Sara Alexandra Luis Nunes
Degree in Biochemistry

Modulation of inflammatory mediators by *Opuntia ficus-indica* and *Prunus avium* bioproducts using an *in vitro* cell-based model of intestinal inflammation

Dissertation to obtain a Master Degree in Biotechnology

Supervisor: Ana Matias, Ph.D IBET/ITQB-UNL
Co-Supervisor: Catarina Duarte, Ph.D IBET/ITQB-UNL

Júri:

Presidente: Prof. Doutor Rui Manuel Freitas Oliveira
Arguente: Doutora Joana Estevão de Matos
Vogal: Doutora Ana Matias

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

September 2011

Sara Alexandra Luis Nunes
Degree in Biochemistry

Modulation of inflammatory mediators by *Opuntia ficus-indica* and *Prunus avium* bioproducts using an *in vitro* cell-based model of intestinal inflammation

Dissertation to obtain a Master Degree in Biotechnology.

Supervisor: Ana Matias, Ph.D, ITQB/IBET
Co-Supervisor: Catarina Duarte, Ph.D, ITQB/IBET

Júri:

Presidente: Prof. Doutor Rui Manuel Freitas Oliveira
Arguente: Doutora Joana Estevão de Matos
Vogal: Doutora Ana Matias

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

September 2011

Modulation of inflammatory mediators by *Opuntia ficus-indica* and *Prunus avium* bioproducts using an *in vitro* cell-based model of intestinal inflammation

Acknowledgments

Gostaria de agradecer a todas as pessoas que directa ou indirectamente contribuiram para a realização deste trabalho e sem as quais seria muito mais difícil conclui-lo.

Em primeiro lugar, gostaria de agradecer à Dra. Ana Matias que foi incansável em todo este processo em que esteve sempre disponível para compartilhar as suas ideias e conhecimentos, tendo sempre uma palavra de incentivo e de apoio quando qualquer coisa corria menos bem. Obrigada Ana, foi um enorme prazer trabalhar contigo.

À Dra. Catarina Duarte por ter acreditado em mim e neste trabalho, por ter sempre uma palavra de incentivo, motivação e de apoio e, também, por todas as portas que me abriu.

A todas as pessoas do laboratório de Nutracêuticos e Libertação controlada que tornaram o ambiente de trabalho impecável. À Dra. Teresa Serra que esteve sempre disponível para ajudar, e sempre com uma palavra válida e excelentes ideias. À Joana Poejo que tornou este ano mais divertido e foi sempre uma grande ajuda, obrigada. À Ana Nunes pela excelente companhia que sempre foi. À Patrícia Almeida pela disponibilidade e por ter sido a primeira pessoa a ensinar-me a trabalhar com culturas de células animais. Ao Agostinho Alexandre por toda a boa disposição durante o processamento dos “Opuntias”. À Dora Pereira, que apesar de já não estar cá ensinou-me imenso e foi sempre uma companhia espectacular. Gostaria também de agradecer à Professora Rosário Bronze e à Catarina Semedo pelo apoio na parte analítica de identificação dos compostos fenólicos.

A todos os meus amigos por terem sido um suporte fundamental, por todos estes anos, as conversas e toda a ajuda, toda a companhia e apoio, por estarem sempre presentes.

Ao Pedro, que foi indispensável pela sua compreensão e apoio incondicional durante todo este percurso, por todo o carinho e bom humor que sempre me transmitiu e por toda a ajuda. Obrigada, sem ti não seria a mesma coisa!

À minha família: avós, tios e primos que sempre acreditaram em mim e que ficaram orgulhosos pelo finalizar desta etapa e que muito contribuíram para que fosse possível. Aos meus pais, que tanto esforço fizeram para que conseguisse chegar até aqui e que sempre acreditaram em mim, obrigada e espero um dia poder retribuir tudo o que já fizeram por mim. Por fim, mas também muito importante, aos meus irmãos que estiveram sempre ao meu lado para me ajudar e incentivar e que foram indispensáveis para tornar possível a concretização de mais uma etapa da minha vida.

Obrigada a todos!

Sara Nunes

Abstract

Inflammatory Bowel Diseases, namely Ulcerative colitis and Crohn's disease, are chronic intestinal inflammatory disorders characterized by an excessive release of pro-inflammatory mediators, intestinal barrier dysfunction and altered permeability and excessive activation of NF-κB cascade that can lead to development of colon cancer. IBD conventional therapy involves multiple medications and long-term up to life-long treatments. Furthermore, these therapies are insufficiently selective and associated to severe side effects. Phenolic compounds are considered to possess antioxidant and anti-inflammatory activities and are a great hope in prevention and treatment of chronic intestinal inflammation. The aim of this study was to evaluate the preventive and prophylactic effects of *Opuntia ficus-indica* (cactus pear) and *Prunus avium* (cherry) polyphenolic-rich extract (PRE) in an *in vitro* cell-model of Inflammatory Bowel Diseases.

The phenolic composition of each PRE is completely different: for cherry's PRE the main compounds founded were anthocyanins while in cactus pear's PRE were isorhamnetin and its glycoside derivates and also a betaxantin.

Cactus pear's and cherry's extract showed similar antioxidant activity in ORAC assay but cherry's PRE has shown to be better in preventing radical formation by metal chelation (HORAC assay) and in inhibiting AAPH-induced LDL-oxidation.

Cherry's PRE exert a most significant protection against intracellular ROS formation, glutathione and protein oxidation than cactus pear's PRE.

Both extracts showed to be effective in reducing inflammatory mediators secretion (IL-8 and NO), attenuating barrier dysfunction and reducing NF-κB pathway activation. However, cherry's PRE were more efficient in both perspectives studied. Both PREs didn't affect the IL-10 secretion.

Anti-inflammatory activity of 5-aminosalicylic acid, a common drug used in IBD therapy, was compared with cherry's PRE. Both agents could modulate barrier dysfunction and permeability alteration and could, also, decrease IL-8 and NO secretion. Moreover, 5-ASA could increase IL-10 secretion.

Obtained results suggest that cherry's PRE could be use as co-therapeutic agent in IBD patients.

Keywords: Inflammatory Bowel Diseases, polyphenols, Antioxidant activity, Anti-inflammatory activity, Caco-2 cells, *Opuntia ficus-indica*, *Prunus avium*

Resumo

As Doenças Inflamatórias Intestinais (DII), nomeadamente a Colite ulcerosa e a doença de Crohn, são doenças inflamatórias crónicas caracterizadas por uma excessiva produção de mediadores pró-inflamatórios, disfunções na barreira intestinal e, consequentemente, alterações na sua permeabilidade e uma excessiva activação do factor de transcrição NF-κB, podendo levar ao aparecimento de cancro do colón. A terapia convencional envolve várias medicações e tratamentos de longo termo ou vitalícios. Estes tratamentos são insuficientemente selectivos e associados a severos efeitos secundários. Os polifenóis são reconhecidos por possuírem actividade anti-oxidante e anti-inflamatória tornando-se uma grande esperança no tratamento e prevenção das DII. O objectivo deste trabalho foi avaliar os efeitos anti-inflamatórios de dois extractos ricos em polifenóis (ERP) derivados de *Opuntia ficus-indica* (figo-da-índia) e *Prunus avium* (cereja) como agentes preventivos ou profiláticos, num modelo celular *in vitro* que mimetize as DII.

Os ERPs possuem uma composição polifenólica diferente: o ERP de cereja é principalmente composto por antocianinas enquanto que o ERP de figo-da-índia possui isoramnetinas e derivados e também uma betaxantina.

Ambos os ERPs possuem actividade anti-oxidante similar no ORAC mas o ERP de cereja apresenta maior HORAC e maior inibição da oxidação da LDL.

Observou-se que o ERP de cereja exerce uma protecção mais significativa contra a formação de radicais intracelulares e contra a oxidação da glutationa e proteinas.

Ambos os extractos estudados mostraram-se capazes de reduzir a secreção de mediadores inflamatórios (IL-8 e NO), atenuar a disfunção da barreira intestinal e reduzir a activação do NF-κB. Contudo, o extracto de cereja foi o mais efectivo nas duas perspectivas estudadas. Nenhum ERP afectou a secreção de IL-10.

A actividade anti-inflamatória do ácido 5-aminosalicilico, um agente comum no tratamento das DII, foi comparada com o ERP de cereja. Ambos são capazes de modular a disfunção da barreira intestinal e as alterações na sua permeabilidade sendo, também, capazes de modular a secreção de IL-8 e de NO. Contudo, apenas o 5-ASA aumentou a secreção de IL-10.

Os resultados obtidos sugerem que o ERP de cereja pode ser usado como agente co-terapêutico em pacientes que sofrem de DII.

Palavras-chave: Doenças Inflamatórias Intestinais, polifenóis, actividade anti-oxidante, actividade anti-inflamatória, células Caco-2, *Opuntia ficus-indica*, *Prunus avium*

Index

Index of figures

Index of tables

List of abbreviations, acronyms and symbols

Abbreviation	Full Form
5-ASA	5-aminosalicylic acid
AAPH	2',2'-Azobis (2-amidinopropane) dihydrochloride
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumine
Caco-2	Caco-2 human colon carcinoma cell line
CAEAC	Caffeic Acid Equivalents Antioxidant Capacity
CD	Cronh's Disease
COX-1	Cyclooxygenase 1
COX-2	Cyclooxigenase 2
CRP	C - reactive protein
CVD	Cardiovascular Diseases
DAD	Diode Array Detector
DCFH-DA	2',7'-Dichlorofluorescin Diacetate
DNA	Deoxyribonucleic acid
DNPH	2,4-dinitrophenylhydrazine
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
ECGC	Epigallocatechin Gallate
ED	Electrochemical Detector
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
EtOH	Ethanol
EU	European Union
FBS	Fetal Bovine Serum
FL	Disodium Fluorescein
GAE	Gallic Acid Equivalents
GPx	Glutathione peroxydase
GSH	Glutathione
GSSG	Glutathione disulfide
HORAC	Hydroxyl Radical Adverting Capacity
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxydase

IBD	Inflammatory Bowel Disease
IEC	Intestinal Epithelial cells
IFN-γ	Interferon gamma
IgG	Imunnogloblulin G
IL-1	Interleukin-1
IL-1β	Interleukin 1-beta
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	inducible Nitric Oxide Synthase
IκB	Inhibitor-κB
IκBα	Inhibitor-κB alpha
LDL	Low Density Lipoprotein
LPMCs	Lamina Propria Mononuclear Cells
LPS	Bacterial Lipopolyssaccharides
MAPK	Mitogen-activated protein kinases
MetOH	Methanol
mRNA	Messenger Ribonucleic acid
NF-κB	Nuclear Factor-κB
NO	Nitric Oxide
OPA	Orthophthalaldehyde
ORAC	Oxygen Radical Absorbance Capacity
Papp	Apparent Permeability
PBS	Phosphate Buffer Saline
PenStrep	Penicillin-Streptomycin
PRE	Polyphenolic-Rich Extract
ROS	Reactive Oxygen Species
rpm	Rotation *per* minute
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
Ser	Serine
SOD	Superoxide Dismutase
TBS	Tris Buffer Saline
TBST	Tris Buffer Saline Tween-20
TEAC	Trolox Equivalent Antioxidant Capacity

TEER	Transepithelial Electrical Resistance
TEMED	*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine
Thr	Threonine
TJ	Tight Junction
TMB	3,3’,5,5’-tetramethylbenzidine
TNF-α	Tumor Necrosis Factor – alpha
TRIS	Tris(hydroxymethyl)aminomethane
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UC	Ulcerative Colitis
UV	Ultra-Violet
WB	Western Blot

1. Introduction

1.1. Diet and Health

The food we consume affects our body beyond just providing essential nutrition and minerals. A diet rich in fruits, vegetables and other plant foods has been associated with a decreased risk of certain diseases such as cardiovascular disease, cancer and inflammatory diseases. Although some specific micronutrients like vitamins or minerals play an important role on this reduction, other plant components, namely polyphenols, may have a strong contribution in beneficial effects related to this kind of food. So, it has been recommended eating at least five servings *per day* of fruits and vegetables in order to reduce the risk of chronic diseases development (Fraga et al, 2010; Paredes-López et al, 2010; Quideau et al, 2011).

1.1.1 Polyphenolic compounds and their bioactivity

Polyphenols are widespread naturally-present constituents of plant-derived food such as fruits, vegetables, cereals, cocoa and beverages like tea, coffee and wine, and are a diverse class of plant secondary metabolites characterized by a polyphenol structure that have one or more aromatic ring with one or more hydroxyl group (Quideau et al, 2011). There are more than 8000 different polyphenols and according to their chemical structure they can be divided into five classes: phenolic acids, flavonoids, stilbenes, lignans and tannins (figure 1.1) (Fraga et al, 2010; Ignat et al, 2011; Liu, 2004).

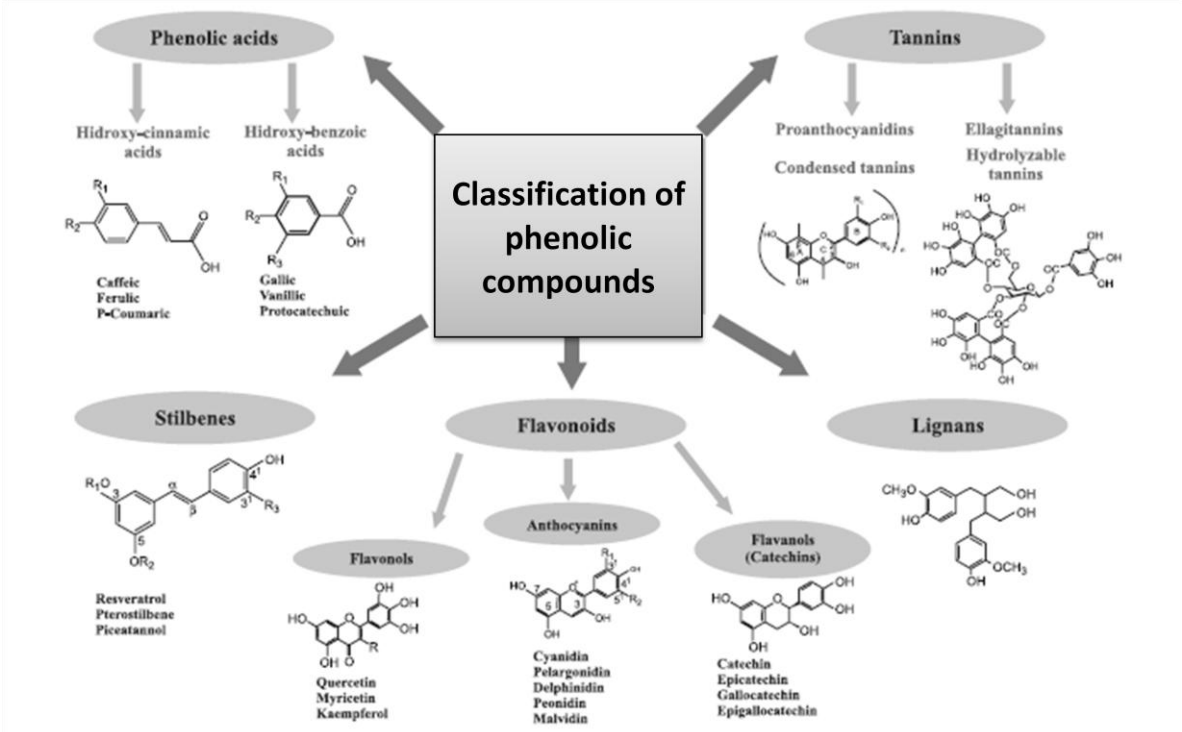


Figure 1.1 – Classification of polyphenols. Adapted from (Paredes-López et al, 2010).

Phenolics, or polyphenolic compounds, have many different functions in plants such as colour of leaves, flowers and fruits, anti-microbial and fungal function, insect feeding deterrence, screening from damage by solar UV radiation, chelation of toxic heavy metals and anti-oxidant protection against free radicals generated during the photosynthetic process (Stevenson & Hurst, 2007).

Total dietary intake of polyphenols is approximately 1g *per* day but only about 10% of this quantity is absorbed by gastrointestinal track (table 1.1) (Williamson & Manach, 2005). Despite of this fact, many health benefits of food are associated with these compounds (figure 1.2), mainly due to their strong antioxidant capacity.

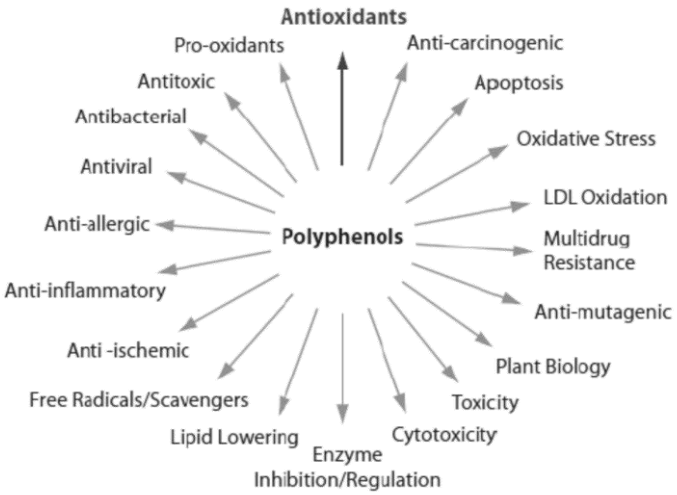


Figure 1.2 – Polyphenols Bioactivities (Alexis-Biochemicals, 2010).

Nowadays, polyphenols, as well as their metabolites, are recognized not only by their protection against oxidative stress, but also by their modulatory actions in cells through direct interaction with receptors or enzymes involved in signal transductions, such as protein and lipid kinases signaling pathways. So, many mechanisms have been described to explain polyphenol's health benefits: antioxidant action by scavenging radicals and induction of endogenous antioxidants (glutathione peroxidase, glutathione reductase, superoxide dismutase, lipooxygenase, xanthine oxidase, among others); iron chelating properties; modulation of genes related to cell survival/death modulation, gene/protein and cell signaling pathway regulatory activity and regulation of mitochondrial function, enzymatic regulations among others (Fraga et al, 2010; Quideau et al, 2011; Rahman et al, 2006). Due to their many ways of actions, polyphenols have potential health benefits in many diseases such as cancer, inflammatory and allergic diseases, cardiovascular diseases, diabetes and neurodegenerative conditions (table 1.1) (Quideau et al, 2011; Rodrigo et al, 2011; Singh et al, 2011; Stevenson & Hurst, 2007). Although, it is important to know that pharmaceuticals drugs have strongest action than polyphenols, but since they are consumed regularly in significantly amounts in an equilibrate diet, they can exhibit noticeable long-term physiological effects (Espín et al, 2007).

Table 1.1 - Summary of main findings from the papers reviewed about Polyphenols (PPs) and their potential health benefits (Stevenson & Hurst, 2007).

Finding
Polyphenolic dietary intake ~1g/day.
~ 90% of dietary polyphenols are not directly bioavailable but are metabolised by the colonic microflora into bioavailable phenolic acids.
Phase II conjugation of polyphenols is extensive *in vivo* and may radically change biological activity.
More polyphenols is not necessarily better; exceptionally high doses have demonstrated detrimental effects.
In vitro, polyphenols can induce the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase.
Polyphenols appear to be pro-oxidants in cell-based studies, but nevertheless appear to protect cultured cells from oxidative stress.
Human trials show correlations between polyphenols (particularly flavonoids) intake and reduced incidence of cancer and cardiovascular disease (CVD), and slower neuro-degeneration.
In vitro regulation of inflammatory pathways by inhibition of signalling molecules such as TNF and by down-regulation of inflammatory genes may e the mechanism of beneficial effects on CVD.
Polyphenols may be able to help regulate blood pressure.
Polyphenols may be able to modulate immune responses in allergic conditions.
Regulation of cell proliferation and differentiation, angiogenesis and apoptosis: may explain anti-cancer effects.
Neuro-protection, by protection of neuronal cells from oxidative stress, induction of antioxidant defences and modulation of signalling cascades and apoptotic processes.
Regulation of metabolic syndrome, via inhibition of glucose uptake and regulation of some metabolic pathways.
Benefits to gut health by growth inhibition of pathogenic gut bacteria and modulation of inflammatory Bowel conditions.

1.1.2 Functional food and Nutraceuticals

Nowadays, it is widely recognized that a diet rich in plant-based food provides health benefits and a reduction of chronic diseases risk (Dai & Mumper, 2010; Kelsey et al, 2010; Shahidi, 2009). Thus, in the last few years there was an increased demand of products rich in bioactive compounds derived from natural matrixes which leads to an increase in consumption of nutraceuticals and functional food (Espín et al, 2007).

The term **nutraceutical** was first defined by DeFelice, director of the Foundation for Innovation in Medicine and it is defined as diet supplements that deliver a concentrated form of a presumed bioactive agent from a food, presented in a non-food matrix, and used with the purpose of enhancing health in dosages that exceed those that could be obtained from normal foods and are sold as pills, extracts, tablets, etc. (Dillard & German, 2000; Espín et al, 2007), while **functional foods** are those that when consumed regularly exert a specific health beneficial effect beyond their nutritional properties (i.e., a healthier status or a lower risk of disease) and this effect must be scientifically proven (Dillard & German, 2000; Espín et al, 2007; Eussen et al, 2011; Shahidi, 2009).

The most common plant-derived bioactive compounds found in the nutraceutical market includes polyphenols such as anthocyanins, proanthocyanidins, phenolic acids, stilbenes, hydroxylcinnamates, coumarins, flavonols, ellagic acid and ellagitannins, isoflavones, lignans (Espín et al, 2007) . The increasing search of this kind of products leads to an effort to develop new nutraceuticals composed by polyphenols and other plant bioactive compounds in order to reduce, delay or prevent chronic diseases like cancer, inflammatory, neurodegenerative and cardiovascular diseases, diabetes, etc (Espín et al, 2007; Kelsey et al, 2010).

1.2. Oxidative Stress

Oxidative stress constitutes a unifying mechanism of injury of many types of diseases such as diabetes, neurodegenerative and cardiovascular diseases, inflammatory bowel disease and cancer, and it is an interest target for prevention and therapeutic of these diseases (Rodrigo et al, 2011; Willcox et al, 2004).

1.2.1 Definition and antioxidant defense system

Oxidation reactions are an essential part of normal metabolism and oxygen is the ultimate electron acceptor in the electron flow system that produces ATP. Oxidative stress occurs when there is a serious imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense systems in the body so that the latter become overwhelmed (Masella et al, 2005; Rodrigo et al, 2011) . In fact, ROS, a family of highly reactive species (table 1.2), are continually produced within the cells as a result of mithocondrial electron transfer processes or as bioproducts of the enzymes xanthine oxidase, lipooxygenases and cycloxygenases and are also generated as a consequence of intracellular xenobiotic's metabolism, activated neutrophils, UV exposure, environmental factors, etc (figure 1.3) (Halliwell, 2011; Masella et al, 2005).

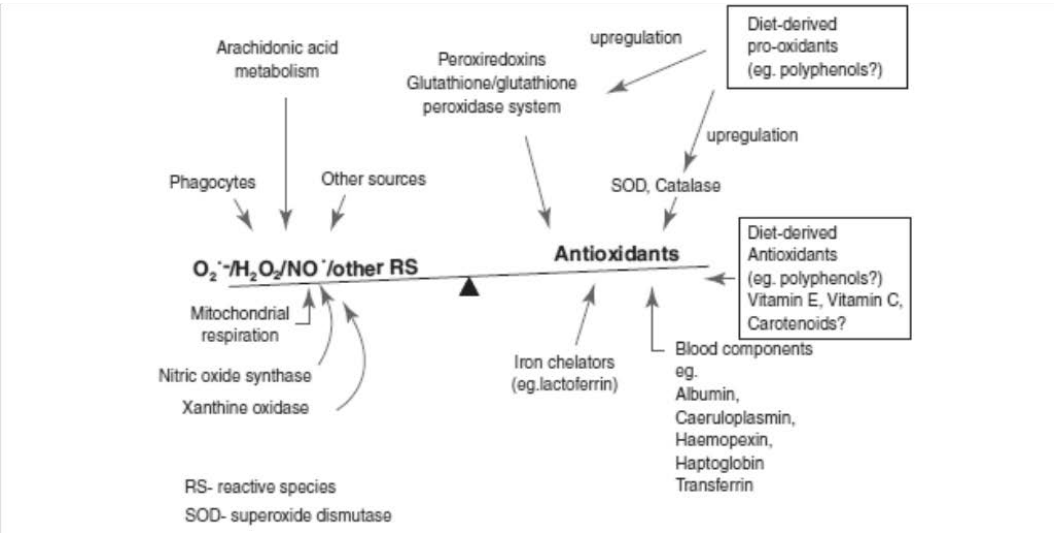


Figure 1.3 –Balance between ROS and antioxidants *in vivo* (Halliwell, 2011).

So, ROS can play two different roles *in vivo*: a positive role being involved in energy production, phagocytosis, cell growth and intercellular signaling regulation; and a negative role being highly damaging since they can attack biological macromolecules, namely, lipids, proteins and DNA leading to membrane and DNA damages and enzyme inactivation (Finley et al, 2011; Masella et al, 2005; Rezaie et al, 2007). In order to avoid a ROS excess in organism, humans have sophisticated protective mechanisms that include enzymatic and nonenzymatic antioxidant defenses produced in body, namely endogenous, and other that can be obtained in diet, namely exogenous (figure 1.3) (Halliwell, 2011; Masella et al, 2005; Sorg, 2004; Willcox et al, 2004).

Table 1.2 – Molecules mediating oxidative stress. Adapted from (Sorg, 2004).

Name	Structure	Main Reactions
Superoxide	•O-O$^-$	Formation of hydrogen peroxyde or peroxynitrite.
Hydrogen peroxide	HO-OH	Formation of hydroxyl radical, enzyme inactivation, oxidation of biomolecules.
Hydroxyl radical	•OH	Hydrogen abstraction, production of free radicals and lipid peroxides; oxidation of thiols
Ozone	$^-$O-O$^+$=O	Oxidation of all kinds of biomolecules, especially those containing double bonds, formation of ozonides and cytotoxic aldehydes.
Singlet oxygen	O=O	Reaction with double bounds, formation of peroxydes, decomposition of amino acids and nucleotides.
Nitric oxid	•N=O	Formation of peroxynitrite; reaction with other radicals.
Peroxynitrite	O=N-O-O$^-$	Formation of hydroxyl radical, oxidation of thiols and aromatic groups, conversion of xanthine dehydrogenase to xanthine oxydase; oxidation of biomolecules.
Hypochlorite	ClO$^-$	Oxidation of amino and sulphur-containing groups; formation of chlorine.
Radical	R•	Hydrogen abstraction; formation of peroxyl radicals and other radicals, decomposition of lipids and other biomolecules.
Peroxyl radical	R-O-O•	Hydrogen abstraction, formation of radicals, decomposition of lipids and other biomolecules.
Hydroperoxyde	R-O-OH	Oxidation of biomolecules, disruption of biological membranes.
Copper and iron ions	CU^{2+}, Fe^{3+}	Formation of hydroxyl radical by Fenton and Haber-Weiβ reaction.

Antioxidants are defined as molecules that protect a biological target against oxidative damage (Halliwell, 2011) and can be divided into three lines: as a first line of body defences are the preventive antioxidants such as peroxidises and metal chelating proteins that suppress the generation of free radicals; in the second line are the radical-scavenging antioxidants such as Vitamin C and E that inhibit the oxidation chain initiation and prevent chain propagation; finally is the third line of defence composed by the repair and *de novo* enzymes (lipases, proteases, DNA repair enzymes and transferases) that can repair the damages and reconstitute biological membranes (figure 1.4) (Finley et al, 2011; Willcox et al, 2004).

Endogenous antioxidants include the most efficient enzymes that catalyse the reduction of ROS such as: Superoxide dismutase (SOD) that catalyses the dismutation of superoxide radical into hydrogen peroxide and oxygen; Catalase that catalyses hydrogen peroxide dismutation into water and oxygen; Glutathione peroxydases (GPx) that reduce hydrogen and organic hydroperoxides, etc. The reduced cofactor glutathione (GSH) plays a central role in intracellular endogenous antioxidant system since it is very important to ROS detoxification acting not only as a enzyme cofactor but also as a radical- scavenger (Finley et al, 2011; Halliwell, 2011; Masella et al, 2005; Rodrigo et al, 2011; Sorg, 2004; Willcox et al, 2004). Exogenous antioxidants are those that are obtained from diet and include compounds such as vitamin E,C and β-caroten, minerals such as zinc and selenium (enzymes cofactors) and polyphenols, among others (Finley et al, 2011; Masella et al, 2005).

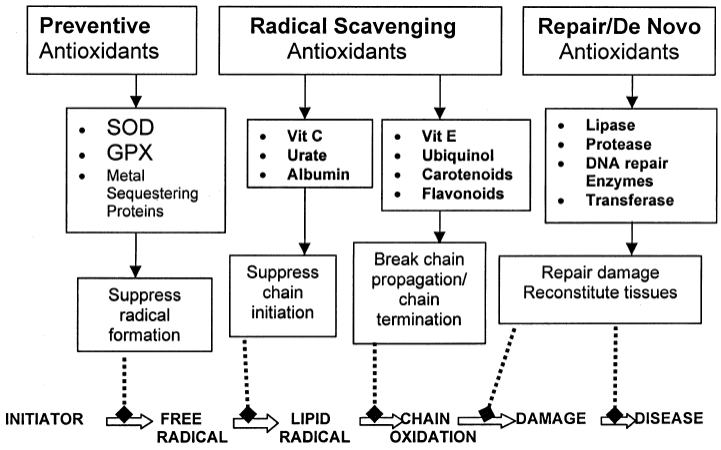


Figure 1.4 – Antioxidant groups and actions (doted line = suppression). Adapted from (Willcox et al, 2004).

1.2.2 Polyphenols as antioxidants

In the recent years there as been a significant increment in scientific knowledge dealing with the beneficial role of polyphenols during oxidative stress and several polyphenols have been demonstrated to have clear antioxidant properties as they can act as chain breakers or radical scavengers depending on their chemical structure. Polyphenols can also act as enzyme modulators, can interact with signal transduction pathway and/or with cell receptors and can offer indirect protection by activating endogenous defense system (D'Archivio et al, 2007; Finley et al, 2011; Fraga et al, 2010; Rahman et al, 2006; Rodrigo et al, 2011).

One of the major antioxidant strategies is the scavenging of free radicals and phenolic compounds are highly efficient in breaking free radical chain reactions since they have the phenolic OH groups that are able to reduce free radicals through a one-electron donation and/or the aromatic structures that allows the stabilization by resonance of radicals. On the other hand, sequestration of metals to prevent metal-catalyzed free radical formation is another important strategy and, once more, polyphenols can be useful since some of these compounds have chemical groups that are centers of high affinity for metal ions (hydroxyl and carbonyl groups, and catechol moieties). Flavonoids, for example, have been identified as potent metal chelating compounds due to their structure: the number of OH group and its position on the ring of molecule determine their antioxidant capacity (Fraga et al, 2010; Rodrigo et al, 2011).

