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O efeito dos extractos de cereja em células humanas do cancro da próstata:
Do cultivo à clínica?

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Dedicatória

Existe um número quase embaraçoso de pessoas sem o apoio das quais eu não teria moral, nem financiamento, nem tampouco a paciência e força de vontade necessárias para escrever qualquer tipo de dissertação.

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“We do not have to visit a madhouse to find disordered minds; our planet is the mental institution of the universe.”

-Johann Wolfgang von Goethe

“Do not wait to strike till the iron is hot; but make it hot by striking.”

-W.B. Yeats

“Fear not for the future, weep not for the past.”

-Percy Bysshe Shelley

Resumo

A proliferação descontrolada das células da próstata é uma condição observada normalmente na população masculina à medida que esta envelhece, e está associada com o desenvolvimento do cancro da próstata. Assim, estratégias que visem diminuir a proliferação dos tecidos prostáticos durante o seu envelhecimento deverão diminuir a prevalêcia desta condição médica. As cerejas são ricas em compostos fenólicos como as antocianinas, com efeitos antioxidantes e anti-proliferativos bem estudados em células cancerígenas como as MCF-7 (cancro da mama) e HCT116 (cancro colo-rectal). O estudo elaborado para esta dissertação pretendeu avaliar o efeito de extractos da cereja *Saco*, uma variedade local da região do Fundão, na viabilidade, apoptose, e metabolismo glicolítico das células não-neoplásicas do epitélio da próstata PNT1A, nas células neoplásicas androgeno-dependentes LNCap, e nas células neoplásicas androgeno-independentes PC3. As cerejas foram descaroçadas, liofilizadas, e a sua extracção efectuada recorrendo a ultrassons e utilizando metanol acidificado com 0,1% HCl como solvente. O extracto obtido foi então dissolvido em meio de cultura celular a várias concentrações (0, 2, 20, 200 e 2000 µg/mL) e utilizado para tratar as células não-neoplásicas e neoplásicas durante 48, 72 e 96 horas. A viabilidade celular nas diferentes condições experimentais foi determinada por ensaios MTT. Globalmente, a viabilidade celular mais baixa foi observada às 72 horas e com 20 µg/mL de extracto de cereja, e estas condições foram utilizadas para avaliar os efeitos dos extractos de cereja na proliferação,apoptose e metabolismo glicolítico das células PNT1A, LNCaP e PC3. A análise à expressão génica e a actividade dos reguladores-alvo destes processos biológicos foram efectuadas através da metodologia de Western Blots e kits de análises bioquimicas, respectivamente. O consumo de glucose e a produção de lactato foram ambos medidos por análise espectofotométrica. Os resultados obtidos demonstraram que a administração dos extractos metanólicos de cereja aumentou a apoptose das células LNCaP, um efeito não-confirmado nas células não-neoplásicas PNT1A. Curiosamente, as células PC3 mostraram uma diminuição da actividade apoptótica na presença dos extractos de cereja, apesar da proliferação diminuída. Quanto ao metabolismo glicolítico, foi observado que as células PNT1A tratadas com extractos de cereja apresentaram uma diminuição parcial deste, enquanto que as células LNCaP demonstraram uma supressão da metabolização da glucose em resposta aos extractos de cereja, com consumo de glucose e produção de lactato ambos diminuídos, acompanhados por alterações da expressão/actividade de transportadores e enzimas glicolíticas. Analogamente aos resultados da apoptose, as células PC3 apresentaram um comportamento distinto das LNCaP relativamente ao metabolismo glicolítico; os extractos de cereja induziram um aumento da glicólise e da produção de lactato. Os presentes resultados indicam que os extractos metanólicos de cereja podem ter efeitos benéficos nas células do cancro da próstata, tanto neoplásicas como não-neoplásicas, diminuindo a sua proliferação e controlando as vias apoptóticas e glicolíticas, produzindo efeitos mais visíveis no caso das células sensíveis à ação dos androgénios, LNCaP.

Palavras-chave

Antocianinas, Apoptose, Cancro da Próstata, Cerejas, Metabolismo Glicolítico, Proliferação

Resumo Alargado

A proliferação descontrolada das células da próstata é uma condição observada normalmente na população masculina à medida que esta envelhece, estando associada com o desenvolvimento do cancro da próstata. Este último é uma das causas de morte oncológica mais frequente nos indivíduos do sexo masculino, com mais de um milhão de mortes e 300.000 novos casos anuais. O envelhecimento geral da população, entre vários outros factores de risco, também contribui para um aumento anual dos ritmos de incidência desta doença.

Assim, estratégias preventivas, que visem diminuir a proliferação dos tecidos prostáticos durante o seu envelhecimento, evitando a progressão de condições como a hiperplasia prostática benigna e posteriormente cancro da próstata, deverão diminuir a prevalêcia desta condição médica de difícil tratamento.

As cerejas são ricas em compostos fenólicos como as antocianinas, com efeitos antioxidantes e anti-proliferativos bem estudados em células cancerígenas como as MCF-7 (cancro da mama) e HCT116 (cancro colo-rectal), além de vários outros benefícios associados à prevenção e atenuamento dos efeitos do cancro e a outras patologias diversas. São também frutos populares, produzidos e consumidos um pouco por todo o mundo.

O estudo elaborado para esta dissertação pretendeu avaliar o efeito de extractos da cereja *Saco*, uma variedade bastante consumida e local da região do Fundão, na viabilidade, apoptose, e metabolismo glicolítico das células não-neoplásicas do epitélio da próstata PNT1A, nas células neoplásicas androgeno-dependentes LNCap, e nas células neoplásicas androgeno-independentes, e comparativamente mais agressivas, PC3.

As cerejas foram descaroçadas, liofilizadas, e a sua extracção foi efectuada recorrendo a ultrassons e usando metanol acidificado com 0,1% HCl como solvente. O extracto obtido foi então dissolvido em meio de cultura celular a várias concentrações (0, 2, 20, 200 e 2000 µg/mL) e utilizado para tratar as células não-neoplásicas e neoplásicas durante 48, 72 e 96 horas. A viabilidade celular nas diferentes condições experimentais foi determinada por ensaios MTT. A viabilidade celular variou consideravelmente entre as linhas celulares, mesmo para as mesmas concentrações e os mesmos periodos de tempo, mas globalmente, os extratos diminuíram a viabilidade celular, sendo esta consistentemente mais baixa em todas as linhas às 72 horas e com 20 µg/mL de extracto de cereja. Estas condições foram então utilizadas para avaliar os efeitos dos extractos de cereja na proliferação, apoptose e metabolismo glicolítico das células PNT1A, LNCaP e PC3. A análise da xpressão génica foi efectuada através da metodologia de Western Blot normalizando a quantidade de proteína com a marcação da ß-actina. A atividade dos reguladores chave da apoptose e metabolismo foi avaliada por ensaios bioquímicos usando kits específicos. O consumo de glucose e a produção de lactato foram medidos por análise espectrofotométrica .

Os resultados obtidos demonstraram que a administração dos extractos metanólicos de cereja aumentou a apoptose das células LNCaP pela via intrínseca, com um aumento no rácio BAX/Bcl-2 e uma maior expressão das caspases 9 e 3. O efeito nas células não-neoplásicas PNT1A foram menos concretos, com um aumento da expressão do gene supressor tumoral p53 mas sem alteração na expressão das caspases-9 e 3. A caspase-8, envolvida na via extrínseca, não se apresentou alterada em respostas aos extratos de cerejas em nenhuma das linhas. Curiosamente, as células PC3 mostraram uma diminuição da actividade apoptótica na presença dos extractos de cereja, com uma diminuição da expressão das caspases 9 e 3, apesar da proliferação diminuída.

Quanto ao metabolismo glicolítico, foi observado que as células PNT1A tratadas com extratos de cereja apresentaram uma diminuição parcial deste, evidente pela diminuição da expressão dos GLUTs 1 e 3 e das enzimas PFK-1 e LDH, apesar de um aumento da produção de lactato. As células LNCaP demonstraram uma supressão da metabolização da glicose em resposta aos extratos de cereja, com uma diminuição da expressão do GLUT3 e MCT4, e com o consumo de glucose e produção de lactato ambos diminuídos. Analogamente aos resultados da apoptose, as células PC3 apresentaram um comportamento distinto das LNCaP relativamente ao metabolismo glicolítico; os extractos de cereja induziram um aumento da glicólise e da produção de lactato, apesar de uma diminuição da expressão da PFK-1 e da actividade da LDH. Os presentes resultados indicam que os extractos metanólicos de cereja podem ter efeitos benéficos nas células do cancro da próstata, tanto neoplásicas como não-neoplásicas, diminuindo a sua proliferação e controlando as vias apoptóticas e glicolíticas, produzindo efeitos mais visíveis no caso das células LNCaP sensíveis à ação dos androgénios.

Abstract

The uncontrolled proliferation of prostate cells is a condition commonly observed in the ageing male population, which is associated with the development of prostate cancer. Thus, strategies to counteract the proliferative feature of aging prostatic tissues should prove beneficial by diminishing the widespread prevalence of this condition. Sweet cherries are rich in phenolic compounds such as anthocyanins, with widely studied anti-proliferative and antioxidant effects in different types of cancer cells such as MCF-7 (human breast) and HCT116 (human colorectal). The present study aimed to evaluate the effect of crude extracts of *Saco* sweet cherry, a local cultivar from the Fundão region (Portugal), on the viability, apoptosis and glycolytic metabolism of non-neoplastic PNT1A prostate epithelial cells, and androgen-sensitive LNCaP cells and androgen-insensitive PC3 prostate cancer cells. Sweet cherries were seeded, freeze-dried, and underwent ultrasound-assisted extraction with methanol acidified with HCl 0.1%. The extract was then dissolved in cell culture medium at several concentrations (0, 2, 20, 200 and 2000 µg/mL) and used to treat non-neoplastic and neoplastic prostate cells for 48, 72 and 96 hours. Cell viability at different experimental conditions was determined by MTT assay. Overall, the lowest viability was observed at 72 hours of culture with 20 µg/mL of cherry extract, and these conditions were used to evaluate the effect of cherry extracts on the cell proliferation, apoptosis and glycolytic metabolism of PNT1A, LNCaP and PC3 cells. Gene expression analysis and activity of target regulators of the aforementioned biological processes was assessed by means of Western blot and biochemical assays, respectively. Glucose consumption and lactate production were measured spectrophotometrically. The obtained results demonstrated that the administration of methanolic cherry extracts increased the apoptotic rate of LNCaP cells, an effect not confirmed in the non-neoplastic PNT1A cells. Curiously, PC3 cells had an overall down-regulation of apoptotic activity in the presence of cherry extracts, in spite of its decreased proliferation. Concerning metabolism, it was found that treated PNT1A cells had a partial down-regulation of glycolytic metabolism, while LNCaP cells markedly displayed a suppression of this process with decreased glucose consumption and lactate production, which was accompanied by altered expression/activity of glycolytic transporters and enzymes. Analogously with the results of apoptosis, PC3 cells had a distinct behavior relatively to LNCaP in what concerns glycolytic metabolism; cherry extracts induced an up-regulation of glycolysis and lactate production. The present findings indicate that cherry methanolic extracts may have beneficial effects on prostate cells, both neoplastic and non-neoplastic, diminishing proliferation and controlling the apoptotic and glycolytic pathways, producing more visible effects in the case of the androgen-sensitive LNCaP cells.

Keywords

Anthocyanins, Apoptosis, Glucose Metabolism, Prostate Cancer, Proliferation, Sweet Cherries.

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Acronym List

1,3BPG	1,3-Bisphosphoglyceric Acid
2PG	2-Phosphoglycerate
3PG	3-Phosphoglyceric acid
Akt	Protein Kinase B
ALT	Alanine Transaminase
ASCT2	ASC amino-acid Transporter 2
ATP	Adenosine Triphosphate
BAK	Bcl-2 Homologous Antagonist Killer
BAX	Apoptosis Regulator BAX
Bcl-2	B-cell Lymphoma 2
BMI	Body Mass Index
BPH	Benign Prostatic Hyperplasia
C3G	Cyanidin-3-Glucoside
DHAP	Dihydroxyacetone Phosphate
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ECL	Electrogenerated Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ErbB2	Receptor tyrosine-protein kinase erbB-2
F-6-P	Fructose 6-Phosphate
FADD	Fas-Associated Protein with Death Domain
FasL	FAS Ligand
FasR	FAS Receptor
GADP	Glyceraldehyde 3-Phosphate
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GLS	Glutaminase
GLUT	Glucose Transporter
HCl	Hydrochloric Acid
Il-6	Interleukin 6
LDH	Lactate Dehydrogenase
LNCaP	Lymph Node Carcinoma of the Prostate
MAPK	Mitogen-activated Protein Kinase
MCP-1	Monocyte Chemoattractant Protein-1
MCT	Monocarboxylate Transporter
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NADH	Nicotinamide Adenine Dinucleotide (reduced)
NF-kB	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
NKX-3.1	Homeobox protein Nkx-3.1
P53	Tumour Suppressor p53
PBS	Phosphate-buffered Saline solution
PCNA	Proliferating Cell Nuclear Antigen
PFK-1	Phosphofructokinase 1
PGI	Glucose-6-Phosphate Isomerase

PGK	Phosphoglycerate Kinase
PGM	Phosphoglycerate mutase
PI3K	Phosphoinositide 3-kinase
PIN	Prostatic Intraepithelial Neoplasia
PMSF	Phenylmethylsulfonyl Fluoride
PPO	Polyphenol Oxidase
PSA	Prostate-specific Antigen
PTEN	Phosphatase and Tensin Homolog
PUMA	p53 Upregulated Modulator of Apoptosis
PVDF	Polyvinylidene Fluoride
RIPA buffer	Radioimmunoprecipitation Assay buffer
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute medium
SDS	Sodium Dodecyl Sulfate
T2D	Type-2 Diabetes
TNF	Tumor Necrosis Factor
TRAIL	TNF-related Apoptosis-inducing Ligand
WR	Working Reagent

Chapter 1 - Introduction

1.1 The anatomy and physiology of prostate

The prostate is an accessory exocrine gland of the male reproductive tract. In a healthy human male prostate has a walnut shape and size. It is located dorsally in regards to the pubic symphysis, anterior to the rectum (being detectable via a rectal examination), and immediately below the urinary bladder. Prostate gland surrounds a portion of the urethra known as the prostatic urethra, and merges it with the two ejaculatory ducts (Fig. 1) [1, 2].

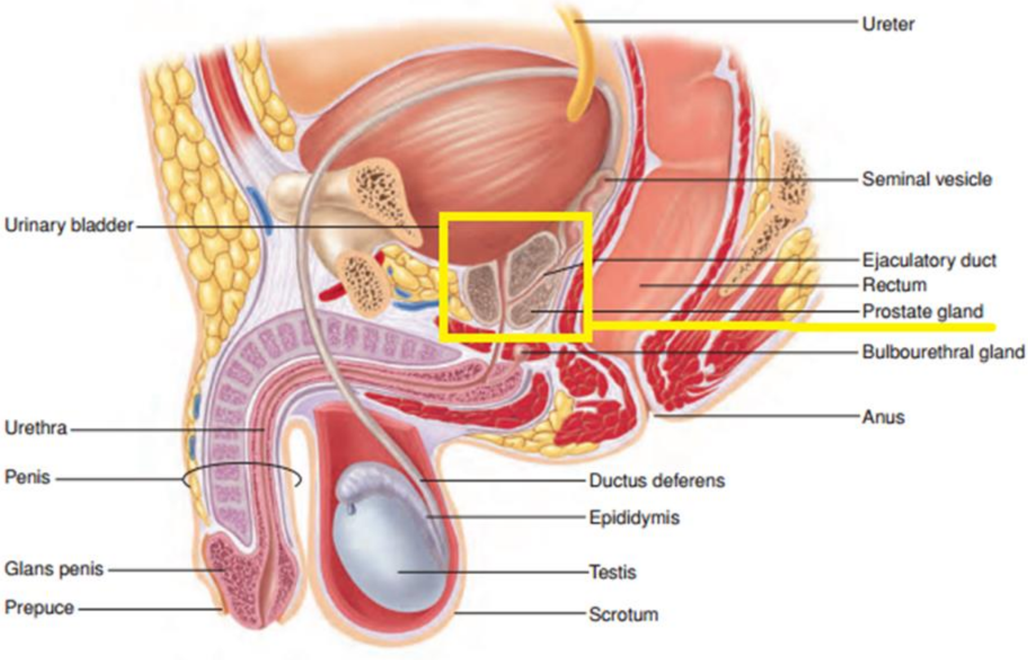


Figure 1 - Sagittal view showing the male reproductive structures. Modified from Seeley's Essentials of Anatomy and Physiology, p.532 [3].

Structurally, prostate can be described as a capsule of fibrous consistency, enveloping smooth muscle cells and a variety of nerves, with a system of veins that provides the entire organ with the necessary nutrients. It can be divided into anterior and apical regions, encompassing the anterior fibromuscular stroma (the anterior part of the prostatic capsule), and the posterior and lateral regions [4].

According to the model proposed by John E. McNeal in 1968, the prostate can be separated into 3 functionally and histologically-different separate zones: i) a central zone, which also comprises the area that envelops the ejaculatory ducts; ii) a transitional zone, enveloping the proximal portion of the prostatic urethra; and iii) a larger peripheral zone that occupies the bulk of the organ and surrounds the aforementioned central zone, contains the urethral ducts and is conical in shape [1, 4-6]. Although not unanimously, this model is generally accepted. Interestingly, the vast majority of prostate cancer cases (75-85%) seems to originate in the peripheral zone, suggesting this area of the prostate is naturally more susceptible to the mutations that might originate the disease [7]. Lastly, there is a "fourth" region that is constituted of fibromuscular stroma and not glandular tissue, adjacent to the bladder and enveloping part of the urethra [5, 8].

In a developmental perspective, the prostate is extremely small in children (roughly 1.2 mL in volume) and ellipsoidal in shape, and its growth starts occurring at puberty reaching the full size in adult. The mass of the prostate is roughly stabilized until the male enters the 30s, when the organ has a tendency to once again start gaining mass, resulting in a condition known as benign prostatic hyperplasia (BPH). This condition affects a sizeable amount (as much as 50%) of the male population aged 51-60 and may cause adverse effects such as several urinary deregulations. BPH is predicted to become a larger issue as the world population has been ageing on average, especially since this growth of the prostate could in many cases evolve into prostate cancer [9–12].

At the cellular level, the prostatic epithelium has four main constituents: i) secretory epithelial cells (mostly luminal cells); ii) stem cells; iii) basal cells and iv) neuroendocrine cells, which promote the growth of the organ. The extra-cellular matrix is non-cellular in nature and contains connective tissue [4, 13].

A correct function of all these components ensure proper prostatic gland activity, which is important to semen constitution and sperm motility. The aforementioned secretory cells produce, in volume, what amounts to roughly 30% of the complete seminal fluid, and are responsible both for maintaining the alkaline pH that protects the sperm from the acidic environment of the female reproductive system, and for providing nutrients and several additional factors such as calcium, zinc, spermine, profibrinolysin. The prostatic fluid also contains clotting enzymes that ensure their good functioning and survival in male and female reproductive systems. [4, 14–16].

Also important is the fact that the prostate produces and releases the prostate-specific antigen (PSA), a protein that is normally found in seminal fluid, but whose concentrations in the bloodstream is used as an indicative of prostatic disease [17].

1.2 Prostate cancer: The overall picture

Prostate cancer is one of the most frequent forms of the oncological diseases, being the overall most frequent in men, and one of the leading causes of cancer death. Incidence is estimated to be over 1.1 million new cases and 300,000 prostate cancer-related deaths a year, and the fact that the average age at which the disease is detected is above 60 means that this prolonged but steady progression makes it a difficult condition to diagnose in a correct and timely fashion, which is troubling as the incidence of the disease is increasing as the population ages. Also notable is the fact that both incidence and mortality associated with prostate cancer have been shown to be significantly higher among males of African descendent, though this factor cannot be completely attributed to race alone [18]. The ratio between the increases in incidence and mortality in men of African descent also seems to be disproportionate, with an increase in incidence of under 60% translating into an increase in mortality of 100-200% [19].

