Joana Margarida de Andrade Poejo

Degree in Biochemistry

Evaluation of *Opuntia spp.* bioactive products as promising natural chemotherapeutical agents- an *in vitro* approach

Dissertation to obtain a Master Degree in Biotechnology

Supervisor: Ana Teresa Serra, Ph.D, IBET/ITQB-UNL
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Presidente: Prof. Doutora Cecília Roque
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Acknowledgments

Gostaria de agradecer a todas as pessoas que directa ou indirectamente contribuíram para me ajudar na realização deste trabalho.

Em primeiro lugar, gostaria de agradecer à Dra. Ana Teresa Serra por me ter acompanhado incansavelmente desde o primeiro ao último dia. Agradeço a constante orientação prestada, os conselhos e ideias novas, a paciência inesgotável, o apoio e o encorajamento demonstrado nas fases mais difíceis. Obrigada por me teres ajudado a crescer tanto profissionalmente como pessoalmente.

À Dra. Catarina Duarte por me ter dado oportunidade para desenvolver o trabalho no seu laboratório. Obrigada pela sua confiança, pelo incentivo e apoio demonstrados durante este ultimo ano.

Agradeço a toda a equipa do laboratório de Nutracêuticos e Libertação Controlada. À Dra. Ana Matias pela sua ajuda e partilha de excelentes conhecimentos e ideias. À Sara Nunes, minha companheira "Opuntiana", agradeço os momentos divertidos e de cumplicidade que facilitaram de algum modo a execução do trabalho. Obrigada também pela tua ajuda e conselhos. À Ana Nunes agradeço a excelente companhia, o apoio, o incentivo e as palavras amigas nos momentos mais solitários do Verão. Agradeço à Patrícia Almeida pela sua preciosa ajuda e por estar sempre disponível. Ao Agostinho Alexandre agradeço pela sua valiosa e indispensável ajuda no projecto "Opuntia". À Dora Pereira agradeço os ensinamentos iniciais, a simpatia e a boa disposição sempre demonstrada. Agradeço à Dra. Maria do Rosário Bronze e à Catarina Semedo pela ajuda prestada na parte analítica do projecto.

Agradeço a todos os meus amigos que de alguma forma me ajudaram a superar os momentos mais difíceis, que sempre me apoiaram e ajudaram quando mais precisei, agradeço especialmente à Sofia Fortalezas.

Ao Filipe pelo seu apoio incondicional e compreensão. Por me ter ajudado a ultrapassar muitos obstáculos, por me ter ouvido quando mais precisei.

À minha família: pais e irmãos especialmente. Agradeço aos meus irmãos pelo apoio, incentivo e motivação. Aos meus pais por sempre me terem apoiado ao longo de todo este percurso, por me incentivarem e acreditarem em mim do princípio ao fim. Obrigada por me terem ajudado a ultrapassar mais uma etapa importante da minha vida.

Obrigada por tudo!

Abstract

Recently, prevention of cancer through dietary intervention has received an increasing interest. In particular, dietary polyphenols mainly found in fruits and vegetables have become very attractive as chemopreventive and therapeutic agents.

The main aim of this work was to investigate the potential activity of Portuguese *Opuntia spp*. on colon cancer therapy, as this fruits have been reported to be rich sources of bioactive compounds.

Opuntia spp. fruits were collected in different sites of Portugal, namely Tramagal, Beja, Marvão Sines and Quarteira and processed in order to obtain fruit juices, polyphenol-rich concentrates and residues extracts from fruit juices production. All *Opuntia* products were investigated for antiproliferative activity of human colon cancer cells (HT29). *Opuntia* juices from Tramagal, Sines and Quarteira were the most effective on cancer cell growth inhibition due to the highest content in total polyphenol content and betalains.

The most promising extracts were derived from Quarteira, Sines and Beja fruit juices residues using conventional solvent extraction, adsorption technology and pressurized liquid extraction, respectively. These extracts showed high antiproliferative effect inducing cell cycle arrest in different checkpoints. Additionally, the same extracts showed to induce ROS production in cancer cells, playing an important role in activation of pro-apoptic cell death mechanisms.

The capacity of natural extracts in reverting chemoresistance of tumor cells was evaluated on a drug-resistant HT29 cell culture. The product derived from Beja residue using pressurized liquid extraction with 60%CO_2 and 40%EtOH showed to increase cells sensitivity to the drug.

Opuntia spp. fruits reveled to be promising natural sources for the production of functional products as well as for the development of novel chemotherapeutic agents.

Keywords: Colon cancer; polyphenols; *Opuntia spp.*; antiproliferative effect; chemoresistance.

Resumo

Recentemente tem surgido um grande interesse na prevenção do cancro através da dieta. Em particular, os polifenóis presentes nas frutas e nos vegetais são reconhecidos como sendo compostos muito atractivos na área da quimioprevenção e terapia do cancro.

O principal objectivo deste trabalho consistiu na avaliação da potencial actividade de frutos de *Opuntia spp.* Portugueses para terapia do cancro do cólon, uma vez que este frutos são reconhecidos como sendo fontes ricas em compostos bioactivos.

Os frutos de *Opuntia spp.* foram recolhidos em diferentes regiões de Portugal, nomeadamente Tramagal, Beja, Marvão, Sines e Quarteira, e foram processados para obter sumos de fruta, extractos concentrados em polifenóis e extractos de resíduo obtidos a partir do processamento dos sumos de fruta. Todos os produtos foram analisados em termos de actividade antiproliferativa numa linha celular humana de cancro do cólon (HT29). Os sumos de fruta provenientes de Tramagal, Sines e Quarteira foram os mais efectivos na inibição do crescimento das células cancerígenas devido ao elevado conteúdo em polifenóis e à presença de betalainas.

Os extractos mais promissores foram obtidos a partir dos resíduos dos sumos de Quarteira, Sines e Beja, usando tecnologias limpas como a extracção convencional, tecnologia de adsorção e a extracção com solventes pressurizados. Os extractos obtidos demonstraram um efeito antiproliferativo elevado e induziram a paragem do ciclo celular em fases distintas. Para além disso induziram a produção de espécies reactivas de oxigénio (ROS) a nível celular, desempenhando assim um papel importante na activação de mecanismos pro-apoptóticos de morte celular.

Neste trabalho foi também avaliada a capacidade dos extractos naturais em reverter a quimioresistência em células resistentes à doxorubicina, uma droga anticancerígena. O extracto obtido por extracção com solventes pressurizados, nomeadamente 60%CO_2 e 40% EtOH, a partir do resíduo de Beja, demonstrou aumentar a sensibilidade à droga nas células resistentes.

Os resultados obtidos nesta tese demonstraram que os frutos de *Opuntia spp.* são fontes promissoras para a produção de produtos funcionais e para o desenvolvimento de novos agentes naturais para aplicação em quimioterapia.

Palavras-chave: cancro do cólon; polifenóis; *Opuntia spp.*; actividade antiproliferativa; quimioresistência.

Index

Index of figures

Index of tables

List of abbreviations, acronyms and symbols

Abbreviation	Full form
AAPH	2‟,2‟-Azobis (2-amidinopropane) dihydrochloride
ACS	American Cancer Society
AD	Adsorption technology
AICR	American Institute of Cancer Research
ASE	Accelerated Solvent Extraction
BSA	Bovine Serum Albumine
Caco-2	Caco-2 human colon carcinoma cell line
CAE	Caffeic Acid Equivalents
CO_2	Carbon dioxide
CSE	Conventional Solvent Extraction
DCFH-DA	2‟,7‟-Dichlorofluorescin Diacetate
DNA	Deoxyribonucleic acid
ED50	Median effective dose
EDTA	Ethylenediamine Tetraacetic Acid
EGCG	Epigallocatechin-3-gallate
EtOH	Ethanol
FBS	Fetal Bovine Serum
FL	Disodium Fluorescein
GAE	Gallic Acid Equivalents
HE	*Hericium erinaceus*
HORAC	Hydroxyl Radical Adverting Capacity

HPLC	High Performance Liquid Chromatography
HT29	HT29 human colon cancer cell line
HT29 dx	HT29 human colon cancer cell line drug-resistant
MDR	Multidrug-Resistance
MPa	MegaPascal
MTT	Methylthiazolyldiphenyl tetrazolium bromide
NCI	National Cancer Institute
NHE	Na^+/H^+ exchanger
NO	Nitric Oxide
ORAC	Oxygen Radical Absorbance Capacity
PBS	Phosphate Buffer Saline
PFE	Pressurized Fluid Extraction
PLE	Pressurized liquid extraction
PRC	Polyphenol-Rich Concentrate
ROS	Reactive Oxygen Species
SFE	Supercritical Fluid Extraction
TCF	Total Flavonoid
Content TPC	Total Polyphenol
Content UV	Ultra - violet
WCRF/AICR	World Cancer Research Fund/American Institute of Cancer Research

1.1 Introduction

1.1 Cancer worldwide

Cancer is a leading cause of mortality in human and a growing health problem all around the world. One defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries and spread to other organs, creating metastasis (Haque, et al., 2010). According to Cancerbase GLOBOCAN, in 2008, more than 12 million new cases of cancer were registered (excluding non-melanoma skin cancer) while 7.5 million people died from cancer worldwide. In 2020 it is estimated that mortality from cancer will increase to more than 10 million (Ferlay J, et al., 2010).

Since there are no particular treatment of cancer, effective preventive measures and cancer awareness among the general population is essential. Many clinical and laboratory studies support that our lifestyle and dietary factors are playing a complex multifaceted role in etiology of cancer (Haque, et al., 2010). Several organizations such as: World Health Organization (WHO), American Cancer Society (ACS), American Institute of Cancer Research (AICR) and National Cancer Institute (NCI) have established dietary guidelines to help people reduce the cancer risk (Surh, 2003).

1.1.1 Colorectal Cancer

1.1.1.1 Epidemiology, etiology and causes

Colorectal cancer (cancer of the colon and/or rectum) is the third most common cancer in men (663 000 cases, 10.0% of the total) and the second in women (571 000 cases, 9.4% of the total) worldwide (Ferlay J, et al., 2010). In 2008, about 608 000 deaths from colorectal cancer were estimated, accounting for 8% of all cancer deaths, making it the fourth most common cause of death from cancer in world. Almost 60% of the cases occur in developed regions, but colon cancer incidence is now increasing in middle and low income countries (see table 1.1) (Ferlay J, et al., 2010).

Colon cancer is defined as any malignant neoplasm arising from the inner lining of the colonic epithelium (Rajamanickam and Agarwal, 2008). According to the 2007 Report of the World Cancer Research Fund/American Institute of Cancer Research (WCRF/AICR), approximately 95 per cent of colorectal cancers are adenocarcinomas. However other types of cancer can occur, including mucinous carcinomas and adenosquamous carcinomas. The occurrence of colon cancer is strongly related with age, 90% of the cases arising in people who are 50 years or older. Survival rates for colon cancer can vary based on a variety of factors, particularly the stage. If the cancer is detected at an early and localized stage, the five-year survival rate (the percentage of people who survive at least five years after the cancer is detected, excluding those who die from other diseases) is 90%. However, when metastasis occur, the 5-year survival rate decrease to 10% (American Cancer Society, 2008).

Etiologically, colorectal cancer may be hereditary or sporadic (Young, et al., 2005). Epidemiological studies have suggested that colon cancer is a manifestation of a number of inherited cancer predisposition syndromes, including familial adenomatous polyposis, hereditary non-polyposis

colorectal cancer, personal or family history of colorectal cancer and/or polyps and inflammatory bowel disease (Rowley, 2005). On the other hand, sporadic colorectal cancers are due to somatic genetic mutations that occur as part of the normal cellular lifespan or because of exposure to environmental factors (Young, et al., 2005). Known causes of colon cancer include tobacco smoking, obesity and lack of exercise, infectious agents, medication, radiation, industrial chemicals, alcohol consumption and diet rich in high fat, red and processed meats. Moreover, inadequate intake of dietary fiber, fruits and vegetables are also associated with the increase of colon cancer risk (Haque, et al., 2010, Kushi, et al., 2006, Rajamanickam and Agarwal, 2008).

Table 1.1. Colorectal Cancer Incidence and Mortality Worldwide in 2008. Numbers are expressed in thousands (adapted from Ferlay J, et al., 2010).

	Men		Women		Both sexes	
	Cases	Deaths	Cases	Deaths	Cases	Deaths
World	663	320	571	288	1234	608
More developed regions	389	165	338	154	727	319
Less developed regions	274	154	232	134	506	288
WHO Africa region (AFRO)	14	11	12	9	26	20
WHO America region (PAHO)	122	46	118	49	240	95
WHO East Mediterranean region (EMRO)	13	9	10	7	23	16
WHO Europe Region (EURO)	238	115	212	107	450	222
WHO South-East Asia region (SEARO)	50	34	47	32	97	66
WHO Western Pacific region (WPRO)	224	101	170	81	394	182
IARC membership (21 countries)	368	151	316	141	684	292
United States of America	79	24	74	26	153	50
China	125	61	95	48	220	109
India	20	14	16	11	36	25
European Union (EU-27)	182	80	151	68	333	148

1.1.1.2 Chemoteraphy and chemoprevention: a perfect combination

Conventional cancer therapies, including surgery, chemotherapy, and radiotherapy have a significant role in the overall treatment of tumors (Sarkar and Li, 2006). In particular chemotherapy has high treatment success rates in various types of cancer. However, the main drawback of this therapy consists on the multidrug-resistance (MDR) effect of cancer cell (De Boo, et al., 2009, Riganti, et al., 2005, Sarkar and Li, 2006, Schonn, et al., 2011). A variety of mechanisms by which cancer cells acquire the MDR phenotype have been described, including the reduced uptake or the increased efflux of the drug, the genetic modification of the drug's specific targets, the increased ability to repair DNA damage, the reduced capacity to enter apoptosis, the different compartmentalization and the increased rate of drug detoxification (Ferreira, et al., 2002, Gottesman, 2002). Thus, the cell resistance to anticancer drugs and the failure of chemotherapy to treat some cancers lead to development of new therapies. The strategies of cancer treatment using combined

therapies or combined agents with distinct molecular mechanisms are considered very promising for higher efficacy, resulting in better survival (Hwang, et al., 2005, Sarkar and Li, 2006). For this reason, chemoprevention, which involves the use of specific natural products or synthetic chemical agents to reverse, suppress or prevent premalignancy before the development of invasive cancer, has appeared as a novel approach for controlling this malignant disease (Haque, et al., 2010, Rajamanickam and Agarwal, 2008, Ramos, 2008).

In terms of molecular perspective, carcinogenesis is a multistage process consisting on initiation, promotion and progression phases which involves sequential generations of cells that exhibit continuous disturbance of cellular and molecular signal cascades (Vincent and Gatenby, 2008) (figure 1.1). The initiation stage comprises the exposure or uptake of cells with a carcinogenic agent causing a genetic alteration. In the promotion stage abnormal cells persists, replicates and may originate a focus of preneoplastic cells. Finally in the last stage – progression - there is an uncontrolled growth of the cells (tumor) that involves the gradual conversion of premalignant cells to neoplastic ones with an increase of invasiveness and metastasis potential, and new blood vessel formation (angiogenesis) (Ramos, 2008). During this multistage sequence of events there are many phases for intervention to inhibit, reverse and/or delay the progress of cancer (Johnson, 2007).

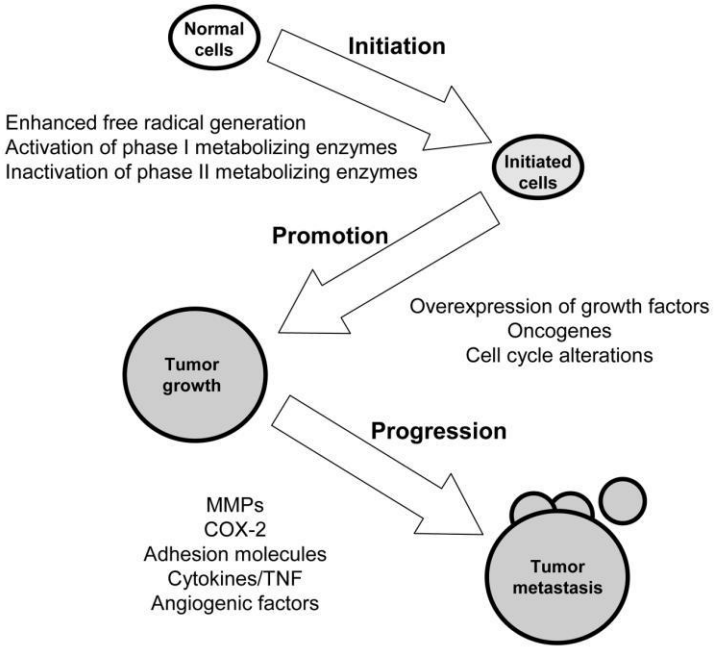

Figure 1.1. Sequence of multistage carcinogenesis process (Ramos, 2008).

In recent years, dietary compounds have been recognized as cancer chemopreventive agents owing to their various health benefits and noticeable lack of toxicity and side effects (Manson, et al., 2005, Sarkar and Li, 2006). Common cancer therapies combined with these dietary compounds may exert enhanced antitumor activity through synergic action or compensation of inverse properties.

Moreover, the combination treatment may decrease the systemic toxicity caused by many chemotherapeutic drugs because lower doses could be used (Sarkar and Li, 2006). It is known that these compounds exert the anticarcinogenic activity through regulation of different cell signaling pathways (figure 1.2). Chemopreventive agents can affect all stages of carcinogenesis by inducing cell cycle arrest and apoptosis, decreasing cell proliferation and angiogenesis, inhibiting tumor cell invasion and metastasis, and modulating various signal transduction as well as COX-2/PGE2 and Wnt/β-catenin pathways, involved in colon cancer development (Dragnev, et al., 2007)

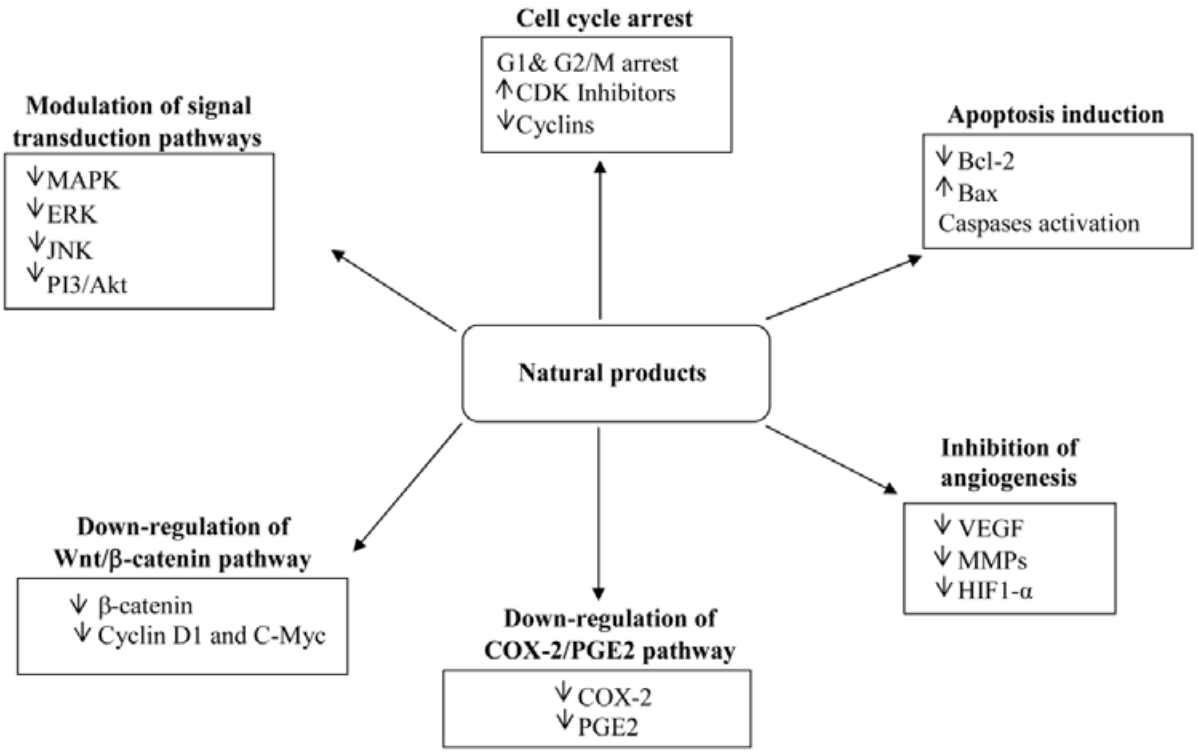

Figure 1.2. Mechanisms of action of natural products in colon cancer chemoprevention (Rajamanickam and Agarwal, 2008).

1.1.1.3 Food and nutrition on cancer chemoprevention

According to WCRF/AICR 2007 report, 30-40% of cancer could be preventable over time by appropriate food and nutrition, regular physical activity, and avoidance of obesity. On a global scale this represents over 3 to 4 million cases of cancer that can be prevented in these ways, every year (WCRF/AICR, 2007). As table 1.2 shows, colorectal cancer are greatly or mostly affected by food and nutrition. It is proven that physical activity and foods containing dietary fiber protect against colorectal cancer while foods, like red and processed meat, alcoholic drinks, body fatness and others factors increase risk of the disease. However, the findings that fruits and vegetables can protect against colon cancer are not yet conclusive, suggesting a limited-suggestive role (WCRF/AICR, 2011). Despite that, several epidemiological studies have reported an inverse correlation between decreased cancer risk and high consumption of vegetables, including cabbage, cauliflower, broccoli, brussels sprout,

tomatoes, and fruits such as, apples, grapes, and berries (Gordaliza, 2007, Vainio and Weiderpass, 2006).

The assessing of the real impact of dietary constituents on human health is difficult because in many cases the exact composition of foods and the bioavailability of active molecules are not known. The most direct evidence of beneficial effects by a particular food rich in certain compounds have come from animal models and in vitro experiments. In fact, cell culture studies constitute a valuable tool for identifying the molecular targets modulated by food compounds in cancer cells and for elucidating the molecular pathways involved in the overall disease process (Ramos, 2008).

Table 1.2. The factors that modify the risk of cancers of the colon and the rectum. Judgments are graded according to the strength of the evidence by the Panel of WCRF/AICR Continuous Update Project 2011 (adapted from WCRF/AICR, 2011).

	Decrease risk	**Increase risk**
Convincing	- Physical activity [1] - Foods containing dietary fiber	- Red meat - Processed meat - Alchololic drinks (men) [2] - Body fatness - Abdominal fatness - Adult attained height [3]
Probable	- Garlic - Milk - Calcium	- Alcoholic drinks (woman) [2]
Limited - suggestive	- Non-starchy vegetables - Fruits - Foods containing folate - Foods containing selenium - Fish - Foods containing vitamin D - Selenium	- Foods containig iron - Cheese - Foods containing animal fats - Foods containing sugars
Limited - no conclusion	- Cereals (grains) and their products; potatoes; poultry; shellfish and other seafood; other dairy products; total fat; fatty acid composition; cholesterol; sugar (sucrose); coffee; tea; caffeine; total carbohydrate; starch; vitamin A; retinol; vitamin C; vitamin E; multivitamins; non-dairy sources of calcium; methionine; beta-carotene; alpha-carotene; lycopene; meal frequency; energy intake	

[1] Physical activity of all types: occupational, household, transport, and recreational. The Panel judges that the evidence for colon cancer is convincing. No conclusion was drawn for rectal cancer.
[2] The judgments for men and women are different because there are fewer data for women. Increased risk is only apparent above a threshold of 30 g/day of ethanol for both sexes.
[3] Adult attained heights are unlikely directly to modify the risk of cancer. It is a marker for genetic, environmental, hormonal, and also nutritional factors affecting growth during the period from preconception to completion of linear growth.

1.2 Polyphenols and health-promoting effects

"Polyphenols" (or phenolic compounds) is a generic term that refers to more than 8000 compounds widely dispersed throughout the plant kingdom (Cartea, et al., 2011). These phytochemicals are secondary metabolites and have considerable physiological and morphological functions. Polyphenols may act as phytoalexins, antifeedants, attractants for pollinators, contributors to plant pigmentation, antioxidants and protective agents against UV light, among others (Naczk and Shahidi, 2006). These bioactive properties play an important role in plant growth and reproduction and providing an efficient protection against pathogens and predators. Additionally, polyphenols contribute to the color and sensory characteristics of many fruits and vegetables (Cartea, et al., 2011, Jaganath and Crozier, 2010, Zitka, et al., 2011).

Polyphenolic compounds derived from phenylalanine and are characterized by having at least one aromatic ring with one or more hydroxyl groups attached. They are divided into several classes according to the number of phenol rings that they contain and the structural elements that bind these rings to one another (D'Archivio, et al., 2007). The main groups of polyphenols are flavonoids (eg: quercetin), phenolic acids (eg: chlorogenic acid), stilbenes (eg: resveratrol) and lignans (enterodiol). Flavonoids and phenolic acids account for 60 and 30%, respectively, of dietary polyphenols (figure 1.3) (Scalbert and Williamson, 2000).