The indirect action of polyphenols as antioxidant can be explained by their capacity to modulate antioxidant enzymes activity and also the expression of these enzymes. Several studies show a positive effect of different classes of polyphenols on some antioxidant enzyme activities such as SOD and GPx. The increase in enzymes activities was accompanied by a significant increase of the glutathione reduced form (GSH) and decrease in lipid peroxidation (Masella et al, 2005; Rahman, 2008; Rahman et al, 2006). Due to their different mechanisms of action, polyphenols are very interesting compounds to prevent, treat or delay diseases related with oxidative stress (Finley et al, 2011; Halliwell, 2011; Masella et al, 2005; Rahman et al, 2006; Rodrigo et al, 2011; Willcox et al, 2004).

1.2.3 *Opuntia ficus-indica*

Cactus pear (*Opuntia ficus-indica*) belongs to *Opuntia spp*. and is the most common cactus and one of the most important members of the *Cactaceae*. It is well adapted to grow in arid or semi-arid regions and produce edible stems and fruits which make this plant a very important food source, due to the agricultural problems in these regions (Guevara-Figueroa et al, 2010; Moussa-Ayoub et al, 2011). Cactus fruits have an important role in prevention of approximately 80 diseases like cancer, cardiovascular diseases, skin disorders, inflammatory diseases, among others (Ching, Ling Soon*, et al*, 2001). Due to their composition, *O.ficus-indica* fruits have been widely used as folk medicine possessing many health-promoting properties mainly because of their rich composition in bioactive antioxidant compounds which makes cactus pear a perfect candidate for nutraceuticals applications (Cayupan et al, 2011; Feugang et al, 2006; Moussa-Ayoub et al, 2011; Stintzing & Carle, 2005). Cactus fruit is a very rich source of flavonoids such as isorhamnetin glycosides, quercetin and their derivates, as well as a very important source of Betalains from two different groups: betaxanthins, yellow and orange coloured, and betacyanins, red to purple coloured (figure 1.5) (Cayupan et al, 2011; Chavez-Santoscoy et al, 2009; Moussa-Ayoub et al, 2011).

Betalains and flavonoids are known by their antioxidant capacity (Obrenovich et al, 2011). Besides antioxidant activity, many other benefic properties are associated with cactus pear, namely anti-inflammatory properties, anti-diabetic effect, cholesterol-lowering properties, analgesic action, among others (Feugang et al, 2006; Moussa-Ayoub et al, 2011).

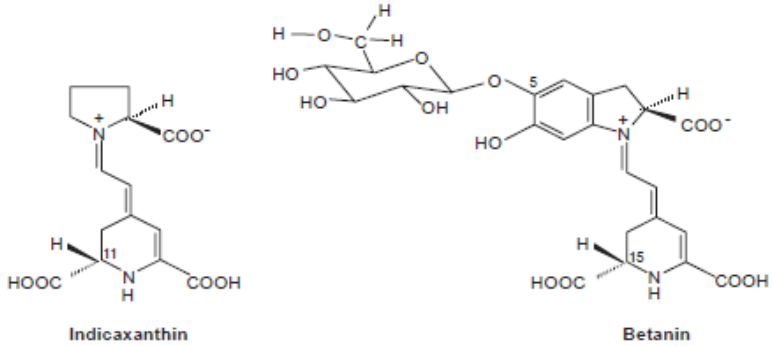


Figure 1.5 - Structures of the major betalains indicaxanthin (yellow) and betanin (red) of cactus pear (Mossammer et al, 2005).

1.2.4 *Prunus Avium*

Sweet cherries are a very attractive fruit to consumers, for their taste and colour and also for their recognized health promoting properties (Serra et al, 2011a). Cherries have a very rich composition in polyphenols including flavonoids (anthocyanins, flavan-3-ols, and flavonols), hydroxycinnamic acids and hydroxybenzoic acids (Ferretti et al, 2010; Serra et al, 2010). Among all of these compounds, anthocyanins, that are responsible for the red colour of fruits, are the most interesting compounds not only because of their high content in this fruit, but also for their high bioactivity (McCune et al, 2011; Usenik et al, 2008). Anthocyanin composition in cherries includes among others, cyanidin 3-glucoside, cyaniding-3-rutinoside, peonidin 3-rutinoside, pelargonidin 3-rutinoside and 3-glucoside (Ferretti et al, 2010; Serra et al, 2011a).

Many health benefits have been reported to this fruit (figure 1.6), including antioxidant activity, anti-proliferative and anti-cancer properties, anti-inflammatory effects, protection against cardiovascular diseases and retarding of aging process (Ferretti et al, 2010; McCune et al, 2011; Serra et al, 2011a).

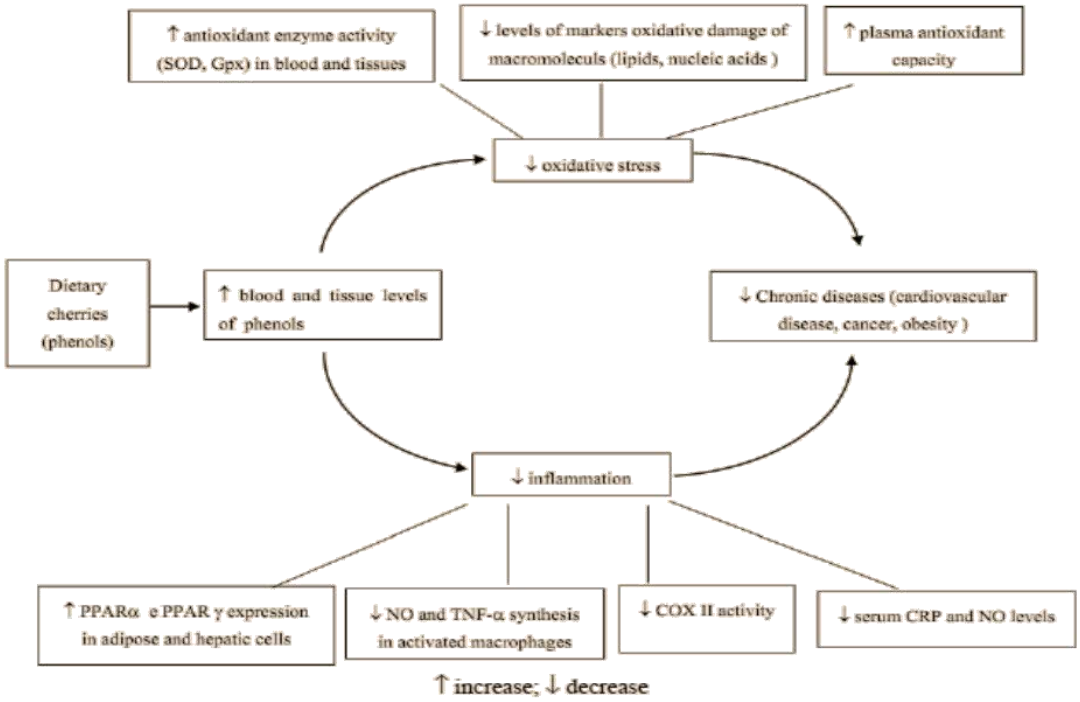


Figure 1.6 - Protective effects exerted by cherry phenols and implication in chronic diseases.

"Saco" cherry is a traditional Portuguese variety that has protected geographical indication (PGI) registration according to EU regulations ("Cova da Beira" Cherry). This variety has a powerful antioxidant capacity and a strong anti-proliferative effect but it is not well accepted by consumers due to it small size and weight (Serra et al, 2011b). Anti-inflammatory activity in Inflammatory Bowel Diseases will be assessed to this cherry variety in this work.

1.3. Inflammatory Bowel diseases

Intestinal inflammation is a natural and protective process, which is crucial to maintain gut integrity and function, although, in some individuals the homeostasis of this process is altered leading to the appearing and development of chronic inflammation, namely, Inflammatory Bowel Diseases (IBD) (Romier et al, 2009; Van De Walle et al, 2010).

1.3.1 Epidemiology, etiology, pathogenesis and causes of IBD

Inflammatory Bowel Diseases encompasses several chronic inflammatory conditions, most significantly Ulcerative Colitis (UC) and Crohn's Disease (CD) (Frontela et al, 2010; Mandalari et al, 2011; Rahimi et al, 2009; Romier et al, 2008). Due to their high incidence and prevalence, these diseases are recognized as a major health complication in developed countries, spreading to the rest of the world, and are thus associated with severe socio-economic problems (Sergent et al, 2010). In Western Europe, IBD affects 0,5-1% of the population with 56 to 104 new cases *per* million inhabitants

per year, and particularly in Portugal, according to IBD Portuguese Association, there are 14 thousands Portuguese habitants affected by these diseases and their incidence is increasing with 140 new cases *per* year (Rahimi et al, 2009; Romier et al, 2009; Romier et al, 2008). It is important to know that one of the worst complications of IBD is the development of colon cancer (Romier et al, 2009).

IBD remains a complex and inadequately understood disease but, although the etiology of these diseases remains unknown, the most accepted hypothesis today implicates more than one factor that contribute for the development of this disease including environmental factors, familial and genetic factors, microbial agents, intestinal immune and non immune systems, etc (figure 1.7) (Atreya & Neurath, 2010; Bruewer et al, 2006; Frontela et al, 2010; Mandalari et al, 2011; Romier et al, 2009; Sergent et al, 2010; Tanoue et al, 2008).

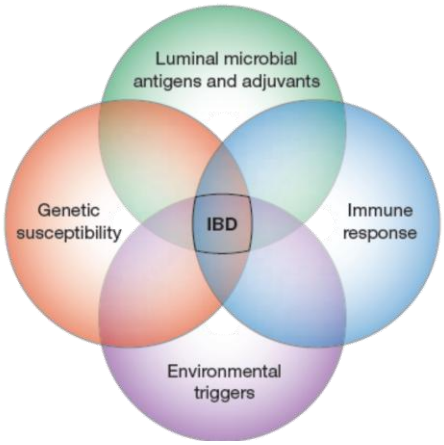


Figure 1.7 – Interaction of various factors that contribute to pathogenesis of Inflammatory Bowel Disease (Sartor, 2006).

So, Inflammatory Bowel Diseases are characterized by chronic and unpredictable attacks of intestinal inflammation causing weight loss, diarrhea, rectal bleeding, abdominal pain, fever and anemia (Van De Walle et al, 2010). Several studies show that these disorders result from dysfunctional epithelial and immune responses against the normal enteric microflora and can involve different mechanisms: (1) an immune response to a specific pathogen resulting in intestinal infection; (2) alterations in normal bacterial content of the intestinal tract; (3) a defective mucosal barrier and overwhelming exposure to resident bacteria and their antigens and endotoxins; and (4) alterations in the intestinal immune response (Edelblum & Turner, 2009; Mandalari et al, 2011; Romier-Crouzet et al, 2009). The IBD characteristic defective epithelial cell barrier functioning and the exaggerate immune activity are thought to cooperate in a self amplifying loop, where the barrier dysfunction causes an increased paracellular permeation of harmful luminal antigens and thus increases activation of mucosal immune cells leading in turn to increased release of inflammatory stimuli, intestinal epithelial cells (IECs) response and further barrier dysfunction. Thus, the inflammatory response is not just responsibility of immune cells but also of IECs (Leonard et al, 2010; Siccardi et al, 2005; Van De Walle et al, 2010).

Various and different mediators are involved in development and maintenance of chronic intestinal inflammation such as cytokines, chemokines, adhesion molecules and free radicals (Danese et al, 2005; Kaplan et al, 2007; Sergent et al, 2010).

1.3.2 Role of cytokines and chemokines in IBD

It is now well known that pro-inflammatory mediators play a crucial role in IBD pathogenesis, particularly cytokines and chemokines that are small (4-15KDa) inducible immune-regulatory proteins (figure 1.8) (Atreya & Neurath, 2010; Romier et al, 2009; Romier-Crouzet et al, 2009; Van De Walle et al, 2010). In both Crohn's Disease and Ulcerative Colitis have been demonstrated alterations in the mucosal cytokine profile resulting in an imbalance between pro- and anti-inflammatory cytokines and consequently an excessive recruitment of effector cells and induction of tissue-damaging (like nitric oxide) and other inflammatory mediators (leukotriens and prostaglandins), which further amplify mucosal inflammation (Atreya & Neurath, 2010).

Chemokines that are chemoattractant cytokines and their corresponding receptors play, also, a key role in the inappropriate recruitment and accumulation of leukocytes into the inflamed gut and stimulate production of metalloproteinases for matrix degradation and oxidative stress which all contribute to chronic intestinal inflammation and mucosal destruction (figure 1.8) (Atreya & Neurath, 2010; Tanoue et al, 2008).

In the case of cytokines, TNF-α (tumor necrosis factor alpha), IFN-γ (interferon gamma) and IL-1β (interleukine 1-beta) are the three major ones involved in IBD pathogenesis (Al-Sadi & Ma, 2007; Atreya & Neurath, 2010; Tanoue et al, 2008; Van De Walle et al, 2010). High levels of TNF-α are consistently observed in these disorders and play a key role in acute phase of inflammation and diverse immunological processes including a decrease in epithelial barrier function and activation of nuclear factor–κB (NF-κB) (see page 12) (Bruewer et al, 2006; Edelblum & Turner, 2009; Erlejman et al, 2008; Satsu et al, 2009; Tanoue et al, 2008; Van De Walle et al, 2010).

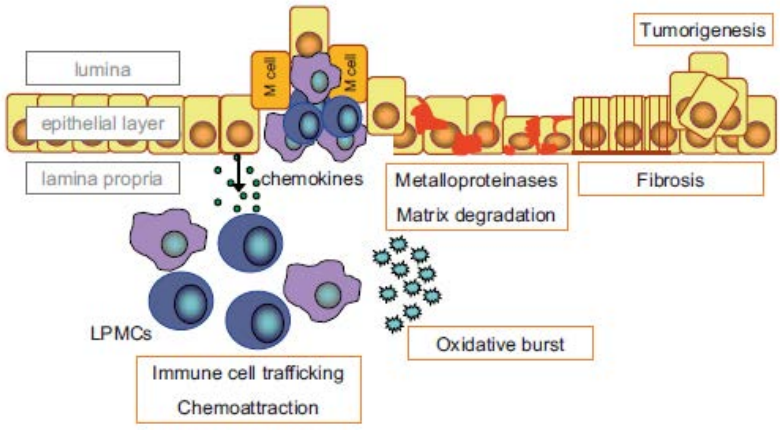


Figure 1.8 – Role of chemokines in IBD. Chemokines and their corresponding receptors contribute to the intestinal inflammatory process in IBD. They control the multistep process of the migration of effector immune cells across the endothelium, modulate the release of lipid mediators and oxygen radicals from leukocytes, regulate the production of metalloproteinases and matrix degradation, influence tissue fibrosis and participate in the process of tumorigenesis. LPMCs = Lamina propria mononuclear cells (Atreya & Neurath, 2010).

IL-1β is an important multifactorial cytokine with functions in the immune system during development, infection, inflammation, cell differentiation, tissue remodeling and cell death, playing a key role in IBD pathogenesis being in very high concentrations in the intestinal tissue of patients with this disease. It mediates important features of this pathology such as the generation of fever, the reduction of appetite, the release of mediators and the recruitment of leukocytes. This cytokine also

increases paracellular permeation of the toxic luminal agents (Al-Sadi & Ma, 2007; Van De Walle et al, 2010). On the other hand, IFN-γ is also overexpressed in IBD patients and is a key cytokine in acute phase of inflammation, mediating e.g. the recruitment, adhesion of leukocytes and the antigen presentation and also the increased permeability of intestinal barrier (Siccardi et al, 2005; Van De Walle et al, 2010).

Many chemokines are involved in IBD development and maintenance, including IL-8 (interleukin-8) (Atreya & Neurath, 2010; Tanoue et al, 2008; Van De Walle et al, 2010). IL-8 is secreted excessively by a variety of cells at the site of inflammation and causes an excessive recruitment and transmigration of neutrophils into inflamed tissues following injury of the epithelium (Atreya & Neurath, 2010; Satsu et al, 2009; Tanoue et al, 2008).

Since chemokines and cytokines are important mediators in IBD pathogenesis, they are interesting targets for treatment of this condition (Atreya & Neurath, 2010).

1.3.3 Role of NF-κB in IBD

Nuclear factor-κB (NF-κB), a transcription factor that is very important in inflammatory response by regulating the expression of various genes encoding pro-inflammatory cytokines and chemokines (TNF-α, IL-6, IL-1, IL-8, etc), adhesion molecules, growth-factors, and inducible enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Erlejman et al, 2008; Mueller et al, 2010; Pan et al, 2009; Rahimi et al, 2009; Romier et al, 2009; Romier et al, 2008; Romier-Crouzet et al, 2009; Van De Walle et al, 2008).

NF-κB exists in an inactive state in the cytosol of cells, complexed to the inhibitor-κB (IκB) (including IκB-α and others) protein. Upon stimulation by various substances (i.e. free radicals, pro-inflammatory cytokines such as IL-1β and TNF-α that are overexpressed in IBD, bacterial lipopolyssaccharides (LPS), UV radiation, etc), this inhibitor becomes phosphorylated and thereafter degraded, allowing nuclear translocation of active NF-κB that binds to specific DNA sequences and induces gene expression, in particular genes involved in inflammatory response (figure 1.9) (Rahimi et al, 2009; Romier-Crouzet et al, 2009).

Excess or inappropriate activation of NF-κB has been observed in human IBD and plays a crucial role in this condition, participating in IBD characteristic self-amplification loop. Thus, IBD are associated with a desregulation of intracellular pathway of NF-κB activation leading to an aberrant activity of this transcription factor (Erlejman et al, 2008; Romier et al, 2008). The inhibition of this pathway at any point of the cascade represses the production of these proteins and/or their reaction products, which thus modulates locally the inflammation and can be an interest target for IBD treatment (Mueller et al, 2010; Romier-Crouzet et al, 2009).

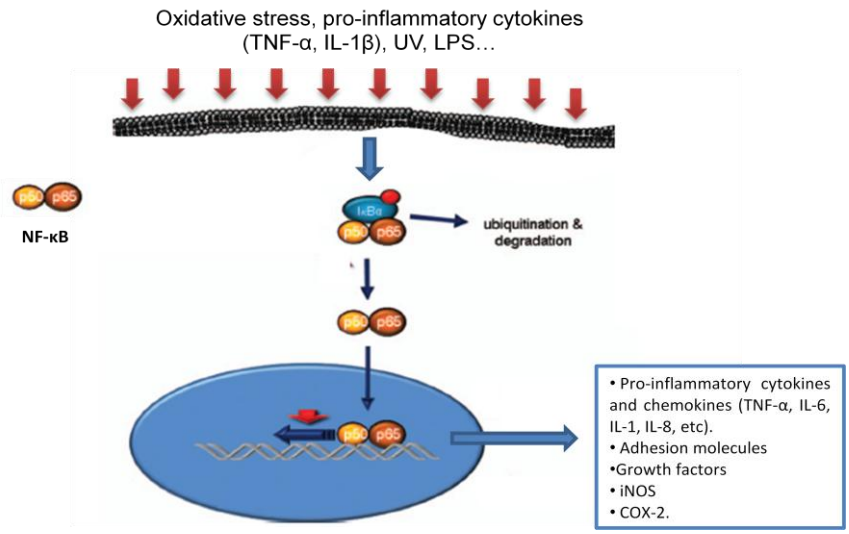


Figure 1.9 – NF-κB activation in Inflammatory Bowel Disease. Adapted from (González et al, 2011).

1.3.4 Role of oxidative stress in IBD

Oxidative stress is thought to play a significant role in the pathogenesis of Inflammatory Bowel Disease (Kaplan et al, 2007; Mandalari et al, 2011; Rahimi et al, 2009; Rahman et al, 2006; Rezaie et al, 2007). Patients with IBD demonstrate excessive oxidized molecules compared with healthy control subjects and a decrease of total antioxidant capacity in blood. Moreover, recent studies have shown that activity of endogenous antioxidant system in IBD patients is also lower than in normal individuals, namely activities of catalase, SOD and GPx that are decreased in 4%, 8% and 45% in intestinal mucosa of these patients (Kaplan et al, 2007; Rahimi et al, 2009). The body's major antioxidant, glutathione (GSH) is depleted in IBD patients and there is an increase in its oxidized form (GSSG) (Kaplan et al, 2007).

ROS can also help to perpetuate inflammatory cascades and cause subsequent tissue damage, and one of the potential mechanisms is through activation of NF-κB. ROS and ROS-mediated mucosal damage can cause alterations in the intestinal barrier integrity and then contribute to the IBD pathogenesis and also contributes for the IBD characteristic self-amplifying loop of inflammation (Erlejman et al, 2008; Kaplan et al, 2007; Rahman, 2008; Rahman et al, 2006). So, prevention and therapy with antioxidants can be useful in this disease (Rahman et al, 2006; Romier et al, 2009).

1.3.5 Role of intestinal epithelium and barrier dysfunction in IBD

Intestinal epithelial cells (IECs), which form a monolayer covering the inside surface of the intestinal tract, are particular important in many intestinal functions, namely in nutrient absorption, barrier functions, signal recognition/transduction, intestinal immune system and production of bioactive compounds (figure 1.10) (Bruewer et al, 2006; Edelblum & Turner, 2009; Shimizu, 2010; Siccardi et al, 2005; Sonier et al, 2009; Van De Walle et al, 2010). They are continuously exposed to high concentration of different substances such as nutrients, non-nutrients, microbes, xenobiotics and chemicals which means that the functions of IECs are affected or even regulated by external factors. Although, they are also regulated by internal factors such as hormones and cytokines (Shimizu, 2010).

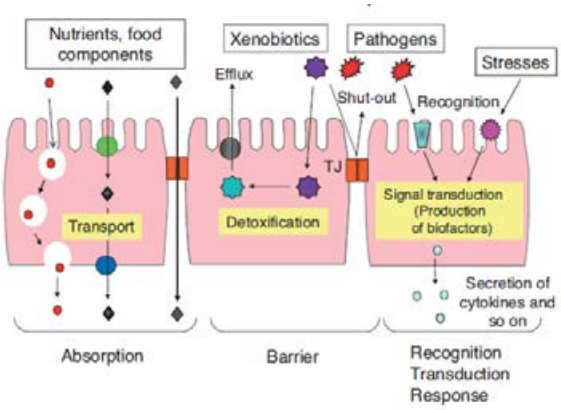


Figure 1.10 – Three major functions of intestinal epithelial cells monolayer (Shimizu, 2010).

IECs participate in mucosal inflammatory response in two ways: they modulate selectively the permeability of the epithelial monolayer and thus immune cell exposure to antigens; on the other hand they have the ability to synthesize and secrete inflammatory mediators themselves. Moreover, IECs also respond to various inflammatory mediators secreted by the immune cells, by modulating the epithelial monolayer permeability and secretion, thus further amplifying or attenuating the inflammatory process (Van De Walle et al, 2010).

Barrier dysfunction is a crucial factor in IBD and tight junctions (TJ) are very important in this condition (Bruewer et al, 2006; Edelblum & Turner, 2009; Shimizu, 2010). Epithelial tight junctions maintain the intestinal barrier while regulating permeability of ions, nutrients and water and are composed by multiple proteins including transmembrane proteins such as occluding, tricellulin, claudins, etc (Edelblum & Turner, 2009). Modifications in TJ function are involved in an increased intestinal permeability in IBD and are not only correlated with an increased expression of proteinases in IBD patients, but also with overexpressed cytokines TNF-α, IFN-γ and IL-1β (Al-Sadi & Ma, 2007; Edelblum & Turner, 2009; Siccardi et al, 2005). These cytokines can downregulate the expression of constitutive proteins of TJ like occludin and induce reorganization of these structures which can lead to abnormal permeability in IBD and a major exposition to the bacterial antigens (Leonard et al, 2010; Siccardi et al, 2005).

Due to their strategic position, excessive stimulation of intestinal epithelial cells is also a key feature in IBD. In response to external stimuli such as bacteria, ROS and inflammatory cytokines, the IECs secrete a large amount of soluble mediators (table 1.3) such as cytokines and chemokines (such as TNF-α, IL-8, IL-1β, IL-6, etc…) that can stimulate immune cells that in turn produce more cytokines, resulting in a amplifying loop that increases even more the barrier dysfunction. Note that IECs not only produce cytokines but also respond to cytokines produced by other cells, namely, immune cells (Al-Sadi & Ma, 2007; Satsu et al, 2009; Shimizu, 2010; Van De Walle et al, 2010).

Table 1.3 – Mediators produced by IECs (Romier et al, 2009).

IECs and their characteristics	Inflammatory products secreted by IECs
Enterocytes	*Secreted epithelial products*
Absorptive cells	Chemokines with chemoattractant and proinflammatory functions:
Represent 80% of total IECs	• IL-8 (or CXCL8)
Apical brush border with microvilli	• ENA-78 (or CXCL5)
	• GRO-α (or CXCL1)
Goblet cells	• MIP-2α (or CXCL2, GRO-β), MIP-2β (or CXCL3, GRO-γ), IP3α
Secrete mucus	• CCL-5 (or RANTES), CCL3 (or MIP-1α), CCL4 (or MIP-1β)
Block penetration of luminal antigens	• MCP-1 (or CCL2)
	Pro-inflammatory cytokines:
	• IL-1α, IL-1β, IL-6, IL-7, IL-15, IL-18
Enteroendocrine cells	• TNF-α
Secrete serotonin, somatostatin, cholecystokinin gastric inhibitory peptide	• GM-CS
Role in gastrointestinal tract mobility	Anti-inflammatory cytokines:
	• TGF-β
Paneth cells	*Cell membrane-associated epithelial products*
Secrete α-defensins (or cryptidyns), phospholipase A2, lysosymes	• MHC class I and II molecules
Role in antimicrobial defense	• ICAM-1
	• Cytokines receptors IL1R, IL2R, IL4R, IL7R, TNFR1, IFNGR
	• Receptors TLRs
M cells	
Lack of brush border of microvilli, presence of microfold	*Intracellular products*
Reduced mucus coating	• Receptor NOD2
Only in follicle-associated epithelium	• Cyclooxygenase-2 (COX-2)
	• Inducible nitric oxide synthase (iNOS)

Abbreviations: CXCL, chemokines (CXC motif) ligand; ENA, epithelial neutrophil-activating protein; GRO, growth-related oncogene; MIP, macrophage inflammatory proteins; CCL, chemokines (C-C motif) ligand; MCP, monocyte chemotactic protein; GM-CS, granulocyte-macrophage colony stimulating factor; TGF, transforming growth factor; MHC, major histocompatibility complex; ICAM, intercellular adhesion molecule; TLRs, Toll-like receptors; NOD2, nucleotide-binding oligomerization domain 2.

1.3.6 IBD treatment

Inflammatory Bowel Disease is traditionally treated with pharmacological agents, which may produce secondary effects such as infections or malignancies (Romier-Crouzet et al, 2009).

5-Aminosalicylic acid (5-ASA) or sulfalazine are among the primary classes of drugs used in treatment of IBD but the precise mechanism of action of 5-ASA still not fully elucidated, but it is known that have multifactorial actions including interaction with the biosynthesis or action of a variety of mediators involved in pathogenesis of IBD such as prostaglandins (products of COX-1 and COX-2), leukotriens, cytokines and chemokines (IFN-γ, IL-1β, IL-2, IL-6), NF-κB activation, and also have antioxidant properties in inflamed intestinal mucosa. Despite of their interesting biological actions, 5-ASA is associated with several side effects (Aguzzi et al, 2011; Sergent et al, 2010).

Other therapies for IBD include corticosteroids, antibody against TNF-α, immunosuppressive and immunoregulatory agents (Aguzzi et al, 2011; Sergent et al, 2010; Siccardi et al, 2005). All of these therapeutics are associated with side effects and high costs and therefore it is really necessary to develop methods and new and safe compounds for prevention and treatment of IBD. Polyphenols represents a great hope for treatment and prevention of this disease (Frontela et al, 2010; Gosslau et al, 2011; Rahimi et al, 2009; Romier et al, 2009; Romier-Crouzet et al, 2009; Sergent et al, 2010).

1.3.7 Polyphenols in IBD

Various dietary polyphenols have been shown to exert anti-inflammatory activity and this activity is beyond their antioxidant properties (table 1.4) (Frontela et al, 2010; González et al, 2011). Polyphenolic compounds can act as modulators of intracellular signaling pathways, including NF-κB pathway, and consequently they decrease expression and secretion of pro-inflammatory cytokines and chemokines, expression of inducible enzymes, etc (figure 1.11) (Romier et al, 2009; Romier et al, 2008). Since only a few studies have been conducted on experimentally induced inflammation in intestinal *in vitro* and *in vivo* models, more investigations are needed to better understand in which mechanisms polyphenols are involved (Romier-Crouzet et al, 2009; Sergent et al, 2010).

For example, kaemferol, quercetin, apigenin, procyanidins, etc, have shown to inhibit activation of NF-κB and reduce mRNA levels or proteins levels of several pro-inflammatory mediators such as IL-8, IL-6, COX-2, iNOS, among others (Frontela et al, 2010; Pan et al, 2009; Romier et al, 2009; Romier et al, 2008; Romier-Crouzet et al, 2009; Sergent et al, 2010).

Although there are only few *in vitro* studies, three main conclusions have been taken for anti-inflammatory activity of polyphenols in IECs: (1) polyphenols are effective in modulating inflammatory transduction cascades and in modifying the production of several inflammatory markers; (2) these effects cannot be generalized to the entire family or even to a class of polyphenols (structure-effect relationship were not yet established) and (3) more investigations are needed to fully understand the mechanisms whereby polyphenolic compounds act on intracellular signaling pathways in intestinal cells (Romier et al, 2009).

Oxidative stress, just like was said before, is also a key factor in IBD, and can be regulated by polyphenols due to their strong antioxidant activity (figure 1.11) (Rahman et al, 2006). Polyphenols, namely four flavonoids (quercetin, genistein, myricetin and epigallocatechin gallate (EGCG)) have also shown protection against barrier dysfunction in IBD, maintaining integrity of TJ (figure 1.12) (Suzuki & Hara, 2011). In this report it will be studied the anti-inflammatory capacity of polyphenolic rich extracts derived from *Opuntia ficus-indica* and *Prunus avium* namely in intestinal barrier integrity, cytokine expression, oxidative stress and NF-κB activation.