In the particular case of Portugal, the latest estimates indicate that by 2020 there will be an expected yearly occurrence of 8600 new cases and 1700 deaths due to prostate cancer. Yet, both in the case of Portugal and the world, mortality associated with prostate cancer seems to be steadily decreasing over time while incidence increases [18, 20].

Also of note is the fact that the study conducted on Portuguese prostate cancer statistics also showed that there is a significant difference in both incidence and mortality (with the former actually decreasing in areas to the north of the country annually) across separate regions of the country [20]. This geographic difference concurs with the global study, as these different regions also differ in their access to adequate medical care.

Prostate cancer is also a highly heterogeneous disease with several associated factors which aren't completely understood. For example, unlike the cases for all other known forms of cancer, the risk of developing prostatic cancer significantly decreases in patients suffering from, or more genetically susceptible to, Type-2 diabetes (T2D) despite studies proclaiming that patients with T2D have an increased risk of prostate cancer mortality [21], [22, 23].

Nevertheless, the main risk factors for prostate cancer, aside from the aforementioned race/ethnicity factors and aging, are hereditary factors, which are considered to be the overall greatest determining factor in both the incidence and expected survivability of patients [24, 25]. Part of this risk is the aforementioned propensity for the prostate to grown with age and the development of BPH, which despite not being an indicative of the onset prostate cancer by itself, has been identified as a possible causal factor for carcinogenesis [26].

Aside from these set factors, there are also other possible risk factors, which have less to do with factors beyond the control of the patient and are more behavioral in nature. Perhaps the most obvious of these factors is the diet, both as a preventive behavior and as a risk factor, since excessive consumption of foods containing saturated fats and red meats seems to be associated with an increased risk of prostate cancer while a diet rich in flavonoids and other similar antioxidant compounds is associated with a decreased risk. Curiously, data regarding the association of obesity, and by extension sedentary routine, and high BMI and prostate cancer risk seems to be inconclusive and even conflicting [27, 28]. However, it is clearly accepted that an obesity condition is associated with poor prognosis and reduced survival times [29].

Sexual behavior and exposure to sexually transmitted diseases also have been implicated as risk factors for prostate cancer [30]. Other risk factors rise from environmental exposure, such as an increase in prostate cancer risk was associated with frequent and routine contact with pesticides, possibly in conjunction with other agricultural occupations [31]. Lastly, chronic inflammation, which can originate from a variety of factors such as disease and exposure to noxious environmental agents, is estimated to be the cause of roughly 20% of all cancer cases, among them prostate cancer [32]. The mechanism through which inflammation drives carcinogenesis is related with an increase in the differentiation from basal to luminal cells, suggesting that the prostate cancer cases augmented by inflammation are basal in nature [32, 33].

Due to its asymptomatic nature, early-detection of prostate cancer is essential. There are two main methods of screening, a physical examination known as a digital rectal examination, and a biochemical examination of the serum content of PSA [34, 35]. It is important to note that neither of these methods of examination can effectively compensate for the other, and that there is a possibility that the PSA test can produce a false positives [36]. For this reason, new tests, such as the 4kscore blood examination that tests four biomarkers instead of just one [37] are being tested. Nevertheless, all these approaches produce preliminary results, with more advanced and indispensable examinations such as a transrectal ultrasound and, a biopsy, being needed to ascertain the presence of cancer tissue with certainty [38].

Finally, there is a wide variety of possible therapies for the treatment of prostate cancer that are dependent on the disease's stage of progression. If the tumour is small, localized and deemed low-risk, a monitoring strategy called "Watchful Waiting", is selectively employed today [39]. General pre-metastatic options include androgen-deprivation therapy, radical prostatectomy, prostate brachytherapy and external beam radiation therapy [40]. Prostate cancer in more advanced, metastatic stages is usually treated with first and second-line hormonal therapy, radiotherapy and chemotherapy [41]. Still, the reliability of these therapies is not overly high, and thus the delineation of innovative therapies is always desirable.

1.2.1 The development of prostate cancer

Prostate cancer generally develops in the basal epithelial compartment of the prostate, more specifically in the transit-amplifying population, a group of slow turnover stem cells. These cells express androgen receptors, and respond to androgen stimuli differentiating into luminal cells [42].

Developmentally, prostate cancer progresses slowly through a variety of developmental stages, beginning with mutations and aberrations at genetic level, that include punctual mutations and alternative splicing events, which are considered as one of the reasons for the disease's wide array of possible phenotypes. Neoplastic focus progress to small and then large scale tissue invasions and alterations [43, 44]. Genomic lesions are also very frequent in prostate cancer cases [45]. As the disease slowly advances, the tissues and channels become less and less organized, become more heterogenic and lose their original structures. Aided by extravasation and neovascularization, tumour cells spread out to the surrounding organs and bones and metastasis occurs [43].

At tissue/cellular level, the first event linked to carcinogenesis is the epithelium atrophy into a structure called a proliferative inflammatory atrophy. Aggravations of this stage will result in the abnormal proliferation of the luminal epithelial cells of the prostate, in a condition knowns as prostatic intraepithelial neoplasia (PIN lesion), which is a recognized precursor of prostate carcinoma [46, 47]. High-grade forms of this type of neoplasia have even been shown to share genetic and molecular markers with prostate cancer [47].

Progression of the disease from this stage on, like in the case of all forms of cancer, is not completely comprehended, but there are several pathways and mechanisms that are known to be altered as the cancer evolves and adapts. For example, phosphoinositide-3-kinase (PI3K), an enzyme associated with cell growth and proliferation, is activated in prostate cancer [48]. This is accompanied by a down/regulation and deletion of the important tumour suppressor phosphatase and tensin homolog (PTEN) [48, 49]. Other contributing factors to the uncontrolled initial growth of prostate cancer include the activation/upregulation of mitogen-activated protein kinases (MAPKs), also associated with cell survival, and mutation/deletion of other important tumour suppressors such as NKX-3.1. [50, 51]. The case of NKX-3.1 is particularly noteworthy since this particular suppressor is known to be an indirect down-regulator of PSA [51]. As cancer cells mutate, they start to release a variety of important growth factors that contribute to the perpetuation their growth [43].

The aforementioned inflammatory process associated with several cases of prostate cancer also aids development by the recruitment and migration of leukocytes and macrophages which release reactive oxygen species (ROS) around the area of the inflammation. This will in turn contribute to further DNA damage and mutations, and this will eventually even result in the loss of essential functions in the androgen receptors of prostate cells [52]. Another essential step in the progression and evolution of prostate cancer is its interaction with the stroma/connective tissue, with the latter providing the cancer with a variety of factors that promote growth and invasiveness [53].

Lastly, perhaps one of prostate cancer’s most defining features is its dependence on essential hormonal regulators, androgens. These steroid hormones exert their actions through the mediation of androgen receptors, and control the development of the healthy prostate from youth [54]. Testosterone, the most abundant androgen, is irreversibly metabolized into the more powerful 5 α-dihydrotestosterone (DHT) by the activity of 5α-reductase. Indeed, it is DHT binding to the androgen receptors that promotes a steady development of prostate gland, and controls cell growth and apoptosis [54].

Prostate cancer is, at initial phases, maintained by androgens and androgen receptors, being considered one of the most hormone-sensitive cancers. This makes the early stages of prostate cancer ideal targets for hormone therapy, as they respond very well to it in general [55].However, prostate cancer cases inevitably reach a hormone-resistant stage if the disease progresses far enough [56].

Indeed, given enough time to mutate, prostate cancer will eventually develop a set of complex sensitizations, reaching a stage where androgen receptor activity will subsist (ensuring continuous development) even in case of castration. This stage is known as hormone-refractory prostate cancer, and it marks a stage where hormone therapy loses almost all its effectiveness [57]. It is achieved by a great amplification of both the expression and sensitivity of androgen receptors, meaning they will continue to function, and promoting proliferation, even at extremely low levels of androgen. In fact, activity can be maintained with androgen levels reduced by as 80% [58]. Cells at this stage can even up-regulate their own levels of 5α-reductase in order to maintain high intratissue levels of DHT so they can function more effectively [59]. In some cases, the androgen receptors themselves mutate in order to allow their stimulation by compounds other than androgens, such as estrogens, and even allow for activation by completely separate methods, such as activation through phosphorylation by the aforementioned MAPKs [60, 61]. Co-activators that would impede cancer progression also seem to be able to be downregulated [62].

Finally, evidence also indicates that, in some malignancies, resistant forms of hormone-refractory prostate cancer can originate from mutated, continuously-renewing stem cells [63].

1.2.2 Apoptosis of prostate cancer cells

In normal, healthy conditions, cell tissue balance is heavily-regulated by apoptotic cell death mechanisms. Apoptosis can be triggered by two pathways, the intrinsic and extrinsic (Fig. 2), i.e., dependent and independent on the release of cytochrome-c from the mitochondria. However, the activation of apoptosis executioner caspase-3 is an end-point in both of these pathways [64].

Essentially, and in simplified terms that better fit the scope of this dissertation, the intrinsic mechanism is initiated through the accumulation of tumour-suppressor p53 in the cell in response to DNA damage and other stress factors (in a mechanism that is also associated with the process of aging) [65]. This will, in turn, up-regulate the expression of other apoptosis regulators, such as BAX, B-cell lymphoma 2 (Bcl-2) homologous antagonist killer (BAK) and PUMA [65, 66]. These apoptosis regulators, especially BAX and BAK, will change their conformation, oligomerize, and be attracted to the mitochondria inducing the creation apoptotic pores in mitochondrial membrane. This will release one of the designated main "killing factors" of the cell, the enzyme cytochrome-c, alongside with another pro-apoptotic protein, Smac/DIABLO, which will aid in caspase activation [67]. The release of cytochrome-c will form a large quaternary protein structure called the apoptosome, resulting in the activation of caspase-9 via cleavage of pro-caspase-9. The activation of initiator caspase-9 will in turn result in the cleavage and activation of the final apoptotic effector, caspase-3 [68–70].

The extrinsic, or mitochondria-independent apoptotic pathway is initiated at cell membrane. The tumor necrosis receptor family member, FAS receptor (FasR), is activated through trimerization induced by binding of the transmembrane protein Fas-ligand (FasL) [71]. FasR will then, through its intracellular “death domain”, proceed with the FADD (Fas-associated protein with death domain)-mediated recruitment and activation of initiator caspase-8, which will then cleave and activate procaspase-3 into caspase-3 [72, 73]. Caspase-3 also feeds back into caspase-8 activity in a positive feedback loop [73].

Interestingly, these two pathways are not completely independent of each-other with, for example, caspase-8 mediated release of cytochrome-c being achieved via the cleaved death ligand tBID [74]. A simplified model of both pathways is observable in Figure 2.

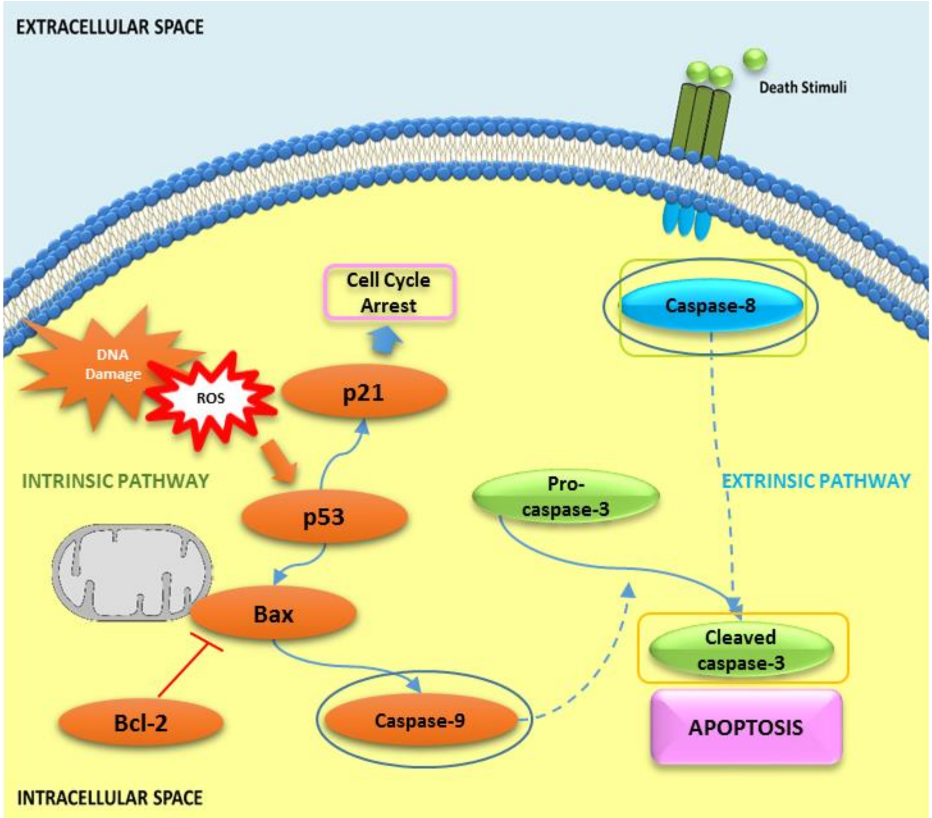


Figure 2- The intrinsic and extrinsic apoptotic pathways. Intrinsic: p53 activation up-regulates Bax, which triggers caspases 9 and 3, resulting in apoptosis. Extrinsic: external death stimuli trigger caspase 8 and 3, resulting in apoptosis.

Cancer cells, display a characteristic resistance to apoptosis and augmented survival rates, which occurs in consequence of the down-regulated expression of these pro-apoptotic factors, usually also associated with the augmented expression of the anti-apoptotic.

Moreover, these features become more pronounced with successive mutations and increasing cancer aggressiveness.

p53 is down-regulated in several forms of prostate cancer, and this pattern is present in roughly 40% of prostate cancer cases [75]. Most noticeably, the aggressive hormone-refractory prostate cancer cell type PC3 is known not to express any quantifiable levels of p53 [76]. BAX expression is also downregulated, though in this case it is directly downregulated by another protein, Bcl-2, an anti-apoptotic protein located in the membrane of the mitochondria and a promoter of cell survival (Fig. 2). This protein is not commonly found in measurable amounts in healthy prostatic tissue, and is only found in very high levels in several cases of prostate cancer (in particular in cases of hormone-refractory prostate cancer), with the ratio of expressed BAX/Bcl-2 being considered a direct indicator of caspase-3 activity and apoptotic cell death [77–79].

Prostate cancer cells also mutate in order to curb the activation of the extrinsic apoptotic pathway, becoming more insensitive to Fas-mediated apoptosis. This kind of apoptosis is notoriously difficult to induce in these cells, with attempts being made with virus infection and FasL agonists [80, 81]. Interestingly, when activation of this pathway is successful, the resulting cytotoxicity causes very high rates of apoptosis, which suggests that the mutations that prostate cancer undergoes do not have a great impact on the effectiveness of this pathway despite hampering their activation [80, 81].

1.2.3 Glycolytic metabolism of prostate cancer cells

Prostate cancer cells, as other cancer cell types, are known by its ability to modify and adapt several metabolic pathways in order to fulfil their energy needs. This includes quantifiable alterations to the metabolism of glucose, glutamine, and lipids [82–85].

In a healthy cell, the glycolytic process is tightly regulated and consists of three stages, the first of which involves glucose uptake from the extracellular space. The importation of glucose across the plasma membrane occurs via the glucose transporters (GLUTs) family of membrane proteins. GLUT1 and GLUT3 isoforms in particular are present across all mammalian cells and are responsible for a considerable amount of the basal glucose uptake [86]. Once inside the cell, this glucose is converted into pyruvate through a chain of reactions – glycolysis (Fig. 3). Firstly, glucose is phosphorylated into glucose-6-phosphate (consuming one molecule of ATP) through the action of the enzyme hexokinase. Glucose-6-phosphate is then converted, in a reversible reaction, into fructose-6-phosphate (F-6-P) through the action of the enzyme phosphoglucose isomerase (PGI). F-6-P in turn is phosphorylated into fructose 1.6-biphosphatase through the action of the enzyme phosphofructokinase-1 (PFK-1). This glycolytic step is of particular importance on the entire process, especially for studies analysing the regulation and good functioning of glycolysis, as PFK-1 is considered to be the main rate-limiting enzyme for the entire glycolytic process [86, 87].

The second stage involves the cleavage of the six-carbon fructose in the fructose 1.6-biphosphatase molecule in order to generate two separate molecules. This is achieved by the enzyme aldolase, and it generates D-glyceraldehyde 3-phosphate (GADP) and dihydroxyacetone phosphate (DHAP). The enzyme triose phosphate isomerase will then convert the DHAP molecule into a second GADP molecule, thus meaning the final glycolytic stage will occur twice [86].

The third and final glycolytic step is characterized by its generation of ATP, known as its “payoff” phase. One GADP molecule will be oxidised by the enzyme glyceraldehyde phosphate dehydrogenase (GAPDH) into D-1,3-bisphosphoglycerate (1,3BPG). One of the phosphate groups from 1,3BPG will then be transferred to an ADP molecule by the enzyme phosphoglycerate kinase (PGK) in order to generate the process’ first molecule of ATP and one molecule of 3-phosphoglycerate (3PG) [86].

The 3PG molecule is isomerized by the enzyme phosphoglycerate mutase (PGM) into 2-phosphoglycerate (2PG). This molecule will then be dehydrated into phosphophenolpyruvate which will, in the last step of the glycolytic process, be converted to pyruvate together with and one final ATP molecule by the action of the enzyme pyruvate kinase [86]. This pyruvate can then be used for several other metabolic pathways, such as the citric acid cycle, which is the common endpoint of this pathway for cells undergoing aerobic respiration, and in normal cellular function this is the pathway given priority to due to its higher ATP yield. The other main possible pathway is the anaerobic pathway, which has a lesser ATP yield and normally occurs in conditions of cellular stress and/or hypoxia. Finally, there are also alternative pathways to pyruvate such as the alanine cycle, which recycles pyruvate back into glucose by shuttling it to the liver [86, 88].

In the anaerobic pathway, pyruvate is converted to lactate by the enzyme lactate dehydrogenase, and is then exported from the cell by monocarboxylate transporters (MCTs), also known as “lactate shuttles”. The MCT4 is the MCTs family member being directly linked to lactate export [89].

It should also be noted that these metabolic pathways are not isolated, and interact with other metabolic systems. For example, glutaminase (GLS), a key enzyme in glutaminolysis that converts glutamine to glutamate, positively regulates glucose uptake [90]. A simplified model is observable in Figure 3.

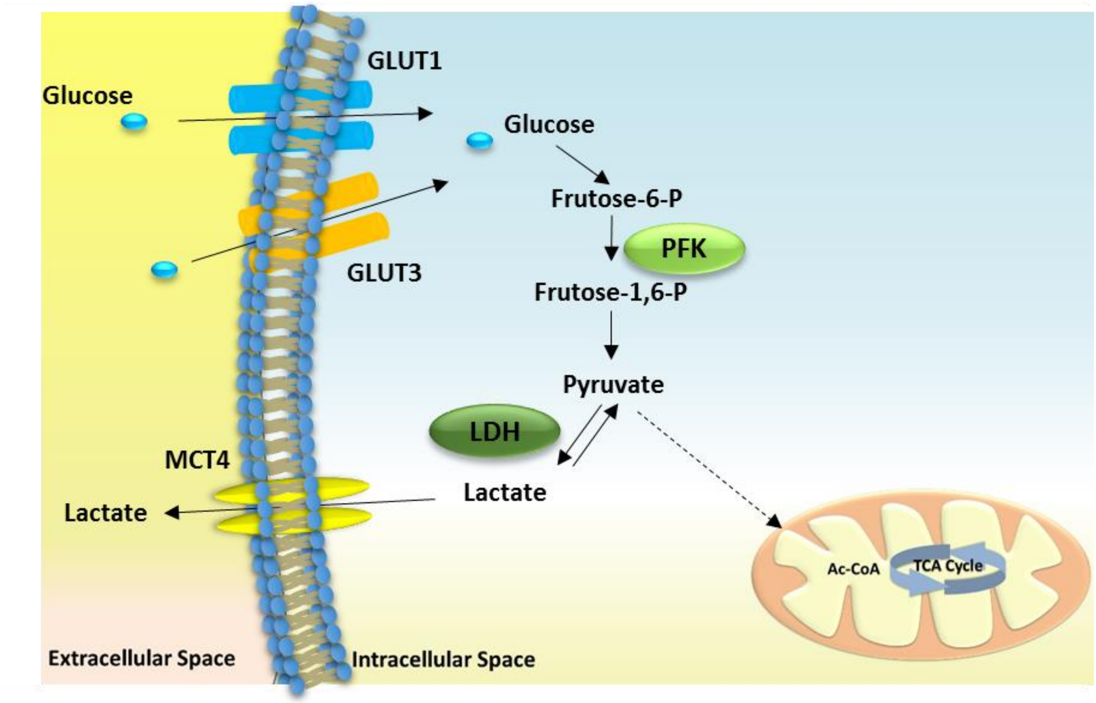


Figure 3 - Glycolysis and the two main possible pathways of pyruvate.

