# UNIVERSITÀ DEGLI STUDI DI TRIESTE

## XXX CICLO DEL DOTTORATO DI RICERCA IN

## NANOTENOLOGIE

# Nanoparticles based delivery System of Flavonoids for Cancer Therapy

Settore scientifico-disciplinare: BIO/10 - BIOCHIMICA

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Heartfelt Thanks to All of You.

# Abstract

Nowadays, cancer remains one of the major public health problems. Although chemotherapeutic drugs efficiently kill cancer cells, these cells can defend themselves from such toxic compounds with a process called cancer multidrug resistance (MDR). Because of unsatisfactory treatment scenario there has been growing interest in the health advantages of using plant-derived compounds for cancer prevention or in the treatment of chemo-resistant cells.

Anthocyanidins are a group of pigments belonging to the family of flavonoids present in red-blue fruits and vegetables. Several studies demonstrated that, together with their glycosylated forms, they exert intense biological activity towards normal and cancer cells, including selective cytotoxicity, capability to interact with extrusion pumps, cell cycle perturbation, anti-proliferation and apoptosis.

Unfortunately, two major concerns have to be pointed out: (i) the concentrations often used to prove the biological effects of such compounds are far from those obtained when the assumption passes only through the ingestion of food rich of phytochemicals; (ii) knowledge on the effects due to a chronic intake of these molecules are missing. Both of these points are related to the low stability and bioavailability of anthocyanins. Moreover, when ingested, the latter is drastically reduced by their poor chemical stability in the weak alkaline conditions of the small intestine, combined with phase II metabolism. Indeed, they usually undergo sulfurylation, methylation and glucuronidation in the small intestine and liver and conjugated metabolites can be found in plasma after flavonoid ingestion. In general, metabolites of flavonoids show controversial bioactivity in comparison to parent compounds, thus challenging the possibility to translate their proven biological effects into therapeutic applications.

Therefore, enhancement of bioavailability would be of utmost importance in order to exert health effects. Nanotechnologies are being developed to afford a solution to the problem. Among available carrier, the application of positively charged polymers is widely studied because of their superior uptake levels as a result of electrostatic attraction with cellular membrane.

In this study, we aimed to:

a) Clarify whether the use of dietary anthocyanins could affect the response to chemotherapy exerted by resistant cancer cells. Long-term treatment with non-toxic delphinidin (DEL) concentration was performed on LoVo/Dx cells (metastatic human colorectal adenocarcinoma cell line, doxorubicin resistant). Interferences with cell cycle, the expression of specific membrane transporters responsible for drug resistance, the accumulation of the drug in the cells and the cellular ATP levels were evaluated. After the

first week, significant results, like cell cycle arrest and increase of doxorubicin accumulation were reported without a corresponding down regulation of efflux pump or cellular energy level modification. Unfortunately, these effects were not maintained in time; therefore, we hypothesized that the failure in chronic treatment with DEL could be attributable to adaptive metabolic response.

b) Improve DEL stability with a nano-delivery system. Chitosan, a natural polysaccharide, has drawn much interest since is biodegradable, biocompatibile, non-immunogenic and is cheap. Different chitosan/tripoliphosphate (TPP) nanoparticle formulations were prepared by ionotropic gelation method and subsequently characterized. Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS) were used to measure size, dispersity and morphology. The DEL - chitosan nanoparticles interaction was investigated through UV Resonant Raman Spectroscopy. Encapsulation efficiency and DEL stability were determined by HPLC. Among the different formulations, we established that both, the amount and the relative proportion of chitosan/TPP/DEL, are involved in particles charge and size as well in anthocyanidin stabilization and encapsulation. The encapsulation efficiency of the nanoparticles was determined to be 73%, and the DEL stability was strongly increased in comparison to the free compound.

c) Demonstrate that nanoencapsulation of DEL (DNPs) with respect to the free form, improves cell bioavailability and increases the biological activity of the natural compound.

   Three colon cancer cell lines (Caco-2, LoVo and Lovo/Dx) were treated with free and encapsulated DEL both at 50μM. Uptake of nanoparticles, cytotoxicity, antioxidant/prooxidant activity were evaluated. Since, DEL was demonstrated to act selectively on normal and cancer cells, the comparison of free DEL with DNPs with respect of cell cycle, apoptosis and uptake of chemotherapeutic drug was performed.

   In addition, preliminary results showed that DNPs treatment exert even a combined reduction of the expression of inflammatory biomarker, such as NF-κB.

In conclusion, the results obtained show that DEL taken through the diet cannot act as a sensitizing agent to chemotherapy. In fact, while showing significant effects in the short term (one week), these subsequently do not persist. One general mechanism of action of phytochemicals that is emerging from recent studies is that they activate adaptive cellular stress response pathways. The increase in the expression or in the enzymatic activity of phase II metabolizing enzymes, could be the mechanism developed by the treated cells to modify the molecule and inhibit its effect.

The stabilization of DEL in biodegradable nanoparticles, has instead positively corresponded the expected results that show the higher performance of DNPs for applications in cancer drug development and might give rise to a new antitumor therapeutic approach avoiding cancer multidrug resistance.

# Sommario

Il cancro resta oggi una tra le maggiori problematiche di salute pubblica. Le tipologie di trattamento utilizzate sono diverse a seconda del tessuto, della tipologia e della stadiazione. Il trattamento con chemioterapico consente una efficiente eliminazione delle cellule tumorali che però spesso riescono a sviluppare un sistema di autodifesa denominato Multi Drug Resistance (MDR).

In uno scenario in cui la terapia farmacologica si rivela talvolta insoddisfacente, l'uso di nutraceutici, sia a scopo preventivo, sia come adiuvante del trattamento chemioterapico, è di crescente interesse.

Le antocianidine sono un gruppo di metaboliti secondari delle piante per le quali è stata dimostrata capacità citotossica selettiva verso le cellule tumorali, interazione con le pompe di estrusione, alterazione del ciclo cellulare con un'azione anti-proliferativa e pro-apoptotica.

Sfortunatamente sono emersi alcuni problemi relativamente l'utilizzo di questi composti: (i) le concentrazioni utilizzate per dimostrarne l'attività biologica sono ben lontane da quelle ottenibili con la sola dieta; (ii) non sono noti gli effetti causati da un possibile utilizzo cronico.

Entrambi questi punti sono correlati alla bassa stabilità e biodisponibilità di questi comosti. Inoltre quando sono ingeriti vanno incontro a degradazione a causa delle condizioni debolmente alcaline dell'intestino tenue, assieme all'azione del metabolismo di II fase che ne causa metilazione, solfatazione e glucuronazione. I metaboliti secondari delle antocianidine sono stati trovati nel plasma, ma i loro effetti biologici sono tendenzialmente più scarsi rispetto a quelli dei composti di origine, rendendo difficilmente traducibile in-vivo l'effetto ottenuto in-vitro e limitandone le possibili applicazioni terapeutiche.

Pertanto, l'aumento della biodisponibilità è di fondamentale importanza per ottenere un effetto sulla salute.

Ad oggi l'utilizzo di nanotecnologie offre una possibile soluzione al problema. Tra i carrier disponibili, l'utilizzo di polimeri carichi positivamente è ampiamente studiato poiché hanno ottimi livelli di assorbimento grazie all'attrazione elettrostatica con la membrana cellulare.

In questo studio abbiamo mirato a:

a) Chiarire se l'uso di antociani derivanti dalla dieta è in grado di influenzare la risposta al chemioterapico esercitata dalle cellule tumorali farmaco-resistenti.

   Per questo è stato effettuato un trattamento a lungo termine con concentrazioni di delfinidina (DEL) sub-tossiche su cellule LoVo/Dx (adenocarcinoma colorettale umano metastatico, resistente alla doxorubicina). Sono state valutate l'interferenza con il ciclo cellulare, l'espressione di specifici trasportatori di membrana responsabili della farmaco-resistenza,

l'accumulo del chemioterapico nelle cellule e i livelli di ATP cellulare. Dopo la prima settimana, sono stati notati risultati significativi, come l'arresto del ciclo cellulare e l'aumento dell'accumulo di doxorubicina senza una corrispondente riduzione dell'espressione della pompa di efflusso o una variazione del livello energetico nella cellula. Sfortunatamente, questi effetti non sono stati mantenuti nel tempo; pertanto, abbiamo ipotizzato che l'inefficacia del trattamento cronico con DEL potrebbe essere attribuibile a una risposta metabolica adattativa.

b) Migliorare la stabilità della DEL con un sistema di nano-incapsulamento. Per farlo è stato usato il chitosano, un polisaccaride naturale, che ha suscitato molto interesse grazie alla sua biodegradabilità, biocompatibilità, non immunogenicità e al basso costo.

   Sono state preparate diverse formulazioni di nanoparticelle di chitosano / tripolifosfato (TPP) mediante gelazione ionotropica e successivamente sono state caratterizzate. Per la valutazione delle dimensioni, della polidispersità e della morfologia sono stati utilizzati il microscopio elettronico a trasmissione (TEM) e il Dynamic Light Scattering (DLS). L 'interazione tra la DEL e il chitosano, nella nanoparticella, è stata studiata mediante spettroscopia Raman Risonante. L'efficienza d'incapsulamento e la stabilità della DEL sono state determinate mediante HPLC. Tra le diverse formulazioni, abbiamo stabilito che sia la quantità assoluta che la proporzione relativa tra chitosano / TPP / DEL sono cruciali per la carica netta, le dimensioni delle particelle e per la stabilizzazione e l'incapsulamento dell'antocianidina. L'efficienza di incapsulamento nella miglior formulazione è stata del 73% e la stabilità della DEL è stata fortemente aumentata rispetto al composto puro.

c) Dimostrare che le nanoparticelle di DEL (DNPs) rispetto alla forma libera, migliorano la biodisponibilità per le cellule e ne aumentano l'attività biologica.

   Sono state utilizzate tre diverse linee di cancro del colon (Caco-2, LoVo e Lovo / Dx) e trattate con DEL sia libera che incapsulata, a una concentrazione finale di 50μM. Sono poi stati valutati l'assorbimento, la citotossicità e l'attività anti/pro-ossidante. Inoltre, poiché, è stato dimostrato che la DEL agisce selettivamente sulle cellule normali e tumorali, sono stati paragonati gli effetti tra DEL libera con quelli delle DNPs per quanto riguarda l'interferenza col ciclo cellulare, l'apoptosi e l'accumulo del chemioterapico.

   Inoltre, dati preliminari hanno dimostrato che il trattamento con DNPs induce anche una riduzione dell'espressione di bio-marcatori infiammatori, come NF-κB.

   In conclusione, i risultati ottenuti mostrano che l'assunzione di DEL attraverso la dieta non è in grado di agire come agente sensibilizzante per il chemioterapico. Infatti seppur ci siano effetti significativi nel breve periodo (una settimana) non persistono nel tempo. Recentemente stanno emergendo studi che indagano l'azione dei fitochimici e che hanno evidenziato meccanismi di adattamento in risposta allo stress cellulare. L'aumento

dell'espressione o dell'attività di enzimi responsabili del metabolismo di fase II potrebbe essere la risposta delle cellule dopo trattamento, per modificare la molecola e inibirne l'effetto.

Al contrario, la stabilizzazione della DEL nelle nanoparticelle biodegradabili, ha invece portato a quei risultati attesi anche a livello biologico, dimostrando la miglior applicabilità delle DNPs per lo sviluppo di farmaci antitumorali e potrebbe dar luogo ad un nuovo approccio terapeutico che eviti la farmacoresistenza del cancro.

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# Abbreviations

AUC  Area Under the Curve

DEL  DEL

DLS  Dinamic Light Scattering

DMEM  Dulbecco's Modified Eagles Medium

DNPs  DEL nanoparticles

FBS  Fetal Bovine Serum

FBS  Fetal Bovine Serum

FITC  Fluoresceine Isotiocyanate

FITC-Cs  FITC – chitosan

GLOBOCAN  International Agency for Research on Cancer, & World Health Organization.

HBSS Hank's Balanced salt Solution

HPLC  High Performance Liquid Chromatography

NF-κB  nuclear factor kappa-light-chain-enhancer of activated B cells

NPs  empty nanoparticles

PBS  Phosphate buffer Saline

PI  Propidium Iodine

ROS  Reactive Oxigen Spieces

TEM  Transmission Electron Microscopy

TPP  tripolyphosphate

UV-RRS  UV-Resonant Raman Spectroscopy

# 1. Introduction

## 1.1. Cancer

Cancer constitutes an enormous burden in both, developed and less economically developed countries.

The burden is expected to grow worldwide due to the growth and aging of the population, particularly in less developed countries, in which about 82% of the world's population resides.

The increase in several less developed and economically transitioning countries is attributable to the adoption of unhealthy western lifestyles that are known to increase cancer risk factors. This lifestyle behavior includes smoking, poor diet and overweight, physical inactivity, changing reproductive patterns (including lower parity and later age at first birth) and are associated with urbanization and economic development. Based on GLOBOCAN estimates (IARC 2014), about 14.1 million new cancer cases and 8.2 million deaths occurred in 2012 worldwide. Over the years, the burden has shifted to less developed countries, which currently account for about 57% of cases and 65% of cancer deaths worldwide.

In Europe there were over 3.4 million new cases of cancer in 2012 (53% occurring in men and 47% in women); the most common were breast cancer for woman (28.8%) and prostate for men (22.8%), followed by lung cancers for men (15.9%) and colorectal cancer (CRC) for both the sex (13.0%). (Siegel et al., 2013; Ferlay et al., 2013).

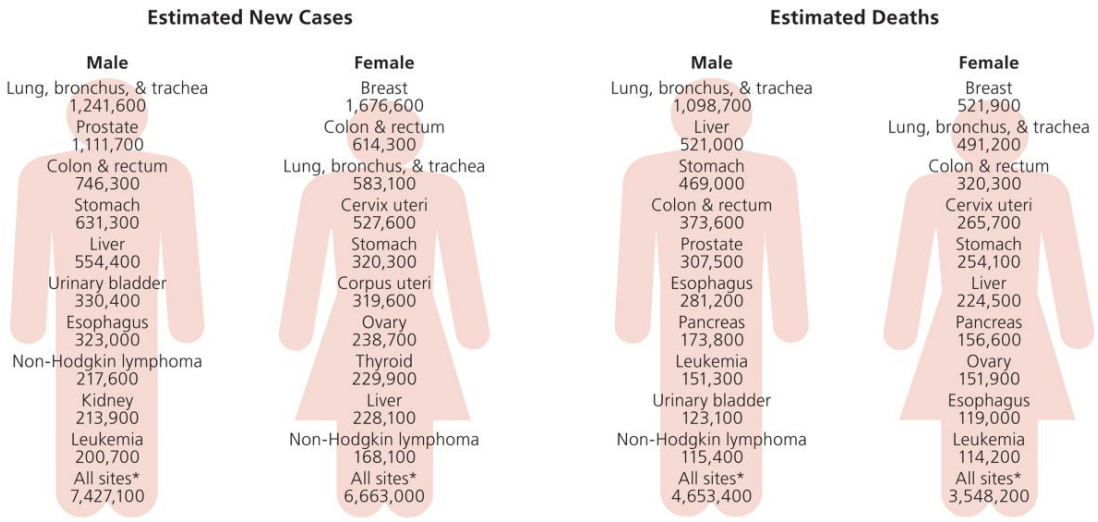



**Figure 1**. Estimated New Cancer Cases and Deaths Worldwide by Sex (excluding non-melanoma skin cancer) GLOBOCAN 2012.

### 1.1.1. Cancer treatment

In cancer therapy, the surgical treatment of primary tumors and associated lymph nodes is the primary choice and also the most effective form of treatment when considered as the only mode. The alternatives to this treatment or to that associated are chemotherapy and radiotherapy, with the big difference that, for each cycle treatment, they can only kill a fraction of the cancer cells. Although radiotherapy is responsible for the cure of 40% of cases, every year, only 45% of all new cases of cancer are treated with this method.

Depending on the type of tumor, radiotherapy can be applied by an external radioactive source, or by liquid systemic injection or by a source placed inside or next to the area requiring treatment (brachytherapy). The degree of invasiveness and side effects of the different methods is highly variable.

Chemotherapy is used alone or in combination with the others. It can be applied before the surgery phase, usually predominantly for colorectal cancer and esophagus, but more often it is applied as post-surgical therapy. The biggest advantage is to increase the eradication of micrometastasis present in the body and not properly highlighted.

Within hormone-dependent tumors, such as breast, thyroid and prostate cancer, chemotherapy treatment has definitely changed the treatment method. In fact, for breast hormone-dependent tumor types, in the past, the only treatment option for pre-menopausal women was the complete ablation of the ovary. The development of drugs that can inhibit the action of aromatases and interact with estrogen receptors, including tamoxifen, completely changed the impact of this pathology. Recently, the development of new third-generation aromatase inhibitors, anastrozole, letrozole and exemestane, with a lesser impact in toxicity, has quickly replaced tamoxifen for breast cancer treatment in postmenopausal women.

In general, systemic chemotherapy has always benefited from the use of high doses of cytotoxic molecules, mostly intercalating DNA molecules, which hatted and killed rapidly divisible cancer cells. There are two main contraindications to this therapy: the first concerns the systemic damage caused by the chemotherapy also on healthy cells to decrease which a recovery period between successive cycles is required; the second is due to the lack of suppression of all tumor cells, their high degree of mutation and the consequent selection of resistant tumor cells. Nowadays, in response to an even deeper knowledge of the molecular mechanisms involved in the pathology, pharmacological therapy tends to be increasingly personalized. Depending on the type and stage of the tumor, alone or in combination, a large number of drugs can be used (Urruticoechea et al., 2010; Carels et al., 2016; Gong et al., 2017; Catenacci et al., 2010). Chemotherapy is usually grouped into several families depending on the mechanism of action

involved. In fact, targets can be related to growth factors, proteins involved in the cell cycle, apoptotic modulators, or molecules involved in the angiogenic process.

To this end, two different approaches are used in clinical practice: (i) the use of monoclonal antibodies whose extracellular action is intended to inhibit the intake or action of growth factors like, humanized monoclonal antibodies such as trastuzumab/herceptin for HER2 (Human Epidermal Growth Factor Receptor 2), cetuximab for EGFR (epidermal growth factor receptor), (ii) the use of molecules acting within tumor cells by blocking signal transduction cascades of specific transcription factors like anti-hormone agents (tamoxifen, flutamide, etc.), anti-metabolites (5-fluorouracil, methotrexate, capecitabine, etc.), anti-microtubule agents (taxanes (paclitaxel/taxol, docetaxel, etc.) and the Vinca alkaloids (vinblastine, vincristine and vindesine) (Lai et al., 2012).

### 1.1.2. Cancer multidrug resistance

Although chemotherapeutic drugs efficiently kill cancer cells, the latter can defend themselves from such toxic compounds when they are used for an extended period or sometimes even after use for a short time, with a process called the cancer multidrug resistance (MDR).

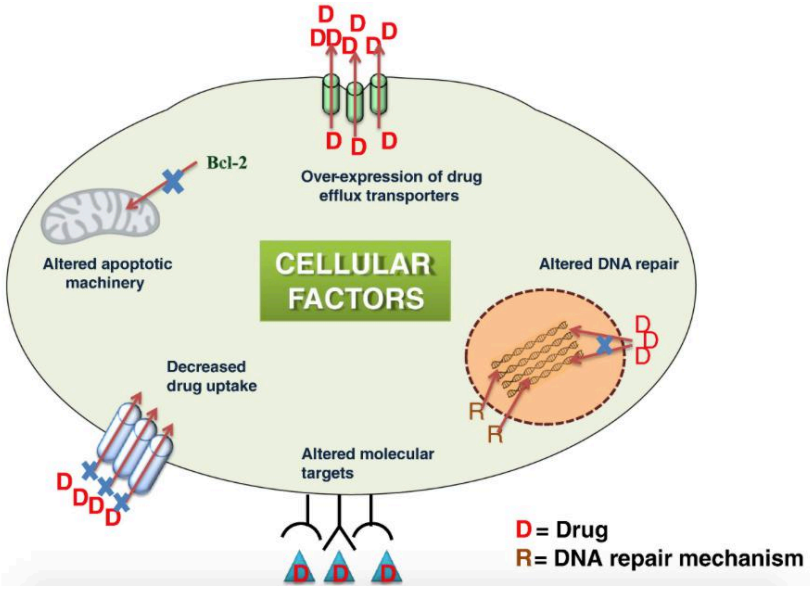



**Figure 2**. Mechanism of cancer MDR (Kibria et al., 2014).

From a physiological point of view, MDR is a mechanism of protection that cell developed against numerous compounds characterized by different chemical structures and by different mechanisms of intracellular activity. Acquired drug resistance can arise during treatment of

tumors that were initially sensitive and can be caused by mutations or by various other adaptive responses, such as increased expression of the therapeutic target and activation of alternative compensatory signaling pathways (Longley and Johnston, 2005). Moreover, it is increasingly recognized that tumors can contain a high degree of molecular heterogeneity, thus drug resistance can arise through therapy-induced selection of a resistant minor subpopulation of cells that was present in the original tumor.

Despite many decades of research, the mechanisms underlying cancer chemoresistance are not completely understood. An example is inflammation, in fact recent evidences suggest that inflammatory tumor microenvironment modulates not only cancer development but also cancer responsiveness and resistance to conventional anticancer therapies (de Visser et al., 2009).

The most investigated mechanisms with known clinical significance (Fig. 2) are: a) activation of transmembrane ATP-dependent proteins involved in the efflux mechanism of xenobiotics; b) activation of the enzymes of detoxification systems; c) alterations of genes and proteins involved in the control of apoptosis (especially p53 and Bcl-2) (Kibria et al., 2014).

#### 1.1.2.1. Mechanisms of drug resistance

**ABC transporter superfamily**

One of the mechanisms involved in chemoresistance depends on a process by which the drug is actively pumped out of the cell reducing its accumulation and effect. Over 10% of all functionally characterized genes encode for transporters or transporter-related proteins, as expected of key players in cellular homeostasis. Indeed, more than 1300 membrane transporter proteins have been identified in the human genome (Ziberna et al., 2014).

Primary active transporters include ATP-binding cassette (ABC) transporters that utilize the free energy of ATP hydrolysis as their driving force.

ABC transporters are found on the extracellular and intracellular membranes of tissue barriers and excretory organs not only in pathologic but also in healthy cells. Members of the ATP binding cassettes (ABC) family are involved in the excretion of drugs and in general of xenobiotics against an electrochemical gradient. (Giacomini et al., 2010; Yu et al., 2013). While maintaining a certain degree of individuality with regard to the protein structure, all members of the family share a nucleotide binding domain and a trans-membrane domain. The transport mechanism through the membrane is in fact dependent on the ATP hydrolysis, which determines a conformational modification and the subsequent transport of the molecule out of the cell. Some major members of this group include P-glycoprotein (P-gp, MDR1/ABCB1), multidrug

resistance-associated proteins (MRPs, ABCC) and breast cancer resistance protein (BCRP, ABCG2).

Chemotherapy forms the mainstay of cancer treatment particularly for patients who do not respond to local excision or radiation treatment. The overexpression of multidrug resistance proteins decreases cellular accumulation of hydrophobic anti-cancer chemotherapeutic drugs by extruding them from cells (Huang, 2007).

The general structural features of ABC transporters have been elucidated based on the study of its most well-characterized member, P-gp, that was the first to be identified (Choudhuri et al., 2016). Is a membrane-bound glycoprotein that is expressed in almost all tissues at low levels, but is found at much higher levels on the surface of epithelial cells that have excretory roles, such as those lining the colon, small intestine, pancreatic and bile ductules and kidney proximal tubules, thus affecting absorption, distribution, metabolism and excretion of xenobiotics.

MDR1 is overexpressed in many tumors, or its expression can be induced by chemotherapy (Holohan et al., 2013) by other mechanisms such as inflammatory response to oxidative stimuli (Ho et al., 2006). Clinical significance of above mentioned carrier is appreciated from the fact that more than fifty percent of existing anti-cancer drugs undergo inhibitable and saturable P-gp mediated efflux. This condition increasingly requires improving pharmacokinetics and anti-tumor activity and simultaneously decrease the systemic toxicity of drugs already present on the market and characterized by inhibitory activity against P-gp.

Flavonoids, a class of secondary plant metabolites, acting as P-gp inhibitors and as antitumor molecules, could be used as third generation drugs used as adjuvants of chemotherapy (Bansal et al., 2009).

**Activation of enzymes of detoxification system**

The increased generation of reactive oxygen species (ROS) in cancer cells makes them highly dependent on antioxidant enzymes to cope with the ROS stress. The sustained oxidative stress due to the presence of constant oncogenic signals and active metabolism requires full utilization of the cellular antioxidant capacity. The formation of additional oxidative stress due to the anti-neoplastic agent may exhaust the cellular antioxidant capacity and drive the cell beyond a “threshold” leading to apoptosis (Kong and Lillehei, 1998; Kong et al., 2000).

Generally, ROS are generated as products of biotransformation of chemotherapeutics and are rapidly reduced to nontoxic “physiological” levels by antioxidant enzymes such as superoxide dismutases (SODs), catalase (CAT), glutathione peroxidases (GPx), peroxiredoxins (PRx), and

non enzymatic antioxidants, such as reduced glutathione (GSH), uric acid, ascorbic acid, and ceruloplasmin, among others (Pelicano et al., 2004).

All these constitutive protective systems are sufficient to manage low levels of xenobiotics or endobiotics. In some cases, chemical stressors like anticancer chemotherapeutics should first be recognized by specific sensors which, in turn, transmit alarm signals to activate or express *de novo* transporting, biotransforming, and detoxifying enzymes (Reuter et al., 2010).

Furthermore, ROS-mediated DNA damage can cause mutations and thus increases the risk of developing drug-resistant cancer cells. (Tan et al., 1998; Feinendegen, 2002).

An increase in ROS is also associated with the activation of the redox sensitive JNK/SAPK signaling pathway, which is often involved in the transcriptional activation of genes and post-translational modifications of proteins necessary for apoptosis (Devi et al., 2001).

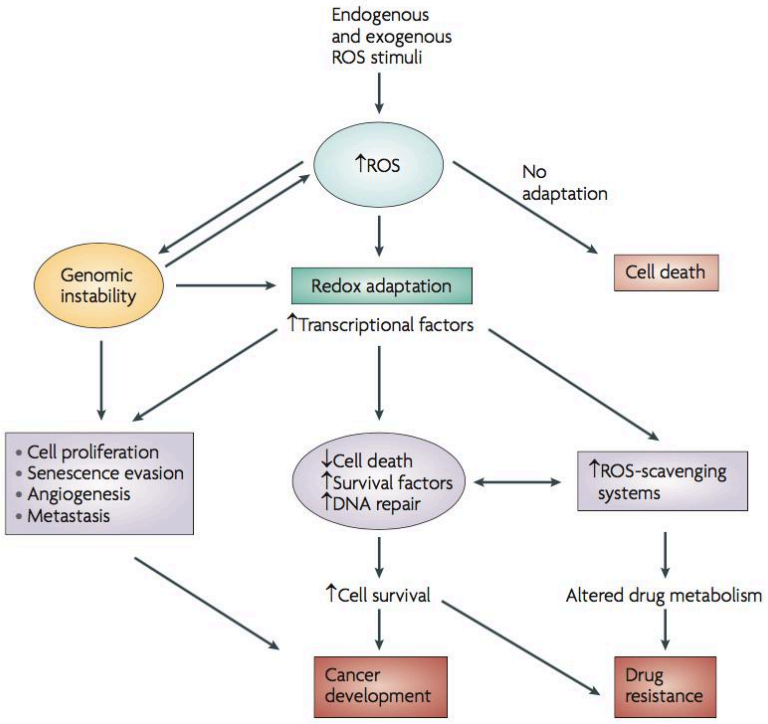



**Figure 3**. Oxidative stress status in anticancer drug resistance. Drug - induced persistent oxidative stress can be overcome by increasing genomic instability and by redox adaption. Both processes are implicated in cancer progression and drug resistance (Reuter et al., 2010).

Interestingly, the BH3-only protein Noxa responds directly to hypoxia-inducible factor-1α (HIF-1α) and seems to play an important role in hypoxia-induced cell death with the participation of ROS (Kim et al., 2004). ROS generation during apoptosis is thought to be associated with the malfunction of the mitochondrial respiratory chain and disengagement of cytochrome *c*, as well

as alteration in mitochondrial transmembrane potential and membrane permeability (Zamzami et al., 1995) influencing the rate of ROS generation and the kinetics of the apoptotic process, and therefore modulating drug-induced apoptosis (Singh et al., 1999; Jiang et al., 1999; Chomyn and Attardi, 2003).

**Defective apoptosis pathway**

Common cancers arise from the progressive accumulation of common genetic errors, most of which subvert the function of normal cell-regulatory genes. If a germline defect is into one of these regulatory genes, often occurs in “tumor suppressor gene”, predisposes to familiar or heritable cancers. For this reason, most chemoterapeutic drugs can induce a series of cellular events that impact on tumor cell proliferation and survival and end with programmed cell death, apoptosis. Now we know that a large number of chemotherapeutic drugs can kill tumor cells by activating common apoptotic pathways. Thus, single mutations that disable apoptosis can produce multidrug resistance (Epstein, 2013).

There exists a large number of genes involved in regulation of cell cycle and apoptosis, but one of the most important checkpoint of this process that have been identified is the protein p53, encoded by the gene TP53.

TP53 is the prototype of tumor suppressor gene in human cancer due to its pro-apoptotic and anti-proliferative function in response to oncogenic stress. Depending on the severity of damage to the genome, p53 can activate genetic programs that halt cell proliferation transiently (G1 and G2 cell cycle arrest) or permanently (senescence), or eliminate the cell altogether (apoptosis); recently, wild-type p53 has been reported to regulate also the expression of microRNAs involved in cell cycle regulation (Wong et al., 2011). The p53 pathway is inactivated in the majority of human malignancies and increased levels of its negative regulators MDM2 and MDM4 downregulate p53 function in many of the rest. Thus, the p53 pathway is most likely disrupted also in a large fraction of wild-type p53- carrying tumors (Muller et al., 2006).