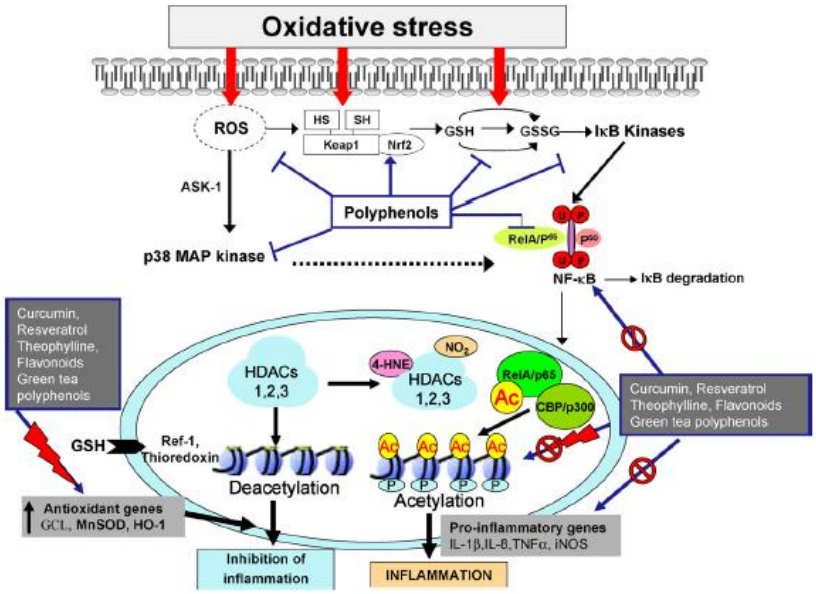


Figure 1.11 - A schematic model for polyphenols and flavonoids mediated modulation of cell signaling: oxidative stress induced inflammation is mediated by NF-kB activation, MAP kinases and affect a wide variety of cellular signaling processes leading to generation of inflammatory mediators and chromatin remodeling. The later allows expression of pro-inflammatory genes such as IL-1β, IL-8, TNF-α and iNOS. On the other hand to counter the effects of oxidative stress, the cells also concomitantly express protective antioxidant genes such as GCL, MnSOD, HO-1. Polyphenols and flavonoids inhibit pro-inflammatory gene expression via inhibition of IkB, thus inhibiting NF-kB transactivation, as well as restoring transrepressive pathways through the activation of histone desacetylases. In addition, expression of antioxidant genes such as GCL, MnSOD, HO-1 via modulation of MAPK-ARE-Nrf pathway are upregulated (Rahman et al, 2006).

Table 1.4 – Effects of polyphenol standards on experimentally induced inflammation in intestinal *in vitro* models. Adapted from (Romier et al, 2009)

Polyphenol standard	Inflammatory effects	Cell type	Stimulus	Polyphenol dose
- Flavonoid class - flavonol subclass				
Kaempferol	Reduced IL-6 secretion	Caco-2 (human)	non-stim	30µM
Quercetin	Reduced IP-10 and MIP-2 mRNA expression, inhibited Akt phosphorylation (no effect on induced IκB phosphorylation/degradation and NF-κB activity), inhibited NF-κB recruitment to IP-10 and MIP-2 gene promoters, inhibited histone acetyl transferase activity.	IEC Mode-K (murine)	TNF-α	40-44µM
	Inhibited NF-κB activation and IL-8 secretion.	HT29, HCT116, SW620 (human)	TNF-α	50-100µM
	Reduced COX-2 mRNA expression, Inhibited COX-2 activity.	Caco-2	non-stim or IL-1β	0,1-10µM
Quercetin 3-glucuronide	Reduced COX-2 mRNA expression, Inhibited COX-2 activity.	Caco-2	non-stim or IL-1β	0,1-10µM
Quercetin 3'-sulfate	Reduced COX-2 mRNA expression, Inhibited COX-2 activity.	Caco-2	non-stim or IL-1β	0,1-10µM
3'-hydroxy-flavone	Inhibited IKK and NF-κB activities, reduced IP-10 secretion, induced p38 MAPK signaling and late caspase-3 cleavage, induce IRF-1 protein degradation.	IEC Mode-K (murine)	TNF-α	100µM
- Flavonoid class - flavone subclass				
Apigenin	Inhibited NF-κB activity, reduced IP10 secretion, inhibited Akt phosporilation/ activity.	IEC Mode-K (murine)	TNF-α	100µM
Chrysin	Inhibited NF-κB transcriptional activity, reduced IκB phosphorilation.	Caco-2	IL-1β, TNF-α, or LPS	50µM
Luteolin	Inhibited NF-κB transcriptional activity, reduced IP10 secretion, inhibited Akt phosporilation/ activity, induced IRF-1 protein degradation.	IEC Mode-K (murine)	TNF-α	100µM
	Inhibited NF-κB transcriptional activity, reduced IκB phosphorilation/degradation, reduced ICAM-1 mRNA expression, inhibited NF-κB recruitment to the ICAM-1 gene promoter, inhibited IKK activity.	IEC-18 (rat)	LPS	10-50µM
- Flavonoid class - isoflavone subclass				
Biochanin-A	Reduced IL-6 secretion.	Caco-2	non-stim.	30µM
Genistein	Reduced IL-6 and IL-6 induced STAT3 nuclear translocation.	Caco-2	non-stim or IL-6	10-50µM
	Reduced IP-10 secretion (but no effect on IL-6 expression, NF-κB activity, IκB phosphorilation/degradation, IRF pathway).	IEC Mode-K (murine)	TNF-α	100µM
	Increased NF-κB transcriptional activity (no effect on IκB phosphorylation), reduced IL-8 secretion.	Caco-2	IL-1β, TNF-α, or LPS	50µM
	Inhibited epithelial permeability.	IEC-6 (rat)	IL-1β + TNF-α + IFN-γ	4µM
	Enhanced neutrophil transmigration across monolayers.	T84 or Caco-2	non-stim.	100µg/mL.
- Flavonoid class - flavonol subclass				
Epigallocatechin-3-gallate (EGCG)	Reduced IL-6 secretion.	Caco-2	non-stim.	30µM
	Reduced IL-8 secretion (no effect on NF-κB activity).	Caco-2	IL-1β, TNF-α, or LPS	50µM
	Reduced IL-8, MIP-3α and PGE_2 secretion, reduced IL-8, MIP-3α, TNF-α, COX-2, GROα and GROγ mRNA expression.	HT29 or T84 (human)	TNF-α	25-50µM
	Inhibited NF-κB activation, IκB phosphorilation and IKK activity, decreased CINC release.	IEC-6	TNF-α	25-200µM
	Supressed NF-κB activation, IκB phosphorylation/degradation, and Akt phosphorylation.	Caco-2	TNF-α	20-80µM
Butein	Inhibited IκB and P38 phosphorylation, reduced IL-8 and MMP-7 mRNA and proteins.	HT29	TNF-α	10-20µM
2',4',6' - Tris (methoxy-methoxy)chalcone	Increased ERK1/2 and P38 phosphorylation (but not JNK), Inhibited NF-κB activity, reduced IL-8 and MMP-7 expression.	HT30	TNF-α	20µM
- Phenolic acid class				
Paeonol	Reduced NF-κB and STAT-1 activation, inhibited iNOS mRNA and protein expression.	CW-2	TNF-α ± IFN-γ	40µM
Ellagic acid	Inhibited NF-κB activity and IκB phosphorilation, reduced IL-8 secretion.	Caco-2	IL-1β, TNF-α, or LPS	50µM
- Stilbene class				
Resveratrol	Induced NF-κB activity (no effect on IκB phosphorilation), increased IL-8 secretion.	Caco-2	IL-1β, TNF-α, or LPS	50µM

Abreviations: Akt - serine/threonine protein kinase B; CINC - chemokine-induced neutrophil chemoattractant; COX - Cyclooxygenase; GRO - growth-regulated oncogen; ICAM-1 - intracellular adhesion molecule-1; IKK - IκB kinase; IP-10 - Interferon-induced protein-10; MIP- macrophage inflammatory protein; MMP-7 - matrix metalloproteinase 7; PGE_2 - prostaglandin E_2, STAT - signal transducer and activator of transcription proteins; non-stim - non stimulated.

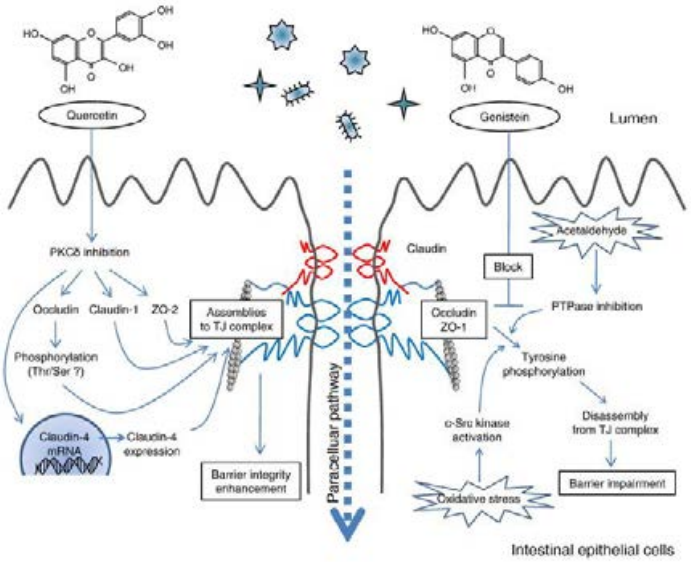


Figure 1.12 - Diagrams showing the molecular mechanisms underlying the flavonoid-mediated effects on intestinal TJ barrier function. Genistein protects intestinal TJ barrier function against oxidative stress and acetaldehyde. Oxidative stress and acetaldehyde induce tyrosine phosphorylation of TJ proteins through c-Src kinase activation or PTP inhibition leading to barrier impairment. Genistein normalizes the tyrosine phosphorylation of TJ proteins by inhibiting protein tyrosine kinases including c-Src kinase. Quercetin enhances intestinal TJ barrier integrity through PKCδ inhibition. Quercetin promotes total claudin-4 expression and the assembly of ZO-2, occludin and claudin-1 without any changes in their total expression levels. The promotion of claudin-4 expression by quercetin is induced at a transcriptional level leading to an increase in assembly. The quercetin-induced occludin assembly is then possibly involved in the phosphorylation of occludin on Ser or Thr residues (Suzuki & Hara, 2011).

1.3.8 Caco-2 model

Cells models are gaining an enormous popularity among scientific research community being widely used in pharmaceutical and cosmetic industries in detriment of *in vivo* models such as animals (Langerholc et al, 2011). Caco-2 cell line is an human IEC line derived from an human colon adenocarcinoma, and is particularly useful because it forms a monolayer with many small intestinal functions. By culturing them for 2-3 weeks, Caco-2 cells are spontaneously differentiated and TJs are formed between the cells, microvillus structures are formed on the apical cell surface and a variety of brush-border digestive enzymes, transporters and receptors are expressed (figure 1.13) (Langerholc et al, 2011; Shimizu, 2010; Van De Walle et al, 2010).

Caco-2 cells can mimic inflamed intestinal epithelium when stimulated with pro-inflammatory cytokines, responding secreting more cytokines, changing their permeability and activating many intracellular pathways, namely NF-κB (Leonard et al, 2010; Sergent et al, 2010; Van De Walle et al, 2010). Although Caco-2 cell line is not a perfect model, since don't include all possible interactions between the innumerous type of cells that are included in IBD pathology and all the complexity of this disease, it can be used to evaluate possible role of polyphenols and their action mechanisms in prevention and treatment of IBD (Langerholc et al, 2011; Leonard et al, 2010; Sergent et al, 2010; Shimizu, 2010; Van De Walle et al, 2010).

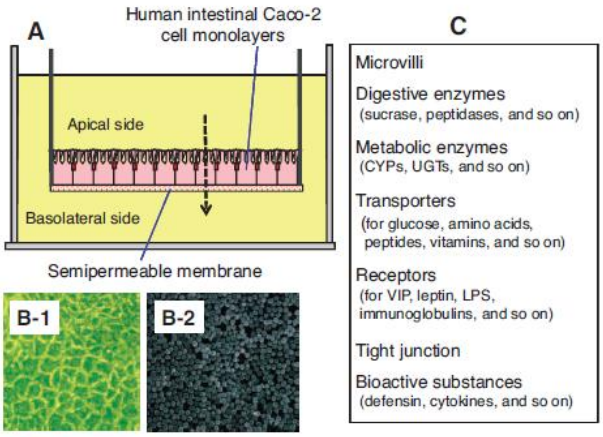


Figure 1.13 – Characteristics of the human IEC Cell line Caco-2. (A) Differentiated cells in confluent Caco-2 monolayer in a semi-permeable membrane; (B1) Light microscopic picture that shows a microvillus structure; (B2) Scanning electron microscopic picture. (C) A variety of molecules that are expressed by Caco-2 cells, which makes it possible to examine many IEC functions *in vitro* (Shimizu, 2010).

1.4. Aim

The main objective of this work was the assessment of the anti-inflammatory activity of two polyphenolic-rich extracts (PRE) derived from *Prunus Avium* and *Opuntia ficus-indica* in an *in vitro* model of Inflammatory Bowel Diseases. To achieve this goal there was defined individual objectives:

- Characterization of both PRE according to their polyphenolic composition and chemical antioxidant activity (ORAC, HORAC and inhibition of LDL oxidation),
- Study of cellular antioxidant activity of these PREs in an *in vitro* model of intestinal epithelial cells (Caco-2 cells) – contribution to glutathione homeostasis, inhibition of intracellular ROS formation and inhibition of protein oxidation.
- Establishment and characterization of an *in vitro* human cell-based model of Inflammatory Bowel Disease in terms of cytokine and chemokines expression, NO (nitric oxide) release, barrier dysfunction and NF-κB activation, using Caco-2 cells.
- Assessment of anti-inflammatory activity of both PRE in a prevention perspective using the established cell model.
- Assessment of anti-inflammatory activity of both PRE in a therapeutic perspective using the established cell model.
- Comparison between anti-inflammatory activity of 5-aminosalisylic acid and cherry's PRE.

2. Materials and Methods

2.1. Material

Methanol, 2',2'-azobis (2-amidinopropane) dihydrochloride (AAPH), 2',7'-dichlorofluorescein diacetate (DCFH-DA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), caffeic acid, ± 98% catechin, bovine serum albumin (BSA), Bradford reagent, gallic acid, phosphate buffer saline (PBS), sodium chloride (NaCl), Griess Reagent modified, 3,3',5,5'-tetramethylbenzidine (TMB), hydrogen peroxide (H_2O_2), Amberlite® XAD 16, ammonium persulfate (APS), Acrylamide/Bisacrylamide 30%, glycerol, glycine, bromophenol blue sodium salt, tris-base, *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED), β-mercaptoethanol, sodium dodecyl sulphate (SDS), sodium hydroxide (NaOH), aluminum chloride ($AlCl_3$), 2,4-dinitrophenylhydrazine (DNPH), Cobalte (II) fluoride (CoF_2), GSH and GSSG standards, orthophthalaldehyde (OPA), Cell Lytic™ M, protease inhibitor cocktail, LPS from *E.coli*, mouse monoclonal Anti-βactin antibody and rabbit anti-DNPH antibody were purchased from Sigma-Aldrich (St. Quentin Fallavier, France), Disodium fluorescein was obtained from TCI Europe (Antwerp, Belgium), Ferrous sulphate ($FeSO_4$) was from Merck (Darmstadt, Germany), Folin Reagent and hydrochloric acid (HCl) were from Panreac (Barcelona, Spain), nitrocellulose membrane 0,45µm, fiber pads and filter paper were purchased from BioRad (California, USA), CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay was obtained from Promega (San Luis Obispo, CA, USA), Streptavidin-HRP was obtained from Millipore (Massachusetts, USA), Low-density lipoprotein (LDL) was purchased from Abcam (Cambridge, UK), FentoMax™ secondary antibody Kit, anti-mouse IgG and anti-rabbit IgG secondary antibodies biotin conjugated, Ultra pure Tween-20, rabbit anti-IL10 antibody, rabbit anti-IL-8 antibody and donkey anti-mouse IgG HRP-labeled secondary antibody were obtained from Rockland (Gilbertsville, PA, USA), mouse monoclonal anti-IκBα primary antibody, mouse monoclonal anti-TNF-α primary antibody, TNF-α and IL-1β were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). All cell culture media and supplements, namely fetal bovine serum (FBS), glutamine, RPMI 1640, RPMI 1640 without phenol-red, Trypsin-EDTA and penicillin-streptomycin (PenStrep) were obtained from Invitrogen (Gibco, Invitrogen Corporation, Paisley, UK).

Phenolic standards used were: chlorogenic acid from Sigma-Aldrich (St. Louis, MO, USA) and catechin, epicatechin, phloridzin, quercetin-3-rhamnoside, cyanidin-3-glucoside, cyanidin-3-rutinoside and Quercetin, Quercetin 3-O-glucoside, Isorhamnetin, Isorhamnetin 3-O-glucoside, Isorhamnetin 3-O-rutinoside were all from Extrasynthèse (Genay, France). Citric acid was purchased from Sigma-Aldrich. Ultra-pure water (18.2 MΩ.cm) was obtained from a Millipore-Direct Q3 UV system (Millipore, USA).

2.2. Raw materials

Cherries of Saco variety were collected at Cova da Beira, Portugal, between May and June 2008 and stored at -18ºC. Raw material used in all extractions was obtained from all fruit with seeds and stalks. Firstly, raw material was crushed in a knife mill (UFESA, Lisbon, Portugal) followed by dehydration in a freeze drier (Freeze Dryer Modulyo, Edwards, UK) at -40ºC, in the absence of light.

After 72h the raw material was milled in a grinder (Braun, KSM 2, Kronberg, Germany) and was extracted in the dark with EtOH:H_2O (50:50 v/v) solution (1:20, w/v), for 2h at room temperature. The extract were then filtered, centrifuged and the supernatant was concentrated in a rotary evaporator under reduced pressure in a water bath thermostated at 40ºC (Buchi, Switzerland; Rotavapor R-210; Heating Bath B-491; Vacuum Controller V-850; Vacuum Pump V-700). Cherry solvent extracts was lyophilized until analysis (Serra, 2010).

Opuntia ficus-indica fruits were collected in Beja, Alentejo, Portugal, in October of 2010. The spikes were removed with a brush and the cactus pear fruits were processed using a kitchen robot (UFESA, LC5005, China). Juice was recovered and centrifuged at 9000rpm for 10 minutes (Avanti J-26 XPI, Beckman Coulter, USA). The supernatant was collected and preserved under frozen storage (-20ºC) until the day of the experiments.

2.3. Preparation of *Opuntia ficus-indica* and *Prunus Avium* polyphenolic-Rich Extracts (PREs)

Food grade macroporous resin Amberlite® XAD-16 was used as adsorbent. This resin is allowed for food applications by the U.S. Food and Drug Administration Code of Federal Regulation Title 21 (Scordino et al, 2003).

The necessary preconditioning of the adsorbent was done by an extensive wash with abundant distilled water to remove salts and impurities, and was then dried at 70ºC for 24 h and then cooled in an oven. The dried resin was immersed in ethanol for 12 h. The ethanol was then replaced by distilled water through washing.

The production of cactus pear and cherry polyphenols rich extracts (PREs) was performed in batch mode as previously described by Silva et al, 2007 with slightly modification. Briefly, aqueous cactus pear and cherry solutions were put in contact with resin (30 mg GAE/g resin), inside flaks protected from light. These flaks were submitted to an agitation at 200 rpm during 4 h. After that, supernatant were removed and resins were washed three times with distilled water in order to remove water soluble constituents like sugars, organic acids and minerals. Polyphenols were then eluted with ethanol (96%). The ethanolic fractions were gently concentrated by evaporation (Buchi, Switzerland; Rotavapor R-210; Heating Bath B-491; Vacuum Controller V-850; Vacuum Pump V-700), transferred into a water phase and, finally, freeze dried. The resulting PREs were kept in a cool, dry and dark environment.

2.4. PRE's analysis

2.4.1 Total phenolic content

Total concentration of phenolic compounds present in both extracts was determined according to the Folin Ciocalteau colorimetric method (Singleton & Rossi, 1965). Briefly, 20µL of the appropriate dilutions of extracts were added to 1480µL of distilled water and oxidized with Folin Ciocalteau reagent (100µL). The reaction was neutralized with 300µL of sodium carbonate and after 30 minutes at 40ºC of incubation the absorbance of samples was measured at 765 nm in a Genesys10uv spectrometer (Thermo Spectronic, New York, USA). Gallic acid was used as standard, and the results were

expressed as means of triplicates (mg of galic acid equivalents per g dry extract –mg GAE/g dry extract).

2.4.2 Total flavonoids content

Measurement of flavonoid content was performed by the $AlCl_3$ complexation method (Michalska et al, 2007) modified by Tavares et al, 2010. To each well of a 96-well plate, water (125 µL), sample (or solvent, in the control, 25 µL) and 5% (w/v) $NaNO_2$ (7,5 µL) was added. The plate was incubated for 6 min at room temperature and then 10% (w/v) $AlCl_3$ (15 µL) was added. After incubation during 5 min, NaOH 1M (100 µL) was added and the solution of each well was mixed. The absorbance was measured at 510nm in a BioTek™ Power Wave XS microplate reader. (+)-Catechin hydrate, minimum 98% (w/w) was used as standard, and the results are expressed as mg catechin equivalents per mg of total polyphenols (mg CE/mg polyphenols) and all samples were analyzed as triplicates.

2.4.3 HPLC analysis

HPLC analyses of cactus pear PRE were performed on a Waters® Alliance 2695 equipped with a quaternary pump, solvent degasser, auto sampler and column oven, coupled to a Photodiode Array Detector Waters 996 PDA (Waters). A pre-column (RP-18, 5µm) and reversed phase column (RP-18 Synergy, 2.5 µm Max-RP from Phenomenex) with oven at at 35°C were used for separation. The gradient mobile phase consisted of 0.5% formic acid p.a in ultra pure water (A): LC-MS grade acetonitrile (B) at a flow rate of 0.30 mL/min. The injection volume was 5µL. Photodiode Array Detector was used to scan wavelength absorption from 210 to 600 nm.

For HPLC quantification of anthocyanins, the mobile phase used consisted of a gradient mixture of eluent A water:formic acid (90:10 v/v) and eluent B acetonitrile:water:formic acid (40:50:10 v/v/v). The following gradient of eluents was used: 0- 15min from 0 until 20% of eluent B; 10 min with 20% eluent B; 25–70 min, from 20 until 70% eluent B; 70–75 min, with 70% of eluent B; 75–85 min from 70 until 100% eluent B; 85–90 min, with 100% eluent B; 90-95 min from 100 to 0% of eluent B; and 95-100 min 100% of eluent A. The solvent flow rate was 0.7 mL/min. Acquisition range was set between 190 and 700 nm and chromatogram was monitored at 527 nm. Cyanidin glucosides were quantified using standard compounds whereas pelargonidin-3-rutinoside, peonidin-3-glucoside and peonidin-3-rutinoside were calculated as cyanidin-3-glucoside equivalents (Serra, 2010).

Coefficients of variation on the HPLC quantifications were <5% and final concentrations were expressed as mg/100g extract.

2.5. PRE's Chemical Antioxidant Activity

2.5.1 Oxygen Radical Absorbance Capacity (ORAC)

Peroxyl radical scavenging capacity was determined by the ORAC method. Briefly, sodium fluorescein (150µL; $2{,}0\times10^{-8}$M in PBS 75mM pH 7,4) and samples or standards (25µL) were added to a 96 well microplate and incubated during 10min at 37ºC. 2,2'-azobis(2-amidopropane)dihydrochloride (25µL,$1{,}28\times10^{-2}$ prepared in PBS 75mM pH 7,4) were injected to each well. The blank contained 75mM phosphate buffer (pH 7.4, 25µL) instead of the sample, whereas for the calibration it contained

5 to 50 µM 6-hydroxy-2,5,7,8-tetramethylchroman-carboxylic acid (Trolox, 25 µL). The decrease of fluorescence was monitored kinetically during 30 min at 37 ºC (excitation: 493 nm; emission 515 nm) on microplate fluorescence reader (FL800 Bio-Tek Instruments). The final results were calculated by the difference of area under the curve of fluorescence's decay between blank and sample and are expressed as µmol Trolox equivalents (TEAC) *per* g of polyphenols and all samples were analyzed as triplicates.

2.5.2 Hydroxyl Radical Adverting Capacity (HORAC)

HORAC assay was based on a previously reported method (Ou et al, 2002) modified for the FL800 microplate fluorescence reader (FL800 Bio-Tek Instruments). This assay evaluates the hydroxyl radical prevention capacity of a sample using fluorescein (FL) as the probe. The hydroxyl radical was generated by a Co(II)-mediated Fenton like reaction and, similarly to ORAC assay, the fluorescence decay curve of FL was used to quantify the HORAC value. Briefly, 10 µL of appropriate dilutions of samples were added to 180µL of FL ($4x10^{-3}$ µM) plus 10µL of H_2O_2 (0,55 M). The reaction was started by the addition of 10µL of CoF_2 to the mixture placed in a 96 well microplate at 37ºC. Fluorescence emitted by the reduced form of FL was measured and recorded every 1 minute during 35 minutes. The FL800 microplate fluorescence reader was used with fluorescence filters for an excitation wavelength of 485±20 nm and an emission wavelength of 530±25 nm, and the plate reader was controlled by software Gen5. Caffeic acid was used as a standard. Data was expressed as micromoles of caffeic acid equivalents (CAE) per gram of polyphenols and all samples were analyzed as triplicates.

2.5.3 Inhibition of LDL oxidation

Prior to oxidation, ethylenediamine tetraacetic acid (EDTA) in the LDL solution was removed by passage through a PD-10 desalting column (GE Healthcare, Buckinghamshire, UK), hydrated and eluted with PBS. Protein concentrations in LDL preparations were determined with Bradford reagent using BSA as a reference standard. For the oxidation assay, 0.1 mg/mL LDL and 500 µM of AAPH with or without PRE (50mg GAE/L) were incubated in a quartz *cuvette* at 37ºC. Light absorbance was read at 234 nm in a Genesys10uv spectrometer (Thermo Spectronic, New York, USA) for a maximum period of 12 hours or until the LDL oxidation reached a plateau. Lag time was calculated by drawing two tangent lines: one through the initial, slowly rising curve which corresponded to the utilization of endogenous antioxidants in the LDL; and the other to a subsequent, rapidly rising curve which corresponded to the rapid LDL oxidation following the exhaustion of endogenous antioxidants. The intersection of two tangents was considered as the lag time, which is expressed in minutes.

2.5.4 Protein determination

Total protein content was determined according to Bradford assay (Bradford, 1976). Briefly,1mL of Bradford reagent and 20µL of sample (LDL or cell lysate) were added in a *cuvette*. The solution was homogenized and was allowed to stay at room temperature for 5 minutes. The absorbance of the samples was measured at 595 nm on a Spectrophotometer (Genesys™ 10UV). Bovine serum albumin

was used as standard and the results were expressed as mg of BSA *per* mL (mg BSA/mL) all samples were analyzed as triplicates.

2.6. PRE's Cell-based Antioxidant Activity

2.6.1 Cell culture

Human colon carcinoma Caco-2 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), and were routinely grown in RPMI 1640 supplemented with 10% of FBS, 2mM of glutamine and 5000U of PenStrep. Stock cells were maintained as monolayers in 175 cm^2 culture flasks and incubated at 37ºC in a 5% CO2 humidified atmosphere.

2.6.2 Cytotoxicity assay

Toxicity assays were performed using Caco-2 cells. Briefly, cells were seeded at a density of $2x10^4$ cells/well in 96 well plate and the medium was changed every 48 hours. The experiments were performed using completely differentiated cells (after reaching confluence- ±96 hours). Caco-2 cells were incubated with 50mgGAE/L of each PRE diluted in culture medium (RPMI 1640 medium with 0,5% FBS and 2mM glutamine) during 48hours (were performed a control where cells were incubated with cell culture medium). After 48h of incubation, the medium was removed and 100µL of the colorimetric reagent MTS (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay) was added to each well for 4hours. MTS is reduced by viable cells to a soluble formazan product that was quantified by measurement of the absorbance at 570nm in a BioTek™ Power Wave XS microplate reader.

The experiments were performed in triplicate and the results are expressed in percentage to the control with culture medium only. Controls were performed incubating cells with culture medium with PBS replacing PREs.

2.6.3 Inhibition of ROS formation

To evaluate the intracellular antioxidant capacity of *Opuntia ficus-indica* and *Prunus Avium* PREs, Caco-2 cells were seeded at a density of $2x10^4$ cells/well in 96 well plate and the medium was changed every 48 hours. The experiments were performed using completely differentiated cells (after reaching confluence- ±96 hours) which are a good model of the intestinal barrier. Cellular antioxidant activity of PREs was evaluated following the formation of reactive oxygen species (ROS) in Caco-2 cells after treatment with two chemical stressors: AAPH and H_2O_2. The formation of intracellular ROS was monitored using the fluorescent probe, DCFH-DA. Briefly, for the pre-incubation assay differentiated Caco-2 cells were washed with PBS and incubated with PREs (50mg GAE/L) and 100 µM of DCFH-DA for 1hour. After removal the PREs with DCFH-DA and further washing, 600µM or 10mM of H_2O_2 were added to the cells for 60 min. For the co-incubation assay, PREs were added to Caco-2 cells at the same time of chemical stressors and after 1hour of incubation with DCFH-DA. For both assays, fluorescence (F) was measured for each sample at 0 and 60 min in a fluorescence microplate reader (FL800 Bio-Tek Instruments, Winooski, VT, USA). Cellular antioxidant activity of PREs was expressed as the percentage of inhibition of intracellular ROS caused by exposure to the

oxidative stressors and was calculated as (1-(F60min-F0min)/F0min) x 100 against a control (cells without PREs). Experiments were done in triplicate and results are expressed as a percentage of the absorbance compared to control cells.

2.6.4 Inhibition of protein oxidation

Caco-2 cells were seeded in 6-well plate at a density of $1x10^5$ cells/well and cultured in standard medium (RPMI – 1640 supplemented with 2mM glutamine, 10% FBS and 1% penstrep) during 21 days to obtain fully differentiated cells. Cells were washed with PBS and incubated with PREs (50mg GAE/L) in PBS for 1 hour except in control wells that were incubated with PBS. Then, the PREs were removed and 10mM of H_2O_2 (stress inductor) was added to all wells, except for control well (without PRE and without stress inductor) for 1hour. The extracellular media was removed and cell Lytic™ supplemented with a proteases inhibitor cocktail was added for 5minutes. Cells were removed by scrapping and centrifuged at 14000g, for 10min at 4ºC (Hettich Zentrifugen MIKRO 220R) and supernatants were freeze at -80ºC to carbonyl proteins determination.