Cancer cells, as is the case of prostate cancer cells, undergo a heavy re-structuring of glycolytic metabolism. The most immediately-noticeable of which is the prioritization of the anaerobic pathway over the transportation of pyruvate to the mitochondria for the citric acid cycle. This happens even in the presence of oxygen, in a phenomenon known as the Warburg Effect [91]. One of the main known reasons for this process being the ability for cancer to use this lactate as fuel to further augment its proliferative capabilities instead of discarding it completely like healthy cells do [92].

As expected from this, more aggressive, androgen-independent strains of prostate cancer produce more lactate when compared to their androgen-dependent counterparts with lesser proliferative capabilities. This is accompanied by increased activity of the enzyme LDH and the transporter MCT4, in order to accommodate this higher lactate production [82].
Not only the prioritization of pathways, but the glycolytic metabolism as a whole seems to be upregulated in prostate cancer cells, with increased expression of the GLUTs and the limiting glycolytic enzyme PFK-1 [93, 94].

1.3 Cherries

Cherries are the generally red-to-yellow drupe fruits of trees of the genus Prunus [95]. They both flower and grow in small bunches and grow in the temperate climates of America, Asia, and especially Europe, though they are grown all over the world in some measure. Despite there being more cherry types than the following overall, the vast majority of edible cherry cultivars are split into sweet cherries (*Prunus avium*) and sour cherries (*Prunus cerasus*).

These two species of cherries do not normally cross-pollinate, though hybrids have been artificially produced [96].

They are a popular fruit consumed all over the world, relatively expensive due to their fragility, and they are very nourishing fruits whose nutritional value is very noticeably impacted, both positively and negatively by the environment they grow in [97, 98].

1.3.1 Chemical composition and biological activities

On a very basic level, cherries can already be recognized as very nutritious (and nutritiously-versatile) fruits, containing a wide array of vitamins, minerals and other dietetically-relevant compounds [97].

They are rich in anthocyanins, a group of pigments that will be described further on, and in hydroxycinnamates, a class of phenolic acids with several biological properties of interest, most notably as strong antioxidants in their own right [97, 99–101].

Other components contained in cherry extracts are, inter alia, carotenoids, which are also pigments with well-established antioxidant potential; melatonin, a hormone widely studied for its positive, therapeutic effects on human sleep patterns, duration and quality across a wide range of ages, as well as other regulatory and immunological benefits. Finally, vitamin C has several modulatory functions in the immune system, associated to building up resistance to several pathogens, though claims of its preventive and therapeutic properties pertaining the common cold are heavily contested and appear to be exaggerated [97, 102–106]. The particular antioxidant properties of vitamin C also made it a focus for dermatological and bone loss studies [107, 108]. Barbados cherries, also known as acerola cherries, in particular have very large quantities of vitamin C [109].

Other components in sweet cherry extracts include fiber and potassium [97]. Both are essential nutrients with beneficial effects on blood pressure and cardiac health, and fiber in particular is very important to the maintenance of a healthy gastrointestinal function [110–112].

Finally, cherries also contain perillyl alcohol, a monoterpene that has been repeatedly noted as a possible chemopreventive and chemotherapeutic agent for a wide variety of different cancers [113–116].

The crude extract has also proven beneficial for the treatment of diabetes, though the degree of effectiveness of these extracts could vary significantly between cherry cultivars due to the fact that different cultivars can contain their active compounds, such as polyphenols, including anthocyanins, in different proportions [117–119].

As an aside, the proportional polyphenolic content and subsequent antioxidant potential in extracts obtained from cherry stems is actually higher than in the extracts obtained from the fruit. However, in the study performed by Bastos et al (2015), only the fruits showed potential anticancer activity the fruits seemed to have more specific anticancer activity , possibly due to the presence of anthocyanins in the fruit and lack thereof in the stem [120]. The stems of certain varieties of cherry, however, are being studied for other purposes, such is the case with the common *Prunus avium* cherry, whose powdered stem has confirmed diuretic effects [121].

Lastly, the stems of a local Portuguese type cultivar of sour cherry called the “Ginja” cherry (*Prunus cerasus austera*), usually used for traditional liqueur infusions, was proved to have antimicrobial effects against both Gram-positive and Gram-negative strains of bacteria [122].

1.4 Classification and properties of phenolic compounds

Phenolic compounds are a set of organic chemicals, classified as aromatic secondary plant metabolites, and they have one characterizing feature among all of them, a hydroxyl group bonded directly to an aromatic hydrocarbon ring [123]. They are the largest group of phytochemicals, and are associated with an array of health benefits [124].

Actually enumerating the possible phenolic compounds would be impossible, as they are a very complex and numerous family of chemicals with over 8000 basic phenolic structures identified to this day [125, 126]. The number of different identified phenolic compounds at this stage is so vast that some have been identified and classified as exclusive to certain plants, such as sesamol (sesame seeds) and raspberry ketone [127, 128].

Their extremely-high variance in complexity also made it challenging to arrange them in a linear classification. Harborne and Simmonds suggested a classification of these phenols into groups in 1964, based on the number of carbons in the molecule, and although several more have been discovered in the interim years, the general classification has remained largely unchanged. Table 1 presents a phenolic compound classification according to the Harborne and Simmonds model [129, 130].

Table 1: The Harborne and Simmonds model**, as seen in "Phenolic Compound Biochemistry" [130].**

Basic Structure	Class(es)
C_6	Simple phenolics
C_6-C_1	Phenolic acids and related compounds
C_6-C_2	Acetophenones and phenylacetic acids
C_6-C_3	Cinnamic acids, cinnamyl aldehydes, cinnamyl alcohols, coumarins, isocoumarins, chromones
C_{15}	Chalcones, aurones, dihydrochalcones, flavans, flavones, flavanones, flavanones, flavanolols, anthocyanidins, anthocyanins
C_{30}	Biflavonyls
C_6-C_1-C_6; C_6-C_2-C_6	Benzophenones, xanthones, stilbenes
C_6, C_{10}, C_{14}	Quinones
C_{18}	Betacyanins
Ligands, neoligands	Dimers or oligomers
Lignin	Polymers
Tannins	Oligomers or polymers
Phlobaphenes	Polymers

Lastly, it should be noted that phenols are a very wide class of chemical compounds with several subgroups, with some proving particularly effective at specific tasks and even boasting comparatively unique properties. Quercetin, which is abundant in cherries, prevents thrombus formation by inhibiting platelet aggregation and selectively lowers blood pressure in hypertensive subjects in addition to boating anti-inflammatory and antioxidant properties similar to those previously-discussed, as well as unique anticancer properties due to its inhibition of mast cell secretion [97, 131–133].

Most interestingly of all, several phenolic compounds have been found to have positive synergies among themselves, meaning that the "extended family" of phenolic compounds benefits greatly from being studied as a whole [134–137].

Out of all the enumerated phenolic compound sub-groups, the largest and possibly the most thoroughly-studied, with over 4000 identified structures is flavonoids, with a three-ringed structure usually with a 15-carbons C_6-C_3-C_6 backbone. The basic flavonoid structure is shown in figure 4 [138].

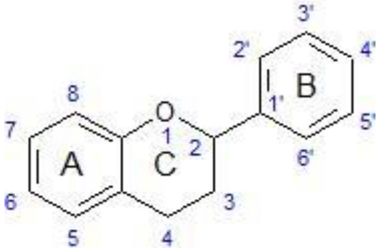


Figure 4 - General flavonoid structure. With two phenyl rings (A, B) and a heterocyclic ring (C) [139].

Much of the available scientific literature seems to indicate that the general consensus on flavonoids and general phenolic compounds is that they contain a wealth of anticancer properties, preventive and therapeutic, observed across a variety of organs and tissues, and acting through several mechanisms, though trends are not always completely clear and more advanced and complex studies are still needed [140–144]. Despite the doubts raised by some of these studies, the anticancer effects of flavonoid compounds is established to the point of there being at least one commercially-available, “pharmaceutical-grade” green tea catechin concentrate with the confirmed capacity to induce both anoikis (programmed cell death) and necroptosis (programmed necrosis) in cancer cells [145].

This trend is likewise extended to other diseases such as cerebral amyloidosis and other neurodegenerative diseases, cardiovascular conditions, ulcer treatments and general antioxidant and anti-inflammatory capabilities [146–150]. A study of general flavonoid, including anthocyanin intake also demonstrated their usefulness in long-term weight maintenance [151].

In spite of these similarities with their parent groups, there are some noticeable differences between flavonoids and other phenolic compounds at large, aside from the preventive and therapeutic parameters that vary even between the elements of smaller sub-groups such as anthocyanins. One of these is the range of stabilities of these compounds when compared to the relatively unstable nature of, for example, anthocyanins by themselves, especially when isolated and purified. A greater number of certain groups (hydroxyl) will decrease stability while others (methoxyl) will increase it. This is verifiable in anthocyanins as well, but the higher structural variability within their enclosing class will result in a greater variability in stability [152, 153].

The use of flavonoids is also, according to present research, comparatively light on side effects due to their moderately low toxicity, though concerns when taking very large daily doses, especially in combination with other drugs that may result in toxic interactions, still apply, and will have an impact in all their future applications [154]. This makes them very desirable elements in treatments such as chemotherapy, where they are gaining relevance as P-glycoprotein inhibitors despite the aforementioned possible contraindications [155].

1.4.1 The particular case of anthocyanins

Anthocyanins are glycosides of athocyanidins, and the most abundant subset of flavonoids, themselves a subset of the larger phenolic compound family, in fruits and vegetables, mostly known as a group of very powerful antioxidants [156]. They are a class of pigments found in several separate sections of a very wide array of plants, and generally found in high quantities in several fruits and vegetables, such as blueberries, blackcurrants, blackberries, eggplants, some peppers, and even in grains, like some varieties of colored rice. Cherries, both sweet and sour, have both been noted as very rich sources of anthocyanins [157, 158].They have been successfully extracted from all these sources, and put to varied uses in several industries, such as in food and pharmaceutical. They have several confirmed anticancer related properties, many of which are shared by other flavonoids, including anti-inflammatory, free-radical-scavenging properties, as well as the induction of apoptosis and differentiation in cancer cells [157, 159–166].

Structurally, anthocyanins present the basic three-ring skeleton of flavonoids, and, most importantly, a positively-charged oxygen atom in their C ring, which confers them unique characteristics. They differ in the positioning/number of their hydroxyl/methoxyl/other active groups, the possible positions of which are marked as “Rs” on figure 5 [167].

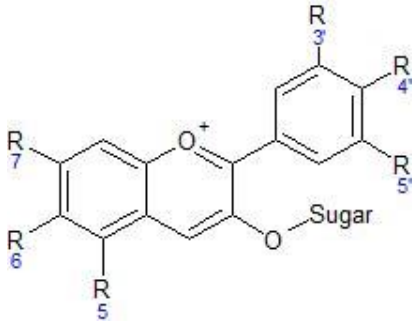


Figure 5 - Basic anthocyanin structure [167].

Research seems to indicate that anthocyanins have distinct behavior in pharmacokinetics, concerning absorption and metabolism, when compared to other flavonoids. Anthocyanins are absorbed as glycosides, although a significant portion of them may be lost during digestion after consumption. This process is dependent on the anthocyanin’s type and degree of glycosylation. In some cases, this can result in low retention and subsequent urinary excretion of entire anthocyanins [153, 168, 169]. Despite this,, recent studies indicate that the stability of anthocyanins can be artificially ameliorated, and that the presumably poor bioavailability that results from this lack of stability might actually be naturally, improved [170, 171].

It should also be said that anthocyanins are only a fraction of the available antioxidant compounds in their plant extracts of origin. As an example, purple wheat is not only rich in anthocyanins, but it also contains noticeable amounts of the antioxidants secoisolariciresinol diglucoside and melatonin, which add to its overall antioxidant capacity and nutritional value [172].

Regarding their chemical stability, anthocyanins are not very stable compounds by themselves under most conditions, and even within the plant cell, where they are synthetized in the cytoplasm, they need to be sequestered and accumulated in the vacuole (which has a very acidic pH) to be properly stabilized [173]. It is important to note, though, that the concentration and stability of these anthocyanins will differ dramatically depending on their source. For example, extracting anthocyanins in high quantities from sweet cherry cultivars is notoriously difficult, as they are considered less stable than their blackberry-borne counterparts in addition to the fact that cherries contain problematic quantities of Polyphenol Oxidase (PPO), a freeze-resistant enzyme that degrades phenolic compounds. This makes a swift albeit careful handling of these cherries very important in obtaining optimal amounts of anthocyanins, despite the fact that they are considered “rich” in phenolic compounds, including anthocyanins [157].

Conversely, strawberries appear to have an excellent amount of antioxidant activity and other nutritional factors even after being left to ripen post-harvest for relatively large periods of time, and other studies indicate that the anthocyanins themselves, along with other phenolic compounds, are generally unstable and hard to store regardless of their source. This does not invalidate the previously-discussed discrepancies in stability [174–176]. Both of these attributes are heavily influenced by the anthocyanin molecule’s unique structure [177].

This disparity in difficulty is not an immutable element, however, as in addition to the calibration and optimization processes inherent to these extractions, several new methodologies are being devised and tested in order to better extract, preserve and keep these compounds from more complicated sources, such as sweet cherries [157, 178, 179]. This is an essential factor to consider when evaluating the viability of cherries as antioxidant sources, and when considering the possible uses for these antioxidants. Cleaner, environmentally-friendly processes with stronger yields will always be desired [178]. The current standard methods are functional, but not ideal, as they yield small doses of anthocyanins, which are very low when compared to similar berries [157].

This dissertation’s focus on sweet cherries, as a source of anthocyanins and other phenols in spite of the aforementioned difficulties in extraction, is due to several reasons. Firstly, they are extremely popular fruits, enjoyed in large quantities all over the world, in some form, frequently processed, all year round despite them being seasonal drupes. Secondly, they are popular in the Cova da Beira region specifically, and their distribution and consumption is considered a part of the local economy. Lastly, they are (as previously-stated) an extremely versatile diet component, rich in several forms of antioxidants not limited to phenolic compounds, like (namely) melatonin.[97, 157].

While studying these compounds, one must also consider the fact that the amounts and proportionalities of these chemicals of interest within the cherries will vary significantly not only across different cultivars and geographical locations, but also according to a host of other factors, most of which can't be controlled, and many can hardly be properly quantified or accounted for. Such factors include, but are not limited to, the amount of ultraviolet light the cherries are exposed to while growing, the storage conditions, the way the cherries are processed and even the aggressiveness of the meteorological conditions the cherries are subjected to while growing; for example, bruising and tearing may rend the fruit, resulting in infection and quality loss [97, 180]. This difference in the levels of active compounds within a certain source are not completely exclusive to cherries, however, as studies have demonstrated that anthocyanin content varies significantly between blueberry genotypes and cultivars, and specific strawberry cultivars [181–183].

Perhaps most importantly of all, thanks to the presence of certain enzymes like the aforementioned PPO that will act on the fruit while it's still ripening, the ripeness of the cherries will severely impact their phenolic, antioxidant and nutritional contents. It can be said that virtually every stage of the cherry's growth and extraction can have noticeable repercussions on its integrity [157, 184].

1.4.1.1 Anthocyanin extraction

As previously discussed, the methods used in the process of extraction will severely impact the amount of anthocyanins and other phenols present in the extract. This makes it necessary to optimize the extraction procedure to best fit the intended component proportions and integrity [157].

Accordingly, there is a wide array of parameters that must be accounted for and controlled in order to yield optimal results. Choosing a fruit's (such as cherry or strawberry) cultivar over another will, as mentioned before, immediately influence both the amount and durability in storage of anthocyanins even before taking into account the entire extraction methodology [183, 185].

The extraction parameters that need to be controlled are varied, and different methodologies will introduce additional parameters, but among the most generalized are mass: solvent ratio, extraction time, type of solvent and extraction temperature [186, 187]. Amass solvent ratio of 1:20 is deemed appropriate, and extraction time will vary significantly between extraction techniques, taking 5 minutes in ultrasound extraction and 60 minutes in enzymatic) [157, 186, 188]; the best anthocyanin yields are generally obtained with an alcoholic based solvent, either ethanol or methanol, and water, possibly acidified a small amount, usually around 1%, of mild-to-strong acid. Studies featuring acetic, trifluoroacetic acid, HCl and gallic acid were observed [157, 161, 186, 188–191]. Data regarding temperature was also varied according to the used method, with some methods, namely accelerated solvent extraction, which inherently requires high temperatures, calling for excessive temperatures of 80 ºC, but for conventional methodologies the anthocyanin degradation that seems to occur around 30-40 ºC proved to be a demonstrable concern, leaving ideal extraction temperatures in the 20-30 degree range [157, 187, 188, 192].

The extract may also be produced from fruits in different forms, from freeze-dried, to dried and powdered, to pomaces, including freeze-dried pomaces, and plain skin and pulp, each with its distinct advantage, such as the powder's increased specific surface area and the fact that the pomace makes it possible to extract anthocyanins from fruits post-juicing. Each form yields better results under different conditions [186, 192–195]. The process of freezing these fruits pre-extraction offers a major benefit, having a much smaller impact on anthocyanin content than simply drying them [194].

In order to arguably measure and thus maximize anthocyanin yield, and to help further optimize some of these parameters, statistical methods such as response surface methodology and the Taguchi method are employed [191, 196, 197].

There are also several additional processes that will facilitate the extraction, such as microwaves, ultrasounds and even enzymatic extraction among other, more complex methods [157, 188, 193, 195, 196, 198] . The main benefit of ultrasound-assisted anthocyanin extraction lies in its ability to significantly reduce extraction time while also necessitating less solvent [157, 196]. Microwave-assisted extraction seems to similarly expedite the process by introducing the added variable of power, which must be carefully controlled. This also proves beneficial for other phenolic compounds and antiradical efficiency, though it also brings some unwanted structural changes to the collected anthocyanins [186, 199]. The benefits of enzymatic extraction proved more mixed, but overall quite positive, with a great increase in the resulting compost stability and in the yield in some anthocyanins, while decreasing the yield of others [188].

In the present work, certain additional precautions would have to be implemented due to the fact that prostate cancer cell models were used, and so anything that could heavily impact the pH and constituency of the cell medium would come into question, since it could affect the correct development of the cells and influence the results. Methanol-based extraction with HCl have been previously used in animal models [140], but the stability of the anthocyanins during prolonged periods in the cell incubator would have to be carefully accounted for, as in a plain water solution they would be deemed unstable except if at very low temperatures and/or pH values, both incompatible with the cellular environment [175]. To this end, dilution in ethanol would be an accepted stabilization buffer during the preparation of the solutions, and serves as a reasonable intermediate as long as the cells themselves aren't exposed to too great a concentration [175, 200].

1.4.1.2 Anthocyanins as anticancer molecules

Anthocyanins have an extremely wide range of uses. These pigments have been frequently featured as the focus of studies analyzing their viability as potential treatments for several forms of cancer, and even completely different, separate conditions.