Induction of cell death by antitumor drugs requires the recognition of DNA damage, activation of specific kinases, stabilization and activation of p53, activation of downstream p53-dependent pathways, and finally execution of cytotoxic programs. Interestingly, resistance in tumor cells harboring wild-type p53 is observed with a large variety of agents, ranging from ionizing radiation to several classes of cytotoxic drugs, such as doxorubicin, paclitaxel, cisplatin, etoposide, and vinblastine. This resistance can occur directly, via factors that reduce or negate the functional activity of p53, or indirectly, by deregulation of pathways downstream of p53.

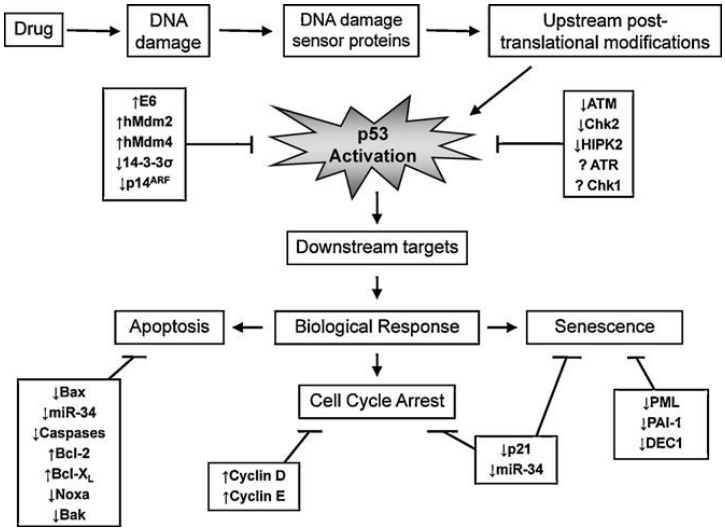



**Figure 4.** The indirect pathways that affect p53 signaling can involve downstream failure either in the activity of target gene products, such as p21, Bax, miRNAs, and caspases, or in the expression or mutation of proteins interacting with p53. Moreover, there are more than one hundred proteins that can bind with p53 to potentially regulate cytotoxic outcome, and a defect in this interaction through alterations in expression or mutation in the binding partner can have serious therapeutic consequences (Martinez-Rivera and Siddik, 2012).

## 1.2. Health benefits of dietary phenolics

Over the last decades, a considerable body of evidence supported the hypothesis that diet and dietary factors play a relevant role in the occurrence of diseases. To date, all the major scientific associations as well as the World Health Organization and the nonscientific organizations place an ever-increasing emphasis on the role of diet in preventing non-communicable diseases (Sofi et al., 2013).

Many studies have evaluated the associations between food groups, foods, or nutrients and chronic diseases, and a consensus about the role of nutritional factors in the etiology of common diseases, such as cardiovascular and neoplastic diseases, has gradually emerged. Indeed, data from analytical and experimental studies indicated a relation between increased consumption of some food categories such as fruits and vegetables, fiber and whole grains, fish, and moderate consumption of alcohol and reduced risk of major chronic degenerative diseases, whereas increased total caloric intake, body weight, meat, and fats are associated with greater risk. However, the appropriate dietary strategy to prevent chronic degenerative diseases remains a challenging and a highly relevant issue (McEvoy et al., 2012; Gerber et al., 2014).

In particular, if we analyze the linkage between diet and cancer risk in the US, where diet rich in fruits and vegetable is very poor, is evident that diet contributes approximately 30–35% of all cancer-related deaths, while tobacco, infections, obesity and other factors contribute approximately 25–30%, 15–20%, 10–20% and 10–15%, respectively.

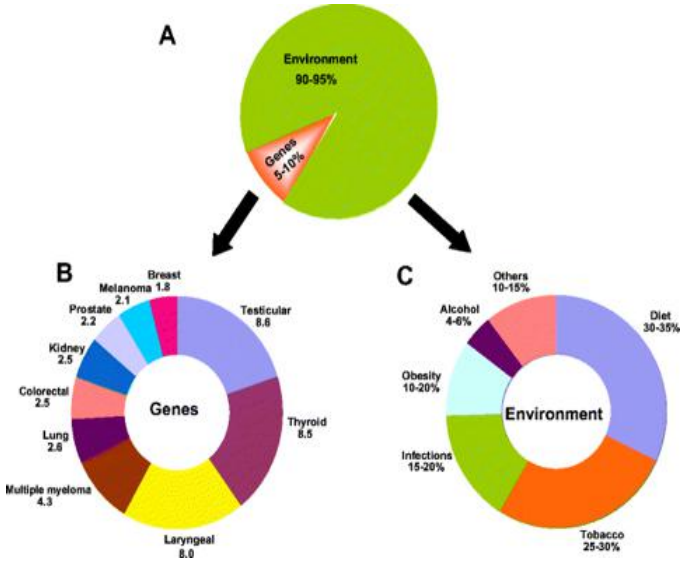



**Figure 5**. The role of genes and environment in the development of cancer. (**A)** The percentage contribution of genetic and environmental factors to cancer. (**B)** Family risk ratios for selected cancers. The numbers represent familial risk ratios, defined as the risk to a given type of relative of an affected individual divided by the population prevalence. Data taken from a study conducted in Utah. (**C)** Percentage contribution of each environmental factor. The percentages represented here indicate the attributable-fraction of cancer deaths due to the specified environmental risk factor.

Plant rich diets, such as mediterranean diet, are characterized by greater consumption of fruit and vegetables containing a high and varied intake of phenolic compounds, including flavonoids and various non-flavonoids such as phenolic acids, stilbenes and lignans, which may offer protective metabolic advantages for both cancer and cardio vascular disease risk.

The traditional Mediterranean Diet term was initially referred to dietary patterns of Mediterranean regions such as Crete, part of the rest of Greece and Southern Italy in the early 1960s (Esposito et al., 2010). It was characterized by a high consumption of plant foods (mostly whole grains products, vegetables, fruits including nuts and legumes), a low to moderate amount of fish, a low intake of eggs, meat and full-fatty dairy (refined carbohydrates were present in small quantities). Wine was drunk during the meals, in low or moderate amount, and olive oil was the only added fat in the diet. As a result, this dietary pattern was low in saturated fatty acids (7–8% of energy) with total fat ranging from <25% to >35% of energy throughout the Mediterranean region (Keys et al, 1986; Buttriss, 2018). Habitual levels of physical activity were high. This dietary pattern has been associated with relatively low rates of coronary heart disease, cardiovascular disease and cancer (Dontas et al., 2007). It shares many characteristics with an anticancer diet that experts have defined.

There is good epidemiological evidence that adherence to Mediterranean Diet has a protective effect against various cancers. The explanation may be the presence of various phytochemicals, including flavonoids and various non-flavonoids, which may offer protective metabolic advantages against cancer (Wang et al., 2015).

Phytochemicals are defined as bioactive “non-nutrient” plant chemicals in fruits, vegetables, grains, and other plant foods that may provide desirable health benefits beyond basic nutrition to

reduce the risk of major chronic diseases. More than 5000 individual phytochemicals have been isolated and identified in fruits, vegetables, and grains, but a large percentage still remains unknown. Furthermore, phytochemical content varies widely between various fruits, vegetables, and whole grains. Therefore, it is suggested that in order to receive the greatest health benefits, one should consume a wide variety of plant-based foods daily (Liu, 2013).

Recently, a prospective pilot study designed to establish the correlation between the long-term flavonoid treatment and colon neoplasia recurrence shown a reduction in recurrence rate especially in those with resected colorectal cancer (Hoensch et al., 2008).

Dietary phytochemicals can be classified into broad categories as phenolics, alkaloids, nitrogen-containing compounds, organosulfur compounds, phytosterols, and carotenoids. Of these phytochemical groups, the polyphenols are the most studied (Liu, 2004).

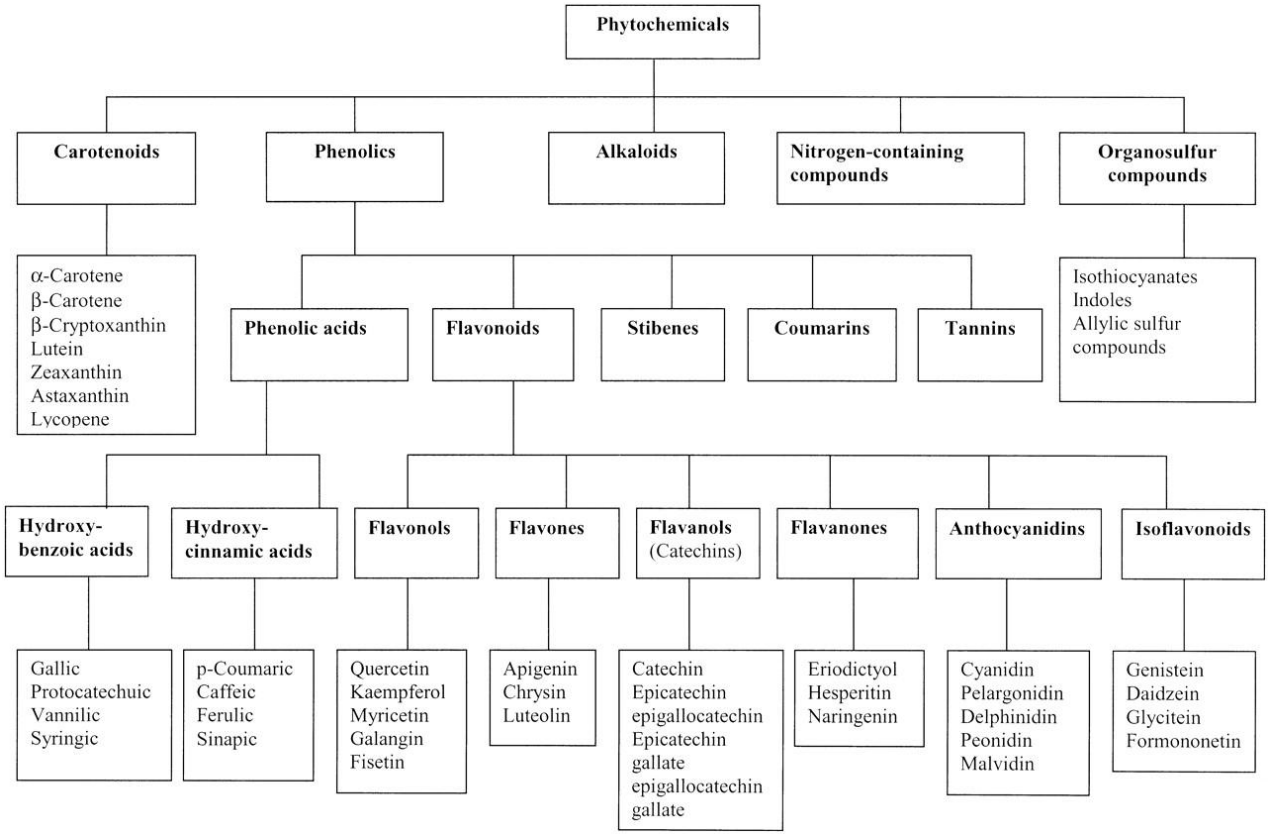



**Figure 6.** Classification of dietary phytochemicals (Liu, 2004).

### 1.2.1. Dietary phenolics

Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens. In food, polyphenols may contribute to the bitterness, astringency, color, flavor, odor and oxidative stability (Kanti and Syed, 2009).

Dietary phenolics or polyphenols constitute one of the most numerous and widely distributed groups of natural products in the plant kingdom. More than 8000 phenolic structures are currently known in various plant species, among them over 4000 flavonoids have been identified, many of which are responsible for the attractive colors of the flowers, fruits and leaves (Harborne et al., 2000; Bravo, 2015; Cheynier, 2005). Although polyphenols are chemically characterized as compounds with phenolic structural features, this group of natural products is highly diverse and contains several sub-groups of phenolic compounds.

All plant phenolic compounds arise from a common intermediate, phenylalanine, or a close precursor, shikimic acid. The majority of polyphenols in plants, occur in conjugated forms indeed they exist as glycosides with one or more sugar residues linked to hydroxyl groups and acylated sugars at different positions of the polyphenol skeletons

Association with other compounds, like carboxylic and organic acids, amines, lipids and linkage with other phenols is also common (Kondratyuket al., 2004).

Polyphenols may be classified into different groups as a function of the number of phenol rings that they contain and on the basis of structural elements that bind these rings to one another. The main classes include phenolic acids, flavonoids, stilbenes and lignans.

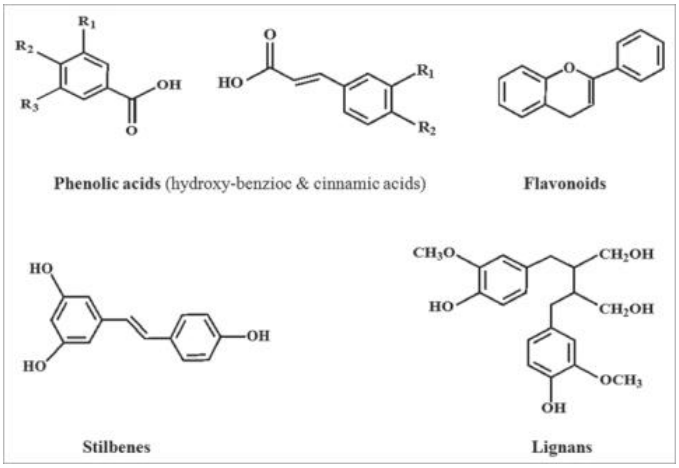



**Figure 7**. Chemical structures of the different classes of polyphenols. Polyphenols are classified on the basis of the number of phenol rings that they contain and of the structural elements that bind these rings to one another. They are broadly dived in four classes; Phenolic acids, Flavonoids, Stilbenes and Lignans**.** Plant polyphenols as dietary antioxidants in human health and disease

**Phenolic Acids:** Phenolic acids are non-flavonoid compounds abundantly found in foods. Can be further divided into two main types, derivatives of benzoic acid and cinnamic acid. While fruits and vegetables contain many free phenolic acids, in grains and seeds, particularly in the bran or hull, phenolic acids are often in the bound form. The hydroxycinnamic acids are more

common than hydroxybenzoic acids and consist chiefly of *p*-coumaric, caffeic, ferulic and sinapic acids.

**Stilbenes:** are naturally occurring compounds and are found in a wide range of plant sources. Are synthesized via the phenylpropanoid pathway and share some structural similarities to estrogen. Stilbenes contain two phenyl moieties connected by a two-carbon methylene bridge. Occurrence of stilbenes in the human diet is quite low. Most stilbenes in plants act as protective agents to defend the plant against viral and microbial attack, excessive ultraviolet exposure, and disease. One of the best studied compound among the cathegory is resveratrol found largely in grapes. A product of grapes, red wine also contains significant amount of resveratrol.

**Lignans:** Lignans are diphenolic compounds that contain a 2,3-dibenzylbutane structure derived from phenylalanine via dimerization of substituted cinnamic alcohol. Several lignans, such as secoisolariciresinol, are considered to be phytoestrogens. The richest dietary source is linseed, which contains secoisolariciresinol and low quantities of matairesinol.

**Flavonoids**: This class of compound comprises the most studied group of polyphenols. This group has a common basic structure consisting of a C6–C3–C6 general structural backbone in which the two C6 units (Ring A and Ring B) are of phenolic nature. Due to the hydroxylation pattern and variations in the chromane ring (Ring C), flavonoids may be divided into six subclasses: flavonols, flavones, flavanones, flavanols, anthocyanins and isoflavones. These basic structures of flavonoids are aglycones; however, in plants, most of these compounds exist as glycosides. Individual differences within each group arise from the variation in number and arrangement of the hydroxyl groups and their extent of alkylation and/or glycosylation. Biological activities of these compounds, including antioxidant activity, depend on both the structural difference and the glycosylation patterns.

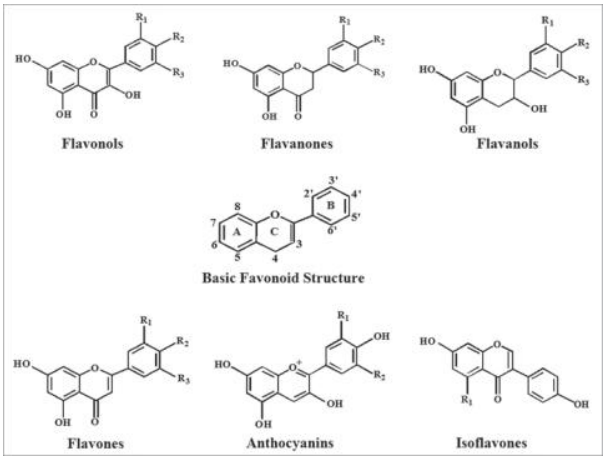



**Figure 8.** Chemical structures of sub-classes of flavonoids. Based on the variation in the type of heterocycle involved, flavonoids are divided into six major subclasses: flavonols, flavanones, flavanols, flavones, anthocyanins and isoflavones.

### 1.2.2. Biological properties of flavonoids

Regarding cancer, cardiovascular disease and generally chronic inflammation correlate diseases recent studies have demonstrated that are related to oxidative stress from reactive oxygen and nitrogen species (Reuter et al., 2010).

An excess of oxidative stress can lead to the oxidation of lipids and proteins, which is associated with changes in their structure and functions.

Polyphenols, especially flavonoids, are the predominant contributor to the total antioxidant activities of fruits, rather than vitamin C (Wang et al., 1996).

Different principle mechanisms of action have been proposed for antioxidants; indeed, they may exert their effect on biological systems by different mechanisms. These mechanisms include electron donation, in fact frequently they act as direct radical scavengers of the lipid peroxidation chain reactions (chain breakers). Chain-breakers donate an electron to the free radical, neutralizing the radicals and becoming themselves stable (less reactive) radicals, thus stopping the chain reactions (Pietta, 2000).

They can act as metal ion chelation preventing oxidation caused by highly reactive hydroxyl radicals. In addition, they can also act as co-antioxidants, involved in the regeneration of essential vitamins.

Another way of exerting antioxidant effects is inducing enzymes such as glutathione peroxidase, catalase and superoxide dismutase that decompose hydroperoxides, hydrogen peroxide and superoxide anions, respectively, and inhibit the expression of enzymes such as xanthine oxidase (Krinsky, 1992).

Among all the biological effects, several flavonoids, have been proposed to interfere with inflammatory pathways (Rull et al., 2010).

Such pathways are extremely complex and provide numerous candidate molecular targets (Figure 10). This complexity helps explain the repeated failures of numerous strategies to assess an exact and simple mechanism of the action of flavonoids on these pathways (González et al., 2011).

An acute inflammatory response is usually considered to be beneficial and is terminated within days. Chronic inflammation, however, lasts for weeks, months or years and results in severe cellular damage, mostly caused by macrophages differentiated by the action of chemokines. Among the chemokines, monocyte chemo-attractant protein-1 (MCP-1) is the most actively involved, both in metabolism and inflammation (Rull et al., 2010), and may be modulated in humans by certain flavonoids (Silva-Beltrán et al., 2015), perhaps independently from the antioxidant effects but it should be included in the equation.

It is particularly important to highlight NO•, which is relevant in the pathogenesis of both atherosclerosis and cancer by acting as a key signaling molecule (Bonavida et al., 2010).
In fact, it interacts with p53, a key molecular node in the inflammatory stress response pathway.

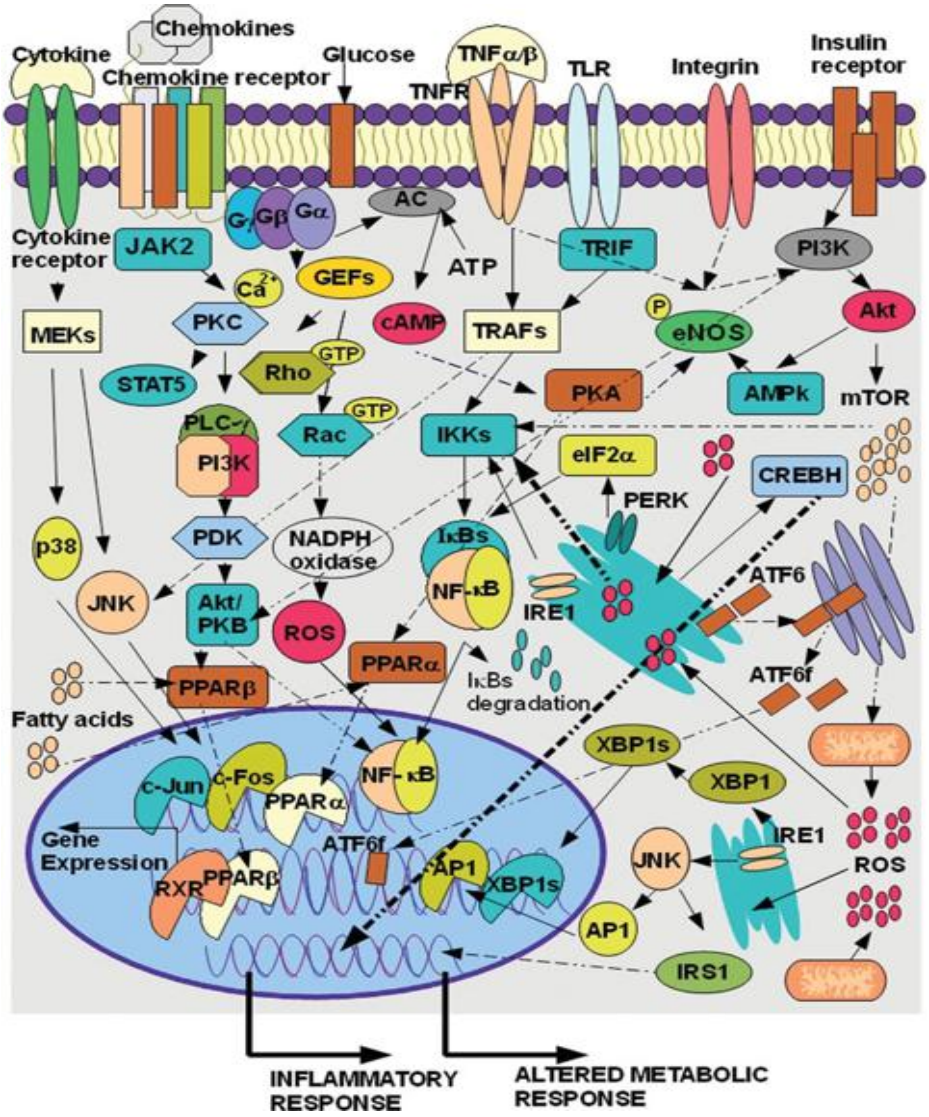



**Figure 9.** Putative molecular targets for polyphenols showing their influence on the cellular inflammatory and metabolic responses. These pathways are extremely complex even when factors being repressed are not depicted to prevent oscillatory or chaotic patterns. Multiple targets, multiple signals, and different dimensions (space-time) hamper the recognition of simple patterns. AC (adenylate cyclase); Akt (v-Akt murine thymoma viral oncogene); AP1 (activator protein 1); ATF6 (activating transcription factor 6); cAMP (cyclic adenosine monophosphate); CREBH (cyclic-AMP responsive element-binding protein); eIF2α (α-subunit of eukaryotic factor 2α); IKK (IκB kinase); IRE1α (phosphorylated inositol-requiring 1α); JAK (Janus kinases); JNK (JUN N-terminal kinase); NF-κB (transcription factor nuclear factor-κB); PDK-1 (phospholipid-dependent kinase-1); PERK (double-stranded RNA-dependent protein kinase (PKR)-like ER kinase); PI3K (phosphatidylinositol-3 kinase); PKB (protein kinase-B); PPARs (peroxisome proliferator-activated receptors); STAT (signal transducers and activators of transcription Factors); TRAF2 (tumor-necrosis factor-α (TNF-α)- receptor-associated factor 2); XBP1 (X-box-binding protein 1) (Joven et al., 2014).

The regulation of its expression activates specific set of genes (Ambs et al., 1998; Hofseth et al., 2003) such as NF-kB or other transcription factors regulating proliferation, differentiation, survival, angiogenesis, cell cycle, and senescence.

For instance, correlation between NO/iNOS production and NF-κB inhibition have been noticed, finding that all active flavonoids that reduced iNOS also were active on NF-κB inhibiting the activation of the signal transducer and activator of transcription 1 (STAT-1), another important transcription factor for iNOS.

An important question then is how exactly NF-κB is inhibited, because different evidences have been noticed, such as the inhibition via blocking IκB-α phosphorylation, but also inhibiting IKK-γ (IKK regulatory subunit) indirectly. Also, inhibition of IKK-β have been noticed, considered the main kinase IKK subunit in the canonical or classical NF-κB pathway, directly upstream of IκB-α (Comalada et al., 2006).

On the other hand, NF-κB is also modulated by other signaling pathways. This is the case of the p38, ERK, and JNK MAP kinases. Flavonoids inhibit these kinases, although not all compounds have the same profile. In macrophages, the activation of NF-κB requires the phosphorylation of IKK-α and IKK-β by Akt. Although this pathway has not received much attention, several flavonoids have been shown to inhibit it directly or indirectly, by actions on PI3K, located upstream of Akt (González et al., 2011).

Because of the experimental approach applied in most studies, it is likely that upstream targets of flavonoids have not been identified yet, an example is the interference with cell surface receptors and proteins that are localized in the so-called lipid rafts.

These structures participate in cellular signal transduction, endocytosis, and the transmembrane translocation of different components (Tarahovsky et al., 2008) and may be important in mediating the antibacterial or anticancer effects of some flavonoids (Cushnie et al., 2008). An example are anthocyanins that can modulate cell surface receptors that are important in the regulation of their anti-inflammatory activity by altering membrane lipid rafts (Kaneko et al., 2008; Xia et al., 2007). In particular, they suppress the accumulation of lipid rafts that occurs as a first step in TNF and TLR4 signaling. Of course, there may be other relatively unexplored pathways.

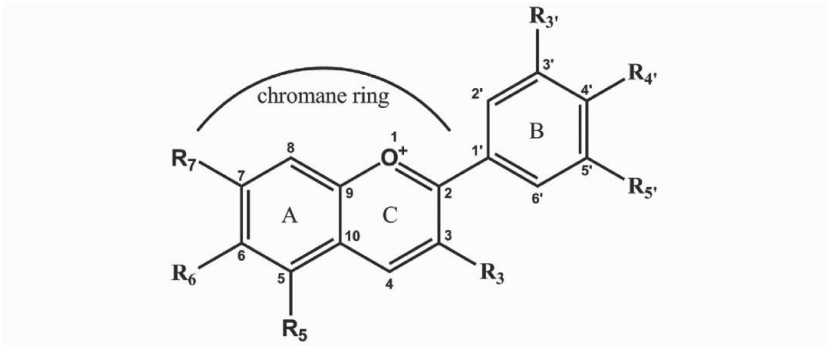
### 1.2.3. Anthocyanins

Among flavonoids, anthocyanins are considered the most abundant and most biologically active compounds, contributing greatly to the high antioxidant potential.

The name came from greek anthos=plant and kyanos=bleu, in fact they are responsible for colors raging from red to violet and blue. They are the most important visible plant pigments dissolved in the vacuolar sap of the epidermal tissues of flowers and fruit in which they play an important role in attracting animals, thereby promoting seed dispersal and pollination. Another important

feature is the absorption of light, contributing to protecting plants from ultraviolet (UV)-induced damages (Castañeda-Ovando et al., 2009).

Anthocyanins are the glycosides of flavonoids with polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavilium cathion (Fig. 10).




| | | |
|---|---|---|
| **1** | Pelargonidin (Pg) | $R_3$=OH; $R_5$=OH; $R_6$=H; $R_7$=OH; $R_{3'}$=H; $R_{4'}$=OH; $R_{5'}$=H |
| **2** | Cyanidin (Cy) | $R_3$=OH; $R_5$=OH; $R_6$=H; $R_7$=OH; $R_{3'}$=OH; $R_{4'}$=OH; $R_{5'}$=H |
| **3** | Delphinidin (Dp) | $R_3$=OH; $R_5$=OH; $R_6$=H; $R_7$=OH; $R_{3'}$=OH; $R_{4'}$=OH; $R_{5'}$=OH |
| **4** | Peonidin (Pn) | $R_3$=OH; $R_5$=OH; $R_6$=H; $R_7$=OH; $R_{3'}$=OMe; $R_{4'}$=OH; $R_{5'}$=H |
| **5** | Petunidin (Pt) | $R_3$=OH; $R_5$=OH; $R_6$=H; $R_7$=OH; $R_{3'}$=OMe; $R_{4'}$=OH; $R_{5'}$=OH |
| **6** | Malvidin (Mv) | $R_3$=OH; $R_5$=OH; $R_6$=H; $R_7$=OH; $R_{3'}$=OMe; $R_{4'}$=OH; $R_{5'}$=OMe |

**Figure 10**. General structure of common anthocyanidin

Anthocyanins are conjugated aromatic system, which are often positively charged, can absorb in the visible range, hence each anthocyanin can be represented by their unique color.

They exist in different chemical forms, both colored and uncolored, according to pH. As visible in Figure 11 these forms are the colored basic flavylium cation and three secondary structures, the quinoidal base(s), the carbinol pseudobase (*syn* chromenol or hemiachetal) and the chalcone.