Carbonylated proteins were determinate according to Ramful et al, 2010 with slightly modifications. Briefly, a total of 40µL of cell lysates (0,6mg/mL of protein) were denatured by 5µL of 12% sodium dodecyl sulfate (SDS) for 10min at room temperature. The protein carbonyls present in the samples were then derivatized using 80µL of 5mM 2,4-dinitrophenylhydrazine (DNPH) in 2M HCl for 20min at room temperature. Samples were diluted and neutralized adding 5µL to 1mL of PBS (coating buffer) followed by addition of 100µL of each sample to wells of an ELISA plate (NUNC Maxisorp) for 3h at 37ºC, blocked with 200µL of Sea Blocking Buffer overnight at 4ºC, and probed with a rabbit anti-DNPH antibody for 3h at room temperature. Then, a HRP conjugated anti-rabbit IgG secondary antibody (donkey) was added for 1h at room temperature. The tetramethylbenzidine (TMB) substrate was then added and allowed to oxidize for 15min at room temperature, leading to the formation of a sapphire blue complex. The reaction was stopped by the addition of 2M HCl and absorbance was measured at 450nm in a BioTek™ Power Wave XS microplate reader. Experiments were done in triplicate and results are expressed as a percentage of the absorbance compared to control cells.

2.6.5 Inhibition of glutathione oxidation

Caco-2 cells were seeded in 6-well plate at a density of $1x10^5$ cells/well and cultured in standard medium (RPMI – 1640 supplemented with 2mM glutamine, 10% FBS and 1% penstrep) during 21 days to obtain fully differentiated cells. Cells were washed with PBS and incubated with PREs (50mg GAE/L) in PBS for 1 hour except in control wells that were incubated with PBS. Then, the PREs were removed and 10mM of H_2O_2 (stress inductor) was added to all wells, except for control well (without PRE and without stress inductor) for 1hour. The extracellular media was removed and cells were detached by adding trypsin followed by inactivation with standard cell medium and centrifugation at 14000g, for 10min at 4ºC (Hettich Zentrifugen MIKRO 220R). Cells were ressuspended in PBS and centrifuged at 14000g, for 10min at 4ºC (Hettich Zentrifugen MIKRO 220R).

To quantify GSH and GSSG, cold 10% (v/v) metaphosphoric acid was carefully added to samples or standards. After incubation (4ºC, 10min) and centrifugation (16000*g*, 20min, 4ºC) supernatants

were transferred into 1.5mL propylene tubes (50µL for determination of GSH and 200µL for determination of GSSG). Derivatization was performed accordingly to Kand'ar et al, 2007, adapted from HISSIN & HILF, 1976. Briefly, for GSH analysis 1mL of 0.1 % (w/v) EDTA in 0.1M sodium hydrogen phosphate, pH 8.0, was added to 50µL of supernatant. To 20µL portion of this mixture, 300µL of 0.1% (w/v) EDTA in 0.1M PBS, and 20µL of 0.1% (w/v) orthophthalaldehyde (OPA) in methanol, were added. Tubes were incubated at 25ºC for 15min in the dark. The reaction mixture was then stored at 4ºC until analysis. For GSSG analysis, 200µL of supernatant was incubated at 25ºC with 200µL of 40mM N-ethylmaleimide (NEM) for 25 min in dark. To this mixture, 750µL of 0.1M NaOH were added. A 20µL portion was taken and mixed with 300µL of 0.1M NaOH and 20µL of 0.1% OPA. Tubes were incubated at 25ºC for 15 min in dark and stored at 4ºC until analysis.

Chromatographic analysis was accomplished using isocratic elution on C18 analytical column (Supelcosil™ ABZ+Plus HPLC Column 15cm x 4.6mm, 3µm (Supelco)) at 40ºC on an Acquity™ Ultra Performance LC system (Waters). The mobile phase consisted of 15% (v/v) methanol in 25mM PBS (v/v), pH 6.0. The flow rate was kept constant at 0.7mL min^{-1}. The excitation and emission wavelengths were set at 350 and 420 nm, respectively. The amount of GSH and GSSG was quantified from the corresponding peak area using Empower® Pro 2.0 software. The concentration of GSH and GSSG in the samples was determined from standard curves with ranges 0-100 µM for GSH and 0-5 µM for GSSG. Values were normalized for total protein content determinate with Bradford reagent using BSA as standard. All samples were analyzed in triplicate.

2.7. Anti-inflammatory activity

2.7.1 5-ASA preparation

Salofalk® was dissolved in cell culture medium (RPMI 1640 supplemented with 0,5% of FBS, 2mM of glutamine) as a 100mM stock, pH was adjusted at 7,0 until stabilization (all 5-ASA were released) with NaOH. The solution was sterile filtrated (0,22µm), and protected from light (Schwab et al, 2009).

At the day of the experiment the solution was diluted in fresh cell culture medium (RPMI 1640 supplemented with 0,5% of FBS, 2 mM of glutamine) to a 30mM concentration.

2.7.2 Cytotoxicity assay

Toxicity assays were performed using Caco-2 cells. Briefly, cells were seeded at a density of $2x10^4$ cells/well in 96 well plate and the medium was changed every 48 hours. The experiments were performed using completely differentiated cells (after reaching confluence- ±96 hours). Caco-2 cells were incubated with inflammatory stimuli (50ng/mL TNF-α, 25ng/mL IL-1β and 10µg/mL LPS) in culture medium (RPMI 1640 medium with 0,5% FBS and 2mM glutamine) during 48hours (were performed a control where cells were incubated with cell culture medium). After 48h of incubation, the medium was removed and 100µL of the colorimetric reagent MTS was added to each well for 4hours. MTS is reduced by viable cells to a soluble formazan product that was quantified by measurement of the absorbance at 570nm in a BioTek™ Power Wave XS microplate reader. The experiments were performed in triplicate and the results are expressed in percentage to the control with culture medium only.

2.7.3 Transepithelial Electrical Resistance and Paracellular permeability

Caco-2 cells were seeded in 12 mm i.d. Transwell® inserts (polycarbonate membrane, 0.4µm pore size, Corning Costar Corp.) in 12-well plates at a density of $1.0x10^5$ cell/well. The basolateral (serosal) and apical (mucosal) compartments contained 1,5 and 0,5mL of culture medium, respectively. Cells were allowed to grow and differentiate to confluent monolayer for 21 days post seeding by changing the medium (RPMI 1640 supplemented with 10% of FBS, 2mM of glutamine and 1% of penstrep) three times *per* week.

Inflammatory stimuli (50ng/mL TNF-α, 25ng/mL IL-1β and 10µg/mL LPS) was added in basolateral or apical compartment at the same time of the addition of cherry's PRE (50mg GAE/L) and 5-ASA (30mM), diluted in RPMI 1640 supplemented with 0,5% of FBS, 2mM of glutamine, in apical side for 48h. Different controls were performed: control without inflammatory stimuli and PRE or mesalazine, control without PRE or 5-ASA with inflammatory stimuli at basolateral side and control without PRE or 5-ASA with inflammatory stimuli at apical side. Transepithelial electrical resistance (TEER) of cells grown in Transwell was measured using EVOM^{TM} voltmeter (WPI, Berlin, Germany). Only monolayers with a TEER value higher than 500 Ωcm^2 were used for experiments. Experiments were done in duplicate and results are expressed as average of obtained values.

2.7.4 Permeability of fluorescein on the Caco-2 cell monolayer

Caco-2 cells were seeded in 12 mm i.d. Transwell® inserts (polycarbonate membrane, 0.4µm pore size, Corning Costar Corp.) at a density of $1.0x10^5$ cell/well. The basolateral (serosal) and apical (mucosal) compartments contained 1,5 and 0,5mL of culture medium, respectively. Cells were allowed to grow and differentiate to confluent monolayer for 21 days post seeding by changing the medium (RPMI 1640 supplemented with 10% of FBS, 2mM of glutamine and 1% of penstrep) three times *per* week.

Permeability of fluorescein on the Caco-2 cell monolayer was determined according to Leonard et al, 2010 method with slightly modifications. Transport was assessed in both absorptive (apical→basolateral) and secretory (basolateral→apical) directions. 1µg/mL of fluorescein disodium (FL) was dissolved in transport buffer (RPMI 1640 without phenol red supplemented with 2mM glutamine and 0,5% FBS). Cell monolayers on Transwell fibers were rinsed gently twice and pre-incubated in transport buffer for one hour at 37ºC and 5% CO_2. Cells were incubated for 4hours with 50mg GAE/L of PREs added to the apical compartment. Wells were washed with transport buffer and FL was added to the donor compartment and transport buffer was added to the acceptor compartment at the same time of the addition of inflammatory stimuli at the basolateral compartment (50ng/mL TNF-α, 25ng/mL IL-1β and 10µg/mL LPS). At different time points 50µL of the samples were taken from the receiver compartment and the volume lost during sampling was replaced with fresh transport buffer. Fluorescein amount in the samples was measured using a microplate fluorescence reader (FL800 Bio-Tek Instruments) at an excitation wavelength of 488nm and emission wavelength of 530nm. Apparent permeability (P_{app}) was calculated according to (equation 1):

$$P_{app} = (dQ/dt)\ (1/A)\ (1/C_0) \quad (1)$$

where dQ/dt is the amount of drug transported *per* time, A is the surface area of the monolayer and C_0 is fluorescein concentration (µg/mL) at time 0. A fluorescein calibration curve was used for fluorescein concentration determination.

Permeability of FL across nonstimulated Caco-2 monolayers was determined as control. TEER of all monolayers was monitored before and after experiment to insure their integrity.

2.7.5 Quantification of NO secretion

Caco-2 cells were seeded in 6 well plates at a density of $2.0x10^5$ cells/well or in 12 mm i.d. Transwell® inserts (polycarbonate membrane, 0.4µm pore size, Corning Costar Corp.) at a density of $1,0x10^5$ cells/well. Cells were allowed to grow and differentiate to confluent monolayer for 21 days post seeding by changing the medium (RPMI 1640 supplemented with 10% of FBS, 2 mM of glutamine and 1% of penstrep) three times *per* week. Cells were pre incubated with PREs or 5-ASA for 4 hours, or co-incubated for 48h with inflammatory stimuli (50ng/mL TNF-α, 25ng/mL IL-1β and 10µg/mL LPS).

Culture medium was removed and centrifuged at 2000g (Hettich Zentrifugen MIKRO 220R) for 10min at 4ºC. Supernatants were harvested and frozen at -80ºC until the day of experiments.

NO was measured using Griess reagent. Briefly 100µL of sample was mixed with 100µL of modified Griess reagent and the absorbance was read at 540nm after 15min using a BioTek™ Power Wave XS microplate reader. Culture medium was used as blank and culture medium of cells without stimulation was used as control. Experiments were done in triplicate and results are expressed as a percentage of the absorbance compared to control cells.

2.7.6 ELISA quantification of IL-8 and IL-10 secretion

Caco-2 cells were seeded in 6 well plates at a density of $2.0x10^5$ cells/well or in 12 mm i.d. Transwell® inserts (polycarbonate membrane, 0.4µm pore size, Corning Costar Corp.) in 12-well plates at a density of $1.0x10^5$ cells/well. Cells were allowed to grow and differentiate to confluent monolayer for 21 days post seeding by changing the medium (RPMI 1640 supplemented with 10% of FBS, 2mM of glutamine and 1% of penstrep) three times *per* week. Cells were pre incubated with PREs or 5-ASA for 4 hours, or co-incubated for 48h with inflammatory stimuli (50ng/mL TNF-α, 25ng/mL IL-1β and 10µg/mL LPS).

Culture medium was removed and centrifuged at 2000g (Hettich Zentrifugen MIKRO 220R) for 10min at 4ºC. Supernatants were harvested, protein concentration was determinate using Bradford assay, and supernatants were frozen at -80ºC until the day of experiments.

Briefly 100µL of supernatants diluted in PBS (5µg of protein) were added to wells of an ELISA plate (NUNC Maxisorp) for 3h at 37ºC, washed 3 times with PBS-T (PBS with 0,05% ultra pure tween-20) and blocked with 200µL of PBS with 1%BSA overnight at 4ºC. Wells were washed 3times with PBS-T and 100µL of anti IL-8 (rabbit antibody) or anti IL-10 (rabbit antibody) were added to each well for 1hour at room temperature, followed by wash and addition of secondary antibody (anti-rabbit IgG Biotin conjugated) for 1hour at room temperature. After this, wells were washed with PBS-T and 100µL of Streptavidin-HRP solution were added for each well for 1hour at room temperature and wells were washed again. The tetramethylbenzidine (TMB) substrate was then added and allowed to oxidize

for 15min at room temperature, leading to the formation of a sapphire blue complex. The reaction was stopped by the addition of 2M HCl and absorbance was measured at 450nm in a BioTek™ Power Wave XS microplate reader. Experiments were done in triplicate and results are expressed as a percentage of the absorbance compared to control cells. All solutions used in ELISA assays are in appendix A.

2.7.7 ELISA quantification of TNF-α

Caco-2 cells were seeded in 6 well plates at a density of $2.0x10^5$ cells/well and were allowed to grow and differentiate to confluent monolayer for 21 days post seeding by changing the medium (RPMI 1640 supplemented with 10% of FBS, 2mM of glutamine and 1% of penstrep) three times *per* week. Cells were pre incubated with PREs for 4 hours, or co-incubated for 48h with inflammatory stimuli (50ng/mL IL-1β).

Culture medium was removed and centrifuged at 2000g (Hettich Zentrifugen MIKRO 220R) for 10min at 4ºC. Supernatants were harvested, protein concentration was determinate using Bradford assay, and supernatants were frozen at -80ºC until the day of experiments.

Briefly 100µL of supernatants diluted in PBS (5µg of protein) were added to wells of an ELISA plate (NUNC Maxisorp) for 3h at 37ºC, washed 3 times with PBS-T (PBS with 0,05% ultra pure tween-20) and blocked with 200µL of PBS with 1%BSA overnight at 4ºC. Wells were washed 3times with PBS-T and 100µL of anti-TNF (mouse antibody) were added to each well for 1hour at room temperature, followed by wash and addition of secondary antibody (anti-mouse IgG Biotin conjugated) for 1hour at room temperature. After this, wells were washed with PBS-T and 100µL of Streptavidin-HRP solution were added for each well for 1hour at room temperature and wells were washed again. The tetramethylbenzidine (TMB) substrate was then added and allowed to oxidize for 15min at room temperature, leading to the formation of a sapphire blue complex. The reaction was stopped by the addition of 2M HCl and absorbance was measured at 450nm in a BioTek™ Power Wave XS microplate reader. Experiments were done in triplicate and results are expressed as a percentage of the absorbance compared to control cells. BioTek™ Power Wave XS microplate reader. All solutions used in ELISA assays are in appendix A.

2.7.8 Western blot quantification of IκBα

Caco-2 cells were seeded in 6 well plates at a density of $2.0x10^5$ cells/well and were allowed to grow and differentiate to confluent monolayer for 21 days post seeding by changing the medium (RPMI 1640 supplemented with 10% of FBS, 2mM of glutamine and 1% of penstrep) three times *per* week. Cells were pre incubated with PREs for 4 hours, or co-incubated for 48h with inflammatory stimuli (50ng/mL TNF-α, 25ng/mL IL-1β and 10µg/mL LPS). The extracellular medium was removed and cell Lytic™ supplemented with a proteases inhibitor cocktail was added for 5minutes. Cells were removed by scrapping and centrifuged at 14000g, for 10min at 4ºC (Hettich Zentrifugen MIKRO 220R) and supernatants were freeze at -80ºC until the day of experiment. Protein concentration was determined using Bradford assay.

Proteins (30µg) were separated by SDS-PAGE in a 12% (w/v) acrylamide gel and transfer onto a nitrocellulose membrane (45µm) using a Mini Trans-Blot® system from BioRad. After blocking with 1%BSA (w/v) in Tris buffered saline solution containing 0,1% (v/v) of ultra pure Tween-20 (TBST) with slightly agitation, the membrane was incubated overnight at 4ºC with primary antibody against IκBα (mouse antibody) or with primary antibody against β-actin. The membrane was washed three times for 5 minutes with TBST and incubated with secondary HRP-labeled antibody for 1hour (FentoMax^{TM} kit) and was revealed according to kit's manufacture protocol using ChemiDoc® from BioRad. Relative intensities were calculated using ImageLab® software from BioRad. All solutions used in SDS-PAGE and Western Blot are in appendix B.

3. Results and discussion

3.1. *Opuntia ficus-indica* and *Prunus Avium* polyphenolic-rich extracts (PRE) characterization

In this work, macroporous adsorption resin Amberlite® XAD16 was used to recover and concentrate polyphenols from *Saco* cherry crude extracts and *Opuntia ficus-indica* juice as described before (material and methods, 2.3). Resin Amberlite® XAD16 was chosen due to its low operation cost, simple handling and high absorption capacity for a large number of compounds, namely compounds with aromatic rings such as phenolics (Di Mauro et al, 2002; Scordino et al, 2003; Silva et al, 2007).

The polyphenolic-rich extracts (PREs) obtained were characterized for their total phenolic and flavonoid content (figure 3.1 and 3.2) and phenolic composition by HPLC and also for their chemical antioxidant activity using three different *in vitro* assays: ORAC, HORAC and inhibition of LDL oxidation (figure 3.3 and 3.4).

3.1.1 Phenolic composition

3.1.1.1 Total phenolic content (TPC)

Both PREs were analyzed for their total polyphenolic content using Folin Ciocalteau colorimetric assay and the obtained results are presented in figure 3.1 and are expressed as mg of gallic acid equivalents (GAE) *per* g of dry extract.

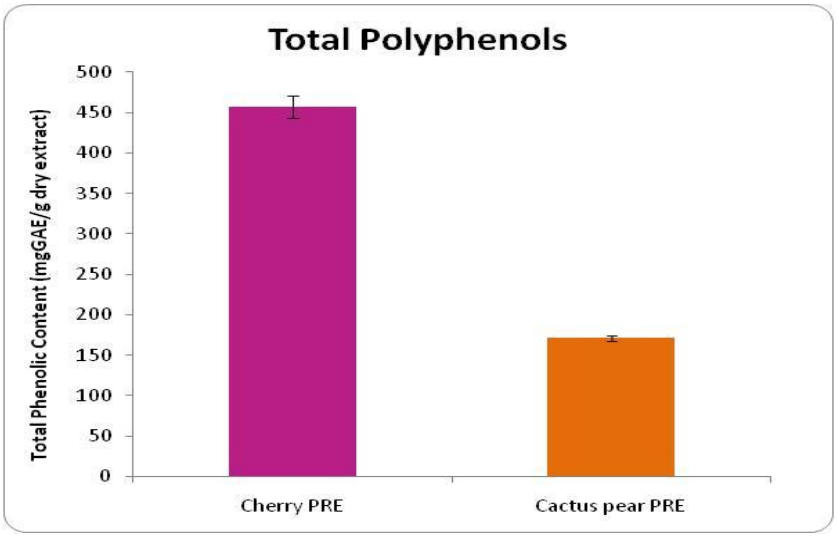


Figure 3.1 – Total polyphenolic content of cherry and cactus pear PREs determined by Folin Ciocalteau colorimetric assay. Results are expressed in mg GAE/g of dry extract.

The process using macroporous adsorption resin Amberlite® XAD16 was more efficient to cherry polyphenols since cherry's PRE presents about three fold higher polyphenolic content (456,9mgGAE/g dry extract) than cactus pear's PRE (171,2mgGAE/g dry extract). This higher efficiency can be due to previous solvent extraction of cherries using a 50:50 mixture of ethanol and water that is a very common method used to extract phenolic compounds (Ignat et al, 2011). Otherwise, the high viscosity of cactus pear's juice could difficult the agitation process reducing the adsorption process efficiency.

3.1.1.2 Total flavonoids content

As mentioned before in introduction section, flavonoids are potent antioxidant and anti-inflammatory compounds (González et al, 2011; Suzuki & Hara, 2011). PREs were studied for their total flavonoids content and the obtained results expressed as mg equivalents of catechin equivalents (CE) *per* mg of polyphenols are presented in figure 3.2. Note that both PRE were analyzed using the some polyphenolic content.

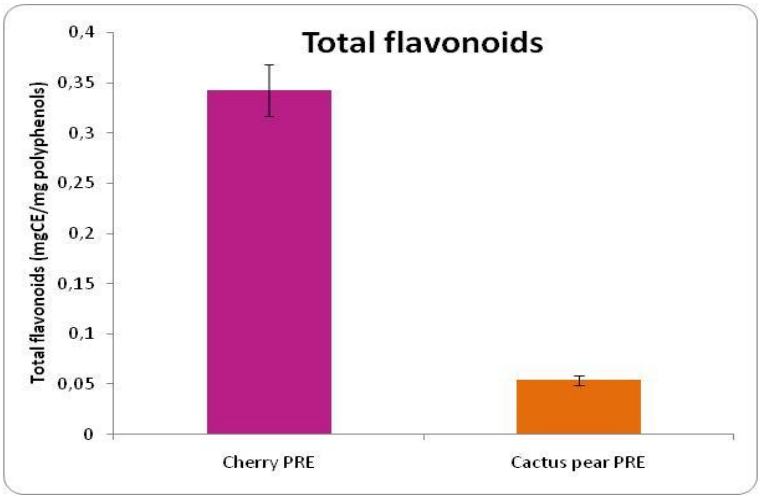


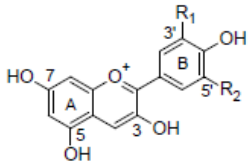
Figure 3.2 – Total flavonoids content of cherry and cactus pear PREs using a colorimetric assay.

Cherry's PRE contain more total flavonoids than cactus pear's one, which could be also due to the previous extraction of cherry fruits as described before and different efficiency of adsorption process, since both fruits are rich in flavonoids (Cayupan et al, 2011; Fernández-López et al, 2010; Ferretti et al, 2010; Feugang et al, 2006; McCune et al, 2011).

3.1.1.3 Phenolic composition by HPLC

PREs also have different polyphenolic composition: anthocyanins were the major compounds identified in cherry's PRE, namely cyanidin-3-glucoside, cyanidin-3-rutinoside and peonidin-3-glucoside, while in cactus pear's PRE were founded mainly isorhamnetin and its glycosides derivates, namely, isorhamnetin-3-O-lyxose-rhamnose-glucoside, isorhamnetin-3-O-lyxose-glucoside and isorhamnetin-3-O-rutinoside. A betalain, namely a betaxantin designed as indicaxantin, was also identified in cactus pear's PRE (see polyphenolic content of both PRE in annex C). All of these compounds are reported as potent antioxidant compounds (Fernández-López et al, 2010; Paredes-López et al, 2010; Quideau et al, 2011; Serra et al, 2010; Stevenson & Hurst, 2007).

Anthocyanins are flavonoids recognized for their promoting health benefits mainly because their strong antioxidant activity (Blando et al, 2004; McCune et al, 2011). Due to their singular structure (figure 3.3), anthocyanins can scavenge ROS and also chelate metals preventing the formation of free radicals by Fenton or Fenton-like reactions (Miguel, 2011). Furthermore, these compounds are, also, reported as anti-inflammatory compounds (Blando et al, 2004; Hou et al, 2004; Karlsen et al, 2007; Lila, 2004; Miguel, 2011; Stintzing & Carle, 2004).



Anthocyanidin	R_1	R_2	Colour
Pelargonidin	H	H	Orange
Cyanidin	OH	H	Orange-red
Delphinidin	OH	OH	Bluish-red
Peonodin	OCH_3	H	Orange-red
Petunidin	OCH_3	OH	Bluish-red
Malvidin	OCH_3	OCH_3	Bluish-red

Figure 3.3 – Structure of major anthocyanins (Miguel, 2011).

Isorhamnetin is a flavonoid, more specifically, it is a quercetin derivate (3'-O-methylquercetin) (Duenas et al, 2011). This compound possesses four hydroxyl groups (figure 3.4) which enhance its antioxidant activity (Liu et al, 2009). It is also reported that isorhamnetin and their conjugates possess other health benefits beyond antioxidant activity, namely anti-inflammatory activity (Boesch-Saadatmandi et al, 2011; Hamalainen et al, 2007; Liu et al, 2009).

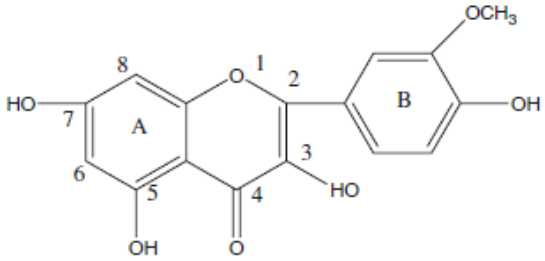


Figure 3.4 – Structure of isorhamnetin (Liu et al, 2009).

Betalains are bioactive compounds present in cactus pear that possess recognized antioxidant and anti-inflammatory activity (Cai et al, 2003; M.C.Azeredo, 2009; Stintzing & Carle, 2004; Stintzing & Carle, 2007).

Due to their rich composition in bioactive compounds, cactus pear's and cherry's PRE could be interesting antioxidant and anti-inflammatory products. In order to evaluate the potential antioxidant activity of PREs, chemical antioxidant activity was studied.

3.1.2 Chemical Antioxidant activity

Chemical antioxidant activity of both PREs were compared using three different and complementary *in vitro* methods: ORAC assay that reflects peroxyl radical absorption/scavenging capacity (figure 3.5), HORAC assay that primarily reflects metal chelating radical prevention ability (figure 3.5) and inhibition of LDL oxidation (figure 3.6). Note that, in all assays, both PRE were analyzed using the some polyphenolic content.

3.1.2.1 ORAC and HORAC assays

ORAC (oxygen radical absorbance capacity) assay was used to evaluate the antioxidant capacity of both PRE towards peroxyl radicals and measure the ability of the antioxidant species present in

cactus pear's and cherry's PRE to inhibit the oxidation of fluorescein catalyzed by AAPH. On the other hand, HORAC evaluates the hydroxyl radical prevention capacity of both PRE using fluorescein as the probe. The hydroxyl radical was generated by a Co(II)-mediated Fenton like reaction and, similarly to ORAC assay, the fluorescence decay of fluorescein was used to quantify the HORAC value. Results of antioxidant activity of both PRE measured by ORAC (μmol TEAC/ g polyphenols) and HORAC (μmol CAEAC/ g polyphenols) assays are presented in figure 3.5.

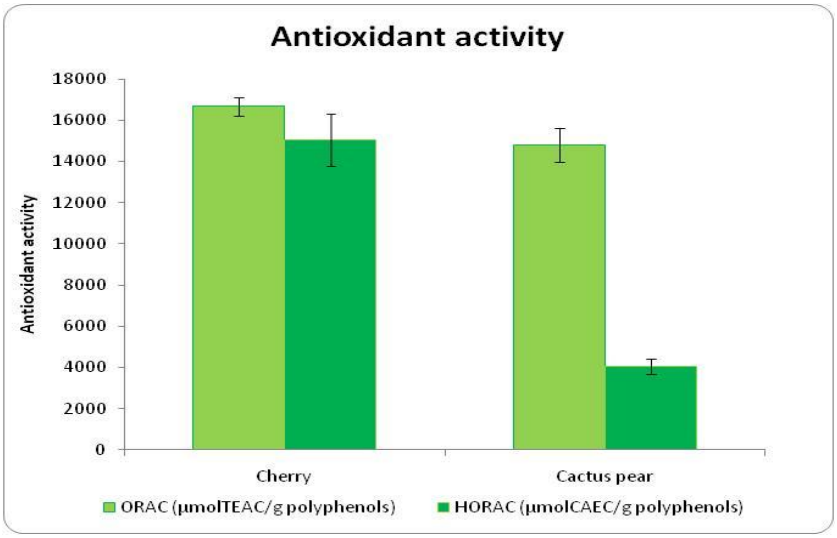


Figure 3.5 – Antioxidant activity of cherry's and cactus pear's polyphenolic-rich extracts (ORAC and HORAC values).

Both PREs have high and similar ORAC but distinct HORAC values. Besides the difference in their composition, polyphenols present in both PRE have similar capacity to scavenge peroxyl radicals.

In turn, cherry's PRE has about 4 times higher HORAC value than cactus pear's PRE which could be due to high anthocyanins content in cherries which have been reported to have a great contribution to the cherry's higher HORAC values than other fruits (Ou et al, 2002; Serra et al, 2011a). The antioxidant effectiveness of anthocyanins is essentially due to the ease with which a hydrogen atom from an aromatic hydroxyl group is donated to a free radical. Moreover, their unique structure allows the chelation of transition metal ions involved in radical-forming processes such as Fenton reactions which could explain their contribution to a higher HORAC value in cherries (Ferretti et al, 2010; Fraga et al, 2010; McCune et al, 2011; Paredes-López et al, 2010; Serra et al, 2011a).

3.1.2.2 Inhibition of LDL oxidation

Inhibition of LDL oxidation was another method used to characterize chemical antioxidant activity of both PREs (figure 3.6). Oxidized LDL is a biomarker of oxidative stress and is one of the alterations observed in atherosclerosis. The antioxidant capacity of cactus pear's and cherry's PRE (50mg GAE/L) against LDL oxidation induced by AAPH was determined following the formation of conjugated dienes at 234nm and results are presented in figure 3.6.

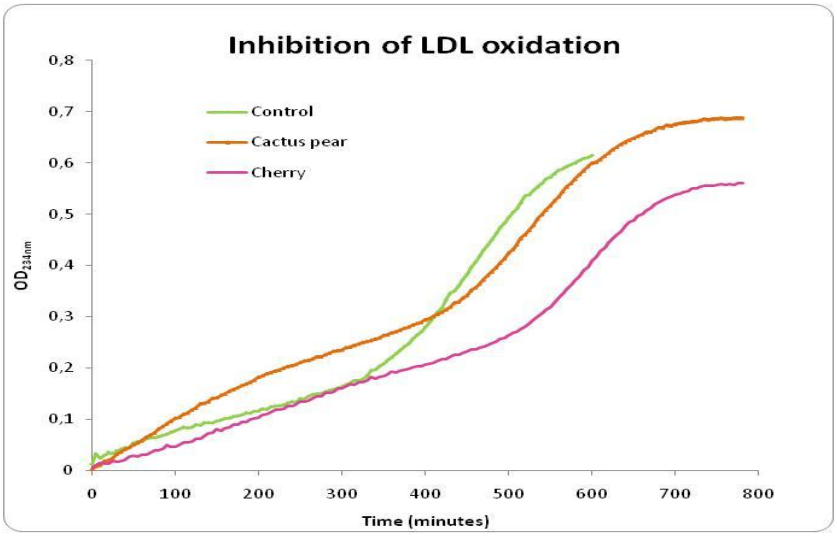


Figure 3.6 - Kinetics of LDL oxidation induced by 500μM of AAPH measured by conjugated dienes (OD_{234nm}) formation in control LDL and LDL in the presence of 50mg GAE/L of cherry's and cactus pear's PRE.

Cherry's extract was, once again, more effective, preventing LDL oxidation in 30,8%, while cactus pear's extract prevent in 20%. The superior content in flavonoids, particularly the high content in anthocyanins, of cherry's PRE can explain this difference, as reported before (Kaliora et al, 2006; Serra et al, 2011a).