The primary field of study involving these compounds remains the study of their varied inhibitory effects on several types of cancer, both in *in vitro* and *in vivo* models. Decreased cell proliferation and biomarker inhibition, including lower expression of cyclooxygenase genes and diminished levels of oxidative DNA damage, were observed in male Fischer 344 rats previously treated with the colon carcinogen azoxymethane, after feeding with anthocyanin-rich extracts [201]. Anthocyanin extracts from black soybeans were tested on rat models with induced-benign prostatic hyperplasia, an enlargement of prostate caused by an increased rate of cell replication, and a possible precursor of malignancy. The mass of the harvested prostates was significantly lower in the rats treated with the extracts containing anthocyanins, demonstrating their ability to noticeably decrease accelerated cell proliferation in prostatic tissue despite the latter not being cancerous at that stage [202].

Black soybean extracts also, similarly to the previously-discussed potato extracts, proved to be effective at causing the apoptosis of cancer cells, though in a completely different cell line; the DU-145 line (moderate metastatic potential), decreasing expression of cancer indicators and androgen receptors, and successfully inhibited the growth of prostate cancer xenografts in mice [203].

The effects of anthocyanins in colorectal cancer chemoprevention have been further confirmed in other rat models via oral administration, with promising but not completely conclusive results, and a study on the comparative effects of identical concentrations of anthocyanins on colon cancer and non-tumorigenic cells demonstrated that while the growth of the latter is inhibited at higher concentrations, more moderate concentrations inhibit the tumorigenic cells exclusively [204, 205]. Further studies on the mechanisms behind this decrease in cell proliferation suggested that the demethylation, or decrease of the methylation-promotion effect, of tumor suppressor genes that was observed in rats fed black raspberries was attributable to anthocyanin intake [206].

Similar effects via diet have also been observed for the proliferation of liver cancer cells, for the inhibition of the formation of skin tumors in mice, via skin application, and the formation and growth of induced esophageal tumors in rats [159, 207–209]. Anthocyanidins have also shown these suppressive effects, acting synergistically, on non-small-cell (less aggressive) lung cancer cells, as well as A549 (Type II) lung cancer cells [210, 211].

Overall, pertaining to their antiproliferative/anticancer properties, anthocyanins as a whole seem to exert a multitude of effects via a varied set of pathways, many of them previously mentioned. These include, among others, a confirmed anti-inflammatory effect enacted by the inhibition of a variety of mediators such as IL-6, TNF and MCP-1, and the regularization of acute phase proteins albumin and transferrin [163, 212]. This anti-inflammatory effect, along with the reduction of oxidative stress, is also applicable to non-cancerous conditions, such as cognitive impairment resulting from neuroinflammation [213].

The already-mentioned antioxidant potential is one of the anthocyanins' most thoroughly-studied properties, which has been analyzed for anthocyanins extracted from different sources using varied methodologies [165, 214, 215]. The prediction of the efficacy as a free radical-scavenging has already begun to be approximated with mathematical models, and is undergoing constant refinement [164, 216]. The antioxidant properties include the activation of phase II enzymes [217] and induction of both apoptosis and cell differentiation, in some cases concurrently. As previously mentioned, the apoptotic pathway triggered by anthocyanins include caspase activation and down-regulation of anti-apoptotic protein Bcl-2, skewing the BAX(proapoptotic)/Bcl-2 ratio. Cell differentiation seems to be promoted by the increased activity of alkaline phosphatase and dipeptidyl peptidase, as well as other protein kinases [160, 166, 218–220].

Anthocyanins have also been shown properties that may contribute to the diminishing propagation and upkeep of fully-formed tumors and metastases, such as anti-angiogenic, i.e., preventing the formation of new blood vessels to nourish the tumor tissue, achieved via, among other processes, the suppression of the expression of vascular growth factors [221]. Another similar property, the prevention of the tumor tissue's invasive potential against the host's healthy tissue, has also been repeatedly verified in (non-small-cell) lung cancer cells, in which the synergistic effects of combining anthocyanins was observed, as their combination proved more efficient in halting migration and invasiveness than the separate anthocyanins [210]. Similar effects have been observed in liver cancer cells [222, 223]. Further studies by the same research group also confirmed that the suppression of Claudins, which are components of tight junctions that are also overexpressed in cancer cells, and the suppression of matrix metalloproteinases, are both important factors in these anti-invasive properties [223, 224].

Studies focusing on specific anthocyanins and anthocyanidins, in lieu of more general plant extracts, also exist. Though these, possibly not counting the cases where more than one anthocyanin is studied simultaneously, obviously exclude the possible synergist effects that these anthocyanins might have with other components in these extracts, and thus might be underestimating their effects [153].

The anthocyanin cyanidin-3-glucoside (C3G, also known as chrysanthemin) has been demonstrated to inhibit the pro-proliferative and pro-metastatic effects of ethanol exposure, (a possible aggravating factor resulting from alcoholism) on breast cancer cells overexpressing ErbB2. ErbB2 is abundantly present in 20-30% of patients, and the quantities of C3G required to trigger this effect are attainable *in vivo* without immediately-noticeable adverse effects.

These quantities are also possibly lower than the quantities required to obtain equivalent effects in *in vitro* cell cultures due to the way C3G is metabolized. C3G extracted from blackberries has also been demonstrated to have chemopreventive and chemotherapeutic properties by decreasing the size and the metastatic potential of tumor xenografts in mice *in vivo* [225–227]. C3G had previously shown a similar antiproliferative potential in breast, prostate and lung cancer cells in combination with another anthocyanin, peonidin 3-glucoside, which has also been individually studied and shown to inhibit lung cancer metastasis by downregulating proteinase and protein kinase activity [228–230].

A similar effect, the inhibition of lung cancer cell migration and invasion, was also observed in a study pairing C3G and cyanidin 3-rutinoside, similar to the previously-mentioned study on synergistic anti-invasiveness [210, 231]. Mitigation of the growth and development of intestinal adenomas was also observed, and a separate study showing that C3G suppressed specific inflammatory responses in human intestinal cells, extending the possible chemopreventive applications of C3G and anthocyanins in general to colorectal cancer [232, 233].

Different forms of C3G have also been demonstrated to show free-radical-scavenging properties when tested in combination [234].

In the case of individual anthocyanidins, the one most thoroughly-studied individually, or at least the one with the most readily-available data, in the context of therapy outside of just prostate cancer seems to be delphinidin. Most notably, aside from its already-explored function in the combat against prostate cancer, as well as having similarly suppressive effects on the growth of other forms of cancer, such as lung and breast cancer, delphinidin has been shown to be a powerful agent against bone loss [235–237]. The antioxidant effects of delphinidin, along with cyanidin and pelargonidin, have been studied for several years [238].
It is important to keep in mind that, while there is very little literature suggesting that anthocyanins by themselves have adverse effects in moderate concentrations, though high concentrations have been observed to have cardiotoxic and other deleterious effects, there is a need to study the potentially unfavorable effects of their interactions with other drugs administered in combined treatments [239–242], which could be a paramount importance considering their possible co-administration with anti-cancer drugs.

Lastly, it can be stated that anthocyanins have been thoroughly-studied on prostate cancer in particular, and have yielded promising results.
Fractioned anthocyanin extracts from a specific type of potato proved to be particularly effective both at stopping the proliferation and at inducing apoptosis through its cytotoxicity in LNCaP and PC3 prostate cancer cells, even at reasonably low concentrations (5 μg/mL). Most importantly, a new pro-apoptotic effect of anthocyanins via the promotion of nuclear uptake of certain pro-apoptotic proteins released from mitochondria was observed for the

first time in any cancer cell line [219]. Similar extracts made from blue maize were also tested on PC3 cells showing a particularly strong anti-proliferative ability (viability below 30% comparatively with the control) [243].

In addition to this, though most studies seem to tackle general anthocyanin extracts or even plant extracts in bulk that contain anthocyanins, there are also studies demonstrating the effect of specific anthocyanins/anthocyanidins in combating prostate cancer. A single study evaluated the inhibition potential for carcinogenesis of three anthocyanins responsible for the color of purple corn (cyanidin-3-glucoside, pelargonidin-3-glucoside and peonidin-3-glucoside) on LNCaP, concluding that the first two were active compounds that noticeably decreased carcinogenesis by themselves [229].

Several anthocyanidins also seem to be the focus of similar specific studies on prostate cancer cells, most notably, delphinidin and cyanidin. Cyanidin was tested on both DU145 and LNCaP prostate cancer cell lines. Antioxidant activity was observed in LNCaP cells whereas anti-proliferative and pro-differentiation properties were observed in both cell lines [244]. Another cyanidin study using LNCap and PC3 cells demonstrated reduced cell viability and a decrease in both the production of specific pro-inflammatory prostanoids and the expression of the enzymes that catalyze their production [245]. This reduction in the cells' pro-inflammatory response is an important anti-prostate cancer property, due to the thoroughly established links between prostate cancer and chronic inflammation [32].

Similar *in vitro* and *in vivo* prostate cancer studies with delphinidin in mice demonstrated the anthocyanidin's ability to specifically curb prostate cancer growth, induce cell cycle arrest and apoptosis in both androgen-dependent and androgen-independent prostate cancer lines [246, 247]. The pathways which are altered by delphinidin were also identified in these studies; was shown the inhibition of NFκB, a nuclear factor that controls DNA transcription; a decrease in proliferation/replication indicated by Ki67 and proliferating cell nuclear antigen (PCNA); caspase activation and an increase in the BAX/Bcl2 ratio were observed indicating a higher rate of cell death [246, 247]. Treatments with delphinidin also further enhance the sensitivity of prostate cancer cells to additional pro-apoptotic treatments, such as TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis [248].

Beyond *in vivo and in vitro* treatments in cell and animal models, intake trends of anthocyanins are also being studied in specific populations such as children and adolescents, in order to extrapolate trends [249], since some of the benefits, such as reduced risk of myocardial infarction in women, seem to be particularly pertinent to young and middle-aged patients [250]. Benefits of a high intake of anthocyanins are numerous, since a consumption of daily portions has been associated with a wealth of health benefits, from lower arterial stiffness and central blood pressure in women, to a decrease in the age-related decline of lung function, particularly in elderly men, to a decreased risk of several chronic diseases resultant from their anti-inflammatory effects, to decreased insulin resistance [251–254].

Anthocyanins have also been isolated as protective and therapeutic agents for a multitude of other conditions, which range from cardiovascular disease, to Alzheimer's and dementia, to nonalcoholic and non-cancerous fatty liver disease [255–258] and diabetes, with *in vivo studies* in diabetic mice reducing blood glucose and increasing glucose tolerance, and promoting insulin secretion from pancreatic ß cells [259, 260]. Early studies also show that they may have beneficial synergies with fructooligosaccharides in regards to gastrointestinal function and metabolism [261], and that these flavonoids can even be used to attenuate muscle damage [262]. Scientific evidence is also mounting for the high consumption of foods rich in specific compounds among which are counted anthocyanins and anthocyanidins, such as assorted fruits, being considered a likely factor in the low incidence of prostate cancer in specific countries in the Mediterranean region, which suggests preventive roles for these compounds in addition to their established potential as sources of new treatments [263].

Chapter 2 – Aim

Prostate cancer is one of the most common cancers in men both in terms of incidence and as a cause of cancer death. It is a very complex condition with a wide array of risk factors, some of which not completely understood, such as its incidence and mortality associations with race, and its relationship with Type 2 Diabetes. Also, like virtually all forms of cancer, prostate cancer risk has a strong association with diet, and several recent studies have started focusing on the analysis of food products both as dietary suggestions and re-purposed as possible innovative medications in order to curb the advancement of the disease.

The aim of this dissertation will be to provide the theoretical foundation for a possible association between the consumption of sweet cherries and health benefits pertaining to the prevention and attenuation of prostate cancer. For this purpose, sweet cherry extracts of the *Saco* cultivar from the Cova da Beira region will be tested *in vitro* on an array of prostate cells representative of the healthy prostatic epithelium, androgen-dependent and the more advanced and aggressive androgen-independent stage of prostate cancer. The effects of cherry extracts on the proliferation and cell viability, apoptosis and glycolytic metabolism of non-neoplastic and neoplastic prostate cells will be evaluated.

Chapter 3 – Materials and Methods

3.1 Cell lines

The prostate cancer cell lines, LNCaP (lymph node carcinoma of the prostate derived from lymph metastasis) and PC3 (derived from metastasis of the bone), and the non-neoplastic PNT1A cell line (human post-pubertal prostate epithelial cell line), were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). LNCaP and PC3 cell lines are both considered to be representative of prostate cancer in its metastatic stages, with LNCaP cells being androgen-dependent and presenting a comparatively lower metastatic potential when compared to PC3, which represent a more aggressive and advanced stage of disease characterized by androgen independency [264–266].

3.2 Cherry extraction

Sweet cherries (*Prunus avium*) from *Saco* cultivar were kindly provided by Cerfundão - Embalamento e Comercialização de Cereja da Cova da Beira, Lda., and were part of a late July crop. This donation was also made possible thanks to a cooperative effort by the Fundão City Hall. These fresh cherries were kept at -20 ºC until sample preparation.

3.2.1 Extraction Procedure

Cherry samples were carefully seeded with a scalpel and placed in 50 mL falcon tubes for freeze-drying. These samples were then stored at -80 ºC for a period of time between 12 and 24 hours.

Falcons containing cherry samples were dipped in liquid nitrogen, slowly and carefully placed inside glass containers, and then freeze-dried (Scanvac CoolSafe 55-9 PRO freeze dryer) for a period of time between 3 and 5 days. After sample lyophilisation, tubes were capped and stored in silica to prevent humidification, until extraction procedure.

Duplicates of freeze-dried cherry samples (2 g) were placed inside 50 mL falcon tubes with 40 mL of extraction solvent (99.9% methanol, 0.1% HCl). The tubes were then secured and partially submerged in an ultrasound bath (under occasional agitation) using a power of 37 KHz, for 30 minutes, always under 30 ºC (Branson 5800 2 ½-gallon ultrasonic cleaner). After extraction, samples were centrifuged at 4 ºC and 8000 rpm for 10 minutes.

The supernatant was stored, and the extraction and centrifugation processes were repeated once with the residues, resulting in four tubes of clean cherry extract in solvent. These underwent solvent evaporation to dryness in a rotary evaporator (Buchi rotavapors; V-850 vacuum unit, R-251 rotary evaporator with a B-491 heating bath model), maintaining the water bath temperature at 33 ºC or less in order to preserve anthocyanin integrity. Cherry extracts were kept at 2 ºC until further analysis.

3.3 MTT Assays and Stimuli

3.3.1 Cell proliferation assays (MTT)

The original cherry solution was prepared by dissolving and homogenizing 40 mg of cherry extract in 0.15 mL H_2O and 0.15 mL ethanol, resulting in a 133 mg/mL extract solution. All other cherry solutions (2, 20, 200 and 2000 µg/mL) were prepared from the original solution non-diluted or diluted 1:10 in the culture medium. In all working solutions the ethanol concentration in the culture medium was ≤ 0,75 % (Table 2).

Table 2. Ethanol content in the different cherry extracts solutions.

Solution	Ethanol content (%)
Original cherry solution (133 mg/mL)	50
2000 µg/mL	0.75
200 µg/mL	0.075
20 µg/mL	7.5×10^{-3}
2 µg/mL	7.5×10^{-4}
0 µg/mL	0

PNT1A, LNCaP and PC3 cell lines were maintained and grown in RPMI 1640 cell culture medium (Gibco, Invitrogen/Life Technologies, Paisley, Scotland), supplemented with 10% foetal bovine serum (FBS, Biochrom AG, Berlin, Germany) and 1% penicillin/streptomycin (Gibco, Life Technologies, at 37 ºC in an air incubator with a humidified atmosphere of 5% CO_2.

For the viability/proliferation MTT assays, prostate cells were grown in 96-well plates with 100 µl of culture medium per well for 48, 72 and 96 hours. Different numbers of cells were seeded for each cell type to ensure comparable growth, 4000 PC3, 6000 PNT1A and 10000 LNCaP cells were seeded. After growing for 24 hours, the cell culture medium was discarded and the medium containing the different concentrations of cherry solutions (Table 2) was added. MTT assay was performed at 48, 72 and 96 hours of culture. In brief, the culture medium was removed and replaced by 200 µl phenol red-free medium plus 10 µl of MTT solution (Sigma-Aldrich, St. Louis, Missouri, USA). The culture plate was then incubated in the dark for 4 hours, after which the medium and MTT solution were carefully removed. The remaining formazan crystals were dissolved in 200 µL DMSO. The plate was wrapped in aluminium foil, agitated for 15-30 minutes for homogenization, and absorbance read at 690 and 570 nm.

A specific time-point and concentration, chosen on the basis of the results obtained in the MTT assay, were selected for subsequent stimuli and analysis. LNCaP, PC3 and PNT1A cells were cultured as described above in 25 cm^2 t-flasks with 5 mL of cell culture medium. After treatment, cells were trypsinized and poured into 50 mL falcon tubes, washed with PBS and centrifuged twice. The obtained pellets were re-suspended in 2 mL PBS and used for protein extraction.

3.3.2 Protein extraction

Prostate cells were homogenized using the radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1 % Nonidet-P40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS, 50 mM Tris pH 8.0, 1 mM EDTA) supplemented with 1 % protease-inhibitor cocktail (Sigma-Aldrich) and 10% PMSF (Sigma-Aldrich). Cells were kept on ice for 20 minutes while being occasionally mixed, and then centrifuged at 14000 rpm, 4 °C for another 20 minutes. The protein-containing supernatant was recovered and stored in a clean separate tube. The protein concentration was determined by the Bradford method [267] using the Bio-Rad protein assay dye reagent (Bio-Rad, Hercules, CA, USA). The standard curve was obtained using serial concentrations of bovine serum albumin.

3.4 Western blot

60µg protein extract were mixed with a previously-prepared loading buffer (with 10% 2-Mercaptoethanol) and de-natured at 100 degrees Celsius for a period of 5 minutes. They were then resolved in a freshly-prepared 12.5% acrylamide gel (with the aid of a 4.7% acrylamide stacking gel) by the sodium dodecyl sulphate polyacrylamide gel electrophoresis method (SDS-PAGE). The electrophoresis proper was performed at 120V for a duration of roughly 90 minutes. Two gels were prepared at a time.

Proteins were then electro-transferred to two previously-activated PVDF membranes (obtained from GE Healthcare in Buckinghamshire, UK). The process was performed at 750mA for a period of 75 to 80 minutes. After this, the membranes were blocked for 1 hour under agitation with a solution made with 5% dried skimmed milk (Regilait). After this, the membrane was incubated overnight with one of several antibodies; anti-rabbit BAX (1:1000, #2772, Cell Signalling Technology), anti-rabbit BCL2 (1:1000, #2876, Cell Signalling Technology), anti-rabbit Caspase9 (1:1000, p35, H-170, SC-8355, Santa Cruz Biotechnology), anti-mouse Caspase8 (1:200, p18, D-8, SC-5263, Santa Cruz Biotechnology), anti-rabbit FasR (1:1000, A-20, SC-1023, Santa Cruz Biotechnology), anti-rabbit FasL (1:1000, C-178, SC-6237, Santa Cruz Biotechnology), anti-rabbit p53 (1:1000, FL-393, SC-6243, Santa Cruz Biotechnology), anti-rabbit Glut-1 (1:1000, CBL242, Millipore), anti-rabbit Glut-3 (1:1000, H-50, SC-30107, Santa Cruz Biotechnology), anti-rabbit LDH (1:5000, EP15664, Abcam), anti-rabbit PFK-1 (1:1000, H-55, SC-67028, Santa Cruz Biotechnology), anti-rabbit MCT4 (1:1000,

H-90, SC-50329, Santa Cruz Biotechnology), anti-rabbit Glutaminase (1:1000, ab93434, Abcam)), and anti-rabbit ASCT2 (1:1000, V501, Cell Signalling Technology).
Each membrane was also incubated with anti-mouse ß-actin (1:10000, A5441, Sigma-Aldritch), in order to serve as a protein loading control to be normalized with each individual antibody's band density.