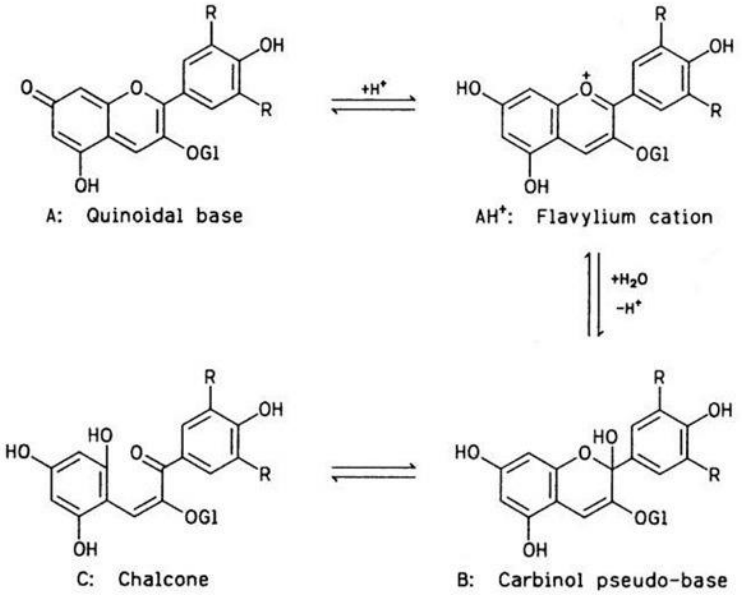



**Figure 11**: Anthocyanin equilibria in aqueous solution (Clifford, 2000).

Since they are highly unstable in the aglycone form (named anthocyanidins), in plants, their degradation is prevented by glycosylation, generally at position 3, and esterification with various organic acids (citric and malic acids) and phenolic acids. In addition, anthocyanins are stabilized by the formation of complexes with other flavonoids (co-pigmentation), improving their resistance to light, pH, and oxidation conditions that are likely to degrade them (Clifford, 2000).

Aglycones are rarely found in fresh plant material. Despite the fact that 31 different monomeric anthocyanidins have been identified (including 3-deoxyanthocyanidins, pyranoanthocyanidins and sphagnorubins), 90% of the naturally occurring anthocyanins are based on only six structures (30% on cyanidin, 22% on delphinidin, 18% on pelargonidin and in summary 20% on peonidin, malvidin and petunidin). These molecules are ubiquitously distributed and are known as common anthocyanidins (Fig. 10) (Jaldappagari et al., 2008).

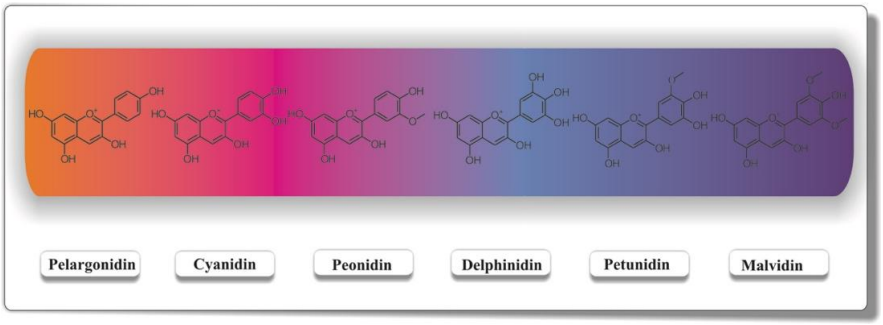



**Figure 12**. Visible color range of common anthocyanidins (Ananga et al., 2013)

The color of anthocyanidins differs with the number of hydroxyl groups, attached on their molecules (especially those substituted in ring B). The visible color of entire molecule shift from orange to violet with the increase of attached hydroxyl groups (Fig. 12).

Glycosylation of anthocyanidins results to additional adjustment of original color anthocyanins, whereas the presence of aliphatic or aromatic acyl moieties causes no color change or slight blue shift, but this modification has significant effect on their stability and solubility (Tanaka et al., 2008). Changes in pH can also cause reversible structural transformations in anthocyanins molecules, which has a dramatic effect on their color (Delgado-Vargas and Paredes-Lopez, 2003).

In the human diet, anthocyanins are found in several foods such as red wine, some varieties of cereals, and some leafy and root vegetables (aubergines, cabbage, beans, onions, radishes), but they are most abundant in fruit.

Usually the content is generally proportional to color intensity and reaches values up to 2–4 g/kg in fruits such as blackcurrants or blackberries. Cyanidin is the most common anthocyanidin in foods. These values increase with the ripening. In fruit, they usually are found mainly in the skin, except for certain types of red fruit, in which they also occur in the flesh (cherries and strawberries). Wine contains ≈200–350 mg anthocyanins/L, and these anthocyanins are transformed into various complex structures as the wine ages (Es-Safi et al., 2002).

Like all the phenolic phytochemical, anthocyanins absorption rate is very low, and bioavailability is limited, as reported in many studies (Table 1).

**Table 1**. Absorption rate of selected phenolic phytochemicals (Zheng et al., 2015).

| Phenolic phytochemicals | Sources | Absorption rate | References |
|---|---|---|---|
| Anthocyanins | Berries | <1% | (Felgines et al., 2003; Wu, Cao, & Prior, 2002) |
| Flavan-3-ols or procyanidins | Tea, chocolate, and grape seed extract | <5% | (Holt et al., 2002; Sano et al., 2003; Zhu, Chen, & Li, 2000) |
| Isoflavones | Soy | <1% | (Cassidy et al., 2006; Izumi et al., 2000; Setchell et al., 2001) |
| Flavonols (Quercetin) | Plant food | 3–7% | (Day et al., 2001; Mullen, Boitier, Stewart, & Crozier, 2004) |
| Flavanones (Hesperidin) | Citrus | 3–9% | (Borges, Lean, Roberts, & Crozier, 2013; Takumi et al., 2012) |
| Stilbenes (Resveratrol) | Grape and wine | <1% | (Rotches-Ribalta, Andres-Lacueva, Estruch, Escribano, & Urpi-Sarda, 2012; Walle, 2011) |
| Hydroxycinnamic acids | Coffee, tomatoes, and cereals | 1–25% | (Bourne & Rice–Evans, 1998; Kern, Bennett, Mellon, Kroon, & Garcia-Conesa, 2003; Rechner, Spencer, Kuhnle, Hahn, & Rice-Evans, 2001) |

For some time, the pharmacokinetic of anthocyanins was unknown and thought to be nearly inexistent as their absorption was considered limited (Nemeth et al., 2007). In general, the absorption percentage, as evident from Table 1, does not exceed a few percent of the ingested dose, as determined by measuring the blood levels of intact anthocyanins and their conjugates. Moreover, the quantification of total anthocyanins is difficult since their molar absorptivity depends on the balance of the different forms, pH values and presence of co-pigments (Atnip et

al., 2017). The main problems in absorption are low aqueous solubility and stability in the gastrointestinal conditions, and the reduced accumulation in the tissues caused not only by the poor absorption but also by the possibility to passively diffuse in the adjacent tissues or the presence in the gastrointestinal tract of many active efflux pumps that decreases the concentration of anthocyanins (McGhie et al., 2007).

Regarding stability in the gastrointestinal conditions we should remember that the digestion process in mammals begins with food intake and ends with defecation and during all these steps the pH varies throughout. Notably, anthocyanins are distinguished from other flavonoids by their re-arrangements in response to pH changes, indeed, the form(s) occurring are not known with certainty. Anthocyanins are more stable at acidic pH, as observed in the stomach, and are present in the form of red flavylium cation. Under basic conditions (pH > 7), such as the conditions that occur in the small intestine, anthocyanins are present as colorless carbinol, which undergoes limited absorption and is possibly metabolized into conjugates that often are not taken into account since they cannot be converted to red flavylium forms (Crozier et al. 2009; McGhie et al., 2007).

It is likely that the cation will exist only in the stomach, and the other forms will be more abundant lower down the gastrointestinal tract and in the tissues if absorbed (Fig. 13).

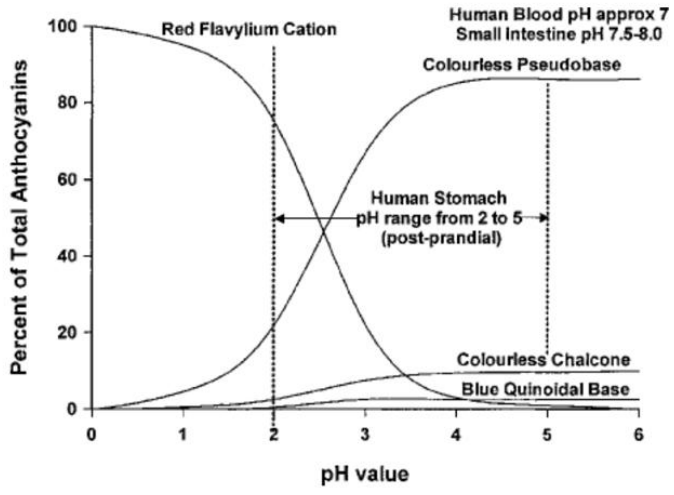



**Figure 13**. The generalized effect of pH value on anthocyanin equilibria in the physiological pH of the gastrointestinal tract (Clifford, 2000).

Several studies correlated uptake and distribution of such natural compounds to the food composition ingested together with the anthocyanins. For instance, proteins may bind to polyphenols reducing their availability or alcohol may improve it as evidenced by the increased uptake of red wine phenolics as compared with levels resulting after the consumption of alcohol-free red wine (Fernandes et al., 2017).

In general, the mechanism for anthocyanins transport across epithelium was principally considered to happen by passive diffusion, including paracellular and transcellular diffusions, in some cases after cleavage of glycoside moiety by lactase (Boyer, Brown, & Liu, 2004, 2005 Ichiyanagi et al., 2008). On the other hand, it was also shown that anthocyanins are partially absorbed by protein transport into the stomach (Passamonti et al., 2003; Passamoni et al., 2009; Atnip et al., 2017)

Prior to their transfer into the bloodstream, the molecule can undergo different fate: first, the aglycones undergo phase II metabolism in the enterocytes, forming sulfate, glucuronide, and/or methylated metabolites respectively through the action of sulfotransferases, uridine-5′-diphosphate glucuronosyltransferases and catechol-O-methyltransferases. This modification may alter tissue uptake and bioactivity and may increase stability (Prior and Xianli, 2009; Charron et al., 2009). Secondly, they can undergo active efflux process, at least some of the metabolites, that causes a decreased accumulation of anthocyanins (Figure 14) (Hollman, 2004; Bravo, 2015). This process is thought to involve members of the ATP-binding cassette family of transporters, that exist on the apical surface of epithelial cells, including P-glycoprotein, MRPs, and BCRPs (Vaidyanathan, 2003). Third, they can be transferred into the portal blood-stream, pass to the liver, and be subjected to further phase II metabolism. Although there is much speculation, as yet there is no compelling evidence that enterohepatic recirculation in human results in substantial recycling of (poly)phenol metabolites back into the small intestine via biliary excretion (Crozier et al., 2009).

In the broad range of modification that anthocyanidin undergoes and that causes a reduction in absorption has to be considered the degradation by the intestinal microflora. The large number of bacteria inhabiting the large intestine forms a highly complex ecosystem called the ‘gut microbiota’.

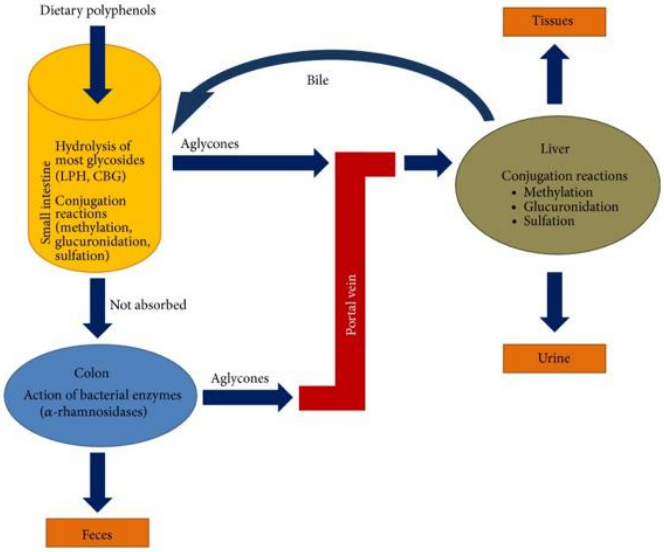



**Figure 14**. Passive diffusion, metabolism, and active efflux of phenolic phytochemicals on epithelial cells- Absorption and metabolism routes for dietary polyphenols and their derivatives in humans (Marín et al., 2015).

Since only a small part of ingested anthocyanins is absorbed at small intestine, large amounts of these compounds are likely to enter the colon, where microbiota has been reported to play a key metabolic role in the biotransformation of anthocyanins. First they are deglycosylated to the aglycone then are converted into smaller molecules such as phenolic and aromatic acids, which are also absorbed (Keppler et al. 2005). The gut microbiota has a high hydrolytic potential and ring scission properties so several anthocyanins degradation products have been identified (Marín et al., 2015).

Data from the limited available trials show that following ingestion there is extensive variability in metabolite levels. Indeed, ingesting such compounds causes the production of a wide range of urinary metabolites with an inter-individual variability in metabolism 15–99% (Czank et al., 2013).

This heterogeneity in responsiveness to anthocyanin intake may relate to a number of factors, including the different phases of metabolism but the microbiome is likely to be critical as it plays a key role in anthocyanin metabolism (Cassidy and Minihane, 2017).

Anthocyanins and their metabolites could also perform a positive modulation of intestinal bacterial populations, altering their composition. On the opposite, microbiota may enhance the metabolism of anthocyanins, but this bidirectional relationship has not yet been addressed (Marín et al., 2015).

In figure 15 a proposed colonic pathway on anthocyanins is presented. Regarding the fission of the B-ring, both phenyl acids and benzoic acids have been reported as microbial metabolites, the further microbial metabolism of which results in the production of different metabolites in respect of the anthocyanis involved, e.g. gallic acid derives from delphinidin glucoside (Mosele et al., 2015).

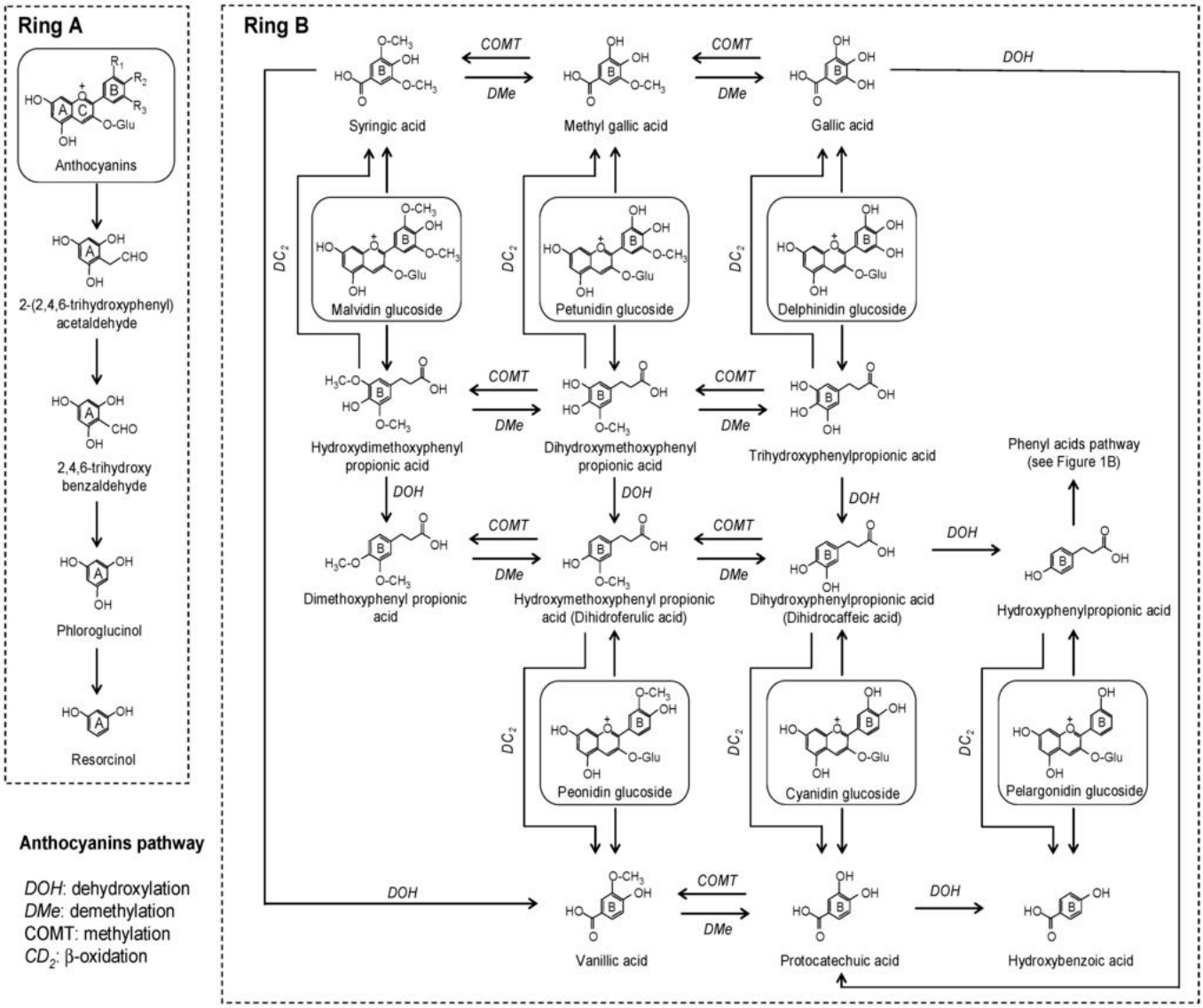



**Figure 15.** Proposed colonic pathways of anthocyanins metabolism (Mosele et al., 2015).

### 1.2.4. Biological role of Anthocyanins

Many beneficial activities have been attributed to anthocyanins thanks to the epidemiological evidences that highlighted an inverse correlation between a high consumption of polyphenols and the incidence of some chronic diseases.

There is a lot of work investigating these activities focuses on the antioxidant characteristics of anthocyanin rich diets and the health benefits. Thanks to their antioxidant capacity anthocyanins, like other flavonoids, are able to prevent oxidative damage caused by (ROS) to DNA, proteins, lipids and other macromolecules.

**Table 2**. Biological activity and health effects of some food-derived anthocyanins in in-vitro and in-vivo studies (Smeriglio et al., 2016).

| Anthocyanins source | Experimental model | Dose | Treatment time | Results | References |
|---|---|---|---|---|---|
| Bilberry | | | *In vitro studies* | | |
| | Isolated rat hearts under ischemia-reperfusion conditions | 0.01–1 mg/L | | ↓ Rate of lactate dehydrogenase (LDH)<br>↑ Post-ischemic coronary flow<br>↓ The incidence and duration of reperfusion arrhythmias | Ziberna *et al.*, 2012 |
| | HT-29 colon cancer cells | 25–75 μg/mL | | ↓ The growth cells | Zhao *et al.*, 2004 |
| | Gram-positive and Gram-negative | 50 μL | | ↓ Growth | Burdulis *et al.*, 2009 |
| | | | *Animal studies* | | |
| | Ischemia-reperfusion hamster model | | | ↓ Microvascular impairments<br>↑ Preservation endothelium<br>↑ Capillary perfusion | Bertuglia *et al.*, 1995 |
| | Type 2 diabetes mouse model | 27 g of BBE/kg diet | | ↓ Hyperglycemia<br>↓ Elevation of blood glucose levels ↑ Insulin sensitivity | Takikawa *et al.*, 2010 |
| | ApcMin mice, intestinal cancer model | 9 mg | 12 weeks | ↓ Adenoma 30% | Cooke *et al.*, 2006 |
| | Fischer 344 male rats | 3.85 g/kg diet | | ↓ Aberrant crypt foci by 26% to 29% | Lala *et al.*, 2006 |
| | | | *In vitro studies* | | |
| | Eight young healthy males | 160 mg (3 times a day) | 21 days | No significant effect observed | Muth *et al.*, 2000 |
| | Double-blind placebo-controlled study with 6 Subjects | | 3 h | Adapted to the light within 6.5 min | Camire 2000 |
| | 50 Patients with senile cataracts | 180 mg (twice a day) | 4 months | Progression of cataracts was stopped in 96% of the subjects treated | Bravetti 1988 |
| Blueberry | | | *In vitro studies* | | |
| | Gram-positive and Gram-negative | 50 μL | | ↓ Growth | Burdulis *et al.*, 2009 |
| | | | *In vitro studies* | | |
| | Old F344 rats | 2% diet | 10 weeks | ↑ HSP70-mediated protection<br>↓ Deleterious effects of ageing | Galli *et al.*, 2006 |
| | F344 rats | 2% diet | 8 weeks | ↓ Inflammation<br>↓ Expression of IL-1b, TNF-α and NF-kβ | Shukitt-Hale *et al.*, 2008 |
| | Mice | 27.8 mg/g | 8 weeks | ↓ Body weight gain | Prior *et al.*, 2008 |
| | Mice | 0.49 mg | 72 days | ↓ Weight gain<br>↓ Body fat accumulation | Prior *et al.*, 2010 |
| | | Human studies | | | |
| | 16 young normal volunteers (single oral dose) | 12, 24 and 36 mg | 24 h | No significant effect observed | Levy and Glovinsky, 1998 |
| | 18 young normal volunteers (twice a day) | 12 and 24 mg | 4 days | No significant effect observed | Zadok *et al.*, 1999 |
| Blackcurrant | | | *In vitro studies* | | |
| | *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecium* | 10.00 mL | | ↓ Growth | Werlein *et al.*, 2005 |
| | | Animal studies | | | |
| | Chick with negative lenses | 50 and 100 mg/kg | 3 days | ↓ Transient myopia<br>↓ Eye fatigue<br>↑ Dark adaptation<br>↑ Retinal blood flow | Lida *et al.*, 2010 |
| | Rats with lung inflammation | 10 and 30 mg/kg | | ↓ Inflammation | Rossi *et al.*, 2003 |
| | | Human studies | | | |
| | Double-blind, placebo controlled crossover design study with 21 slightly myopic subjects, 2 h of continuous work on a personal computer | 50 mg | | ↓ Diopter values | Nakaishi *et al.*, 2000 |
| | 30 glaucoma patients | 50 mg/day | 6 months | ↑ Retinal blood flow | Ohguro *et al.*, 2007 |
| Strawberry | | | *In vitro studies* | | |
| | CAL-27, KB, HT29, HCT-116, LNCaP and DU145 cancer cells | 100 μg/mL | | ↓ Cell viability<br>↓ Cell growth | Zhang *et al.*, 2008 |
| Strawberry | | | *Animal studies* | | |
| | Mice | 2.9 mg/g | 8 weeks | ↓ Body weight gain | Prior *et al.*, 2008 |
| | | | *Human studies* | | |
| | Eight elderly women | 240 g | | ↑ Antioxidant capacity in serum and urine<br>↑ Plasma vitamin C level | Cao *et al.*, 1998 |
| Grape | | | *In vitro studies* | | |
| | HT-29 colon cancer cells | 25–75 μg/mL | | ↓ The growth cells | Zhao *et al.*, 2004 |
| | | | *Animal studies* | | |
| | Rats | 21 mg/kg | 6 weeks | ↓ Triglycerides<br>↑ HDL | Al-Awwadi *et al.*, 2005 |
| | Fischer 344 male rats | 3.85 g/kg diet | | Aberrant crypt foci by 26% to 29% | Lala *et al.*, 2006 |
| | Rats (female) | 10, 15 and 20 mg | 20 weeks | ↓ Mammary tumours | Singletary *et al.*, 2003 |
| | Rats (female)<br>↓DMBA DNA adducts | 830 mg/L drinking water | 3 weeks | ↓ Final tumour mass | Jung *et al.*, 2006 |
| | | | *Human studies* | | |
| | 22 hemodialysis patients | 50 mL (twice daily) | 14 days | ↓ Oxidized LDL<br>↓ Activity of NADPH oxidase<br>↑ HDL | Castilla *et al.*, 2008 |
| | 20 subjects | 7 mL/kg | 14 days | ↓ Platelet aggregation<br>↑ NO ↓ O2- | Freedman *et al.*, 2001 |
| | Eight men | 50 g | | ↓ LDL oxidation | Kamiyama *et al.*, 2009 |
| | Older adults with memory decline | 6 and 9 mL/kg | 12 weeks | ↑ Verbal learning<br>↑ Cognitive function | Krikorian *et al.* 2010 |
| | 60 patients with asthenopia | 85 mg (twice daily) | 4 week | 73% of patients<br>↑ symptoms | Lee *et al.*, 2005 |

It has been postulated that anthocyanins act on two levels. They have a systemic action, since they are absorbed and circulate in the blood, and it is in this circulating form that they act upon different target tissues in the human body.

These features reflect in a broad range of positive effects on health, interfering with several diseases. In Table 2 are reported some studies concerning the health effects of anthocyanins on different types of disease (Smeriglio et al., 2016).

As visible from the table, these molecules have a broad range effects on pathologies such as diabetes (Jin-Ming et al., 2003), obesity and cardiovascular disease (García Alonso et al., 2014), but seems also to be protective in neurons and in hepatocytes too (Kyu-Ho et al., 2006) even ocular effects have been identified (Fursova et al., 2005). Recently has been investigated also antimicrobial activity but needs further investigation to understand fully the mechanisms underlying this capacity (Nohynek et al., 2006).

To us the most important effects exerted by this family of molecules is the preventing and inhibiting cancer growth (Cooke et al., 2005; Wang and Stoner, 2008; Thomasset et al., 2009) that have been widely investigated in this years.

The anticancer properties of anthocyanins have been largely based on evidence from in vitro cell-based assays and may be due to multiple and sometimes additive mechanisms. All together the evidences obtained in this years led to think that may be both an anticancer effect and a chemopreventive effect, in particular regarding cancers of the gastrointestinal tract-related organs and colon, probably due to the high availability of these compounds at this level (Shih et al., 2005; Jian and Giusti, 2010).

The main interactions noticed regards: cell cycle arrest (G1/G0 and G2/M) and induction of apoptosis (Weiguang et al., 2005) as well as anti-angiogenesis effects (Bagchi et al., 2004).

A lot of effects are linked to the antioxidant capacity of these molecules such as the inhibition of DNA oxidative damage (Webb et al., 2008), induction of detoxification phase II enzymes (Srivastava et al., 2007), anti-mutagenic effects (Ohara et al., 2004) and anti-carcinogenic effects (Shih et al., 2005) and inhibition of COX-2 pathway (Yong et al., 2017). The anti-proliferative activity of anthocyanins occurs in different cell types (Zhao et al., 2004; Zhang et al., 2006; Yi et al., 2005). They block various cell cycle stages that influence regulatory proteins as well as selectively inhibiting cancer cell growth.

Anthocyanidins have been demonstrated to be better inhibitors of cell proliferation than anthocyanins (Zhang et al., 2006), with delphinidin having the best growth inhibition property. This may be linked to the presence of hydroxyl groups on the B ring of the anthocyanidin core, which seems to facilitate this molecule to exert its effect by blocking activation of the MAPK

pathway (Hou, 2003). From previous studies conducted in our laboratory cyanidin and delphinidin have shown growth inhibition properties via inactivation of the glutathione antioxidant system and promotion of oxidative stress, while malvidin have not shown any such effect (Cvorovic et al., 2010). Similar findings are reported in other studies on human leukemia cells (HL-60) and in JB6 mouse epidermal cells model, respectively. In the first, the authors concluded that delphinidin, petunidin and cyanindin induced apoptosis and DNA fragmentation, while pelargonidin, peonidin and malvidin showed no induction of apoptosis. Once again, the highest anticancer activity was shown by delphinidin and involved gene expression and activation of caspase-3 (pro-apoptotic gene) and activation of oxidative stress-induced signalling pathways (Hou, 2003).

Again, delphinidin among the tested anthocyanidins had the strongest inhibitory effect on the MAPK signalling pathway and through a synergistic effect with superoxide dismutase enzyme leding to an inhibition of cell transformation. These results suggest that the ortho-dihydroxyphenyl structure on the B-ring may be essential (De-Xing et al., 2003).

The beneficial effects of many anthocyanins/anthocyanidins in vitro studies previously reported suggest that these molecules exert their beneficial effects in a concentration range of $10^{-6}$ to $10^{-4}$ M. Even though the daily intake of anthocyanins is 9-fold higher than the other flavonoids (Wang et al., 2008), some human studies on anthocyanin uptake have suggested that they reach levels of $10^{-8}$ to $10^{-7}$ M in human blood, concentrations far below those required to exhibit *in vitro* anticarcinogenic effects. Thus, it is not clear whether the *in vivo* concentrations are sufficient to induce anticancer effects on humans or if anthocyanins exert chemopreventive effects by themselves or must be hydrolysed to aglycon forms to exert their properties (Cooke et al., 2005). These properties have been extensively reviewed by various authors but to date, results are still controversial (Bansal et al., 2009).

## 1.3. Nanoparticles

As described in the previous paragraph flavonoids can exert a wide range of biological effects in vitro, but their in-vivo activity is still not clear due to their poor bioavailability.

The low bioavailability is mainly due to the low absorption in the human gastrointestinal tract following consumption, extensive biotransformation within the gut and rapid clearance from the body.

Therefore, enhancement of bioavailability would be of utmost importance in order to exert health effects, in vivo. In this regard, numerous attempts have been made to increase bioavailability

such as: improving the intestinal absorption via use of absorption enhancers, novel delivery systems; improving metabolic stability; changing the site of absorption from large intestine to small intestine. All of which are achievable by a nano-technological approach.

Nowadays, nanotechnology has attending significant attention due to the advances in material science and nano-engineering leading to significant progress in several fields. Nanosystems have become very attractive for their applications in biology and medicine, including their use as carrier for drugs, to improve solubility, minimize degradation process, reduce toxicity, and control the active absorption and biological response of compounds such as flavonoids.

Many sources define nanomaterials as particles of size ranging between 1 nm and 100 nm, but its definition is more complicated since the benefits are due to the range of properties and interactions that are unique to the nanoscale structure. Thus, particles >100 nm can exhibit these unique properties and can be considered nanomaterials. For example, polymer nanoparticles between 10 nm and 1,000 nm in diameter can have the characteristics desired for a successful delivery system (Bonifácio et al., 2014).

The most common types of nanoparticles used for drug delivery are polymeric nanoparticles, solid lipid nanoparticles (SLNs), crystal nanoparticles, liposomes, micelles, and dendrimers, each of which has its own advantages and disadvantages (Fig. 16).