All results obtained for cherry's and cactus pear's PRE characterization are summarized in table 3.1.

Table 3.1 – Polyphenolic content and antioxidant activity of cactus pear's and cherry's polyphenolic-rich extracts

	Cherry PRE	**Cactus pear PRE**
Total polyphenols (mg GAE/ g extract)	456,9 ± 13,8	171,2 ± 3,3
Total flavonoids (mgCE/mg polyphenols)	0,34 ± 0,03	0,05 ± 0,00
ORAC (μmol TEAC/g polyphenols)	16657,9 ± 466,2	14793,9 ± 825,0
HORAC (μmol CAEAC/g polyphenols)	15044,9 ± 1284,8	4049,0 ± 395,4
Inhibition of LDL oxidation (%)	30,8	20,0

Thus, cherry's PRE has shown to be better in scavenging peroxyl radicals (measure by ORAC assay), preventing radical formation by metal chelation (HORAC assay) and inhibiting AAPH-induced LDL oxidation (table 3.1). Rich composition in flavonoids, namely anthocyanins may contribute for higher chemical antioxidant activity observed for cherry's extract (Ferretti et al, 2010).

3.2. Cellular antioxidant activity of PREs in an *in vitro* human intestinal cell-based model

As mentioned before, oxidative stress is a key factor in many chronic diseases such as cardiovascular and neurodegenerative diseases, *diabetes*, asthma, Inflammatory Bowel diseases,

among others. Despite the existence of an endogenous antioxidant system in human body, exogenous antioxidants, such as polyphenols, can contribute to oxidative homeostasis by preventing radical formation, scavenging radicals or by stimulation of endogenous antioxidant system (Halliwell, 2011; Masella et al, 2005; Obrenovich et al, 2011; Rodrigo et al, 2011).

As first approach, it was important to assess the PREs cytotoxicity in Caco-2 model in the selected concentration (50mg GAE/L) and results are presented in figure 3.7. The selected concentration of polyphenols used for both PRE in all subsequent assays (50mg GAE/L) was defined taking into account that the average daily intake of polyphenols is estimated in approximately 1g/day which means that polyphenolic concentration in the gastrointestinal tract can reach levels up to several hundred µmol/L (about 10% of daily intake). Therefore, 50mgGAE/L is an expectable intestinal concentration (D'Archivio et al, 2007; Romier et al, 2009; Williamson & Manach, 2005).

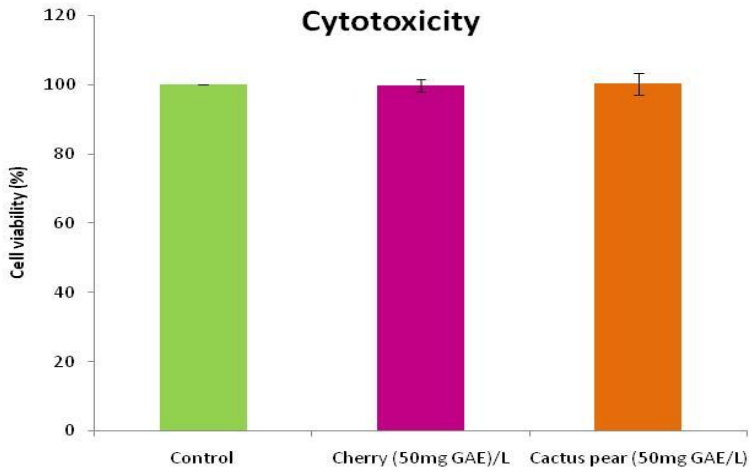


Figure 3.7 – Cell viability (%) after incubation with PREs during 48h.

Incubation with cherry's and cactus pear's PREs (50mg GAE/L) for 48h doesn't lead to any cytotoxicity, since cell's viability is approximately 100% in both cases (figure 3.7) which was expected because it was used a physiological concentration.

Using Caco-2 model, as described in material and methods section, intracellular antioxidant activity was evaluated through three different ways: i) inhibition of ROS for pre-incubation and co-incubation of PREs in cells submitted to two kind of stress inductors (H_2O_2 and AAPH) (figure 3.8); ii) protection of PREs against GSH oxidation (figure 3.9) and iii) protection of PREs against protein oxidation (measured by level of carbonyl proteins in cells) (figure 3.10).

3.2.1 Inhibition of ROS formation

AAPH is an azo compound which generates peroxyl radicals by thermal decomposition (Serra et al, 2011a) while H_2O_2 can generated hydroxyl radicals by Fenton or Fenton-like reactions (Wijeratne et al, 2005). Both are stress inductors capable to generate oxidative stress in Caco-2 cells with an increase in ROS production. Polyphenols are known for their capacity to prevent or decrease oxidative stress and in figure 3.8 is presented the capacity of cherry's and cactus pear's PRE to inhibit ROS (inhibiting its formation or scavenging it) in presence of both stress inductors and in two different

conditions: co-incubation (when cells were co-incubated with PREs plus stress inductors) and pre-incubation (when cells are incubated with PREs previously to the stress induction).

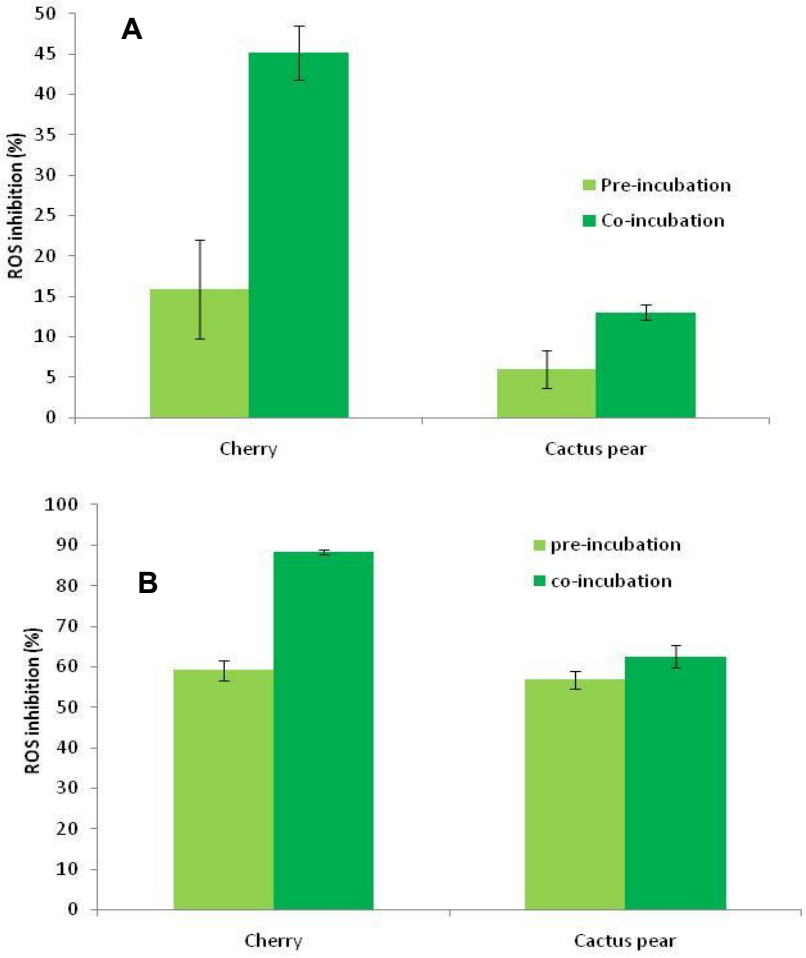


Figure 3.8 - Effects of cherry and cactus pear extracts (50 mg GAE/L) on (A) H_2O_2 and (B) AAPH induced ROS in Caco-2 cells, measured by 2',7'-dichlorofluorescin oxidation. Comparison between co- and pre-incubation assays.

When hydroxyl radicals production were induced, cherry's PRE was more efficient than cactus pear's PRE, in both conditions, pre- and co-incubation, which is in agreement with HORAC assay that shows higher antioxidant activity against hydroxyl radicals for cherry PRE. In fact, cherry's PRE has about 3,5 fold higher cellular antioxidant activity against hydroxyl radicals that cactus pear's PRE which is practically the some fold difference obtained for both PRE for HORAC assay. Co-incubation values were higher than pre-incubated in both PREs which could indicate that are compounds that didn't be cellular uptake by Caco-2 cells but could act against extracellular free radicals injury. This explanation could be emphasized for cherry's PRE for pre- and co-incubation assays where were obtained different values, which can be due to the presence of anthocyanins that are compounds with lower cellular uptake in Caco-2 monolayer but are powerful scavengers of extracellular free radicals (Paredes-López et al, 2010; Serra et al, 2011a; Yi et al, 2006).

When peroxyl radicals production were induced, both PRE shows similar protection in pre-incubation assay which is in agreement with values obtained in ORAC assay. For cactus pear's PRE there are no significant differences between pre- and co-incubation which means that phenolic compounds with higher permeability in Caco-2 cells or those that can interact with cellular membrane

can be responsible agents for the cellular antioxidant response. Once again, cherry's PRE presented higher antioxidant activity in co-incubation assay which can be due to the high anthocyanins content in this extract that cannot be uptaked by Caco-2 monolayer. Note that in both cases (cherry's and cactus pear's PRE) the protection against oxidative stress is higher using AAPH as stress inductor than using H_2O_2 which indicate a higher protection of both PRE against peroxyl radicals.

3.2.2 Inhibition of glutathione oxidation

Glutathione plays crucial roles in biological systems: it is important in regeneration of other antioxidants, in the detoxification of potentially harmful endogenous xenobiotics and other compounds and is an enzyme cofactor for many enzymes, namely antioxidant enzymes. Glutathione (GSH) can readily be oxidized to it disulfide (GSSG) and the ratio of both forms is crucial for the characterization of oxidative stress in cells (Kand'ar et al, 2007; Masella et al, 2005). Cactus pear's and cherry's PREs (50mg GAE/L) were analyzed for their capacity to prevent H_2O_2-induced glutathione oxidation in Caco-2 cells and the results are presented in figure 3.9.

It has been widely reported that H_2O_2 is highly deleterious to cells, and that its accumulation causes the oxidation of cellular targets such as DNA, several proteins, and lipids, leading to mutagenesis and cell death. Depletion in GSH and consequently decrease of [GSH]/[GSSG] ratio is one of the consequences of oxidative stress generated by H_2O_2 in Caco-2 cells, which is in agreement with our results that shows a decrease in this ratio when cells were exposed to this stress inductor – negative control (figure 3.9) (Cilla et al, 2008; Wijeratne et al, 2005).

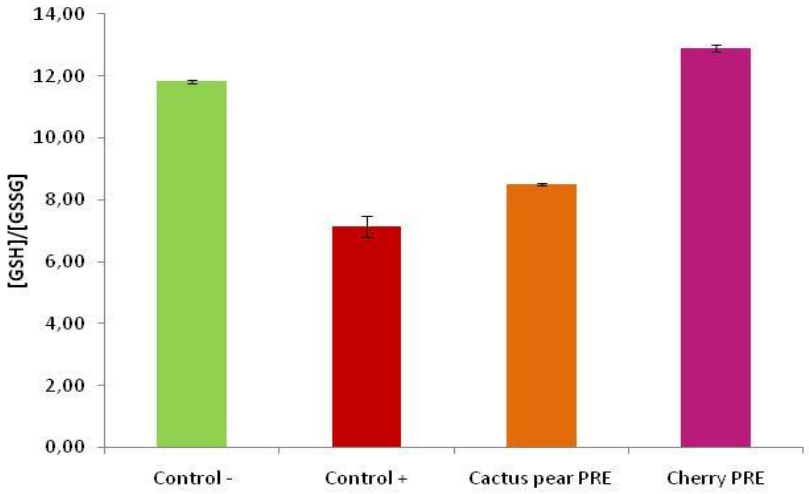


Figure 3.9 – Effect of cactus pear's and cherry's PRE (50mg GAE/L) in the ratio between GSH and its oxidized form, GSSG, concentration. Negative control (–) consists in cells without stress inductor (H_2O_2) and positive control (+) consists in cells incubated with stress inductor.

When cells were pre-treated with cactus pear the reduction in [GSH]/[GSSG] ratio due to stress injury was less accentuated. Moreover, cells treated with cherry PRE presented [GSH]/[GSSG] ratio similar to the basal one (negative control). Previous reports showed the capacity of cherries and cherry's extracts to reduce directly the stress oxidative damages by their capacity to scavenge or prevent radicals formation and indirectly by inducing endogenous antioxidant system, namely by increasing GSH levels and enzymes related with glutathione antioxidant system (Cvorovic et al, 2010;

Ferretti et al, 2010; McCune et al, 2011). As far as is known, there are no reports that study the influence of cactus pear's polyphenols in glutathione homeostasis. Obtained results indicate that polyphenols present in cactus pear's PRE can prevent against GSH oxidation.

3.2.3 Inhibition of protein oxidation

Protein oxidation is another biological marker of oxidative stress and can be measured by the formation of carbonyl proteins (Dalle-Donne et al, 2003; Ramful et al, 2010). In this work the prevention against H_2O_2 – induced protein oxidation by the two PREs was evaluated using Caco-2 cells pre-incubated with both PREs and the results expressed as percentage of carbonyl proteins content relatively to positive control are presented in figure 3.10.

Oxidative stress induced by H_2O_2 leads to protein oxidation, which can be observed in figure 3.10 where cells from positive control (cells incubated with stress inductor) has a two-fold increase in carbonyl proteins content when compared to negative control (cells without stress inductor). These results are in agreement with previous studies where hydrogen peroxide increases protein oxidation in Caco-2 cells (Cecarini et al, 2007; Chen et al, 2009).

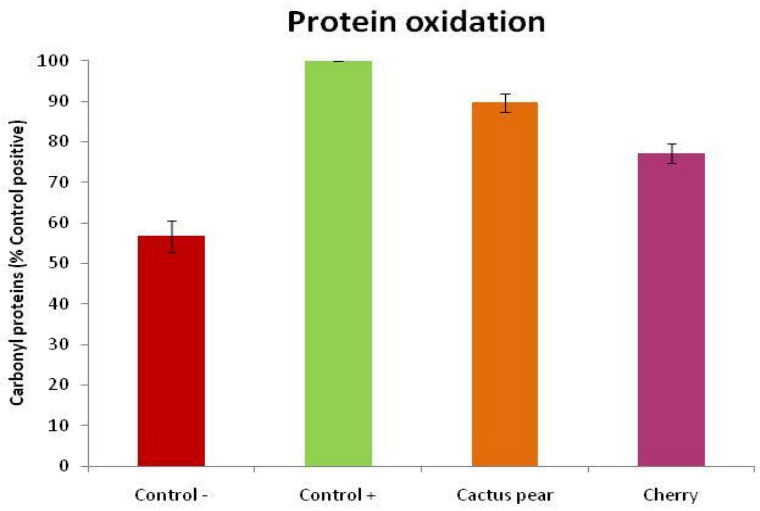


Figure 3.10 – Prevention of Cactus pear and Cherry PRE against protein oxidation. This capacity was measured by carbonyl proteins formation using H_2O_2 as stress inductor. Negative control (-) represents cells without stress inductor and positive control (+) represents cells with stress inductor and without pre-incubation with any PRE.

Pre-incubation with both PRE decreases carbonyl proteins content when compared to positive control, protecting against oxidative stress – mediated protein oxidation. However, due to their composition and in agreement with other antioxidant results, cherry's PRE was more efficient in prevention against carbonyl protein formation (Ferretti et al, 2010; McCune et al, 2011; Serra et al, 2011a).

All results obtained for cellular antioxidant activity of *Opuntia ficus-indica* and *Prunus avium* polyphenolic-rich extracts are summarized in table 3.2.

Table 3.2 – Cellular antioxidant activity of cactus pear's and cherry's polyphenolic-rich extracts

	Negative control*	Positive control**	Cherry PRE	Cactus pear PRE
ROS inhibition (%) pre-incubation/ H_2O_2	----------	----------	15,9 ± 6,1	6,0 ± 3,3
ROS inhibition (%) co-incubation/ H_2O_2	----------	----------	45,1 ± 3,3	13,0 ± 0,9
ROS inhibition (%) pre-incubation/ AAPH	----------	----------	59,1 ± 2,3	56,8 ± 2,2
ROS inhibition (%) co-incubation/ AAPH	----------	----------	88,3 ± 0,7	62,6 ± 2,8
[GSH]/[GSSG]	11,8 ± 0,1	7,1 ± 0,4	12,9 ± 0,1	8,51 ± 0,0
Inhibition of protein oxidation (%)	56,7 ± 3,9	100,0 ± 0,0	77,1 ± 2,4	89,7 ± 2,4

* Cells without stress inductor.
**Cells incubated with stress inductor and without PRE.

PREs were analyzed for their intracellular antioxidant activity using the some polyphenolic concentration (50mg GAE/L). In the case of ROS inhibition, for cherry's PRE there are compounds that aren't uptaked by Caco-2 cells but could protect against oxidative damages (most of them can be located at cellular membrane). Otherwise, cactus pear's PRE presented similar results in pre and co-incubation assay. These results emphasize the different composition of both PRE.

For all assays, protection against oxidative stress exerted by cherry's PRE is higher than that observed for cactus pear's PRE.

3.3. Anti-inflammatory activity of PREs in an *in vitro* human cell-based model of intestinal inflammation

Inflammatory Bowel Diseases (IBD), such as Crohn's disease and Ulcerative colitis, are incurable diseases related with chronic intestinal inflammation and their incidence is increasing worldwide which are generating a huge concern in scientific community (Rahimi et al, 2009; Shimizu, 2010; Van De Walle et al, 2010). Moreover, IBD is classically treated with pharmacological agents that are known for their secondary effects and also for their high costs. Thus, nowadays has emerged the necessity of search and development of new, safe and low cost compounds, preferably natural compounds that could be use for prevention, treatment or delay of this kind of diseases. Polyphenols appear as perfect candidates for this application due to their reported antioxidant and anti-inflammatory activity (Aguzzi et al, 2011; Jung et al, 2009; Kaplan et al, 2007; Leonard et al, 2010; Mandalari et al, 2011; Pan et al, 2009; Rahimi et al, 2009; Romier et al, 2009; Romier et al, 2008; Romier-Crouzet et al, 2009; Sergent et al, 2010; Sonier et al, 2009).

3.3.1 Establishment and characterization of intestinal inflamed cell model

Although different cell types in the gut contribute to the inflammatory response *in vivo*, Caco-2 cells were chosen to establish a valid model to evaluate anti-inflammatory activity of cherry and cactus pear PRE in IBD, since intestinal epithelial cells play a crucial role in IBD pathogenesis. The Caco-2 cell line was selected since differentiated Caco-2 cells express functional tight junction (TJ), brush border characteristics and biotransformation enzymes and their differentiation process is thought to reflect the maturation occurring *in vivo*, which makes these cells probably the best *in vitro* model of human enterocytes currently available (Leonard et al, 2010; Shimizu, 2010).

The intestinal inflammatory response requires the correct integration of various signals that are released or received by different cell types in intestinal mucosa, so it is important to choose the right inflammatory stimuli that can mimic IBD characteristics like: cytokine and chemokine secretion, barrier dysfunction and inflammatory pathways activation such as NF-κB pathway.

TNF-α, IL-1β and LPS were selected to stimulate Caco-2 cells and mimic the IBD main characteristics since TNF-α and IL-1β are cytokines whose mucosal levels are increased during IBD as well as LPS due to the increased permeability to bacteria (Al-Sadi & Ma, 2007; Leonard et al, 2010; Van De Walle et al, 2010).

The concentrations of pro-inflammatory cocktail selected (50ng/mL TNF-α, 25ng/mL IL-1β and 10µg/mL LPS) were determined according to the literature (Al-Sadi & Ma, 2007; Erlejman et al, 2008; Leonard et al, 2010; Romier et al, 2008; Romier-Crouzet et al, 2009; Sergent et al, 2010) and are physio-pathologically relevant, reflecting the much localized increase in cytokine production in the gut mucosa during active IBD. The chosen inflammatory stimuli were verified for their cytotoxicity using fully differentiated Caco-2 cells and results are presented in figure 3.11.

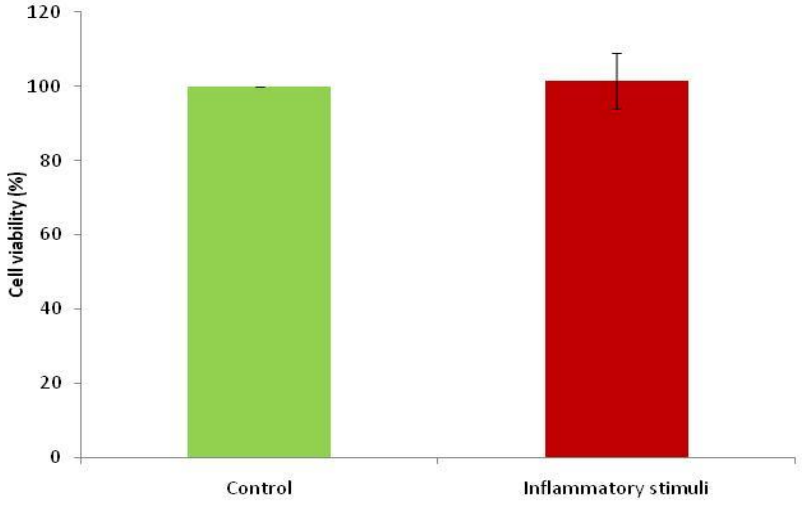


Figure 3.11 – Cell viability (%) after incubation with inflammatory stimuli during 48h.

The inflammatory stimuli didn't alter cell viability, which means that they are not cytotoxic (figure 3.11).

In this study were assessed different Caco-2 response parameters to inflammatory stimuli selected:

- IL-8 secretion: IL-8 is a chemokine attracting neutrophils during the early stage of intestinal inflammation,

- NO secretion: NO is a mediator produced through the conversion of L-arginine to L-citruline by the nitric oxide synthase (NOS) and constitutively synthesized in gut to regulate gastro-intestinal blood flow, motility and secretion but in inflammatory condition, namely in IBD, there is a large increase in NO production through upregulation of the expression of inducible-NOS (iNOS) isoform, and this can lead to altered permeability and motility, cytokine production, oxidative damage and tissue injury (Van De Walle et al, 2010),
- IL-10 secretion: IL-10 is an anti-inflammatory cytokine that is constitutively expressed but their secretion is supposed to increase in response to inflammatory stimuli (Sergent et al, 2010);
- NF-κB activation: NF-κB is a transcription factor responsible for the transcription of many pro-inflammatory genes (Romier et al, 2008),
- Barrier dysfunction, an important biomarker of IBD, which could be measure by TEER decrease and permeability alterations.

Note that is really important to characterize the established model since IBD have singular inflammatory characteristics that need to be mimic in order to obtain a valid *in vitro* model for this disease.

The first task was to define the localization (apical or basolateral) and time (24 and 48h) of inflammatory stimuli in terms of induction of barrier dysfunction using Caco-2 cells seeded in Transwell® inserts. Experimental setup used for this task is presented in figure 3.12.

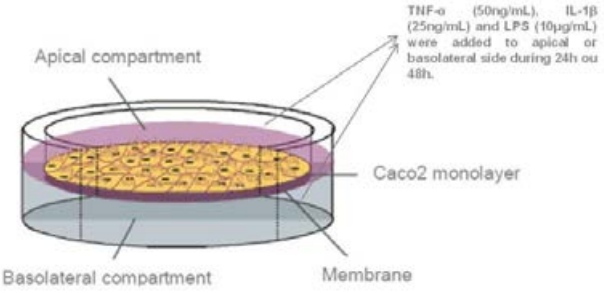


Figure 3.12 – Experimental setup study for definition of inflammatory stimuli location (basolateral or apical) and time (24 and 48h) in fully differentiated Caco-2 cells.

The defective intestinal epithelial TJ barrier function has been proposed as an important pathogenic factor leading to the intestinal inflammation. IBD patients demonstrate increased intestinal paracellular permeability, which reflects decreased epithelial barrier function (Edelblum & Turner, 2009). Furthermore, cytokine-induced increase in intestinal TJ permeability represents an important pro-inflammatory mechanism (Al-Sadi & Ma, 2007). Since an increased paracellular permeability is a hallmark of intestinal inflammation the effect of inflammatory stimuli selected on the Caco-2 cell barrier integrity was evaluated through the measurement of TEER (transepithelial electrical resistance), which quantifies the measurement of ions across the paracellular pathway and obtained results are presented in figure 3.13.

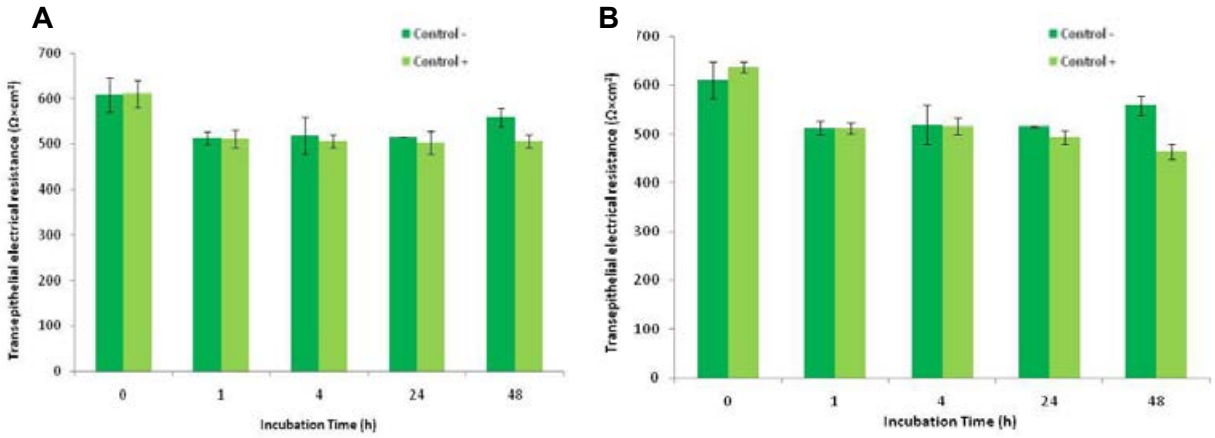


Figure 3.13 – TEER values of Caco-2 monolayer stimulated with the pro-inflammatory cocktail selected (50ng/mL TNF-α, 25ng/mL IL-1β and 10µg/mL LPS) in A) apical compartment and B) in basolateral compartment both during 48h. Negative control (-) represents cells without inflammatory stimuli and positive control (+) represents cells incubated with inflammatory stimuli.

Observing the results presenting in figure 3.13 it is easily seen that pro-inflammatory stimulation in apical compartment didn't had a visible impact in TEER values, while stimulation in basolateral side shown a decrease in transepithelial resistance values. These results are in agreement with previous reports that indicate the polarized location of cytokines receptors in intestinal epithelial cells (IECs) mostly in basolateral side (Leonard et al, 2010; Sergent et al, 2010; Van De Walle et al, 2010). Moreover, this TEER decrease is not visible at 24h of incubation with inflammatory stimuli, becoming evident at 48h of incubation. So, it was decided to continue our studies using inflammatory stimuli on basolateral side.

The barrier dysfunction was assessed by evaluation of fluorescein permeability across inflamed Caco-2 monolayer stimulated on basolateral side and obtained value for apparent permeability of fluorescein are presented in table 3.3.

Table 3.3 – Apparent permeability (P_{app}) of fluorescein across inflamed Caco-2 monolayer in both directions (apical to basolateral and basolateral to apical side) during 24h and 48h.

	P_{app} 24 hours (cm/s)		**P_{app}48 hours (cm/s)**	
	Apical→ Basolateral	**Basolateral → Apical**	**Apical→ Basolateral**	**Basolateral → Apical**
Control - *	$4{,}31 \times 10^{-7}$	$3{,}74 \times 10^{-7}$	$6{,}04 \times 10^{-6}$	$4{,}58 \times 10^{-6}$
Control + **	$4{,}39 \times 10^{-7}$	$4{,}28 \times 10^{-7}$	$7{,}39 \times 10^{-6}$	$8{,}47 \times 10^{-6}$

* Cells without inflammatory stimuli.
** Cells with inflammatory stimuli.

As mentioned in section 2.6.4 of material and methods, apparent permeability was calculated using equation 1.

$$P_{app} = (dQ/dt)\ (1/A)\ (1/C_0) \quad (1)$$

where dQ/dt is the amount of drug transported *per* time, A is the surface area of the monolayer and C_0 is fluorescein concentration (µg/mL) at time 0.

After 24hours of incubation with the selected pro-inflammatory stimuli and compared with negative control, there are no significant differences in P_{app} of fluorescein across inflamed Caco-2 monolayer in both directions. This fact is in agreement with TEER results that also exhibit no significant differences.

After 48h of incubation with selected inflammatory stimuli there was a slightly increase in permeability of fluorescein in apical to basolateral direction while permeability of fluorescein in basolateral to apical direction was increased in two-fold. Note that one important feature of IBD is the excessive recruitment of immune cells to the intestinal mucosa which could explain the higher obtained increase of permeability in basolateral to apical direction. Moreover, these results are in tune with those obtained for TEER measurement that showed a evident decrease after 48h of incubation with selected pro-inflammatory cocktail and are in agreement with previous reports (Leonard et al, 2010).

Morphologic alterations caused in Caco-2 cells by the selected inflammatory stimuli were also studied and pictures of inflamed monolayer are present in figure 3.14.

It was observable more pronounced morphological alterations in Caco-2 monolayer when stimuli were added to the basolateral compartment during 48h, confirming permeability alteration results (figure 3.14). It is visible that inflamed cells after 48h of stimulation are smaller and present open tight junctions which are not so observable after 24hours. These results justify the permeability alteration seen in TEER measurements and fluorescein permeability assays. Moreover, these alterations are not so visible when stimuli were added to the apical compartment, which reinforce the main localization of cytokine receptors at the basolateral side.