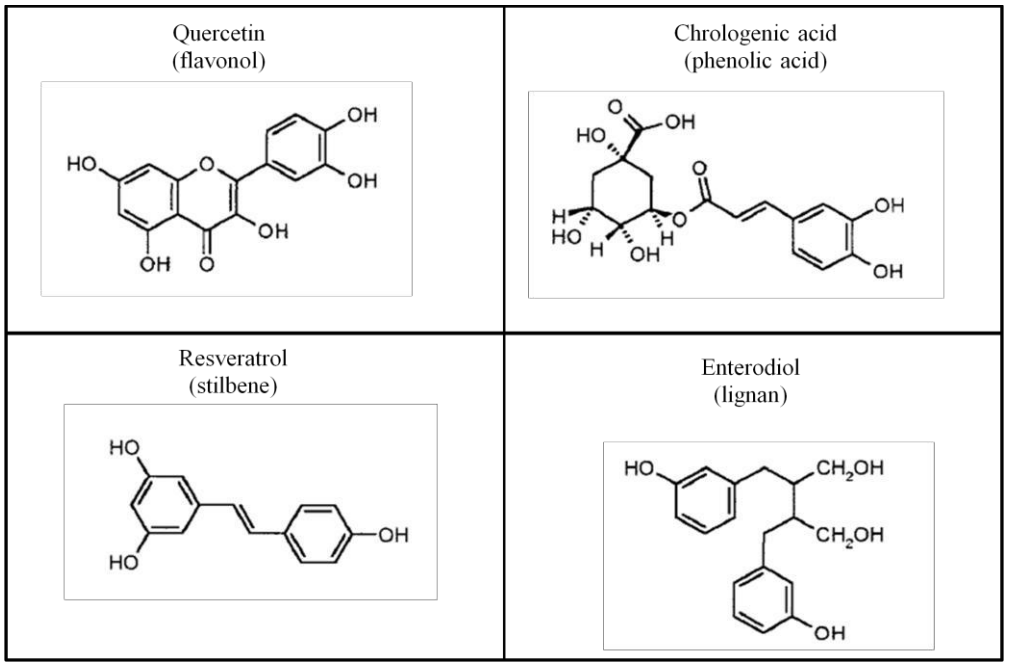

Figure 1.3. Chemical structures of the main classes of polyphenols (adapted from Scalbert and Williamson, 2000).

Polyphenols are widespread constituents of fruits, vegetables, cereals, olive, dry legumes, chocolate and beverages, such as tea, coffee and wine (D'Archivio, et al., 2007). They have a great interest in the industry because they have many applications as food preservatives and colorants or in

production of paints, papers and cosmetic (Ignat, et al., 2011). However, in last decade, polyphenols have received much more attention for their health benefits. The most well-know property of polyphenols is their antioxidant capacity - the ability of a food constituent to enhance the endogenous antioxidant defense system (Lila, 2007). Reactive oxygen species (ROS), derived from oxidation processes, are an important part of the defense mechanisms against infection, but excessive generation of free oxygen radicals may damage the tissue. When there is an imbalance between ROS and antioxidant defense mechanisms, the ROS lead to the oxidative modification of cellular membranes or intracellular biomolecules and result in the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides (Cartea, et al., 2011). Therefore, as antioxidants, polyphenols can trap ROS in the human body, protecting cells against oxidation damage and consequently limit the risk of several pathologies associated to oxidative stress (Cartea, et al., 2011, D'Archivio, et al., 2007, Lila, 2007, Ramos, 2008). In more detail, polyphenolic compounds can prevent the DNA-damage caused by free radicals or carcinogenic agents through different mechanisms: (i) direct radical scavenging (Alía, et al., 2006, Sonee, et al., 2004), (ii) chelating divalent cations involved in Fenton reaction (Nakagawa, et al., 2004) and (iii) modulation of enzymes related to oxidative stress (glutathione peroxidase, glutathione reductase, nitricoxide sinthase, lipooxygenase, xanthine oxidase, etc.) (Alía (a), et al., 2006, Alía(b), et al., 2006). Besides their antioxidant activity, several studies have revealed that polyphenols exhibit an extensive spectrum of others biological activities such as, stimulation of the immune system, antibacterial, antiviral, anti-hepatotoxic, anti-ulcer, anti-inflammatory , anti-mutagenic, and anticancer effects (figure 1.4) (Zitka, et al., 2011).

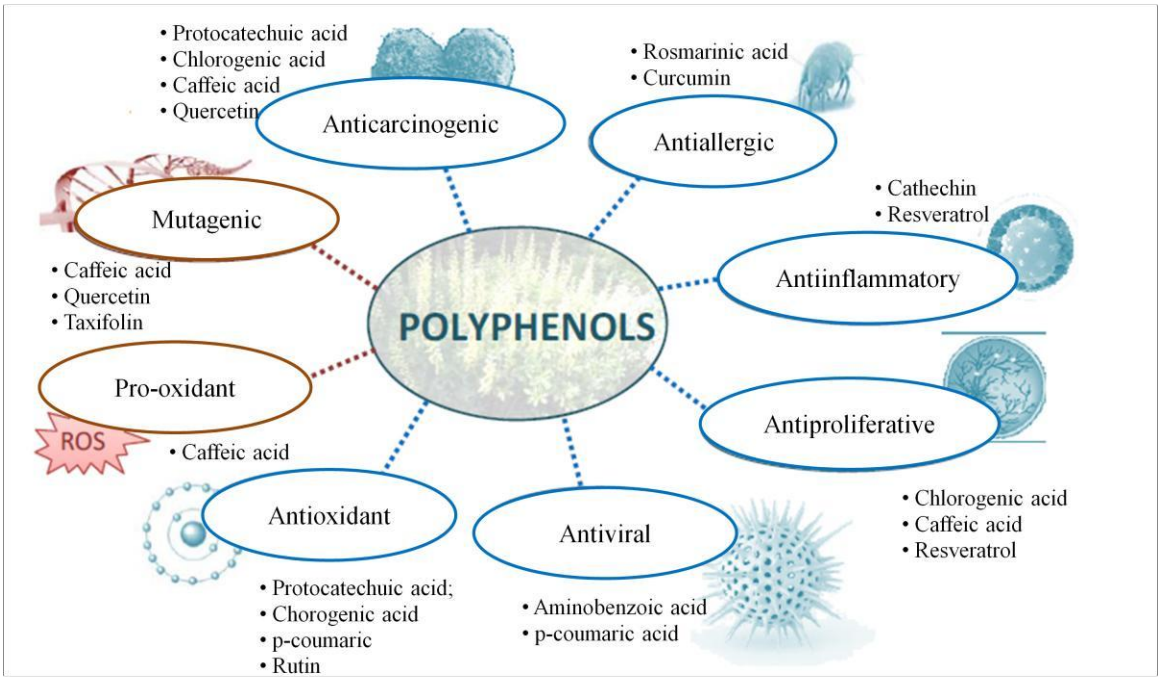

Figure 1.4. Polyphenols and their biological properties (adapted from Zitka, et al., 2011).

Dietary polyphenols can also act as pro-oxidants depending on the cell type, dose and/or time of treatment, having opposite effects on basic cell physiological processes: if as antioxidants they improve cell survival, as pro-oxidant they may induce apoptosis and block cell proliferation (Lambert, et al., 2005). Because of these several effects and properties, polyphenols may confer protection against several pathologies, such as cardiovascular disease, cancer, osteoporosis, diabetes mellitus and neurodegenerative diseases (Parkinson`s and Alzheimer`s disease) (Scalbert, et al., 2005).

Many studies in different cell lines and animal models suggest a protective role of dietary polyphenols against colorectal cancer (table 1.3) (Araújo, et al., 2011). According to Ramos (2008), there are 6 main common chemopreventive effects that polyphenols can exert on cancer cells: (1) antioxidant effect; (2) antiproliferation and antisurvival effect; (3) induction of cell cycle arrest, (4) induction o apoptosis, (5) anti-inflammatory effect, and (6) inhibition of angiogenesis and metastasis.

It is important to note that phenolic compounds are less potent that pharmaceutical drugs but since they are ingested regularly in significant amounts as part of the diet, they may have a noticeable long-term physiological effect (Espín, et al., 2007). The average dietary intake of polyphenols varied between 0.1 and 1 g/d in occidental Europe and the United States population but only 10% are absorbed upper gastrointestinal track (Manach, et al., 2004). For that reason, when compared with other organs and tissues, the intestinal region has been pointed as a promising site for chemoprevention due to the higher exposition doses of dietary polyphenols (Manach, et al., 2004, Scalbert and Williamson, 2000). As example, after the ingestion of 250 to 500 mg of polyphenol supplements the lumen of the colon can be exposed to concentrations around 0.1 to 3 mmol/L (Dihal, et al., 2006, Scalbert and Williamson, 2000) whereas the plasma concentrations are around 1µmol/L (van der Woude, et al., 2003).

Table 1.3. Mechanisms involved in the chemopreventive effect of polyphenols in colorectal cell lines (adapted from Araújo, et al., 2011).

Polyphenol	Cell type	Cellular mechanism
Quercetin	HT29, Caco2, SW480, HCT-116, IEC-6, FHC, VACO-235, COLO 201, LS-174T, T84, DLD-1, LT97, rat and mouse colonocytes	Cell growth inhibition and cytotoxic activity; reduction or stimulation of cell proliferation; decrease of cell migration; induction of cell cycle arrest, differentiation, apoptosis, and autophagy
Rutin	HT29 and Caco2	Inhibition or no alteration of cell proliferation, differentiation, and apoptosis
Myricetin	HT29, Caco2, SW480, T84, VACO-235, COLO 205	Cell growth inhibition; reduction of cell proliferation; induction of apoptosis; antimetastatic properties
Chrysin	HT-29, Caco-2, SW480	Cell growth inhibition; reduction of cell proliferation; induction of cell cycle arrest and apoptosis
EGCG	HT29, Caco2, SW480, SW837, SW426, HCT-116, FHC, T84, murine colon 26-L5, mouse colon 26	Inhibition of cell growth, proliferation, neoplastic transformation, invasion, and angiogenesis; induction of cell cycle arrest and apoptosis
Epicatechin	HT29	Weak or absent growth-inhibitory and apoptotic activity
Catechin	HT29	Weak or absent growth-inhibitory and apoptotic activity
Resveratrol	HT29, SW480, Caco2, SW620, HCT-116, CCL 220.1, WiDr	Inhibition of cell growth; induction of apoptosis; arrest of proliferation, cell cycle, and neoplastic transformation
Xanthohumol	HT29, CCL 220.1, HCT-116–derived 40-16	Inhibition of cell growth, proliferation, invasiveness, and angiogenesis; induction of apoptosis, terminal differentiation, and cell cycle arrest
Combination of polyphenols	HT29, Caco2, SW480	Inhibition of cell growth and proliferation; induction of apoptosis
Combination of polyphenols with therapeutic drugs	HT29, mouse colon 26	Inhibition of cell growth and proliferation; induction of apoptosis

1.3 *Opuntia spp.*

The prickly pear cactus (*Opuntia spp.*) or cactus pear belongs to the *Cactaceae* family (Feugang, et al., 2006) and is a long-domesticated crop critically important in agricultural economies throughout the arid and semiarid parts of the world, where water can be a limitation for cultivation (Griffith, 2004). It is widely distributed in Europe, Southwestern United States, Northern Mexico, much of Latin America, South Africa and the Mediterranean countries (Utkarsha, et al., 2010).

Two parts of the plant have been used for food: the „„nopal"" or cladodes (stems) and the fruits or the prickly pears (figure 1.5). In particular, cladodes are consumed in Mexican regions as a constituent of salads (Medina, et al., 2007) while *Opuntia spp.* fruits are known to be fresh and sweet fruits that can be eaten fresh, dried or preserved in jams, syrups or processed into candy-like products (Galati, et al., 2003, Medina, et al., 2007).

Figure 1.5. Images of *Opuntia spp.* cladodes and fruits.

Opuntia fruits are fleshy and elongated berries, varying in shape, size and color (orange, yellow, red, purple, green, white) and have a consistent number of hard seeds (Piga, 2004). This fruit was ignored by the scientific community until the beginning of the 1980s when several lab reports and studies demonstrated their biological activity. Recently, investigations on the chemical components and the nutritional value of *Opuntia spp*. have attracted attention in the food, nutritional and pharmacological field (Feugang, et al., 2006, Hernández-Pérez, et al., 2005, Piga, 2004, Saenz, 2000, Stintzing, et al., 2001). Nowadays it is known that *Opuntia spp.* fruits are sources of several bioactive and nutritionally valuable compounds (Cayupán, et al., 2011, Utkarsha, et al., 2010) and their concentrations are dependent on the climate, cultivation site and respective fruit variety (Feugang, et al., 2006). *Opuntia* fruits are rich in polyphenolic compounds, namely flavonoids such as quercetin, kaempferol, isorhamnetin (Tesoriere (a), et al., 2005) and betalains, (Stintzing, et al., 2001). Betalains are nitrogen-containing vacuolar pigments and can be yellow-orange (betaxanthins) or red-violet

(betacyanins). Recently, betalains have received much more interest due to their possible use as natural food colorants in industry (Azeredo, 2009) . *Opuntia* fruits also contain vitamins E, K_1 and C (Ramadan and Morsel, 2003, Stintzing, et al., 2001), amino acids, especially proline and taurine (Stintzing, et al., 2001, Tesoriere, et al., 2005), minerals, namely calcium, potassium and magnesium (Gurrieri, et al., 2000, Lee, et al., 2005, Piga, 2004, Stintzing, et al., 2001) and sugars, such as glucose and fructose, (Galati, et al., 2003, Piga, 2004).

As numerous fruits and vegetables, *Opuntia* fruits have been reported to be beneficial to health. Recent studies reported that *Opuntia* fruits may prevent oxidative stress by acting as scavengers of free radicals (Butera, et al., 2002, Chavez-Santoscoy, et al., 2009, Galati, et al., 2003, Kuti, 2004, Tesoriere, et al., 2004). In particular, betalains have shown to be more potent antioxidants than ascorbic acid (Stintzing, et al., 2005). The anticancer effect of *Opuntia* fruits extracts has also been reported *in vitro* using ovarian, cervical and bladder cancer cells and in vivo using a nude mice ovarian cancer model (Zou, et al., 2005). The antiproliferative effect of *Opuntia* was also reported in human cancer cells of gliobastomas (Hahm, et al., 2010) and leukemia (Sreekanth, et al., 2007). Additionally, *Opuntia* fruits have shown others beneficial effects such as antiulcerogenic (Galati, et al., 2003), antinflammatory and analgesic (Loro, et al., 1999).

Due to the great number of potentially active nutrients and their multifunctional properties *Opuntia spp.* fruits can be considered as perfect candidates for the production of health-promoting food and food supplements.

1.4 Nutraceuticals and functional foods

In the past few years, there has been an increase demand by consumers for health-promoting food products and two new concepts appear in the market: nutraceuticals and functional food.

In 1989, a new hybrid term between nutrients and pharmaceuticals, "nutraceuticals", has been coined by Stephen DeFelice, MD, founder and chairman of the Foundation for Innovation in Medicine (Brower, 1998). Nutraceuticals can be found in presentations similar to drugs (pills, extracts, tablets, etc) (Espín, et al., 2007) and is defined as diet supplements that deliver a concentrated form of a presumed bioactive agent from a food (Zeisel, 1999).

Functional foods represents a type of food that when consumed regularly exert a specific health-beneficial effect (i.e., a healthier status or a lower risk of disease) beyond their nutritional properties, and this effect must be scientifically proven (Espín, et al., 2007).

Some of the most common bioactive ingredients found in the nutraceutical and functional food market are polyphenols such as anthocyanins, proanthocyanidins, flavonols, stilbenes, hydroxycinnamates, coumarins, ellagic acid and ellagitannins, isoflavones, lignans, etc. (Espín, et al., 2007). Most of these compounds are isolated from natural sources using several extraction techniques.

1.4.1 Extraction methods

In recent years, extraction, isolation and purification of phytochemicals from natural sources, provide business opportunities and offers additional environmental and economic incentives for food industry. In order to obtain such valuable compounds, extraction techniques have been widely investigated. For that, it is of critical importance to select efficient extraction procedure/method and maintain the stability of phenolic compounds. Conventional solvent extractions (CSE) are the most commonly used procedures to prepare extracts from plant materials due to their ease of use, efficiency, and wide applicability (Dai and Mumper, 2010). The yield of chemical extraction depends on several factors such as the type of solvents, extraction time and temperature, sample/solvent ratio as well as on the chemical composition and physical characteristics of the samples (Dai and Mumper, 2010, Xu and Chang, 2007). Organic solvents, such as methanol, ethanol, acetone, ethyl acetate and their combinations with water have been widely used for the extraction of phenolics from plant materials (Xu and Chang, 2007). However, due to the low selectivity and extraction yields (unwanted substances such as sugar, organic acids and/or fats may be also recovered) of CSE additional steps may be required to successful isolate the bioactive ingredients (Dai and Mumper, 2010). Additionally, CSE employed large amounts of toxic solvents (Dai and Mumper, 2010) and longer extraction times Therefore nmerous others extraction methods have been proposed such as: adsorption technology (AD) (Soto, et al., 2011) microwave, ultrasound-assisted extractions, and techniques based on use of compressed fluids such as: supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) (Dai and Mumper, 2010).

The adsorption technology (AD) is attractive for its relative simplicity of design, operation and scale up, high capacity and favorable rate, insensitivity to toxic substances, ease of regeneration and low cost. Additionally, it avoids using toxic solvents and minimizes degradation (Soto, et al., 2011). Adsorption is a process of accumulation of molecules from a bulk solution onto the external and internal surfaces of the adsorbent. This process involves various chemical interactions such as hydrophobic, electrostatic attraction and hydrogen bonding. The sorption capacity of the adsorbents strongly depends on the surface area, contact time, polarity, concentration and the degree of hydrophobicity in the adsorption system (Barkakatia, et al., 2010). There are several types of adsorbents that can be used, including activated carbons, minerals and resins (Soto, et al., 2011). In particular, the use of resins (synthetic polymeric adsorbents with hydrophilic or hydrophobic nature) has many advantages, such as: chemically inert, durable and stable, high adsorption capacity, efficiency, selectivity and ease of regeneration, with relatively low cost and limited toxicity.

Other technique with growing interest for extraction of phytochemicals is pressurized liquid extraction (PLE) (or accelerated solvent extraction (ASE) or pressurized fluid extraction (PFE)) , and it partly derives from supercritical fluid extraction (Camel, 2001) . In this technology carbon dioxide can be used in combination with water and/or an alcohol (forming a gas-expanded liquid) to extract pytochemicals (Seabra, et al., 2010). The combined use of high pressures (3.3-20.3 MPa) and

temperatures (40-200°C) provides faster and efficient extractions that require small amounts of classic solvents (Dai and Mumper, 2010). Moreover high pressure extraction has other advantages such as the fact that native enzymes, which degrade phenolic compounds, are inhibited by extraction pressure increasing and CO_2 addition. Besides that supercritical fluid processed materials do not require additional sterilization steps (Seabra, et al., 2010). In recent years, PLE has been successfully applied to the extraction of phenolic compounds from different plant materials such as grape seeds and skin (Piñeiro, et al., 2006), apples (Alonso-Salces, et al., 2001) spinach (Howard and Pandjaitan, 2008), and eggplants (Luthria and Mukhopadhyay, 2006).

1.5 Aim and rational of the thesis

This thesis focus on the evaluation of the bioactive effect of several Portuguese *Opuntia* fruits derived from two different species (*O. ficus-indica* and *O. robusta*) with potential application in colon cancer therapy. To achieve this, an integrated approach was developed by: i) investigating functional properties of *Opuntia spp.* fruit juices, and ii) developing and/or evaluating the anticancer activity of *Opuntia spp*. natural extracts.

Within this context, the work was divided into three parts, as esquematly presented in figure 1.6.

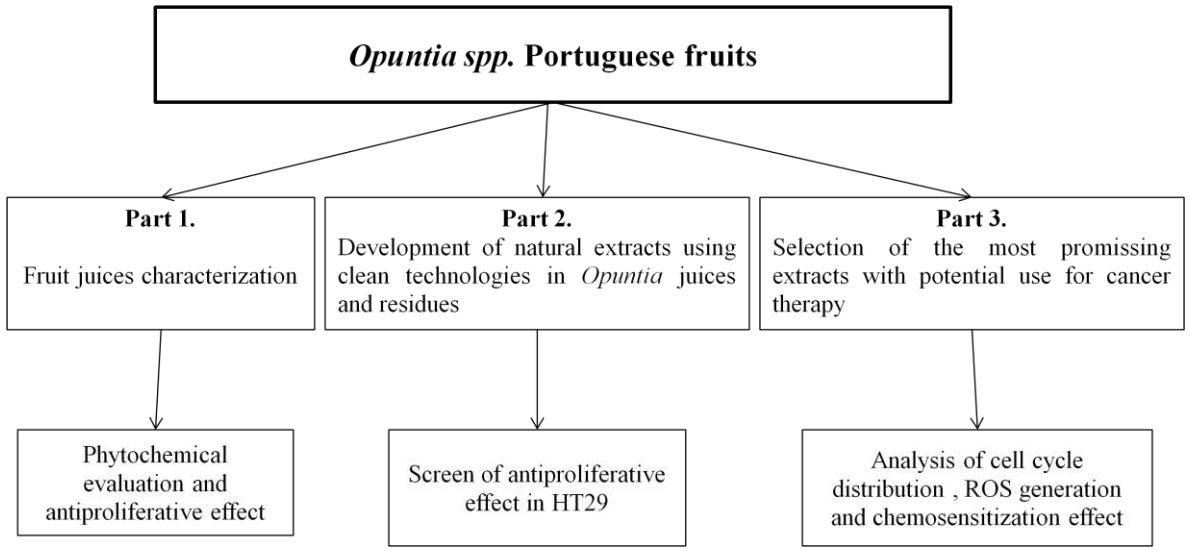

Figure 1.6. Structure of the thesis.

In part 1, five *Opuntia* fruit juices were evaluated in terms of phenolic compounds and bioactivity in an effort to distinguish promising functional beverages. In part 2, the development of *Opuntia* natural extracts were performed by processing the most effective *Opuntia* juices and the most interesting fruit juice residues with clean technologies. All extracts were screened for their antiproliferative effect in HT29 cells and the most promising ones were further selected to be fully characterized in Part 3. In this part, the analysis of cell cycle distribution and ROS generation were

performed, in order to better understand the mechanisms underlying HT29 cell death. Additionally, a drug-resistant cell line (HT29 dx) was created to evaluate the chemosensitization effect of natural extracts.

2. Experimental procedure

2.1 Chemicals

EtOH 96% (AGA, Lisbon, Portugal), distillated water (ITQB, Lisbon), food grade macroporous resin Amberlite XAD-16 (Sigma-Aldrich, St Louis, USA) and were used for extraction experiments.

For phytochemical characterization: sodium carbonate (Na_2CO_3) were purchased from Sigma-Aldrich (St Quentin Fallavier, France), Folin Ciocalteau reagent was acquired from Panreac (Barcelona, Spain) and gallic acid was purchased from Fluka (Germany). Sodium nitrite ($NaNO_2$) were from Riedel-de-Haën (Seelze, Germany), aluminium chloride (*A*lCl_3) and sodium hydroxide (NaOH) was obtained from Sigma-Aldrich, in Germany. (+)-Catechin hydrate was from Sigma (Japan).

Chemicals used for antioxidant activity assays were: 2‟,2‟-Azobis (2- amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox), caffeic acid ($C_9H_8O_4$), cobalt floride tetrahydrate (CoF2), hydrogen peroxide (H_2O_2) and picolinic acid ($C_6H_5NO_2$) were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Disodium fluorescein (FL) was from TCI Europe (Antwerp, Belgium). Sodium chloride (NaCl), potassium chloride (KCl) and monopotassium phosphate (KH_2PO_4) were from Sigma-Aldrich (St Quentin Fallavier, France) and sodium phosphate dibasic dehydrate ($Na_2HPO_4{\cdot}2H_2O$) from Riedel-de-Haën (Seelze, Germany) were used for phosphate buffer solution preparation (PBS).

All cell culture media and supplements, namely fetal bovine serum (FBS), glutamine, RPMI 1640 and trypsin/EDTA were obtained from Invitrogen (Gibco, Invitrogen Corporation, Paisley, UK). Moreover, chemicals used for cell-based assays were: MTS tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) from Promega Corporation (Madison, USA), dimethyl sulphoxide (DMSO) (99.5%, Panreac, Barcelona, Spain), ribonuclease A (Calbiochem, Darmstadt, Germany), 2‟,7‟- dichlorofluorescin diacetate (DCFH-DA), methylthiazolyldiphenyl - tetrazolium bromide (MTT), Triton X ($C_{14}H_{22}O(C_2H_4O)_n$), propidium iodide ($C_{27}H_{34}I_2N_4$), doxorubicin hydrochloride ($C_{27}H_{29}NO_{11}{\cdot}HCl$), bovine serum albumin (BSA) and Bradford reagent all from Sigma-Aldrich (St Quentin Fallavier, France). PBS for cells was purchased from Sigma-Aldrich (St. Louis, USA) and CyQUANT Cell Proliferation Assay Kit was from Invitrogen (Carlsbad, California, USA).