Polymeric nanoparticles have been the most tested in combination with natural products; the carrier materials are natural polymers or synthetic biodegradable high molecular polymers. The latters usually include Poly(lactic-co-glycolic acid) (PLGA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), poly-l-lactic acid (PLA). The natural ones are usually divided into two classes: polysaccharides and proteins. Polysaccharides include compounds from plant origin, such as pectin, cellulose, starch and its derivatives, and from microbial and animal origin, such as and chitosan. Proteins are albumin, gelatine, soy proteins, casein.

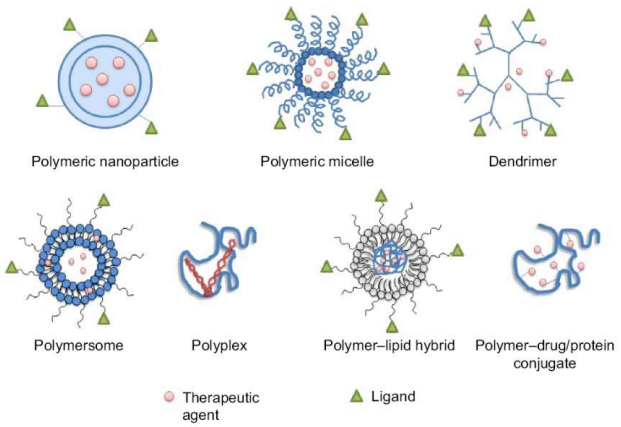



**Figure 16**. Schematic illustration of polymeric nanoparticles (Prabhu et al., 2015).

The principal advantage of polymeric nanocarriers is their robust structural characteristics imparting very high stability in the gastrointestinal tract. Furthermore, the hydrophobicity and hydrophilicity within the polymeric system can be manipulated to accommodate wide variety of drug molecules (Plapied et al., 2011). The polymeric nanoparticles tend to show very high degree of sustained release of drug molecules, which could be of special significance for oral delivery to guarantee that no drug is released from the formulation till it reaches systemic circulation thereby bypassing various physiological barriers to oral delivery of difficult-to deliver drugs.

Nanoparticles made of polysaccharides, due to their unique properties are promising carriers to deliver and protect the physiological properties of flavonoids and have been successfully applied as drug-delivery systems (Liu et al., 2008).

The type of nanoparticles used in a given delivery application can be selected based on the physicochemical properties of the drug of interest. As natural biomaterials, polysaccharides are stable, safe, non-toxic, hydrophilic and biodegradable. In addition, polysaccharides have abundant resources in nature and low cost in their processing.

The nano-encapsulation process refers to several methods based on chemical, physical, and physiochemical principles. Chemical nanoencapsulation (e.g. interfacial and in situ polymerization methods) requires the polymerization of monomers at the interface of two immiscible substances through the addition of a cross-linker in the external phase.

Physical processes (e.g. air-suspension method, pan coating, spray drying, spray congealing etc.) involve the interaction of the vector material with the molecules to be encapsulated when both are aerosolized or atomized (Wais et al., 2016).

Finally, physicochemical processes (e.g. coacervation, phase separation, complex emulsion, meltable dispersion and nanoprecipitation) form stable nanometer size drug nano-suspensions or nanoparticles through particle size reduction approaches.

The different process opportunity, combined with the ability to load high quantities of compounds, maintain them stable and delivering them directly to the target site, make physicochemical methods interesting approaches to overcome the challenge of poor absorption, especially for oral delivery, and enhancing the chemopreventive effects of a broad variety of molecules.

One of the major problems with nanoparticle delivery systems is their potential toxicity. This is a major concern with nanoparticles, partly because they can cross biological membranes, such as cellular membranes and, in some special cases, the Blod Brain Barrier (Loureiro et al., 2014). Cells with phagocytosis ability can take up nanoparticles between 100 nm and 1,000 nm whereas

nanoparticles <100 nm, can be taken up via endocytosis and could potentially cause systemic toxicity. The harm caused by their biodistribution is not controlled, due to the possible content of toxic polymers or drugs or in case of formation of toxic metabolites. Nanoparticles with sizes >20 nm are then degraded in lysosomes and no therapeutic effects are achieved. Furthermore, another issue that arises using nanoparticles as drug delivery systems is that they may undergo changes in the body. Indeed, it is known that the surface of nanoparticles changes as the particles move through different membranes, tissues, and organs in the body (Watkins et al., 2015).

Another area of concern is with the makeup of the nanoparticles. It is important that after the drug has been delivered, the nanoparticle either is eliminated afterward or is biodegradable, especially in the treatment of chronic human diseases. Accumulation of non-degradable nanoparticles in the body over time could lead to unwanted toxicity and cell death (Kamaly el al., 2012).

### 1.3.1. Nano-formulations for delivery of phenolic phytochemicals

New concepts from the field of nanotechnology have been utilized to overcome the challenge of poor absorption especially for oral delivery and enhancing the chemopreventive effects of a broad variety of polyphenols.

Different types of nano-formulations have been designed for the improvement of bioavailability of agents such as curcumin, EGCG and resveratrol, such as polymeric micelles, liposome/phospholipid, nano-/micro-emulsions, nanogels, solid lipid nanoparticles and polymer conjugates (Caddeo et al., 2008; Yallapu et al., 2012; Wang et al., 2012; Khan et al., 2014).

Nanoparticles can interact with phenolic phytochemicals by hydrogen bonds and hydrophobic interactions, which can enhance aqueous solubility and stability. Sufficient surface charges and suitable hydration property keep phenolic phytochemical encapsulated in nanoparticles stable in aqueous system, and due to their interparticle repulsions and hydration they remain stable in dispersion system. Different types nanoparticles were reported to encapsulate curcumin to enhance its aqueous solubility (Anand et al., 2010; Duan et al., 2010; Luz et al., 2012; Mukerjee & Vishwanatha, 2009). The hydrogen bonds and hydrophobic interactions occurring between nanoparticles and polyphenols may also be capable of preventing oxidation/degradation of phenolics, that during the transition in the gastrointestinal tract are rapidly degraded in both low acidic and neutral conditions (Onoue et al., 2011).

More importantly, nanoparticles can be taken directly up by epithelial cells in small intestine, which significantly increases absorption and bioavailability of phenolic phytochemicals (Zheng et al., 2015). Indeed, nanoparticles are capable to penetrate the mucus and be uptaken from small intestinal epithelial cells (Hariharan et al., 2006; Roger et al., 2010).

In particular mucus (a layer that is considered a physical barrier to protect and lubricate the epithelial surfaces) that is negatively charged, easy interacts with positive charged nanoparticles by electrostatic interactions (Ensign, Cone, & Hanes, 2012).

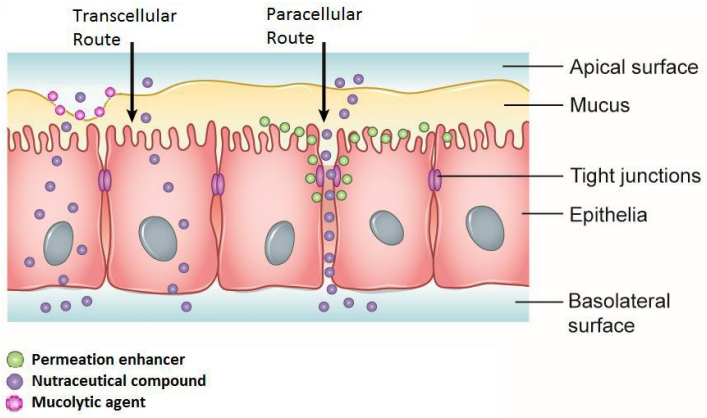



**Figure 17**. Cellular uptake of nanoparticles by epithelial cells.

Once reached the epithelial cells nanoparticles have been reported to be penetrated across small intestinal epithelium by either paracellular or transcellular pathway (Fig. 17); paracellular transport refers to a passive diffusion through intercellular spaces among the epithelium whereas transcellular pathways is associated with energy input (Sonaje et al., 2012). These transport ways enhance absorption of phenolic phytochemicals encapsulated in the gastrointestinal tract.

Cellular uptake process of nanoparticles is conducted by macropinocytosis or clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin/caveolae independent endocytosis (Wang et al., 2011).

After being internalized in epithelial cells, nanoparticles may be translocated to endo/lysosome and further degraded in lysosome to release bioactive compounds. These bioactive compounds may continue to undergo the metabolism process in epithelial cells (Ma et al., 2011). Nanoparticles may potentially remain intact in endolysosome and enter blood circulation by exocytosis. Additionally, nanoparticles may escape the accumulation in endo/lysosome and can be translocated to the endoplasmic reticulum and Golgi complex. These nanoparticles may further experience the exocytosis across the membrane (Qian et al., 2009).

# 2. Aim of the thesis

Nowadays, cancer remains one of the major public health problems. Although chemotherapeutic drugs efficiently kill cancer cells, these cells can defend themselves from such toxic compounds with a process called cancer multidrug resistance (MDR). Because of unsatisfactory treatment scenario there has been growing interest in the health advantages of using plant-derived compounds for cancer prevention or in the treatment of chemo-resistant cells.

Anthocyanidins are a group of pigments belonging to the family of flavonoids present in red-blue fruits and vegetables. Several studies demonstrated that, together with their glycosylated forms, they exert intense biological activity towards normal and cancer cells, including selective cytotoxicity, capability to interact with extrusion pumps, cell cycle perturbation, anti-proliferation and apoptosis.

Unfortunately, two major concerns have to be pointed out: (i) the concentrations often used to prove the biological effects of such compounds are far from those obtained when the assumption passes only through the ingestion of food rich of phytochemicals; (ii) knowledge on the effects due to a chronic intake of these molecules are missing. Both of these points are related to the low stability and bioavailability of anthocyanins. Moreover, when ingested, the latter is drastically reduced by their poor chemical stability in the weak alkaline conditions of the small intestine, combined with phase II metabolism. Indeed, they usually undergo sulphation, methylation and glucuronidation in the small intestine and liver and conjugated metabolites can be found in plasma after flavonoid ingestion. In general, metabolites of flavonoids showed controvertial bioactivity in comparison to parent compounds, thus challenging the possibility to translate their proven biological effects into therapeutic applications.

Therefore, enhancement of bioavailability would be of utmost importance in order to exert health effects. Nanotechnologies are being developed to afford a solution to the problem. Among available carrier, the application of positively charged polymers is widely studied because of their superior uptake levels as a result of electrostatic attraction with cellular membrane.

In this study, we aimed to:

a) Clarify whether the use of dietary anthocyanins could affect the response to chemotherapy exerted by resistant cancer cells. Long-term treatment with non-toxic

delphinidin (DEL) concentration will be performed on LoVo/Dx cells (metastatic human colorectal adenocarcinoma cell line, doxorubicin resistant). Interferences with cell cycle, expression of specific membrane transporters responsible for drug resistance, accumulation of the drug in the cells and the cellular ATP levels will be evaluated.

b) Improve DEL stability and bioavailability with a nano-delivery system. Chitosan, a natural polysaccharide, has drawn much interest since is biodegradable, biocompatibile, non-immunogenic and is cheap. Different chitosan/tripoliphosphate (TPP) nanoparticle formulations will be prepared by ionotropic gelation method and subsequently characterized. Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS) will be used to measure size, dispersity and morphology. The DEL - chitosan nanoparticles interaction will be investigated through UV Resonant Raman Spectroscopy. Encapsulation efficiency and DEL stability were determined by HPLC

c) Demonstrate that nano-encapsulation of delphindin (DNPs) with respect to the free form, increases the biological activity of the natural compound.

   Three colon cancer cell lines (Caco-2, LoVo and Lovo/Dx) will be treated with free and encapsulated delphindin. Uptake of nanoparticles, cytotoxicity, antioxidant/prooxidant activity will be evaluated. Since, DEL was demonstrated to act selectively on normal and cancer cells, the comparison of free DEL with DNPs with respect of cell cycle, apoptosis and uptake of chemotherapeutic drug will be performed.

# 3. Experimental section

## 3.1. CHAPTER I

### *Long-term treatment with delphinidin on doxorubicin resistant colon cancer cells*

#### 3.1.1. Aim of the work

Drug resistance represents one of the major challenges to effective chemotherapeutic interventions against cancer. Potential bioactive effects exerted by natural compounds led us to investigate whether the use of dietary anthocyanins could affect the response to chemotherapy exerted by resistant cancer cells. Previous studies showed that DEL, one of the most important compound of the anthocyanidin family, had the most efficient activity in terms of antioxidant capacity, known as one of the most relevant function of this compound class. Moreover, it has been proved to have a superior effect compared to others of the same class acting selectively on normal and cancer cells. We decided to mimic the every-day intake by treating doxorubicin-resistant metastatic colon cancer cells (LoVo/Dx) with low non-cytotoxic concentrations of DEL for five weeks.

#### 3.1.2. Materials and methods

##### 3.1.2.1. Chemicals

Materials used are listed in Appendix

##### 3.1.2.2. Cell lines

Metastatic human colorectal adenocarcinoma cell line (LoVo) and their doxorubicin resistant subclone (LoVo/Dx) maintained in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, L-glutamine and doxorubicin (200 ng/mL). 7 days before each experiment

doxorubicin was removed from the medium.

#### 3.1.2.3. Cytotoxicity Assay

IC50 values of doxorubicin and DEL were determined by using MTS assay (CellTiter 96 Aqueous One Solution Assay, Promega, USA) based on mitochondrial reduction of MTS tetrazolium compound by viable cells to generate a colored formazan product that is soluble in cell culture media. The amount of formazan product was quantified using absorbance at 490 nm.
LoVo/Dx cells were seeded at $1x10^4$ in a 96-well plate in complete culture medium. After 24 hours, the medium was removed from the wells by aspiration and the cells were treated with designed concentrations of doxorubicin 1 nM - 1 mM and/or DEL 1-100 µM, for 48 hours at 37°C in a humidified chamber. The total medium volume in each well was 200 µL. Each concentration of each substance was repeated in 8 wells. MTS reagent (20 µL, 10 mg/mL in PBS) was added to each well and incubated for 1 additional hour. Absorbance was recorded on a microplate reader at 490 nm. Data were analyzed using GraphPad Prism 7. IC50 were calculated using a 4-parameter dose/response curve.

#### 3.1.2.4. Long-term treatment (5 weeks with DEL) of LoVo/Dx cells

LoVo/Dx cells were seeded at $3x10^5$ in 25 $cm^2$ flasks treated with designed nontoxic concentration of DEL (2.5, 5 and 12 µM). Corresponding flask with untreated cells served as a control. All the concentrations were repeated in triplicate. Total volume of medium in each flask was 5 mL.
The medium was changed twice a week (on the 3rd and on the 5th day after every seeding); in control cells the medium was changed and replaced with fresh medium without DEL.
Once a week each different flask was trypsinized, counted and divided in 3 parts: one part was seeded again ($3x10^5$ in a new flask) and treated in the same way as described above; one part was seeded for further experiments, whereas the remaining amount for each flask was divided in two parts and collected in a sterile conical tube by centrifugation. The supernatants were discarded and the remaining pellet were stored at -80°C and used to extract proteins and RNA.

#### 3.1.2.5. Doxorubicin accumulation in LoVo/Dx after long-term treatment

Every week, the cells were trypsinized and plated at $1x10^4$/well in 96-well plates and treated

with or without DEL (2.5, 5 and 12µM) in completed DMEM. After 24 hours, the medium was removed from the wells by aspiration, the cells were washed with PBS after which were treated with HBSS alone (in the case of control wells) or with 25 µM doxorubicin dissolved in the same medium. Each treatment was performed for 60 minutes and repeated in 8 wells. The uptake was then stopped by aspiration of the solutions.

All wells were washed with ice-cold PBS after which, 100 µL of 0.3 N HCl/50% ethanol was added in each well and incubated for 60 minutes at +4°C in order to lysate cells and extract all the intracellular doxorubicin amount. Doxorubicin content in the resulting cell lysate was measured at wavelengths $\lambda_{excitation}$= 485 nm and $\lambda_{emission}$= 535 nm. To obtain the doxorubicin standard curve, aliquots of freshly prepared doxorubicin dissolved in 0.3 N HCl/50% ethanol were used, to give final concentrations ranging from 0.02 µM to 10.0 µM). Values were expressed as increased folds with respect to the control.

#### 3.1.2.6. Cell cycle analysis

Every week LoVo/Dx cells were plated at $5x10^5$/well in 6-well plates and treated without or with DEL (2.5, 5 and 12 µM) in the complete medium. After 24 hours, the medium was removed from the wells by aspiration and $0.5 \times 10^6$ cells were fixed in 70% ethanol, washed twice with PBS, and allowed to balance in PBS for 1 h. Cells were stained overnight with 0.5 mL of a PBS solution containing 10 µg PI, 0.25 ng FITC, and 4 µg RNase (all chemicals were purchased from Sigma-Aldrich, Italy). Cell cycle assessment was done using flow cytometry measurements carried out on a Cytomics FC500 (Beckman Coulter Inc., Fullerton, CA), equipped with an argon laser (488 nm, 5 mV) and standard configuration with photomultiplier tube (PMT) fluorescence detector for green (525 nm, FL1), orange (575 nm, FL2), or red (610 nm, FL3) filtered light. After acquisition, of at least 10,000 events per each run, data are stored as list mode files and analyzed with the FCS Express V3 software or, the FL3 saved histograms, and were submitted to the analysis of the cell cycle, performed by the MultiCycle software.

#### 3.1.2.7. Protein quantification and Western Blot Analysis

LoVo/Dx cells were lysed with lysis buffer (PBS 1x – SDS 1%), and protein concentration were determined by Bicinconinic Acid Assay (BCA) (Smith et al., 1985) using Bovine serum albumin (BSA) as standard.

An equal amount of protein (40 µg) for each sample were loaded on an 10% SDS-PAGE and

transferred to a PVDF membrane (Millipore, USA).

The membranes were incubated for 2 hours with blocking solution (4% milk, 0,2% BSA in PBS-0,05% Tween100). Primary antibodies were diluted in the same solution, 1:250 mouse Anti-P-gp (Cat. # sc-55510, Santa Cruz Biotechnologies) and β-actin (Cat. # A-5060, Sigma Aldrich) and incubated over-night at 4°C. The secondary antibodies were diluted in blocking solution and incubated one hour at room temperature. Peroxidase labeled α-mouse was used as a secondary antibody for P-gp (Cat. # 1858413, Pierce) and peroxidase labeled α-rabbit was used as a secondary antibody for β-actin (Cat. # 32460, Thermo Scientific, MA).

Between the two antibodies the membranes were washed three times for 5 minutes with PBS-tween 0.05%. Bands were visualized using SuperSignal WestDura (Thermo Scientific, MA).

#### 3.1.2.8. RNA Extraction

Total RNA was extracted from LoVo/Dx cells using the EuroGold Total RNA Kit (Euroclone, Italy) following the manufacturer's instruction from $3x10^6$ sedimented cells.

The total RNA isolated in this way was reverse transcribed into complementary DNA (cDNA) according to standard protocols.

#### 3.1.2.9. qRT_PCR

Following reverse transcription, cDNA was used for real-time PCR employing TaqMan® assay (Applied Biosystems, USA). The relative expression of the target gene (MDR1, P-gP) in treated vs. non-treated (control) cells was determined by taking the ratio of the β-actin-normalized P-gp expression in the abovementioned samples (the most stable housekeeping gene between GAPDH and β-actin was chosen after a one-week treatment with DEL). The $2^{-\Delta\Delta Ct}$ method was used to perform the relative gene expression analysis determining first the amplification efficiencies of the target and the reference genes. Once we established that the target and reference genes had similar and nearly 100% amplification efficiencies, we determined the relative difference in expression level of the target genes in treated vs. non-treated Lovo/Dx cells using the following formulas:

$$\Delta Ct = Ct_{GOI} - Ct_{HK}$$

(GOI = gene of interest, target gene – P-gp in our case; HK = housekeeping gene – β-actin in our case)

$$\Delta\Delta Ct = Ct_{treated} - Ct_{control}$$

Finally, the fold change in expression of target gene in treated vs. non-treated cells was calculated as:

$$2^{-\Delta\Delta Ct}$$

### 3.1.2.10. ATP determination in LoVo/Dx cells

The whole amount of ATP in cells was performed by using the ATP determination kit from Molecular Probes (Thermo Scientific, MA). After every week of treatment, cells were seeded at $5x10^5$/well in 6-well plates and treated without or with DEL (2.5, 5 and 12 μM) for 24 hours. After medium aspiration, cells were scraped on ice and centrifuged at 4,500 × g for 5 min at 4°C. ATP was extracted from cell pellets with 1% Trichloroacetic acid (TCA)/4 mM EDTA solution for 10 min on ice. Cell extracts were centrifuged at 12,000 × g for 10 min at 4°C and used for ATP determination as indicated by the manufacturer. Interference of TCA on ATP determination was avoided by dilution of sample at least 1:10. The amount of ATP was determined by comparison with ATP standard curve.

### 3.1.2.11. Statistical analysis

Data are presented as means ± s.d. of independent experiments. The statistical significance was analyzed using one way analysis of variance (ANOVA) or two-way ANOVA followed by Bonferroni post hoc test. All data analyses were performed using the Prism software 7 (GraphPad Software, USA). $p < 0.05$ were considered statistically significant.

## 3.1.3. Results and Discussion

### 3.1.3.1. Cytotoxicity of delphinidin and doxorubicin in LoVo/Dx cell line

Cytotoxic effect of DEL and doxorubicin was determined in Lovo/Dx cells after a 48h exposure. IC50 value of DEL was 80 μM, therefore in this study, concentrations far from the cytotoxic ones were used mimicking the diet conditions.

IC50 for doxorubicin was 53 μM underlining the high level of resistance. Toxicity of same compound in non-doxorubicin resistant cell line, LoVo cells, was also evaluated. We measured an IC50 of 4.5 μM proving the superior resistance of their subclone.

These data are coherent with those found in literature where the reported IC50 is 10-70 times higher in LoVo/Dx cells in comparison to LoVo. Values with a broad range of variability are reported depending on culture conditions, days of exposure to the chemotherapeutic drug and most important the sub-clone used (Scala et al 1991; Colombo et al 2011; Broggini; 1988; Capolongo et al., 1990; Conforti et al., 1995).

In order to reduce the variability due to this behavior all the experiments have been performed with the same sub-clone.

### 3.1.3.2. Delphinidin effects on doxorubicin accumulation in LoVo/Dx cells after long-term treatment

Intracellular drug accumulation is a complex process including drug uptake, retention and distribution, as well as the efflux from the cell. At any given time, the net accumulation of a drug in cells is the difference between the amount of drug up taken and pumped out.

The ABC transporters (P-pg, BCRP, MRP2 etc.) are drug efflux mediators acting by decreasing the intracellular net accumulation and leading cell to the acquisition of drug resistance. Many inhibitors produced against their transport activity have been developed. Their use will result in an increased net accumulation of drugs and greater clinical efficacy of chemotherapeutic agents in tumors overexpressing P-gp. On the other hand, their low in vivo activity and high required doses led to unacceptable toxicity (Shen et al., 2008). We measured the doxorubicin accumulation in these cells, knowing that doxorubicin is not the “gold standard” for colon, but using it as a model drug to assess possible interferences with the drug resistance process.

In order to test if DEL could increase the accumulation of anti-neoplastic drugs through the interaction and/or inhibition of ABC transporters, the intracellular doxorubicin content was evaluated comparing Lovo/Dx cells treated with different DEL concentrations with untreated

cells.

As previously described, the treatment has been performed for 5 weeks and doxorubicin accumulation have been measured at the beginning and at the end of the long-term treatment. We choose to measure the drug accumulation even in the intermediate point (3rd week) in order to understand if there were changes in the behavior in-between the treatment.

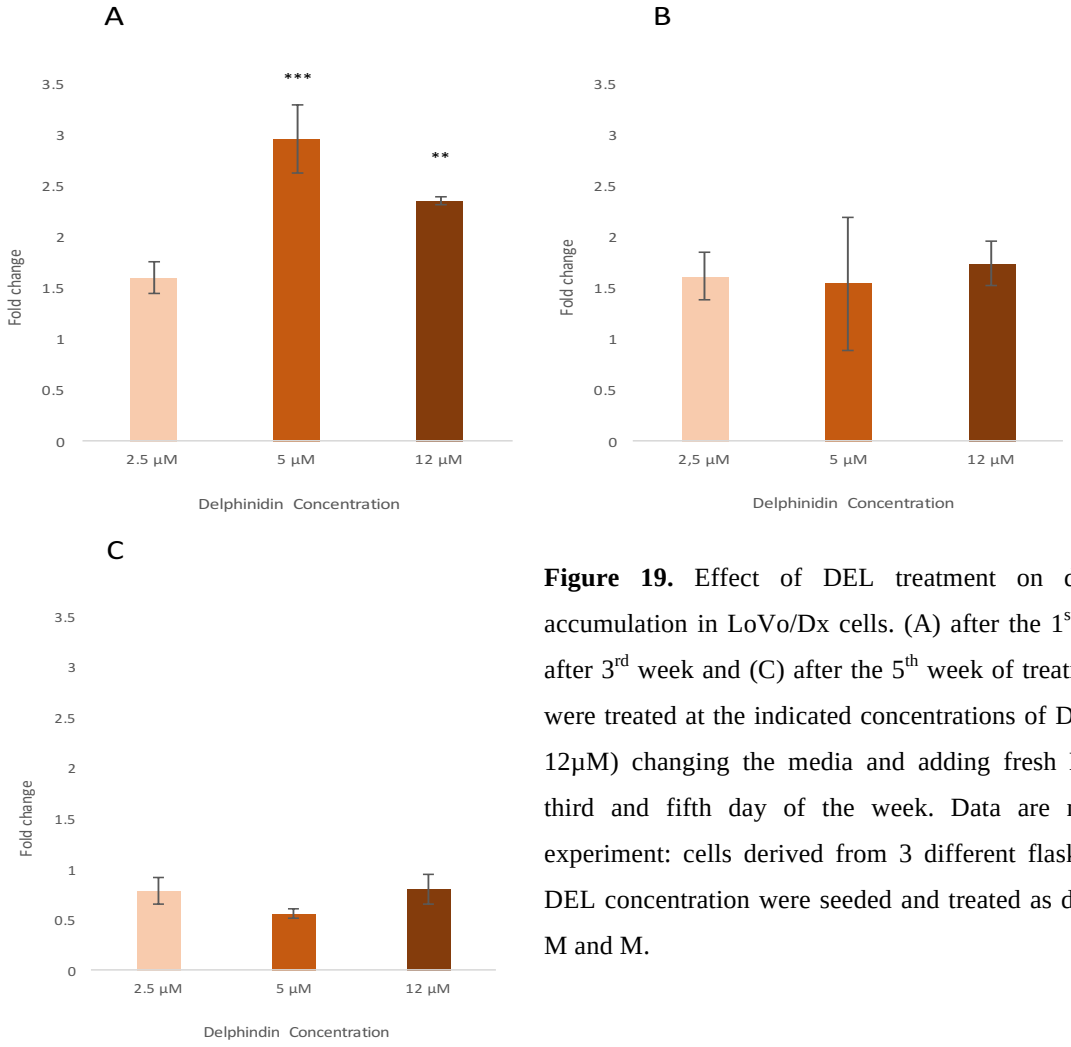



**Figure 19.** Effect of DEL treatment on doxorubicin accumulation in LoVo/Dx cells. (A) after the 1st week, (B) after 3rd week and (C) after the 5th week of treatment. Cells were treated at the indicated concentrations of DEL (2.5, 5, 12µM) changing the media and adding fresh DEL every third and fifth day of the week. Data are mean of 1 experiment: cells derived from 3 different flasks for each DEL concentration were seeded and treated as described in M and M.

As shown in Figure 19A, after the first week of treatment we saw a significant increase of doxorubicin content, with respect to the control but not in a concentration dependent manner. Moving further with the treatment and measuring the doxorubicin accumulation at the intermediate point (3rd week), the increased intracellular concentration of doxorubicin was significantly decreased in comparison to the effects previously obtained (Fig. 19B). This is even more evident observing the effects at the end of the long-term treatment, where there seems to be a complete reduction of those DEL effects (Fig. 19C).

Although the structure-activity relationships of flavonoids on P-gp functions have been

studied, they are still not clear, especially concerning their effects in whole cells (Shuji, 2006).

In literature are reported various and contradictory evidences for the effect of flavonoids family on multidrug resistance depending on several factors, such us concentration used, incubation time and cell lines. Anthocyanidins have been reported to alter pharmacokinetics of drugs that are BCRP substrates (Dreiseitel et al., 2009), and to inhibit P-gp activity in a dose dependent manner (Vrzal, 2016).

Several mechanisms may be involved in the interference of flavonoids with ABC transporters, such as the reduction of the expression of membrane transport protein (Miao et al., 2016; Satonaka et al., 2017) or the direct interaction with the nucleotide-binding domain consequently blocking the pump (Conseil et al., 2988; Ma, 2016).

#### 3.1.3.3. P-gp expression measured by Real-Time PCR and Western Blot Analysis

The results obtained from the measurement of the doxorubicin intracellular accumulation proved that non-cytotoxic doses of DEL were capable of increasing the intracellular content of doxorubicin in a LoVo/Dx cells. Since the effects were lost at the end of the treatment, expression of P-gp both at the transcriptional and protein level were considered, in order to make clear the possible role of protein in adaptive response.

To assess if there was an alteration of the P-gp expression at a transcription level, total RNA from long-term treated cells were extracted and used for Quantitative Real-Time PCR (qPCR).

The qPCR results showed that the level of transcription was not modified in none of the treated samples at none of the different time points measured (data not shown).

Possible modifications at the translational level of the protein was performed by Western Blot analysis. In Figure 20, we reported the fold increase of P-gp protein in treated cells with respect to untreated control. Western blot analysis did not show any changes in the protein level either.

Our data suggest that increased intracellular accumulation of doxorubicin may not be caused by a change in the membrane transport protein expression, but may due to other types of interference of DEL with the cancer physiology. Our evidences do not correlate with results obtained by other groups.