In order to prevent overlapping bands and to maximise both the mileage of the membranes and the clarity of the results, several membranes were punctually stripped with a mild, acidified stripping solution produced with glycine, SDS and Tween20.

After the primary incubation period elapsed, and after proper cleansing of the membranes, they were incubated for 1 hour with one of two secondary antibodies; goat anti-rabbit (1:40000, IgG-HRP, SC-2004, Santa Cruz Biotechnology) or goat anti-mouse (1:40000, IgG-HRP, SC-2005, Santa Cruz Biotechnology). After this, membranes were incubated with an ECL substrate (BioRad) for 5 minutes, and then scanned with their ChemiDoc™ MP Imaging System (BioRad). This raw data was posteriorly visualized with, and the band densities processed by volumetric analysis tool from, Bio-Rad's Image Lab 5.1 software. This data was then normalized with each membrane's respective ß-actin values.

3.5 Biochemical assays

Caspase-3 activity assays were performed with protein samples obtained from every separate supernatant. In a 96-well plate, 25 µg total protein extract were incubated with an appropriate volume of the kit's designated reaction buffer (25 mM HEPES, 0.1% 3-[(3-holamidopropyl) dimethylammonio]-1-propanesulfonate, 10% sucrose, 10 mM Dithiothreitol (DTT), with a pH of 7.5), with an added 200 µM of the kit's caspase-3 substrate Ac-DEVD-pNA). Negative controls without this substrate were performed for each separate sample. The incubation was undertaken for 2 hours at 37 degrees Celsius, with measurements being taken spectrophotometrically (405 nm) at 30, 60, 90 and 120 minutes. The assay's active principle involves the cleaving of the caspase-3 substrate (Ac-DEVD-pNA), which releases its p-amilo-nitrile group (pNA) and gives the solution a yellowish colour which is quantified by these periodic spectrophotometric readings, and calculated by their extrapolation. The amount of free pNA produced (and thus the intensity of the yellow pigment) is proportional to each sample's caspase-3 activity.

LDH assays were also performed, also in 96-well plates. The working reagent (WR) was prepared by mixing the assay kit's set buffer (imidazole, 65 mmol/L and pyruvate, 0,6mmol/L) with an NADH (0,18 mmol/L) in a ratio of 4:1. In each well, 5 µl of protein supernatant were added to 300 µl of WR, and the plate was both mixed and incubated for 1 minute at 37 degrees Celsius in a Bio Rad xMark™ Microplate Absorbance Spectrophotometer. An initial absorbance was then read, followed by subsequent readings every minute for 3 minutes, still maintaining a constant temperature of 37 degrees. All readings were taken at 340 nm.

The assay's principle consists of the fact that LDH will catalyse the reduction of pyruvate by NADH (both present in the working reagent) into lactate. It's these varying levels of NADH which are measured at 340 nm and monitored over 3 minutes. This consumption ratio was then extrapolated upon and used to gauge LDH activity.

Lastly, the used (spent) cell culture medium were analysed for both their glucose and lactate levels, to determine the glucose consumed and lactate produced by each cell type during the stimuli. Spinreact assay kits were used in both cases.

For glucose quantification, a bottle of the kit's R1 buffer (92 mmol/L TRIS, pH 7.4 and 0.3 mmol/L phenol) was used to dissolve a determined, sealed measure of R2 powdered enzyme mix (15000 u/L glucose oxidase, 1000 u/L peroxidase, 2.6 mmol/L 4-aminophenazone). In a 96-well plate, 1 µl from all 12 (6 stimulated, 6 unstimulated) cell medium samples (per cell type), alongside fresh samples of cell culture medium (with and without cherry extract), were each mixed with 100 µl of the previously mixed working reagent, and both a blank with just working reagent and a standard with 1 µl of the aqueous glucose calibration solution were also prepared alongside the samples. The plate was mixed and incubated for 10 minutes at 37 degrees Celsius, and then its absorbance values were read at 505 nm. The blanks were subtracted from the other results and the amount of glucose consumed for each sample was calculated from its proportionality to the standard.

The principle behind this assay lies in the oxidation of the glucose present in the samples by the glucose oxidase present in the working reagent. This will form hydrogen peroxide, which will in turn react with phenol and aminophenazone (in the presence of phenol oxidase) to form quinone, which gives the wells a red/violet colour, which is the measured parameter.
The lactate assay kit had a similar methodology, and was also prepared in a 96-well plate; 10 mL of R1 buffer (50 mmol/L PIPES, pH 7.5, 4 mmol/L 4-chlorophenol) were used to dissolve a bottle of R2 enzyme powder (800 u/L lactate oxidase, 2000 u/L peroxidase, 0.4 mmol/L 4-aminophenazone). 100 µl of this working reagent were mixed with 1 µl of each sample (6 culture medium from stimulated cells, 6 culture medium from unstimulated cells, 1 fresh cell culture medium and 1 fresh cell culture medium with cherry extract) in individual wells of a 96-well plate, along with a sample-less blank (just 100 µl working reagent) and a standard (100 µl working reagent and 1 µl aqueous lactate calibration solution). The plate was mixed and incubated at 37 degrees Celsius for 5 minutes, and the absorbance values were read at 505 nm. The blank was subtracted from all values and the amount of lactate produced in each sample is calculated based on the ratio between said sample and the lactate standard.

The principle behind this assay is very similar to the previously-discussed glucose kit. The lactate in each sample will be oxidised by the lactate oxidase present in the working reagent, resulting in pyruvate and peroxide, the latter of which will be turned into quinone by the action of peroxidase (alongside 4-aminophenazone and 4-chlorophenol). The production of the violet/red quinone compound is the measured parameter which is then associated with lactate production.

3.6 Statistical analysis

All data obtained from assay kits and western blots was processed with the GraphPad Prism v6.00 software (GraphPad Software, San Diego, California). Statistically significant differences between individual groups were evaluated by means of unpaired t-tests with Welch's correction, which are a feature of the aforementioned software. P values beneath 0.05 were deemed to be statistically significant and, considering significance, $p<0.05$ = (*), $p<0.01$= (**) and $p<0.001$ = (***). All experimental data is shown as mean ± SEM.

Chapter 4 – Results

4.1 MTT assays

Exposure to *saco* sweet cherry extracts decreased the viability of human prostate cells

The viability of non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) human prostate cells in response to different concentrations (2, 20, 200 and 2000 µg/mL) of *saco* sweet cherry extracts (late July crop) was determined by the MTT assay.

In the case of PNT1A cells (Fig. 6), a diminished viability of cells exposed to cherry extracts was observed at all experimental time-points. For 48 hours of treatment no significant difference was observed with the 2 µg/mL concentration when compared to the control group. This was followed by a noticeable decrease in proliferation of roughly 60% in the group exposed to 20 µg/mL; the higher concentrations (200 and 2000 µg/mL) showed higher cell proliferation than this. The results obtained at 72 hours showed that all concentrations of cherry extracts were effective supressing the viability of PNT1A cells with a dose-dependent effect for concentrations of 2 to 20 µg/mL. The lowest proliferation was achieved for 200 µg/mL, approximately 50% of that observed in the control. This trend was also observed for 96 hours of exposure, with roughly 29, 43, and 45 % reduction of proliferation being observed in 2 µg/mL, 20 µg/mL, and 200 µg/mL, respectively. No significant diminution of proliferation occurred when using 2000 µg/mL of cherry extract.

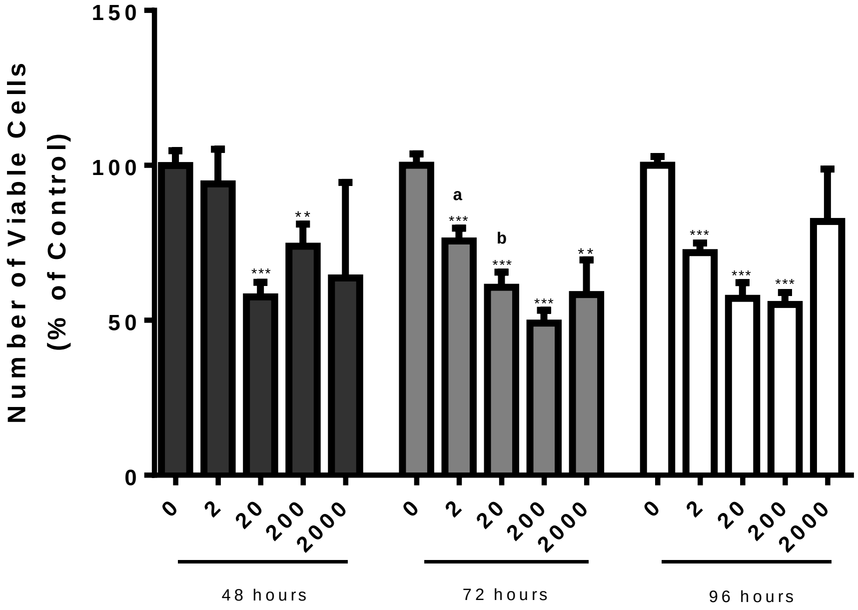


Figure 6 - Number of viable non-neoplastic human prostate epithelial PNT1A cells after exposure to different concentrations of *saco* sweet cherry extracts (2, 20, 200, and 2000 µg/mL) for 48, 72 and 96 hours determined by the MTT assay. Results are expressed as % of control. Error bars indicate mean ±_S.E.M (n≥6). ** P<0.01; *** P<0.001 when compared with the control group.

Relatively to the prostate cancer cell line LNCaP (Fig. 7), treatment for 48 hours with sweet cherry extracts concentrations above 2 µg/mL showed a dose-dependent effect diminishing cell proliferation. Cell proliferation was significantly decreased by roughly 25, 50 and 60 % comparatively with the control for 20, 200 and 2000 µg/mL concentrations, respectively. For 72 hours of treatment, 2-200 µg/mL concentrations were effective supressing cell viability (proliferation around 60%, $P<0.01$) whereas the highest concentration (2000 µg/mL) resulted in an increased (150 % when compared to the control group, $P<0.001$). Similarly, the highest cherry extract concentration 2000 µg/mL augmented proliferation of LNCaP cells for 96 hours of exposure (120% when compared to the control group, $P<0.05$). Cell viability results obtained after 96 hours of exposure with 2 and 20 µg/mL concentrations were not significantly altered, and the only decrease in proliferation was observed at 200 µg/mL (80% when compared to the control group, $P<0.05$).

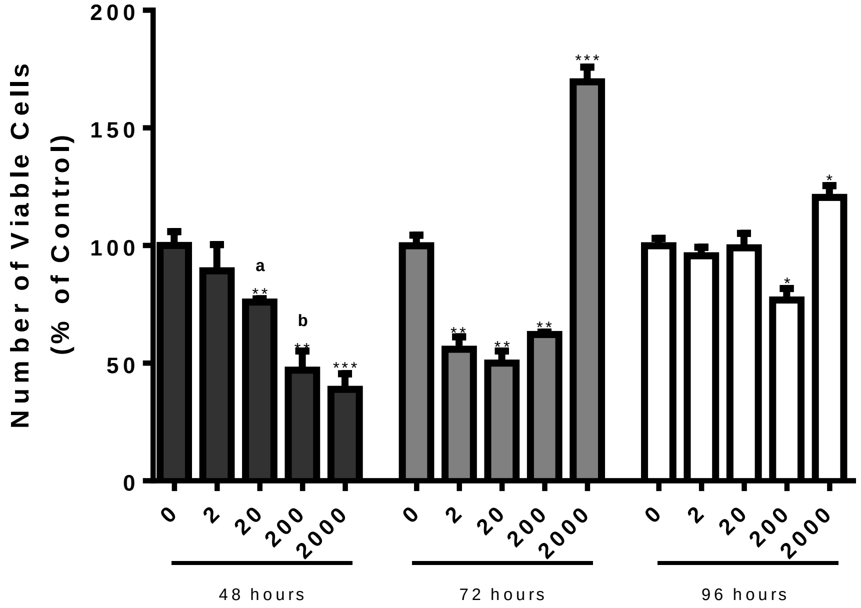


Figure 7 - Number of viable human prostate cancer LNCaP cells after exposure to different concentrations of *saco* sweet cherry extracts (2, 20, 200, and 2000 µg/mL) for 48, 72 and 96 hours determined by the MTT assay. Results are expressed as % of control. Error bars indicate mean ±_S.E.M (n≥6). * $P<0.05$; ** $P<0.01$; *** $P<0.001$ when compared with the control group.

Finally, concerning the neoplastic prostate cancer cells PC3 (Fig. 8), all cherry extract concentrations showed significantly decreased cell proliferation for 48 hours of exposure. Concentrations from 2 to 200µg/mL supressed cell proliferation by approximately 40% whereas at 2000 µg/mL cell proliferation remained only 10% of that of control. However, and similarly to that observed in PNT1A cells, treatment with 2000 µg/mL for 72 and 96 hours showed no significant decrease of cell viability. Also, no dose-dependent effect was found. Cell proliferation in the 2-200 µg/mL concentrations was significantly decreased both for 72 and 96 hours of exposure, being, respectively, around 50 % and 40 % of that of the control group.

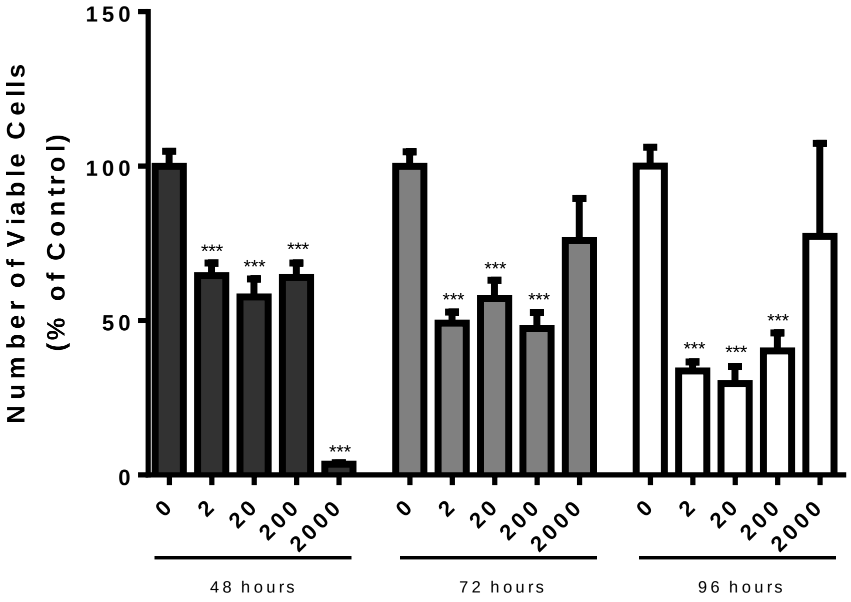


Figure 8 - Number of viable human prostate cancer PC3 cells after exposure to different concentrations of *saco* sweet cherry extracts (2, 20, 200, and 2000 µg/mL) for 48, 72 and 96 hours determined by the MTT assay. Results are expressed as % of control. Error bars indicate mean ±_S.E.M (n≥6). *** P<0.001 when compared with the control group.

Integrating the results of cell viability obtained for the three cell lines under study, the concentration of 20 µg/mL and the incubation time of 72 hours were selected for subsequent analysis of apoptosis and metabolism.

Additionally, it should be stated that controls without cherry extracts added and with ethanol concentrations equivalent to that found in the experimental culture medium (Table 2) were run. Including for the highest ethanol concentration present in the cherry extracts, this compound has demonstrated a negligible effect on cell proliferation at the different time-frames.

4.2 Apoptotic pathways

Exposure to sweet cherry extracts was underpinned by the up-regulated expression of pro-apoptotic genes in PNT1A cells

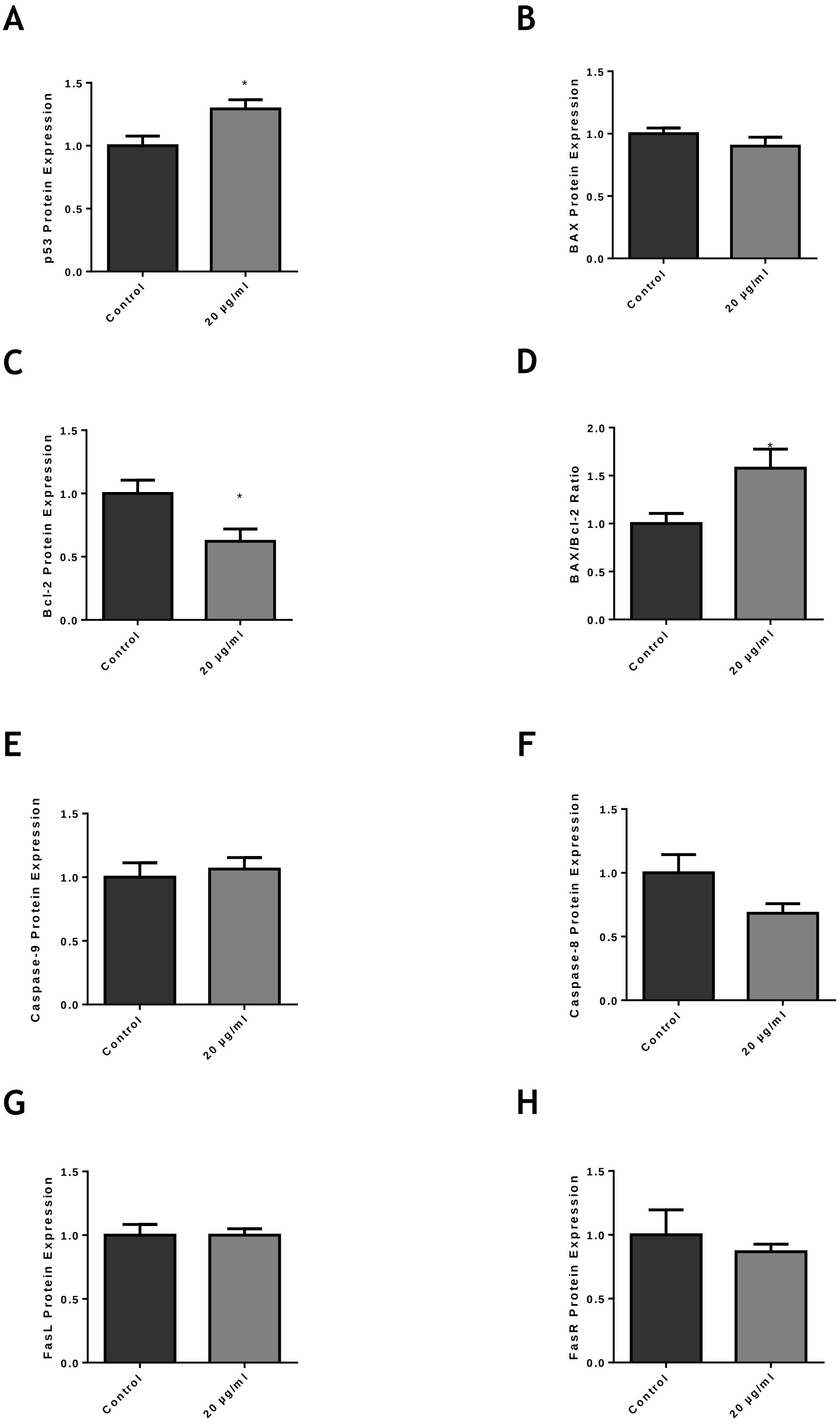
The expression levels of the pro-apoptotic tumour suppressor p53 protein, whose heightened expression marks one the initiation of accelerated extrinsic apoptosis [268], were significantly increased in PNT1A cells exposed to sweet cherry extracts (almost a 30% increase when compared to the control group, P=0.0305, Fig. 9A).

