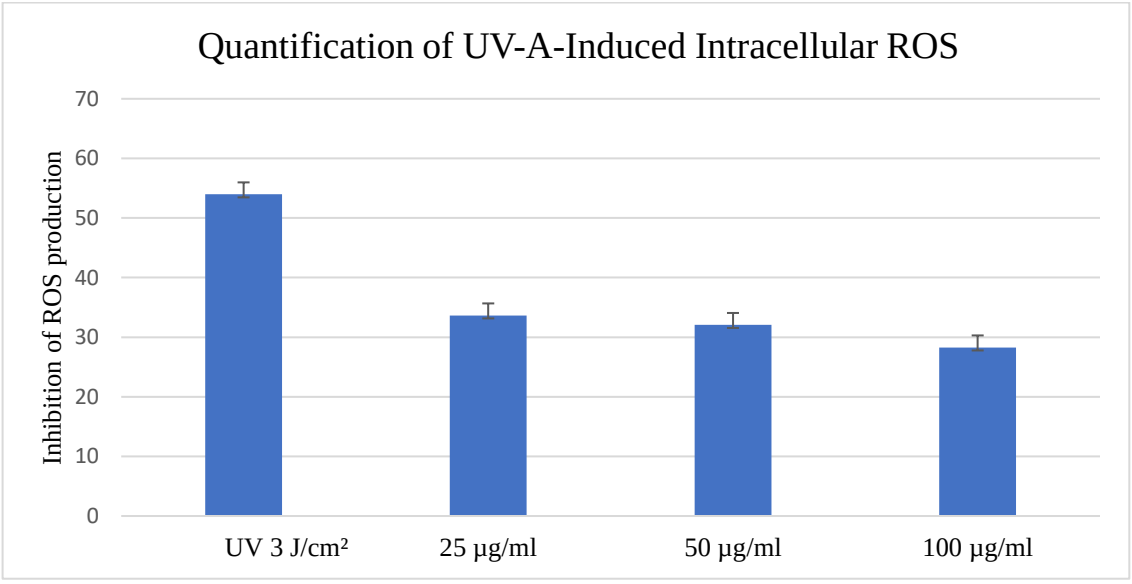

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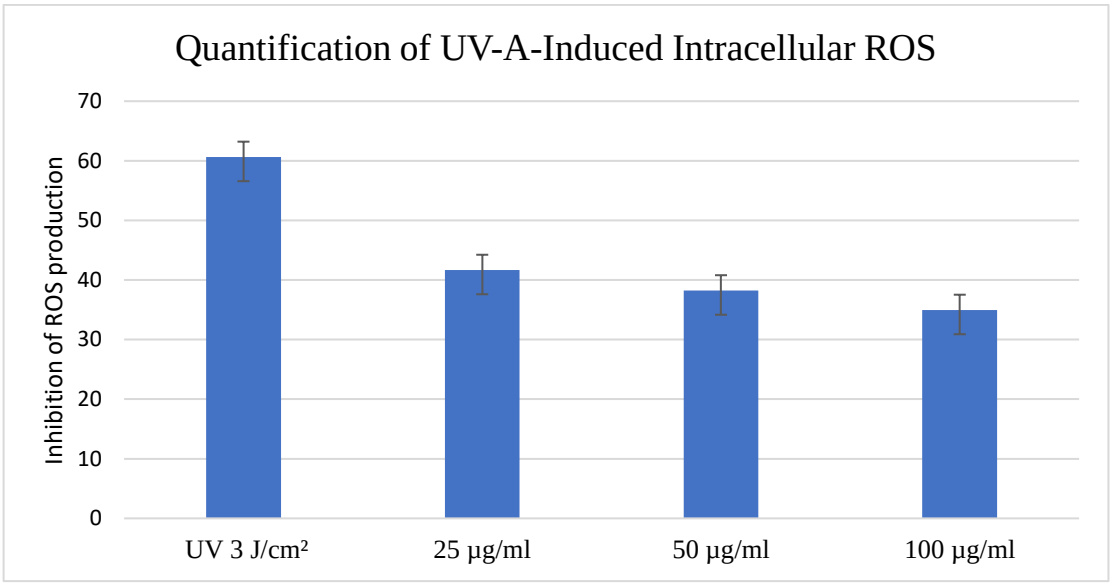

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Figure 5.18 Dose-dependent effect of (a) B, (b) N, (c) P polyphenols extracts on intracellular ROS caused by a dose UV-A light (3 J cm³) in HSFs. ROS production was measured using the CellROX deep red reagent. All values are the means ± SD from three independent experiments.