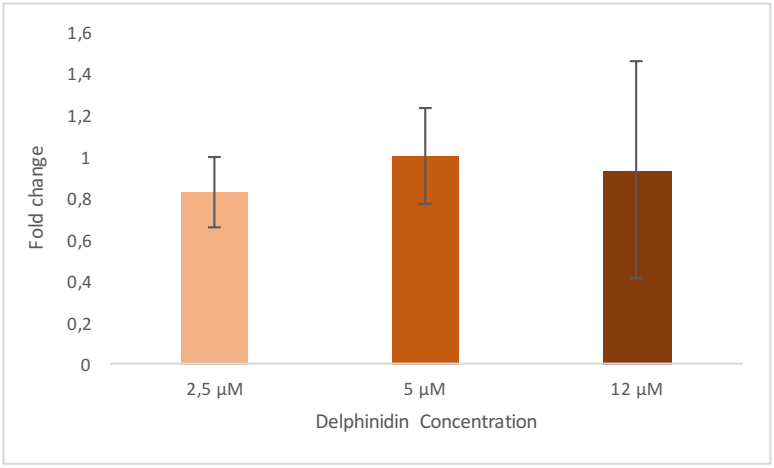



**Figure 20**. P-gp expression after 1st week treatment measured by Western Blot analysis. Cells were treated at the indicated concentrations of DEL (2.5, 5, 12µM) changing the media and adding fresh DEL every third and fifth day of the week. The protein expression has been normalized using β-actin. Graph report the fold change of P-gp expression compared to the untreated sample. No significant differences have been measured in none of the the tested samples. Data are mean of 3 different experiments: cells derived from 3 different flasks for each DEL concentration were seeded and treated as described in M and M.

Previous studies concerning the effect of several flavonoids on P-gp expression after long-term treatment in Caco-2 cells proved an adaptation response, but unlike our study the response was mediated by an increase on the P-gp levels (Lohner et al., 2007). Furthermore, other drug transporter protein could be involved.

#### 3.1.3.4. Effects on cell cycle and ATP determination

There are several evidences that anthocyanidins can affect cell cycle, in fact they have been shown to interrupt the cell cycle at G1 or G2/M phase and to have a pro-apoptotic effect in different tumor cell lines (Lazzè et al., 2004; Hsu et al., 2012; Bin Hafeez et al., 2014; Yun et al., 2009). Assessing cell cycle distribution and cell proliferation is important for studying cell growth differentiation, senescence and apoptosis. The effect of DEL long-term treatment on cell proliferation was evaluated. LoVo/Dx cells have been treated with different DEL concentration (2.5-12 µM) up to 5 weeks and at the end of the 1st, the 3rd and the 5th week, finally cells has been stained with PI in order to measure the DNA content of the cells by flow cytometry.

The data presented in Table 3 reported the results for each time point. Notably we detected a low but significant reduction in cellular proliferation (decreased %G2M value) after the 1st and the 3rd week of treatment at any concentration. The ratio of the percentage of cell cycle at G1 phase to that in G2/M phase gives information about the inhibitory effect of the test compound in G1

phase. After the first week, the ratios were 2.77, 3.5, 3.4 and 4.41 respectively from untreated to 12 μM DEL, while in the third week the values were 3.64, 7.9, 5.8 and 6.25 showing at both time point an inhibition of the G1 phase cycle progression. Indeed, it was demonstrated that DEL in prostate cancer cells was able to induce an increased expression of p21 protein, well known modulator of cell cycle arrest (Hafeez et al., 2008). Unfortunately, at the end of the treatment, we didn't measure any further alteration in cell cycle (G1/G2M mean for all conditions was 3.65 ± 0.11). Interestingly, no apoptotic peak (sub G1), for any treatment were evidenced. Similarly to the previous results, mechanisms occurring in the chronic treatment leads the cells to activate adaptive mechanisms annulling the effects detectable when cells are treated in acute conditions (Lazzè et al., 2004; Hsu et al., 2012; Bin Hafeez et al., 2014; Yun et al., 2009).

**Table 3**. Percentage of cells in the different phases of cell cycle. Cells were treated at the indicated concentrations of DEL (2.5, 5, 12μM) changing the media and adding fresh DEL every third and fifth day of the week. After the 1st week and the 3rd week of treatment there is a significant decrease of the proliferating cells, but this feature is not maintained in time and disappears after the 5th week. Data are mean of 1 experiment: cells derived from 3 different flasks for each DEL concentration were seeded and treated as described in M and M.

| | [ μM ]Delphinidin | % G1 | %S | %G2M |
|---|---|---|---|---|
| 1st week | Ctrl | 42.4 | 42.3 | 15.3 |
| | 2.5 μM | 43.3 | 44.4 | 12.3* |
| | 5 μM | 46.2 | 40.3 | 13.5 |
| | 12 μM | 45.5 | 44.2 | 10.3* |
| 3rd week | Ctrl | 54 | 31.1 | 14.8 |
| | 2.5 μM | 58.7 | 33.9 | 7.4* |
| | 5 μM | 58.3 | 31.7 | 10.0* |
| | 12 μM | 65.3 | 24.2 | 10.4* |
| 5th week | Ctrl | 57.1 | 27.3 | 15.5 |
| | 2.5 μM | 55.7 | 29.5 | 14.7 |
| | 5 μM | 54.8 | 30.2 | 15 |
| | 12 μM | 55.2 | 29.1 | 15.7 |

It has been proposed that flavonoids may induces cell cycle perturbation by provoking mitochondrial alterations characteristic of mitochondrial permeability transition (MPT) induction (Salvi et al., 2002).

This mitochondrial alteration may lead to ATP dysfunction in cells with a consequent effect on cell cycle perturbation. This may be the explanation of the increased doxorubicin uptake without a correspondent reduction of the P-gp expression (Hu et al., 2016).

Many polyphenols have been shown to bind and inhibit ATP synthase, suggesting that apoptotic process could be in part linked to the inhibition of ATP synthesis in tumor cells (Dadi et al., 2009).

In order to evaluate the effect of DEL on energy level, we measured the total intracellular ATP of the treated samples.

Interestingly no significant alterations in ATP levels were observed (data not shown), however it has to be taken into account that one of the most important property of invasive cancers is to alter glucose metabolism in order to increase ATP levels via glycolisis (Gatenby and Gillies, 2004).

#### 3.1.3.5. Effects of simultaneous administration of DEL and doxorubicin

As described in the Methods, the long-term protocol for cell processing was to change medium with fresh DEL on the third and the fifth day. In this way, all the experiments (cell cycle, uptake etc.) occurred when the cells were without fresh compound for at least 48 hours.

Recent studies have described how in the culture medium, the degradation of DEL under our experimental conditions, was completed already after one hour (Long et al., 2010). On the other hand, the absorption and metabolism of cyanidine-3-glucoside (C3G) in endothelial cells proved to be even faster: indeed, after one minute of treatment it was possible to measure a significant increase of C3G in the cells and its methylated derivative (3-glucoside pelargonide) in the medium (Ziberna et al., 2012).

Therefore, to see if DEL effect could be limited by its stability/cellular metabolism, new experiment, just one week long was performed again using a new cell aliquot. This time after the first week, doxorubicin accumulation was determined also by adding anthocyanidin immediately before uptake assay as described in materials and methods.

Figure 21 shows doxorubicin accumulation in LoVo/Dx cells when treated immediately before the doxorubicin administration. Data presented show that when cells are treated with DEL toghether with doxorubicin, we have a concentration dependent increase of the intracellular content of chemotherapeutic drug, twice higher compared to cells that only received the pretreatment for 1 week.

To assess whether the effect of DEL may be affected by the stability of the molecule, we have decided to repeat the treatment for only one week. This time, prior to the doxorubicin accumulation assay, the cells were divided into two aliquots, one of which receive the same concentration of fresh DEL (Fig. 21B) while the other not (Fig. 21A). The data clearly

demonstrate that concomitant treatment with DEL and doxorubicin results in an increased accumulation of drug in a concentration dependent manner.

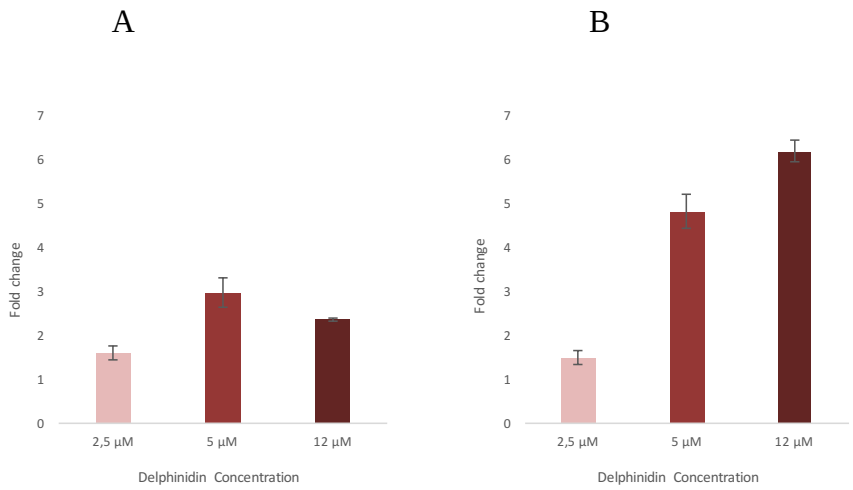



**Figure 21.** Doxorubicin accumulation in LoVo/Dx cells treated for 1 week with DEL (2,5-12 µM). 25 µM doxorubicin was subsequently added without (A) and together with fresh DEL (B). Data are mean of 1 experiment: cells derived from 3 different flasks for each DEL concentration were seeded and treated as described in M and M.

It is undeniable that DEL does affect the doxorubicin (well-known substrate for ABC transporters) accumulation in LoVo/Dx cells.

The intracellular concentration of the drug remains high after short-term treatment, without alterations of protein level, suggesting a possible direct interaction of DEL with membrane transport proteins (Ma et al., 2016; Conseil et al., 1998). Indeed, inhibition of ATPase activity by DEL was already described (Dreiseitel et al., 2009).

On the other hand, others efflux transporters involved in doxorudicin resistance, such as MRP1, MRP2 and BCRP, and expressed in LoVo/Dx cells, may be involved and modulated (Krawczenko et al., 2017).

We underlined that dietary-like intake condition is able to interfere with cell cycle and with chemotherapeutic accumulation, but is not able to maintain its effects in time. It has to be mention that a characteristic found in invasive cancers is their adaptive mechanisms to environmental conditions acquired during carcinogenesis (Gatenby et al., 2004).

Although flavonoids are considered to be health-promoting ingredients of the diet, they may also be seen as xenobiotics for which a variety of mechanisms exist to limit its uptake and to increase clearance and excretion from the body (Lohner et al., 2007; Redan et al., 2017). As previously mentioned, flavonoids were also shown to alter the activities and/or expression levels of phase-II enzymes (Shih et al., 2007) in the intestinal tract, with the consequent alteration of the plasma

levels of xenobiotics.

The evidences reported so far makes us suppose that in our experimental conditions, in which cells are constantly maintained in the presence of low concentrations of DEL, can stimulate a mechanism of adaptation by the cells themselves. As flavonoids coming from the diet, our findings address a very important area of drug–food interactions.

Further investigations are required to precisely identify the molecular mechanisms involved. Transport mechanism is a very challenging issue since membrane transporter show broad and overlapping substrate pattern. In particular it would be crucial to understand if DEL interacts directly with the ABC transporters such as P-gp in cells or in more complex models.

# 3.2. CHAPTER II

## *Preparation and characterization of DEL loaded chitosan nanoparticles*

### 3.2.1. Aim of the work

In order to protect anthocyanidin from natural degradation, we produced a non-toxic nanoparticle-based delivery system. The goal was to enhance DEL stability and bioavailability in order to increase its biological activity and to make it more efficient. In this chapter are presented the results regarding the preparation and characterization of DEL nanoparticles.
To encapsulate DEL, chitosan-TPP nanoparticles have been prepared using ionotropic gelation method and characterized in terms of loading efficiency, dimension, stability, antioxidant capacity and uptake from cells.

### 3.2.2. Materials and Methods

#### 3.2.2.1. Materials

Materials used are listed in Appendix

#### 3.2.2.2. Nanoparticles Preparation

The nanoparticles were prepared following the procedure described by Calvo et al. (1997). A 10 mg/mL chitosan solution was prepared by dissolving chitosan in a 0.05% (v/v) acetic acid solution and leaving it under stirring for 24 hours. The pH was adjusted to 4 with a 0.5 M HCl solution and diluted in deionized water to the final desired concentrations. TPP was dissolved in deionized water to a final concentration of 1 mg/mL and then diluted to the final desired concentrations. TPP and chitosan solutions were filtered through a 0.22 µm membrane (Millipore) prior to use.
Chitosan solution was mixed with DEL (dissolved in MeOH/0,8% Perchloric acid (v/v)) both at fixed concentration, then, TPP solution was added drop wise in order to prepare different chitosan : TPP ratios.

Preparations of DEL nanoparticles (DNPs) and empty nanoparticles (NPs) were made under 700 rpm magnetic stirring at room temperature. The resulting suspension was then left to gelify for 60 min. NPs were prepared with the same procedure adding the same volume of MeOH/Perchloric acid used to reach the desired concentration of DEL. The DEL concentration of different DNPs preparations was determined by spectrophotometric analysis (Varian Cary 4E UV/Vis spectrometer) and HPLC-DAD analysis.

#### 3.2.2.3. Preparation of fluorescent nanoparticles

The synthesis of FITC-labeled chitosan (FITC-Cs) was based on the reaction between the isothiocyanate group of FITC and the primary amino group of chitosan (Onishi et al., 1999; Roula and Mansoor 1999). 20 ml of 1 mg/ml FITC in dehydrated ethanol was added to 20 ml 1% (w/v) chitosan in deionized water. After 3 hours of reaction in the dark at room temperature, the FITC-Cs was precipitated by raising the pH with 0.2 M NaOH. The unreacted FITC was washed with deionized water and separated by centrifuge. The FITC-Cs dissolved in 20 ml deionized water was then transferred in Amicon Ultra-15 Centrifugal Filter Units (Cutoff 10.000 Da, Merck Millipore, USA) and centrifuged at 4000 g per 35 minutes. This procedure was repeated until no fluorescence was detected in the flow-through.
FITC-Cs nanoparticles were prepared by ionotropic gelation as previously described.

#### 3.2.2.4. Particles size and morphology

*Dinamic light scattering:* NP size and ζ potential of both NPs and DNPs was determined using Dynamic Light Scattering (DLS) on a Zetasizer Nano ZS (Malvern Instruments, UK), operating at scattering angle of 173°, in 10 mm path length cells, at 25°C.
The mean hydrodynamic diameter of the particles, dh, was computed from the intensity of the scattered light using the Malvern software package by multiple mode analysis, based on the theory of Brownian motion and the Stokes–Einstein equation:

$$D=kT3\pi\eta d_h$$

where D is the diffusion coefficient (the primary parameter obtained from DLS measurements), k the Boltzmann constant, T the temperature, and η the solvent viscosity.
All nanoparticles preparations were diluted with deionized water (1:10) to adequate scattering

intensity prior to the measurements, then placed in disposable polystyrene cuvettes and the scatter intensity was measured. The size is expressed as the Z-average hydrodynamic diameter obtained by a cumulative analysis of the correlation function using the viscosity and refractive index of deionized water in the calculations.

*Transmission Electron Microscopy*: 10 µl of diluted nanoparticles were deposited on a carbon coated copper grid, then 10 µl of uranyl acetate (1% in deionized water) were added as contrasting agent. After 10 minutes, the time needed for deposition of the uranyl acetate, the excess acetate was absorbed with filter paper and the sample was kept 20 minutes to dry completely. All the measurements were carried out using an EM 208-Philips TEM (PhilipsEindhoven, Netherland) equipped with a Quemesa camera (Olympus Soft Imaging Solution, Germany). The analysis was performed at Electronic Microscopy Center (Department of Life Science of University of Trieste).

#### 3.2.2.5. Loading efficiency of DEL in DNPs

Preliminary DEL encapsulation efficiency was measured by spectrophotometric analysis using the absorbance at 520 nm.
The accurate assessment of DEL concentration loaded within the nanoparticles have been performed by HPLC-DAD analysis by injecting the complete formulation and the sediment (10 minutes centrifugation at 12.000 *xg*, Biofuge A, Haereus, Germany) resuspended in MeOH/0.8% perchloric acid.

#### 3.2.2.6. Stability of free VS encapsulated DEL in aqueous solution

The evaluation of DEL concentration in an aqueous solution comparing the free and the encapsulated form was performed. DEL and DNPs at 50 µM final concentration were diluted in HBSS. At different incubation times, aliquots were collected and diluted 1:1 in MeOH/0.8% perchloric acid. Each time point was repeated in triplicate and DEL quantification was performed by HPLC analysis.

#### 3.2.2.7. Free radical scavenging activity by ABTS assay

Antioxidant capacity was determined using ABTS assay. The procedure followed the method of Re et al. (1999) with some modifications.

The pre-formed radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants. Antioxidants in the sample suppress the production of ABTS•+ in a concentration- dependent manner, and the color intensity decreases proportionally.

ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation (ABTS•) was produced by allowing ABTS stock solution to react with 2.45 mM potassium persulfate (final concentration) in the dark at room temperature for 12–16 h before use.

This solution was stable in this form for two days. The solution was then diluted 1:30 in deionized water to obtain an absorbance of 0.700 ± 0.021 units at 734 nm. Fresh ABTS solution was prepared for each assay.

The influences of both the pH of DEL solution and duration of reaction on the inhibition of the radical cation absorption was taken into account when determining the antioxidant activity. In each experiment, ascorbic acid was used as internal control.

DEL and DNPs were diluted to desired concentrations in order to obtain between 20%–80% inhibition of the blank absorbance. Reaction was allowed to proceed for 1 h at room temperature. The ABTS scavenging capacity of the extract was expressed as percentage of inhibition with respect to the blank and calculated as follow:

$$\% = [(\text{Abs blank} - \text{Abs sample}) / (\text{Abs blank})] \times 100$$

where $Abs_{sample}$ is the absorbance of ABTS radical solution mixed with DEL or DNPs at given concentrations and $Abs_{blank}$ is the absorbance of the appropriate blanks. The solvent blanks were MeOH/0.8% Perchloric acid for DEL and empty nanoparticles for DNPs. All determinations were performed in triplicate (n = 3).

#### 3.2.2.8. Cell lines

• Human colonic adenocarcinoma isolated from primary tumor (Caco-2), metastatic human colorectal adenocarcinoma cell line (LoVo) and their doxorubicin resistant subclone (LoVo/Dx) maintained in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, L-glutamine and doxorubicin (200ng/mL). 7 days before each experiment doxorubicin was removed from the medium.

#### 3.2.2.9. Free and encapsulated DEL uptake in cells

The uptake of DNPs and free DEL was performed using LoVo cells as a model, measuring the differences in DEL intracellular concentration at different time points according to the method Kern et al. (2007). LoVo cells were seeded in 24-well plates and treated with complete medium. After 24 hours, the medium was removed from the wells by aspiration, the cells were washed with PBS, and treated for different times with DEL and DNPs at 50 μM in HBSS. Each time point was repeated in triplicate. Culture wells without cells for each time points were used as control. After 30, 60, 90 and 120 minutes the supernatant was collected and diluted 1:1 in MeOH/0.8% Perchloric Acid. Cells were washed once and lysed adding directly MeOH/0.8% Perchloric Acid to extract the intracellular DEL and directly injected onto HPLC-DAD.

#### 3.2.2.10. HPLC analysis

An Agilent 1100 HPLC with DAD, coupled to an Agilent NDS ChemStation (Agilent Technologies, USA), was used for DEL quantification. A C18 column (250x4.6 mm, 5 μm; Purospher, Merck, Germany) with a C18 guard column (4x4 mm, 5 μm; Purospher) maintained at 35 °C was used. The mobile phase consisted of solvent A (0.8 % Perchloric Acid in deionized water) and solvent B (100 % methanol). The gradient conditions were linear starting at 35 % B, to 60 % B in 20 min, to 100 % B in 1 min, 100 % B for 3 min and to 35 % B in 1 min. The column was equilibrated for 5 min prior to each analysis. The flow rate was 0.55 ml/min and the injection volume was 20 μl. The Uv-vis spectra were recorded from 200 to 700 nm, with the detection at 520 nm. Calibration curve based on the concentration of DEL using the absorption at wavelength 520 nm. Six-point standard calibration curve was linear in the range of 10 to 200 μM with the coefficient of correlation 0.99 and in the range 0.1 to 10 μM with the coefficient of correlation 0.99. The limit of detection (signal to noise S/N = 3) and limit of quantification (S/N = 10) were 0.01 μM and 0.04 μM respectively.

#### 3.2.2.11. Nanoparticles internalization in different colon cancer cell lines

The nanoparticles uptake was performed on 3 different colon cancer cell lines; namely, LoVo, LoVo/Dx and Caco-2 cells were plated at 5 x $10^5$/well in 6-well plates and treated with the

complete medium. After 24 hours, the medium was removed from the wells by aspiration, the cells were washed with PBS, and treated for 30 minutes with DNPs-FITC (50μM final DEL concentration) or NPs-FITC in HBSS, each treatment was repeated in 3 wells.
After treatment cells were trypsinized and placed immediately into ice to block cell membranes. In order to measure only the intracellular emission any extracellular fluorescence was quenched by adding Trypan Blue (TB) at a final concentration of 0.05% to cell suspension, since this dye cannot permeate through the living cells with integer membranes. TB quenches the FITC fluorescence shifting the emission from green to red, allowing us to discriminate the fluorescence caused by the intracellular and extracellular particles. FITC_DNPs and NPs fluorescence, in the presence and absence of TB were used as control.
The nanoparticles uptake assessment was done with a FACScalibur instrument (Becton Dickinson, USA). After acquisition, of at least 10,000 events per each run, data are stored as list mode files and analyzed with the CellQuest software (Becton Dickinson).

#### 3.2.2.12. UV Resonance Raman Spectroscopy

In order to assess the potential interactions between DEL and chitosan-TPP, UV Resonant Raman Spectroscopy has been used. We exploited a technique that allows us to monitor small changes in molecule structure resulting from tiny ~0.001 nm bond length changes, such as IR or Raman vibrational spectroscopies, that detect vibrations of any molecules in gas, liquid and solid samples. Unfortunately, the use of IR absorption spectroscopy is challenging for molecules in aqueous solutions due to the overwhelming IR absorption of $H_2O$. One of the main advantages of the Raman spectroscopy in the investigation of aqueous solutions is that water is a poor "Raman scatterer"; as a consequence, this technique is suitable for the analysis of wet biomacromolecular compounds (e.g., polysaccharides) and hydrophilic molecules in general (Bruno, 2009).
The physical effect is based on the inelastic scattering of light (Stokes and Anti-Stokes scattering) due to the interaction between an electromagnetic wave and the sample. The energy difference between the incident photon and the scattered photon corresponds to the vibrational energy of the scattering molecules. The resulting Raman spectrum consists in the plot of the signal intensity of the scattered light versus the Raman shift. The Raman shift is defined as the difference between the frequency of the incident and scattered photons and is expressed as $cm^{-1}$ ($\lambda\nu$ = c, 1 eV = 8065.54 $cm^{-1}$).
This phenomenon is extremely weak (approximately one photon in $10^5$-$10^7$) with respect to the elastic process (Rayleigh scattering), but it is still detectable providing a vibrational spectrum

that can be theoretically simulated. To overcome some limits in low intensity peaks, the Resonance Raman mode can be used to enhance the signal intensity as long as the sample contains chromophore groups that can be properly excited. The condition is to use a laser excitation source with a frequency matching or approaching the electronic transition energy of the compound under examination (Butler et al., 2016). A better knowledge of the resonance Raman properties of anthocyanins and anthocyanidins should also allow a finer structural analysis of the in vitro spectroscopic data. Since anthocyanidins in their flavylium cationic form absorbs in the ultraviolet region of the light spectrum, the UV - Resonance Raman spectroscopy has been chosen.

*UV Resonance Raman scattering measurements and data analysis*

Ultraviolet (UV) resonance Raman scattering experiments were carried out at the BL10.2–IUVS beamline at the Elettra Synchrotron Laboratory in Trieste. (ref. UV resonant Raman scattering facility at Elettra)

The isotropic and anisotropic Raman spectra were acquired at fixed temperature of 310 K at wavelength $\lambda$ = 266 nm. This wavelength was chosen according to the UV absorption (Results - Fig. 22) characteristics of the integer form of the molecules as a flavylium cation (272 nm) as visible from Figure 12 in introduction.

The experimental resolution was set to 2 $cm^{-1}$. DEL and DNPs in aqueous solution were placed in a UV-grade quartz cell. Data acquisition was made with a proprietary software which included that of the CCD camera, selecting all protocol conditions (accumulation time, number of spectra, sample oscillation, etc).

The samples were measured at a final DEL concentration of 450 μM (pH = 5) for DNPs with an acquisition time of 5 minutes collecting spectra for an overall time of 240 minutes. The concentration of the free DEL solutions was 1 mM (pH=5). Solutions were freshly prepared, since the compound undergoes chemical modifications (bleaching) once mixed in aqueous solution. For each sample, the same number of spectra as for DNPs has been acquired.

Spectra processing was performed using the Igor software and the data analysis with the hyperSpec package for R software (for statistical computing and plotting) (R: a language and environment for statistical computing 2013; Claudia Beleites and Valter Sergo).

In particular, the processing workflow was built as follows:

1- removal of cosmic ray peaks

2- dark subtraction

3- fluorescence subtraction

4- solvent subtraction

5- quartz cell correction

For the baseline correction, a third-order polynomial baseline has been subtracted to each spectrum of the dataset using the function spc.fit.poly.below from the package hyperSpec.

The kinetic information has been obtained observing the evolution of the area versus time of concentration sensitive peaks (in particular, the peaks with maxima at: 1110 cm-1 have been analyzed) (Merlin et al., 1994; Merlin et al., 1993).

## 3.2.3. Results and discussion

### 3.2.3.1. DEL absorbtion properties

DEL like the other anthocyanidins is sensitive to environmental conditions, such as pH (Hurtado et al., 2009), temperature (Fischer et al., 2013), oxygen (Fleschhut et al., 2006) and light (Chiste et al., 2010).

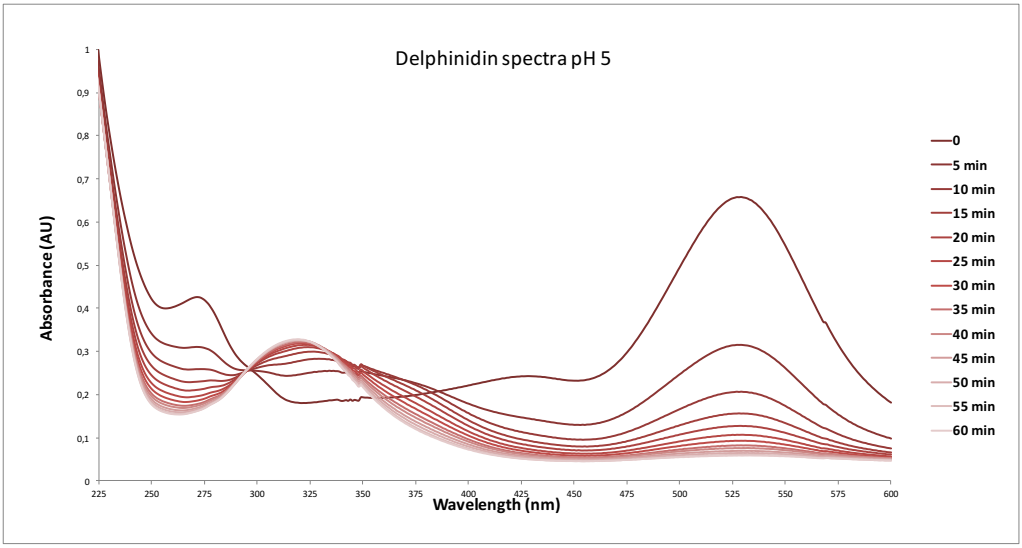



**Figure 22.** Delphindin spectra at a final concentration 25 µM, pH 5, at room temperature.

For this reason, the absorbtion spectrum of 25 µM DEL at the pH 5 (the same of nanoparticles preparation) was investigated, in order to better understand its behavior once mixed in an aqueous solution. This was an important starting point to follow DEL stability for further analysis.

It has been observed that, the molecule in its flavylium cation form has two main absorption peaks at 272 (characteristic of the aromatic rings) and 520 nm; once the molecule starts gradually to hydrate a decrease in concentration of the flavylium cation and a consequent decrease in color intensity is observed, the solution became clearer due to the production of a undesired colorless carbinol pseudo-base and chalcone, with a decrease of the peak at 520 nm, characteristic for the pink-red colored form. This process was completed after 60 minutes. Starting from the spectra acquired at min=0 (Fig. 22), an isosbestic point appears resulting from the equilibrium of the two different forms of the molecule in the aqueous solution, coherently with what found in literature (McClelland and Gedge1980; Vayupharp and Laksanalamai 2015).

In fact, the strong absorption band at 272 nm characteristic of the integer chroman C ring, shifts to 315 nm while the structure of the molecule starts to modify. This peak appearing is likely to correspond to a chalcone structure (Brouillard and Delaporte, 1977; Brouillardet al., 1978; Brouillard and El Hage Chahine, 1980).

### 3.2.3.2. Nanoparticles Preparation

DNPs were prepared using chitosan and TPP with the ionotropic gelation method taking advantage of the intermolecular linkages created between the negative groups of TPP and the amino groups of chitosan (positively charged) allowing the gelation process (Calvo et al., 1997). The concentration of the polysaccharide and the cross-linker were kept as low as possible to avoid the formation of aggregates. Different chitosan and TPP concentration (ratio 2.2:1 v/v) were used as follows:

A) Chitosan 0.15% and TPP 0.1% + DEL final concentration 250 µM
B) Chitosan 0.25% and TPP 0.2% + DEL final concentration 250 µM
C) Chitosan 0.25% and TPP 0.1% + DEL final concentration 250 µM
D) Chitosan 0.25% and TPP 0.2% + DEL final concentration 500 µM

Control nanoparticles have been prepared in the same way, but omitting DEL.