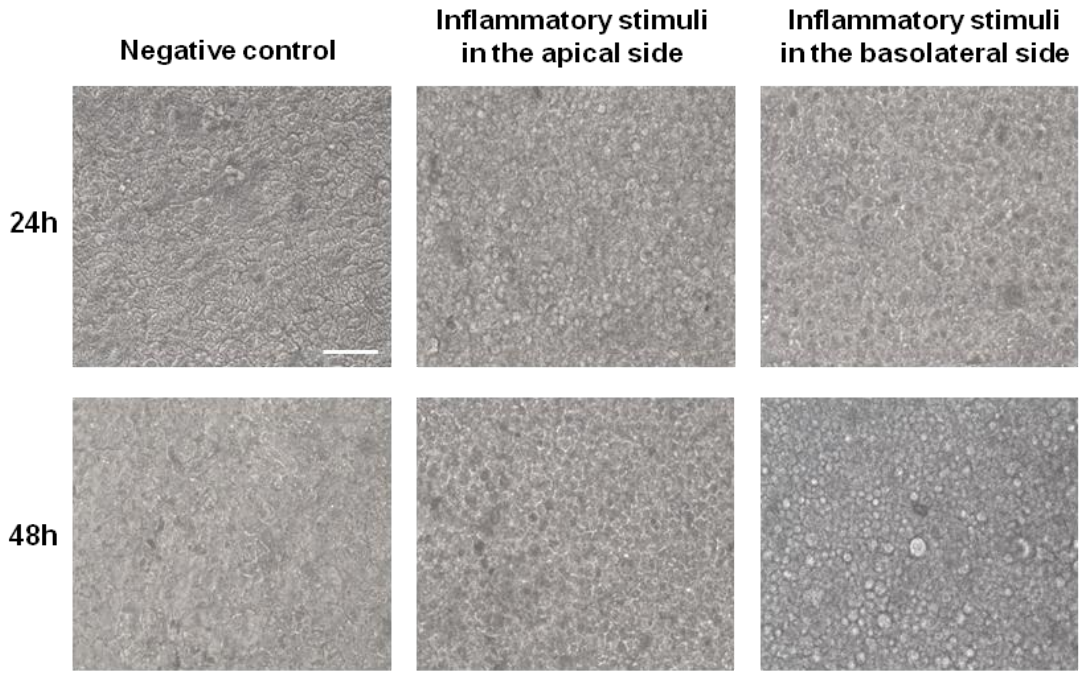


Figure 3.14 - Inflammation-induced morphological alterations in Caco-2 monolayer. Phase contrast images of cell cultures incubated with pro-inflammatory stimuli in the apical or basolateral side during 24h or 48h. Negative control culture was performed by incubating cells in standard culture medium without pro-inflammatory stimuli. Scale bar: 100µm.

Obtained results showed that a TEER decrease (figure 3.13) is accompanied by an increase in cellular permeability (table 3.3) as well as more defined and open TJs (figure 3.14) confirming that the selected pro-inflammatory stimuli is adequate to mimic the barrier dysfunction that is a biomarker of IBD pathogenesis.

Taking into account the obtained results to inflammatory stimuli barrier dysfunction induction, all subsequent studies were performed with inflammatory stimulation in the basolateral side during 48hours.

In order to verify if the selected pro-inflammatory stimuli composed by 50ng/mL TNF-α, 25ng/mL IL-1β and 10µg/mL LPS can mimic IBD main characteristics, such as an oversecretion of IL-8 by IECs, IL-8 secretion by inflamed Caco-2 cells was evaluated and results, expressed as percentage of IL-8 secretion relatively to negative control, are presented in figure 3.15.

Results obtained showed that IL-8 are constitutively expressed by fully differentiated Caco-2 cells but their secretion increase with inflammation induction, which indicates that selected stimuli can upregulate IL-8 secretion. Moreover, the increase in IL-8 secretion is more evident in basolateral compartment, which indicates that cells preferentially secreted this cytokine to basolateral side due to the subepithelial recruitment of immune cells to the intestinal mucosa (Sergent et al, 2010). Thus, the selected inflammatory stimuli can increase IL-8 secretion by inflamed IECs, and it can mimic other biomarker of IBD.

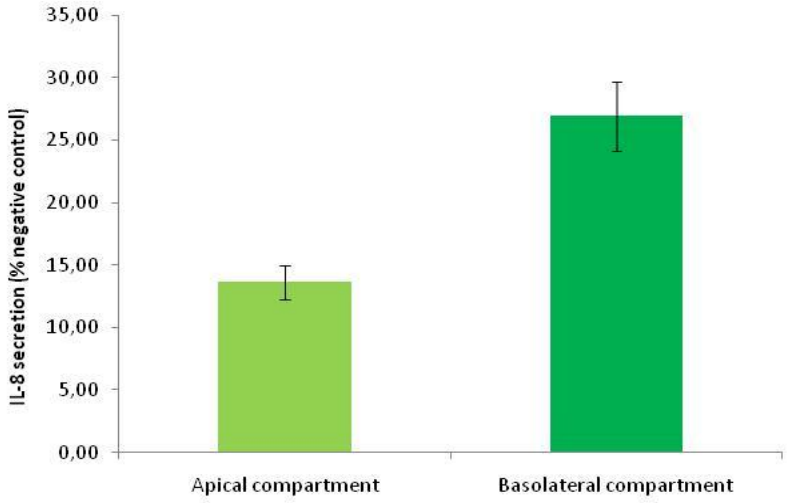


Figure 3.15 – IL-8 secretion by inflamed Caco-2 cells to the apical and basolateral compartment. Results are expressed in % to the negative control (cells without inflammatory stimuli).

IL-10 is an anti-inflammatory cytokine that, contrary to normal inflammation, isn't induced or is only slighted induced in IBD condition, which reinforce another hallmark of chronic intestinal inflammation: an unbalance production of cytokine/chemokine in favor of pro-inflammatory mediators (Mueller et al, 2010; Sergent et al, 2010). In order to find if the selected inflammatory stimuli induce IL-10 expression, IL-10 secretion was determining in inflamed Caco-2 cells and results, expressed as percentage of IL-10 secretion relatively to negative control, are represented in figure 3.16.

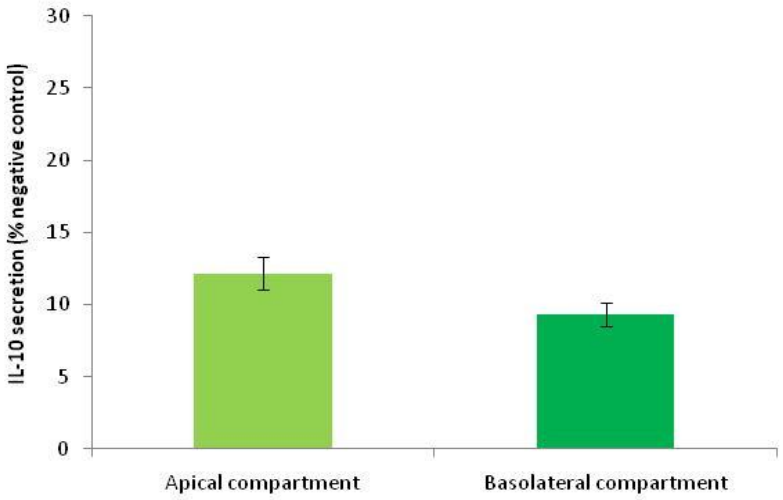


Figure 3.16 – IL-10 secretion by inflamed Caco-2 cells to the apical and basolateral compartment. Results are expressed in % to the negative control (cells without inflammatory stimuli).

Obtained results indicate that fully differentiated Caco-2 cells constitutively secret IL-10 and it secretion was slightly increased upon 48h of inflammatory stimuli. Thus, these results are in agreement with what is observed in case of chronic intestinal inflammation, namely, a low induction in IL-10 secretion.

NO is another important mediator in inflammatory processes, regulating blood flow, pain transmission and activation of the immune system (Mandalari et al, 2011; Van De Walle et al, 2010). Nitric oxide is over-secreted in IBD, and its secretion was therefore evaluated in inflamed Caco-2 cells and results, expressed as percentage of NO secretion relatively to negative control, are represented in figure 3.17.

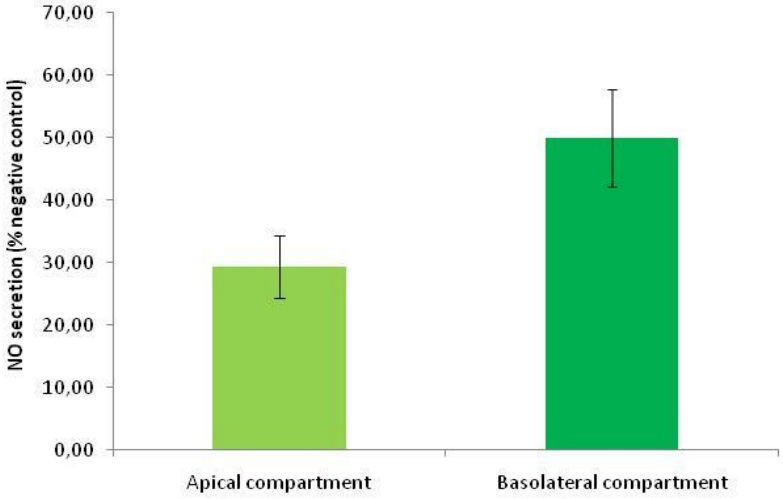


Figure 3.17 – NO secretion by inflamed Caco-2 cells to the apical and basolateral compartment. Results are expressed in % to the negative control (cells without inflammatory stimuli).

NO is also constitutively expressed in unstimulated Caco-2 cells but its secretion increased in presence of the selected inflammatory stimuli. This secretion is, once again, mostly to the basolateral side confirming the role of this side in inflammatory modulation. NO results are in agreement with previous reports that indicate an overexpression of iNOS and, consequently, an increase in NO

secretion in IBD conditions. (Mueller et al, 2010; Romier-Crouzet et al, 2009; Sergent et al, 2010; Van De Walle et al, 2010).

Activation of NF-κB pathway is another key feature in IBD and can be indirectly measure by IκBα detection. IκBα is an inhibitor of NF-κB that sequester it in cytosol, preventing NF-κB migration to the nucleus where it can bind to DNA and promote pro-inflammatory genes transcription (Erlejman et al, 2008; Romier et al, 2008; Van De Walle et al, 2008). In presence of pro-inflammatory stimuli, IκBα is phosphorylated and degraded by the proteassome, allowing NF-κB migration to the nucleus (Erlejman et al, 2008). It is now well known that IBD patients have hyper activation of NF-κB pathway due to a lack in its regulation (Romier et al, 2008). In order to assess the establishment of an cell-based model that can mimic IBD condition is important to determine if selected inflammatory stimuli can activate NF-κB cascade in Caco-2 model and results are presented in figure 3.18.

Figure 3.18 – Influence of pro-inflammatory stimuli on NF-κB pathway activation. (A) Immunoblot of IκBα and β-actin (30µg protein *per* well). Negative control (-) represents cells without inflammatory stimuli and positive control (+) represents cells incubated with pro-inflammatory stimuli.

After 48h of incubation of fully differentiated Caco-2 cells with a pro-inflammatory stimuli composed by 50ng/mL TNF-α, 25ng/mL IL-1β and 10µg/mL, it is observable a huge decrease in IκBα signal which indicate activation of NF-κB pathway.

In conclusion, obtained results show that fully differentiated Caco-2 cells incubated with pro-inflammatory stimuli composed by 50ng/mL TNF-α, 25ng/mL IL-1β and 10µg/mL and applied at the basolateral compartment for 48h can mimic IBD main characteristics:

- Induction of barrier dysfunction,
- Increase in IL-8 secretion,
- Low induction of IL-10 secretion.
- Increase in NO secretion,
- NF-κB activation.

So, it was established a simple, valid and consistent *in vitro* model that mimics the physio-pathological behavior of IECs during the acute phase of IBD and that can be used as tool to assess anti-inflammatory activity of many agents, namely natural products.

3.3.2 Effects of PREs against intestinal inflammation as prevention agents

In order to determine preventive effect of both PRE in IBD, fully differentiated Caco-2 cells were pre-incubated with 50mgGAE/L of each extract for 4 hours and then stimulated with the pro-inflammatory cocktail composed by 50ng/mL TNF-α, 25ng/mL IL-1β and 10µg/mL for 48hours. Anti-inflammatory effects of cactus pear's and cherry's PRE were evaluated following the secretion of inflammatory markers IL-8 (figure 3.19), IL-10 (figure 3.20) and NO (figure 3.21), NF-κB activation

(figure 3.22) and barrier dysfunction measured by alterations in fluorescein permeability across inflamed Caco-2 monolayer (table 3.4). As far as we know, this is the first time that anti-inflammatory activity of cactus pear's and cherry's extracts were assessed using an *in vitro* model of inflamed intestinal epithelium.

Although barrier dysfunction *per si* can't cause IBD pathology, it is an important biomarker of IBD becoming an interesting target for IBD prevention and therapy (Al-Sadi & Ma, 2007; Edelblum & Turner, 2009). Effects of both PRE in barrier dysfunction were assessed by evaluation of fluorescein permeability across inflamed Caco-2 monolayer and results are presented in table 3.4.

Table 3.4 – Permeability of fluorescein across inflamed Caco-2 monolayer after pre-incubation with Cactus pear and Cherry PRE (50mg GAE/L).

	P_{app} 48 hours (cm/s)	
	Apical → Basolateral	**Basolateral → Apical**
Control - *	$6{,}04 \times 10^{-6}$	$4{,}58 \times 10^{-6}$
Control + **	$7{,}39 \times 10^{-6}$	$8{,}47 \times 10^{-6}$
Cactus pear PRE	$5{,}58 \times 10^{-6}$	$6{,}41 \times 10^{-6}$
Cherry PRE	$6{,}38 \times 10^{-6}$	$5{,}40 \times 10^{-6}$

* Negative control represents cells without incubation with pro-inflammatory stimuli.
** Positive control represents cells with incubation with pro-inflammatory stimuli.

Obtained results indicate that cactus pear's and cherry's PRE protect IECs against barrier dysfunction induced by pro-inflammatory stimuli (table 3.4). In apical to basolateral direction, cactus pear's extract was more effective preventing fluorescein transport returning P_{app} values to the basal values (negative control) while cherry's PRE also reduce P_{app} value of fluorescein transport in this direction but was not so effective as cactus pear's PRE. On the other hand, in basolateral to apical direction, cherry's PRE was more effective, approaching P_{app} value to the basal value while cactus pear's PRE, in spite of the ability to reduce P_{app} of fluorescein, it is not so effective as cherry's PRE.

There is a huge lack in literature concerning with polyphenols effects against intestinal barrier dysfunction, and obtained results are a valid contribute to this issue, showing that, in fact, polyphenolic compounds can reduce inflammatory damages at barrier level (table 3.4). Although some reports focus a potential role of polyphenols, namely flavonoids, on TJ function (Suzuki & Hara, 2011), these effects in case of inflammation are not well explored. For example, incubation of Caco-2 cells with 100μM of quercetin increase claudin-4 expression, an important TJ protein, in normal cells but this effect was not assessed in inflamed Caco-2 cells (Sergent et al, 2010; Suzuki & Hara, 2011).

Many reports mentioned the capacity of polyphenols from different sources to modulate IL-8 secretion in inflammatory conditions (Rahman et al, 2006; Romier et al, 2009; Romier-Crouzet et al, 2009; Satsu et al, 2009; Sergent et al, 2010). Effects of pre-incubation with both PRE in IL-8 secretion by inflamed Caco-2 cells were assed and results, expressed as percentage of IL-8 secretion relatively to negative control, are presented in figure 3.19.

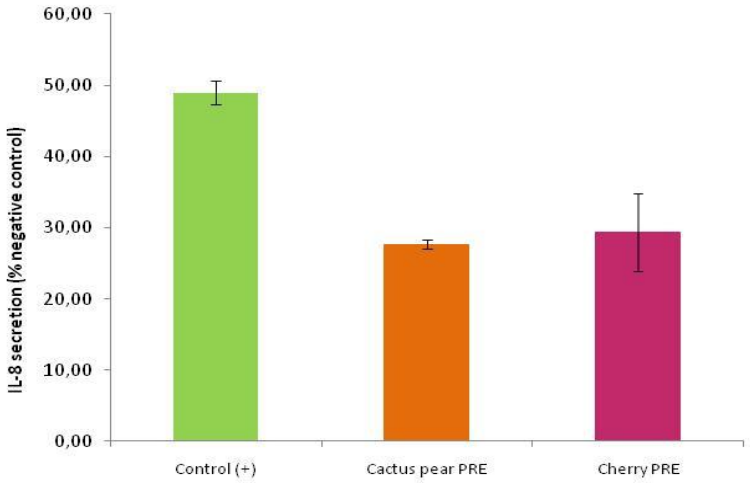


Figure 3.19 – Effects of Cactus pear's and Cherry's PRE (50mg GAE/L) on IL-8 secretion by inflamed Caco-2 cells. Results are expressed in % to the negative control (cells without inflammatory stimuli).

Cactus pear's and cherry's PRE can reduce IL-8 secretion in a similar percentage (figure 3.19) upon pro-inflammatory stimuli. This suggests that these extracts could contribute to a reduce efflux of inflammatory cells at the inflamed intestine, since IL-8 is a chemoattractive chemokine. These results are in agreement with studies that demonstrated that cherry intake can inhibit inflammatory pathways, namely due to their high content in anthocyanins (Ferretti et al, 2010; McCune et al, 2011). These effects cannot be generalized to the entire flavonoids family since structure-effect relationship were not yet established (Romier et al, 2009).

There are evidences that polyphenols can increase IL-10 expression (González et al, 2011) but there are few reports that confirm these evidences, namely in inflamed Caco-2 cells. The effect of both PRE in IL-10 secretion by inflamed Caco-2 cells was assessed and results, expressed as percentage of IL-10 secretion relatively to negative control, are presented in figure 3.20.

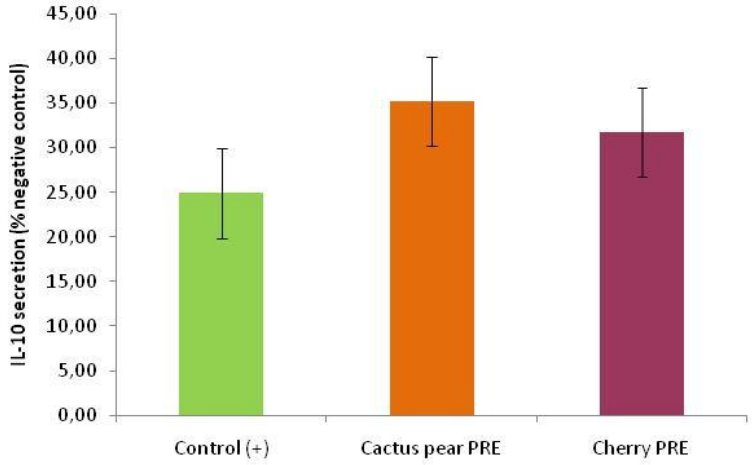


Figure 3.20 – Effects of Cactus pear's and Cherry's PRE (50mg GAE/L) on IL-10 induced secretion by inflamed Caco-2 cells. Results are expressed in % to the positive control (cells with inflammatory stimuli).

After 4h of pre-treatment with 50mg GAE/L of cactus pear's and cherry's PRE, IL-10 secretion slightly increase (figure 3.20) in order to reduce intestinal inflammation. Once again, since effects of

polyphenols in IL-10 secretion are scarce, obtained results can help to understand the mechanisms behind anti-inflammatory activity of polyphenols, namely for those present in cactus pears and cherries.

NO is another inflammatory mediator that has demonstrated to be modulated by polyphenolic compounds in different ways: inhibiting iNOS transcription or decreasing iNOS and NOS activity (Mueller et al, 2010; Romier-Crouzet et al, 2009). In order to assess the effects of both PRE in inflamed cells, NO secretion was evaluated and results, expressed as percentage of NO secretion relatively to negative control, are presented in figure 3.21.

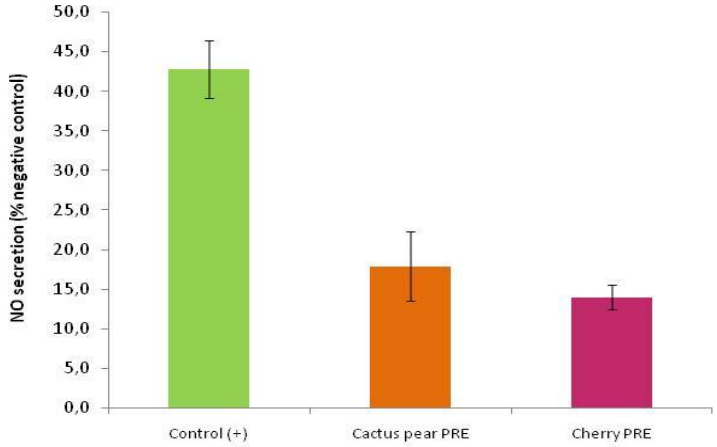


Figure 3.21 – Effects of Cactus pear's and Cherry's PRE (50mg GAE/L) on NO secretion by inflamed Caco-2 cells. Results are expressed in % to the positive control (cells with inflammatory stimuli).

Cactus pear's and cherry's PRE showed to decrease NO secretion (figure 3.21). These results are in accordance with other studies that suggest that polyphenols can protect against NO secretion (Romier-Crouzet et al, 2009). Cherry's polyphenols have been reported as strongest anti-inflammatory compounds, namely by decrease NO secretion and COX-2 activity (Ferretti et al, 2010; McCune et al, 2011). Despite betalains and flavonoids are reported as anti-inflammatory compounds, until now there are no available studies that assess anti-inflammatory activity of cactus pear's polyphenols modulating all studied inflammatory mediators (Feugang et al, 2006; González et al, 2011).

The hypothesis that anti-inflammatory properties of PREs could be mediated through modulation of the NF-κB activation pathways in the intestine was also studied. The activation of this pathway was measured indirectly by quantification of IκBα in Caco-2 cells pre-treated with 50mg GAE/L for 4hours followed by incubation with pro-inflammatory stimuli composed by 50ng/mL TNF-α, 25ng/mL IL-1β and 10µg/mL for 48hours and results are presented in figure 3.22.

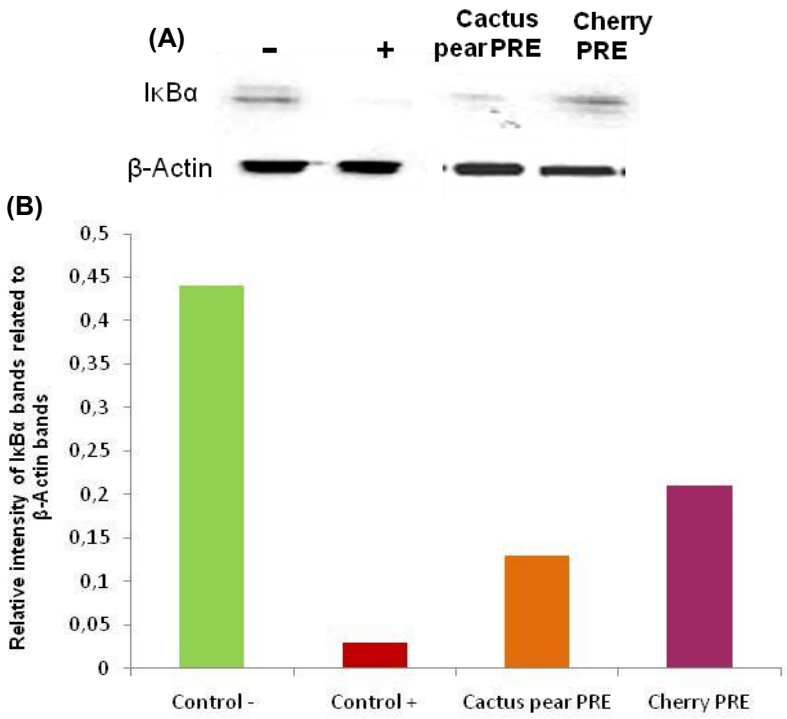


Figure 3.22 – Effect of pre-incubation with cactus pear's and cherry's PRE (50mg GAE/L) on NF-κB activation in Caco-2 cells incubated with pro-inflammatory stimuli.(A) Immunoblot of IκBα and β-actin (30µg protein *per* well) (B) Intensity of IκBα band relative to correspondent β-actin band expressed as relative intensity. Negative control (-) represents cells without inflammatory stimuli and positive control (+) represents cells incubated with pro-inflammatory stimuli.

Upon a 48h of stimulation with pro-inflammatory stimuli, results shown in figure 3.22 demonstrated that cactus pear's and cherry's PRE can modulate NF-κB activation since both PREs lead to a higher level of IκBα when compared with positive control. However, none of the PRE can approximate to basal levels of IκBα obtained for the negative control. Oxidative stress is known as a strong inducer of NF-κB cascade and both PRE showed the ability to decrease oxidative stress injuries. Thus, one possible mechanism which polyphenols present in cactus pear's and cherry's PRE modulate NF-κB activation is the decrease of oxidative stress.

Isorhamnetin have been reported as an inhibitor of NF-κB activation (Boesch-Saadatmandi et al, 2011; Hamalainen et al, 2007) and its presence in cactus pear's PRE could be related with the modulation of NF-κB pathway evidenced by this extract. On the other hand, anthocyanins also have the capacity to modulate NF-κB activation (Karlsen et al, 2007; Miguel, 2011). Furthermore, obtained results indicate that anthocyanins present higher capacity to inhibit NF-κB activation than polyphenols present in cactus pear's PRE which can be correlated with the higher antioxidant activity observed to cherry's PRE.

The promoter of the IL-8 and NO genes contain binding sites to NF-κB and this transcription factor is essential to regulate the expression of both genes (González et al, 2011; Romier et al, 2009; Romier et al, 2008; Romier-Crouzet et al, 2009). The obtained results suggest that PREs could control IL-8 and NO secretion through a mechanism depended on NF-κB. This mechanism of action was proved and reported for some polyphenols, namely quercetin; apigenin, luteolin, among others

(González et al, 2011; Romier et al, 2008; Romier-Crouzet et al, 2009) and polyphenols presented on cactus pear and cherry can also act in this pathway.

Since TNF-α is an important and crucial mediator of IBD pathogenesis and in order to determine if both PREs can reduce the secretion of this cytokine, Caco-2 cells were pre-incubated with cactus pear's and cherry's polyphenolic rich extracts followed by incubation with 50ng/mL of IL-1β used as inflammatory stimuli and results, expressed as percentage of TNF-α secretion relatively to negative control, are presented in figure 3.23. The stimuli with IL-1β was chosen due to its increased concentration in intestinal mucosa of IBD patients and also for its proven capacity to induce TNF-α secretion by a NF-κB depended mechanism (Al-Sadi & Ma, 2007; Van De Walle et al, 2010).

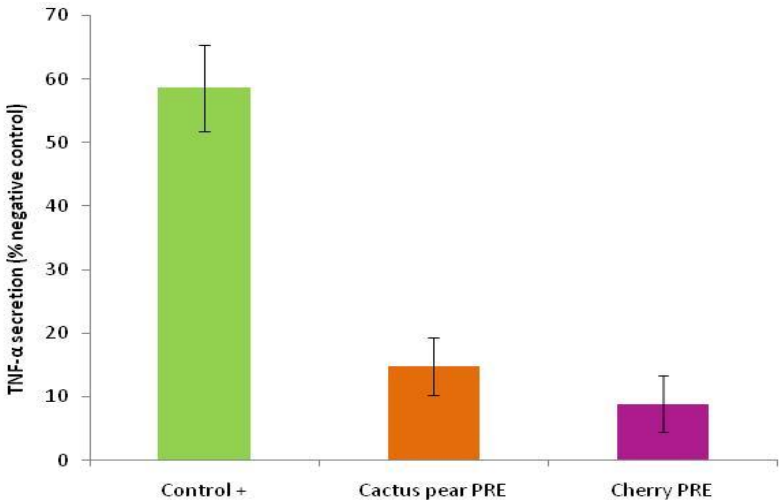


Figure 3.23 – Effects of Cactus pear's and Cherry's PRE (50mg GAE/L) in TNF-α secretion by inflamed Caco-2 cells. Results are expressed in % to the negative control (cells without inflammatory stimuli).

The obtained results suggest that cactus pear's as well as cherry's PRE can decrease TNF-α secretion by inflamed Caco-2 cells. TNF-α is an important mediator responsible for many issues in inflammatory response, namely it is capable to increase barrier dysfunction (Al-Sadi & Ma, 2007; Van De Walle et al, 2010) and it is a target for IBD therapy. Although the applied stimuli was different, results obtained for barrier dysfunction, where both PRE showed the capacity to modulate permeability across inflamed Caco-2 monolayer, could be correlated with results obtained for TNF-α secretion. Thus, modulation of barrier dysfunction of both PRE could be due to a decrease in TNF-α secretion, that is a cytokine reported as an important agent in induction of permeability alterations observed in IBD condition.

TNF-α gene has also a binding site to NF-κB in its promoter (Rahman et al, 2006), which means that regulation of TNF-α expression by cherry's and cactus pear's PRE can be done by modulation of NF-κB pathway.

Since both PREs can modulate TNF-α secretion and other important inflammatory mediators (IL-8 and IL-10), they can be interesting products to delay and control IBD.

3.3.3 Effects of PREs in intestinal inflammation as therapeutic agents

There is a huge necessity of new and safe compounds that can treat or help to treat inflammatory attacks in IBD and polyphenols appear as an interesting alternative to combat this disease (Sergent et al, 2010). In this context, some assays were performed in order to evaluate the potential benefits of using this extracts as therapeutic agents. Inflamed Caco-2 cells were co-incubate with cherry and cactus pear PRE and selected pro-inflammatory stimuli for 48h. IL-8 secretion was assessed and results, expressed as percentage of IL-8 secretion relatively to negative control, are presented in figure 3.24.

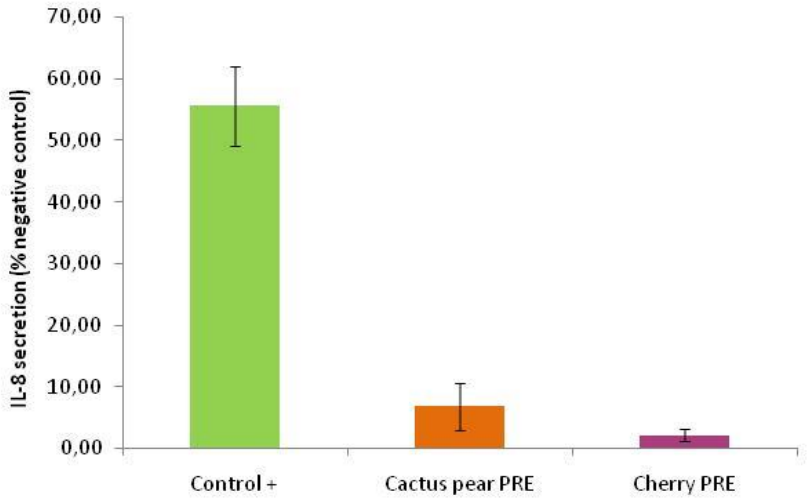


Figure 3.24 – Effect of co-incubation of Cactus pear's and Cherry's PRE (50mg GAE/L) with pro-inflammatory stimuli in IL-8 secretion by Caco-2 cells. Results are expressed in % to the negative control (cells without inflammatory stimuli).