Accompanying this result, the expected increase in the expression of pro-apoptotic protein BAX was, however, not observed. BAX expression showed a minor, non-significant decrease of under 10% (P=0.2913) (Fig. 9B). However, the expression of the anti-apoptotic protein Bcl-2, which is known to down-regulate the expression of BAX [269] was significantly decreased in PNT1A cells upon exposure to cherry extracts (a 38% reduction comparatively with the control, P=0.0250, Fig. 9C). This decrease in Bcl-2 expression resulted in an overall significant increase in the BAX/Bcl-2 ratio of over 55% (P=0.0396) (Fig. 9D), which itself is a known indicator of a pro-apoptotic state [270].

Despite the obtained results on the mitochondrial regulators of apoptosis, the expression of caspase-9 (P=0.6922, Fig. 9E), the initiator caspase associated with the intrinsic pathway of apoptosis wasn't significantly altered.

Quantifications were also made for the expression of three proteins associated with the extrinsic pathway of apoptosis, caspase-8 (Fig. 9F), FasL (Fig. 9G) and FasR (Fig. 9H), though no statistically significant differences were perceived.

Also, the activity of caspase-3, a major indicator of apoptosis modulated by both the intrinsic and extrinsic pathways, was not significantly altered in PNT1A cells by the administration of cherry extracts (Fig. 10).


A
p53 Protein Expression
0.0
0.5
1.0
1.5
*
Control
20 µg/ml
B
BAX Protein Expression
0.0
0.5
1.0
1.5
Control
20 µg/ml
C
Bcl-2 Protein Expression
0.0
0.5
1.0
1.5
*
Control
20 µg/ml
D
BAX/Bcl-2 Ratio
0.0
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*
Control
20 µg/ml
E
Caspase-9 Protein Expression
0.0
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Control
20 µg/ml
F
Caspase-8 Protein Expression
0.0
0.5
1.0
1.5
Control
20 µg/ml
G
FasL Protein Expression
0.0
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Control
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FasR Protein Expression
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1.5
Control
20 µg/ml

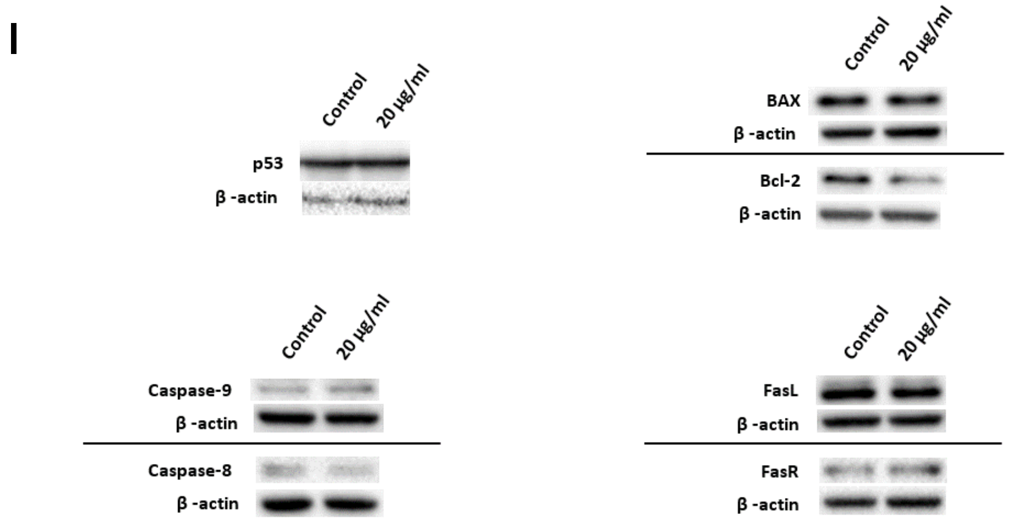


Figure 9 - Expression of apoptosis regulators (A-H) in the human prostate epithelial PNT1A cells after exposure to 20 µg/mL of *saco* (July crop) cherry for 72 hours, determined by Western blot analysis after normalization with ß-actin. Results are expressed as fold-variation relatively to control. Error bars indicate mean ±_S.E.M (n≥4). * P<0.05. Representative blots are shown in panel I.

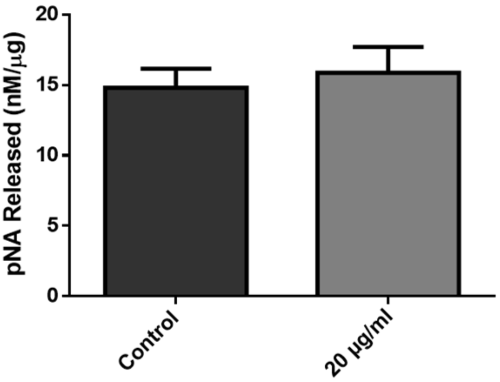


Figure 10 - Activity of Caspase-3 in the human prostate PNT1A epithelial cells after exposure to 20 µg/mL of *saco* (July crop) cherry for 72 hours, determined by a specific assay kit. Error bars indicate mean ±_S.E.M (n≥4).

Up-regulated expression of pro-apoptotic genes and increased activity of caspase-3 in LNCaP cells treated with sweet cherry extracts

Despite no significant differences being observed on the expression of tumour suppressor p53 (Fig. 11A) and pro-apoptotic regulator BAX proteins (Fig. 11B), the expression levels of the anti-apoptotic Bcl-2 protein were significantly lowered in the LNCaP-stimulated cells when compared to the respective control (31% decrease, P=0.0145, Fig. 11C). This significant decrease in the expression of Bcl-2 together with the unaltered BAX expression resulted in an overall increase of over 50% in the BAX/Bcl-2 ratio (Fig. 11D).

Also, the expression of caspase-9 (Fig. 11E) was increased in LNCaP cells treated with cherry extracts (36% increase when compared to the control group, P=0.0210) and, most importantly, a noticeable increase in the activity of caspase-3 was observed (71%, P=0.0471,

Fig. 12). Regarding the extrinsic pathway of apoptosis, measuring and quantifying of both caspase-8 and FasR were attempted without any quantifiable results.

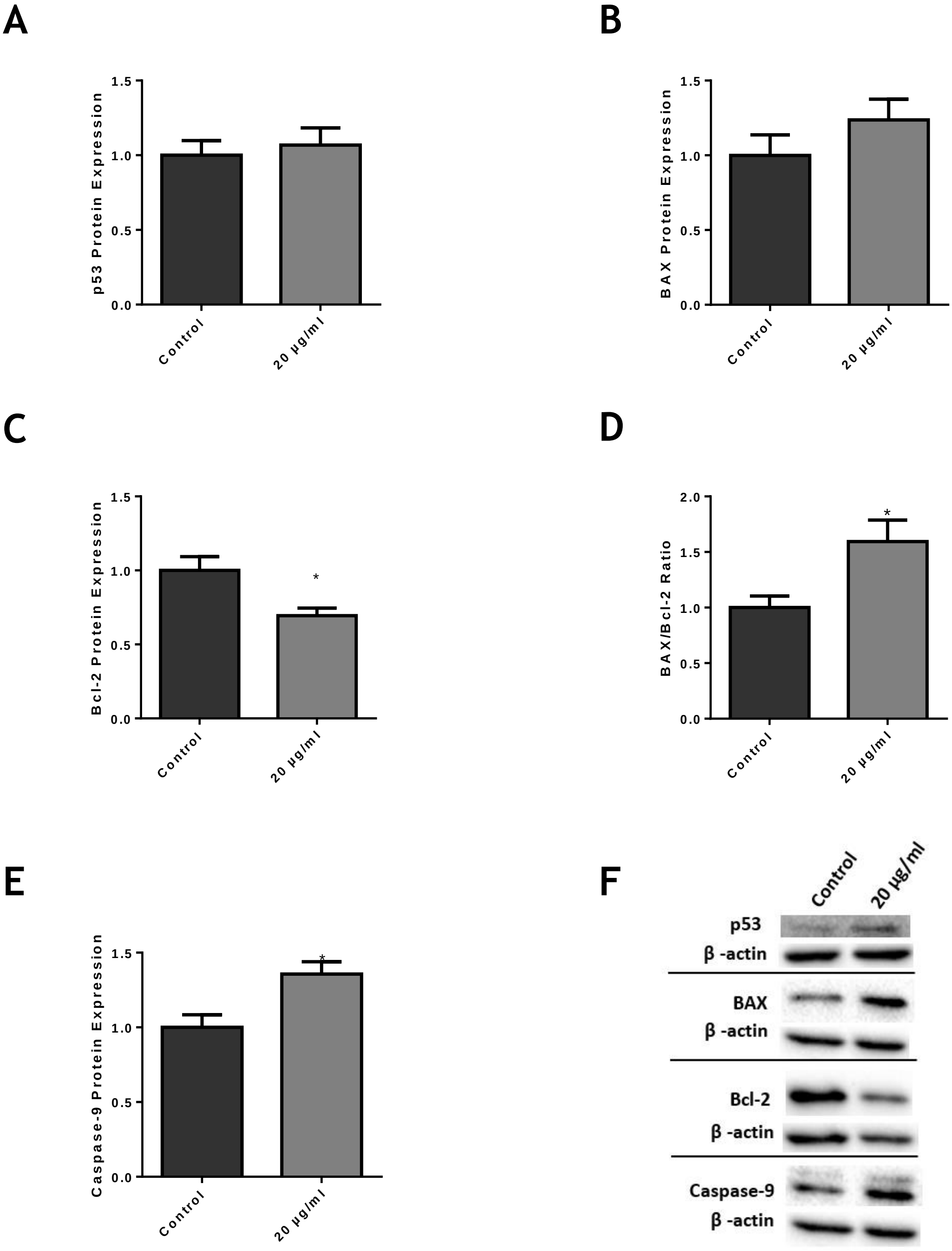


Figure 11 - Expression of apoptosis regulators (A-E) in the human prostate cancer LNCaP cells after exposure to 20 µg/mL of *saco* (July crop) cherry for 72 hours, determined by Western blot analysis after normalization with ß-actin. Results are expressed as fold-variation relatively to control. Error bars indicate mean ±_S.E.M (n≥4). * P<0.05. Representative blots are shown in panel F.

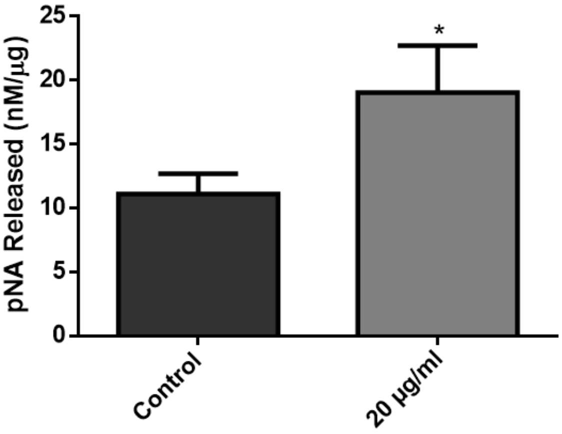


Figure 12 - Activity of Caspase-3 in the human prostate cancer LNCaP cells after exposure to 20 µg/mL of *saco* (July crop) cherry for 72 hours, determined by a specific assay kit. Error bars indicate mean ±_S.E.M (n≥5). * P<0.05.

Expression of pro-apoptotic genes is unaltered or down-regulated in PC3 cells after exposure to sweet cherry extracts

Unlike PNT1A and LNCaP, PC3 cells do not normally express the p53 protein [271], thus, there was no attempt of its quantification in this cell line.

Concerning other apoptosis regulators, neither BAX (Fig. 13A) nor Bcl-2 (Fig. 13B) expression displayed any statistically-significant difference between PC3-treated and untreated cells. As a result, the BAX/Bcl-2 ratio (Fig. 13C) was also unaltered in response to the administration of cherry extracts.

The expression of caspase-9 was significantly decreased in PC3 cells exposed to cherry extracts (approximately a 20% reduction when compared with the control group, P=0.0415, Fig. 13D), whereas no significant difference could be perceived on the expression of the extrinsic apoptosis pathway-associated caspase-8 (Fig. 13E).
Nevertheless, caspase-3 activity was significantly decreased in the PC3 cells under stimulation of cherry extracts (Fig. 14). A reduction of roughly 50% relatively to the control group was observed (P=0.0383).

FasR labelling and quantification were attempted but the obtained signals were too faint to be processed.

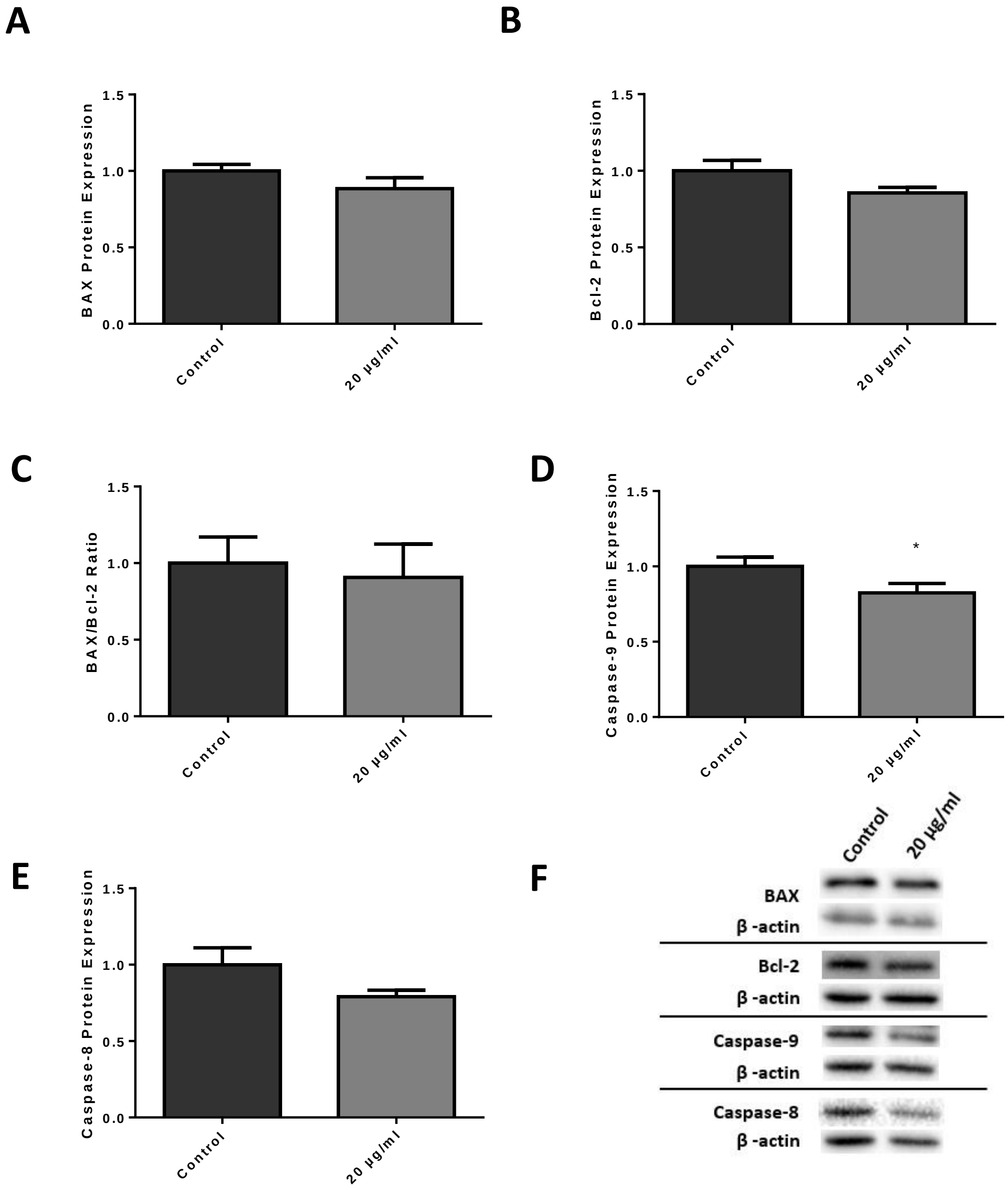


Figure 13 - Expression of apoptosis regulators (A-E) in the human prostate cancer PC3 cells after exposure to 20 µg/mL of *saco* (July crop) cherry for 72 hours, determined by Western blot analysis after normalization with ß-actin. Results are expressed as fold-variation relatively to control. Error bars indicate mean ±_S.E.M (n≥4). * P<0.05. Representative blots are shown in panel F.

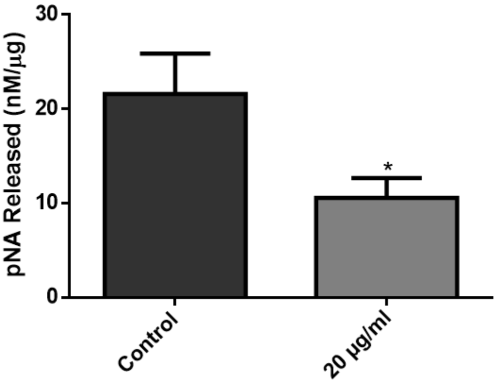


Figure 14 - Activity of Caspase-3 in the human prostate cancer PC3 cells after exposure to 20 µg/mL of *saco* (July crop) cherry for 72 hours, determined by a specific assay kit. Error bars indicate mean ±_S.E.M (n≥5). * P<0.05.

4.3 Glucose metabolism

Sweet cherry extracts augmented lactate export in PNT1A cells

The biochemical assay measuring glucose concentration in cell culture medium allowed to demonstrate that no significant changes exist in the amount of glucose consumed (Fig. 15A) by the control and cherry extract-stimulated PNT1A cells (P=0.9653). However, the amount of lactate exported by PNT1A cells (Fig. 15B) was significantly increased by over 35% in the presence of cherry extracts (P=0.0092). Although glucose consumption was unaltered in PNT1A cells under cherry extracts stimulation, a significantly decreased expression of both GLUT1 (Fig. 16A) and GLUT3 (Fig. 16B) (approximately 17% (P=0.0239) and 27% (P=0.0402), respectively) was observed. Concerning the metabolization of the internalized glucose, we evaluated the expression of PFK-1 (Fig. 16C), which was also significantly decreased, by almost 12% (P=0.0461) in PNT1A-treated cells when compared to the respective control group. The end-product of glycolysis pyruvate can be converted to lactate, that is then exported to the extracellular space by the activity of MCTs, specifically the MCT4, whose expression was significantly decreased in PNT1A cells exposed to cherry extracts (Fig. 16D, roughly 33% reduction comparatively with the control group). Despite the augmented lactate export in PNT1A cell in response to the administration of cherry extracts, no significant alterations were found in the expression of LDH (Fig. 16E), the enzyme responsible for the conversion of pyruvate into lactate (P=0.3438). However, the activity of LDH (Fig. 17) was significantly increased in PNT1A-treated cells, an increase over 100% was observed relatively to the control values.

Lastly, the expression of glutaminase was also analysed. Glutaminase is a key enzyme in the metabolism of glutamine, but its increased expression has been associated with increased glucose utilization by prostate cancer cells [90]. No significant differences were found in glutaminase expression (Fig. 16F, P=0.5837) between treated and untreated-PNT1A cells. No positive Western blot result with quantifiable signals could be obtained for the glutamine transporter ASCT2.