2.2 *Opuntia spp.* samples and fruit juices preparation

Opuntia spp. fruits were collected in five different regions of Portugal, named Tramagal, Beja, Marvão, Sines and Quarteira. The plants are grown without any agronomical inputs and fruits were harvested by hand during October 2010.

Firstly, for sample preparation, spikes were removed with a brush and prickly pears were processed in a food processor (UFESA, LC5005, China) to collect the fruit juice and the

corresponding residue, which are mainly composed by hard seeds and part of the peel. Fruits juices were centrifuged (Avanti J26 XPI, Beckman Coulter®, California, USA) at 9000 rpm for 10 minutes and the supernatant was collected and filtrated in a 0.22 µm filter (Sarstedt, Nümbrecht, Germany). The resulting juices and residues were separated and preserved under frozen storage (-20ºC) until analyses.

2.3 *Opuntia spp.* extracts

2.3.1 *Opuntia spp.* extracts from fruit juices: adsorption technology (AD)

Opuntia juices were used for production of polyphenols-rich concentrates (PRCs) through adsorption technology. For that a food grade macroporous resin Amberlite XAD-16 was used. This resin is allowed to food applications by the U.S. Food and Drug Administration Code of Federal Regulation Title 21 (Scordino, et al., 2003). Before the experimental procedure it was necessary the resin preconditionation. This was realized by an extensive wash of the resin with distilled water to remove salts and impurities as described by Serra (2010). Then the resin was dried for 24 h at 70ºC and cooled in desiccators and further immersed in ethanol (96%). After 12 h later ethanol was replaced by distilled water through washing. Once prepared to use, *Opuntia spp.* juices were put in contact with resin XAD-16 inside flaks (equivalent to 30 mg of polyphenols/g of resin). These flaks were protected from light and submitted to an agitation at 200 rpm for 4 h. Afterwards the supernatant were removed and resins were washed three times with distilled water in order to remove water soluble constituents (i.e. sugars, organic acids and minerals). Polyphenols were recovered from resins in the elution step with ethanol (96%). The ethanolic fractions were vacuum filtered through one layer of filter paper (Filter-lab, Barcelona, Spain) and concentrated in a rotary evaporator (BUCHI Rotavapor R-210, Flawil, Switzerland) under reduced pressure at 40ºC (BUCHI Heating Bath B-491, Flawil, Switzerland). Finally, extracts were transferred into a water phase and freeze dried (Modulyo freeze dried, Eduards, Sussex, UK) at -20ºC, in the absence of light, during 48h. The resulting PRCs were kept in a cool, dry and dark environment until analyses. The final extracts were designated as PRCs according with sample origin (Quarteira, Sines and Tramageal).

2.3.2 *Opuntia spp.* extracts from fruit juices residues: Conventional solvent extraction (CSE)

Opuntia fruits residues from Sines, Quarteira and Beja were extracted using CSE under the conditions described below.

Opuntia fruits residues from Sines and Quarteira were extracted in the dark with EtOH:H_2O (50:50 v/v) solution (1:20, w/v), for 2 h at room temperature in constant agitation (IKA® dual-speed mixer RW 20.n, Aldrich, St. Luis, USA). The extracts were then centrifuged at 9000 rpm for 10 minutes and the supernatants were concentrated in a rotary evaporator (BUCHI Rotavapor R-210, Flawil, Switzerland) in a water bath at 40ºC and under reduced pressure, in order to remove ethanolic

fraction. The final extracts were designated as Q1 (from Quarteira residue) and S1 (from Sines residue).

Opuntia residue from Beja was extracted in a 1:20 (w/v), for 2 hours, in the dark, at room temperature in constant agitation (IKA® dual-speed mixer RW 20.n, Aldrich, St. Luis, USA) using different solutions: i) 100% EtOH; ii) 100% H_2O; iii) 60%EtOH:40%H_2O and iv) 70%EtOH:30%H_2O. The extracts were then filtered, centrifuged at 9000 rpm for 10 minutes and the supernatants were concentrated in a rotary evaporator in a water bath at 40ºC under reduced pressure. Finally, extracts were freeze dried (Modulyo freeze dried, Eduards, Sussex, UK) at -20ºC, in the absence of light, during 48h. The final extracts were designated as A (100% EtOH), B (100% H_2O) C (60%EtOH:40%H_2O) and D (70%EtOH:30%H_2O).

2.3.3 *Opuntia spp.* extracts from fruit juices residues: adsorption technology (AD)

Conventional extracts obtained from Quarteira and Sines fruit juice residues (Q1 and S1) were submitted to adsorption technology using the adsorbent Amberlite XAD-16 resin. The experimental procedure was the same as described for fruit juices (section 2.3.1). The final extracts were designated as Q2 and S2.

2.3.4 *Opuntia spp.* extracts from fruit juices residues: Pressurized liquid extraction (PLE)

Pressurized solvent extracts from *Opuntia ficus-indica* fruit residue from Beja were kindly provided by Dr. Hermínio de Sousa and his laboratory team group (Laboratory of Polymer Processing and Supercritical Tecnology, Universidade de Coimbra, Portugal). Ten extracts obtained at 313K and 20MPa and using diverse CO_2/EtOH/H_2O mixtures were provided (table 2.1).

Table 2.1. Extracts obtained from Beja residue using PLE technology

Sample ID	Molar fracctions		
	EtOH	CO_2	H_2O
1	1	0	0
2	0.4	0.6	0
3	0.4	0	0.6
4	0.7	0.3	0
5	0.4	0.3	0.3
6	0.7	0	0.3
7	0.8	0.1	0.1
8	0.5	0.4	0.1
9	0.5	0.1	0.4
10	0.6	0.2	0.2

2.4 Phytochemical characterization

2.4.1. Polyphenols

Total phenolic content by Folin Ciocalteau method

Total concentration of phenolic compounds present in *Opuntia spp.* extracts were determined according to the Folin Ciocalteau colorimetric method (Singleton and Rossi, 1965) as described by Serra (2010). Briefly, 20µL of the appropriate dilutions of *Opuntia* samples were added to 1580µL of distilled water and oxidized with 100 µL of Folin Ciocalteau reagent. The reaction was neutralized with 300 µL of sodium carbonate solution (Na_2CO_3) and incubated at 40ºC for 30 minutes. The absorbance of samples were measured at 765 nm in a spectrometer (Genesys10uv, Thermo Spectronic, New York, USA) and gallic acid was used as standard (0-800 mg/L) for the calibration curve. The results were expressed as means of triplicates (mg of gallic acid equivalents per gram of extract - mgGAE/g).

HPLC analysis

HPLC analysis of *Opuntia spp.* juices were performed by Analytical Group of IBET (Oeiras, Portugal) coordinated by Dr. Rosário Bronze. Briefly the analysis were performed on a Waters® Alliance 2695 (Waters) 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 35ºC 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. Total polyphenol content of fruit juices were assayed using acid gallic as standard. Solutions of 5, 20, 40, 60, 80 and 100 ppm of gallic acid were used for calibration curve. Results were expressed as mg GAE/ L of juice fruit.

Identification of polyphenol compounds was done by HPLC/MS. A triple quadrupole mass spectrometer MicroMass® Quattro micro (Micromass, Waters) outfitted with electrospray ionization source (ESI) was used in tandem at temperature of 120ºC and capillary voltage of 3.0 kV. The compounds were ionized in both negative and positive ion mode and spectra of the column elute were recorded in the range *m/z* 100-1000. High purity nitrogen (N_2) was used both as drying gas and as a nebulising gas. Ultrahigh-purity Argon (Ar) was used as collision gas at a pressure of 1×10^{-4} mBar. MassLynx software was used for data acquisition and processing.

2.4.2 Flavonoid content

The measurement of flavonoid content was performed by the $AlCl_3$ complexation method described by (Zhishen, et al., 1999) modified for the reader microplate (Powerwave XS Microplate Spectrophotometer, Biotek Instruments, Winooski, USA) according to Tavares, et al., 2010 . To each well of a 96-well microplate was added 125 µL of distilled water, 25 µL of *Opuntia spp.* fruit juice and

7.5 µL of $NaNO_2$ 5% (w/v). The mixture was allowed to stay at room temperature and after 6 minutes were added 15 µL of $AlCl_3$ 10% (w/v). After 5 min of incubation, 100µL of NaOH (1M) was added and the solution of each well was mixed by pippetting up and down. The absorbance was measured at 510 nm. (+)-Catechin hydrate, minimum 98% (w/w) was used as standard, and the results are expressed as mg catechin equivalents per L of juice (mg CE/L).

2.5. Antioxidant activity

2.5.1 Oxygen radical absorbance capacity (ORAC)

ORAC assay was carried out by following the method previously described (Huang, et al., 2002) modified for the FL800 microplate fluorescent reader as described by Serra et al., 2010. This assay measures the ability of the antioxidant species present in the sample to inhibit the oxidation of disodium fluorescein (FL) catalyzed by peroxyl radicals generated from AAPH.

Briefly, in a 96-well microplate were added 25 µL of the appropriate sample dilution and 150 µL of disodium fluorescein (2×10^{-7} mM). The microplate was put in a fluorescent reader and allowed to incubat at 37ºC, for 10 minutes. The reaction was started with 25 µL of AAPH (153 mM) added through the injector. Fluorescence emitted by the reduced form of FL was measured in an FL800 microplate fluorescent reader (Bio-Tek Instruments, Winooski, VT, USA) and recorded every 1 min at the emission wavelength of 530±25 nm and excitation wavelength of 485±20 nm for a period of 30 min. Phosphate buffer (75 mM, pH=7.4) was used to prepare AAPH and FL solutions and was used as blank. Solutions of 5, 10, 20, 40, and 50 µmol/L of Trolox were used as control standards. All samples, including the blank and the controls, were analyzed as triplicates. Final ORAC values were calculated by a regression equation between the Trolox concentration and the net area under the FL decay curve and were expressed as Trolox Equivalents per litre of *Opuntia* juice or gram of extract (µmol TE/L of juice or µmol TE/g of extract).

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 (Bio-Tek Instruments, Winooski, VT, USA) as described by Serra et al., 2010. 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 180 µL of FL ($4x10^{-3}$ µM) in a 96 well microplate. After 10 minutes of incubation at 37ºC were added 10µL of H_2O_2 (0.55 M). The reaction was started with 10 µL of CoF_2 added through the injector. 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. Caffeic acid was used as a standard and 100, 200, 300, 400, 600 μM solutions in PBS were used to perform the calibration curve. Data was expressed as micromoles of caffeic acid equivalents (CAE) per litre of juice or g of extract and all samples were analyzed as triplicates.

2.6. Cell-Based Assays

2.6.1 Cell culture

Human colon cancer cell lines, HT29 and Caco2, were obtained from American Type Culture Collection (ATCC, USA) and Deutsche Sammlung von Microorganismen und Zellkulturen (Barunshweig,Germany), respectively. Both cell lines were grown in RPMI 1640 medium supplemented with 10% of fetal bovine serum (FBS) and 2 mM of glutamine. Stock cells were maintained as monolayers in 175 cm^2 culture flasks and incubated at 37ºC with 5% CO_2 in a humidified atmosphere. All cell culture media and supplements were obtained from Invitrogen (Gibco, Invitrogen Corporation, Paisley, UK).

2.6.2 Citotoxicity assay

Citotoxicity assays of *Opuntia spp.* samples were performed using completely differentiated Caco2 cells which were obtained from a human colon adenocarcinoma and are a good model of the intestinal barrier. This cell line undergoes in culture a process of spontaneous differentiation that leads to the formation of a monolayer of cells, expressing several morphological and functional characteristics of the mature enterocyte (Sambuy, et al., 2005).

The assay was performed as described by Serra 2010. Briefly, cells were seeded at a density of $2x10^4$ cells/well in 96 well microplate and the medium was changed every 48 h. After confluence (about 72 h after seeding), Caco2 cells were incubated with different concentrations of *Opuntia spp.* samples diluted in culture medium (RPMI medium with 0.5% FBS and 2mM glutamine). Control cells were performed with 100% of RPMI 1640 medium or a mixture of sample solvent and medium. After 24, 48, 72 or 96 hours of incubation, the citotoxicity was analyzed using cell viability assays such as Cell Titter® AQueous One Solution Cell Proliferation Assay and CyQUANT® Cell Proliferation Assay Kit (see appendix A). Results were calculated in terms of percentage of cellular viability relative to control (%). Experiments were performed in triplicate and results were expressed in terms of % of cellular viability relative to control.

2.6.3 Antiproliferative assay

The antiproliferative activity of *Opuntia spp.* samples was assayed on human colon carcinoma cells, HT29, which are a widely used model for in vitro colorectal cancer studies.

The assay was performed as described previously (Serra, 2010). Briefly, cells were cultured in 96-well microplates at a density of $1x10^4$ cells/well. After 24 hours incubation at 37ºC in 5% CO_2 atmosphere, the medium of each well was removed and cells were incubated with *Opuntia* samples diluted in RPMI medium with 0.5% FBS. After 24, 48, 72 or 96 hours the medium was remove and the cell viability was determined using commercially available assay kits, such as colorimetric Cell Titter® AQueous One Solution Cell Proliferation Assay or CyQUANT® Cell Proliferation Assay Kit (see appendix A). Results were expressed in terms of % of cellular viability relative to control (cells without *Opuntia* samples). Additionally the amount of sample necessary to decrease 50% of the cellular viability, ED50 (Effective Dose), was calculated through concentration logarithm. The experiment was performed in triplicates.

2.6.4 Cell Cycle analysis

Cell cycle analysis was performed as described by Serra (2010) with some modifications. Briefly, HT29 cells were seeded in 25 cm^2 culture flasks at a density of $1,96x10^5$ cell/flask. After 24 hours, cells were treated with *Opuntia* extracts diluted in culture RPMI medium (0.5% FBS and 2mM glutamine) for 24, 48 or 72 hours. Control cells were performed using culture medium alone or with the sample solvent. After incubation time, cells were detached with 1 mL of trypsin/EDTA and resuspended in 1 mL of RPMI medium (0.5% FBS) and centrifuged at 200 g (Mikro 220R, Hettich, Tuttlingen, Germany) for 10 minutes. Then, cells were washed with cold PBS and centrifuged again (200 g) for 10 minutes. Afterwards, supernatant was discarded and 1 mL of a solution containing propidium iodide (50 mg/L), 1.5% Triton – X (1.5%), ribonuclease A (0.700 U/mL) and NaCl (0.01M) was added to $1x10^6$cells. Cell suspensions were incubated for 2 hours at room temperature and then at 4ºC overnight. The samples were sorted by flow cytometer CyFlow Space (Partec, Germany) and cell cycle analysis was done with FlowMax cell cycle platform (Partec, Germany).

2.6.5 Detection of ROS generation

Intracellular ROS level were determined in HT29 cultured cells using the method described by (Wolfe and Liu, 2007) with some modifications. HT29 cells were seeded in a 96-well microplate ($1x10^4$ cells/well) in RPMI medium (10% FBS and 2 mM glutamine) and allowed to incubate at 37ºC in a 5% CO_2 atmosphere. After 24 hours the medium was replaced by 0.5 mg/ml of *Opuntia* extracts and incubated for more 24 hours. Controls were performed with RPMI medium only. After incubation, *Opuntia* extracts were removed and cells were washed with PBS. After that 100 µL of DCFH-DA (25 µM) was added to each well and cells were incubated for 10 minutes. Fluorescence were measured (excitation 492 nm; emission 540 nm) using a FL800 microplate fluorescent reader (Bioteck Instruments, USA). The experiment was performed in triplicates and results were presented in percentage of fluorescence intensity relatively to the control.

2.6.6 Chemosensitization effect

2.6.6.1 Development of a subpopulation drug-resistant (HT29-dx)

A subpopulation of HT29 cells, named HT29 dx, was created according to the method of Riganti et al., 2005. Briefly, HT29 cells were cultured in RPMI medium (10% FBS and 2 mM of glutamine) supplemented with 68 nmol/L of a common chemotherapeutic drug (doxorubicin) during 20 to 25 passages. After that HT29 cells were subsequently cultured in RPMI medium containing 34 nmol/L of doxorubicin as "maintenance dose".

2.6.6.2 Cellular sensitivity to drug

Cellular sensitivity to doxorubicin was evaluated by growth-inhibition assay after 1 hour of drug exposure, according to the method previously described (Ravizza, et al., 2004). Briefly, HT29 and HT29-dx cells were seeded onto 96-well microplates ($1x10^4$ cells/well) and allowed to grow for 24 hours prior to treatment. Then, the medium was removed and several concentrations of doxorubicin were added (0.16-20 µM) in each cell line, for 1 hour. After exposure, doxorubicin was removed; cells were washed with PBS and incubated with RPMI medium (10% FBS and 2 mM glutamine). After 72 hours of incubation, cell growth inhibition was assayed by the MTT assay (Supino, 1998) (see appendix A). Experiments were performed in triplicate and results were expressed in terms of % of cellular viability relative to control.

2.6.6.3 Intracellular doxorubicin accumulation

The intracellular doxorubicin accumulation in HT29 and HT29 dx cells were determined according to the method described previously (De Boo, et al., 2009). Both cell lines were seeded in 25 cm^2 culture flasks at a density of $1.46x10^5$ cell/flask in RPMI medium (10% FBS). After 24 hours the medium was removed and cells were washed twice with PBS and incubated in RPMI medium (without phenol red) containing 5 µmol/L doxorubicin. Controls were performed with RPMI medium, only. After 24 hours, cells were washed twice with PBS and detached with 2 mL of trypsin/EDTA. The cells were centrifuged for 30 seconds at 13000 *g* (Mikro 220R, Hettich, Tuttlingen, Germany) and resuspended in 1 mL of a 1:1 mixture of ethanol/0.3 N HCl. Cells were disrupted with cycles of thawing and heating. The protein content of cell lysates was measured through Bradford assay (Bradford, 1976) and the amount of intracellular doxorubicin was detected using a FL800 microplate fluorescent reader (Bioteck Instruments, USA) at excitation and emission wavelengths of 475 and 553 nm, respectively. A blank was prepared in the absence of cells in each set of experiments and its fluorescence was subtracted from that measured in each sample. Fluorescence was converted in ng doxorubicin/mg cells proteins using a calibration curve previously prepared.

2.6.6.4 Chemosensitization activity

The effect of *Opuntia* extracts in combination with doxorubicin on HT29 and HT29 dx cells growth was assessed by the MTT assay (see appendix A). Briefly, cells were seeded in 96-well microplate ($1x10^4$ cells/well) and allowed to grow for 24 hours. Three different treatment schedules were used a) 24h RPMI medium, 1h doxorubicin (5-20 µM) followed by 72 h incubation in drug-free medium; b) 24 h of *Opuntia* extract (9 and 6 mg/ml for Q1 extract and 12 and 9 mg/ml for extract 2 of PLE) followed by 72 h incubation in drug-free medium; c) 24 h of *Opuntia* extract (9 and 6 mg/ml for Q1 extract and 12 and 9 mg/ml for extract 2 of PLE) followed by 1 h doxorubicin (5-20 µM) and 72 h incubation in drug-free medium. Results were expressed in terms of % of cellular viability relative to control.

3. Results and discussion

Opuntia spp. fruits were harvested in Portugal from five different regions, namely Tramagal, Beja, Marvão, Sines and Quarteira and were processed and separated into juice and residue.

Firstly, *Opuntia spp*. juices were characterized in terms of phytochemical composition, antioxidant activity and antiproliferative effect on human colon cancer cell line (section 3.1- Part 1). Then, the most effective juices and the most interesting residues were processed by clean technologies for the developed functional ingredients (section 3.2. - Part 2). All extracts were screened for their antiproliferative effect and the most promising ones were further selected to be fully characterized (section 3.3 – Part 3).

3.1 PART 1 - Looking for anticancer activity in *Opuntia spp.* juices

3.1.1 Phytochemical characterization

In this work two different species of *Opuntia spp*., namely *O. ficus-indica* and *O. robusta* were analyzed. The main morphological difference between the fruits of these two species is related with the spikes: *O. ficus-indica* have smaller spikes than *O. robusta* (figure 3.1). Concerning the juices, the color of samples varied according with fruit origin (table 3.1 and figure 3.2). Additionally, it was noted that *O. robusta* pulp juice fruit is more viscous than *O. ficus-indica*.

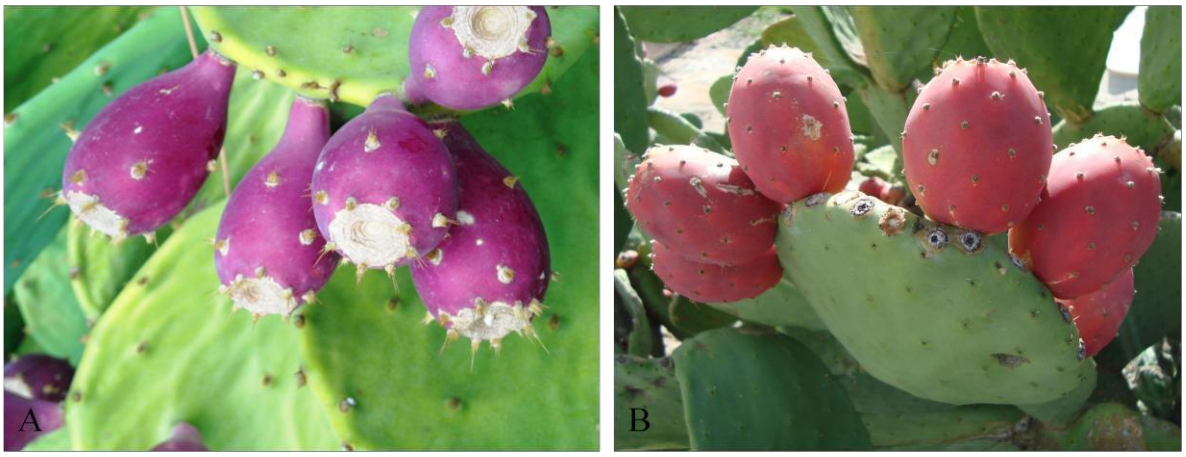

Figure 3.1. *Opuntia spp.* fruits from: A) *O. robusta* (Quarteira) and B) *O. ficus-indica* (Marvão).

Table 3.1. *Opuntia spp.* fruits varieties, color juice and phytochemical characterization.

Sample ID	***Opuntia* specie**	**Fruit juice color**	**TPC**[1] (mg GAE/L)	**TFC**[2] (mg CE/L)
Tramagal	*Opuntia ficus-indica*	Dark yellow	409.4	86.2
Beja		Yellow	337.1	220.2
Marvão		Orange-red	384.5	158.4
Sines		Dark yellow	494.3	79.6
Quarteira*	*Opuntia robusta*	Purple	45.0	80.0

*All fruits were processed with peel, except *O. robusta*

[1] TPC: Total Polyphenolic Content; [2] TCF: Total Flavonoid Content

Figure 3.2. *Opuntia spp.* fruit juices from Tramagal, Beja, Marvão, Sines and Quarteira.

In a first approach, *Opuntia* juices were analyzed for their content and composition of bioactive compounds. For that HPLC was used to identify the main phenolic compounds as well as to quantify the total content. Additionally, the total flavonoid concentration was determined using a colorimetric assay. As shown in table 3.1, the five *Opuntia* juices present different phytochemical content. The total phenolic content ranged from 45 to 494 mg GAE/L and flavonoid between 80 and 220 mg CE/L, which is in accordance with the literature (Chavez-Santoscoy, et al., 2009, Kuti, 2004, Stintzing, et al., 2005). When compared with other beverages *O. ficus-indica* has a polyphenol total content similar to some types of teas (i.e. cammolmile, lemon thyme and black tea) and orange and apple juice (Neveu, et al., 2010, Seeram, et al., 2008). Among all samples, the fruit juices from Sines and Tramagal presented higher phenolic content whereas the sample from Beja contained the highest flavonoid concentration. Moreover, it is important to note that flavonoid concentrations varied within the juices of the same specie (*Opuntia ficus-indica*) and this is in agreement with previously works (Kuti, 2004). Despite that, there are some reports where flavonoid content is much lower than the values reported in this work for *O. ficus-indica* (Fernández-López, et al., 2010, Kuti, 2004, Tesoriere, et al., 2005). This result could be related with the part of fruit used for making the juice. In this work *O. ficus-indica* samples were processed with the peel whereas the other authors used the fruit pulp only. This justification is in accordance with data reported in literature related with phenolic composition of the peel and pulp of several fruits (Tomás-Barberán, et al., 2001). Phenolics might tend to accumulate in the dermal tissues of plant body due to their potential role in protection against UV radiations, acting as attractants in fruit dispersal, and as defense chemicals against pathogens and predators (Tomás-Barberán, et al., 2001).

Among all fruit juices, the sample from Quarteira (*O.robusta*) showed the lowest concentrations in phenolic compounds which could be related with the fact that this fruit was processed without peel. Moreover, this *Opuntia* specie is already recognized to have lower phenolic content than *Opuntia ficus-indica* (Chavez-Santoscoy et al., 2009).