Chapter 6

Discussion

Until recently, the relationship between diet and risk of developed different diseases, including cancer, has been hardly understood. In the last few years the scientific literature that study plant foods, containing phytochemicals that confer remarkable health benefits, is rising. Flavonoid and carotenoid rich foods, based on their surprising health effects. are known as superfoods. These include all plant origin foods mainly tea, fruit, vegetables, grains, legumes, nuts, and wine. In this context, tomato represents an important source of bioactive compounds, among which stand out carotenoids and polyphenols; both these molecules have the capacity to react with Reactive Oxygen Species (ROS) and to prevent DNA possible mutation.

After careful analysis based on content of polyphenols and antioxidant capacity of many tomato extracts, we decided to use extracts from three tomato cultivars (Brandywine, Cherry Purple and New Yellow) because their high polyphenols concentration and high antioxidant properties. Here we presented our findings on a possible role of tomatoes on inhibit carcer chacteristics in a metastatic melanoma cell line. We used total tomato polyphenolic extracts, which better mimics dietary tomato intake, without isolating single compounds. The tomatoes employed for methanolic (Brandywine, New Yellow and Cherry Purple) extractions are grown in a totally organic agriculture, since the presence of pesticides could alter the properties of the vegetable.

After analyzed the physical-chemical tomato characteristics and obtained the value of the most abundant polyphenols contained in our tomatoes extracts, we first investigated the possible biological effects expecially on cancer cell viability and on formation of metastasizing colonies. Data obtained from two viability assays showed how the treatment with all three tomato polyphenolic extracts reduce cancer cells viability in a dose-dipendent way. This inhibition of cellular divion is confirmed also by colony formation assy and could be explained by a block of cell cycle (increase of Go/G1 phase and, subsequently, decrese of cell in S phase). Consistently, at the molecular level, we

found that cell treatment with all tomato extracts upregulated different tumor suppressor proteins invoved in cell cycle regulation. In particular in our study we observed an upregulation of the pocket protein pRb2/p130 that, together with the increase of two cell cycle inhibitors, p21/^{Cip1} and p27/^{Kip1}, controlled cell cycle causing cell block in the G0/G1 phase. We never see any effects on cell viability on no-tumoral cells (HSFs), but when human fibroblasts were exposed to UV-A rays, the co-treatment with our tomato extracts have the ability to reduce the production of ROS UV-A induced. These last data highlighted the possible preventive role of polyphenols in malignant transformation, because these compounds seem to inhibit the release of free radicals, harmul for cells. Until a few years ago, most studing analyzing tomatoes as a superfood with anti-oxidant and antitumoral properties focused on the most impostant class compounds carotenoids and leaving out the other class of compounds namely polyphenols. Nowdays, multiple scientific studies on different tumors (such as MCF-7 breast cancer) cells support our results [Alimohammadi M et al 2017] , on the basis of which the polyphenols are very important for their antioxidant and antitumoral activity both *in vitro* and *in vivo*. In conclusion there are several studies that emphasize the importance of a correct nutrition, particularly rich in fruits and vegetables. Our data suggests that a consumption of tomatoes (Brandywine, New Yellow and Cherry Purple) could be useful in preventing the development of tumor thanks to their strong antioxidant activity. Despite the amount of the two most abundant polyphenols (Rutin and Naringenine Chalcone) varies comparing the three tomato varieties, we do not notice great differences in their anticancer activity. This makes us assume that it is not the single compound that operates but the right balance between all the substances present in our whole poliphenolic tomato extracts. In future it could be interesting to study both *in vitro* (melanoma cell lines) and *in vivo* (rats) the additive or synergistic effects of tomato polyphenols to increase the efficacy of chemotherapy drugs or cancer immunotherapy. In the future, it would also be very interesting to evaluate whether after treatment with the polyphenolic extract there is an increase in the expression of the *OGG1* repair gene, which is involved in the repair by excision of 8-Oxoguanine, a mutagenic form of oxidized guanine in DNA (mutations that are induced by ROS) .