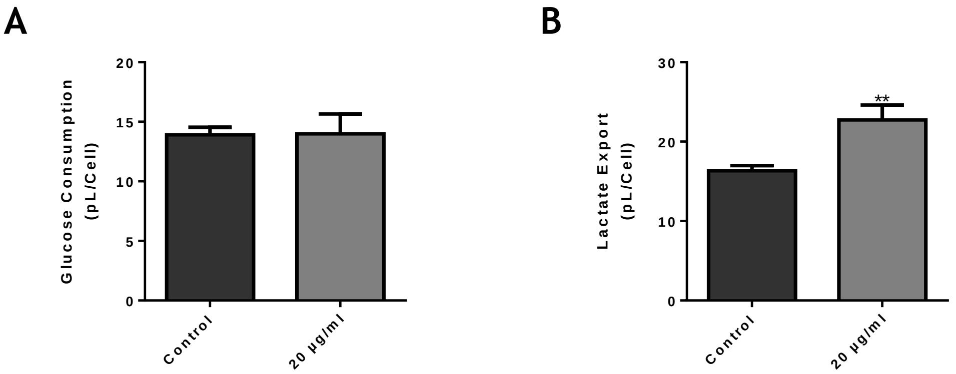
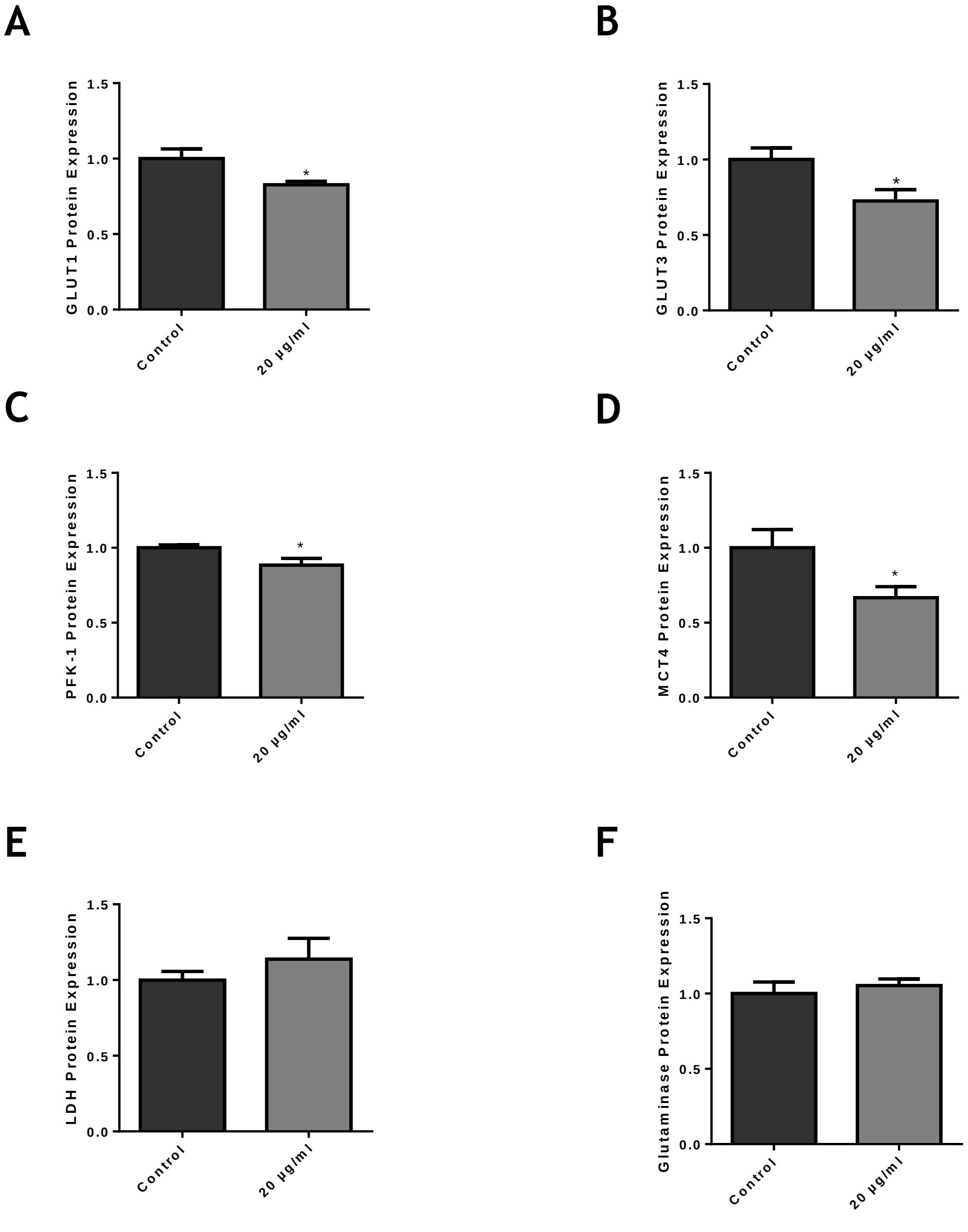


Figure 15 - Glucose consumption (A) and lactate export (B) in human prostate PNT1A epithelial cells after exposure to 20 µg/mL of *saco* (July crop) cherry for 72 hours, determined by spectrophotometric assays. Error bars indicate mean ±_S.E.M (n≥6). ** P<0.01.



G

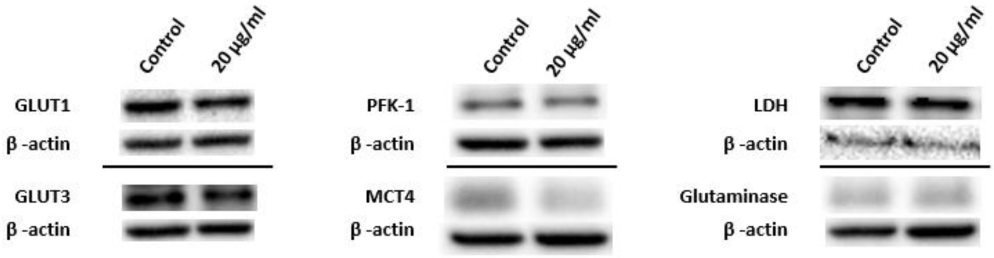


Figure 16 - Expression of metabolism-associated proteins (A-F) in human prostate PNT1A epithelial cells after exposure to 20 µg/mL of *saco* (July crop) cherry for 72 hours, determined by Western blot analysis after normalization with ß-actin. Results are expressed as fold-variation relatively to control. Error bars indicate mean ±_S.E.M (n≥5). * P<0.05. Representative blots are shown in panel G.

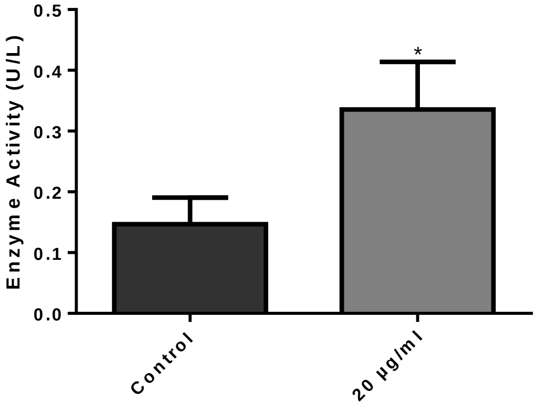


Figure 17 - LDH enzymatic activity in human prostate PNT1A epithelial cells after exposure to 20 µg/mL of *saco* (July crop) cherry for 72 hours, determined by spectrophotometric assays. Error bars indicate mean ±_S.E.M (n≥4). * P<0.05.

The glycolytic metabolism of LNCaP cells is supressed in the presence of sweet cherry extracts

Both glucose consumption (Fig. 18A) and lactate export (Fig. 18B) were significantly decreased in LNCaP cells under stimuli of cherry extracts (respectively, 78% (P=0.0053) and 26% (P=0.0159) reduction when compared with the respective controls).

In spite of this marked change, expression of the glucose transporter GLUT1 (Fig. 19A) seemed to be statistically-significantly unchanged from its control value (P=0.4205). Expression of GLUT3, however, had a very significant decrease of roughly 45% (P=0.0002, Fig. 19B).

The expression of PFK-1 (Fig. 19C), the enzyme that limits the glycolytic flux, was significantly increased by over 100% (P=0.0295) in LNCaP cells in the presence of cherry extracts.

The expression of the lactate exporter MCT4 (Fig. 19E) was also significantly altered, with a decrease of over 35% (P=0.0233).

Although LDH expression (Fig. 19D) was unaltered (P=0.5898) between treated and untreated cell, the activity of the LDH (Fig. 20) was significantly decreased in LNCaP cells in response to the administration of cherry extracts (roughly 50% variation relatively to the control, P=0.0287).

No positive Western blot assays could be obtained for glutaminase and ASCT2.

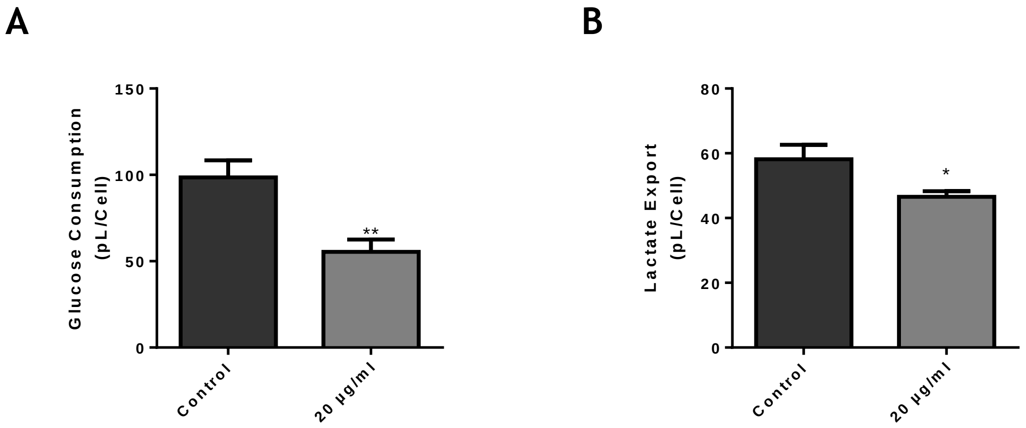


Figure 18 - Glucose consumption (A) and lactate export (B) in human prostate cancer LNCaP cells after exposure to 20 µg/mL of *saco* (July crop) cherry for 72 hours, determined by spectrophotometric assays. Error bars indicate mean ±_S.E.M (n≥6). * $P<0.05$; ** $P<0.01$.

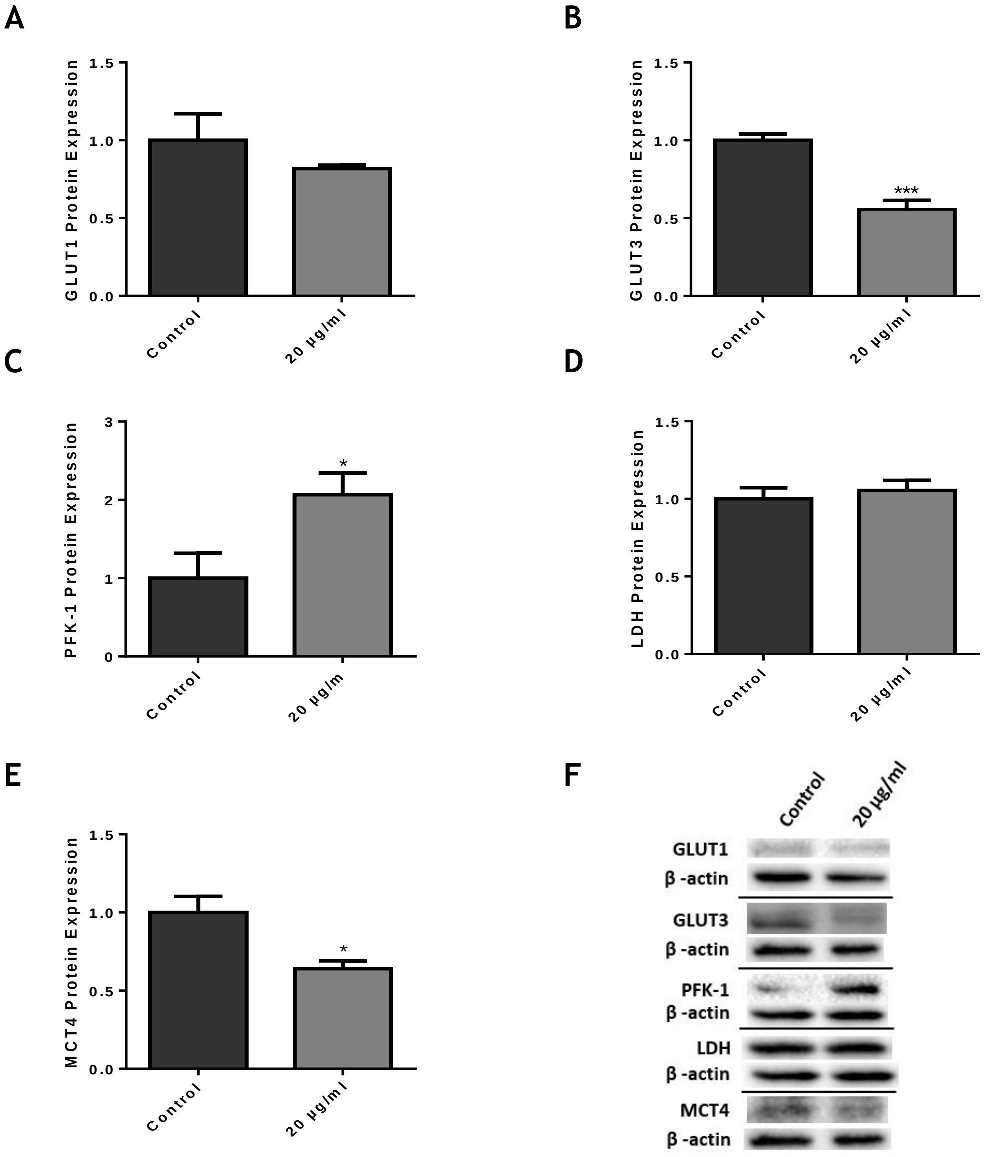


Figure 19 - Expression of metabolism-associated proteins (A-E) in human prostate cancer LNCaP cells after exposure to 20 µg/mL of *saco* (July crop) cherry for 72 hours, determined by Western blot analysis after normalization with β-actin. Results are expressed as fold-variation relatively to control. Error bars indicate mean ±_S.E.M (n≥4). * P<0.05; *** P<0.001 Representative blots are shown in panel F.

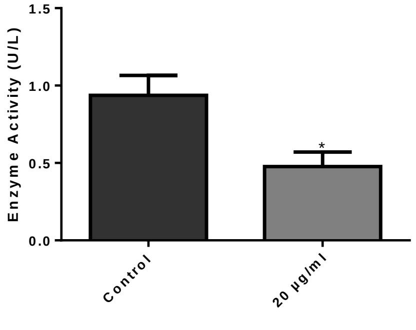


Figure 20 - LDH enzymatic activity in human prostate cancer LNCaP cells after exposure to 20 µg/mL of *saco* (July crop) cherry for 72 hours, determined by spectrophotometric assays. Error bars indicate mean ±_S.E.M (n≥4). * P<0.05.

Sweet cherry extracts enhanced glucose consumption and lactate export in PC3 cells

Unlike the observed in the other non-neoplastic (PNT1A) and neoplastic (LNCaP) cell lines, both glucose consumption (Fig. 21A) and lactate export (Fig. 21B) were significantly increased in PC3 cells exposed to sweet cherry extracts (respectively, 100% (P<0.0001) and roughly 40% (P=0.0477) when compared to the respective controls).

Despite the augment in glucose consumption, no significant changes could be perceived in the protein expression of both GLUT1 (Fig. 22A) and GLUT3 (Fig. 22B) (P=0.5140 and P=0.3951, respectively). The glycolytic enzyme PFK-1, however, displayed significantly-decreased expression in PC3 cells under stimuli of cherry extracts (roughly 23% relatively to the control, P=0.0468, Fig. 22C).

Concerning lactate-regulating proteins, the expression of LDH (Fig. 22D) and MCT4 (Fig. 22E), were also unaltered in response to cherry extracts (P=0.3261 and P=0.1788, respectively). Nevertheless, LDH activity was significantly decreased in PC3-treated cells when compared to the control group (roughly 60% variation, P=0.0063, Fig. 23).

Lastly, expression of the complementary glutaminase enzyme was also unaltered (P=0.9023, Fig. 22F).

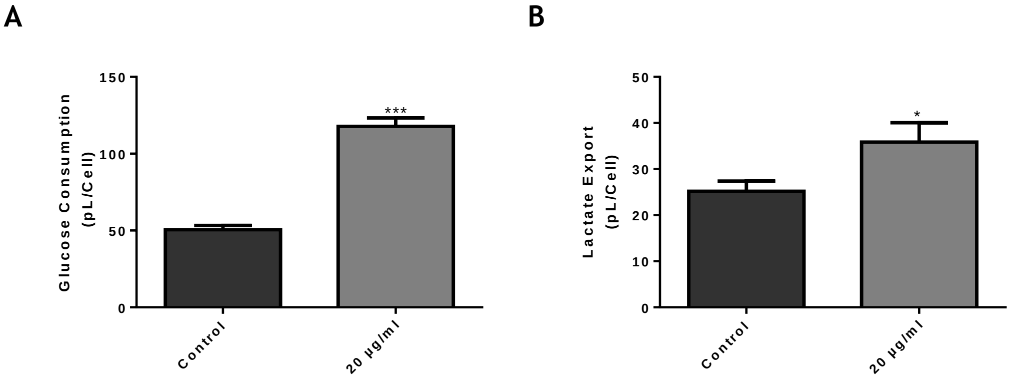


Figure 21 - Glucose consumption (A) and Lactate export (B) in human prostate cancer PC3 cells after exposure to 20 µg/mL of *saco* (July crop) cherry for 72 hours, determined by spectrophotometric assays. Error bars indicate mean ±_S.E.M (n≥5). * P<0.05; *** P<0.001.

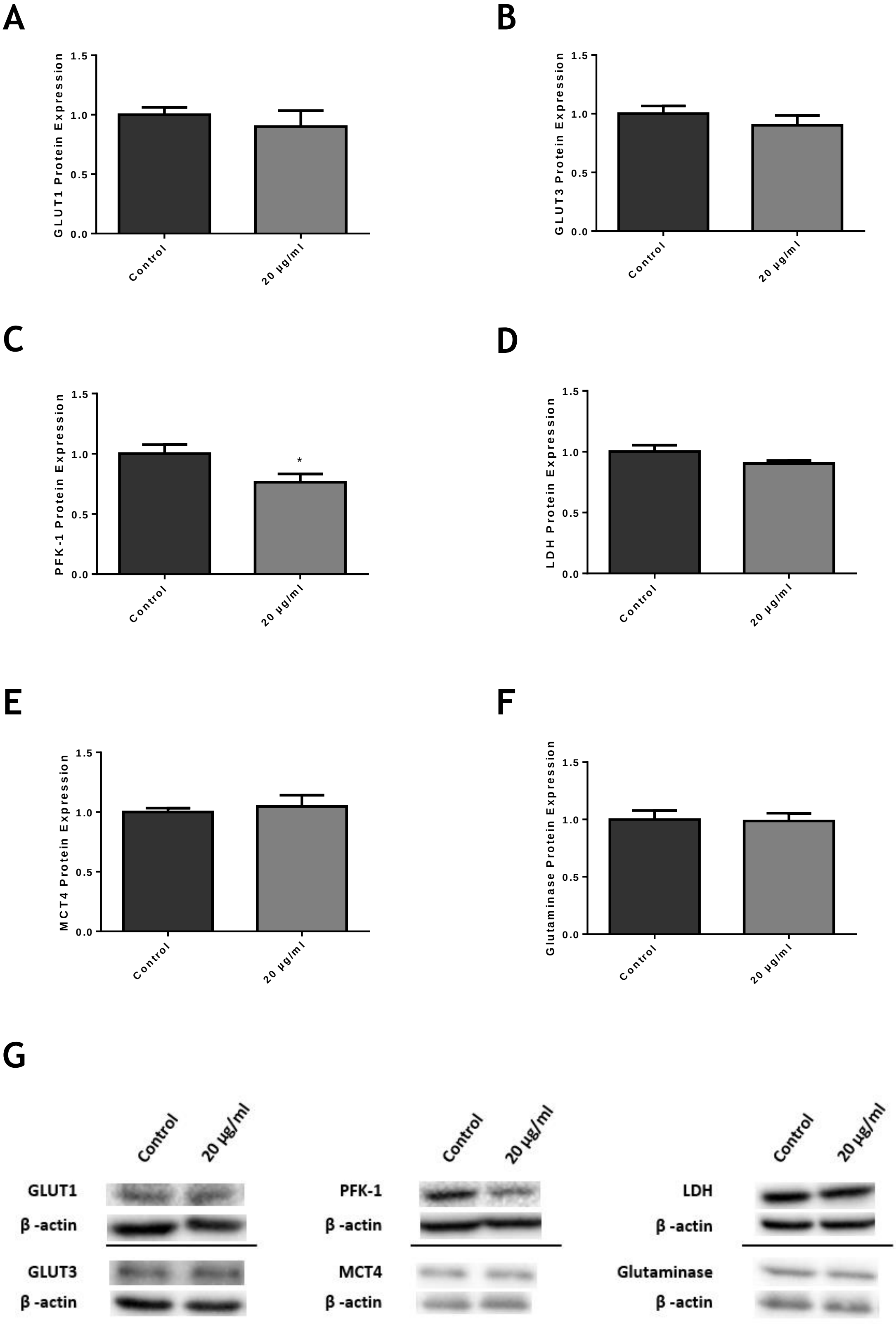


Figure 22 - Expression of metabolism-associated proteins (A-F) in human prostate cancer PC3 cells after exposure to 20 µg/mL of *saco* (July crop) cherry for 72 hours, determined by Western blot analysis after normalization with ß-actin. Results are expressed as fold-variation relatively to control. Error bars indicate mean ±_S.E.M (n≥4). * P<0.05. Representative blots are shown in panel G.