In order to analyzed and compare the polyphenol composition of samples HPLC was performed at different UV absorptions, namely 280nm, 360nm, 480nm and 535nm which are the characteristic

wavelength of phenolic compounds, flavonoids, betaxanthin (yellow-orange pigment) and betacyanin (red-purple pigment), respectively. As show in figure 3.3 *Opuntia* juices presented different phenolic content and composition. Some compounds were identified including: citric acid, gallic acid, isorhamnetins (isorhamnetin-3-O-rhamnose-lyxose-glucose, isorhamnetin-3-O-rutinoside, isorhamnetin-3-O-lyxose-glucose) and some betaxanthin and betacyanin pigments (betalain indicaxantin and leucine-bx). The difference between *O. ficus-indica* and *O. robusta* species is evident in all chromatographic profiles. Moreover, fruit juice from Marvão distinguished from the others *Opuntia ficus-indica* juices for the presence of betaxanthin and betacyanin pigments.

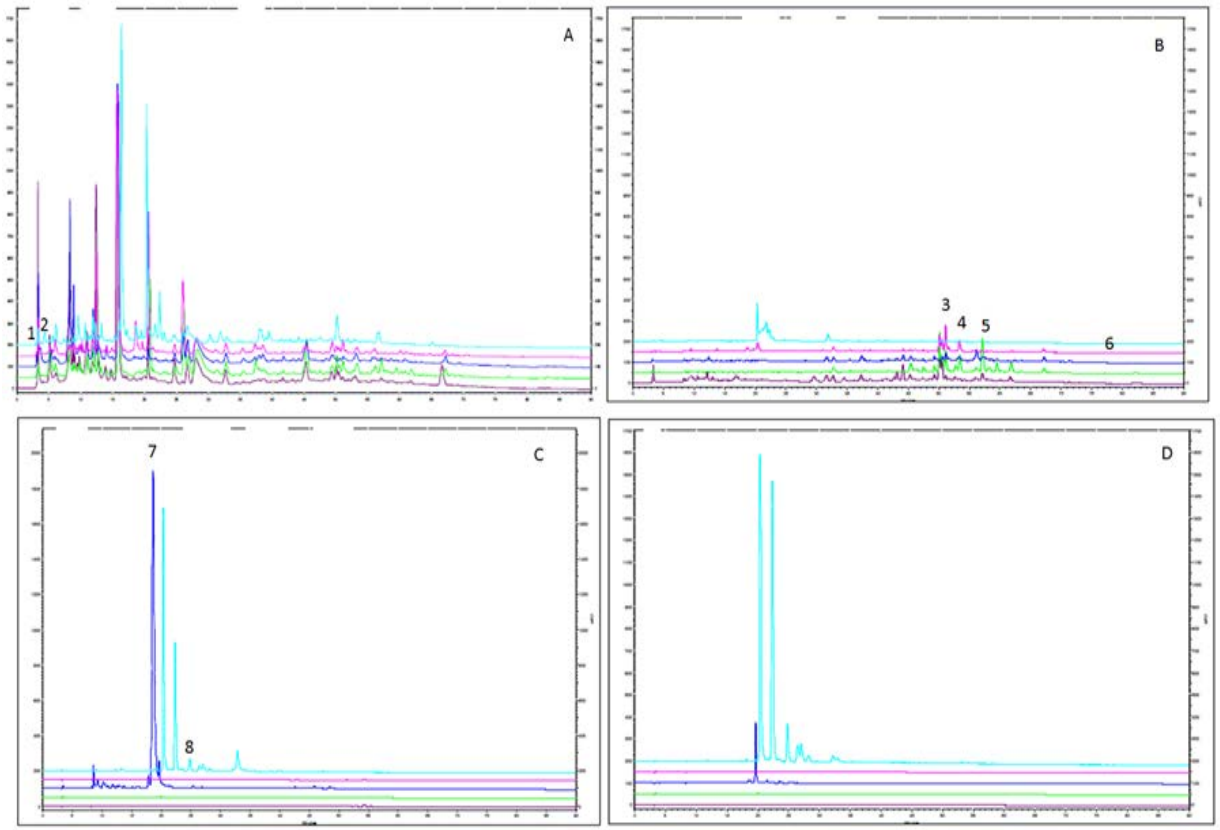

Figure 3.3. Chromatograms profiles of *Opuntia spp*. juices: Tramagal, Beja, Marvão, Sines and Quarteira at A) 280nm, B) 360nm, C) 480nm and D) 535nm. Legend: 1- citric acid, 2 – gallic acid, 3- isorhamnetin-3-O-rhamnose-lyxose-glucose, 4- isorhamnetin-3-O-lyxose-glucose, 5- isorhamnetin-3-O-rutinoside, 6- isorhamnetin, 7- indicaxantin, 8- leucina-bx.

3.1.2 Antioxidant activity

In order to evaluate the antioxidant activities of *Opuntia spp.* juices, two different assays were performed: ORAC and HORAC. The ORAC assay analyzes the potential to scavenge harmful oxygen reactive species, namely peroxyl, while HORAC assay measures the efficacy of the sample to prevent hydroxyl radical formation. ORAC and HORAC values obtained for *Opuntia spp*. juices range from 9949 to 33449 µmol TE/L and 9224 and 18285 µmol CAE/L, respectively (figure 3.4).

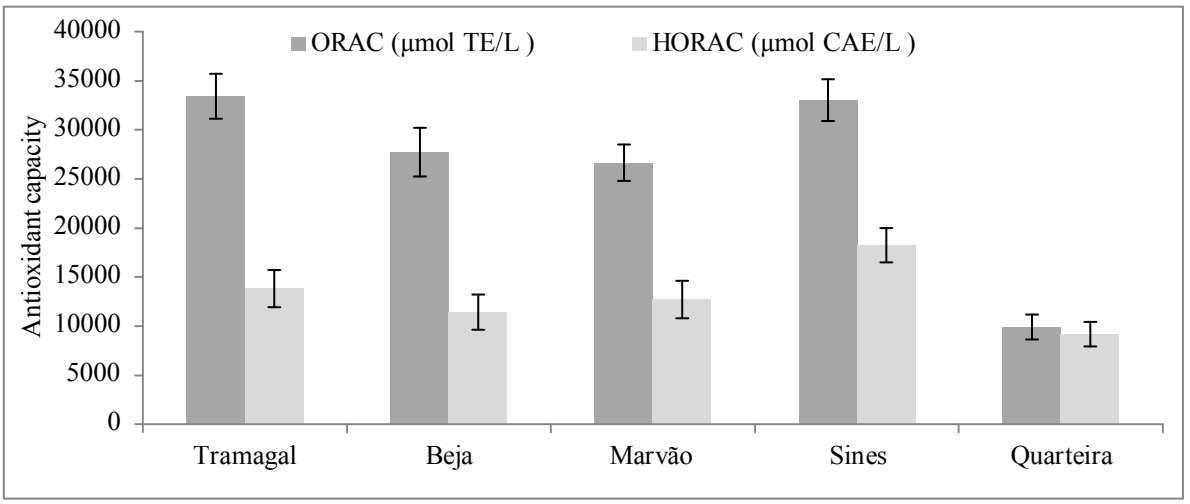

Figure 3.4. Antioxidant capacity (ORAC and HORAC values) of *Opuntia spp* fruit juices varieties.

Tramagal and Sines juices were the varieties that presented the highest ORAC and HORAC values while *O.robusta* juice from Quarteira showed the lowest result. It is important to note that ORAC values of *O.ficus-indica* samples are higher than those previously reported by other authors (Chavez-Santoscoy, et al., 2009, Kuti, 2004), probably due to the inclusion of the peel in the juice. When compared with other products, Portuguese *O. ficus-indica* juices have similar ORAC value as red wine, concord grape, blueberry juice and black cherry and contain at least a twice higher ORAC value that orange, apple, and black, green and white iced tea (Seeram, et al., 2008).

Concerning HORAC results, this is the first report where this values are presented for *Opuntia spp.* It is important to note that the antioxidant activity is mainly related with the phenolics present in the juice. In fact, high correlations were obtained for total phenolic composition and ORAC (R^2=0.944) and HORAC assay (R^2 = 0.742).

3.1.3 Antiproliferative effect

In order to evaluate the anticancer potential of *Opuntia spp.* juices, antiproliferative assay were performed using a human colon cancer cell line (HT29). Cells were exposed to *Opuntia* juices for 24, 48, 72 and 96 h and cell viability was evaluated using MTS assay. Since the inhibition of cancer cell growth was much more effective after 96 hours of exposure, the antiproliferative effect of all *Opuntia* juices were compared at that time. Dose-dependent curves are presented in figure 3.5.

Results showed that juices from Tramagal and Sines had the highest antiproliferative effect on human colon cancer cells. This result could be related with the highest content in polyphenols and antioxidant activity. The amount of sample necessary to decrease 50% of the cellular viability (ED50) was also determined and Tramagal proved to be the most effective in inhibiting cancer cell growth (sample with the lowest ED50 value) followed by Sines juice (table 3.2).

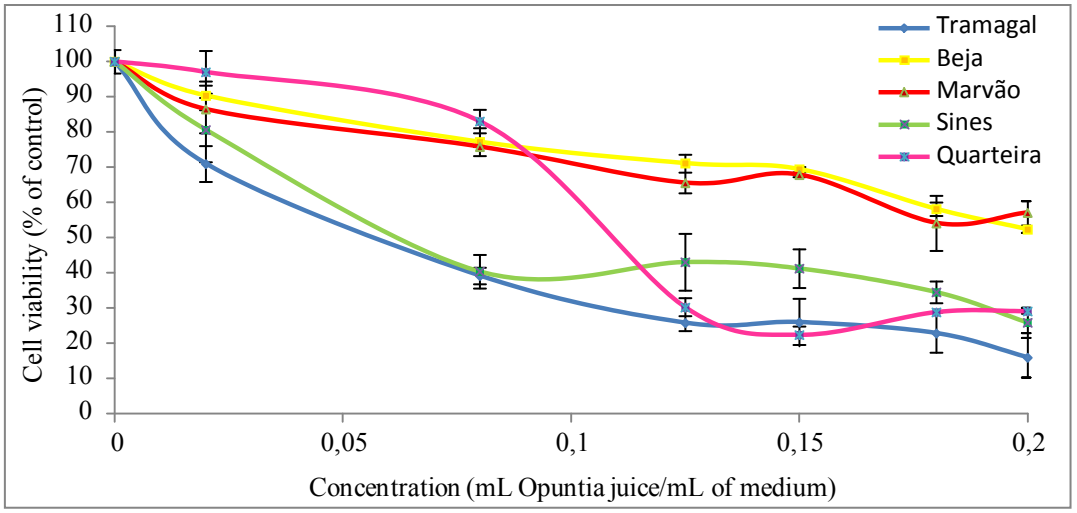

Figure 3.5. Antiproliferative effect of Portuguese *Opuntia spp.* juices on human colon cancer cells (HT29) (incubation time: 96 hours).

Surprisingly, the juice obtained from *O. robusta* fruit (Quarteira), which is the one that showed lowest content in polyphenols and antioxidant effect, was the third most effective in inhibiting HT29 cell growth. This result suggests that there are some compounds in this juice that contribute to this effect. Comparing with Tramagal and Sines juices, chromatograms profiles of Quarteira at 480nm and 535 nm show the presence of several compounds from betalains family. There are reports that suggest the anticancer effect of betalains, namely the betacyanin (red-purple pigment) that showed anticancer effect on Human Chronic Myeloid Leukemia Cell Line-K562 (Sreekanth, et al., 2007).

Table 3.2. The amount of sample necessary to decrease 50% of the cellular viability (ED50) after 96 h of *Opuntia* juice exposure to HT29 cell line.

***Opuntia spp* juice**	**ED 50 (ml/ml)**
Tramagal	0.058 ± 0,01
Beja	> 0.2
Marvão	> 0.2
Sines	0.075 ± 0.02
Quarteira	0.12 ± 0.01

Moreover, it is important to note that no *Opuntia ficus-indica* juices showed cytotoxic on Caco2 cell model. However, the juice from Quarteira showed some cytotoxicity after 96h of treatment. The half maximal inhibitory concentration (IC50) was determined and Quarteira juice is cytotoxic at high concentrations (IC50 = 0.17 ml/ml).

Until now, this is the first report concerning the antiproliferative effect of *Opuntia* juices against HT29 cell line. *Opuntia spp.* fruit extracts and juices are already reported to inhibit the growth of other cancer cell lines derived from human cervical, ovarian, bladder, prostate, hepatic, colon and mammary cancer (Chavez-Santoscoy, et al., 2009, Zou, et al., 2005)

3.2 PART 2: Development of functional ingredients from *Opuntia spp.* and screening of their anticancer effect

In this part of the work, the efforts were directed to develop promising anticancer *Opuntia* extracts that can be used as natural ingredients in chemotheraphy and chemopreventation of colon cancer. The use of different extraction methods such as conventional solvent extraction (CSE), adsorption technology (AD) and pressurrized liquid extraction (PLE) was explored and applied to different raw material: *Opuntia* juice fruits and residues. All extracts were analyzed in terms of phytochemical content, antioxidant activity and antiproliferative effect in order to select the most promising ones.

3.2.1 Extracts from *Opuntia spp.* juices: adsorption technology (AD)

In order to obtain polyphenol-rich concentrates (PRCs) with anticancer properties, the most effective Portuguse *Opuntia spp.* juices, namely Tramagal, Sines and Quarteira, were selected and processed with macroporous adsorption resin Amberlite XAD16, as described in experimental procedure (section 2.3.1). The products obtained were analyzed for their phytochemical composition, antioxidant capacity and antiproliferative effect on HT29 cell line.

3.2.1.1 Phytochemical and antioxidant characterization

Figure 3.6 shows the total phenolic content of PRCs from the three *Opuntia spp*. juices. As it can be see, PRC-Sines presented the highest polyphenolic content. When compared with the juices, all PRCs have a much higher phenolic content (Table 3.3). This improvement was approximately 38, 42 and 318 times for Tramagal, Sines and Quarteira samples.

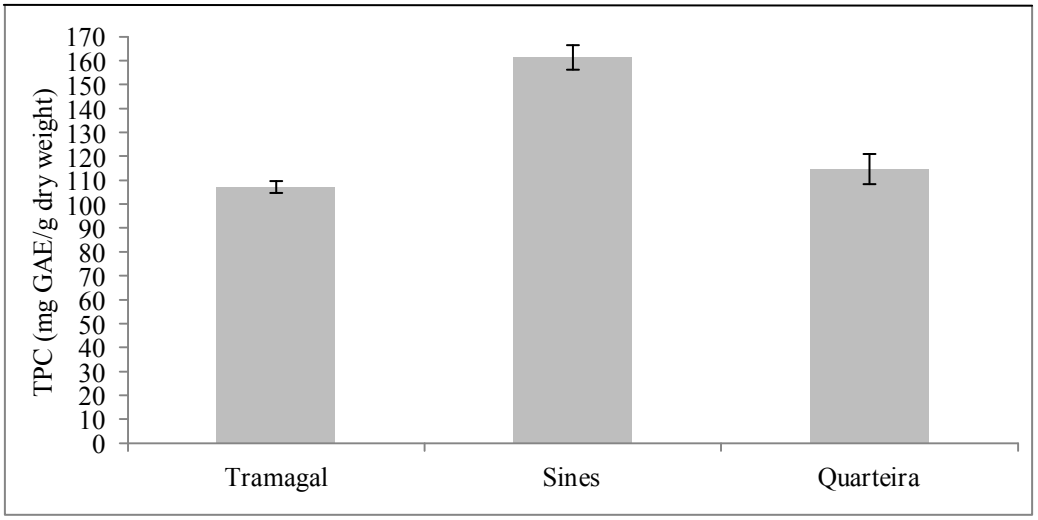

Figure 3.6. Total polyphenol content (mg GAE/g dry weight) of Sines, Quarteira and Tramagal PRCs.

Table 3.3. Total polyphenol content (mg GAE/dry weight) of PRCs and juices.

Sample ID	PRCs	Juices	Concentration factor (PRCs/juices)
	TPC (mg GAE/g dry weight)		
Tramagal	107.1 ± 2.5	2.80	38
Sines	161.3 ± 5.1	3.84	42
Quarteira	114.6 ± 6.3	0.36	318

Chromatograms profiles obtained at 280, 360, 480 and 535nm show different phenolic composition (figure 3.7). Results showed that piscidic acid is the main phenolic acid present in Quarteira and Sines PRC samples (figure 3.7A). At 360 nm (figure 3.7B) it is show the absence of flavonoids in PRC-Quarteira. Furthermore, betaxanthin compounds were identified in both PRC-Sines and PRC-Quarteira samples (figure 3.7C), whereas betacyanin pigments were only present in the last one (Figure 3.7D).

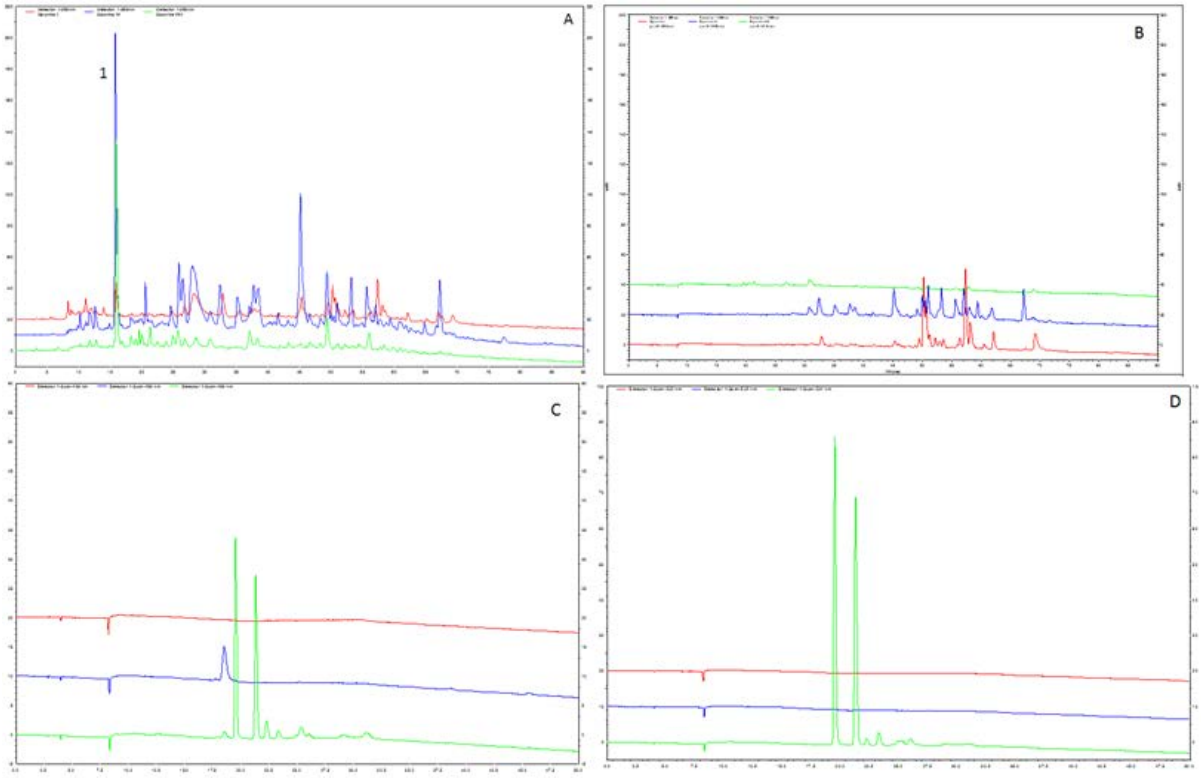

Figure 3.7. Chromatograms of *Opuntia* PRCs from Tramagal, Sines and Quarteira (1mg/mL) at (A) 280nm, (B) 360 nm, (C) 480nm and (D) 535 nm. Legend: 1 – piscidic acid.

The antioxidant capacity of PRCs was evaluated by ORAC and HORAC assays. Table 3.4 shows that PCRs present higher antioxidant activity than *Opuntia* juices, and this difference is more pronounced in the ORAC value. As verified for total polyphenolic content, PRC-Sines had the highest

antioxidant activity (see figure 3.8). This result was expected because phenolic compounds are recognized as potent antioxidants.

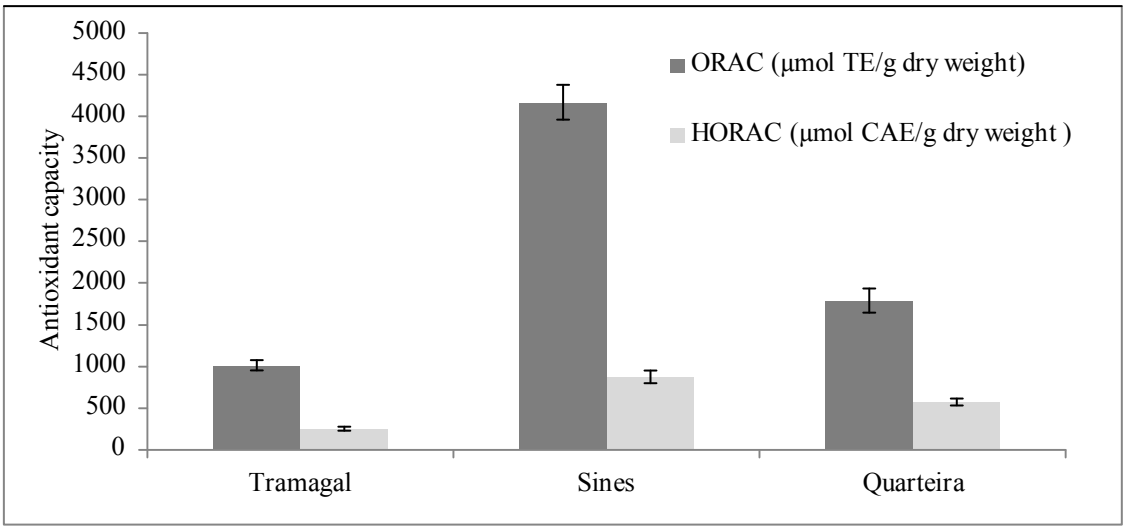

Figure 3.8. Antioxidant capacity (ORAC and HORAC assays) of PRCs (Sines, Tramagal and Quarteira).

Table 3.4. Anitoxidant capacity (ORAC and HORAC) of juices and its PRCs.

	ORAC (µmol TE/g dry weight)			**HORAC (µmol CAE/g dry weight)**		
Sample ID	**Juices**	**PRCs**	**Concentration factor**	**Juices**	**PRCs**	**Concentration factor**
Tramagal	228.9 ± 15.7	1011.1 ± 61.9	4	94.8 ± 12.9	251.5 ± 24.2	3
Sines	257.1 ± 16.5	4164.5 ± 209.3	16	142.2 ± 13.6	872.5 ± 76.1	6
Quarteira	79.7 ± 10.2	1786.3 ± 144.76	22	73.9 ± 10.0	571.3 ± 41.6	8

3.2.1.2 Antiproliferative effect and cell cycle analysis

The next step of this work was to evaluate the antiproliferative effect of PRCs and select the most promising extracts for cell cycle distribution analysis.

The antiproliferative activity was evaluated in HT29 cell line, after 72 hours of *Opuntia* PRCs treatment (1-6 mg PRCs/mL). As shown in figure 3.9, PRC-Sines and PRC-Quarteira presented higher antiproliferative effect on HT29 cells. These results were expected because these two extracts had higher concentration of polyphenols and antioxidant effect, as reported above.

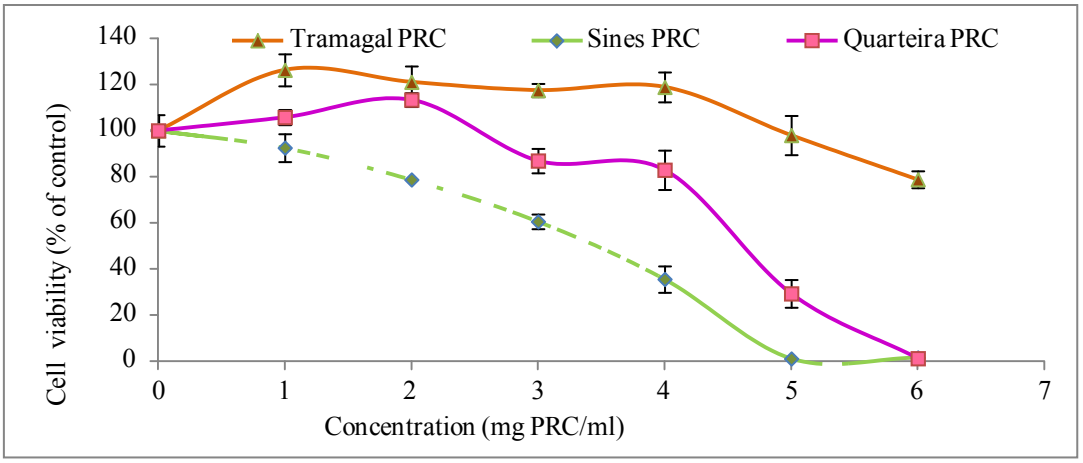

Figure 3.9. Antiproliferative activity of PRCs on HT29 cell line (Incubation time= 72h).

