UNIVERSIDADE DA BEIRA INTERIOR
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# The effect of plant extracts in human prostate cells: implications in cancer

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“For artists, scientists, inventors, schoolchildren, and rest of us, intrinsic motivation - the drive to do something because it is interesting, challenging and absorbing - is essential for high levels of creativity “

- Dan Pink

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Manifesto a minha gratidão e o meu maior reconhecimento:

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# Resumo

O cancro da próstata é uma das doenças neoplásicas mais frequentes no sexo masculino e a segunda causa de morte oncológica, nos países ocidentais. Apesar dos desenvolvimentos ocorridos nas últimas décadas, ainda são necessárias novas abordagens terapêuticas para fazer face ao desenvolvimento e progressão desta patologia. Desde a antiguidade que os produtos naturais são utilizados para fins terapêuticos. E ao longo dos anos tem havido crescentes evidências de que as plantas medicinais desempenham um papel crucial na prevenção e mitigação de diferentes doenças humanas. Os compostos fenólicos constituem um dos grupos mais extensos de produtos químicos no reino vegetal, aos quais têm sido atribuídas propriedades medicinais. Estes compostos apresentam efeitos antiproliferativos e antioxidantes, aos quais têm estimulado um grande interesse na investigação da sua aplicabilidade no controlo do crescimento de células cancerígenas. Contudo, são ainda muito pouco conhecidos os efeitos dos compostos fenólicos no controlo de outras propriedades das células cancerígenas, como por exemplo, sobre a sua capacidade de contrariar o metabolismo híper-glicolítico e a reprogramação do metabolismo. *Crataegus monogyna* e *Arbutus unedo* são exemplos de plantas ricas em compostos fenólicos. O *C. monogyna,* "pirliteiro", é uma espécie da classe *Mognoliopsida*, ordem *Rosales*, família *Rosaceae*, que se encontra amplamente distribuído na Península Ibérica, Europa Ocidental e Central e Norte da África. É uma planta com interesse farmacológico, devido aos seus diversos tipos de benefícios, tais como no tratamento da hipertensão, distúrbios cardíacos e distúrbios digestivos. O *A. unedo*, "medronheiro", é uma espécie da classe *Mognoliopsida*, ordem *Ericales*, da família *Ericaceae*. É uma planta que cresce espontaneamente ao redor da bacia do Mediterrâneo, bem como noutras regiões com verões quentes e invernos amenos e chuvosos. Esta planta tem vindo a ser utilizada desde há muito tempo na medicina popular, e a sua aplicação surge de diversas características promotoras da saúde, tais como no tratamento de problemas gastrointestinais e urológicos, hipertensão, doenças cardíacas, diabetes, assim como agente anti-inflamatório, entre outras. O presente estudo teve como objetivo investigar a atividade dos extratos de *C. monogyna* e *A. unedo* no controlo do crescimento e metabolismo de células da próstata humana não neoplásicas (PNT1A) e neoplásicas (LNCaP e PC3). Extratos metanólicos das folhas secas de ambas as plantas foram obtidos através de uma extração assistida por ultra-som. As células PNT1A, LNCaP e PC3 foram tratadas com diferentes concentrações dos extratos purificados (0, 10, 50, 100, 200, 500 e 1000 µg / ml) durante 24, 48 e 72 h. A viabilidade celular nas diferentes condições experimentais foi determinada pelo ensaio MTT. Integrando os resultados de viabilidade celular obtidos para as três linhas celulares de próstata em estudo na presença das diferentes concentrações de ambos os extratos, foi seleccionada a concentração de 200 µg / ml e o tempo de incubação de 72 h para análise posterior da apoptose e do metabolismo glicolítico. As alterações na expressão proteica de