Upon 48h of inflammatory stimulation in presence of each PRE the IL-8 secretion decreased (figure 3.24). Moreover, this reduction is more effective than that observed when Caco-2 cells were pre-incubated with the same PRE since IL-8 secretion almost return to the basal level. These results were expectable since in case of co-incubation there is a significantly contribution of bioavailable polyphenols and those that cannot cross Caco-2 monolayer. Glycosilated polyphenols are, often, no bioavailable, but enteric microflora can metabolize these compounds in their respective aglycones (polyphenol without sugars) making them bioavailable, being absorbed in colon (Williamson & Manach, 2005). On the other hand, it is important to note that those compounds that cannot be metabolized by intestinal microflora and cannot become bioavailable, can exert their actions in intestinal lumen or in cellular membrane (González et al, 2011).

The obtained results for IL-8 secretion can also be correlated with oxidative stress results where co-incubation leads to a higher inhibition of ROS formation than pre-incubation (figure 3.8) and oxidative stress is known as an important factor in IBD pathogenesis due to its capacity to perpetuate and accentuate inflammatory cascade (Frontela et al, 2010; Mandalari et al, 2011; Rahman, 2008; Rahman et al, 2006; Rezaie et al, 2007). Polyphenols such as chlorogenic acid, caffeic acid and isoflavone fractions have shown suppressive effects on IL-8 secretion in Caco-2 cells by two different mechanisms: elimination of ROS in cells and modulation of NF-κB pathway (Erlejman et al, 2008;

Satsu et al, 2009; Shimizu, 2010). The PREs studied can also inhibit IL-8 secretion based in these two mechanisms.

Effect of co-incubation with both PRE and selected pro-inflammatory stimuli on IL-10 secretion was also assessed and results, expressed as percentage of IL-10 secretion relatively to negative control, are presented in figure 3.25.

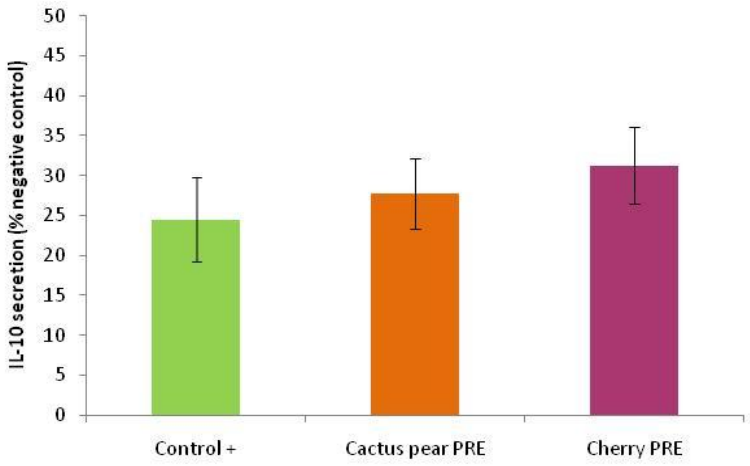


Figure 3.25 – Effect of co-incubation of Cactus pear's and Cherry's PRE (50mg GAE/L) with pro-inflammatory stimuli in IL-10 secretion by Caco-2 cells. Results are expressed in % to the negative control (cells without inflammatory stimuli).

After 48h of co-incubation of inflammatory stimuli and PREs there wasn't an evident increase in IL-10 secretion. Moreover, obtained results are similar to those obtained for pre-incubation assay.

These results are in agreement with a previous report where authors co-incubate different polyphenols (quercetin, genistein, resveratrol, chrysin, curcumin and ellagic acid) with a pro-inflammatory cocktail (IL-1β+TNF-α+IFN-γ+LPS) and didn't observe any significant change in IL-10 secretion (Sergent et al, 2010).

NO secretion, another biomarker of IBD pathogenesis, was assessed upon 48h of co-incubation of Caco-2 cells with both PREs and selected pro-inflammatory stimuli and obtained results, expressed as percentage of NO secretion relatively to the negative control, are presented in figure 3.26.

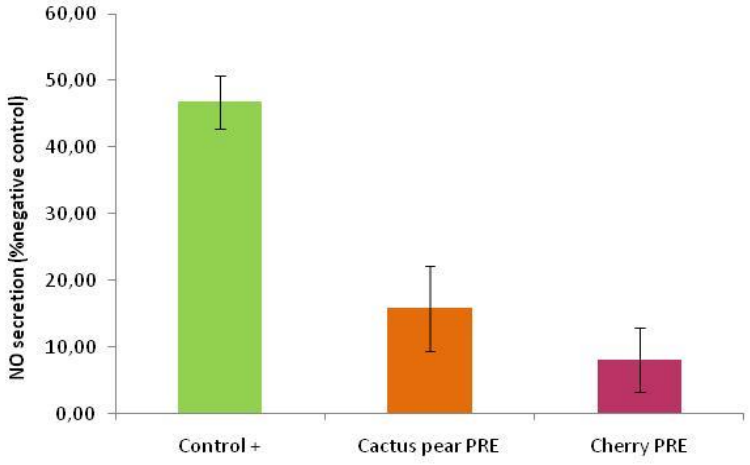


Figure 3.26 – Effect of co-incubation of Cactus pear's and Cherry's PRE (50mg GAE/L) with pro-inflammatory stimuli in NO secretion by Caco-2 cells. Results are expressed in % to the negative control (cells without inflammatory stimuli).

NO secretion also decreased when Caco-2 cells were co-incubated with both PREs and pro-inflammatory stimuli (figure 3.24). Indeed, cherry's PRE was more effective, decreasing NO secretion almost to the basal level. Once again, modulation of NO secretion by both PRE is more accentuated than when Caco-2 cells were pre-incubated with PREs which can be due to the higher inhibition of ROS formation in case of co-incubation and also for the stimulation of endogenous antioxidant defense, namely, glutathione system.

Isorhamnetin has shown the ability to inhibit NO secretion by inhibiting iNOS expression through modulation of NF-κB activation (Hamalainen et al, 2007). Inhibition of iNOS expression was also observed for anthocyanins (Miguel, 2011). The presence of these compounds in cactus pear's and cherry's PRE, respectively, could explain their anti-inflammatory activity against NO secretion.

A decrease in NF-κB activation promoted by cactus pear's and cherry's PRE can also be an explication for these results. To verify this hypothesis, the activation of NF-κB was evaluated when Caco-2 cells were co-incubated with both PREs and selected pro-inflammatory stimuli and results are presented in figure 3.27.

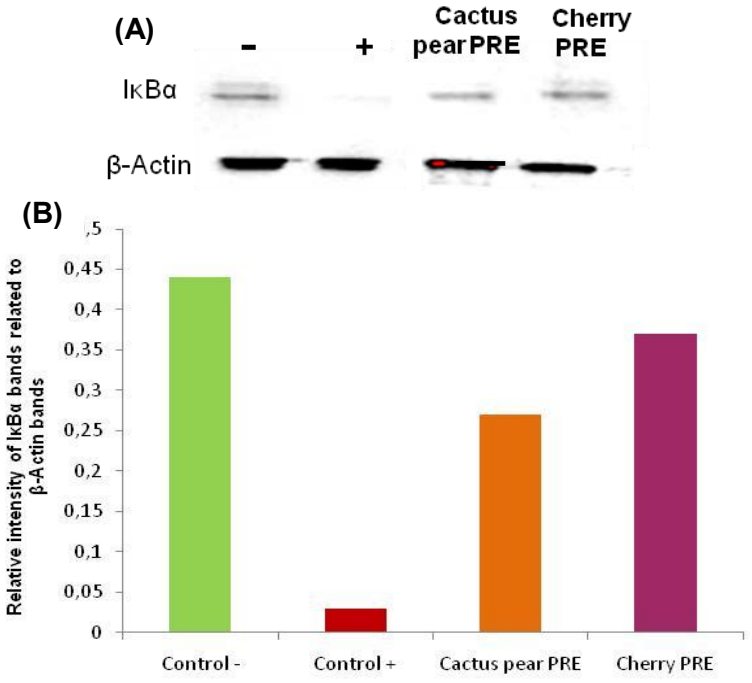


Figure 3.27 – Effect of co-incubation of cactus pear's and cherry's PRE (50mg GAE/L) with pro-inflammatory stimuli on NF-κB activation in Caco-2 cells.(A) Immunoblot of IκBα and β-actin (30μg protein *per* well) (B) Intensity of IκBα band relative to correspondent β-actin band expressed as relative intensity. Negative control (-) represents cells without inflammatory stimuli and positive control (+) represents cells incubated with pro-inflammatory stimuli.

Co-incubation of fully differentiated Caco-2 cells with both PREs and pro-inflammatory stimuli leads to a lower activation of NF-κB than the observed when cells were pre-incubated with cherry's and cactus pear's extract (figure 3.27). Cherry's PRE was also more effective in NF-κB modulation which can be correlated with higher inhibition of ROS formation and higher enhancement of endogenous antioxidant system observed to this extract. As was mentioned before, oxidative stress is a strongest inducer of NF-κB activation resulting in an increase of inflammatory response (Rahman et al, 2006;

Romier et al, 2008), Thus, PREs can modulate this transcription factor by decreasing oxidative stress through all the different mechanisms studied in point 2 of Results and Discussion section.

In order to determine if both PREs can reduce the secretion of TNF-α, Caco-2 cells were co-incubated with cactus pear's and cherry's polyphenolic rich extracts (50mg GAE/L) and 50ng/mL of IL-1β (figure 3.28).

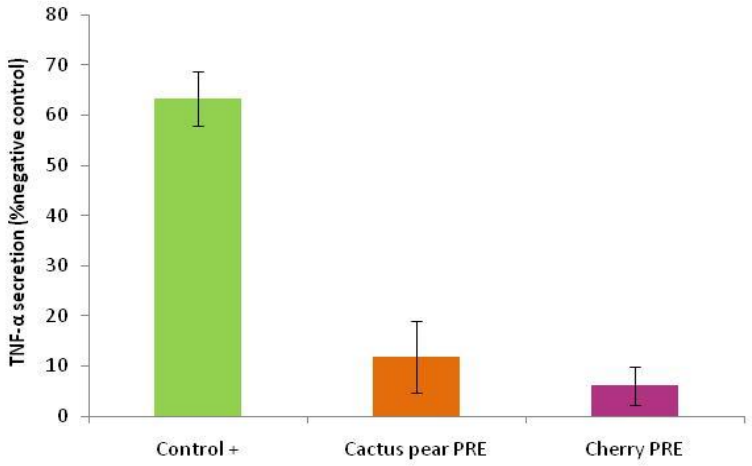


Figure 3.28 – Effect of co-incubation of Cactus pear's and Cherry's PRE (50mg GAE/L) with 50ng/mL of IL-1β in TNF-α secretion by Caco-2 cells. Results are expressed in % to the negative control (cells without inflammatory stimuli).

TNF-α secretion was reduced almost to the basal level by both PREs (figure 3.28). Once again it was observed a higher decrease of TNF-α secretion in co-incubation assay when compared with pre-incubation assay. These results can be also correlated with oxidative stress that shows a higher inhibition of ROS formation when Caco-2 cells were co-incubated with both PRE.

Since cherry's PRE was more effective in suppressing inflammatory mediators and also in modulate NF-κB activation in a therapeutic perspective, cherry's extract was chosen to compare with anti-inflammatory activity of 5-ASA, a common drug used to treat IBD.

3.3.4 Comparison between anti-inflammatory activity of *Prunus avium* PRE and 5-aminosalicylic acid (5-ASA)

In order to compare anti-inflammatory activity of *Prunus Avium* polyphenolic-rich extract and 5-aminosalicylic acid (5-ASA), Caco-2 cells were seeded in Transwell® inserts and were cultured for 21 days to ensure that cells were fully differentiated. The next step was the co-incubation of 30mM of 5-ASA and 50mgGAE/L of cherry's PRE in the apical compartment with pro-inflammatory stimuli (50ng/mL TNF-α, 25ng/mL IL-1β and 10μg/mL LPS) introduced in the basolateral compartment for 48hours. The anti-inflammatory activity of both agents was determined by the assessment of barrier dysfunction (figure 3.29), morphological alterations (figure 3.30) and measuring of IL-8 (figure 3.31), IL-10 (figure 3.32) and NO (figure 3.33) secretion. Note that 30mM of 5-ASA is a predictable concentration of this agent in intestinal mucosa (Aguzzi et al, 2011; Desreumaux & Ghosh, 2006; Schwab et al, 2009).

It was suggested that barrier dysfunction may predispose or enhance IBD progression and therapies targeted to specifically restore or improve the barrier function may provide an alternative or

supplement current therapies (Edelblum & Turner, 2009). The capacity of 5-ASA and cherry's PRE to modulate barrier dysfunction was assessed by measurement of TEER and results are presented in figure 3.29.

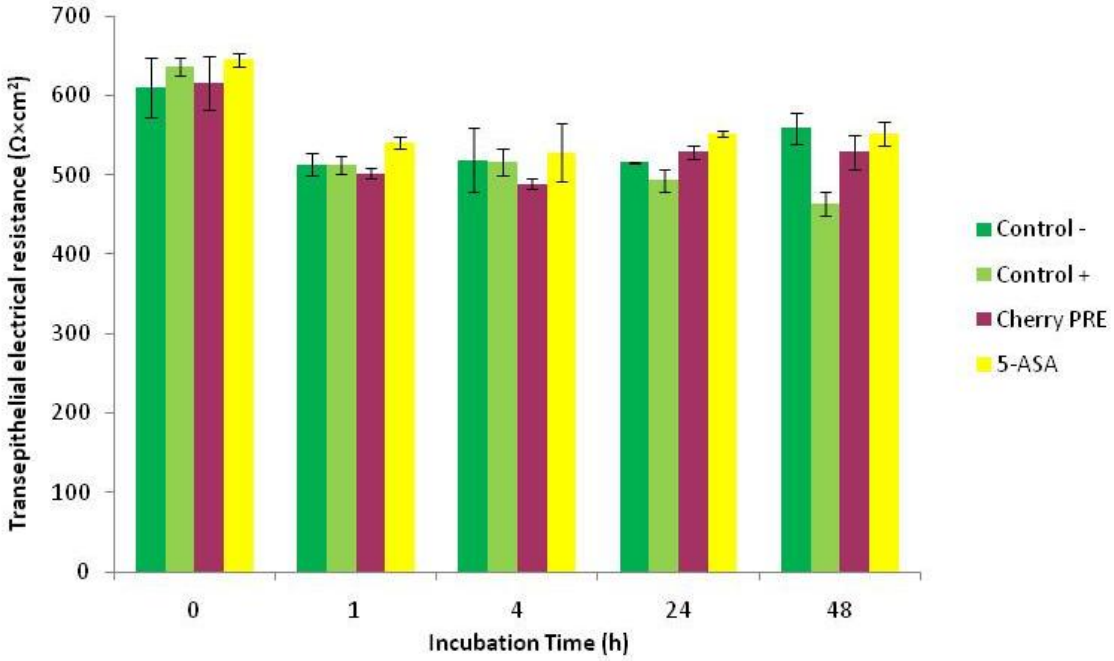


Figure 3.29 – Cherry's PRE (50mgGAE/L) and 5-ASA (30mM) effects in TEER of Caco-2 monolayer stimulated with pro-inflammatory cocktail (50ng/mL TNF-α, 25ng/mL IL-1β and 10μg/mL LPS) in the basolateral side for 48h. Negative control (-) represents cells without inflammatory stimuli and positive control (+) represents cells incubated with inflammatory stimuli.

Cherry's PRE and 5-ASA can modulate barrier dysfunction since TEER values obtained for both anti-inflammatory agents are similar to those obtained in negative control after 48h of pro-inflammatory stimuli (figure 3.29). Although the precise mechanism of action of 5-ASA remains unknown, it is known that it can directly stimulate intestinal epithelial wound repair (Aguzzi et al, 2011). The results suggest that one possible mechanism of action of 5-ASA is the attenuation of barrier dysfunction. Moreover, cherry PRE can also be able to promote barrier integrity in case of inflammation.

As it is possible to see in figure 3.30, morphological changes induced by pro-inflammatory stimuli also seems to be attenuated by both anti-inflammatory agents.

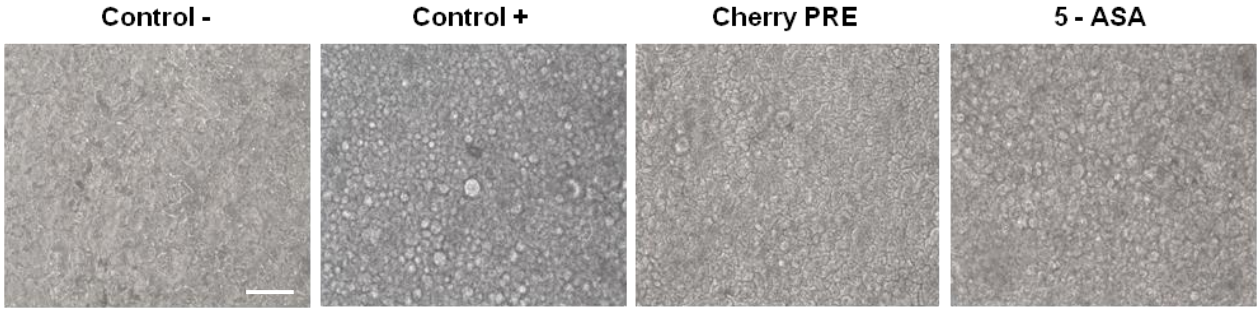


Figure 3.30 – Cherry's PRE and 5-ASA effects in inflammation-induced morphological alterations in Caco-2 monolayer. Phase contrast images of cell cultures incubated with pro-inflammatory stimuli in the basolateral side during 48h. Negative control culture was performed by incubating cells in standard culture medium without pro-inflammatory stimuli. Scale bar: 100μm.

Morphological appearance of Caco-2 cells treated with cherry's PRE seems to be similar to negative control while cells treated with 5-ASA seems to be more affected. The appearance of Caco-2 monolayer treated with 5-ASA can be due to the side effects reported to this anti-inflammatory activity.

A reduction in IL-8 secretion is another important effect that an IBD anti-inflammatory agent should possess. Thus, effects of both agents in IL-8 secretion was determined and results, expressed as percentage of IL-8 secretion relatively to negative control, are presented in figure 3.31.

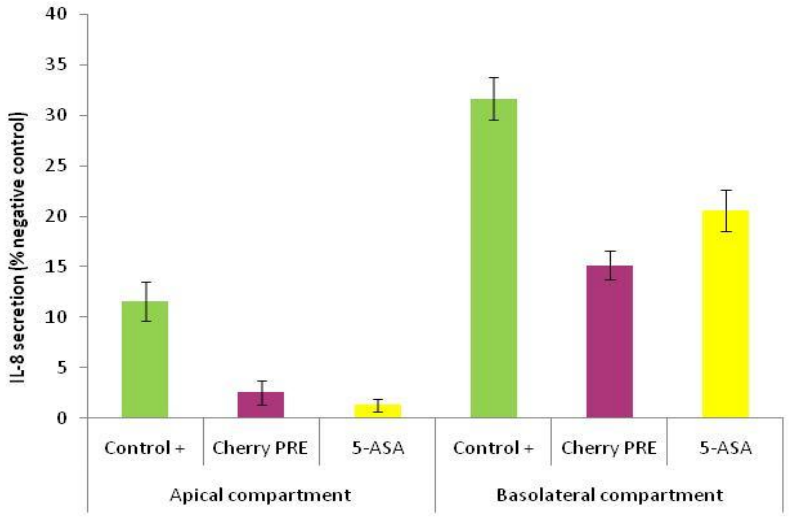


Figure 3.31 – Effect of co-incubation of Cherry's PRE (50mg GAE/L) and 5-ASA (30mM) with pro-inflammatory stimuli in IL-8 secretion by Caco-2 cells to basolateral and apical compartments. Results are expressed in % to the negative control (cells without inflammatory stimuli).

Cherry PRE demonstrate a higher reduction in IL-8 secretion when compared with 5-ASA (figure 3.31). 5-ASA is reported as a potent intracellular antioxidant that it is capable to modulate NF-κB activation and consequently pro-inflammatory genes transcription (Aguzzi et al, 2011; Desreumaux & Ghosh, 2006; Schirbel & Fiocchi, 2010; Schwab et al, 2009; Shimizu, 2010). So, it is possible that 5-ASA induced reduction in IL-8 secretion is due to modulation of NF-κB and oxidative stress. Obtained results are in agreement with previous reports which indicate that this classical therapeutic agent in IBD is able to inhibit IL-8 secretion in IBD models (Aguzzi et al, 2011; Desreumaux & Ghosh, 2006; Wallace, 2003). Furthermore, cherry's PRE can also act by these two mechanisms: reduction of oxidative stress and modulation of NF-κB pathway activation.

An increase in IL-10 secretion in order to counteract established inflammation in IBD could help to treat and delay chronic inflammation attacks. Thus, effects of cherry's PRE and 5-ASA in IL-10 secretion by inflamed Caco-2 cells were assessed and results, expressed as percentage of IL-10 secretion relatively to negative control, are presented in figure 3.32.

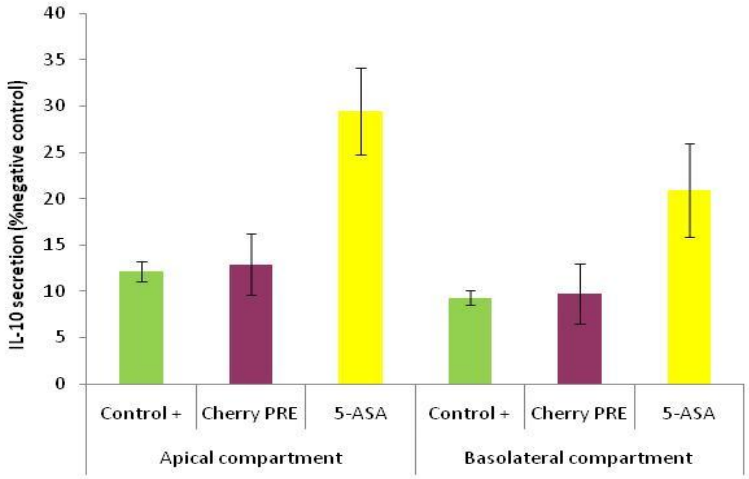


Figure 3.32 – Effect of co-incubation of Cherry's PRE (50mg GAE/L) and 5-ASA (30mM) with pro-inflammatory stimuli in IL-10 secretion by Caco-2 cells to basolateral and apical compartments. Results are expressed in % to the negative control (cells without inflammatory stimuli).

In IL-10 secretion case (figure 3.32), cherry's PRE didn't altered this cytokine level. On the other hand, 5-ASA demonstrates the ability to induce IL-10 secretion to both compartments. As far as we know, there are no reports that confirm induction of IL-10 secretion by 5-ASA in an *in vitro* IBD model composed by Caco-2 cells stimulated with a pro-inflammatory cocktail. Moreover, since action mechanisms of 5-ASA are not fully elucidated, IL-10 induction can be a possible anti-inflammatory effect of this pharmacological agent.

In order to further evaluate the anti-inflammatory activity of both agents, their effects were assessed on NO secretion by Caco-2 cells and results, expressed as percentage of NO secretion relatively to negative control, are presented in figure 3.33.

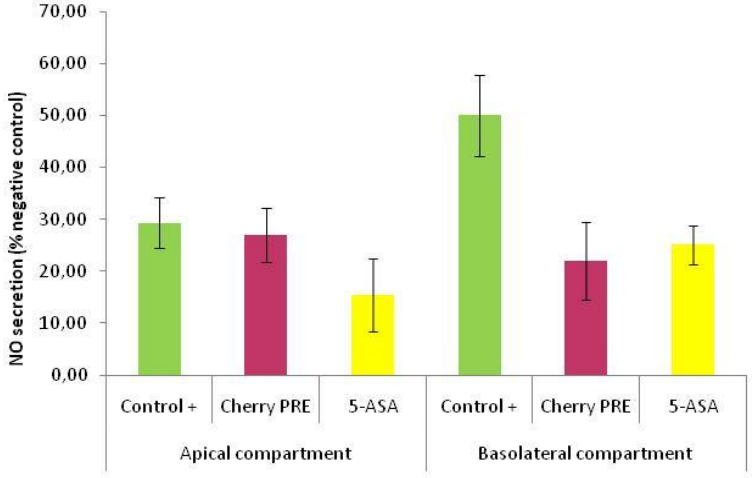


Figure 3.33 – Effect of co-incubation of Cherry's PRE (50mg GAE/L) and 5-ASA (30mM) with pro-inflammatory stimuli in NO secretion by Caco-2 cells to basolateral and apical compartments. Results are expressed in % to the negative control (cells without inflammatory stimuli).

Nitric oxide is oversecreted in IBD and 5-ASA is thought to reduce NO secretion by a mechanism dependent on NF-κB (Aguzzi et al, 2011; Desreumaux & Ghosh, 2006). Obtained results (figure 3.33) confirm that 5-ASA can decrease NO secretion for both compartments (apical and basolateral). Moreover, cherry's PRE and 5-ASA showed similar decrease of NO secretion in the basolateral

compartment and this effect can be due to an induced reduction in oxidative stress and in NF-κB activation.

Most patients suffering from IBD normally respond to conventional therapy consisting on 5-ASA and corticosteroids. However, some do not respond to this standard treatment or although they can respond it leads to several side effects. So, alternative therapies such as combination of standard pharmacological agents with natural products can be helpful in the treatment of these intestinal disorders (Schwab et al, 2009; Sergent et al, 2010). These results suggest that cherry PRE can be use for this propose since their anti-inflammatory activity seems to be comparable and complementary to 5-ASA. Cherry's PRE and 5-ASA can decrease IL-8 and NO secretion and can, also, attenuate barrier dysfunction. However, only 5-ASA can increase IL-10 expression, which suggests that these two agents can be used simultaneously to treat IBD patients. It is important to note that these results are from an *in vitro* model and need to be studied in human clinical trials.

4. Conclusions

Inflammatory Bowel Diseases (IBD), the collective name for Crohn's disease and Ulcerative colitis, are chronic inflammatory diseases which incidence worldwide is increasing (Rezaie et al, 2007; Romier et al, 2008). Although the etiology of these diseases remains unknown, many factors have been associated to IBD, namely genetic, environmental and immunological factors (Edelblum & Turner, 2009; Mandalari et al, 2011). Moreover, the pathogenesis of these diseases is not fully elucidated but it is known that includes a dysfunction of the intestinal mucosa due to a decrease in tight junction barrier function, an overproduction of pro-inflammatory mediators including cytokines and chemokines and, also, an aberrant activation of NF-κB pathway (Al-Sadi & Ma, 2007; Atreya & Neurath, 2010; Edelblum & Turner, 2009; Van De Walle et al, 2010). Oxidative stress is another key factor in IBD pathogenesis that amplifies and perpetuates inflammatory cascades (Rezaie et al, 2007). One of the worst complications of these diseases is the increasing risk of colon cancer development (Romier et al, 2008; Sergent et al, 2010). Current treatment of IBD includes drugs like 5-aminosalysilic acid, corticosteroids or immunosuppressive agents but these therapies are associated with severe side effects and high costs (Aguzzi et al, 2011).

Polyphenols are among the most secure and reliable substances available for preventing various diseases, including disorders related to oxidative stress and inflammation, and are an alternative natural source for prevention and treatment of IBD (Frontela et al, 2010; Romier et al, 2009). Current evidences for the protective action of polyphenols against chronic diseases have generated enormous expectations which make polyphenols a huge attraction for the food and nutritional supplement industry regarding to the development of polyphenol-rich products (Dai & Mumper, 2010; Espín et al, 2007; Quideau et al, 2011).

The objective of the present work was to evaluate the anti-inflammatory activity of two polyphenolic-rich extracts (PRE) derived from *Prunus avium* and *Opuntia ficus-indica* (Portuguese varieties) in an *in vitro* cell-based model of intestinal inflammation.

Cactus pear's and cherry's PRE were obtained using a macroporous adsorption resin Amberlite® XAD16 in order to obtain natural extracts, rich in polyphenols that can be used as anti-inflammatory agents. The main compounds identified in cherry's PRE were anthocyanins, namely cyanidin-3-glucoside, cyanidin-3-rutinoside and peonidin-3-glucoside. Anthocyanins are known as powerful antioxidant compounds due to their singular structure and they are also reported as anti-inflammatory agents (Ferretti et al, 2010; McCune et al, 2011; Paredes-López et al, 2010; Usenik et al, 2008). Cactus pear's PRE is mainly composed by isorhamnetins and derivates that are known by possessing a strong antioxidant capacity and also anti-inflammatory properties (Boesch-Saadatmandi et al, 2011; Hamalainen et al, 2007; Liu et al, 2009). Cactus pear's PRE also possesses in its composition a betalain, namely a betaxantin designed by indicaxantin, known for its strong antioxidant properties (Cai et al, 2003; M.C.Azeredo, 2009; Stintzing & Carle, 2004; Stintzing & Carle, 2007).

To further characterize the obtained PREs and in order to evaluate the potential antioxidant activity, the chemical antioxidant activity of cactus pear's and cherry's PRE was assessed using three different but complementary assays: ORAC, HORAC and inhibition of LDL oxidation. Both PREs presented similar antioxidant activity in ORAC assay, while in HORAC assay and in inhibition of LDL oxidation,

cherry's PRE were more effective. Thus, cherry's PRE presents higher chemical antioxidant activity probably due to their high content in anthocyanins.

As mentioned before, oxidative stress plays a major role in IBD pathology. Thus, intracellular antioxidant activity was assessed for the developed PREs using three different assays: inhibition of ROS formation, of glutathione oxidation and protein oxidation. Except for prevention against peroxyl radicals formation, where cactus pear's and cherry's PRE presented similar effect, cherry's PRE was more effective in all antioxidant assays performed. It is possible to conclude that cherry's polyphenols are more potent antioxidants than those presented in cactus pear's and even those who have low cellular uptake by Caco-2 cells, had potent scavenging capacity against extracellular ROS. Moreover, it was showed that antioxidant activity of polyphenols-rich extracts derived from the two fruits in study are not only derived by scavenging or inhibiting ROS formation but it is also indirectly due to stimulation of endogenous antioxidant system, namely enhancing glutathione antioxidant system.

In order to evaluate anti-inflammatory activity of cactus pear's and cherry's PRE, it was necessary to establish and characterize an *in vitro* cell-based model of inflamed intestine. Since intestinal epithelial cells (IEC) actively participate in IBD pathogenesis, Caco-2 cells were selected to be used as IECs model (Shimizu, 2010). The pro-inflammatory stimuli selected to apply in fully differentiated Caco-2 cells need to mimic what happens in intestinal mucosa during IBD. The selected inflammatory stimuli, composed by 25ng/mL IL-1β, 50ng/mL TNF-α and 10μg/mL LPS, were chosen taking into account that TNF-α and IL-1β are cytokines whose mucosal levels are increased during IBD as well as LPS due to the increased permeability of intestinal barrier and consequently the major permeation of bacteria and other harmful luminal antigens (Van De Walle et al, 2010).