The anticancer effect of PRCs could be related with the presence of some phenolic compounds, namely betalains and piscidic acid. In fact betalains that were only identified in PRC-Sines and PRC-Quarteira (figure 3.7C and D) are already recognized to be powerful antiproliferative compounds (Sreekanth, et al., 2007, Stintzing, et al., 2005, Stintzing, et al., 2001). Piscidic acid, a phenolic acid present in higher amounts in PRC-Sines followed by PRC-Quarteira (figure 3.7A), could also be considered as one of the compounds responsible for the antiproliferative effect. ED50 values were also determined for each PRC samples (table 3.5). PRC-Sines showed the lower ED50 value (3.5 mg/mL) followed by PRC-Quarteira and PRC-Tramagal. For this sample ED50 value was not be determined being superior to the highest concentration tested (6mg/mL). Furthermore is important to note that all PRC samples were not cytotoxic on Caco2 cell model.

Table 3.5. ED50 values of PRCs in HT29 cells (Incubation time= 72h).

PRCs	**ED50 (mg extract/mL)**
Tramagal	> 6
Sines	3,5
Quarteira	4,6

In order to better understand the antiproliferative effect of PRC-Sines and PRC-Quarteira, flow cytometry was performed for cell cycle arrest analyzes. For that, HT29 cells were treated with 5 mg/mL of each PRC during 48 and 72 h (figure 3.10). Control was performed with RPMI medium and water.

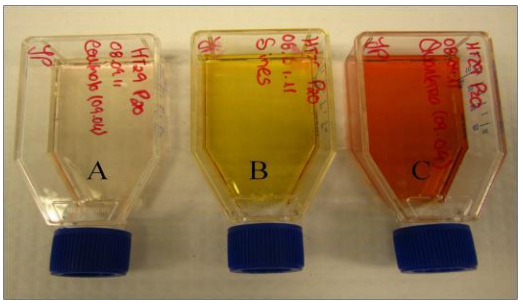

Figure 3.10. HT29 cells, in 25 cm^2 flask, treated with: A) Control; B) PRC-Sines; C) PRC- Quarteira.

Results obtained (figure 3.11) show that, after 48 and 72 hours of incubation, both PRCs increase HT29 population in G1 phase (cell growth phase). At 48h, this increase was more pronounced in PRC-Quarteira, and was accompanied by a decrease in both S (DNA replication) and G2/M (cell division) phases. After 72 hours of treatment, PRC-Sines showed a significant increase in G1 phase, with no cell population in G2/M phase. Phase contrast images of cells after 48h of PRCs exposure confirm that PRC-Sines is more effective in inhibiting cancer cell growth (figure 3.12). PRC-Sines showed to have the highest inhibitory effect on cancer cell growth inhibition.

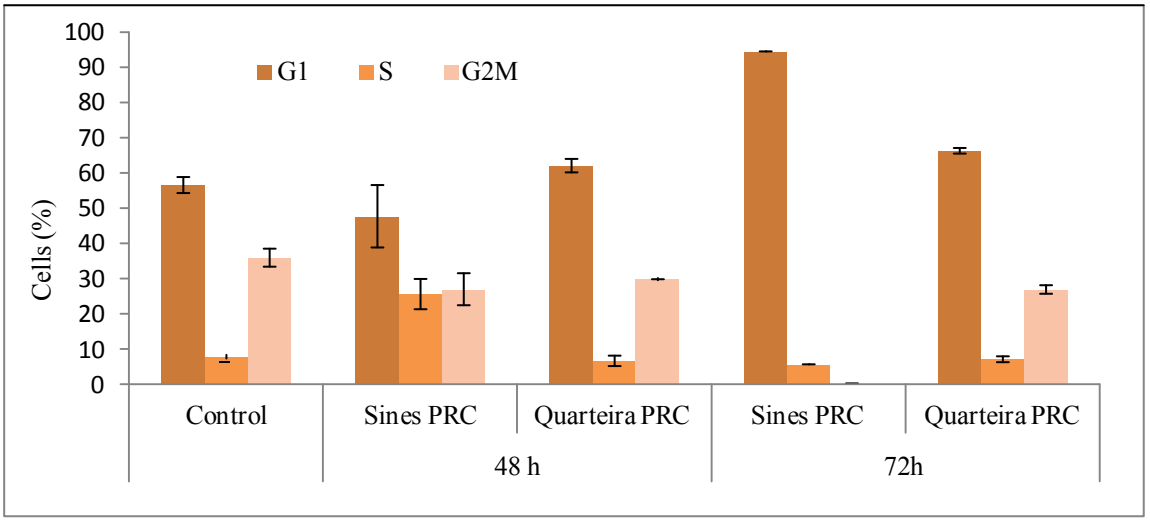

Figure 3.11. Cell cycle distribution of HT29 cells incubated with PRC samples (5mg/mL) during 48 and 72 hours.

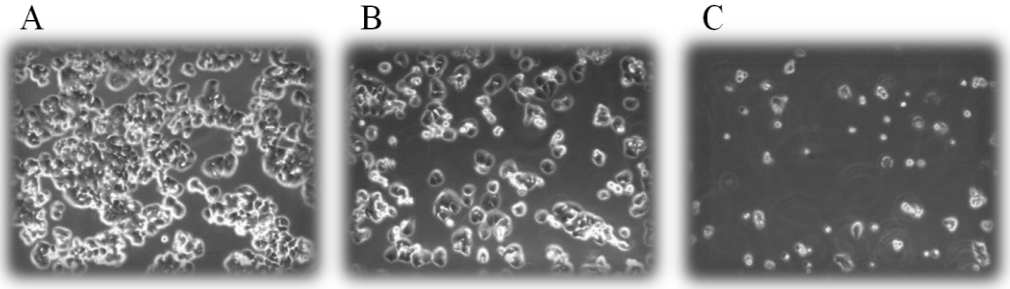

Figure 3.12. Phase contrast images of cells. A) Control (RMPI medium with solvent); B) PRC-Quarteira; C) PRC-Sines. Scale bar: 200µm

The induction of cell cycle arrest into G1 phase by *Opuntia spp.* fruit extracts is already reported by other authors. (Yoon, et al., 2009) showed that *Opuntia* fruit extracts induced U87MG Human Glioblastoma cell cycle arrest in this cell cycle checkpoint after 24h of exposure. Yoon, et al., 2009 published a similar result using water extracts of *O. humifusa* fruits in MCF-7 human breast cancer cells. Additionally, Zou et al., (2005) showed an increased G1 phase population and decreased cells in G2/M and S phases in ovarian and bladder cancer cells incubated with *Opuntia* extracts.

In a previous work, doxorubicin, a common drug used in cancer chemotherapy, showed to induce HT29 cell cycle arrest in G2/M phase (Serra, 2010). Therefore, the results obtained in this work are very promising for the use of these PRCs as chemotherapeutic agents for colon cancer since it has been demonstrated that potent inhibition of tumor survival is achieved when combining drugs with different cell cycle check points (Chang, et al., 2001, Li, et al., 1999).

3.2.2 Extracts from *Opuntia spp.* fruit juice residues: conventional solvent extraction (CSE) and adsorption technology (AD)

The processing of many fruits results in the accumulation of large quantities of residues. Proper utilization of these residues could reduce waste disposal problems and serve as a potential new source of pytochemicals that can be used in nutraceutical and functional food market.

The results obtained in this work (section 3.2.1) showed that Sines and Quarteira juices are the most promising for the development of PRCs with anticancer effect. For this reason, fruit residues of that samples were selected and processed with clean technologies such as conventional solvent extraction (CSE) and adsorption technology (AD) as described in experimental procedure (2.3.2. and 2.3.3 sections). At the end, four different extracts were obtained, namely S1, S2, Q1 and Q2 (figure 3.13 and table 3.6), and all were characterized in terms of phytochemical composition, antioxidant activity and antiproliferative effect.

Table 3.6. Extracts obtained from Sines and Quarteira residues and its PRCs.

Fruit	Extract description	Abbreviation
Sines	CSE – Extract (EtOH:H_20) obtained from Sines residue	S1
	AD – PRC obtained from residue extract	S2
Quarteira	CSE - Extract (EtOH:H_20) obtained from Quarteira residue	Q1
	AD - PRC obtained from residue extract	Q2

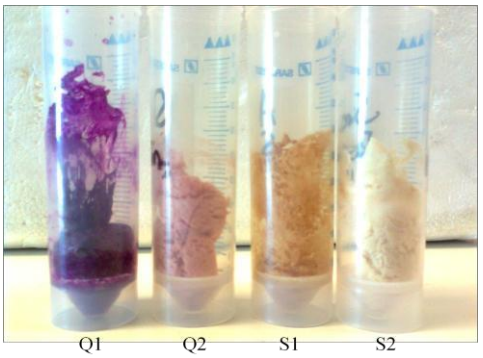

Figure 3.13. Dried extracts obtained from Sines (S1) and Quarteira (Q1) fruit residues and its PRCs (S2 and Q2, respectively).

3.2.2.1 Phytochemical and antioxidant characterization

Figure 3.14 shows the total polyphenol content of all extracts. Results showed that the extracts obtained after AD application (S2 and Q2) present higher phenolic content than the extracts derived from CSE (S1 and Q1) with improvements of 5 and 8 times for Sines and Quarteira samples, respectively. The total phenolic content varied between 17.5mg GAE/g and 142.8 mg GAE/g according with the sample: Q1-17.5 mg GAE/g; S1-26.3 mg GAE/g; Q2-142.8; S2-119.0 mgGAE/g.

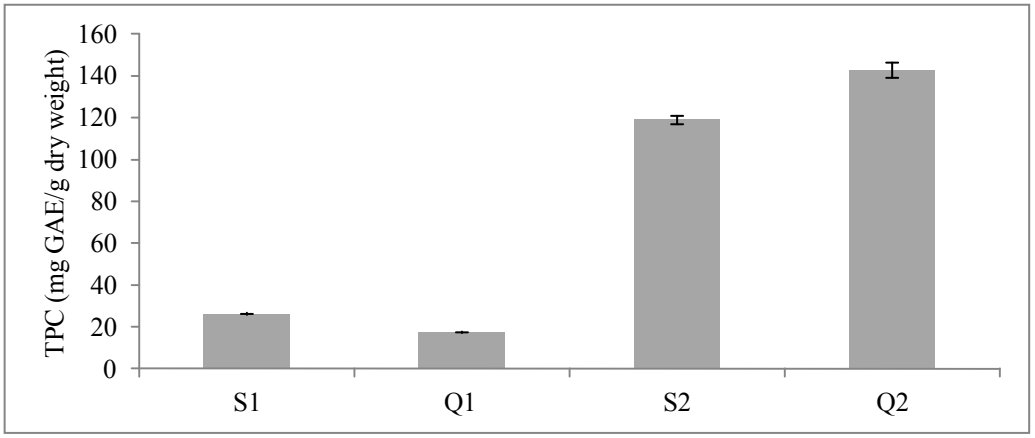

Figure 3.14. Total polyphenol content of extracts derived from Sines (S1 and S2) and Quarteira (Q1 and Q2) residues.

In figure 3.15 are compared the phenolic profiles of all extracts according with type of residue used. In particular, piscidic acid, a phenolic acid that is already identified in PRCs juices, seems to be also present in higher amounts in fruit residues (t_{ret}=16 min). As shown in both chromatograms, the content of piscidic acid is higher in S1, Q1 and S2 extracts than in S2. Regarding S2 and Q2 extracts, it is important to mention that these ingredients present higher concentration of flavonoid compounds (tret=25 to 70 min) than S1 and Q1. This result is related with the application of Amberlite Xad16 resin, which concentrated all this compounds. Moreover, Q1 extract had more betalain compounds than the others, as show in figure 3.15 C and D.

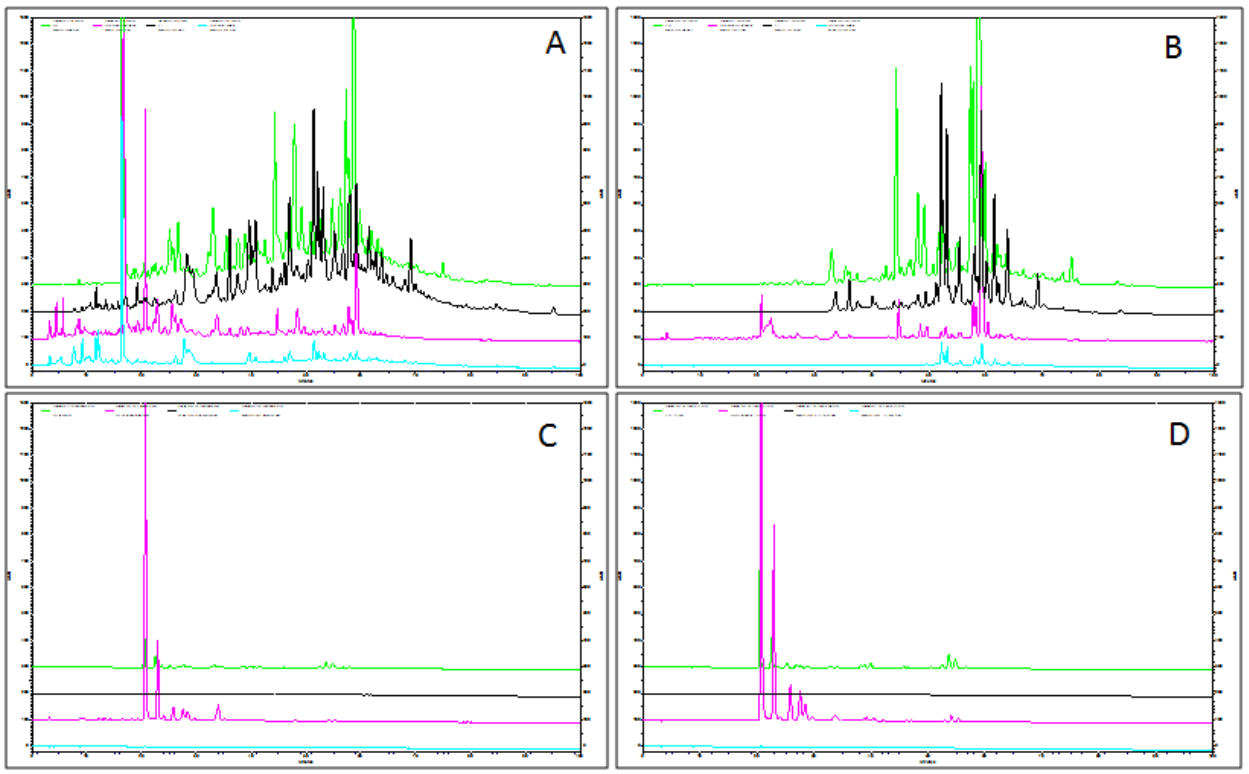

Figure 3.15. Chromatograms profiles of S1 (50 mg/ml), **S2** (10 mg/ml), Q1(50 mg/ml) and Q2 (10 mg/ml) at A) 280 nm; B) 360 nm; C) 480 nm; D) 520 nm. Legend: 1- piscidic acid.

The antioxidant capacity of residues extracts were performed using ORAC and HORAC assays and results are shown in figure 3.16. As expected S2 and Q2 had the highest values of antioxidant activity.

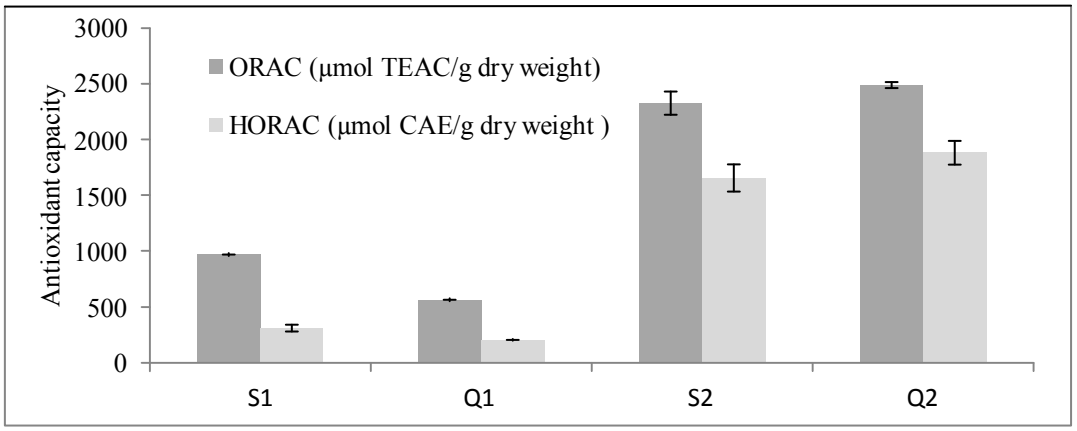

Figure 3.16. Antioxidant capacity (ORAC and HORAC) of residues extracts from Sines (S1) and Quarteira (Q1) and its respective PRCs (S2 and Q2).

In table 3.7, the total polyphenol content, ORAC and HORAC results between Sines and Quarteira *Opuntia* juices residues and their respectives PRCs are compared. In summary, juices were the samples with the lowest content in phenolic compounds and ORAC and HORAC values. This result demonstrated that fruit residues, constituted mainly of peel, are richer sources of bioactive

compounds that can be easily extracted by CSE. Therefore, it is more advantageous to process the fruit residues than pulp juices, which are mainly composed by other compounds such as sugars, fibers, proteins and minerals. Another very important fact is that there was a high improvement in TPC, ORAC and HORAC values in both *Opuntia* juices and residues after submitted to AD technology, proving that Amberlite XAD16 resin is a good approach to enhance the bioactive compounds of a sample.

Table 3.7. Comparison of total polyphenols and antioxidant activity between juices, residues and respective PRCs of Sines and Quarteira *Opuntia spp.* fruits.

Sample ID		**TPC (mgGAE/g of dry weight)**	**ORAC (µmol TE/g dry weight)**	**HORAC (µmol CAE/g dry weight)**
Sines	Juice	3.84	257± 16	142± 13
	Juice PRC	161.3 ± 5.1	4164± 209	872± 76
	Residue (S1)	26.3 ± 0.5	968 ± 13	309± 30
	Residue PRC (S2)	119.0 ± 2.0	2326 ± 104	1655± 122
Quarteira	Juice	0.36	79 ± 10	73 ± 10
	Juice PRC	114.6 ± 6.3	1786± 144	571.3 ± 41
	Residue (Q1)	17.5 ± 0.4	562± 13	202 ± 8
	Residue PRC (Q2)	142.8 ± 3.6	2489± 27	1881 ± 106

3.2.2.2 Antiproliferative effect

In order to evaluate the anticancer effect, HT29 cells were exposed to all extracts, for 24 hours and cell grown inhibition was accessed using CyQuant assay. Dose-dependent curves are presented in Figures 3.17 and 3.18.

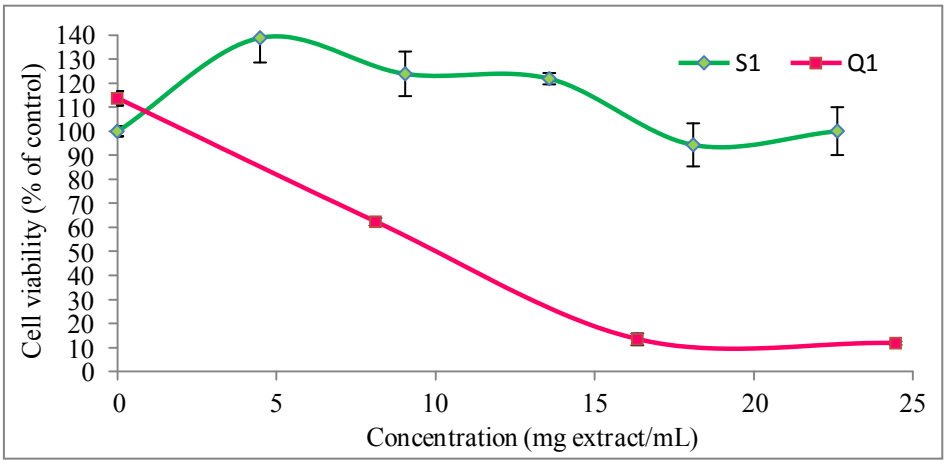

Figure 3.17. Antiproliferative effect of S1 and Q1extracts on HT29 cells. (Incubation time= 24 hours).

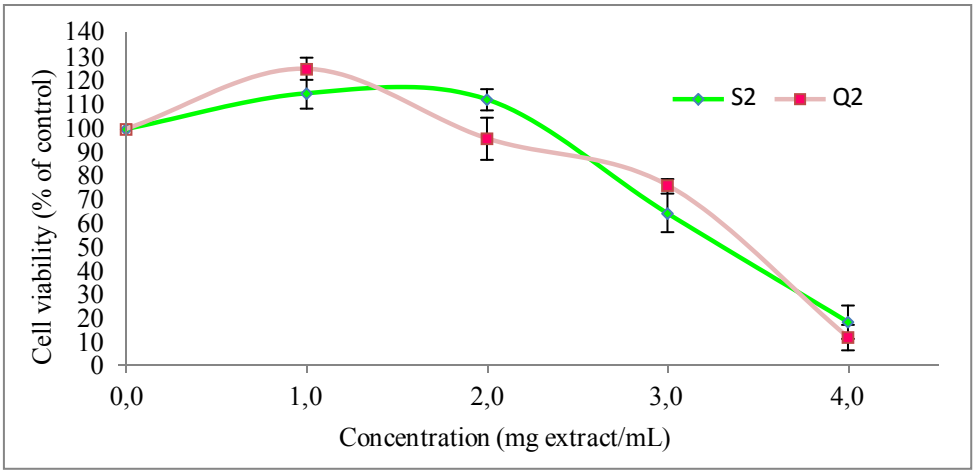

Figure 3.18. Antiproliferative effect of S2 and Q2on HT29 cells (Incubation time: 24 hours).

Among all extracts analyzed, S2 and Q2 showed to be the most effective in inhibiting cancer cell growth, because lower concentrations were required to reduce cancer cell viability. Between the two samples, S2 had the lowest effective dose value (Table 3.8).

Table 3.8. ED50 values in HT29 cells after 24h of residues extracts and PRCs exposure.

	ED50 values	
Sample ID	mg extract/ml	mg GAE/mL
S1	> 22,6	-
Q1	9,59	0,168
S2	2,40	0,343
Q2	4,12	0,490

Curiously, for the concentrations tested, S1 extract did not show antiproliferative effect. Despite the similar values of TPC, ORAC and HORAC values, between S1 and Q1, and the presence of a peak at 16 min (piscidic acid, probably) in chromatogram profile, S1 did not inhibit cancer cell growth. This result indicates that other compounds can be responsible for the antiproliferative effect. In fact, after processed by AD technology, the resulting extract (S2) presented lower quantity of this phenolic acid but exhibit higher antiproliferative effect. However further lab investigations will be needed to elucidated and find the main responsible compounds for this bioactivity.

When compared the antiproliferative effect in terms of polyphenolic concentration (figure 3.19), it can be conclude that Q1 contains the most effective compounds. In fact for the same polyphenolic concentration range, this extract showed to induce lower cell viabilities. Accordingly the ED50 value in terms of polyphenols concentration of Q1 extracts was the lowest. The presence of betalain compounds in this extract can be responsible for the antiproliferative effect.

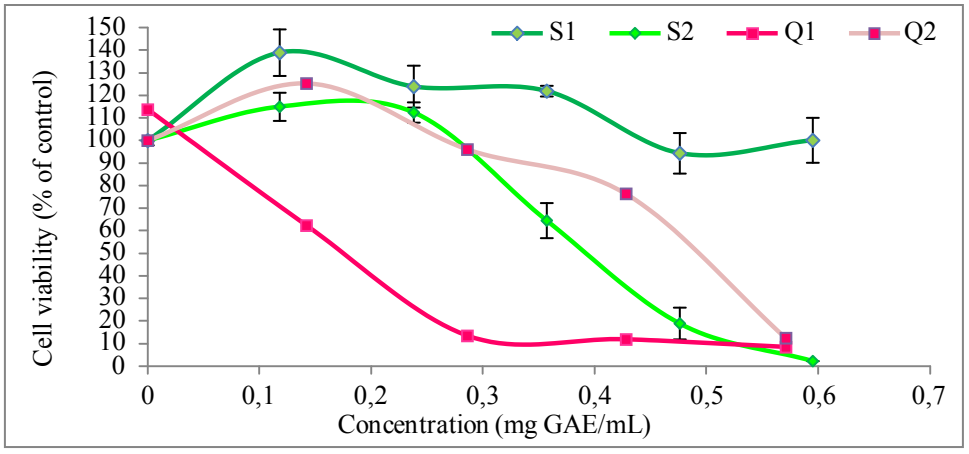

Figure 3.19. Anticancer effect on HT29 cells after residues extracts of Sines and Quarteira and PRCs exposure for 24 hours. The results were presented in total polyphenol content of extracts (mg GAE/mL).

3.2.3 Extracts from *Opuntia spp.* fruit juice residues: pressurized liquid extraction (PLE)

In order to overcome the main drawbacks of conventional solvent extraction, other extraction methods have been proposed including pressurized liquid extraction (PLE). In PLE, carbon dioxide is used in combination with water and/or ethanol at high pressures (forming a gas-expanded liquid) to extract pytochemicals from natural sources. In this work, ten extracts obtained with PLE from *Opuntia ficus-indica* fruit residues from Beja were evaluated in terms of bioactive effect. The extracts were produced using different type of solvents. Moreover, in this work, conventional solvent extractions with water and/or ethanol were performed (section 2.3.2.1) in order to compare which method is better to extract compounds with anticancer effect. Table 3.9 summarized all the samples analysed.