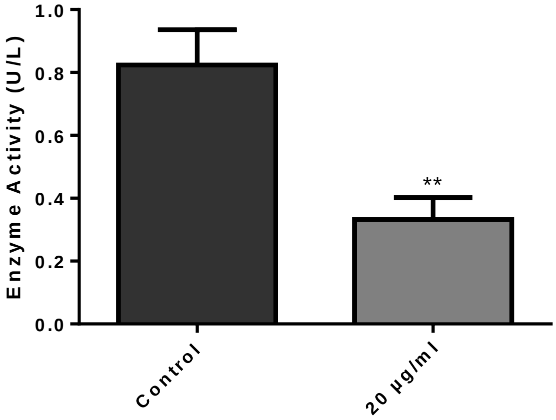


Figure 23 - LDH enzymatic activity in human prostate cancer PC3 cells after exposure to 20 µg/mL of *saco* (July crop) cherry for 72 hours, determined by spectrophotometric assays. Error bars indicate mean ±_S.E.M (n≥6). * P<0.05.

Chapter 5 – Discussion

The present thesis investigated the effect of *Saco* sweet cherry extracts from a late July crop in the proliferation, apoptosis and glycolytic metabolism of neoplastic (PNT1A) and non-neoplastic (LNCaP and PC3) prostate cells. PNT1A cells are immortalized cells representative of a non-neoplastic prostatic epithelium, whereas LNCaP and PC3 cells are representative of prostate cancer of moderate metastatic potential (androgen-dependent) and of elevated metastatic potential (androgen-independent), respectively. These cell line models have been widely used to study the behaviour of prostate cancer cells in multiple aspects including the evaluation of glycolytic metabolism [82, 272, 273].

The results obtained in the MTT proliferation assays allowed firstly to conclude that, in a general sense, all three cell types behaved consistently with what was observed in the literature in similar cases, showing a general tendency for decreased proliferation in the presence of cherry extracts [219, 274]. Cherry extracts are characterized by containing large quantities of anthocyanins, minerals, and other assorted nutritional components [97].The present findings also resemble studies performed with similar fruit extracts on different cancer cell lines [275, 276], as well as different anthocyanin-rich extracts on prostate cancer cell lines [219, 277].

Despite cherry extracts diminish the proliferation of all prostate cell lines used in this study, there were noticeable differences between them. It is noteworthy that the anti-proliferative effect of cherry extracts was more pronounced in the PC3 cells when compared to the LNCaP or non-neoplastic prostate epithelial cells PNT1A. Cancer cells usually have accelerated rates of cell division and stimulated metabolic activity, thus it is expected that PC3 cells naturally have higher division rates comparatively with their non-neoplastic counterparts, which would result in faster metabolization of extracts and enhanced effects.

Also, it cannot be excluded from the discussion the fact that not always a dose-dependent effect could be reported, with some unexpected cell viability values being observed in LNCaP cells treated with cherry extracts for 96 hours. At this time point, proliferation of LNCaP cells exposed to different concentrations of cherry extracts was almost unaltered, except for the highest concentration (2000 µg/mL) that resulted in increased proliferation values. This unpredictable augment of cell proliferation with the 2000 µg/mL concentration was also seen for 48 hours of exposure. There is no definitive explanation for both these phenomena but the fact that the cherry extracts did not undergo any purification process, as well as the possibility that some of the useful compounds such as anthocyanins could have been partially degraded during the MTT process into other compounds (including sugars) that were also unaccounted for, could possibly contribute to the findings obtained. In addition, some difficulties were experienced while elaborating the MTT proliferation assays in LNCaP cells, which also may be underpinning the lower consistency of results obtained in this cell line. Taking all this into consideration, a concentration of 20 µg/mL and an exposure time of 72 hours were selected for the subsequent stimuli of PNT1A, LNCaP and PC3 cells.

The diminished viability of PNT1A- and LNCaP-treated cells determined by the MTT assays, was accompanied by the altered expression and activity of target regulators of apoptosis, generally confirming the anti-proliferative effects of cherry extracts.

In PNT1A cells, the upregulated expression of pro-apoptotic protein p53, together with the down-regulation of anti-apoptotic Bcl-2 protein, and the subsequent increase in the BAX/Bcl-2 ratio, would be indicative of an increased apoptotic activity in these cells after exposure to 20 µg/mL late-crop *saco* sweet cherry extracts for 72 hours. However, the expression of other apoptosis regulators was not significantly altered. Moreover, no changes were observed in the activity of caspase-3, an end-point of apoptosis triggered by both the intrinsic and extrinsic apoptotic pathways, which hampers the conclusion that apoptosis was stimulated in PNT1A cells in the presence of cherry extract. Due to the complexity of the apoptotic mechanism, and on the basis of the results obtained, it is not possible to conclude that the reduced cell proliferation induced by cherry extracts in these cells is consequence of augmented apoptosis. This issue could lie with a variety of factors, such as possible interference by the activation of the cell's growth factor receptors, or deficient cytochrome c release due to interference of other modulating factors. Both of these phenomena have been observed in different cell types under different circumstances [278–280] .

Results obtained for the LNCaP cell line conform closely to the expected results based on the available scientific literature showing that certain cherry extracts stimulate apoptosis of multiple types of cancer cells [275, 276]. Firstly, the fact that there was no increase in the expression of p53 would seem to indicate that the intrinsic apoptotic pathway was not implicated, since the accumulation of p53 in response to stress-induced DNA damage is one of the initiator steps of the intrinsic apoptotic pathway. However, the downregulation of Bcl-2 (and subsequent increase in the BAX/Bcl-2 ratio), the upregulation of caspase-9 and the augmented activity of caspase-3 demonstrated that the aforementioned pathway was indeed active in LNCaP-treated cells. Moreover, these findings indicate that LNCaP cells in the presence of cherry extracts were undergoing apoptosis at more accelerated rates than the controls, in agreement with the decreased proliferation/cell viability.

In the PC3 cells the decreased expression of caspase-9 and the diminished activity of caspase-3, in spite of the fact that there were no observable changes to either BAX or Bcl-2, demonstrated that, in the particular case of this cell line, cherry extracts suppressed the activity of the intrinsic apoptotic pathway. The fact that PC3 cells express little or no p53 (one of the initiators of the intrinsic pathway) [281], suggests an innate resistance to any attempts to upregulate this pathway. On the other hand, the unchangeable expression of caspase-8 in response to the administration of cherry extracts does not support the involvement of the extrinsic pathway of apoptosis contributing to the diminished viability observed in PC3 cells. Also, there were no attempts made at assaying the activation of caspase-independent cell death pathways, but these could be a possibility, as these pathways have been triggered in PC3 cells through treatment with other compounds [282].

In the last years, the reprogramming of cell metabolism has been recognized as a hallmark of cancer and an interesting point of intervention for treatment [283]. It is widely known that highly proliferative cancer cells rely on an accelerated rate of glycolysis to produce the energy needed for their accelerated rates of proliferation [284]. Most importantly, cancer cells prioritize the usually anaerobic pathway, which results in the production of very large amounts of lactate without the usual requirement of a hypoxic environment [285, 286]. This phenomenon is also documented in the particular case of prostate cancer [272]. Previous findings from our research group have shown that PC3 and LNCaP cells have a distinct glycolytic metabolism comparatively with PNT1A cells, and that androgens are important regulators of this metabolic pathway [82, 272].

In order to create a more complete picture of the reasons behind the decrease in proliferation observed in PNT1A, LNCaP and PC3 cells under cherry extracts-stimulation, the glycolytic metabolism was also studied.

The decreased proliferation observed in the non-neoplastic PNT1A cells treated with cherry extracts, was not fully in agreement with the unaltered glucose consumption observed in these cells, despite the downregulated expression of GLUTs, GLUT1 and GLUT3. Glucose intake into the cell is ensured by specific GLUTs, of which GLUT1 and GLUT3 are the most well-characterized and frequently-studied in prostate cells. A lower expression of these two transporters will usually signify a lower glucose intake, and incongruences in this scheme should be explained by opposing expressions of other glucose transporters. The presence of other GLUTs isoforms, namely, GLUT12 has been reported in prostate cancer cases and its role as one of the main glucose suppliers to glycolytic metabolism has been proposed [287-289]. Therefore, it is liable to speculate that an altered expression of GLUT12 would be influencing the overall glucose consumption of PNT1A cells. Still, the downregulated expression of other key player of the glycolytic metabolism suggest that there is less energy available due to the downregulation of glycolysis. In fact, the expression of PFK-1 was significantly decreased in PNT1A-treated cells. PFK-1 is one of the most important enzymes in the glycolytic process due to the fact it catalyses a limiting step, the conversion of fructose 6-phospate into fructose 1-6 biphosphate. Thus, decreased expression of PFK-1 would signify a cell decreased capacity to produce pyruvate via the glycolytic pathway, which is demonstrative of the suppression of this energy route.

The glycolytic metabolism itself, as previously-discussed, is a long string of reactions that converts glucose into pyruvate, and said pyruvate may then be transported for mitochondrial respiration or, as is the case of rapidly-dividing cells, be converted into lactate through a process of fermentation that is less efficient but faster in its production of ATP. This conversion into lactate is achieved by the activity of LDH, and this lactate is then exported into the extracellular environment by the MCTs, most notably MCT4 [290].

Herein, the results obtained showed that, the administration of cherry extracts though not affecting glucose consumption, augmented the lactate production by the non-neoplastic PNT1A cells. These findings were supported by the upregulated activity of LDH since MCT4 expression was decreased. In this context, it should be considered that the enhanced exportation of lactate would be occurring by the up-regulated expression of another lactate exporter, namely MCT1, which has been shown to be expressed in both non-neoplastic and neoplastic prostate cells [273, 291, 292].

Most importantly, there is the question of how increased amount of lactate was produced, when glucose consumption was unaltered and the downregulation of PFK-1 was observed. There are several possibilities, with the most immediate being reliant on the increased activity of the LDH enzyme resulting in a higher lactate turnover despite the lower pyruvate production. However, as it has been previously discussed, this lactate overproduction via pyruvate is typical of neoplastic cells. In the non-neoplastic PNT1A cells, other likelier explanation would be that lactate was being generated as a by-product of other metabolic processes, namely, glutaminolysis, the process of cleaving glutamine into glutamate that could result in the production of pyruvate, which leaves the mitochondria generating lactate. Also, the activity of the enzyme alanine transaminase (ALT) that catalyses the reversible reaction of alanine conversion into pyruvate could be responsible for the increased lactate levels, although only the measurement of intracellular levels of pyruvate and ALT activity would allow to conclude this with certainty.

Overall, it can be stated that the rate of apoptosis of the non-neoplastic PNT1A cells remained unaltered, while their glycolytic metabolism was down-regulated despite the consumption of glucose not being impacted and the production of lactate actually being augmented. The importance of lactate in non-neoplastic cells needs clarification but the supressed glycolytic metabolism is in agreement with the diminished proliferation observed in PNT1A-treated cells.

Regarding the glycolytic metabolism of LNCaP cells, no significant differences were found in GLUT1 protein levels whereas GLUT3 expression was significantly decreased in the presence of cherry extracts, which followed the observed diminished glucose intake by these cells. In this way, despite PFK-1 expression was increased, a diminution of lactate production was observed, concomitantly with the diminished activity of LDH and decreased expression levels of MCT4. The enhanced lactate production is a typical feature of cancer cells that has been related with increased metastasis, tumor recurrence, and poor outcome [293]. Thus, the decrease in lactate yield together with the observed diminished proliferation and augmented apoptosis observed in LNCaP-treated cells can be viewed as a positive effect of cherry extracts over these cancer cells, raising the curiosity whether these compounds would ameliorate the response to anti-cancer therapies. The obtained findings are also consistent with the metabolic alterations recorded in several consulted scientific papers describing the antioxidant properties of anthocyanin/phenol-rich extracts and their effects in prostate cancer cell and other cancer cell types [203, 244, 294].

The results pertaining to the glycolytic metabolism of PC3 cells, showed their distinct behaviour comparatively with LNCaP. Administration of cherry extracts significantly increased glucose consumption by PC3 cells, in spite of unaltered expression levels of GLUTs (both GLUT1 and 3), in opposition with the diminished glucose consumption reported in LNCaP cells. As already discussed for PNT1A cells, it cannot be excluded the possibility that other GLUTs, namely GLUT12, may be involved in augmented uptake of glucose by PC3 cells. Also, lactate production was increased in PC3-treated cells, which is not supported by the lower PFK-1 expression, lower LDH activity and unaltered MCT4 levels. Conclusions are impossible to draw without intracellular glucose, pyruvate and lactate measurements, but is liable to speculate that lactate can be originated from alternative metabolic routes, such as that of alanine and glutamine, which are also severely upregulated in cancer cells [295–297]. Nevertheless, cherry extracts did not alter glutaminase expression in PC3 cells.

The contradictory response of PC3 cells comparatively with other prostate cancer models has been described for other experimental conditions and stimuli. Cardoso et al. have found that the tyrosine kinase inhibitor imatinib diminished viability and augmented apoptosis of neoplastic DU145 cells, as expected, whereas promoting the opposite effects in PC3 cells [298]. A distinct behaviour with conflicting results stimulating or inhibiting was also reported for PC3 cells adhesion induced by Akt [299, 300].

Interestingly, the effects in the cell viability and apoptotic cell death of prostate cancer cells under stimulation with cherry extracts were underpinned by alterations in the glycolytic metabolism, which displayed a different pattern between non-neoplastic (PNT1A) and neoplastic prostate cells. Moreover, as mentioned above, a distinct response was observed in both prostate cancer cell lines under study, LNCaP and PC3 cells. These observations are summarized in Table 3.

As a whole, these results point to an overall decrease in proliferation for all three cell lines after they were treated with sweet cherry extracts (as stated in previously-referenced literature), though increased caspase-dependent apoptosis was only detected in LNCaP. It should be noted that other phenolic compounds have been proven to induce apoptosis, differentiation and sensitization to other treatments in LNCaP [301, 302].

Table 3 - Synthesis of the effect of cherry extracts in proliferation, apoptosis and glycolytic metabolism of non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) cells

	Prolif	Apopt	Glycolytic Metabolism							
			GLUT1	GLUT3	PFK-1	MCT4	LDH Expr.	LDH Act.	Glucose Cons.	Lactate Exp.
PNT1A	↓	-	↓	↓	↓	↓	-	↑	-	↑
LNCaP	↓	↑	-	↑	↓	↓	-	↓	↓	↓
PC3	↓	↓	↓	-	↓	-	-	↓		↑

Subtitle: ↓ Downregulated ↑ Upregulated - Unaltered

Regarding PC3 cells, a purer anthocyanin fraction would be expected to yield more conclusive results on the extrinsic apoptotic pathway, as other purified anthocyanin-rich extracts have been demonstrated to activate both caspase-dependent and caspase-independent apoptotic pathways in these cells [219]. With the exception of some of the alterations to the glycolytic metabolism of the PC3 cells, all other ascertained metabolic alterations can be easily rationalized.

The fact that the PNT1A cell line was the least affected overall is supported by published research that indicates that not only are normal epithelial cell lines less impacted by anthocyanin supplementation, but also that the latter can in some cases (such as in andropausal models) even ensure their survival and proper function [246, 303].

In sum, it seems that cherry extracts despite diminishing the proliferation of all prostate cells under study, only were effective enhancing the apoptosis of cancer cells, specifically the LNCaP cells, which was concomitant with the attenuation of the glycolytic metabolism that is typical enhanced in cancer cells.

Chapter 6 – Conclusions and future prospects

The present dissertation demonstrated that anthocyanin-rich sweet cherry extracts effectively decreased the proliferation of both neoplastic and immortalized non-neoplastic cells from the prostatic epithelium. The mechanisms through which this was achieved were also analysed.

The apoptotic rates of the non-neoplastic PNT1A cells weren't increased in the presence of cherry extracts, which suggested that the extracts weren't particularly cytotoxic to healthy epithelial cells, although it wasn't possible to identify the precise mechanism which caused the number of viable cells to decrease.

The sweet cherry extracts revealed to be most effective in LNCaP cells, with the diminished proliferation followed by increased apoptotic rates and a heavy down-regulation of the Warburg effect (enhanced metabolization of glucose with excessive lactate production). LNCaP-treated cells displayed diminished glucose consumption with reduced lactate production.

The effect of sweet cherry extracts on PC3 cells produced less clear results. Cell proliferation was decreased, but apoptosis activity was down-regulated and glycolysis and lactate production were upregulated. Thus, sweet cherry extracts maintained the resistance to apoptosis and enhanced the glycolytic metabolism typical of cancer cells. Overall, the effect of sweet cherry extracts on these highly aggressive, highly-metastatic prostate cancer cells seemed to be beneficial based on the MTT assays alone, but considering the apoptosis and metabolism results no definitive idea about the benefits (or not) could be discerned.

The present findings indicate that the compounds present in the studied cherry extracts are cytotoxic to prostate cancer cells with moderate metastatic potential (LNCaP cells) while being less harmful to the healthy epithelium, which raises the curiosity whether the consumption of sweet cherries could prove beneficial to prostate cancer patients. To start shedding light on this question *in vivo* studies will be performed to assess the effect of cherry extracts administration in the development of carcinogen-induced tumours in rat animal models.

Also, and before any definitive statement can be made on the benefits of cherry consumption in regards to preventing or attenuating the progression of prostate cancer, there are still certain parameters that must be analysed. First of all, it will be determinant to analyse the expression of other apoptosis-related proteins, as well as, the target players in the caspase-independent cell death pathways. Secondly, other metabolic routes, also known to be enhanced in cancer cells, should be explored. This includes the analysis of lipids and glutamine metabolism, which could all give a more complete vision of the effect of these cherry extracts on prostate cells. Also, the expression levels of other glucose and lactate transporters could also be assayed to paint a clearer picture of how the altered glycolytic processes of these cells are again altered by cherry extracts. Thirdly, it should prove beneficial to repeat the assays and tests realized during this dissertation with purified anthocyanin fractions on all studied cell lines. The results obtained from a purer and more concentrated extract, deprived of most extraneous components, could also be deprived of several negative and positive synergies, and thus differences between these sets of data could slowly optimize these extracts for greater efficiency.

Lastly, a caveat has to be added that all these possible discoveries would yield little benefit if they weren't demonstrably applicable in a patient context. In order to definitively prove that consumption of sweet cherries or ingestion of cherry-based supplements are both beneficial for prostate cancer prevention and attenuation, the necessity to assay the bioavailability of these compounds in the possible affected areas inside the human body, post-digestion will remain a crucial aspect.

Chapter 7 – References

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Chapter 8 - Publications and Communications

Oral Communication

Silva G.R., Vaz C.V., Duarte A.P., Socorro S. “Beneficial effects of sweet cherry extracts on human prostate cells.” XI Annual CICS-UBI Symposium. 30th June and 1st July 2016, Covilhã, Portugal.