reguladores chave da apoptose e do metabolismo foram analisadas pela técnica do Western Blot. O consumo de glicose, a produção de lactato, a atividade do lactato desidrogenase (LDH) e a atividade da Caspase-3 foram avaliados por ensaios bioquímicos usando *kits* específicos com posterior análise espectrofotométrica. Diferentes respostas foram observadas nas células prostáticas não neoplásicas (PNT1A) e neoplásicas (LNCap e PC3) sob o efeito dos extractos de *C. monogyna* e *A. unedo*. A diminuição significativa da viabilidade das células LNCaP e PC3 foi observada a partir da presença de 100 e 200 µg / ml de extratos de plantas durante 72 h de tratamento. Os resultados da proliferação das células PNT1A foram diferentes, demonstrando uma diminuição significativa da proliferação apenas a partir da concentração de 500 µg / ml. No geral, os resultados obtidos demonstraram que ambos os extratos diminuíram significativamente a proliferação das células da próstata. Considerando a morte celular, apenas a atividade dos extratos de *A. unedo* aumentou a taxa apoptótica nas células PNT1A, esta por sua vez foi sustentada pela expressão alterada de reguladores chave da apoptose. Nas células neoplásicas LNCaP e PC3 não se observou qualquer efeito na atividade apoptótica na presença dos extratos. Em relação ao metabolismo glicolítico, as células LNCaP tratadas por extratos de *C. monogyna* apresentaram uma supressão do metabolismo glicolítico com diminuição da atividade da LDH e produção de lactato, o que foi acompanhado pela expressão/atividade alterada dos transportadores glicolíticos e enzimas. Curiosamente, nas células PC3, no global, os extractos de *C. monogyna* e *A. unedo* demonstraram regular negativamente tanto a atividade apoptótica como o metabolismo glicolítico, apesar da diminuição da proliferação. Os presentes resultados indicam que os extratos de *C. monogyna* e *A. unedo* podem ter efeitos benéficos sobre as células neoplásticas e não neoplásticas da próstata, diminuindo a proliferação e controlando as vias apoptóticas e glicolíticas. Estes efeitos foram mais pronunciados no caso das células sensíveis aos androgénios, as células LNCaP.

## Palavras-chave

Cancro da Próstata, Apoptose, Metabolismo Glicolítico, Compostos Fenólicos, *Crataegus monogyna*, *Arbutus unedo*

# Resumo Alargado

O cancro da próstata é uma das doenças neoplásicas mais frequentes no sexo masculino e a segunda causa de morte oncológica, nos países ocidentais. Apesar dos desenvolvimentos ocorridos nas últimas décadas, ainda são necessárias novas abordagens terapêuticas para fazer face ao desenvolvimento e progressão desta patologia. O envelhecimento geral da população, entre outros factores de risco, contribuem para um aumento anual da 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, têm um grande interesse para a diminuição da prevalência desta doença de difícil tratamento.

Desde a antiguidade que os produtos naturais são utilizados para fins terapêuticos. E ao longo dos anos tem havido crescentes evidências de que as plantas medicinais desempenham um papel crucial na prevenção e mitigação de diferentes doenças humanas.

Os compostos fenólicos constituem um dos grupos mais extensos de produtos químicos no reino vegetal, aos quais têm sido atribuídas propriedades medicinais. Estes compostos apresentam efeitos antiproliferativos e antioxidantes, os quais têm estimulado grande interesse na investigação da sua aplicabilidade no controlo do crescimento de células cancerígenas. Contudo, são ainda muito pouco conhecidos os efeitos dos compostos fenólicos no controlo de outras propriedades das células cancerígenas. Uma vez que o metabolismo energético tumoral apresenta um elevado consumo de glicose e libertação de lactato, mesmo na presença de oxigénio (Efeito Warburg) e altera outras vias metabólicas, processo conhecido como reprogramação metabólica, é pertinente descobrir se há compostos com capacidade de contrariar o metabolismo híper-glicolítico e a reprogramação do metabolismo. Sendo assim é de grande interesse científico identificar novos agentes anticancerígenos a partir de fontes naturais e descobrir se os compostos fenólicos poderão ter acção ao nível do metabolismo. As espécies *Crataegus monogyna* e *Arbutus unedo* são exemplos de plantas ricas em compostos fenólicos.

O *C. monogyna,* "pirliteiro", é uma espécie da classe *Mognoliopsida*, ordem *Rosales*, família *Rosaceae*, que se encontra amplamente distribuída na Península Ibérica, Europa Ocidental e Central e Norte da África. É uma planta com interesse farmacológico, devido aos seus diversos tipos de benefícios tais como no tratamento da hipertensão, distúrbios cardíacos e distúrbios digestivos. Já foi demonstrado o seu efeito antiproliferativo em células do cancro do pulmão, NCI-H460 74, e também em células do cancro da mama, MCF-7.

O *A. unedo*, “medronheiro”, é uma espécie da classe *Mognoliopsida*, ordem *Ericales*, da família *Ericaceae*. É uma planta que cresce espontaneamente ao redor da bacia do Mediterrâneo, bem como noutras regiões com verões quentes e invernos amenos e chuvosos. Esta planta tem vindo a ser utilizada desde há muito tempo na medicina popular, e a sua aplicação surge de diversas características promotoras da saúde, tais como no tratamento de problemas gastrointestinais e urológicos, hipertensão, doenças cardíacas, diabetes, assim como agente anti-inflamatório, entre outras. O seu efeito antiproliferativo já foi constatado em células do cancro do cólon, HCT 116.