After 48 h from the DNPs preparation the absorption characteristics of the solutions was investigated in order to understand if the behavior was similar to free DEL. DNPs were diluted 10 times with deionized water. Absorbance of all the DNPs preparation was then measured at 520 nm. The UV-vis absorption analysis allowed also to understand which might have been the best preparation to encapsulate and stabilize DEL.

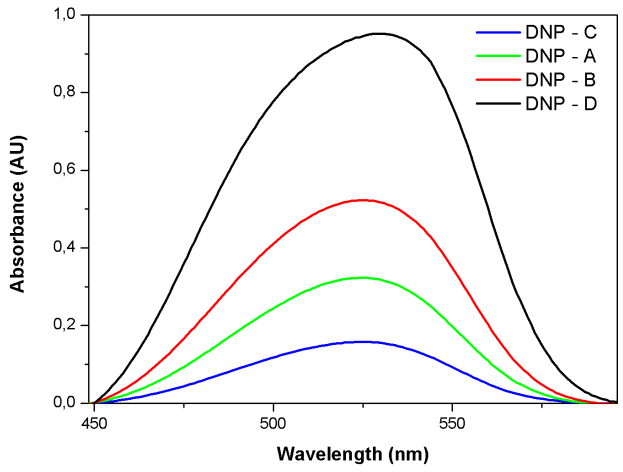



**Figure 23**. Absorbance peaks values at 520 nm of the different DNPs formulations read 48 h after their preparation (10 times diluted in pH 5 deionized water).

The first evidence of the stabilizing effect of the DNPs on anthocyanidin structure was the permanence in time of 520 nm absorbtion peak. It should be noticed, however, that the four formulations did not give the same degree of stabilization. In fact, as shown in Figure 23, the concentration of encapsulated DEL increases with the chitosan/TPP amount (A vs B) used for the nanoparticles preparation. In literature, similar data concerning the encapsulation of polyphenols are reported, suggesting that the amount of chitosan could strength the chitosan skeleton surrounding the polyphenolic compound (Ficai, 2017).

Furthermore, even the chitosan:TPP molar ratio appeared to be important for a good stabilization (C vs B). Indeed, starting from the same DEL concentration, the C formulation (more concentrated in chitosan and less in TPP) showed the lower absorption, suggesting a poorer stabilization efficiency of the anthocyanidins.

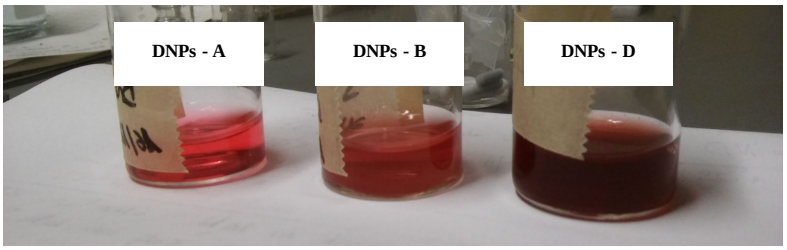



**Figure 24**. Nanoparticles formulations: color and turbidity increase with increase of chitosan (A vs B) and DEL concentration (B vs D).

Due to the low absorption and color stability, the formulation C was not used for further investigation.

### 3.2.3.3. Size distribution, morphology and ζ potential

Size distribution and ζ potential were measured with a Malvern Zeta Sizer in order to evaluate how the relative amount of the compounds could interfere with particles formation.

**Table 4**. Particles size, zeta potential, PDI and physical appearance of chitosan/TPP nanoparticles formulations.

| | Dimension (nm) | Zeta potential (mV) | PDI | Physical appearance and opacity |
|---|---|---|---|---|
| **DNP – A** CS 0.15%, TPP 0.1% Del 250 µM | 430 | + 35 | Broad | Transparent |
| **DNP - B** CS 0.25%, TPP 0.2% Del 250 µM | 270 | + 40 | Broad | Opalescent |
| **DNP - D** CS 0.25%, TPP 0.2% Del 500 µM | 150 | + 65 | 0.3 | Opalescent |
| **NPs** CS 0.25%, TPP 0.2% | 120 | + 5 | 0.2 | Transparent |

Collected data (Table 4) suggest that the amount of encapsulated DEL (according to the absorbance spectra of the different formulations) is crucial for the resulting charge. Indeed, empty nanoparticles are almost neutral (ζ = +5 mV), whereas the net charge of DNPs increases with the amount of encapsulated molecule, with a corresponding decrease in dimension. The positive charge of all the formulations is very important considering that cationic surface is desirable as it promotes interaction of the nanoparticles with the cells and hence increases the rate and extent of internalization (Shenoy et al., 2005).

As previously reported, DEL is present in aqueous solution in four different forms, among which the flavylium cation. We hypothesize the involvement of this positively charged form in the nanoparticles formation.

Regarding particles dimension, the amount of chitosan and TPP used seems to affect the size of the nanoparticles only partially. Even in this case, the amount of encapsulated DEL appears to be decisive. The size of the nanoparticles, in fact, varies in an inverse proportion manner to the optical density measured (Table 4, Fig.23).

Moreover, it has been described that aromatic ring structures could interact through π-π stacking between molecules (Castañeda-Ovando et al., 2009). This mechanism, depending on DEL concentration, could establish a more hydrophobic environment, the subsequent removal of water molecules from the nanoparticle and a resulting decrease in the size of the particle itself.

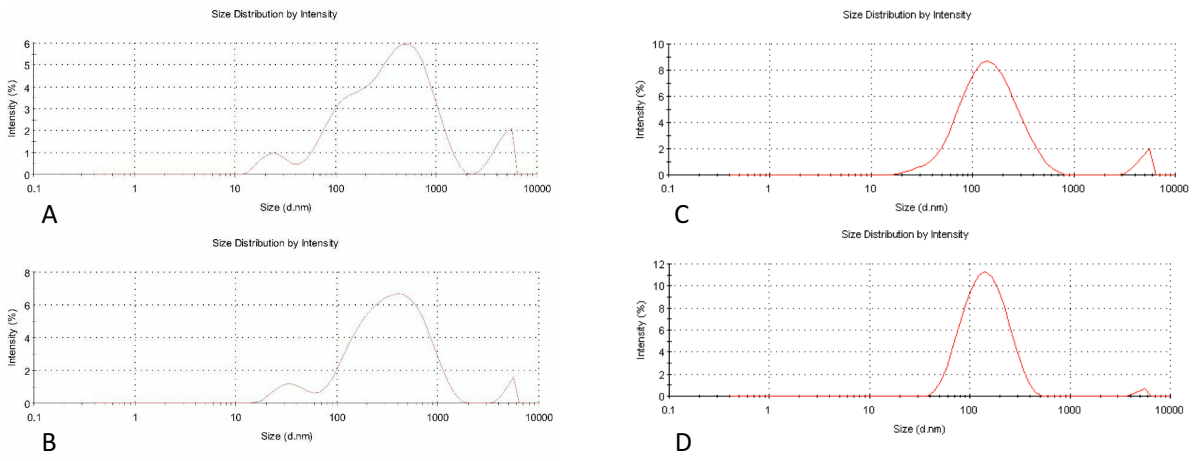



**Figure 25**. Size distribution intensity graph of the different nanoparticles formulations reported in Table 4. (A) formulation A; (B) formulation B; (C) formulation D; (D) empty nanoparticles.

Transmission electron microscopy (TEM) measurements have been provided to confirm the data obtained with Zeta Sizer regarding particles size, knowing that without additional control the use of only DLS may lead to artifacts.

In Figure 26, TEM images of the different preparations are presented. Electron micrographs show that only DNPs B and D (Fig. 26B and 26C) have a spherical morphology and a more homogeneous distribution. DNPs A (Fig. 26A) have bigger dimensions and a complete different shape appearing more polydisperse (confirming the data obtained by DLS). The structure looks spongier and seems less reticulated compared to the other preparations.

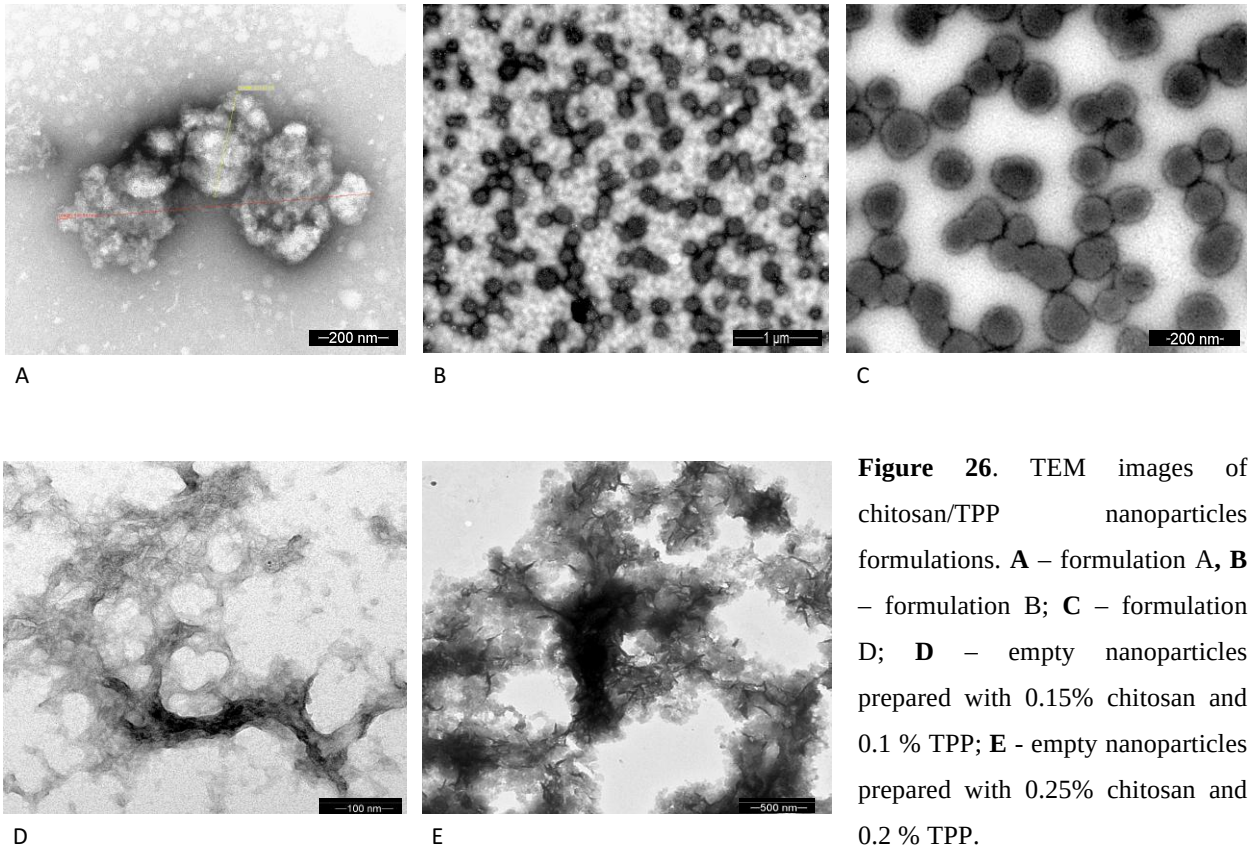



**Figure 26**. TEM images of chitosan/TPP nanoparticles formulations. **A** – formulation A**, B** – formulation B; **C** – formulation D; **D** – empty nanoparticles prepared with 0.15% chitosan and 0.1 % TPP; **E** - empty nanoparticles prepared with 0.25% chitosan and 0.2 % TPP.

Unexpectedly, NPs showed different morphology. Our data suggest low nanoparticles formation in the absence of anthocyanidin, further supporting the involvement of DEL in nanoparticles development.

The formulation D has to be considered optimum in terms of particles dimension and charge taking into account that our purpose is to test them on colon cancer cells. Indeed, data from literature reported high uptake rates with spherical nanoparticles between 20 and 50 nm (Gao et al., 2005; Lu et al., 2009), but it has also been described that enterocytes are an exception since they preferentially ingest particles in the range between 100 and 200 nm (Win and Feng, 2005).

### 3.2.3.4. Encapsulation efficiency

As previously reported, anthocyanins undergo molecular rearrangements in response to the pH of the chemical environment (Mazza, and Miniati, 1993). Hydration of the flavylium cation yields a colourless carbinol pseudo-base. This species undergoes tautomerization, through opening of the C ring, to generate a yellow chalcone. Importantly, the ability to detect anthocyanins by HPLC is based on the ability to transform colorless forms back into the flavylium cation (McGhie et al., 2003).

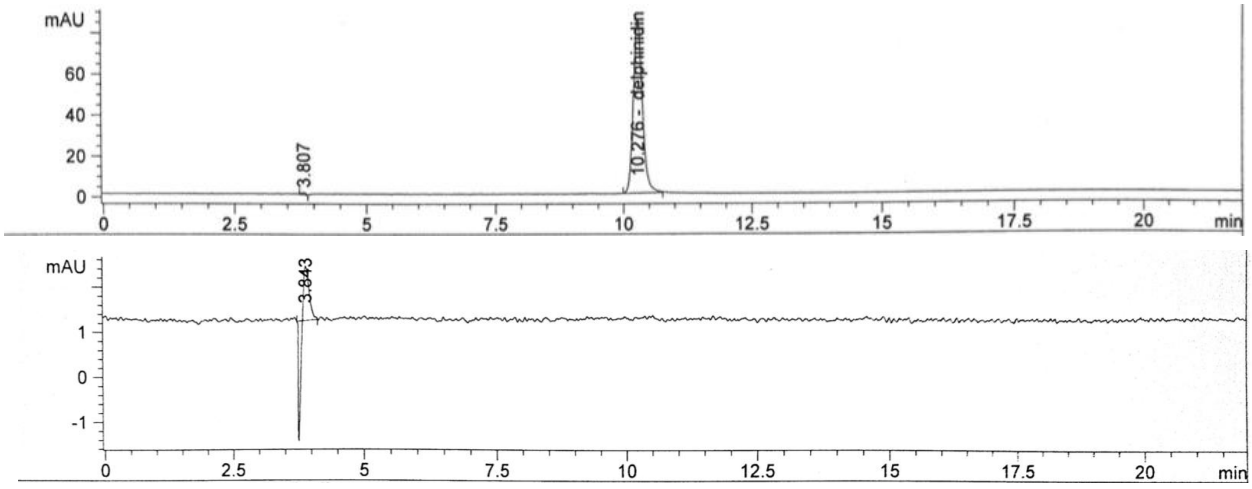



**Figure 27**. HPLC chromatograms of fresh (A) and 1 week old (B) DEL standard solution (10 μM) diluted in MeOH/0.8% Perchloric Acid and measured at 520 nm.

In Figure 27 chromatograms of fresh (A) and one month old (B) free DEL are presented.
The absence of characteristic peak in the chromatogram of old solution, detected at 520 nm, is shown. In our experimental condition, DEL, once in the chalcone form, when diluted in acid solution, it not able to form back the flavylium cation and it is therefore not detectable by HPLC. Moreover, both glycosides and aglycons can spontaneously degrade to a phenolic acid and phloroglucinol aldehyde.

The encapsulation efficiency of DNPs was evaluated using HPLC analysis and results are reported in Table 5. Nanoparticles solution and their sediment fraction diluted 1:1 in MeOH/0.8% Perchloric Acid were directly injected into the HPLC column.
These data confirmed that anthocyanidin content in DNPs increases with the increase of chitosan amount (A vs B and D). Interestingly, by comparing formulation B and D, the encapsulation efficiency increased with DEL concentration, suggesting again a direct involvement of the natural compound in particles formation that results in the enhancement of DEL stability.

On the other hand, considering the formulations A and D, where the relative ratio of all its components is the same, the improvement of stabilization of DEL depends not only on the ratio of the components involved, but also on their absolute content (Fig. 24).

**Table 5**. Quantification of DEL encapsulated within the different formulations. Efficiencies of stabilization and encapsulation are reported as percentage (%) of delphinid referred to the starting concentration.

| | **Delphinidin** | **Stabilized delphinidin** | | **Encapsulated delphinidin** | |
|---|---|---|---|---|---|
| | **[µM]** | **[µM]** | **%** | **[µM]** | **%** |
| **DNP – A** CS 0.15%, TPP 0.1% | 250 | 93 | 37 | 5.5 | 2.2 |
| **DNP - B** CS 0.25%, TPP 0.2% | 250 | 175 | 70 | 133 | 53 |
| **DNP - D** CS 0.25%, TPP 0.2% | 500 | 440 | 88 | 365 | 73 |

As well described in literature, polymeric nanoparticles are solid colloidal systems in which a target compound may be entrapped, encapsulated, or adsorbed inside the resulting polymer matrix. Chitosan nanoparticles form matrix systems in which the compound is dispersed throughout the particles (Prabhu et al., 2015). It is important to note that, the different formulations when centrifuged shown different behavior. Indeed, while the absence of sediment was constantly observed in all NPs formulations, under similar centrifugation conditions, the higher the DEL encapsulated, the higher the sediment obtained.

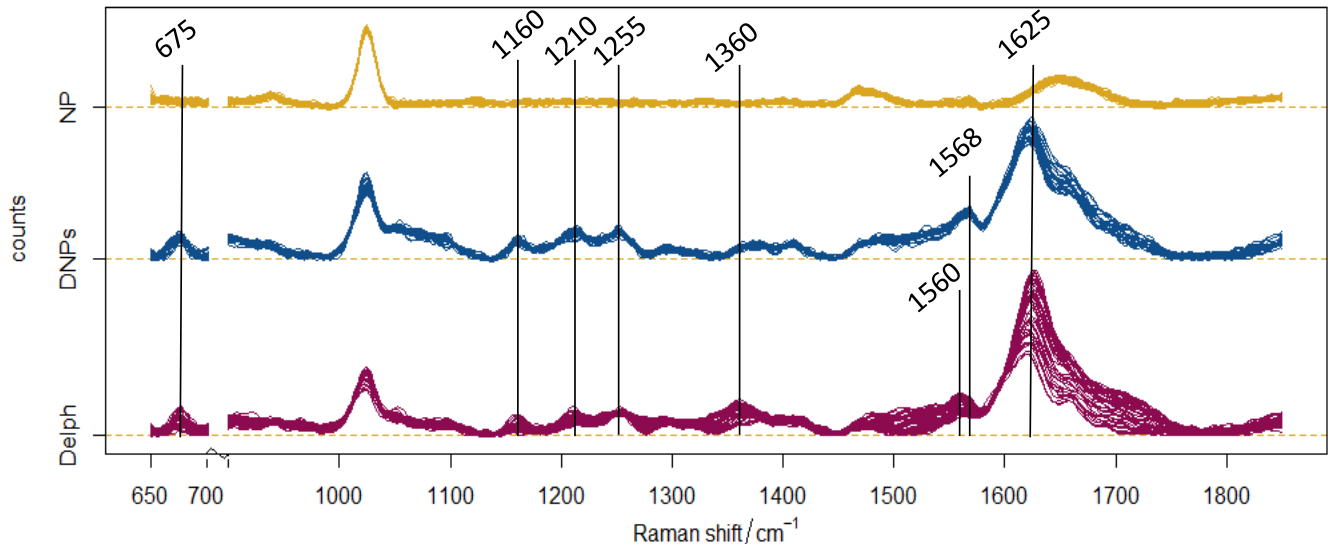
In addition, due to their polydispersity, all supernatants still contained a small portion of pink-red nanoparticles, therefore the real encapsulation efficiency could be higher than the one reported.

The time stability of encapsulated DEL concentration was estimated by injecting the same formulation one month later, obtaining the same values.

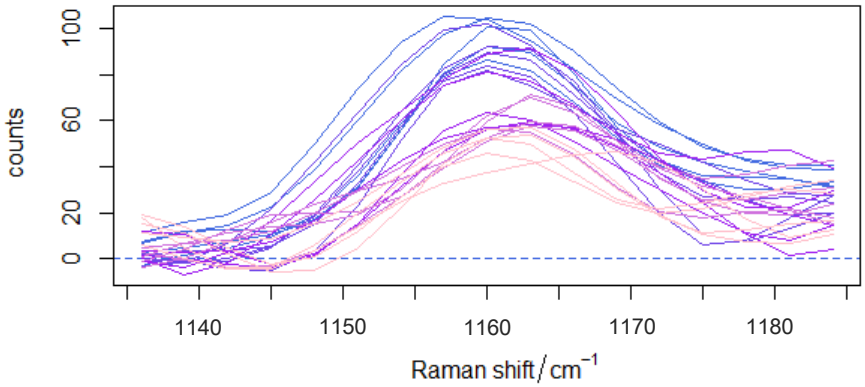
#### 3.2.3.5. UV Resonant Raman Spectroscopy

The Resonance Raman Spectroscopy has been used to evaluate the stability of the DEL in its flavylium form in time, through the evolution of its spectral features. In particular, we were able to excite the sample at a wavelength close to its UV absorption peak (272 nm) with a laser at $\lambda$ = 266 nm. The transformation of DEL in aqueous solution leading to the bleaching of the solution (Fig. 12, Fig. 22) is described in literature (Clifford, 2000).

As a result, the modified molecule is no longer capable to resonate at this frequency. Taking advantage of this behavior, DEL has been used as probe to evaluate in which chemical form it is present in the DNPs.




**A**




**B**

**Figure 28**. NN UV-Resonant Raman spectra of the (A) empty nanoparticles, the 450 µM encapsulated DEL and the 1 mM pure DEL (orange, blue and purple, respectively); (B) detail of the pure DEL spectra: the intensity of the peak at 1160 cm$^{-1}$ decreases with time (from blue to pink).

The time evolution of each series of spectra is shown in Figure 28, where the smoothed fitting curves are reported in order to provide a reasonable representation of changes with the time. Several peaks can be attributed to DEL vibrations according to the work of Merlin et al (Merlin et al., 1994; Merlin et al., 1993). It should be emphasized that all recorded UV resonance spectra where affected by a much smaller fluorescence interference when compared with the data of Merlin et al., who used visible excitation wavelength from 457.9 to 568.2 nm.

Furthermore, peak attribution was in general confirmed by quantum chemistry computations carried out by Dr. Di Fonzo using the Gaussian 09 software package (Frisch et al., 2009), according to the procedure described for caffeine in Tavagnacco et al., (2016). Structural data from literature were used in the available .pdb format. For the analysis of the peaks obtained by simulation, the GaussView module turned out very instructive in identifying C-C and C-H vibrations extended on the two fused aromatic rings (A and C), on the ring B only or the whole molecule. Based on these observations, the peak at ca. 1160 cm-1 was selected as a marker of ring C integrity. The time evolution of this peak is reported in Figure 29.

However, the analysis of the spectra revealed also other differences that could not be fully understood without a critical reading of the literature. A brief survey of the physico-chemical properties of concentrated flavylium solutions is necessary in order to provide the basis for an insight on the mechanism of stabilization of these molecules.

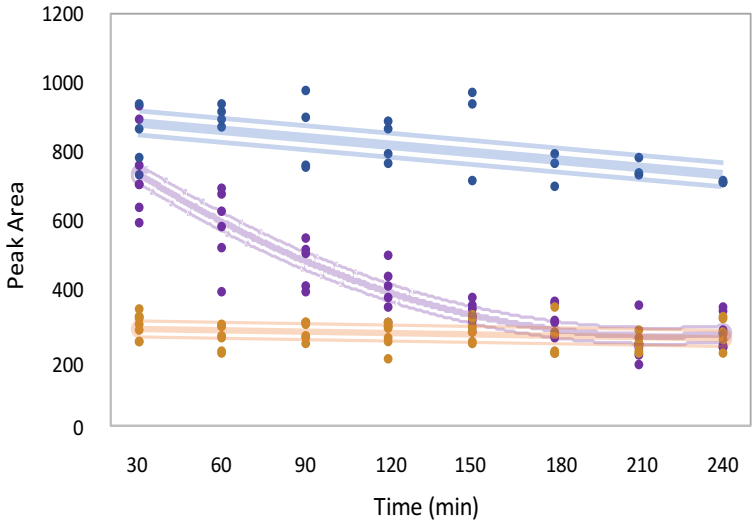



**Figure 29**. Evolution of the area of the peak at 1160 $cm^{-1}$ with time for empty nanoparticles, the 500 uM encapsulated DEL and the 1 mM pure DEL (orange, blue and purple, respectively). The kinetic curve demonstrates how the nanoparticles protect the DEL from the hydration effect, that leads to the opening of the chromane ring. Instead, for the pure DEL the spectra decay to a final plateau where no more anthocyanidine is present in its integer form (see the empty nanoparticles as control).

Previous computational studies carried out at density functional theory levels provided reliable structural information about structural tautomers of anthocyanins and their sugar derivatives in aqueous solution either in the neutral form (deprotonated at C4′), or in the most stable anion form (deprotonations at C5 and C4′) (Mosquera et al., 2015).

However, other studies by NMR and UV spectroscopies clearly underlined that in aqueous media, anthocyanins undergo several structural transformations and exist in a series of equilibria. Interestingly, some investigation suggest that co-pigmentation process of anthocyanins could occurs. This is a phenomenon in which the pigments and other colorless organic compounds form molecular or complex association generating a change or an increment in color intensity (Boulton, 2001).

The interaction could involve other anthocyanidins, nucleotides, polysaccharides and alkaloids and the phenomenon has been interpreted in terms of hydrophobic reinforced '$\pi$-$\pi$' stacking of anthocyanin and co-pigment molecules in the aqueous environment (Castañeda-Ovando et al., 2009).

These results on stacking ability of the anthocyanins stimulated further investigations on the conformational planarity of the molecule. All recent studies point to coplanarity for flavylium cations in both the gas and aqueous phases, although a reasonable number of slightly twisted quinoidal bases may be found. It has been proposed that the non-planar conformers of flavylium salts may play an important role in determining the fluorescence quantum yield due to relaxation to a twisted intramolecular charge transfer state. Similar conclusion was reached in another very recent investigation on quercetin and its derivatives, where the planar conformation and the hydroxyl groups in the B and C rings where claimed to play a pivotal role in the antioxidant capacity (George et al., 2001). Thus, we can conclude that not only for most common situations the optimized near-planar structure is adequate to describe the solution state, but this conformation is the more suitable for extensive association processes, further stabilizing the molecule as in a natural reservoir.

Although still speculative for the absence of other experimental data and the full analysis of the Raman spectral data, the hypothesis can be made that DEL at high concentration is stabilized in chitosan-TPP NPs by filling the gel micro-cavities in a highly associated form. This micro-droplet-like form can favorably interact with the residual charges of TPP and the slightly apolar side of acetyl-glucosamine moieties, in principle even at a van der Waals distance. The visual inspection of the spectra and their time evolution, in particular, can support this hypothesis that needs more work, including molecular dynamics simulation studies.

#### 3.2.3.6. DEL stability in aqueous solution

An important goal of encapsulation is to stabilize the molecule in physiological solutions where a different pH and the presence of salts can modify the structure of the nanoparticle itself.

In this regard, we prepared 7 different solutions of DEL or DNPs D at 50 µM with pH from 1 to 7 and let them to balance over time. 72 hours later we evaluated the changes that took place.

Figure 30 shows DNPs (D) and DEL behavior at different pH after 72 hours at room temperature; different pH conditions drive the pure compound (Fig. 30A) from the flavylium cation form (pH 1-3) to other conformations: in particular, it is possible to see how, from pH 4 to pH 7, the DEL equilibrium shifts from the pseudo-colorless carbinol-base to chalcone (increase

in yellow color).

On the other hand (Fig. 30B), DNPs D in the same conditions show to maintained the DEL form constantly between the red and colorless hydrated form, preventing the opening of the ring. Moreover, on the bottom of the cuvettes, where the solutions were kept, it was possible to observe the formation of a precipitate indicating maintenance of the nanoparticles structure.

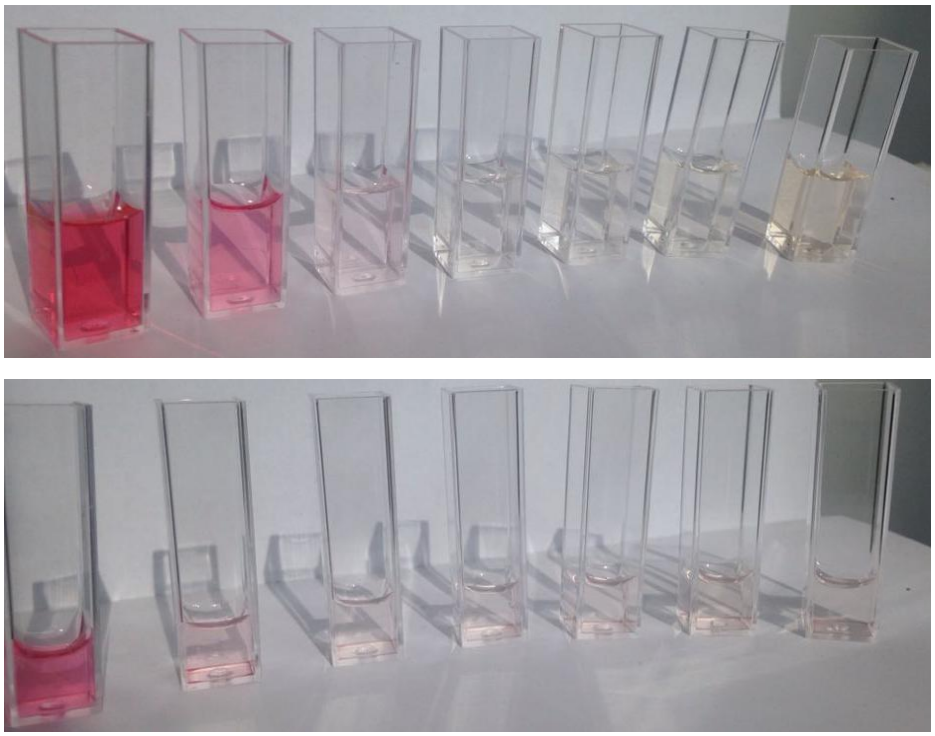



**Figure 30**. (A) DEL solution in deionized water at pH from 1, on the left, to 7, on the right, and (B) DNPs diluted in the same way. The picture was taken after 72 hours; as is visible the DEL at neutral pH became a calchone (light yellow color), whereas the DNPs remained pink and started to precipitate.