Table 3.9. Extracts obtained from Beja residue using PLE and CSE.

Extraction method	**Sample ID**	**Molar fracctions**		
		EtOH	**CO_2**	**H_2O**
Pressurized liquid extraction (PLE)	**1**	1	0	0
	2	0.4	0.6	0
	3	0.4	0	0.6
	4	0.7	0.3	0
	5	0.4	0.3	0.3
	6	0.7	0	0.3
	7	0.8	0.1	0.1
	8	0.5	0.4	0.1
	9	0.5	0.1	0.4
	10	0.6	0.2	0.2
Conventional sovent extraction (CSE)	**A**	1	0	0
	B	0	0	1
	C	0.4	0	0.6
	D	0.7	0	0.3

3.2.3.1 Phytochemical and antioxidant characterization

In figure 3.20 is represented the total phenolic content of all CSE and PLE extracts. It appears that CSE method was more effective in extract polyphenolic compounds than PLE technology. Extracts B, C, D from CSE and extract 5 from PLE were the ones that presented the highest amount of phenolic compounds.

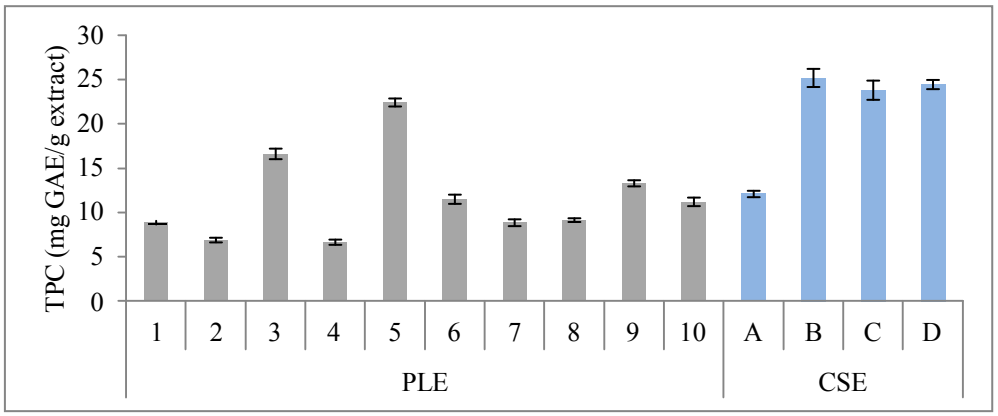

Figure 3.20. Total polyphenol content of extracts obtained from Beja residue, using PLE and CSE.

Until now, no penolic compounds were identified in all extracts. However, when comparing HPLC profiles at 280nm and 360 nm (Figure 3.21) some differences were observed between CSE and PLE derived products.

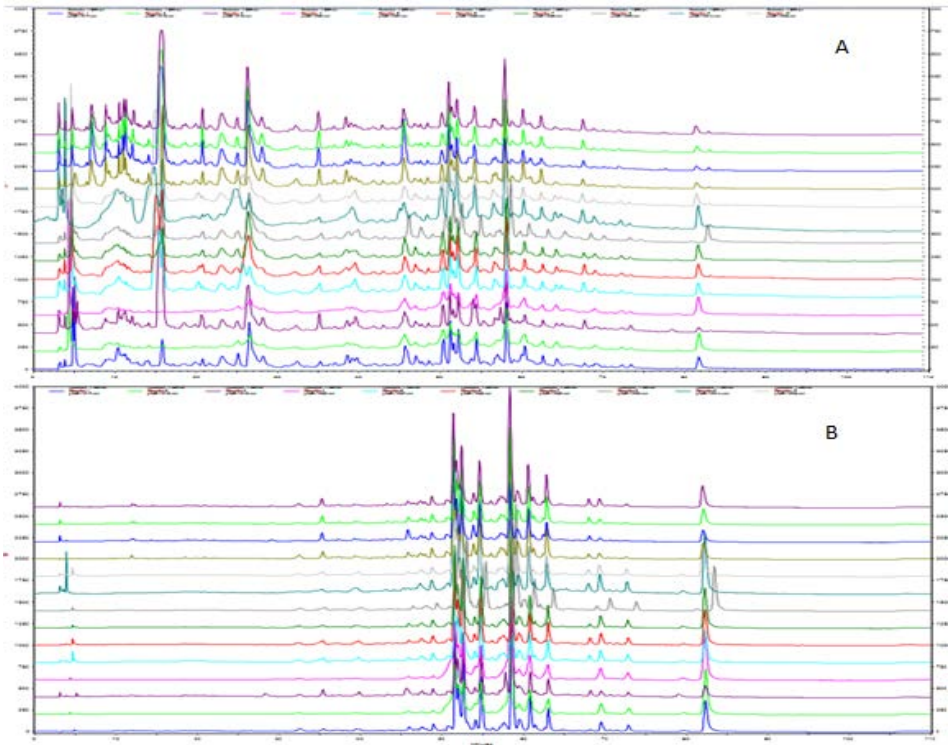

Figure 3.21. Chromatograms profiles of PLE and CSE extracts (200 mg/ml) at: A) 280nm and B) 360nm. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, A, B, C e D.

Overall, the phenolic profiles of CSE extracts are similar between the four samples (A-D). In the case of PLE extracts, the major difference occurred in extracts 2 and 4. Apparently these two extracts seemed to have less compounds than the others indicating that the use of these extract conditions: 60%CO_2:40%EtOH and 30%CO2:70%EtOH, contribute to obtain ingredients with lower polyphenolic content. Figures 3.22, 3.23 and 3.24 shows in more detail the difference between CSE and PLE ingredients for the same extracting solvent.

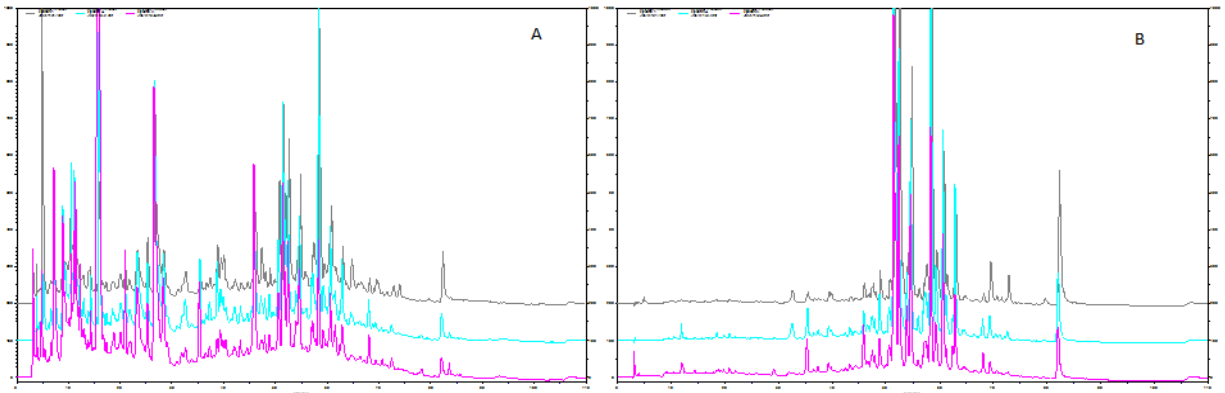

Figure 3.22. Chromatograms profiles of 1, A and B extracts at: A) 280 nm and B) 360 nm

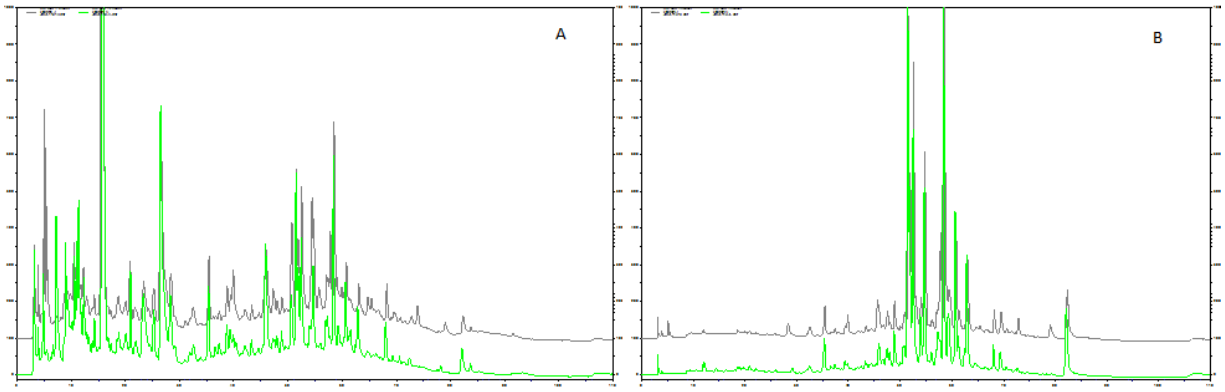

Figure 3.23. Chromatograms profiles of 3 and C extracts at: A) 280 nm and B) 360 nm.

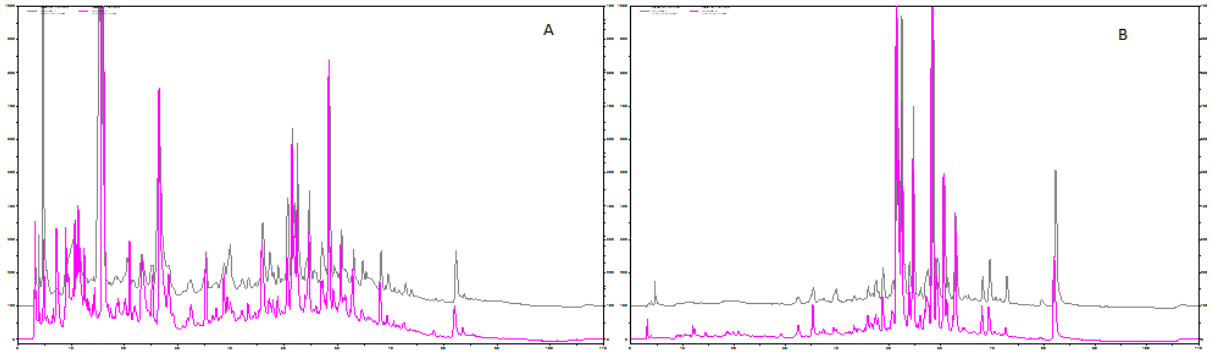

Figure 3.24. Chromatograms profiles of 6 and D extracts at: A) 280 nm and B) 360 nm.

In general, chromatograms profiles of CSE extracts show a higher content in polyphenolic compounds as demonstrated earlier in TPC quantification (figure 3.20). On the other hand, PLE extracts seems to have more flavonoid compounds (t ret =70-80 min).

Regarding to antioxidant activity, the results obtained were different than would be expected. As mentioned previously, for all *Opuntia* juices and *Opuntia* extracts analyzed in this work, ORAC values are always higher than HORAC values. This result was verified in CSE extracts (figure 3.25) but not in PLE extracts. Curiously, all PLE extracts had higher HORAC values than ORAC values, except for extract 10 where both antioxidant results are similar. Among all, extract 2 has the highest HORAC values. These results suggest that PLE and CSE have the ability to extract completely different compounds from Beja residue, with different bioactivities. While CSE have mostly radical chain breaking antioxidants, PLE extracts are mainly constitute for preventive antioxidants.

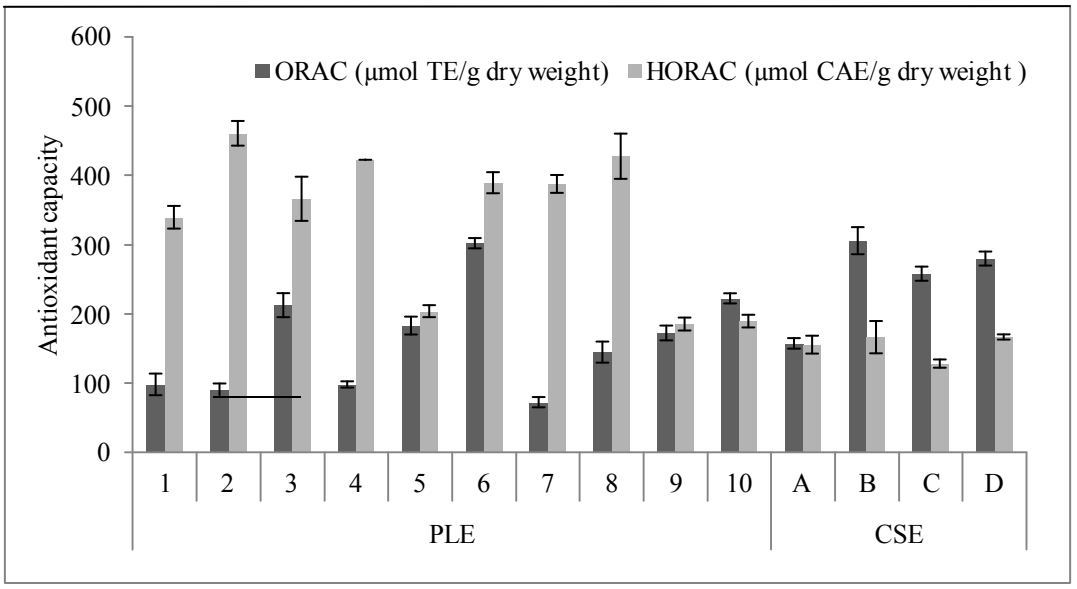

Figure 3.25. Antioxidant capacity of extracts obtained from Beja residue, using PLE and CSE.

3.2.3.2 Antiproliferative effect

In order to compare the anticancer effect between PLE and CSE extracts, HT29 cells were exposed, for 24 hours, at the same extract concentration (0-24 mg/mL) and cell viability was accessed.

As show in figure 3.26, CSE extracts did not show significant antiproliferative effect. Only extract B (extract with 100% of water) have inhibited cell growth at the highest concentration tested - cell viability decreased about 50% relative to the control. In contrast, almost all PLE extracts showed anticancer effect, except extract 1 and 3 (figure 3.27). It is important to note that these two extracts were obtained using 100% ethanol and 40%EtOH:60%H_2O, indicating that these solvent combinations are not effective in extracting anticancer compounds using the both extraction methods - CSE and PLE. On the other hand, PLE – 6 extract, which was extracted using the same type of solvent as CSE-D extract - 70% EtOH; 30%H2O2, showed significant antiproliferative effect. This result indicates that PLE method contributes to extract more or other compounds with antiproliferative effect.

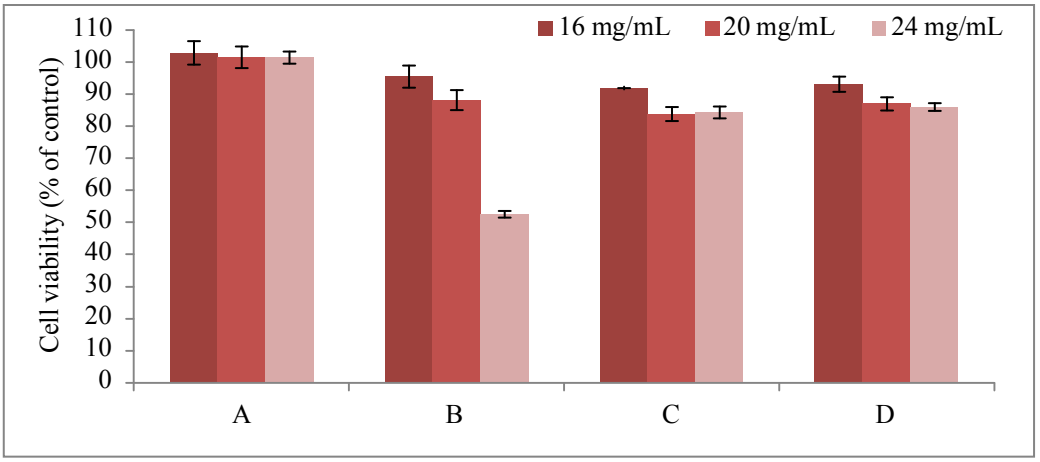

Figure 3.26. Antiproliferative effect on HT29 cell line, after 24 hours of CSE extracts treatment.

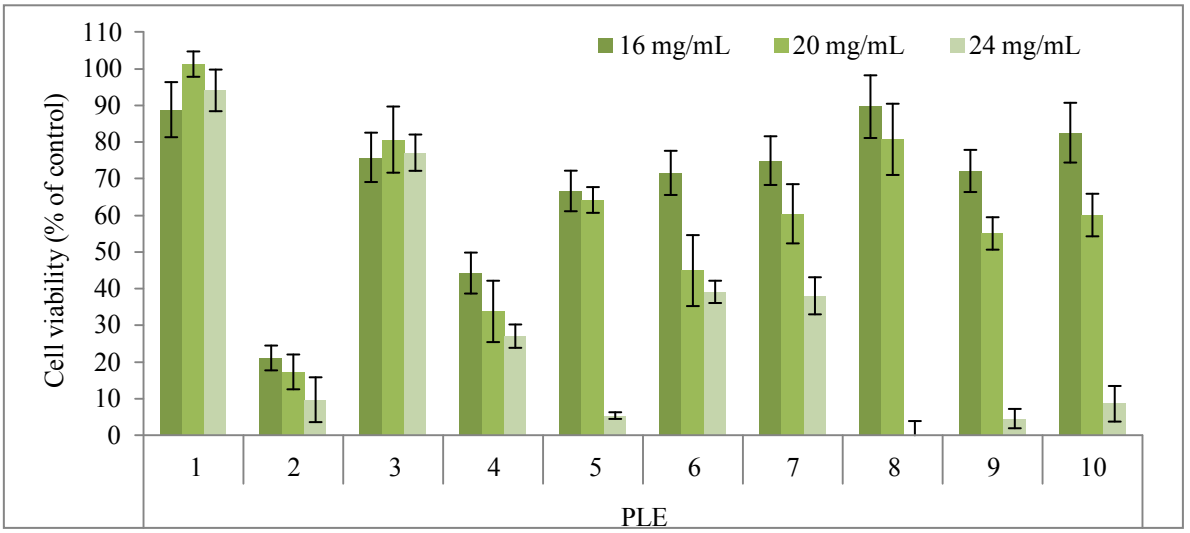

Figure 3.27. Antiproliferative effect on HT29 cell line, after 24 hours of PLE extracts treatment.

Among all PLE, extracts 2 (60%CO_2:40%EtOH) and 4 (30%CO2:70%EtOH) showed the highest antiproliferative activity indicating that the use of CO_2 and EtOH only, is beneficial for the extraction of anticancer compounds from Beja residues. The lower TPC values and the fewer content in compounds in chromatogram profiles indicate that this anticancer effect can be responsible for other compounds that aren`t polyphenols.

Between the two samples, extract 2 is more effective in inhibiting cancer cell growth. The concentration of this extracts required to reduce 50% of cell viability was also determined - 12.3 mg extract/ml (figure 3.28). Furthermore, it is important to note that this concentration was not cytotoxic, which was evaluated in Caco2 cell model (data not shown).

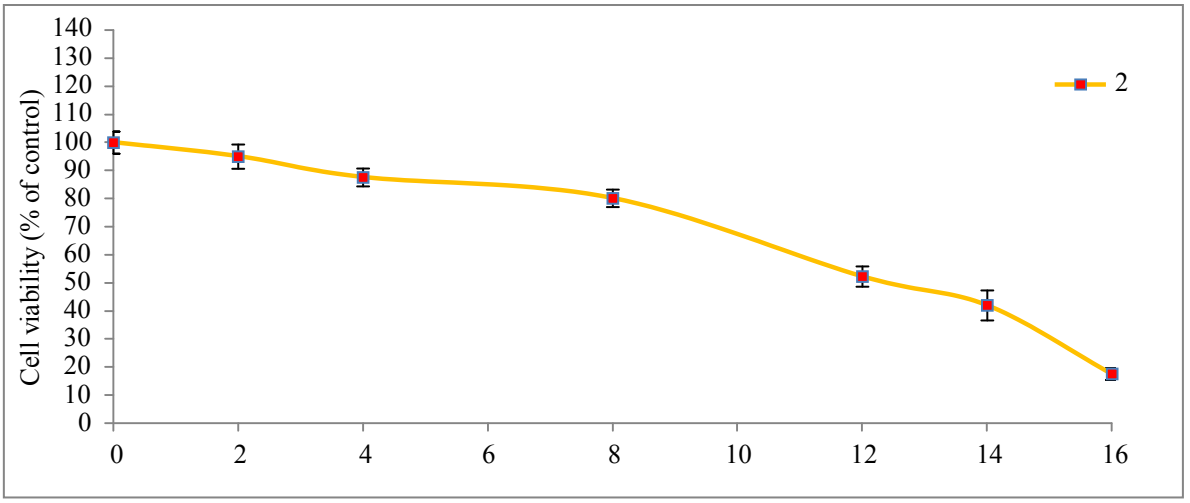

Figure 3.28. Antiproliferative effect on HT29 cell line, after 24 hours of treatment with extract 2.

3.3. PART 3: Evaluation of *Opuntia spp.* extracts as promising bioactive ingredients for colon cancer therapy

In this part of the work, the four more promising *Opuntia spp*. residues extracts described previously (section 3.2), namely Q1 (extract obtained through CSE from Quarteira residue), Q2 (PRC from Quarteira CSE extract), S2 (PRC from Sines CSE extract) and PLE-2 (extract 2 obtained from Beja residue through PLE) were characterized for their anticancer effect. For all, the induction of cell cycle arrest was evaluated as well as the generation of intracellular ROS. Additionally, the capacity of natural extracts in reverting chemoresistance of tumor cells was studied on a doxorubicin-resistant HT29 cell culture.

3.3.1. Cell cycle arrest and monitorization of ROS production

In order to analyze the induction of cell cycle arrest of selected extracts, flow cytometry was performed in human colon cancer cell line. HT29 cells were treated with the amount of extract that correspond to each ED50 value (Q1=9.6; Q2= 4.1; S2=2.4 and PLE-2= 12.3 mg extract/mL) during 24 hours.

Results obtained show that extracts had different behaviors in cell cycle arrest (figure 3.29). Q1 extract clearly induced cell cycle arrest in the G1 phase (cell growth phase). This effect was similar to those previously verified for the extracts obtained from Quarteira fruit juices with adsorption technology (section 3.2.1), suggesting that both products may contain the same bioactive ingredients. On the other hand, S2 extract induced cell cycle arrest in G2/M phase (cell division phase). Results of Q2 and PLE-2 extracts showed that the population distribution is similar in all phases of the cell cycle. Herein it is important to note that, relatively to control, there is an increase in S phase population in Both Q2 and PLE-2 extracts.

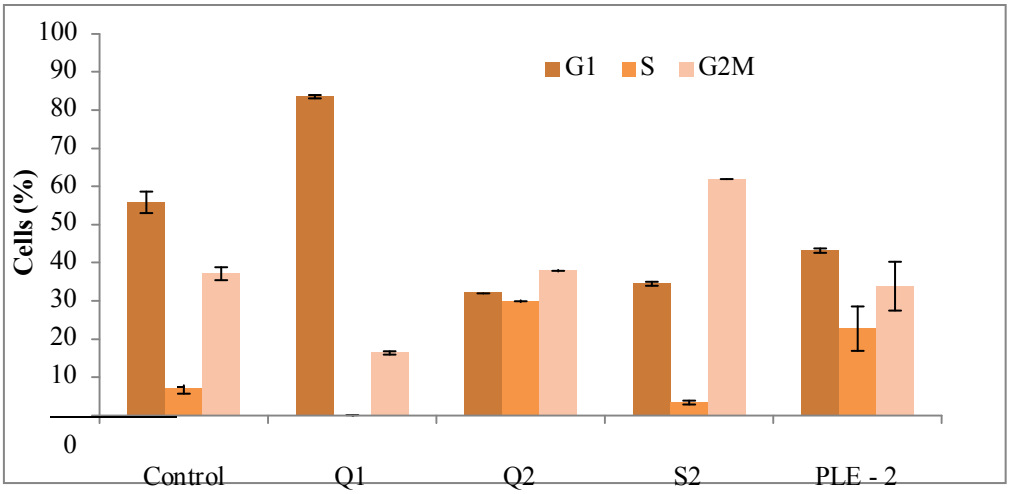

Figure 3.29. Cell cycle distribution of HT29 cells incubated for 24h with different *Opuntia spp.* extracts.

The evaluation of cell cycle analysis allowed to conclude that *Opuntia* extracts obtained from different fruits sources and extraction methods have distinct bioactive compounds that are responsible for different bioactivities in human colon cancer cell line.

Since ROS were found to play a pivotal role in the activation of pro-apoptic cell death signals under stress conditions such as chemotherapeutic agent treatments (Benhar, et al., 2002, Jackson and Loeb, 2001, Pelicano, et al., 2004, Swamy and Huat, 2003), the effect of the four *Opuntia* extracts on ROS generation in HT29 cells was evaluated. For that HT29 cells were treated with 0.5 mg/mL of each extract, during 24 hours and ROS production was assessed by DCFH-DA fluorimetric assay. When compared to control, Q1, S2 and PLE-2 exhibited a significant increase in ROS production (figure 3.38). Among all, S2 induced the highest cellular ROS formation. On contrary, Q2 extract did not show ROS induction in HT29 cells. Probably this happen because the macropouros resin (Amberlite XAD16) was not effective in isolating the responsible compounds for ROS generation in Q2 extract. Overall, the results obtained in this study suggested that *Opuntia* extracts (with Q2 exception) induce cell death through ROS dependent mechanism.