O presente estudo teve como objetivo investigar a atividade dos extratos de *C. monogyna* e *A. unedo* no controlo do crescimento e metabolismo de células da próstata humana não neoplásicas (PNT1A) e neoplásicas (LNCaP e PC3).

Extratos metanólicos das folhas secas de ambas as plantas foram obtidos através de uma extração assistida por ultra-som. As células PNT1A, LNCaP e PC3 foram tratadas com diferentes concentrações dos extratos purificados (0, 10, 50, 100, 200, 500 e 1000 µg / ml) durante 24, 48 e 72 h. A viabilidade celular nas diferentes condições experimentais foi determinada pelo ensaio MTT. Integrando os resultados de viabilidade celular obtidos para as três linhas celulares de próstata em estudo na presença das diferentes concentrações de ambos os extratos, foi seleccionada a concentração de 200 µg / ml e o tempo de incubação de 72 h para análise posterior da apoptose e do metabolismo glicolítico. As alterações na expressão proteica de reguladores chave da apoptose e do metabolismo foram analisadas pela técnica do Western Blot. O consumo de glicose, a produção de lactato, a atividade do lactato desidrogenase (LDH) e a atividade da Caspase-3 foram avaliados por ensaios bioquímicos usando *kits* específicos com posterior análise espectrofotométrica.

Diferentes respostas foram observadas nas células prostáticas não neoplásicas (PNT1A) e neoplásicas (LNCap e PC3) sob o efeito dos extractos de *C. monogyna* e *A. unedo*. A diminuição significativa da viabilidade das células LNCaP e PC3 foi observada a partir da presença de 100 e 200 µg / ml de extratos de plantas durante 72 h de tratamento. Os resultados da proliferação das células PNT1A foram diferentes, demonstrando uma diminuição significativa da proliferação apenas a partir da concentração de 500 µg / ml. No geral, os resultados obtidos demonstraram que ambos os extratos diminuíram significativamente a proliferação das células da próstata. Considerando a morte celular, apenas a atividade dos extratos de *A. unedo* aumentou a taxa apoptótica nas células PNT1A, esta por sua vez foi sustentado pela expressão alterada de reguladores chave da apoptose. Nas células neoplásicas LNCaP e PC3 não se observou qualquer efeito na atividade apoptótica na presença dos extratos. Em relação ao metabolismo glicolítico, as células LNCaP tratadas por extratos de *C. monogyna* apresentaram uma supressão do metabolismo glicolítico com diminuição da atividade da LDH e produção de lactato, o que foi acompanhado pela expressão/atividade alterada dos transportadores glicolíticos e enzimas. Curiosamente, nas células PC3, no global,

os extractos de *C. monogyna* e *A. unedo* demonstraram regular negativamente tanto a atividade apoptótica como o metabolismo glicolítico, apesar da diminuição da proliferação.

Os presentes resultados indicam que os extratos de C. monogyna e A. unedo podem ter efeitos benéficos sobre as células neoplásticas e não neoplásticas da próstata, diminuindo a proliferação e controlando as vias apoptóticas e glicolíticas. Estes efeitos foram mais pronunciados no caso das células sensíveis aos androgénios, as células LNCaP.