In order to evaluate the maintenance and the amount of DEL in physiological solution in time, pure compound and DNPs at the same DEL concentration were added to HBSS.

After 1h incubation at 37 °C, aliquot was diluted 1:1 in methanol/0.8% perchloric acid and injected for HPLC analysis.

Figure 31 shows the data obtained by resuspending the free and encapsulated DEL in HBSS. The results are expressed as a percentage of the starting concentration. All three nanoparticle formulations increase the stability of DEL over time with respect to the pure compound. Formulation D in particular shows the 40% stabilization of the molecule after 1h.

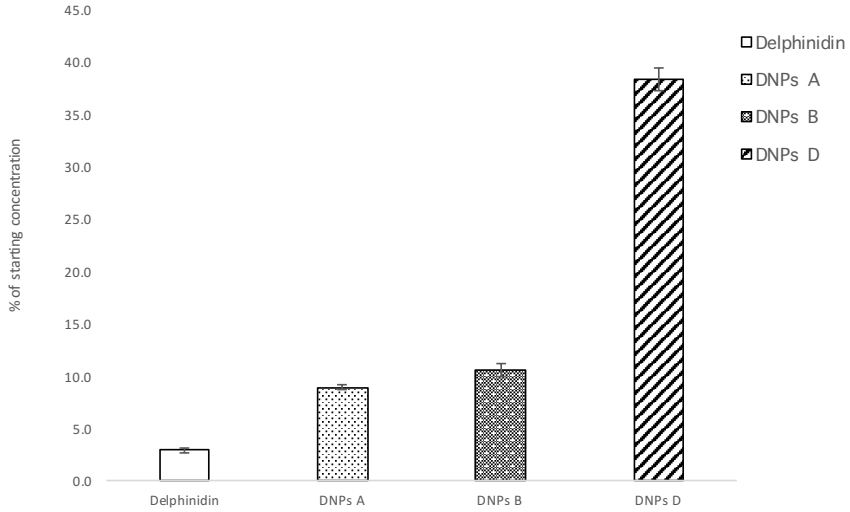



**Figure 31.** Relative amount of DEL in HBSS solution after 1 h incubation. All DNPs formulations stabilize DEL with respect to the free form. In particular the D formulation increased significantly the amount of DEL still available after 1h. Data are expressed as percentage from the starting concentration. Data are mean of 3 different experiment.

In order to deeper investigate the stabilization capacity of the formulation D, the incubation time in HBSS was prolonged to 2 hours as described by Kern et al. (2007). Even in this case presence of DEL in the solution was detected (8% with respect to the starting concentration) (Fig. 32). This data correlates with that previously published (Kern et al. 2007) where the limited stability in cell culture medium was described.

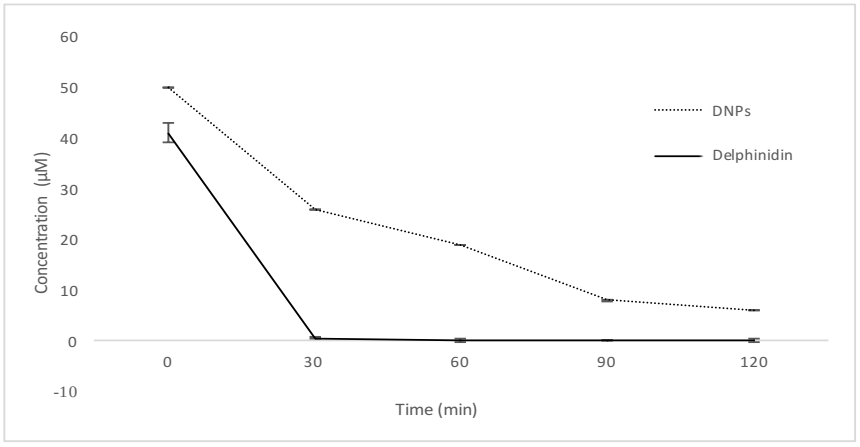



**Figure 32**. DEL concentration (μM) detected in HBSS after 2 h incubation of free DEL and DNPs D.

### 3.2.3.7. ABTS scavenging activity

Known that antioxidant activity is one of the most relevant function of this compound class, the assessment of the possible interference with this feature due to the encapsulation process was performed using ABTS assay.

DEL antioxidant capacity have been tested at different pH assessing that the maximum effect is reached when the solution is left to incubate at pH 5, the same of the nanoparticles formulation. All the different nanoparticles preparations were tested for the antioxidant capacity and were diluted to the same concentration in deionized water at pH 5, after their quantification.

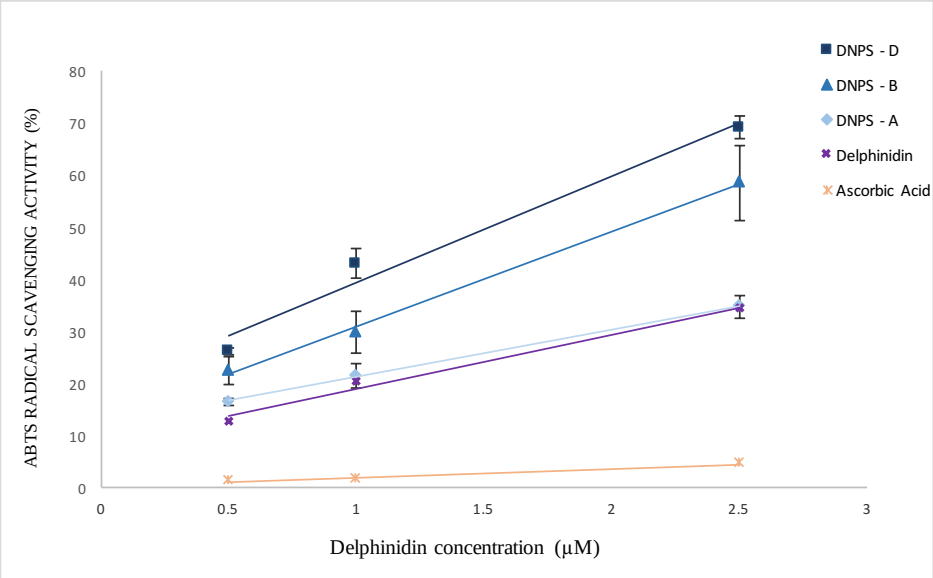



**Figure 33**. ABTS radical scavenging activity (%) of free DEL and nanoparticle formulations (DNP A, B, D).at different concentrations.

In Figure 33 are plotted the results obtained from the scavenging activity of 1 h incubation of free DEL compared to the encapsulated form. The DNPs – D reaches almost 70 % of ABTS scavenging capacity whereas free DEL never reaches values bigger than 40% at the same concentrations.

It has to be noticed that even though the final concentration was the same for all the DNPs (based on HPLC analysis), it appears that the particles that were assessed to have a better encapsulation efficiency were those with the best antioxidant activity and it is even more visible when we compare the antioxidant capacity to that of ascorbic acid used as internal control. Since the samples were allowed to react for 1 hour in the aqueous ABTS solution, the differences in antioxidant capacity may be determined by the increased stability of the internalized compound

in the different formulations, considering the poorer stability of the formulations A and B compared to the D. Taking into account that encapsulation process both, prolongs DEL permanence in acqueous solution (Fig. 32) and slows its release from the nanoparticles by virtue of the hypothesized hydrophobic interactions, the quantity of compound available for the antioxidant activity is proportionally higher.

#### 3.2.3.8. DEL and DNPs uptake in cells

Having demonstrated the greater stability of encapsulated DEL over the time in physiological solutions, therefore we further evaluated its absorption in the cells by comparing it with the free compound. Uptake of DEL in free and D formulation in LoVo cells was performed. For this experiment, we have chosen to use only one formulation as it is the best in terms of size, charge, efficacy of encapsulation and stabilization. Cells have been incubated in 0.2 mL of 50 μM DEL in free and encapsulated form for 2 hours. In Figure 34 shows the amount of DEL in cells measured at different time point and express as nanomoles/$10^6$ cells. Intracellular DEL concentration was significantly higher when cells were treated with DNPs in comparison to those treated with free compound. The improvement of absorption remains for all the experimental period.

Several studies obtained from decades of research in this field have demonstrated nanoparticles uptake by gastrointestinal tissues can increase the delivery of different types of compounds.

It particular, chitosan properties have been studied proving that it can adhere to cellular surfaces in the mucosa and can enhance the absorption of several compounds (Schnitzer, 2001; Jung, 2000).

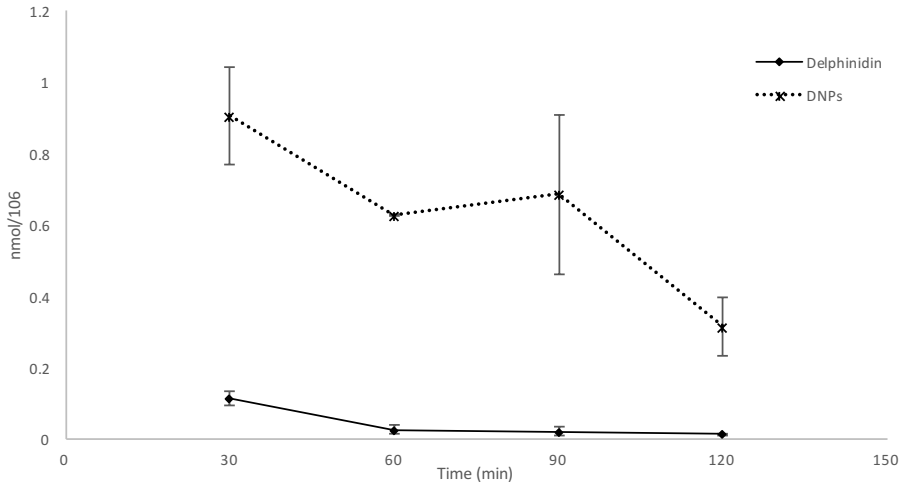



**Figure 3**4. Cellular uptake of DEL in free and encapsulated form. Data are mean of three different experiment. ($p<0,0001$).

In literature, the increased in vitro intestinal absorption of some polyphenols when encapsulated in chitosan nanoparticles has been demonstrated, mainly regarding tea catechins; investigations indicated that this enhancement most likely occurs as a result of the stabilization of these compounds, which increases their bioavailability; the application for other similar compounds which exhibit low bioavailability due to poor gastrointestinal stability have been suggested (Dube et al., 2010).

To confirm that the DEL amount measured in the cells was effectively inside the cells and not adherent to the extracellular surface, the internalization of the DNPs was evaluated after their conjugation with FITC. Flow cytometry assessment of fluorescence emitted from cells treated for 30 minutes with DNPs-FITC was performed. Trypan Blue (TB), that is excluded from living cells, was added in order to quench extracellular fluorescence signal as described in the Methods section.

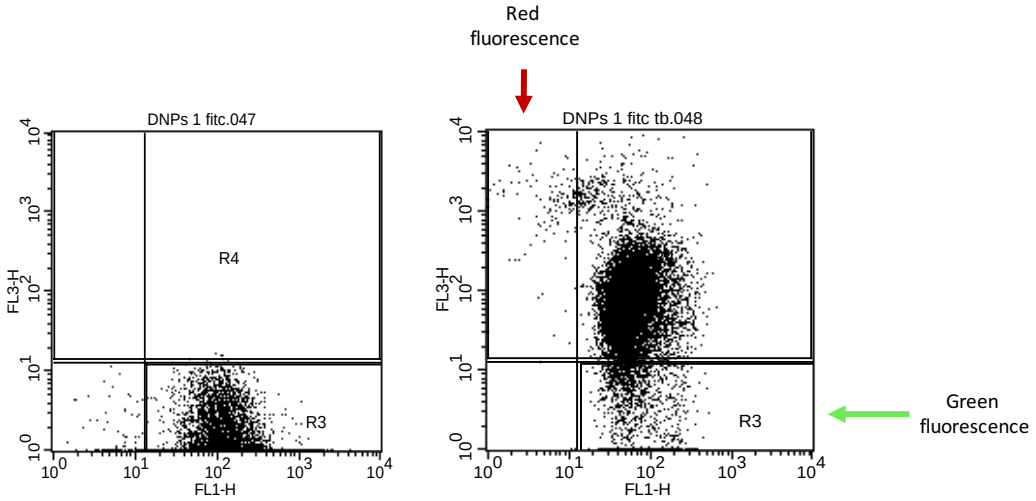



**Figure 3**5. Fluorescence detected from DNPs-Fitc in DMEM (A) emitting only green fluorescence and (B) emitting red/green fluorescence after quenching by Trypan Blue 0,5%. Representative data are shown.

In the set-up of the method the assessment of the proper concentration and incubation time for the experiment confirmed that the addiction of TB was capable of shifting the emission of nanoparticles from green toward red fluorescence (Fig. 35). One-minute incubation was enough to quench more than 90% of the particles. This allowed to discriminate the extracellular (red) nanoparticles from the intracellular (green).

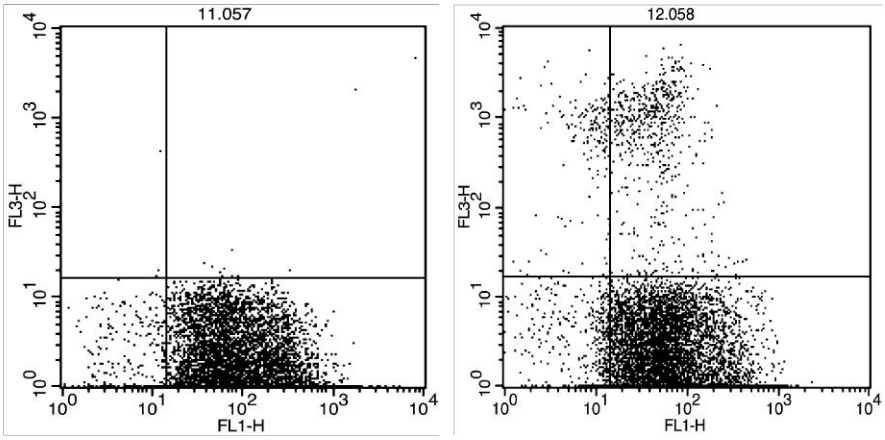



**Figure 3**6. Nanoparticles uptake in colon cell lines. Cells treated for 30 min with DNPs-FITC in DMEM were collected and immediately analyzed by Flow Cytometry. Fluorescence signal was obtained in the absence (A) and in the presence (B) of trypan blue to quench extracellular fluorescence. Representative results are shown.

The internalization of the nanoparticles in 3 different colon cancer cell lines (Caco-2, LoVo, LoVo/Dx) was performed obtaining almost the same result: the internalization after 30 minutes is very high, close to 95 % in all the tested cells. An example obtained from incubation of LoVo cells, before and after the addiction of TB, is reported in Figure 36.

# 3.3. CHAPTER III

## *Biological effects of DEL nanoparticles*

### 3.3.1. Aim of the work

The results showed in chapter II demonstrated that encapsulated DEL has higher stability in physiological solution compared to the free form, it maintains and improve the antioxidant activity in vitro and it enters with higher efficiency into the cells. In this section, the evaluation of the biological effects of DNPs has been performed on culture cells.

The effects on the interference with drug resistance have been evaluated, together with the antioxidant activity performed in cells and the effects on inflammation marker. All the experiments have been performed after a 2 hours treatment with DNPs on human colonic adenocarcinoma cells. We chose to test the effects on colon knowing that approximately 50% of human cancers express P-gp at levels sufficient to confer drug resistanceand that colon cancer is insensitive to most chemotherapeutic agents from the beginning of therapy.

### 3.3.2. Matherials and method

#### 3.3.2.1. Materials

Materials used are listed in Appendix

#### 3.3.2.2. Cell lines

Human colonic adenocarcinoma isolated from primary tumor (Caco-2), metastatic human colorectal adenocarcinoma cell line (LoVo) and their doxorubicin resistant subclone (LoVo/Dx) maintained in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, L-glutamine and doxorubicin (200ng/mL). 7 days before each experiment doxorubicin was removed from the medium.

#### 3.3.2.3. Cytotoxicity Assay

Cytotoxicity was determined by using MTS assay (CellTiter 96 Aqueous One Solution Assay

(Promega, USA) as described in Methods of Chapter I.

DEL and DNPs cytotoxicity was performed seeding LoVo/Dx and LoVo cells at $1x10^4$ and Caco-2 cells at $2x10^4$ in a 96-well plate in complete culture medium. After 24 hours, the cells were treated with designed serial dilution of DNPs or DEL (5 – 200 μM) for 2 hours and cytotoxicity was measured immediately and after 24 hours. Same volume dilution of NPs was used to evaluate chitosan nanoparticles cytotoxicity. Untreated cell/wells were added to each plate as control. Each drug concentration was assayed in 6 wells and repeated 3 times.

Doxorubicin cytotoxicity was performed measuring the effects of doxorubicin alone or after 2 h pretreatment with DEL, DNPs or NPs.

LoVo/Dx cells were seeded at $1x10^4$ in a 96-well plate in complete culture medium. After 24 hours, the cells were treated with DNPs or DEL (final concentration 50 μM) for 2 hours, after which doxorubicin serial dilution from 1nM to 1mM was added for 48 hours at 37°C in a humidified chamber. The effect of same volume of NPs was also evaluated. The total medium volume in each well was 200μL. Each concentration of each substance was repeated in 8 wells and repeated 3 times.

Data were analyzed using GraphPad Prism 7.0. IC50 values were calculated using a four-parameter dose-response curve.

#### 3.3.2.4. Cellular antioxidant activity (CAA) assay

Intracellular ROS production was detected modifying the method of Wolf et al (2007). The proposed principle is shown in Figure 37.

Namely, LoVo, LoVo/Dx and Caco-2 cells were seeded at a density of $1x10^4$/well (LoVo, LoVo/Dx) or $2x10^4$/well (Caco-2) on a 96-well microplate in 100μL of growth medium/well. The outside wells of the plate were not used. 24 hours after seeding, the growth medium was removed and the wells were washed with 100μL PBS.

After this step, the cells were treated with 100μL of DEL, DNPs (final dephinidin concentration was 25 and 50μM) or NPs (the same volume used to reach the desired concentration of DNPs) in HBSS. The supplemented buffer alone was used as control. After 2 hours, the treatment solutions were removed and wells were washed with PBS, after which 2μM chloromethyl-$H_2$DCFDA (CM-$H_2$DCFDA) in HBSS was added and left in incubator.

Thirty minutes later, the solutions were removed by aspiration, and the cells were washed with PBS. Then 1mM $H_2O_2$ in HBSS was added. Samples treated with HBSS by omitting $H_2O_2$ were used as blanks. Kinetic fluorescence production was followed at 37°C into a Microplate Reader (Bio-Tek Instrument, USA).

Emission at 538 nm was measured with excitation at 485 nm every 5 minutes for 1h. For all sample condition 8 replicates were used.

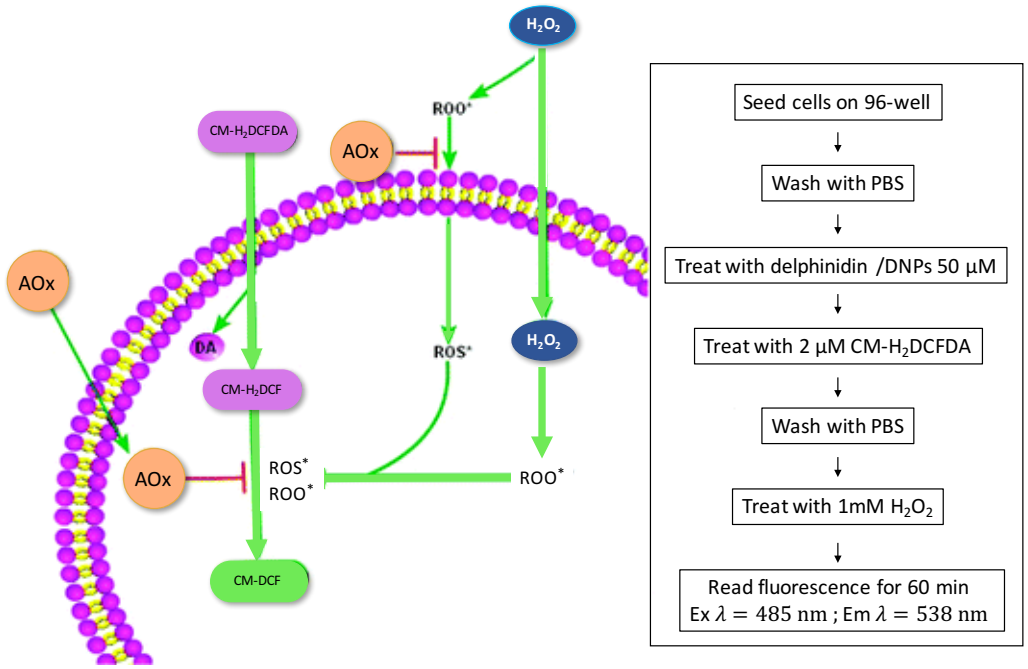



**Figure 37**. Method and proposed principle of the cellular antioxidant activity (CAA) assay. CM-H2DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular thiols. Subsequently cells were treated with $H_2O_2$, that oxidizes the intracellular CM-$H_2$DCFH to the fluorescent DCF leading to the formation of a fluorescent adduct inside the cell. Antioxidants prevented oxidation and reduced the formation of CM-DCF. Pro-oxidants, on the other hand, caused an increase in ROS accumulation which led to the oxidation of CM-$H_2$DCFDA and increased the formation of the fluorescent CM-DCF. Readapted from Wolf et al. 2007.

*Quantification of CAA*

After blank subtraction, the area under the curve of fluorescence versus time was integrated to calculate the CAA value at each experimental condition as follows:

$$\text{CAA unit} = 100 - (\int SA / \int CA) \times 100$$

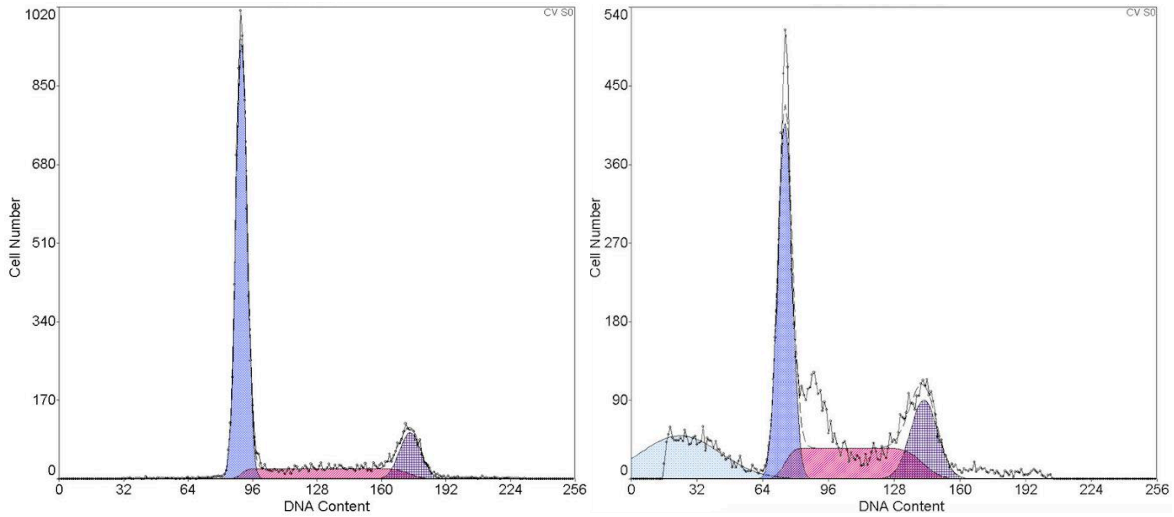
where $\int$ SA is the integrated area under the sample fluorescence versus time curve and $\int$ CA is the integrated area from the control curve.

#### 3.3.2.5. Cell cycle analysis and PI staining

LoVo, LoVo/Dx and Caco-2 cells were plated at $5x10^5$/well in 6-well/plates in complete medium. After 24 hours, the medium was removed from the wells by aspiration, the cells were

washed with PBS, and treated for 2 hours with DEL, DNPs (50µM dephinidin) or NPs (the same volume used for DNPs) in HBSS, each treatment was repeated in triplicate. Untreated cells were used as control.

After 24 hours from treatment $0.5 \times 10^6$ cells were fixed in 70% ethanol, washed twice with PBS, and allowed to balance in PBS for 1 hour. Cells were stained overnight with 0.5 mL of a PBS solution containing 10 µg PI, 0.25 ng FITC, and 4 µg RNase (all chemicals were purchased from Sigma-Aldrich, Italy). Cell cycle analysis is based on nucleic acids staining by PI to evaluate total DNA content. In fact, when the cells are in G1 phase they are diploid, whereas in G2/M they are tetraploid. Assessment of apoptosis is also possible do to DNA fragmentation and subsequent generation of low fluorescence emitting G1 population (sub-G1) (Fig. 38).




A B

**Figure 3**8. Example of the DNA histogram showing cell cycle peak distribution obtained with Flow Cytometer; (A) Normal (B) Apoptotic. In panel B, apoptotic sub-G1 peak is visible.

Cell cycle assessment was done using flow cytometry measurements carried out on a Cytomics FC500 (Beckman Coulter Inc., USA), equipped with an argon laser (488 nm, 5 mV) and standard configuration with photomultiplier tube (PMT) fluorescence detector for green (525 nm, FL1), orange (575 nm, FL2), or red (610 nm, FL3) filtered light. After acquisition, of at least 10,000 events per each run, data are stored as list mode files and analyzed with the FCS Express V3 software or, the FL3 saved histograms, and were submitted to the analysis of the cell cycle, performed by the MultiCycle software.

#### 3.3.2.6. Doxorubicin accumulation

LoVo/Dx cells were plated at $1x10^4$/well in 96-well plates and treated with the complete medium. After 24 hours, the medium was removed from the wells by aspiration, the cells were washed with PBS, and treated for 2 h as follows (each treatment was repeated in 8 wells):

*Control*: the cells were incubated in 100µl/well of HBSS for 180 min

*Doxorubicin alone*: the cells were incubated in 100µl/well of HBSS for 120 min after which it was aspirated and doxorubicin (25µM) dissolved in HBSS was added and left for another 60 minutes.

*Verapamil + doxorubicin*: the cells were incubated in 100µl/well of HBSS for 90 min after which it was aspirated and verapamil (50 µM) was added. After 30 min incubation with verapamil doxorubicin (25µM final concentration) dissolved in HBSS was added and left for another 60 minutes.

*DEL + doxorubicin*: the cells were incubated in 100µl/well of 25 or 50µM DEL in HBSS for 120 minutes after which it was aspirated and cells were washed 3 times with PBS. Then doxorubicin (25µM) dissolved in HBSS was added and left for another 60 minutes.

*DNPs + doxorubicin*: the cells were incubated in 100µl/well of DNPs (final DEL concentration 25µM and 50µM) in HBSS for 120 minutes after which it was aspirated and cells were washed 3 times with PBS. Then doxorubicin (25µM) dissolved in HBSS was added and left for another 60 minutes.

*NPs + doxorubicin*: the cells were incubated with empty nanoparticles (adding the same volume as DNPs) in HBSS for 120 minutes after which it was aspirated and cells were washed 3 times with PBS. Then doxorubicin (25µM) dissolved in HBSS was added and left for another 60 minutes.

Uptake was stopped by aspiration of the solutions from the wells and washing each well with ice-cold PBS after which 100µl of 0.3 N HCl/50% ethanol was added in each well and left incubate for 60 minutes at +4°C. The resulting cell lysate was transferred in a black plate to record fluorescence. Emission was measured at $\lambda$=535nm with excitation at $\lambda$=485nm every 5 min for 1h.

#### 3.3.2.7. Western blot analysis

Caco-2, LoVo and LoVo/Dx cells were plated at $5x10^5$/well in 6-well plates and treated with the complete medium. After 24 hours, the medium was removed from the wells by aspiration, the

cells were washed with PBS, and treated for 2 hours with DEL, DNPs (final DEL concentration was 50μM) or NPs (the same volume used for DNPs) in HBSS, each experimental condition was made in triplicate. After 24 hours from treatment cells were washed with PBS 3 times and lysed with lysis buffer (PBS 1x – SDS 1%).

For each well the protein concentration was determined using Bicinconinic Acid Assay (BCA) according to method described by Smith et al., 1985.

After quantification, 20 μg of total protein were loaded on an 10% acrylamide gel for SDS-PAGE, and then transferred to a PVDF membrane.

The membranes were incubated for 2 hours with blocking solution (4% milk, 0,2% BSA in PBS-tween 0,05%). Primary antibody mouse anti-NF-κB (Cat. # ALX-210-574-R100, Enzo Lifescience) was diluted in the same solution and incubated over-night at 4°. Peroxidase labeled α-rabbit was used as a secondary antibody for NF-κB (Cat. # 32460, Thermo Scientific); it was diluted in blocking solution and incubated one hour at room temperature.

Between the two antibodies the membranes were washed three times for 5 minutes with PBS-tween 0.05%.

Antibody staining was detected using SuperSignal WestDura (Thermo Scientific, USA). Normalization of the signal have been done using the total protein content, staining the membrane with Red Ponceau to stain all the proteins before the addition of the blocking solution.

#### 3.3.2.8. Statistical analysis

Data are presented as means ± S.E. of independent experiments. The statistical significance was analyzed using one way analysis of variance (ANOVA) followed by Dunnet's post hoc test or two-way ANOVA followed by Bonferroni post hoc test. All data analyses were performed using the Prism software 7 (GraphPad Software, USA). $p < 0.05$ were considered statistically significant.