When the selected pro-inflammatory stimuli were applied in fully differentiated Caco-2 cells at basolateral compartment for 48h, it was possible to observe a decrease in TEER measurements and an increase in permeability across inflamed Caco-2 monolayer. Furthermore, morphologic alterations in Caco-2 monolayer were evident since inflamed cells were smaller and possess open and preeminent tight junctions. IL-8 and NO secretion increased, IL-10 was slightly induced and NF-κB was strongly activated. These facts lead to the conclusion that the pro-inflammatory stimuli selected are able to mimic the main features of IBD and can be used as a tool to assess the anti-inflammatory activity of the developed PRE.

Anti-inflammatory activity of both PREs was assessed in two different perspectives: preventive and/or therapeutic. In the first case, fully differentiated Caco-2 cells were pre-incubated for 4h with each PRE followed by the addition of the selected inflammatory stimuli. Both PREs can decrease barrier dysfunction by modulation of monolayer permeability and can also decrease pro-inflammatory mediators secretion (IL-8 and NO). NF-κB activation was inhibited by both PRE while IL-10 was not induced by any PRE. Cherry's PRE were more effective in prevention against inflammation injury. These results can be correlated with obtained results for oxidative stress assays where cherry's PRE showed higher intracellular antioxidant activity. Moreover, IL-8 and NO gene have a binding site for NF-κB which suggests that both PRE could modulate the secretion of pro-inflammatory by modulation of NF-κB activation. Thus, cherry's and cactus pear's polyphenols can modulate inflammation reducing oxidative stress and decreasing the activation of NF- κB.

Caco-2 cells were stimulated with 50ng/mL of IL-1β after pre-incubation with both PRE, TNF-α secretion was reduced but, once again, cherry's PRE were more effective. TNF-α is a cytokine with a major role in barrier dysfunction and it is possible that the modulation of barrier permeability by cactus pear's and cherry's polyphenols can be due to a reduction of TNF-α release.

When fully differentiated Caco-2 cells were co-incubated with both PRE and pro-inflammatory stimuli (therapeutic perspective) there was a decrease in the secretion of pro-inflammatory mediators (IL-8 and NO) and a reduction in NF-κB activation. However, this reduction was superior than that observed for pre-incubation assays. These results are due to the contribution of polyphenols that are bioavailable and also of those that are not bioavailable but can scavenge extracellular free radicals. These results are in agreement of oxidative stress results where co-incubation assay reveals a higher inhibition of ROS formation. Cherry's PRE was, again, more effective in modulation of pro-inflammatory mediators. TNF-α secretion by fully differentiated Caco-2 cells stimulated with IL-1β decrease in the presence of both PRE.

The presence of isorhamnetin and anthocyanins in cactus pear's and cherry's PRE, respectively, could be correlated with the anti-inflammatory founded to both PRE. Their capacity to modulate NF-κB activation and, consequently, pro-inflammatory secretion, and also their anti-oxidant activity, can be responsible for anti-inflammatory activity observed for cactus pear's and cherry's PRE.

Since cherry's PRE had strongest anti-inflammatory activity, it was chosen to be compared with anti-inflammatory activity of 5-ASA. Both agents presented similar decrease of NO and IL-8 secretion and in attenuation of barrier dysfunction which can be due to the high antioxidant activity and capacity to modulate NF-κB activation of both anti-inflammatory agents. Only 5-ASA could increase IL-10.

Obtained results suggests that both PRE could be use to prevent or delay the development of IBD. Furthermore, cherry's PRE could be use as co-therapeutic in IBD patients.

However, it is important to note that these results are obtained for an *in vitro* model using a single culture. The next step is the assessment of anti-inflammatory activity in an *in vitro* model using co-cultures of different cell types such as dendritic cells, macrophages and IECs in order to mimic the complexity and the innumerous interactions that occurs in IBD pathogenesis. The effects of cactus pear's and cherry's PRE should be studied in other signal pathways involved in IBD such as MAPK cascades in order to better understand the mechanisms behind the health benefits of these extracts. Action of these PREs in TJ, namely in expression of important tight junction protein as well as in their assembly need to be assessed in order to understand how both PREs act in barrier dysfunction. Furthermore, individual anti-inflammatory activity of polyphenols present in both extracts should be assessed to understand their contribution for the whole anti-inflammatory activity of PREs. Co-incubation of cherry's PRE and 5-ASA will be interesting in order to try to diminish 5-ASA dose and, consequently, its side effects.

It is important to mention that this is the first time that a cherry and a cactus pear polyphenolic-rich extract was evaluated for their anti-inflammatory activity in IBD. Moreover, this work provides a valid and an important contribution to understand polyphenolic actions in chronic intestinal inflammation.

5. References

Aguzzi C, Ortega A, Bonferoni MC, Sandri G, Cerezo P, Salcedo I, Sánchez R, Viseras C, Caramella C (2011) Assessement of anti-inflammatory properties of microspheres prepared with chitosan and 5-amino salicylic acid over inflamed Caco-2 cells. *Carbohydrate polymers* **85:** 638-644

Al-Sadi RM, Ma TY (2007) IL-1beta causes an increase in intestinal epithelial tight junction permeability. *J Immunol* **178:** 4641-4649

Alexis-Biochemicals. (2010) Polyphenols: flavonoids, stilbenoids and phenolic acids. International Edition Catalog.

Atreya R, Neurath MF (2010) Chemokines in inflammatory bowel diseases. *Dig Dis* **28:** 386-394

Blando F, Gerardi C, Nicoletti I (2004) Sour cherry (Prunus cerasus L) anthocyanins as ingredients for functional foods. *Journal of Biomedicine and Biotechnology***:** 253-258

Boesch-Saadatmandi C, Loboda A, Wagner A, Stachurska A, Jozkowicz A, Dulak J, Doring F, Wolffram S, Rimbach G (2011) Effect of quercetin and its metabolites isorhamnetin and quercetin-3-glucuronide on inflammatory gene expression: role of miR-155. *Journal of Nutritional Biochemistry* **22:** 293-299

Bradford M. (1976) A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, Vol. 72, pp. 248-254.

Bruewer M, Samarin S, Nusrat A (2006) Inflammatory bowel disease and the apical junctional complex. *Ann N Y Acad Sci* **1072:** 242-252

Cai Y, Sun M, Corke H (2003) Antioxidant activity of betalains from plants of the Amaranthaceae. *Journal of Agricultural and Food Chemistry* **51:** 2288-2294

Cayupan Y, Ochoa M, Nazareno M (2011) Health-promoting substances and antioxidant properties of Opuntia sp. fruits. Changes in bioactive-compound contents during ripening process. *Food Chemistry* **126:** 514-519

Cecarini V, Gee J, Fioretti E, Amici M, Angeletti M, Eleuteri A, Keller J (2007) Protein oxidation and cellular homeostasis: Emphasis on metabolism. *Biochimica Et Biophysica Acta-Molecular Cell Research* **1773:** 93-104

Chavez-Santoscoy RA, Gutierrez-Uribe JA, Serna-Saldívar SO (2009) Phenolic composition, antioxidant capacity and in vitro cancer cell cytotoxicity of nine prickly pear (Opuntia spp.) juices. *Plant Foods Hum Nutr* **64:** 146-152

Chen J, Zhou X, Feng L, Liu Y, Jiang J (2009) Effects of glutamine on hydrogen peroxide-induced oxidative damage in intestinal epithelial cells of Jian carp (Cyprinus carpio var.Jian). *Aquaculture* **288:** 285-289

Cilla A, Laparra J, Alegria A, Barbera R, Farre R (2008) Antioxidant effect derived from bioaccessible fractions of fruit beverages against H2O2-induced oxidative stress in Caco-2 cells. *Food Chemistry* **106:** 1180-1187

Cvorovic J, Tramer F, Granzotto M, Candussio L, Decorti G, Passamonti S (2010) Oxidative stress-based cytotoxicity of delphinidin and cyanidin in colon cancer cells. *Archives of Biochemistry and Biophysics* **501:** 151-157

D'Archivio M, Filesi C, Di Benedetto R, Gargiulo R, Giovannini C, Masella R (2007) Polyphenols, dietary sources and bioavailability. *Ann Ist Super Sanita* **43:** 348-361

Dai J, Mumper RJ (2010) Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules* **15:** 7313-7352

Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R (2003) Protein carbonyl groups as biomarkers of oxidative stress. *Clinica Chimica Acta* **329:** 23-38

Danese S, Semeraro S, Marini M, Roberto I, Armuzzi A, Papa A, Gasbarrini A (2005) Adhesion molecules in inflammatory bowel disease: Therapeutic implications for gut inflammation. *Digestive and Liver Disease***:** 811-818

Desreumaux P, Ghosh S (2006) Review article: mode of action and delivery of 5-aminosalicylic acid - new evidence. *Aliment Pharmacol Ther* **24 Suppl 1:** 2-9

Di Mauro A, Arena E, Fallico B, Passerini A, Maccarone E (2002) Recovery of anthocyanins from pulp wash of pigmented oranges by concentration on resins. *Journal of Agricultural and Food Chemistry* **50:** 5968-5974

Dillard C, German J (2000) Phytochemicals: nutraceuticals and human health. *Journal of the Science of Food and Agriculture* **80:** 1744-1756

Duenas M, Surco-Laos F, Gonzalez-Manzano S, Gonzalez-Paramas A, Santos-Buelga C (2011) Antioxidant properties of major metabolites of quercetin. *European Food Research and Technology* **232:** 103-111

Edelblum KL, Turner JR (2009) The tight junction in inflammatory disease: communication breakdown. *Curr Opin Pharmacol* **9:** 715-720

Erlejman A, Jaggers G, Fraga C, Oteiza P (2008) TNF alpha-induced NF-kappa B activation and cell oxidant production are modulated by hexameric procyanidins in Caco-2 cells. *Archives of Biochemistry and Biophysics* **476:** 186-195

Espín JC, García-Conesa MT, Tomás-Barberán FA (2007) Nutraceuticals: facts and fiction. *Phytochemistry* **68:** 2986-3008

Eussen SR, Verhagen H, Klungel OH, Garssen J, van Loveren H, van Kranen HJ, Rompelberg CJ (2011) Functional foods and dietary supplements: Products at the interface between pharma and nutrition. *Eur J Pharmacol*

Fernández-López JA, Almela L, Obón JM, Castellar R (2010) Determination of antioxidant constituents in cactus pear fruits. *Plant Foods Hum Nutr* **65:** 253-259

Ferretti G, Bacchetti T, Belleggia A, Neri D (2010) Cherry antioxidants: from farm to table. *Molecules* **15:** 6993-7005

Feugang J, Konarski P, Zou D, Stintzing F, Zou C (2006) Nutritional and medicinal use of Cactus pear (Opuntia spp.) cladodes and fruits. *Frontiers in Bioscience***:** 2574-2589

Finley J, Kong A, Hintze K, Jeffery E, Ji L, Lei X (2011) Antioxidants in Foods: State of the Science Important to the Food Industry. *Journal of Agricultural and Food Chemistry* **59:** 6837-6846

Fraga CG, Galleano M, Verstraeten SV, Oteiza PI (2010) Basic biochemical mechanisms behind the health benefits of polyphenols. *Mol Aspects Med* **31:** 435-445

Frontela C, Canali R, Virgili F (2010) [Use of dietary phenols to modulate inflammatory bowel response]. *Gastroenterol Hepatol* **33:** 307-312

González R, Ballester I, López-Posadas R, Suárez MD, Zarzuelo A, Martínez-Augustin O, Sánchez de Medina F (2011) Effects of flavonoids and other polyphenols on inflammation. *Crit Rev Food Sci Nutr* **51:** 331-362

Gosslau A, Li S, Ho CT, Chen KY, Rawson NE (2011) The importance of natural product characterization in studies of their anti-inflammatory activity. *Mol Nutr Food Res* **55:** 74-82

Guevara-Figueroa T, Jimenez-Islas H, Reyes-Escogido M, Mortensen A, Laursen B, Lin L, De Leon-Rodriguez A, Fomsgaard I, de la Rosa A (2010) Proximate composition, phenolic acids, and flavonoids characterization of commercial and wild nopal (Opuntia spp.). *Journal of Food Composition and Analysis***:** 525-532

Halliwell B (2011) Free radicals and antioxidants - quo vadis? *Trends in Pharmacological Sciences* **32:** 125-130

Hamalainen M, Nieminen R, Vuorela P, Heinonen M, Moilanen E (2007) Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-kappa B activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-kappa B activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. *Mediators of Inflammation***:** -

Hissin P, Hilf, R (1976) FLUOROMETRIC METHOD FOR DETERMINATION OF OXIDIZED AND REDUCED GLUTATHIONE IN TISSUES. *Analytical Biochemistry* **74:** 214-226

Hou D, Fujii M, Terahara N, Yoshimoto M (2004) Molecular mechanisms behind the chemopreventive effects of anthocyanidins. *Journal of Biomedicine and Biotechnology***:** 321-325

Ignat I, Volf I, Popa V (2011) A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry* **126:** 1821-1835

Jung M, Triebel S, Anke T, Richling E, Erkel G (2009) Influence of apple polyphenols on inflammatory gene expression. *Molecular Nutrition & Food Research***:** 1263-1280

Kaliora A, Dedoussis G, Schmidt H (2006) Dietary antioxidants in preventing atherogenesis. *Atherosclerosis* **187:** 1-17

Kand'ar R, Zakova P, Lotkova H, Kucera O, Cervinkova Z (2007) Determination of reduced and oxidized glutathione in biological samples using liquid chromatography with fluorimetric detection. *Journal of Pharmaceutical and Biomedical Analysis* **43:** 1382-1387

Kaplan M, Mutlu EA, Benson M, Fields JZ, Banan A, Keshavarzian A (2007) Use of herbal preparations in the treatment of oxidant-mediated inflammatory disorders. *Complement Ther Med* **15:** 207-216

Karlsen A, Retterstøl L, Laake P, Paur I, Kjølsrud-Bøhn S, Sandvik L, Blomhoff R. (2007) Anthocyanins Inhibit Nuclear Factor-kB Activation in Monocytes and Reduce Plasma Concentrations of Pro-Inflammatory Mediators in Healthy Adults. The Journal of Nutrition, pp. 1951-1954.

Kelsey NA, Wilkins HM, Linseman DA (2010) Nutraceutical antioxidants as novel neuroprotective agents. *Molecules* **15:** 7792-7814

Langerholc T, Maragkoudakisb PA, Wollgastb J, Gradisnikc L, Cencica A. (2011) Novel and established intestinal cell line models - An indispensable tool in food science and nutrition. Trends in Food Science & Technology, pp. 1-10.

Leonard F, Collnot EM, Lehr CM (2010) A three-dimensional coculture of enterocytes, monocytes and dendritic cells to model inflamed intestinal mucosa in vitro. *Mol Pharm* **7:** 2103-2119
Lila M (2004) Anthocyanins and human health: An in vitro investigative approach. *Journal of Biomedicine and Biotechnology***:** 306-313

Liu P, Deng T, Hou X, Wang J (2009) Antioxidant Properties of Isolated Isorhamnetin from the Sea Buckthorn Marc. *Plant Foods For Human Nutrition* **64:** 141-145

Liu RH (2004) Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J Nutr* **134:** 3479S-3485S

M.C.Azeredo H. (2009) Betalains: properties, sources, applications, and stability – a review. International Journal of Food Science and Technology, Vol. 44, pp. 2365–2376.

Mandalari G, Bisignano C, Genovese T, Mazzon E, Wickham MS, Paterniti I, Cuzzocrea S (2011) Natural almond skin reduced oxidative stress and inflammation in an experimental model of inflammatory bowel disease. *Int Immunopharmacol* **11:** 915-924

Masella R, Di Benedetto R, Vari R, Filesi C, Giovannini C (2005) Novel mechanisms of natural antioxidant compounds in biological systems: Involvement of glutathione and glutathione-related enzymes. *Journal of Nutritional Biochemistry* **16:** 577-586

McCune L, Kubota C, Stendell-Hollis N, Thomson C (2011) Cherries and Health: A Review. *Critical Reviews in Food Science and Nutrition* **51:** 1-12

Michalska A, Ceglinska A, Zielinski H (2007) Bioactive compounds in rye flours with different extraction rates. *European Food Research and Technology* **225:** 545-551

Miguel MG. (2011) Anthocyanins: Antioxidant and/or anti-inflammatory activities. Journal of Applied Pharmaceutical Science, Vol. 06, pp. 07-15.

Mossammer M, Stintzing F, Carle R (2005) Development of a process for the production of a betalain-based colouring foodstuff from cactus pear. *Innovative Food Science & Emerging Technologies* **6:** 221-231

Moussa-Ayoub T, El-Samahy S, Kroh L, Rohn S (2011) Identification and quantification of flavonol aglycons in cactus pear (Opuntia ficus indica) fruit using a commercial pectinase and cellulase preparation. *Food Chemistry***:** 1177-1184

Mueller M, Hobiger S, Jungbauer A (2010) Anti-inflammatory activity of extracts from fruits, herbs and spices. *Food Chemistry***:** 987-996

Obrenovich ME, Li Y, Parvathaneni K, Yendluri BB, Palacios HH, Leszek J, Aliev G (2011) Antioxidants in health, disease and aging. *CNS Neurol Disord Drug Targets* **10:** 192-207

Ou B, Hampsch-Woodill M, Flanagan J, Deemer E, Prior R, Huang D (2002) Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. *Journal of Agricultural and Food Chemistry* **50:** 2772-2777

Pan MH, Lai CS, Dushenkov S, Ho CT (2009) Modulation of inflammatory genes by natural dietary bioactive compounds. *J Agric Food Chem* **57:** 4467-4477

Paredes-López O, Cervantes-Ceja ML, Vigna-Pérez M, Hernández-Pérez T (2010) Berries: improving human health and healthy aging, and promoting quality life--a review. *Plant Foods Hum Nutr* **65:** 299-308

Quideau S, Deffieux D, Douat-Casassus C, Pouységu L (2011) Plant polyphenols: chemical properties, biological activities, and synthesis. *Angew Chem Int Ed Engl* **50:** 586-621

Rahimi R, Mozaffari S, Abdollahi M (2009) On the use of herbal medicines in management of inflammatory bowel diseases: a systematic review of animal and human studies. *Dig Dis Sci* **54:** 471-480

Rahman I (2008) Dietary polyphenols mediated regulation of oxidative stress and chromatin remodeling in inflammation. *Nutr Rev* **66 Suppl 1:** S42-45

Rahman I, Biswas SK, Kirkham PA (2006) Regulation of inflammation and redox signaling by dietary polyphenols. *Biochem Pharmacol* **72:** 1439-1452

Ramful D, Tarnus E, Rondeau P, Da Silva C, Bahorun T, Bourdon E (2010) Citrus Fruit Extracts Reduce Advanced Glycation End Products (AGEs)- and H2O2-Induced Oxidative Stress in Human Adipocytes. *Journal of Agricultural and Food Chemistry* **58:** 11119-11129

Rezaie A, Parker R, Abdollahi M (2007) Oxidative stress and pathogenesis of inflammatory bowel disease: An epiphenomenon or the cause? *Digestive Diseases and Sciences* **52:** 2015-2021

Rodrigo R, Miranda A, Vergara L (2011) Modulation of endogenous antioxidant system by wine polyphenols in human disease. *Clin Chim Acta* **412:** 410-424

Romier B, Schneider YJ, Larondelle Y, During A (2009) Dietary polyphenols can modulate the intestinal inflammatory response. *Nutr Rev* **67:** 363-378

Romier B, Van De Walle J, During A, Larondelle Y, Schneider YJ (2008) Modulation of signalling nuclear factor-kappaB activation pathway by polyphenols in human intestinal Caco-2 cells. *Br J Nutr* **100:** 542-551

Romier-Crouzet B, Van De Walle J, During A, Joly A, Rousseau C, Henry O, Larondelle Y, Schneider Y (2009) Inhibition of inflammatory mediators by polyphenolic plant extracts in human intestinal Caco-2 cells. *Food and Chemical Toxicology***:** 1221-1230

Sartor RB. (2006) Mechanisms of Disease: pathogenesis of Crohn's disease and ulcerative colitis. Nature Clinical Practice Gastroenterology & Hepatology, Vol. 3, pp. 390-407.

Satsu H, Hyun JS, Shin HS, Shimizu M (2009) Suppressive effect of an isoflavone fraction on tumor necrosis factor-alpha-induced interleukin-8 production in human intestinal epithelial Caco-2 cells. *J Nutr Sci Vitaminol (Tokyo)* **55:** 442-446

Schirbel A, Fiocchi C (2010) Inflammatory bowel disease: Established and evolving considerations on its etiopathogenesis and therapy. *J Dig Dis* **11:** 266-276

Schwab M, Reynders V, Steinhilber D, Stein J (2009) Combined treatment of Caco-2 cells with butyrate and mesalazine inhibits cell proliferation and reduces Survivin protein level. *Cancer Letters* **273:** 98-106

Scordino M, Di Mauro A, Passerini A, Maccarone E (2003) Adsorption of flavonoids on resins: Hesperidin. *Journal of Agricultural and Food Chemistry* **51:** 6998-7004

Sergent T, Piront N, Meurice J, Toussaint O, Schneider YJ (2010) Anti-inflammatory effects of dietary phenolic compounds in an in vitro model of inflamed human intestinal epithelium. *Chem Biol Interact* **188:** 659-667

Serra A, Duarte R, Bronze M, Duarte C (2011a) Identification of bioactive response in traditional cherries from Portugal. *Food Chemistry* **125:** 318-325

Serra A, Matias A, Almeida A, Bronze M, Alves P, de Sousa H, Duarte C (2011b) Processing cherries (Prunus avium) using supercritical fluid technology. Part 2. Evaluation of SCF extracts as promising natural chemotherapeutical agents. *Journal of Supercritical Fluids* **55:** 1007-1013

Serra A, Seabra I, Braga M, Bronze M, de Sousa H, Duarte C (2010) Processing cherries (Prunus avium) using supercritical fluid technology. Part 1: Recovery of extract fractions rich in bioactive compounds. *Journal of Supercritical Fluids* **55:** 184-191

Serra AT (2010) Valorization of Traditional Portuguese Apples and Cherries - Biochemical characterization and development of functional ingredients. Ph.D Thesis, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa,

Shahidi F (2009) Nutraceuticals and functional foods: whole versus processed foods. *Trends in Food Science & Technology* **20:** 376-387

Shimizu M (2010) Interaction between food substances and the intestinal epithelium. *Biosci Biotechnol Biochem* **74:** 232-241

Siccardi D, Turner J, Mrsny R (2005) Regulation of intestinal epithelial function: a link between opportunities for macromolecular drug delivery and inflammatory bowel disease. *Advanced Drug Delivery Reviews***:** 219-235

Silva EM, Pompeu DR, Larondelle Y, Rogez H. (2007) Optimization of the absorption of polyphenols from Inga edulis leaves on macroporous resins using an experimental design methodology. Sep Purif Technol, Vol. 53, pp. 274-280.

Singh A, Holvoet S, Mercenier A (2011) Dietary polyphenols in the prevention and treatment of allergic diseases. *Clin Exp Allergy*

Singleton VL, Rossi JA. (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic, Vol. 16, pp. 144-158.

Sonier B, Patrick C, Ajjikuttira P, Scott FW (2009) Intestinal immune regulation as a potential diet-modifiable feature of gut inflammation and autoimmunity. *Int Rev Immunol* **28:** 414-445

Sorg O (2004) Oxidative stress: a theoretical model or a biological reality? *Comptes Rendus Biologies* **327:** 649-662

Stevenson DE, Hurst RD (2007) Polyphenolic phytochemicals--just antioxidants or much more? *Cell Mol Life Sci* **64:** 2900-2916

Stintzing F, Carle R (2004) Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends in Food Science & Technology* **15:** 19-38

Stintzing F, Carle R (2005) Cactus stems (Opuntia spp.): A review on their chemistry, technology, and uses. *Molecular Nutrition & Food Research***:** 175-194

Stintzing F, Carle R (2007) Betalains - emerging prospects for food scientists. *Trends in Food Science & Technology* **18:** 514-525

Suzuki T, Hara H (2011) Role of flavonoids in intestinal tight junction regulation. *Journal of Nutritional Biochemistry* **22:** 401-408

Tanoue T, Nishitani Y, Kanazawa K, Hashimoto T, Mizuno M (2008) In vitro model to estimate gut inflammation using co-cultured Caco-2 and RAW264.7 cells. *Biochemical and Biophysical Research Communications***:** 565-569

Tavares L, Carrilho D, Tyagi M, Barata D, Serra A, Duarte C, Duarte R, Feliciano R, Bronze M, Chicau P, Espirito-Santo M, Ferreira R, dos Santos C (2010) Antioxidant Capacity of Macaronesian Traditional Medicinal Plants. *Molecules* **15:** 2576-2592

Usenik V, Fabcic J, Stampar F (2008) Sugars, organic acids, phenolic composition and antioxidant activity of sweet cherry (Prunus avium L.). *Food Chemistry***:** 185-192

Van De Walle J, Hendrickx A, Romier B, Larondelle Y, Schneider Y (2010) Inflammatory parameters in Caco-2 cells: Effect of stimuli nature, concentration, combination and cell differentiation. *Toxicology in Vitro***:** 1441-1449

Van De Walle J, Romier B, Larondelle Y, Schneider YJ (2008) Influence of deoxynivalenol on NF-kappaB activation and IL-8 secretion in human intestinal Caco-2 cells. *Toxicol Lett* **177:** 205-214

Wallace JL (2003) Nitric oxide-releasing mesalamine: potential utility for treatment of inflammatory bowel disease. *Dig Liver Dis* **35 Suppl 2:** S35-40

Wijeratne SS, Cuppett SL, Schlegel V (2005) Hydrogen peroxide induced oxidative stress damage and antioxidant enzyme response in Caco-2 human colon cells. *J Agric Food Chem* **53:** 8768-8774

Willcox J, Ash S, Catignani G (2004) Antioxidants and prevention of chronic disease. *Critical Reviews in Food Science and Nutrition* **44:** 275-295

Williamson G, Manach C (2005) Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am J Clin Nutr* **81:** 243S-255S

Yi W, Akoh CC, Fischer J, Krewer G (2006) Absorption of anthocyanins from blueberry extracts by caco-2 human intestinal cell monolayers. *J Agric Food Chem* **54:** 5651-5658

Appendixes

Appendix A – ELISA solutions

- PBS 1% BSA

Dissolve 1g of BSA in 100mL of PBS.

- PBS 0,05% Tween-20

Add 50µL of ultra-pure Tween-20 to 100mL of PBS.

Appendix B – SDS-PAGE and Western Blot solutions

- Loading buffer 2%

Dissolve 0,91g of Tris, 2mL glycerol, 0,01g Bromophenol Blue, 1,2g SDS in 15mL. Correct pH to 6,8 and make up volume to 20mL. Add 0,9mL of β-mercaptoethanol (5%).

- Running Buffer

Dissolve 30g of Tris, 144g of Glycine and 10g of SDS to 2L of MiliQ water.

- Transfer buffer

Add 6,06g of Tris, 28,8g of glycine, 1g of SDS to 1,6L of miliQ water. Add 600mL of methanol.

- Tris buffered saline with Tween-20 (TBS-T)

Add 800mL of miliQ water and dissolve 12,1g of Tris base, 8,8g of NaCl. Adjust pH to 7,5 with HCl. Add 1,0mL of ultra pure Tween-20. Adjust volume to 1,0L with miliQ water.

- TBS-T with 1,0% (w/v) BSA

Add 100mL of TBS-T and dissolve 1,0g of BSA.

Appendix C – Phenolic composition

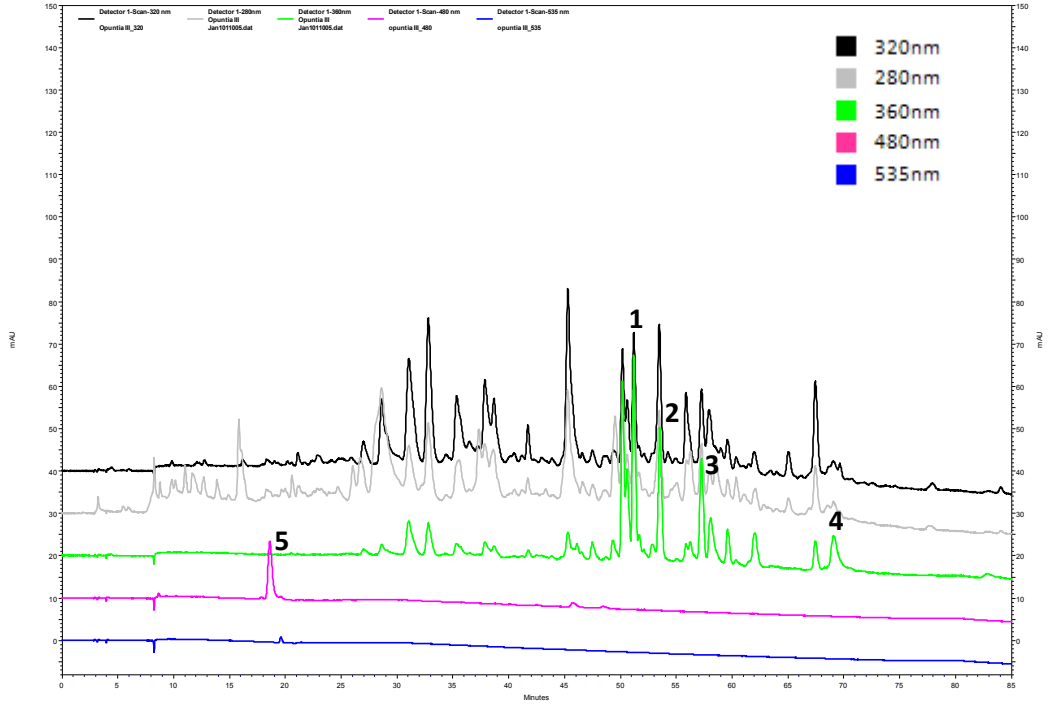


Figure C.1 – HPLC profile of cactus pear's PRE at different wavelengths: 320,280,360,480 and 535nm. 360nm corresponds to flavonoids while 480nm corresponds to Betaxantins. Legend: 1- Isorhamnetin-3-O-rha-lyx-glu; 2- Isorhamnetin-3-O-Lyx-glu; 3 – Isorhamnetin-3-O-rutinoside; 4 – Isorhamnetin and 5-Betaxantin.

Table C.1 – Phenolic content of cactus pear's PRE.

Phenolic compound	Concentration (ppm)
1- Isorhamnetin 3-O-rha-lyx-glu	36,58
2- Isorhamnetin-3-O-Lyx-glu	30,55
3- Isorhamnetin-3-O-rutinoside	26,35
4- Isorhamnetin	18,02

Table C.2 – Phenolic content of cherry's PRE.

Phenolic compound	Concentration (mg/g)
Cyanidin-3-glucoside	26,82
Cyanidin-3-rutinoside	114,80
Peonidin-3-glucoside	4,83