# Abstract

Prostate cancer is one of the most frequent malignancies diagnosed in men and the second cause of cancer-related deaths in Western countries. Despite several developments in the last decades, better approaches are still needed for the treatment and management of this pathology.  Natural products have been used as a therapeutic resource since ancient times. Over the years there has been increasing evidence that medicinal plants play a crucial role in the prevention and mitigation of different human diseases. Polyphenols are a large class of chemicals found in plants that have attracted much attention in the last decades due to their antiproliferative properties and antioxidant effects, which has increased the interest on the investigation of its applicability controlling the growth of cancer cells. Much less is known considering the effects of polyphenols controlling other specific features of cancer cells, as their ability to suppression of highly glycolytic metabolism and the reprogram metabolism. *Crataegus monogyna* and *Arbutus unedo* are examples of plants rich in phenolic compounds. The *C. monogyna*, "pirliteiro", is a species of the class *Mognoliopsida*, order *Rosales*, family *Rosaceae*, widely distributed in the Iberian Peninsula, Western and Central Europe and North of Africa. This plant has been studied due to its pharmacological interest, having various benefits in hypertension, heart and digestive disorders. The *A. unedo*, "medronheiro", is a species of the class *Mognoliopsida*, order *Ericales* and family *Rosaceae*. It grows spontaneously around the Mediterranean basin, as well as, in other regions with hot summers and mild rainy winters. This plant has been long used in folk medicine, on traditional remedies, due to its several health-promoting characteristics, for treatment of gastrointestinal and urological problems, hypertension, cardiac diseases, and diabetes and as antiinflammatory agent, among other interesting properties. The present study investigated the activity of *C. monogyna* and *A. unedo* extracts controlling human non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) prostate cells growth. Methanolic extracts of dry leaves were obtained by ultrasound-assisted extraction. PNT1A, LNCaP and PC3 cells were treated with different concentrations of the purified extracts (0, 10, 50, 100, 200, 500 and 1000 μg/ml) for 24, 48, and 72 h. Cell viability at different experimental conditions was determined by the MTT assay. Integrating the results of cell viability obtained for the three prostate cell lines under study in the presence of both plant extracts, the 200 μg/ml concentration and the incubation time of 72 h were selected for the subsequent analysis of apoptosis and metabolism. The alteration on the protein expression of key apoptosis and metabolic regulators was analyzed using the Western Blot. The glucose consumption, lactate production, activity of lactate dehydrogenase (LDH) and activity of Caspase-3 were evaluated by biochemical assays using specific kits and subsequent spectrophotometric analysis. Distinct responses were observed in non-neoplastic (PNT1A) and neoplastic (LNCap and PC3) prostate cells upon exposure to *C. monogyna* and *A. unedo* extracts. For both extracts, the viability of

LNCaP and PC3 cells started to decrease from 100 and 200 µg/ml on, for 72 h of treatment. PNT1A cells proliferation was significantly different, starting only at a concentration of 500 µg/ml. Nevertheless, the administration of both plants extracts decreased prostate cell proliferation. Considering cell-death, *A. unedo* extracts increased the apoptotic rate of PNT1A cells, which was underpinned by the altered expression of key apoptosis regulators. An effect not observed in the apoptotic rate of the neoplastic LNCaP and PC3 cells. LNCaP cells treated by *C. monogyna* extracts markedly displayed a suppression of glycolytic metabolism with decreased LDH activity and lactate production, which was accompanied by altered expression/activity of glycolytic transporters and enzymes. Curiously, PC3 cells had an overall down-regulation of apoptotic and metabolic activity in the presence of *C. monogyna* and *A. unedo* extracts, despite the decreased proliferation. The present findings indicate that *C. monogyna* and *A. unedo* extracts may have beneficial effects on neoplastic and non-neoplastic prostate cells, diminishing proliferation and controlling the apoptotic and glycolytic pathways. These effects were more pronounced in the case of the androgen-sensitive LNCaP cells.

# Keywords

Prostate cancer, Apoptosis, Glycolytic Metabolism, Proliferation, Phenolic compounds, *Crataegus monogyna*, *Arbutus unedo*

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# List of Abbreviations

| | |
|---|---|
| 1,3BPG | 1,3-Bisphosphoglyceric Acid |
| 2PG | 2-Phosphoglycerate |
| 3PG | 3-Phosphoglyceric acid |
| ADP | Adenosine diphosphate |
| ADT | androgen deprivation therapy |
| AP | Acid phosphatase |
| APAF-1 | Apoptotic protease activating factor 1 |
| AR | Androgen Receptor |
| ATP | Adenosine triphosphate |
| BAK-1 | Bcl-2 Homologous Antagonist Killer |
| Bax | Apoptosis Regulator Bax |
| Bcl-2 | B cell lymphoma 2 |
| BID | Bcl-2 Homologous Antagonist Killer |
| BPH | Benign Prostatic Hyperplasia |
| $Ca^{2+}$ | Cálcio |
| CGA | Chlorogenic Acids |
| CK | Cytokeratin |
| CRPC | Castration-resistant prostate cancer |
| DHAP | Dihydroxyacetone Phosphate |
| DHT | Dihydrotestosterone |
| DNA | Deoxyribonucleic Acid |
| FADD | Fas-Associated Protein with Death Domain |
| FAS | Ligand acts as a prototypic death factor |
| Fructose-1,6-p | Fructose- 1,6-Phosphate |
| Fructose-6-p | Fructose 6-Phosphate |
| GADP | Glyceraldehyde 3-Phosphate |
| GADPH | Glyceraldehyde 3-Phosphate Dehydrogenase |
| GLUT | Glucose transporter |
| $H_2O$ | Água |
| HOXB13 | Gene associate with an increased risk for autosomal dominant prostate cancer |
| IFN-$\gamma$ | Interferon-$\gamma$ |
| LDH | lactate dehydrogenase |