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External Evaluation of PhD thesis by Dr Constantinos Demonacos Thesis title: "Evaluation of anti-proliferative and antioxidant potential of tomato extract against melanoma"

The thesis investigates the potential tumor suppressive effects of the constituents of tomato. In order to approve or disapprove this hypothesis the candidate explored the levels of compounds contained in the extract of three different types of tomato and investigated their effects on one melanoma cancer cell line and in healthy skin fibroblasts. The methodology used included analytical chemistry cellular and molecular biology and biochemistry approaches, which are appropriate for this type of research. The results are presented clearly and the conclusions are justified supported by experimental evidence.

Minor comments the candidate could take into account to improve the thesis are listed below:

1. A section providing the full explanation of the abbreviations should be included.
2. Abstract of the thesis should be included.
3. Few terms should be introduced the first time they appear in the thesis (for example amelatonic melanomas in the page 9 TERT in page 10 etc.).
4. Statistics indicating the p values should be included in all figures as appropriate.
5. In the figures 5.13, 5.14 and 5.15 cell cycle profiles should be included I available.
6. In the figure 5.16 please insert the pRb2/p130 phospho-isoforms if available.
7. Please explain the difference between the panels a, b and c in the figure 5.18.
8. Please add a section indicating future directions of the research.
9. Please add reference where indicated in the thesis especially pages 30 and 35.
10. Minor grammatical and typos should be corrected.

The thesis is good and I agree to admit the candidate to the defense.

Yours sincerely

Dr. Constantinos Demonacos
Senior Lecturer & Principal Investigator

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25th Feb 2021

External Evaluation of PhD thesis by Professor Marija Krstic-Demonacos
Thesis title: Evaluation of anti-proliferative and antioxidant potential of tomato extract against melanoma

Student: Costantino D'Angelo, Supervisor: Prof. Antonio Giordano
University of Siena – Department of Medical Biotechnologies, Doctorate in Genetics, Oncology and Clinical Medicine (GenOMeC)

General comments
This interesting thesis investigates effects of tomato extracts on cancer cells, in particular melanoma. Thesis is well presented, clearly written and meets the aims proposed at the start, as well as contributing to new knowledge. Thesis meets the standards of European PhD.

Further comments are outlined below and in the attached document
Student should obtain Turnitin report before submitting the thesis and share it with supervisor-this is standard practice to eliminate similarities issues in introduction and other written parts of the thesis. Thesis should provide list of abbreviations at the beginning of the thesis and have an abstract. Unless figure is made by the student, references must be provided where figure was taken from in the figure legend and added that to the reference list-this applies to all figures in the PhD introduction.

In methods, please provide more detailed explanation about plants, microorganisms used, conditions of growing, where plants were obtained from etc. Also, few more sentences are needed about cell culture protocols and SDS PAGE.

In results, for all tables place table legends above the table, for all figures place figure legend below the figure. For all figures indicate if p value is equal/less or more than 0.05 and if it is indicated by star (for example figure 5.2 and 5.4 etc). If results are not significant, please indicate that fact in results description. Western blots should be quantified using Image J.

Discussion needs substantial addition of comparison of data obtained and published literature-what other reports have found in this and other cancers, how that compares with data obtained here etc. Also limitations of the methods used need to be discussed as well as future directions-use of individual and combined components, use of more cell lines, animal studies, combination with chemotherapy drugs and phospho antiobodies for cdk targets etc

Thesis is excellent and I agree to admit the candidate to the PhD defence. Please don't hesitate to contact me if I can be of any further assistance.

Sincerely yours

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