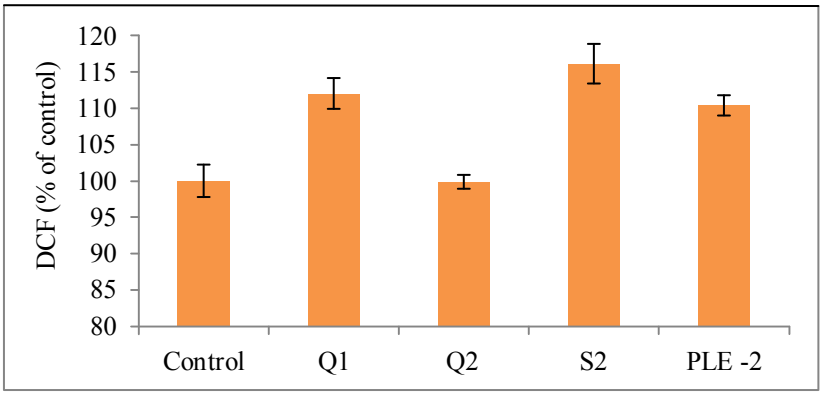

Figure 3.30. Effect of *Opuntia* extracts (0.5 mg/mL) on reactive oxygen species (ROS) accumulation in HT29 cells, after 24h incubation.

3.3.2. Chemosensitization effect in a drug resistant cell sub-population (HT29 dx)

Multi-drug resistance (MDR) is actually one of the major factors impairing the success of pharmacological treatments of several tumors, such as colon cancer. Recently, several approaches to revert it have been studied. In cancer cell models, one of the most studied mechanisms is the overexpression of several energy-dependent drug efflux pumps that belong to the ATP-binding cassette family of transporters, such as the P-glycoprotein (Pgp) and the MDR associated proteins (MDRs) (Riganti, et al., 2005). Evidence that drug efflux pumps play a significant role in clinical drug resistance has stimulated the introduction of new chemical inhibitors compounds into the clinical arena such as: Pgp inhibitors (Thomas and Coley, 2003) and NHE (Na^+/H^+ exchanger) inhibitors, which are involved in the homeostasis of intracellular pH (Miraglia, et al., 2005). On the other hand, there are studies that suggest the utilization of chemical inducers, such as inducers of NO synthesis (i.e cytokines), which may increase drug accumulation and a marked reduction on doxorubicin efflux rate (De Boo, et al., 2009, Riganti, et al., 2005). Furthermore, a more recent study suggests that the inactivation of RhoA gene (small interfering RNA) subsequently lead to a decrease of RhoA protein, which was associated with the increased sensitivity to doxorubicin in HT29 cells and the complete reversion of HT29 dx (Doublier, et al., 2008). These solutions seems to be good enough to reverse doxorubicin resistance, however, they have some drawbacks. The use of inducers or inhibitors, consequently leads to the consumption of others chemical drugs, which are responsible for many adverse effects and have several contraindications (i.e atorvastatin). Besides that, gene therapy (RhoA silencing) is a complex and costly method, which require other complementary studies for their potential clinical application in humans.

To overcome these issues, recently, natural compounds or extracts had been used for chemiosensitization effect. It has been demonstrated that the *Hericium erinaceus* (HE) mushroom extract in combination with doxorubicin serves as an effective tool for treating drug-resistant human hepatocellular carcinoma (Lee and Hong, 2010).

In this work, the chemosensitization effect of Opuntia-based natural extracts was evaluated in a drug-resistant cell line subpopulation (HT29 dx). The aim is to reduce the necessary dose of drug decreasing the toxicity associated with it.

3.3.2.1 Cellular sensitivity to drug and intracellular accumulation of doxorubicin

The human colon carcinoma resistant cell line (HT29 dx) was created through addition of a doxorubicin dose on culture medium during several passages, as previously described in experimental procedure. In the end, to evaluate the sensitivity to doxorubicin, both cell lines (HT29 and HT29 dx) were exposed to several drug concentrations (0.16-20 µM), for 1 hour. After three days in a drug-free medium, cell viability was performed using MTT assay and IC50 values (the half maximal of inhibitory concentration of a substance) for each cell line were determined.

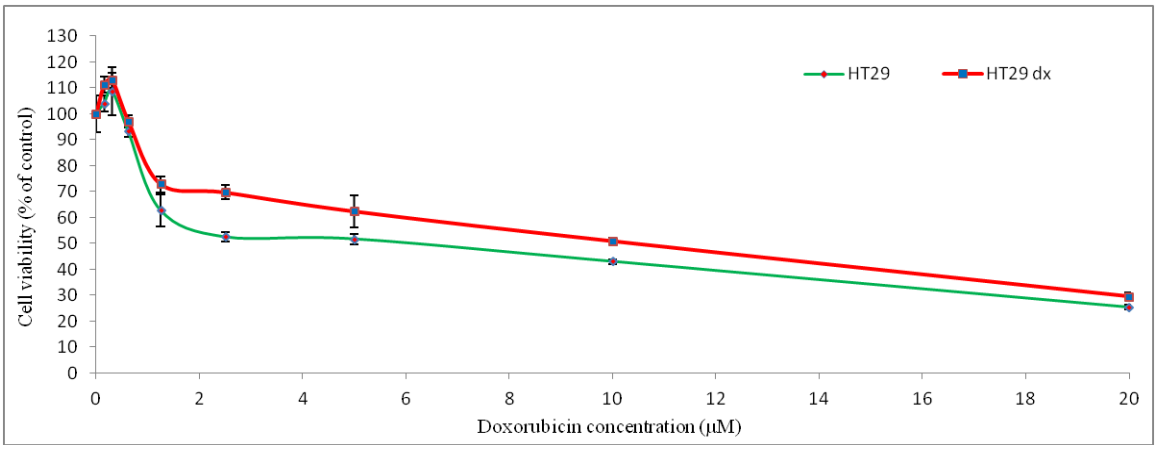

Figure 3.31. Inhibitory effect of doxorubicin exposure during1 hour, in HT29 cell line and HT29 dx.

Table 3.10. IC50 values determined for HT29 cell line and HT29 dx, after 1h drug exposure.

	IC50 (µM Doxorubicin)
HT29	6.03
HT29 dx	10.23
R.I*	1.7

*The resistance index (R.I.) was calculated as the ratio between the IC50 values obtained

In figure 3.31, is show that the cell viability line of HT29 dx is above the other line (HT29), which demonstrated that effectively HT29 dx was more resistant to doxorubicin. IC50 values are presented in table 3.10, being approximately 6 µM for HT29 and 10.2 µM of doxorubicin for HT29 dx.

To ensure the resistance of HT29 dx, intracellular drug accumulation was evaluated and compared in both cell lines. This was accomplished by incubating cell lines with 5µmol/L of drug during 24 hours. After incubation time the fluorescent drug was quantified fluorimetrically and cell protein was determined according to Bradford assay. Fluorescence was converted in ng doxorubicin/mg cell protein using a calibration curve prepared previously, as show in figure 3.32. Results show that HT29 normal cell line accumulated significantly more doxorubicin (almost 50%) than the resistant cell line, as expected. These results are a little bit higher than reported in literature (De Boo, et al., 2009).

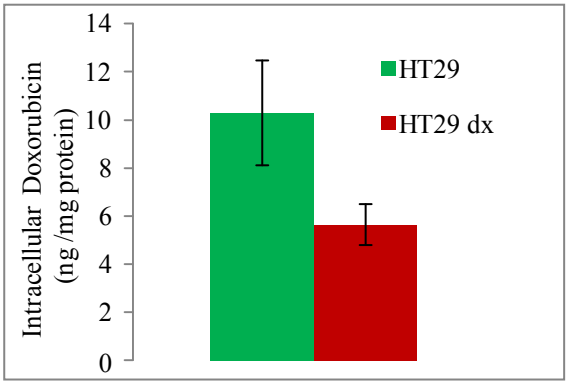

Figure 3.32. Intracellular doxorubicin accumulation in HT29 and HT29 dx.

3.3.2.2 Chemosensitization activity

After the development of HT29 dx sub population, the next step of this work was the evaluation of the capacity of *Opuntia* natural extracts to revert chemoresistance of this resistant cell population. For that, HT29 dx cells were exposed to *Opuntia* extracts (6 mg/ml) for 24 hours, further 1h of drug (0-20µM) incubation. After 72 of proliferation, cell viability was accessed and compared. The extracts selected for this study were: Q1 extract (extract obtained of CSE with 50%EtOH:50%H_2O from Quarteira residue) and PLE-2 (extract obtained from Beja residue using PLE with 60%CO_2:40% EtOH).

In figure 3.33 is presented the toxicity of *Opuntia* extracts conjugated with doxorubicin in HT29 dx cells. As it can be seen, Q1 extract had the lowest effect on HT29 dx viability. When combined with doxorubicin, cell viability was higher or similar to the drug alone, indicating that this ingredient do not present chemosensitization effect.

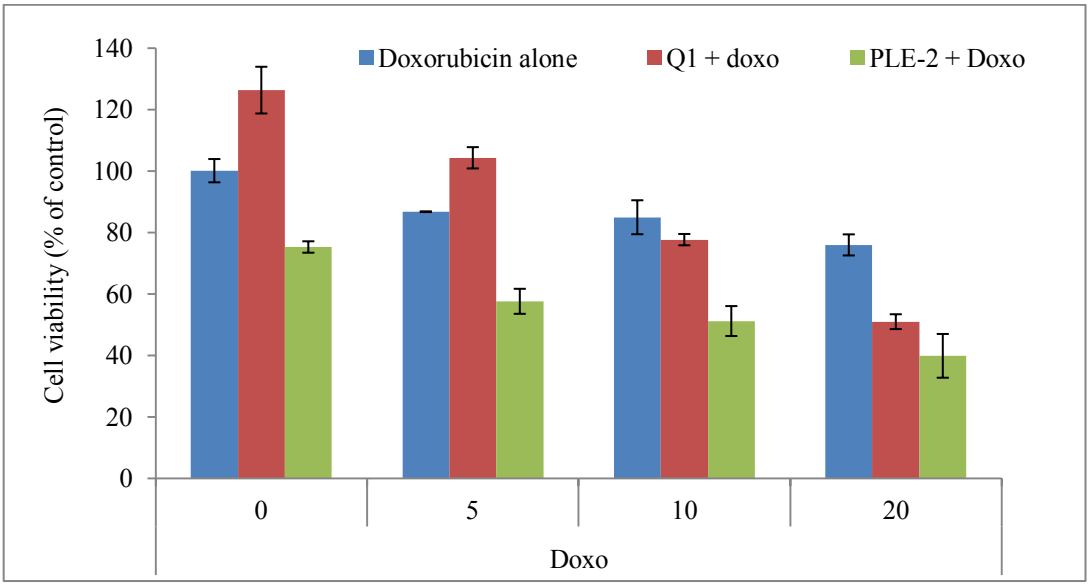

Figure 3.33. Toxicity of Q1 and PLE-2 extracts and /or doxorubicin (0-20 µM) on HT29dx cells.

On the other hand, PLE-2 extract had the strongest effect on HT29 dx viability. As shown in figure 3.33, 6 mg/mL of PLE-2 combined with 5, 10 or 20 µM of doxorubicin reduced HT29 dx viability below 80%, which is the effect verified for the highest concentration of drug alone. Moreover, when subtracting the toxicity effect of PLE-2 extract it was observed that PLE-2 increased drug sensitivity on HT29 dx cell population (figure 3.34). It is important to note that this effect is closer than those verified for doxorubicin alone on HT29 normal cell population.

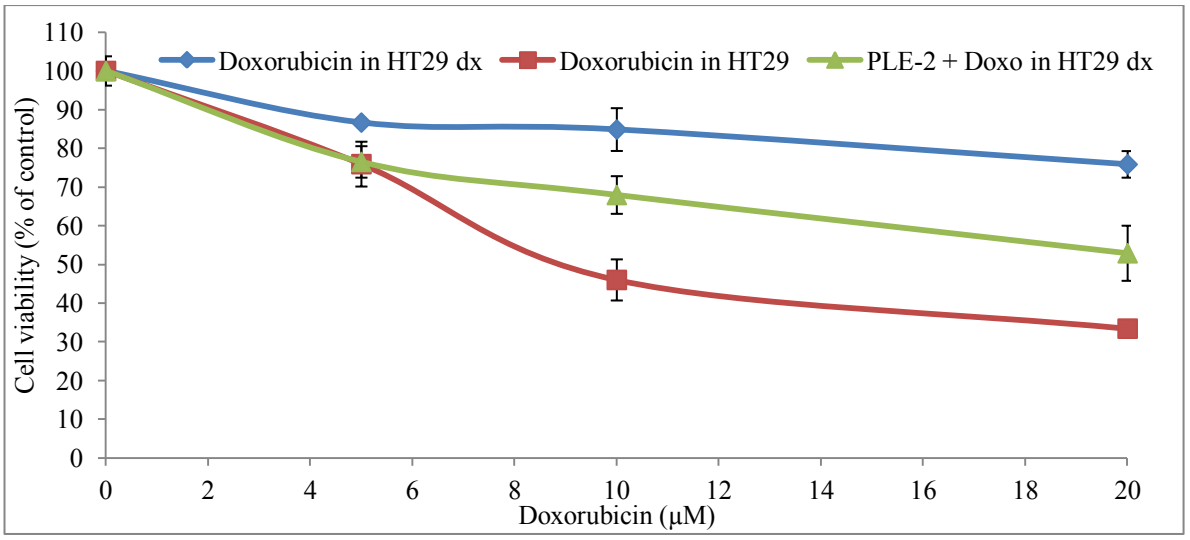

Figure 3.34. Toxicity effect of PLE-2 extract plus doxo or using only doxo in HT29 or HT29 dx.

The chemosensitization effect of PLE-2 extract observed in HT29 dx cell population suggests that combining this *Opuntia* extract with doxorubicin may be beneficial to prevent chemoresistance of cancer cells. Therefore, PLE-2 is a promising natural extract to be used in combination with the drug in colon cancer treatments.

4.Conclusions

In this work, the bioactive effects of *Opuntia spp.* fruit juices and *Opuntia spp.* - based extracts were investigated in order to recognize their high added-value for colon cancer therapy application. It was demonstrated that *Opuntia ficus*-indica and *Opuntia robusta* cultivated in Alentejo and Algarve can be considered as functional fruits with promising activity in chemotherapy of colon cancer.

Five Portuguese *Opuntia spp.* fruit juices were screened for their bioactivity. Two species of *Opuntia spp*. were studied, namely *Opuntia ficus-indica* from Tramagal, Sines, Marvão and Beja, and *Opuntia robusta* collected in Quarteira. Among all *Opuntia ficus-indica* samples, the juices from Tramagal and Sines fruits have the highest antiproliferative effect in human colon cancer cells, and this is mainly related to the highest content in polyphenols. Despite of *Opuntia robusta*, have the lowest concentration in polyphenols, showed a significant antiproliferative effect on HT29 cells, maybe due to the presence of betalains, that are compounds identified as anticancer substances (Sreekanth, et al., 2007).

Within this work, efforts were made to develop *Opuntia spp.* bioactive products such as polyphenol-rich concentrates (PRCs) and natural extracts using *Opuntia* fruit juices and their residues. For that different clean technologies were explored such as adsorption technology (AD), conventional solvent extraction (CSE) with biocompatible solvents and pressurized liquid extraction (PLE) with CO_2:EtOH:H_2O different mixtures. In particular, polyphenols-rich concentrates were obtained with juices from Tramagal, Sines and Quarteira using the macroporous adsorptive resin Amberlite XAD16. This method allowed the enrichment in polyphenol content and antioxidant capacity of juices, mainly in PRC-Quarteira. Only the PRCs from Sines and Quarteira showed significant cancer cell growth inhibition and this effect seemed to be related with the presence of some phenolic compounds namely betalains and piscidic acid. The same process was used to isolate polyphenols-rich concentrates from Sines and Quarteira fruit juices residues and the products obtained demonstrated an improvement in antiproliferative activity in HT29 cell line. For Sines and Quarteira residues conventional extractions using 50%EtOH:50%H_2O were also performed. Results showed that, in terms of polyphenols compounds, Quarteira conventional extract showed to be more effective in inhibiting cell growth than PRC from Quarteira. Although adsortive resins contribute to increase the polyphenol content of the final product, some bioactive polyphenol compounds with anitproliferative activity were not recovered using this process.

For fruit juice residues from Beja pressurized liquid extraction was explored. Ten different extracts obtained with different CO_2:EtOH:H_2O ratios were analyzed and the product derived from 60%$CO_2$and 40%EtOH extraction was the most effective in inhibiting cancer cell growth. Additionally, when comparing with conventional extraction, the use of pressurized solvents contributes to obtain extracts with enhanced antiproliferative effect.

In this thesis, the mechanism behind the antiproliferative effect of *Opuntia spp*. extracts was investigated in terms of cell cycle arrest and ROS generation. The most promising extracts are the ones derived from Quarteira, Sines and Beja fruit juices residues using conventional solvent extraction, adsorption technology and pressurized liquid extraction with 60%$CO_2$and 40%EtOH, respectively. These extracts induce cell cycle arrest in different cell cycle checkpoints and increase ROS generation in HT29, playing an important role in activation of pro-apoptic cell death mechanisms. Finally, exctract from Beja residue showed to be a promising natural ingredient to be used in cancer therapy due to its capacity in reverting chemoresistance of tumor cells. In fact, when resistance cancer cells were pré-treated with this extract, their sensitization to the drug increases more than 4 times. Moreover, it is important to highlight that this effect is more pronounced than that observed in normal cancer cells with dorxorubicin alone, suggesting that some synergetic effects occurs between Beja extract and doxorubicin

Despite the evidence provided by cell culture studies presented in this work, the molecular mechanisms responsible for the anticarcinogenic effect of the *Opuntia* bioactive ingredients need to be more clarified. Further studies will be necessary to identify more compounds responsible for anticancer effect and what mechanisms are underlying HT29 cell death. Moreover, others future investigations should also be performed such as: (a) studies about the combined chemopreventive effects of *Opuntia* functional ingredients and doxorubicinin should be further explored through the use of extracts with different compositions, using cell culture, animal models and clinical trials; (b) studies on polyphenol bioavailability (eg, intestinal absorption and metabolism) in animal models, to better elucidate the chemopreventive properties of its intestinal metabolites; and (c) clinical studies in human subjects to fully confirm and quantify the bioavailability, safety, efficacy, and chemotherapeutic properties *Opuntia* polyphenols.

Overal, this thesis demonstrate that Portuguese *Opuntia* fruits can be recognized as products with bioactivity or as rich sources of high added-value ingredients with promising application in food and pharmaceutical industries.

5. References

Alonso-Salces, R. M., E. Korta, A. Barranco, L. A. Berrueta, B. Gallo, and F. Vicente. 'Pressurized Liquid Extraction for the Determination of Polyphenols in Apple.', *J Chromatogr A* Vol. 933, No. 1-2, 37-43, 2001.

Alía (a), M., R. Mateos, S. Ramos, E. Lecumberri, L. Bravo, and L. Goya. 'Influence of Quercetin and Rutin on Growth and Antioxidant Defense System of a Human Hepatoma Cell Line (Hepg2).', *Eur J Nutr* Vol. 45, No. 1, 19-28, 2006.

Alía, M., R. Mateos, S. Ramos, E. Lecumberri, L. Bravo, and L. Goya. 'Influence of Quercetin and Rutin on Growth and Antioxidant Defense System of a Human Hepatoma Cell Line (Hepg2).', *Eur J Nutr* Vol. 45, No. 1, 19-28, 2006.

Alía (b), M., S. Ramos, R. Mateos, A. B. Granado-Serrano, L. Bravo, and L. Goya. 'Quercetin Protects Human Hepatoma Hepg2 against Oxidative Stress Induced by Tert-Butyl Hydroperoxide.', *Toxicol Appl Pharmacol* Vol. 212, No. 2, 110-8, 2006.

American Cancer Society. 'Cancer Facts & Figures', American Cancer Society, 2008.

Araújo, J. R., P. Gonçalves, and F. Martel. 'Chemopreventive Effect of Dietary Polyphenols in Colorectal Cancer Cell Lines.', *Nutr Res* Vol. 31, No. 2, 77-87, 2011.

Azeredo, Henriette M.C. 'Betalains: Properties, Sources, Applications, and Stability – a Review', *International Journal of Food Science and Technology* Vol. 44, 2365–2376, 2009.

Barkakatia, Pranab, Ashma Beguma, Makhan Lal Dasb, and Paruchuri Gangadhar Rao. 'Adsorptive Separation of Ginsenoside from Aqueous Solution by Polymeric Resins: Equilibrium, Kinetic and Thermodynamic Studies', *Chemical Engineering Journal* Vol. 161, 34–45, 2010.

Benhar, M., D. Engelberg, and A. Levitzki. 'Ros, Stress-Activated Kinases and Stress Signaling in Cancer.', *EMBO Rep* Vol. 3, No. 5, 420-5, 2002.

Bradford, Marion M. 'A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding', *Analytical Biochemistry* Vol. 72, 248-254, 1976.

Brower, V. 'Nutraceuticals: Poised for a Healthy Slice of the Healthcare Market?', *Nat Biotechnol* Vol. 16, No. 8, 728-31, 1998.

Butera, D., L. Tesoriere, F. Di Gaudio, A. Bongiorno, M. Allegra, A. M. Pintaudi, R. Kohen, and M. A. Livrea. 'Antioxidant Activities of Sicilian Prickly Pear (Opuntia Ficus Indica) Fruit Extracts and Reducing Properties of Its Betalains: Betanin and Indicaxanthin.', *J Agric Food Chem* Vol. 50, No. 23, 6895-901, 2002.

Camel, V. 'Recent Extraction Techniques for Solid Matrices-Supercritical Fluid Extraction, Pressurized Fluid Extraction and Microwave-Assisted Extraction: Their Potential and Pitfalls.', *Analyst* Vol. 126, No. 7, 1182-93, 2001.

Cartea, M. E., M. Francisco, P. Soengas, and P. Velasco. 'Phenolic Compounds in Brassica Vegetables.', *Molecules* Vol. 16, No. 1, 251-80, 2011.

Cayupán, Yanina S. Coria, María J. Ochoa, and Mónica A. Nazareno. 'Health-Promoting Substances and Antioxidant Properties of Opuntia Sp. Fruits. Changes in Bioactive-Compound Contents During Ripening Process', *Food Chemistry* Vol. 126, 514–519, 2011.

Chang, W., J. J. Kang, K. Y. Lee, K. Wei, E. Anderson, S. Gotmare, J. A. Ross, and G. D. Rosen. 'Triptolide and Chemotherapy Cooperate in Tumor Cell Apoptosis. A Role for the P53 Pathway.', *J Biol Chem* Vol. 276, 2221-2227, 2001.

Chavez-Santoscoy, R. A., J. A. Gutierrez-Uribe, and S. O. Serna-Saldívar. 'Phenolic Composition, Antioxidant Capacity and in Vitro Cancer Cell Cytotoxicity of Nine Prickly Pear (Opuntia Spp.) Juices.', *Plant Foods Hum Nutr* Vol. 64, No. 2, 146-52, 2009.

D'Archivio, M., C. Filesi, R. Di Benedetto, R. Gargiulo, C. Giovannini, and R. Masella. 'Polyphenols, Dietary Sources and Bioavailability.', *Ann Ist Super Sanita* Vol. 43, No. 4, 348-61, 2007.

Dai, J., and R. J. Mumper. 'Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties.', *Molecules* Vol. 15, No. 10, 7313-52, 2010.

De Boo, S., J. Kopecka, D. Brusa, E. Gazzano, L. Matera, D. Ghigo, A. Bosia, and C. Riganti. 'Inos Activity Is Necessary for the Cytotoxic and Immunogenic Effects of Doxorubicin in Human Colon Cancer Cells.', *Mol Cancer* Vol. 8, 108, 2009.

Dihal, A. A., R. A. Woutersen, B. van Ommen, I. M. Rietjens, and R. H. Stierum. 'Modulatory Effects of Quercetin on Proliferation and Differentiation of the Human Colorectal Cell Line Caco-2.', *Cancer Lett* Vol. 238, No. 2, 248-59, 2006.

Doublier, S., C. Riganti, C. Voena, C. Costamagna, E. Aldieri, G. Pescarmona, D. Ghigo, and A. Bosia. 'Rhoa Silencing Reverts the Resistance to Doxorubicin in Human Colon Cancer Cells.', *Mol Cancer Res* Vol. 6, No. 10, 1607-20, 2008.

Dragnev, K. H., Q. Feng, Y. Ma, S. J. Shah, C. Black, V. Memoli, W. Nugent, J. R. Rigas, S. Kitareewan, S. Freemantle, and E. Dmitrovsky. 'Uncovering Novel Targets for Cancer Chemoprevention.', *Recent Results Cancer Res* Vol. 174, 235-43, 2007.