## 3.3.3. Results and Discussion

### 3.3.3.1. Cytotoxicity of DNPs after 2h treatment

In order to compare the effect on cell viability of DEL as pure compound or as chitosan formulation, we decided to firstly evaluate its cytotoxicity in Caco-2, LoVo and Lovo/Dx cells. Serial dilution of pure DEL, DNPs and NPs (as DNPs control) were added to cells for 2 hours. Cytotoxicity was evaluated immediately and after 24h of recovery. In this experimental condition, none of the assayed compound showed a cellular toxic effect on none of cell line tested.

### 3.3.3.2. Cellular antioxidant activity in colon cancer cell lines

Comparison between DEL and DNPs in their capability to interfere with the cellular oxidation levels were assessed in Caco-2, LoVo and LoVo/Dx cell lines.

As shown in Figure 39, treatment behaved differently in the different cell lines. Pure DEL acts as an antioxidant on Caco-2 and LoVo cells at both concentration tested, whereas in LoVo/Dx it has an increasing pro-oxidant activity, proportional to the DEL amount. On the other hand, DNPs act as antioxidant only on Caco-2 but showed an opposite behavior in LoVo and LoVo/dx cell lines, even at the lower concentration tested. Even empty NPs has different effect, indeed in Caco-2, have a low antioxidant activity, whereas in LoVo/Dx take part to the pro-oxidant effect.

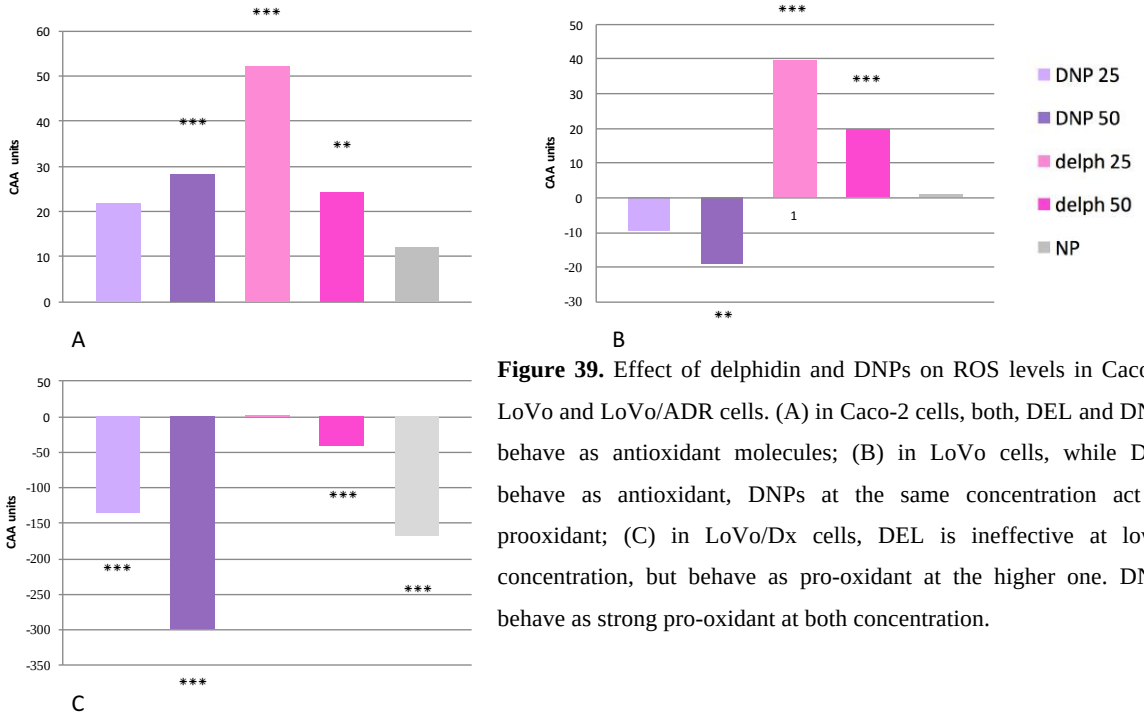



**Figure 39.** Effect of delphidin and DNPs on ROS levels in Caco-2, LoVo and LoVo/ADR cells. (A) in Caco-2 cells, both, DEL and DNPs behave as antioxidant molecules; (B) in LoVo cells, while DEL behave as antioxidant, DNPs at the same concentration act as prooxidant; (C) in LoVo/Dx cells, DEL is ineffective at lower concentration, but behave as pro-oxidant at the higher one. DNPs behave as strong pro-oxidant at both concentration.

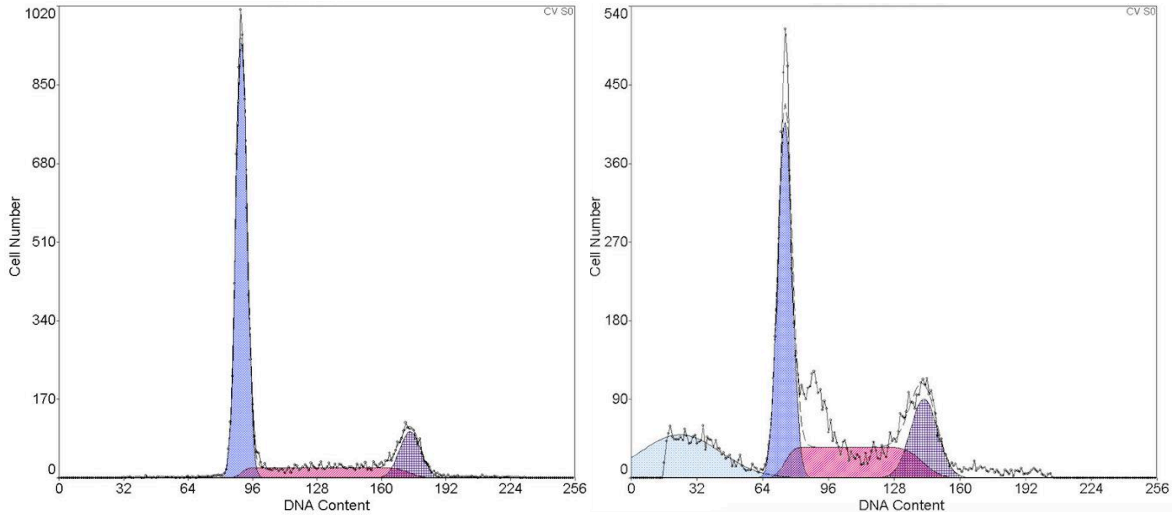
It was described that prooxidant compounds enhance the cellular quantity of ROS to cytotoxic levels in cancer cells but not in normal ones (Eghbaliferiz and Iranshahi, 2016; Kwok et al., 2016). These data are coherent with those obtained in our laboratory testing the effects of free DEL on the same cell lines. Indeed, DEL acted as an anti/pro-oxidant molecule depending on the concentration and type of the treated cells (Cvorovic et al., 2010). This effect can be due by the higher concentration of copper ions and greater metabolic activity found in cancer cells compared with normal ones. The most significant difference with the new data obtained concerns the effective concentrations. Our results show that DEL, once encapsulated in chitosan nanoparticles behave strongly at lower concentration.

The evaluation of antioxidant activities of several anthocyanidins have been performed in other studies suggesting a possible involvement in the inhibition of a Fenton reagent •OH generating system, is plausible to link it with the chelation of ferrous ion, but also with the scavenging of superoxide radicals. Others suggest that anthocyanidin act indirectly through the activation of phase II enzymes involved in the antioxidant responses (Shih et al., 2007). Together with the anti-oxidative properties in reactive oxygen species (ROS) scavenging, other studies suggested a possible pro-oxidant effect correlating it to the pro-apoptotic activity exerted by anthocyanindins. Scheit et al. indicated that cyanidin, but similarly also other anthocyanidins, indirectly induces oxidative stress and apoptosis via the inhibition of extracellular catalase, rather than directly via its prooxidant activity. Indeed, anthocyanidins addition cause singlet oxygen generation immediately after their addition to the tumor cells and indirectly target tumor cell protective catalase and thus reactivate inter-cellular apoptosis-inducing ROS signaling (Scheit and Bauer, 2015).

A relationship between the structure of the different anthocyanidins and the activity performed have been demonstrated. Both the antioxidant and the prooxidant activity seem to be dependent on the number of hydroxyl groups in the aglycon, in particular the B-ring. DEL among all the anthocyanidin is the one with most OH substituents and for this reason appears to be the one with the stronger antioxidant and pro-oxidant activities both in vitro and in cellular-systems (Noda et al., 2002; Tsuda et al., 1996 Ramirez-Tortosa, et al, 2001; Tsuda et al., 1999; Tsuda et al 2000; Hou, 2003).

### 3.3.3.3. Cell cycle perturbation

Cell cycle analysis is based on nucleic acids staining by PI to evaluate total DNA content. In fact, when the cells are in G1 phase they are diploid, whereas in G2/M they are tetraploid. Assessment of apoptosis is also possible do to DNA fragmentation and subsequent generation of low fluorescence emitting G1 population (sub-G1) (Fig. 40).




A B

**Figure** 40. Example of the DNA histogram showing cell cycle peak distribution obtained by Flow Cytometer; (A) Normal (B) Apoptotic.

Known that the alteration of cell cycle might be one of the effects exerted by anthocyanidins this possible feature was evaluated on the three different colon cancer cell lines as previously described.

Cells were treated for 2 hours at a concentration 50 μM with free or encapsulated DEL; empty NPs were used to evaluate any possible effect cause by chitosan using the same volume of that of DNPs.

**Table 6**. Cell Cycle phase distribution of colon cancer cells. Cells deriving from 2 h treatment with DEL 50 μM, DNPs 50 μM and NPs (the same volume needed to reach the concentration of DNPs) were analyzed by Flow Cytometry. No significant effects on cell cycle were observed

| | CaCo 2 | | | LoVo | | | LoVo/Dx | | |
|---|---|---|---|---|---|---|---|---|---|
| | G1 | S | G2/M | G1 | S | G2/M | G1 | S | G2/M |
| Control | 44.2 ± 0.8 | 40.7 ± 2.5 | 15.2 ± 2.7 | 49 ± 0.3 | 38.3 ± 0.2 | 12.7 ± 0.1 | 55.2 ± 1.7 | 28.5 ± 0.4 | 16.3 ± 2.1 |
| NPs | 44.7 ± 2.7 | 40.7 ± 3.2 | 14.5 ± 0.6 | 46.5 ± 4.1 | 38.5 ± 4.9 | 14.9 ± 2.5 | 54.4 ± 1.9 | 27.3 ± 0.6 | 18.4 ± 1.2 |
| Delphinidin | 43.8 ± 0.9 | 45.3 ± 1.04 | 10.9 ± 1.9 | 51.3 ± 3.0 | 38.6 ± 4.5 | 10.4 ± 1.0 | 56.9 ± 0.6 | 27 ± 2.1 | 16.1 ±1.4 |
| DNPs | 40.8 ± 0.9 | 49.7 ± 2.1 | 9.5 1.1 | 48.8 ± 1.9 | 31.5 ± 3.7 | 19.7 ± 2.1 | 57.7 ± 0.1 | 27.7 ± 0.7 | 14.6 ± 0.6 |

As reported in Table 7 regarding Caco-2 treatment only DNPs were able to significantly increase the amount of cell in the S phase. Since this increase is coupled with a slight decrease in the G2/M phase, a cell cycle arrest in the S phase is hypothesized. Unlike the data we obtained, previous studies have shown that DEL treatment resulted in a significant arrest in G2/M phase of HCT 116 cells. It should be noted, however, that the incubation time and the concentrations used were significantly different from our (Yun et al., 2009). Although no other statistically significant modification was highlighted in the LoVo and LoVo/Dx cell cycle for any of the treatments performed, it is interesting to note that DNPs treatment in both LoVo and LoVo/Dx induced apoptosis. In fact, in the samples treated for 2 hours with 50 µM DNPs, a sub-G1 peak of 7% and 19% respectively in LoVo and LoVo / Dx cells was found ($p < 0{,}001$) (Fig. 41).

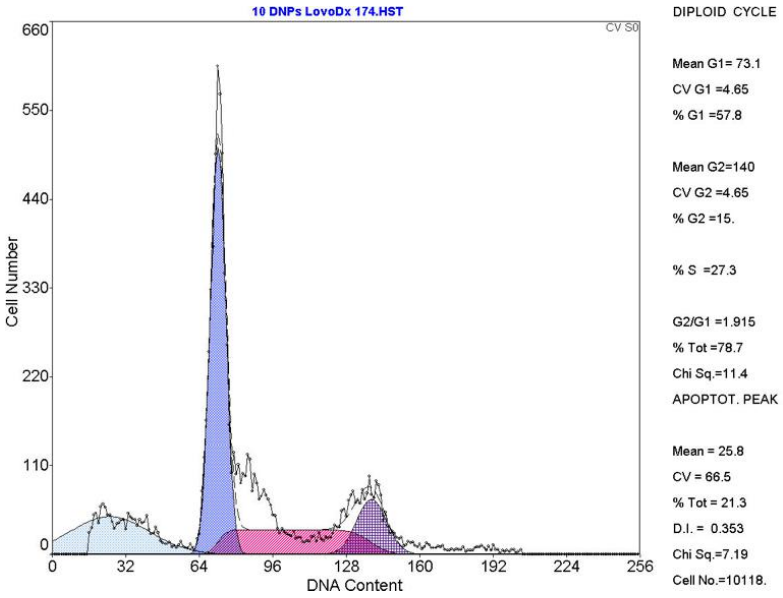



**Figure 41. The DNA histogram** representative of a sample of LoVo/Dx treated with DNPs. As is visible there are evident the 3 main peaks: G1 (blue), S (pink) and G2/M (purple).
On left of the G1 peak there is a population with fragmented DNA, meaning that a portion of the population is apoptotic.

The effect may be attributed to the increased amount of DEL internalized by cells and once more suggests an improved biological activity of DNPs in comparison with the free compound.
Apoptosis is a complex event characterized by several mechanisms that leads to programmed cell death. Two major apoptosis pathways are closely regulated to induce cell destruction via a cascade that finally activates caspases: one is the activation of death domains and death effector domains on the cell surface and the second is the intrinsic cytotoxic mitochondrial-mediated apoptosis after mitochondrial membrane permeabilization (Parrish et al., 2013). The second one seems to be more likely (Lopez and Tait, 2015). Cells are stimulated to trigger cell death by apoptosis or necrosis when cells fail to repair (Yoshida et al., 2008).
DEL, together with cyanidin, but not its isolated glycosides have been reported to diminishing the catalytic activity of topoisomerases stimulating the formation of DNA breaks (Esselen et al., 2011; Habermeyer et al., 2005; Fritz et al., 2008).

Recent studies have demonstrated that reactive oxygen species (ROS) and the resulting oxidative stress play a pivotal role in apoptosis. Bcl-2, an endogenously produced protein, has been shown to prevent cells from dying of apoptosis apparently by an antioxidative mechanism (Kannan and Sushil, 2000).

Furthermore, DEL have been reported to decrease the expression of Bcl-2 in colon cancer cells inducing an increase in Bax level with a consequent activation of caspases (Yun et al., 2009; Huang et al., 2011). As previously reported, DNPs behave as prooxidant in LoVo and Lovo/Dx cells, suggesting oxidative stress apoptotic process.

#### 3.3.3.4. NF-κB expression after 2 h treatment with DNPs and DEL

Accumulating evidence shows that chronic inflammation is associated with increased risk of cancer and extensive investigations have uncovered many of the important mechanistic pathways underlying cancer-related inflammation.

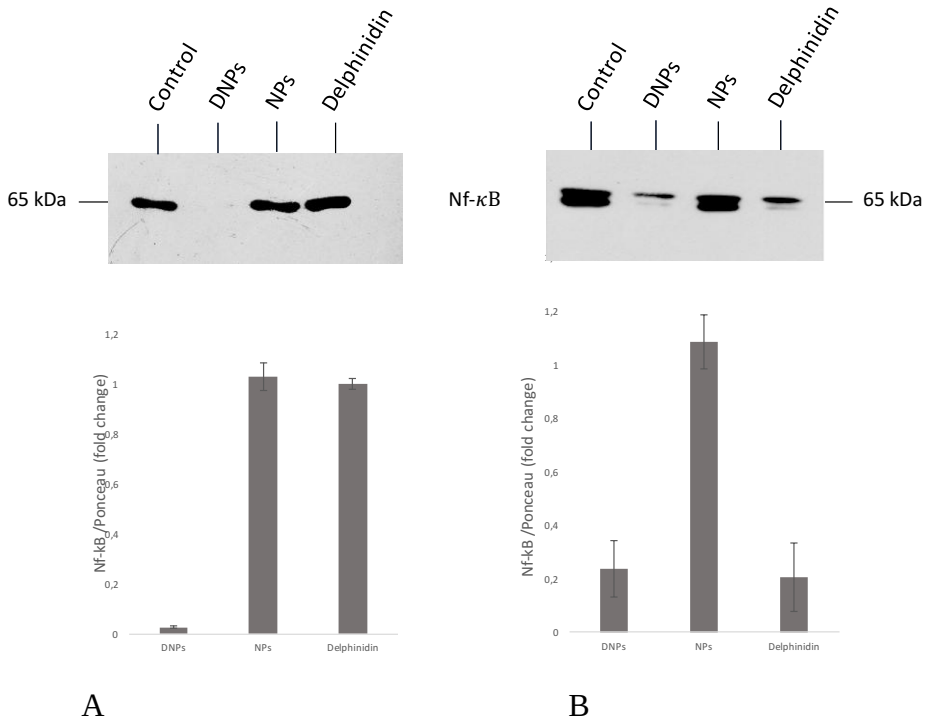



**Figure 4**2. NF-κB expression in LoVo (A) and LoVo/Dx cells (B) after 2h pretreatment with 50 μM DEL or DNPs. Cell lysate have been collected after 24 h from pretreatment. Normalization have been performed using total protein amount detected with red Ponceau. Data are means of 3 independent experiments.

The expression of one of the main players in proliferation and survival of malignant cells, Nuclear Factor kappa-B (NF-κB), was evaluated in LoVo and LoVo/Dx cell lines by Western blot analysis.

Cells were treated for 2 hours with DEL and DNPs at the same compound concentration and with NPs to evidence the effect of the encapsulation material.

In Figure 42A, NF-κB protein expression in Lovo cells at different experimental conditions is reported. Data are expressed as fold change of control and normalized to total protein loaded. Only DNPs treatment was able to decrease of more than 80% NF-κB expression. Whereas, in LoVo/Dx (fig 42B) not only DNPs, but also DEL had the same decreasing effect.

In Caco-2 cell we were not able to detect a basal level of NF-kB in any experimental conditions. These findings may be reasonably related to the fact that NF-κB is a transcription factor involved in cell cycle regulation by promoting cell proliferation in cancer cells. Caco-2 cells are tumor cells with a low degree of proliferation and a low potential for malignancy (Cvorovic et al., 2010).

One of the proposed mechanisms by which DEL may induce apoptosis is the inhibition of inflammatory NF-κB pathway via phosphorylation and degradation of IκBα (Yun et al., 2009). Furthermore, other interfering mechanisms have been reported such as the inhibition of activation of I kappa B kinase α (IKKα), in turn involved in the activation of IκBα (Verma et al., 2012).

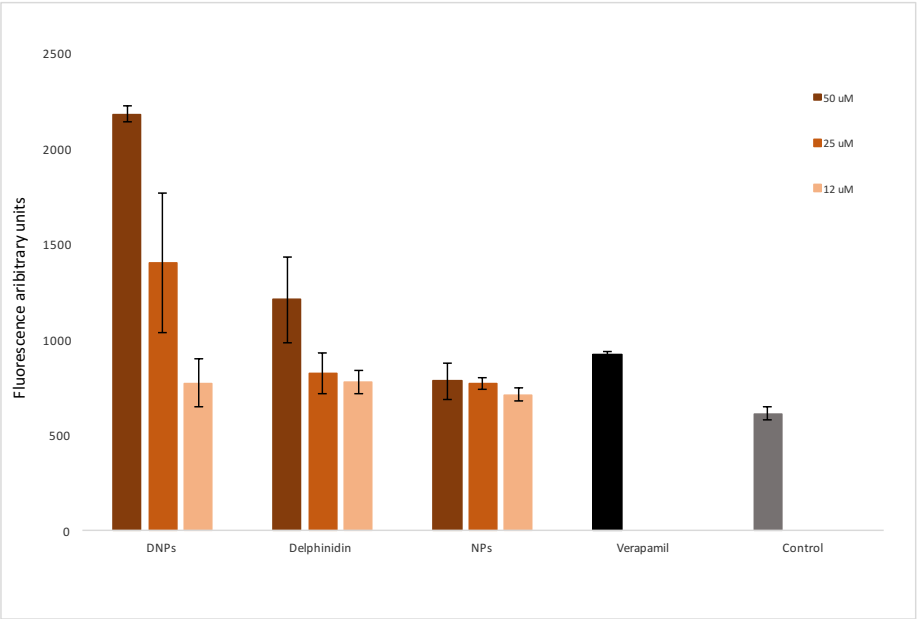
These events led to an inhibited phosphorylation of NF-κB p65 by DEL with a consequent induction of apoptosis (de Sousa Moraes et al., 2017).

#### 3.3.3.5. Doxorubicin accumulation

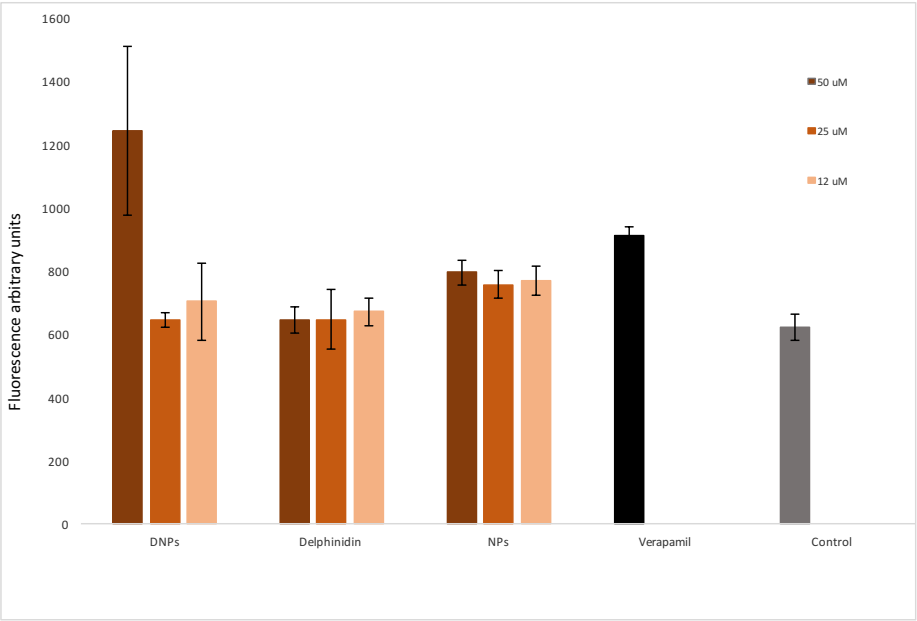
Health advantages of DEL use as adjuvant in chemotherapeutics treatment, considering the important decrease of doxorubicin cytotoxicity obtained, prompted us to evaluate the effect of DNPs on drug accumulation in resistant cells.

Two possible treatment protocols were evaluated on isolated cells: in one case, doxorubicin was given immediately after 2h pretreatment with DEL or DNPs (concentration), whereas in the second case the chemotherapeutic drug was administered 24h after it.

As shown in Figure 43A both DNPs and free DEL induce an increase in drug accumulation in a concentration dependent manner but DNPs show to dramatic improve this effect. Most interestingly, when the doxorubicin uptake was performed the day after (Fig 43B), the effect on drug accumulation was maintained only in cells treated with DNPs and not with free compound, demonstrating once more its improved stability and bioavailability. The longer lifetime of the encapsulated DEL prolonged the effect of the molecule.




**A**




**B**

**Figure 4**3. Doxorubicicn uptake in LoVo/Dx cells. Effect of DEL or DNPs on doxorubicin accumulation in LoVo/Dx cells after 2 h pre-treatment with a concentration 50 µM. In Figure A is reported doxorubicin accumulation measured immediately after the pretreatment. In Figure B is doxorubicin accumulation 24h after DEL pre-treatment.

Several studies have demonstrated the role of ABC transporters in the resistance of cancer. Usually the proposed mechanisms for the interference of drug resistance reported in literature regards a reduction of membrane protein levels that results in increased cytotoxicity of the drug (Miao et al., 2016; Satonaka et al., 2017).

As previously reported, P-gp is an integral membrane protein with a rather long half-life. The 2 h

treatment used in our experiments is certainly not long enough to alter the expression of the protein. Therefore, drug accumulation could be referred to a different molecular mechanism.
Indeed, Dreiseitel and colleagues (Dreiseitel et al., 2009) reported that DEL, but not its glycosylated form, is able to inhibit (IC50=104 μM) P-gp transport activity mainly connected with ATP hydrolysis.

### 3.3.3.6. Doxorubicin cytotoxicity after pretreatment with DNPs and DEL.

**Table 7. Effect of DEL on doxorubicin cytotoxicity.** Cytotoxicity of doxorubicin detected using MTS Assay. After 2 h pre-treatment with 50 μM DEL/DNPs, LoVo/Dx cells were incubated with different concentration of doxorubicin for 48 h.

| Doxorubicin IC50 (μM) | | | |
|---|---|---|---|
| Control | DNPs | Delphinidin | Nps |
| 53 | 28 | 37 | 44 |

Subsequently, the effect of 2 hour treatment of 50 μM DEL, both pure and encapsulated, on doxorubicin cytotoxicity in LoVo/Dx cells was evaluated.
It is interesting to note that pure DEL is already able to decrease the IC50 value of doxorubicin from 53 μM (control) to 37 μM. This data is in line with previous data obtained and reported in Chapter 1 (Table 7). Indeed, after 1 week of DEL treatment, even at low concentrations, increasing accumulation of doxorubicin in Lovo/Dx cells was obtained. However, this effect was obtained without any clear variation in P-gp expression.
Even more important is the dramatic decrease in IC50 value following treatment with DNPs which dropped to 28 μM. Empty nanoparticles were used too, and the resulting IC50 value was 44 μM, proving that the increased cytotoxicity was caused by DEL and not by the nanostructures. These results could be explained considering that we demonstrated, as DNPs are able to transfer and maintain a higher DEL content in cells.

# 4. Concluding remarks

Oxidative stress seems to be involved in many chronic and degenerative diseases such as Parkinson and Alzheimer diseases, Diabetes and Cancer. Flavonoids, a class of secondary metabolites of plants, thanks to their strong antioxidant activity have attracted great interest for their nutritional and pharmacological application. Anthocyanidins belong to the class of flavonoids and are responsible for the red-purple color of flowers and fruits. These compounds, beyond the antioxidant effect, exert anti-proliferative and anti-inflammatory activity. In addition, their inhibition activity on the efflux pumps present in cancer cell membranes, and responsible for drug resistance mechanisms, has been described. Many of the studies carried out, show biological effects obtained under experimental conditions that are difficult to translate into physiological ones mostly due to the high concentration applied. In fact, due to low stability and poor bioavailability, the concentrations tested were far from that introduced with the daily food intake.

The main goals reached by the research activity presented in this thesis are listed:

- A long-term treatment with non-toxic doses of DEL protract for 5 weeks was performed, mimicking the dietary intake of the molecule on a doxorubicin-resistant colon cancer cell line. These experiments showed that the treatment caused cell cycle perturbation and increased the intracellular concentration of the chemotherapeutic agent. Unfortunately, mechanisms of adaptation took place impeding the flavonoid to maintain its effects in time, suggesting a higher suitability of action in acute conditions.

- DEL nanoparticles (DNPs) were produced using biodegradable and biocompatible polysaccharide, testing different formulations. The assessment of the physical chemical properties was performed. In the best formulation obtained the encapsulation efficiency was 73% and the stability of the molecule in physiological solution was strongly increased compared to the free molecule. The intracellular concentration of the compound was significantly increased when administrated as DNPs. Even the antioxidant performance was higher when tested in comparison to the free molecule. Furthermore, UV Resonant Raman Spectroscopy suggested that the encapsulated DEL form is in equilibrium between the flavylium cation and the carbinol-pseudobase and cannot move to the chalcone.

- Investigating the biological activity on different colon cancer cell lines, a dramatic increase of doxorubicin uptake in doxorubicin-resistant colon cells (LoVo/DX) was noticed together with an improvement of its cytotoxicity. Results after 2 hours treatment with DNPs suggested a reduction of the expression of biomarkers of inflammation and the induction of apoptosis. Antioxidant properties were maintained in cells with low basal metabolic rate, whereas in malignant cells, with active metabolism they behave as pro-oxidants.

These results show the superior performance of nano-encapsulated DEL for applications in cancer drug development and, due to the multilevel effect noticed, suggest the suitability of this nanoparticles for several applications, not only for an oral use but also considering the possibility to administer nanoparticle-based therapeutics via different routes, such as pulmonary, nasal dermal and parenteral, exploring the proper coating system for the specific tissue-targeted delivery.

It would be interesting to test the interference with drug efflux using different types of anticancer drugs as substrates to assess if the interference with the multidrug resistance mechanism is extended to other transporters or chemicals. Moreover, preliminary data regarding anti-inflammatory properties suggest a possible application in the treatment of inflammatory bowel disease.

# Appendix

Colorectal Adenocarcinoma cell lines LoVo, Caco-2 (ATCC) and LoVo/Dx (kindly proided by Prof. Decorti) were cultured in DMEM supplemented with 10% FBS, 1% L-glutammine and 1% Penicillin/Streptomicyn.

All components for cell culture, toghether with Trypsin, RNAse, FITC, PI, PVDF membranes, Kodak films, Developer and Fixer solutions, $H_2O_2$, were purchased from Merk-Sigma (USA).

DEL was purchased from Extrasynthes (FR); Doxorubicin was purchased from Merk-Sigma (USA); stock solutions were prepared by dissolving the substances in either MeOH/PerchloricAcid 0.8% (DEL), or distilled water (doxorubicin), and stored at –20°C.

Medium molecular weight chitosan with a deacetilation degree >60% was used for NPs preparation mixing it with Tripolyphosphate pentasodium (TPP) (Merk-Sigma).

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