Espín, J. C., M. T. García-Conesa, and F. A. Tomás-Barberán. 'Nutraceuticals: Facts and Fiction.', *Phytochemistry* Vol. 68, No. 22-24, 2986-3008, 2007.

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, and Parkin DM. 'Cancer Incidence and Mortality Worldwide: Iarc Cancerbase, *GLOBOCAN 2008*, International Agency for Research on Cancer, 2010.

Fernández-López, J. A., L. Almela, J. M. Obón, and R. Castellar. 'Determination of Antioxidant Constituents in Cactus Pear Fruits.', *Plant Foods Hum Nutr* Vol. 65, No. 3, 253-9, 2010.

Ferreira, C. G., M. Epping, F. A. Kruyt, and G. Giaccone. 'Apoptosis: Target of Cancer Therapy.', *Clin Cancer Res* Vol. 8, No. 7, 2024-34, 2002.

Feugang, J. M., P. Konarski, D. Zou, F. C. Stintzing, and C. Zou. 'Nutritional and Medicinal Use of Cactus Pear (Opuntia Spp.) Cladodes and Fruits.', *Front Biosci* Vol. 11, 2574-89, 2006.

Galati, E. M., M. R. Mondello, D. Giuffrida, G. Dugo, N. Miceli, S. Pergolizzi, and M. F. Taviano. 'Chemical Characterization and Biological Effects of Sicilian Opuntia Ficus Indica (L.) Mill. Fruit Juice: Antioxidant and Antiulcerogenic Activity.', *J Agric Food Chem* Vol. 51, No. 17, 4903-8, 2003.

Gordaliza, M. 'Natural Products as Leads to Anticancer Drugs.', *Clin Transl Oncol* Vol. 9, No. 12, 767-76, 2007.

Gottesman, M. M. 'Mechanisms of Cancer Drug Resistance.', *Annu Rev Med* Vol. 53, 615-27, 2002.

Griffith, M. Patrick. 'The Origins of an Important Cactus Crop, Opuntia Ficus-Indica (Cactaceae): New Molecular Evidence', *American Journal of Botany* Vol. 91, No. 11, 1915–1921, 2004.

Gurrieri, S., L. Miceli, C. M. Lanza, F. Tomaselli, R. P. Bonomo, and E. Rizzarelli. 'Chemical Characterization of Sicilian Prickly Pear (Opuntia Ficus Indica) and Perspectives for the Storage of Its Juice.', *J Agric Food Chem* Vol. 48, No. 11, 5424-31, 2000.

Hahm, S. W., J. Park, and Y. S. Son. 'Opuntia Humifusa Partitioned Extracts Inhibit the Growth of U87mg Human Glioblastoma Cells.', *Plant Foods Hum Nutr* Vol. 65, No. 3, 247-52, 2010.

Haque, N., U. Salma, T. R. Nurunnabi, A. K. Haque, I. J. Mukti, S. Pervin, and R. Nahar. 'Lifestyle Related Causes of Cancer and Chemoprevention through Phytonutrients.', *Pak J Biol Sci* Vol. 13, No. 19, 916-26, 2010.

Hernández-Pérez, T., A. Carrillo-López, F. Guevara-Lara, A. Cruz-Hernández, and O. Paredes-López. 'Biochemical and Nutritional Characterization of Three Prickly Pear Species with Different Ripening Behavior.', *Plant Foods Hum Nutr* Vol. 60, No. 4, 195-200, 2005.

Howard, L., and N. Pandjaitan. 'Pressurized Liquid Extraction of Flavonoids from Spinach.', *J Food Sci* Vol. 73, No. 3, C151-7, 2008.

Huang, D., B. Ou, M. Hampsch-Woodill, J. A. Flanagan, and R. L. Prior. 'High-Throughput Assay of Oxygen Radical Absorbance Capacity (Orac) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format.', *J Agric Food Chem* Vol. 50, No. 16, 4437-44, 2002.

Hwang, J. T., J. Ha, and O. J. Park. 'Combination of 5-Fluorouracil and Genistein Induces Apoptosis Synergistically in Chemo-Resistant Cancer Cells through the Modulation of Ampk and Cox-2 Signaling Pathways.', *Biochem Biophys Res Commun* Vol. 332, No. 2, 433-40, 2005.

Ignat, Ioana, Volf Irina, and Valentim I. Popa. 'A Critical Review of Methods for Characterisation of Polyphenolic Compounds in Fruits and Vegetables', *Food Chemistry* Vol. 126, 1821-1825, 2011.

Invitrogen. 'Cyquant Cell Proliferation Assay Kit', In Invitrogen detection technologies Molecular Probes, 2006.

Jackson, A. L., and L. A. Loeb. 'The Contribution of Endogenous Sources of Dna Damage to the Multiple Mutations in Cancer.', *Mutat Res* Vol. 477, No. 1-2, 7-21, 2001.

Jaganath, Indu B., and Alan Crozier. *Dietary Flavonoids and Phenolic Compounds. Plant Phenolics and Human Health: Biochemistry, Nutrition, and Pharmacology*, John Wiley & Sons, Inc., 2010.

Johnson, I. T. 'Phytochemicals and Cancer.', *Proc Nutr Soc* Vol. 66, No. 2, 207-15, 2007.

Kushi, L. H., T. Byers, C. Doyle, E. V. Bandera, M. McCullough, A. McTiernan, T. Gansler, K. S. Andrews, M. J. Thun, and American Cancer Society 2006 Nutrition and Physical Activity Guidelines Advisory Committee. 'American Cancer Society Guidelines on Nutrition and Physical Activity for Cancer Prevention: Reducing the Risk of Cancer with Healthy Food Choices and Physical Activity.', *CA Cancer J Clin* Vol. 56, No. 5, 254-81; quiz 313-4, 2006.

Kuti, Joseph O. 'Antioxidant Compounds from Four Opuntia Cactus Pear Fruit Varieties', *Food Chemistry* Vol. 85, 527–533, 2004.

Lambert, J. D., J. Hong, G. Y. Yang, J. Liao, and C. S. Yang. 'Inhibition of Carcinogenesis by Polyphenols: Evidence from Laboratory Investigations.', *Am J Clin Nutr* Vol. 81, No. 1 Suppl, 284S-291S, 2005.

Lee, J. S., and E. K. Hong. 'Hericium Erinaceus Enhances Doxorubicin-Induced Apoptosis in Human Hepatocellular Carcinoma Cells.', *Cancer Lett* Vol. 297, No. 2, 144-54, 2010.

Lee, Y.-C., Y.-H. Pyo, C.-K. Ahn, and S.-H. Kim. 'Food Functionality of Opuntia Ficus-Indica Var. Cultivated in Jeju Island', *J Food Sci Nutr* Vol. 10, 103-110, 2005.

Li, C. J., Y. Z. Li, A. V. Pinto, and A. B. Pardee. 'Potent Inhibition of Tumor Survival in Vivo by Beta-Lapachone Plus Taxol: Combining Drugs Imposes Different Artificial Checkpoints.', *Proc Natl Acad Sci U S A* Vol. 96, No. 23, 13369-74, 1999.

Lila, M. A. 'From Beans to Berries and Beyond: Teamwork between Plant Chemicals for Protection of Optimal Human Health.', *Ann N Y Acad Sci* Vol. 1114, 372-80, 2007.

Loro, J. F., I. del Rio, and L. Pérez-Santana. 'Preliminary Studies of Analgesic and Anti-Inflammatory Properties of Opuntia Dillenii Aqueous Extract.', *J Ethnopharmacol* Vol. 67, No. 2, 213-8, 1999.

Luthria, D. L., and S. Mukhopadhyay. 'Influence of Sample Preparation on Assay of Phenolic Acids from Eggplant.', *J Agric Food Chem* Vol. 54, No. 1, 41-7, 2006.

Manach, C., A. Scalbert, C. Morand, C. Rémésy, and L. Jiménez. 'Polyphenols: Food Sources and Bioavailability.', *Am J Clin Nutr* Vol. 79, No. 5, 727-47, 2004.

Manson, M. M., P. B. Farmer, A. Gescher, and W. P. Steward. 'Innovative Agents in Cancer Prevention.', *Recent Results Cancer Res* Vol. 166, 257-75, 2005.

Medina, E.M. Días, E.M. Rodríguez Rodríguez, and C. Díaz Romero. 'Chemical Characterization of Opuntia Dillenii and Opuntia Ficus Indica Fruits', *Food Chemistry* Vol. 103, 38–45, 2007.

Miraglia, E., D. Viarisio, C. Riganti, C. Costamagna, D. Ghigo, and A. Bosia. 'Na+/H+ Exchanger Activity Is Increased in Doxorubicin-Resistant Human Colon Cancer Cells and Its Modulation Modifies the Sensitivity of the Cells to Doxorubicin.', *Int J Cancer* Vol. 115, No. 6, 924-9, 2005.

Naczk, M., and F. Shahidi. 'Phenolics in Cereals, Fruits and Vegetables: Occurrence, Extraction and Analysis.', *J Pharm Biomed Anal* Vol. 41, No. 5, 1523-42, 2006.

Nakagawa, H., K. Hasumi, J. T. Woo, K. Nagai, and M. Wachi. 'Generation of Hydrogen Peroxide Primarily Contributes to the Induction of Fe(Ii)-Dependent Apoptosis in Jurkat Cells by (-)-Epigallocatechin Gallate.', *Carcinogenesis* Vol. 25, No. 9, 1567-74, 2004.

Neveu, V., J. Perez-Jiménez, F. Vos, V. du Crespy, L. Chaffaut, L. Mennen, C. Knox, R. Eisner, J. Cruz, D. Wishart, and A. Scalbert. 'Phenol-Explorer: An Online Comprehensive Database on Polyphenol Contents in Foods'. *Phenol-Explorer: an online comprehensive database on polyphenol contents in foods*, 2010.

Ou, B., M. Hampsch-Woodill, J Flanagan, E. K. Deemer, R. L. Prior, and D. Huang. 'Novel Fluorometric Assay for Hydroxyl Radical Prevention Capacity Using Fluorescein as the Probe', *J. Agric. Food Chem* Vol. 50, 2772-2777, 2002.

Pelicano, H., D. Carney, and P. Huang. 'Ros Stress in Cancer Cells and Therapeutic Implications.', *Drug Resist Updat* Vol. 7, No. 2, 97-110, 2004.

Piga, Antonio. 'Cactus Pear: A Fruit of Nutraceutical and Functional Importance', *Journal of the Professional Association of Cactus Development*, 9 - 22, 2004.

Piñeiro, Z., M. Palma, and C. G. Barroso. 'Determination of Trans-Resveratrol in Grapes by Pressurised Liquid Extraction and Fast High-Performance Liquid Chromatography.', *J Chromatogr A* Vol. 1110, No. 1-2, 61-5, 2006.

Promega Corporation. 'Celltiter 96® Aqueous One Solution Cell Proliferation Assay', In Promega Corporation, 2009.

Rajamanickam, S., and R. Agarwal. 'Natural Products and Colon Cancer: Current Status and Future Prospects.', *Drug Dev Res* Vol. 69, No. 7, 460-471, 2008.

Ramadan, Mohamed Fawzy, and Jorg-Thomas Morsel. 'Oil Cactus Pear (Opuntia Ficus-Indica L.)', *Food Chemistry* Vol. 82, 339–345, 2003.

Ramos, S. 'Cancer Chemoprevention and Chemotherapy: Dietary Polyphenols and Signalling Pathways.', *Mol Nutr Food Res* Vol. 52, No. 5, 507-26, 2008.

Ravizza, R., M. B. Gariboldi, L. Passarelli, and E. Monti. 'Role of the P53/P21 System in the Response of Human Colon Carcinoma Cells to Doxorubicin.', *BMC Cancer* Vol. 4, 92, 2004.

Riganti, C., E. Miraglia, D. Viarisio, C. Costamagna, G. Pescarmona, D. Ghigo, and A. Bosia. 'Nitric Oxide Reverts the Resistance to Doxorubicin in Human Colon Cancer Cells by Inhibiting the Drug Efflux.', *Cancer Res* Vol. 65, No. 2, 516-25, 2005.

Rowley, P. T. 'Inherited Susceptibility to Colorectal Cancer.', *Annu Rev Med* Vol. 56, 539-54, 2005.

Saenz, Carmen. 'Processing Technologies: An Alternative for Cactus Pear (Opuntia Spp.) Fruits and Cladodes', *Journal of Arid Environments* Vol. 46, No. 209–225, 2000.

Sambuy, Y., I. De Angelis, G. Ranaldi, M. L. Scarino, A. Stammati, and F. Zucco. 'The Caco-2 Cell Line as a Model of the Intestinal Barrier: Influence of Cell and Culture-Related Factors on Caco-2 Cell Functional Characteristics.', *Cell Biol Toxicol* Vol. 21, No. 1, 1-26, 2005.

Sarkar, F. H., and Y. Li. 'Using Chemopreventive Agents to Enhance the Efficacy of Cancer Therapy.', *Cancer Res* Vol. 66, No. 7, 3347-50, 2006.

Scalbert, A., C. Manach, C. Morand, C. Rémésy, and L. Jiménez. 'Dietary Polyphenols and the Prevention of Diseases.', *Crit Rev Food Sci Nutr* Vol. 45, No. 4, 287-306, 2005.

Scalbert, A., and G. Williamson. 'Dietary Intake and Bioavailability of Polyphenols.', *J Nutr* Vol. 130, No. 8S Suppl, 2073S-85S, 2000.

Schonn, I., J. Hennesen, and D. C. Dartsch. 'Ku70 and Rad51 Vary in Their Importance for the Repair of Doxorubicin- Versus Etoposide-Induced Dna Damage.', *Apoptosis* Vol. 16, No. 4, 359-69, 2011.

Scordino, M., A. Di Mauro, A. Passerini, and E. Maccarone. 'Adsorption of Flavonoids on Resins: Hesperidin.', *J Agric Food Chem* Vol. 51, No. 24, 6998-7004, 2003.

Seabra, Inês J., Mara Elga Braga, Maria Teresa Batista, and Hermínio C. Sousa. 'Effect of Solvent (Co2/Ethanol/H2o) on the Fractionated Enhanced Solvent Extraction of Anthocyanins from Elderberry Pomace', *J. of Supercritical Fluids* Vol. 54, 145–152, 2010.

Seeram, N. P., M. Aviram, Y. Zhang, S. M. Henning, L. Feng, M. Dreher, and D. Heber. 'Comparison of Antioxidant Potency of Commonly Consumed Polyphenol-Rich Beverages in the United States.', *J Agric Food Chem* Vol. 56, No. 4, 1415-22, 2008.

Serra, Ana Teresa. 'Valorization of Traditional Portuguese Apples and Cherries. Biochemical Characterization and Development of Functional Ingredients', ITQB-UNL/IBET Nutraceuticals and Controlled Delivery Laboratory, 2010.

Singleton, V.L., and J. A. Jr Rossi. 'Colorunetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents.', *Amer. J. Enol. Viticult.* Vol. 16, 144-58, 1965.

Sonee, M., T. Sum, C. Wang, and S. K. Mukherjee. 'The Soy Isoflavone, Genistein, Protects Human Cortical Neuronal Cells from Oxidative Stress.', *Neurotoxicology* Vol. 25, No. 5, 885-91, 2004.

Soto, María Luisa, Andrés Moure, Herminia Domínguez, and Juan Carlos Parajó. 'Recovery, Concentration and Purification of Phenolic Compounds by Adsorption: A Review', *Journal of Food Engineering* Vol. 105, 1-27, 2011.

Sreekanth, D., M. K. Arunasree, K. R. Roy, T. Chandramohan Reddy, G. V. Reddy, and P. Reddanna. 'Betanin a Betacyanin Pigment Purified from Fruits of Opuntia Ficus-Indica Induces Apoptosis in Human Chronic Myeloid Leukemia Cell Line-K562.', *Phytomedicine* Vol. 14, No. 11, 739-46, 2007.

Stintzing, F. C., and R. Carle. 'Cactus Stems (Opuntia Spp.): A Review on Their Chemistry, Technology, and Uses.', *Mol Nutr Food Res* Vol. 49, No. 2, 175-94, 2005.

Stintzing, F. C., K. M. Herbach, M. R. Mosshammer, R. Carle, W. Yi, S. Sellappan, C. C. Akoh, R. Bunch, and P. Felker. 'Color, Betalain Pattern, and Antioxidant Properties of Cactus Pear (Opuntia Spp.) Clones.', *J Agric Food Chem* Vol. 53, No. 2, 442-51, 2005.

Stintzing, Florian C., Andreas Schieber, and Reinhold Carle. 'Phytochemical and Nutritional Significance of Cactus Pear', *Eur Food Res Technol* Vol. 212, 396–407, 2001.

Supino, R. 'Mtt Assays', In O'Hare SM and Atterwill CK. (Eds.), *In in Methods in Molecular Biology: In Vitro Toxicity Testing Protocols*, Humana Press, 1998.

Surh, Y. J. 'Cancer Chemoprevention with Dietary Phytochemicals.', *Nat Rev Cancer* Vol. 3, No. 10, 768-80, 2003.

Swamy, S. M., and B. T. Huat. 'Intracellular Glutathione Depletion and Reactive Oxygen Species Generation Are Important in Alpha-Hederin-Induced Apoptosis of P388 Cells.', *Mol Cell Biochem* Vol. 245, No. 1-2, 127-39, 2003.

Tavares, L., D. Carrilho, M. Tyagi, D. Barata, A. T. Serra, C. M. Duarte, R. O. Duarte, R. P. Feliciano, M. R. Bronze, P. Chicau, M. D. Espírito-Santo, R. B. Ferreira, and C. N. dos Santos. 'Antioxidant Capacity of Macaronesian Traditional Medicinal Plants.', *Molecules* Vol. 15, No. 4, 2576-92, 2010.

Tesoriere (a), L., M. Fazzari, M. Allegra, and M. A. Livrea. 'Biothiols, Taurine, and Lipid-Soluble Antioxidants in the Edible Pulp of Sicilian Cactus Pear (Opuntia Ficus-Indica) Fruits and Changes of Bioactive Juice Components Upon Industrial Processing.', *J Agric Food Chem* Vol. 53, No. 20, 7851-5, 2005.

Tesoriere, L., D. Butera, A. M. Pintaudi, M. Allegra, and M. A. Livrea. 'Supplementation with Cactus Pear (Opuntia Ficus-Indica) Fruit Decreases Oxidative Stress in Healthy Humans: A Comparative Study with Vitamin C.', *Am J Clin Nutr* Vol. 80, No. 2, 391-5, 2004.

Tesoriere, L., M. Fazzari, M. Allegra, and M. A. Livrea. 'Biothiols, Taurine, and Lipid-Soluble Antioxidants in the Edible Pulp of Sicilian Cactus Pear (Opuntia Ficus-Indica) Fruits and Changes of Bioactive Juice Components Upon Industrial Processing.', *J Agric Food Chem* Vol. 53, No. 20, 7851-5, 2005.

Thomas, H., and H. M. Coley. 'Overcoming Multidrug Resistance in Cancer: An Update on the Clinical Strategy of Inhibiting P-Glycoprotein.', *Cancer Control* Vol. 10, No. 2, 159-65, 2003.

Tomás-Barberán, F. A., M. I. Gil, P. Cremin, A. L. Waterhouse, B. Hess-Pierce, and A. A. Kader. 'Hplc-Dad-Esims Analysis of Phenolic Compounds in Nectarines, Peaches, and Plums.', *J Agric Food Chem* Vol. 49, No. 10, 4748-60, 2001.

Utkarsha, U. Shedbalkar, S. Adki Vinayak, P. Jadhav Jyoti, and A. Bapat Vishwas. '*Opuntia* and Other Cacti: Applications and Biotechnological Insights', *Tropical Plant Biol.* Vol. 3, 136-150, 2010.

Vainio, H., and E. Weiderpass. 'Fruit and Vegetables in Cancer Prevention.', *Nutr Cancer* Vol. 54, No. 1, 111-42, 2006.

van der Woude, H., A. Gliszczyńska-Swigło, K. Struijs, A. Smeets, G. M. Alink, and I. M. Rietjens. 'Biphasic Modulation of Cell Proliferation by Quercetin at Concentrations Physiologically Relevant in Humans.', *Cancer Lett* Vol. 200, No. 1, 41-7, 2003.

Vincent, T. L., and R. A. Gatenby. 'An Evolutionary Model for Initiation, Promotion, and Progression in Carcinogenesis.', *Int J Oncol* Vol. 32, No. 4, 729-37, 2008.

WCRF/AICR. 'World Cancer Research Fund / American Institute for Cancer Research. Food, Nutrition, Physical Activity, and the Prevention of Cancer: A Global Perspective', 2007.

WCRF/AICR, (b). 'Continuous Update Project Interim Report Summary. Food, Nutrition, Physical Activity, and the Prevention of Colorectal Cancer' ', World Cancer Research Fund / American Institute for Cancer Research, 2011

Wolfe, K. L., and R. H. Liu. 'Cellular Antioxidant Activity (Caa) Assay for Assessing Antioxidants, Foods, and Dietary Supplements.', *J Agric Food Chem* Vol. 55, No. 22, 8896-907, 2007.

Xu, B. J., and S. K. Chang. 'A Comparative Study on Phenolic Profiles and Antioxidant Activities of Legumes as Affected by Extraction Solvents.', *J Food Sci* Vol. 72, No. 2, S159-66, 2007.

Yoon, JA., SW. Hahm, J. Park, and YS. Son. 'Total Polyphenol and Flavonoid of Fruit Extract of *Opuntia Humifusa* and Its Inhibitory Effect on the Growth of Mcf-7 Human Breast Cancer Cells', *J Korean Soc Food Sci Nutr* Vol. 38, 1679–1684, 2009.

Young, G. P., Y. Hu, R. K. Le Leu, and L. Nyskohus. 'Dietary Fibre and Colorectal Cancer: A Model for Environment--Gene Interactions.', *Mol Nutr Food Res* Vol. 49, No. 6, 571-84, 2005.

Zeisel, S. H. 'Regulation Of "Nutraceuticals".', *Science* Vol. 285, No. 5435, 1853-5, 1999.

Zhishen, Jia, Tang Mengcheng, and Wu Jianming. 'The Determination of Flavonoid Contents in Mulberry and Their Scavenging Effects on Superoxide Radicals', *Food Chemistry* Vol. 64, 555-559, 1999.

Zitka, O., J. Sochor, O. Rop, S. Skalickova, P. Sobrova, J. Zehnalek, M. Beklova, B. Krska, V. Adam, and R. Kizek. 'Comparison of Various Easy-to-Use Procedures for Extraction of Phenols from Apricot Fruits.', *Molecules* Vol. 16, No. 4, 2914-36, 2011.

Zou, D. M., M. Brewer, F. Garcia, J. M. Feugang, J. Wang, R. Zang, H. Liu, and C. Zou. 'Cactus Pear: A Natural Product in Cancer Chemoprevention.', *Nutr J* Vol. 4, 25, 2005.

6. Appendix

Appendix A – Cellular viability assays

- **CellTiter 96® AQueous One Solution Cell Proliferation Assay**

The CellTiter 96® AQueous One Solution Cell Proliferation Assay is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. This assay contain the MTS tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), which is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium (Promega Corporation, 2009). Briefly, after removed the medium was added 100 µL of a MTS solution (16.6% MTS diluted 10x and 83,3% of RPMI medium 0.5%FBS) to each well. After 4 hours at 37ºC in a humidified, 5% CO2 atmosphere the absorbance was read in a 96-well plate reader at 490nm (Powerwave XS Microplate Spectrophotometer, Biotek, Winooski, USA). The quantity of formazan product measured by the absorbance at 490nm is directly proportional to the number of living cells in culture.

- **CyQUANT Cell Proliferation Assay Kit**

The CyQUANT Cell Proliferation Assay Kit provides a fast, sensitive, and convenient method both for counting cells in a population and for measuring their proliferative activity. The CyQUANT assay uses a green-fluorescent CyQUANT® GR dye, which exhibits strong fluorescence enhancement when bound to cellular nucleic acids (Invitrogen, 2006). Briefly, the culture medium was removed and wash 2X with phosphate buffered saline, pH=7.4 (PBS). The cells were freeze in the microplate and store at -70ºC until samples are to be assayed. The freezing step is important for efficient cell lysis in the CyQUANT assay. One day after, microplates were thaw at room temperature and was added 200 µL of the CyQUANT® GR dye/cell lysis buffer to each sample well and mixed gently. Samples were incubated ate room temperature for 2-5 minutes, protected from light. The fluorescence was measured on a FL800 microplate fluorescent reader (Bioteck Instruments, USA) with filters appropriate for ~480nm excitation and ~520 nm emission maxima.

- **MTT**

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) is reduced to a purple formazan product by mitochondrial reductase enzymes active cell viability in viable cells and therefore the amount of formazan product is proportional to the number of viable cells. Briefly, the medium was removed and 100 µL of the colorimetric reagent MTT (0.5mg/mL) was added to each well and the microplate was incubated for 4 hours. The reaction was stopped with

DMSO (150µL/well) and formazan was quantified by measurement of the absorbance at 570 nm in a microplate reader Spectrophotometer Powerwave XS, Biotek Instruments (Winooski, USA)