| | |
|---|---|
| LNCaP | Human cell line of prostate cancer derived from lymph node metastasis |
| MCT | Monocarboxylate transporter |
| MDM2 | Mouse double minute 2 |
| MTT | 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide |
| NOXA | Proapoptotic protein |
| nsSNPs | Non-synonymous single nucleotide polymorphisms |
| p21 | Cyclin-dependent kinase inhibitor |
| p53 | Tumour Suppressor p53 |
| P63 | Tumor protein |
| PAP | Prostatic acid phosphatase |
| PC3 | Human cell line of prostate cancer derived from bone metastasis |
| PCa | Prostate Cancer |
| PCA3 | Prostate Cancer Gene 3 |
| PFK-1 | Phosphofructokinase-1 |
| PGI | Enzyme phosphoglucose isomerase |
| PGK | Mono Phosphoglycerate Kinase |
| PGM | Phosphoglycerate mutase |
| PNT1A | Human cell line of prostate |
| PSA | Prostate specific antigen |
| PUMA | p53 Upregulated Modulator of Apoptosis |
| RNA | Ribonucleic acid |
| SNP | Single nucleotide polymorphism |
| tBID | truncated Bid |
| TCA | Tricarboxylic acid |
| TNF | Tumor necroses factor |
| TRAIL | TNF-related Apoptosis-inducing Ligand |
| WAF1 | p21 |

# Chapter I - General Introduction

## 1. Brief overview of prostate anatomy and physiology

The prostate is a compound tubule alveolar exocrine gland that is part of the male reproductive system (figure 1). The prostate gland develops after puberty as a result of the testosterone surge. It reaches a size of three centimeters in length, four centimeters in width and two centimeters in depth, in the average adult. It's the largest gland of the male reproductive system and it weighs about twenty grams, being detectable via a rectal examination [1].

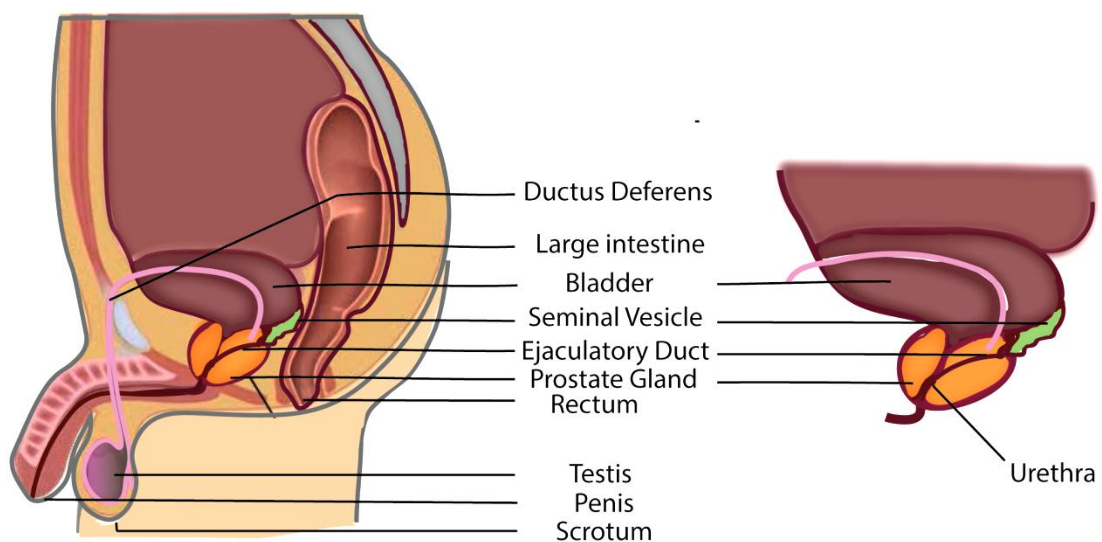


**Figure 1. Sagittal view showing the male reproductive structures.**

This gland is located just below the bladder and it lies above the urogenital diaphragm between the rectum and the symphysis pubis. It surrounds the ejaculatory ducts, and the beginning of the urethra, which goes right through the center of the prostate and is known as the prostatic urethra [1][2]. A simplified representation of prostate position in the male reproductive structures is observable in Figure 1. The prostate is composed by three kinds of cells: glandular cells, which secrete the prostatic fluid part of the semen; muscle cells, which regulate urine flow and ejaculation; and fibrous cells, which support the gland. The glandular cells of the prostate comprise the luminal cells, basal cells and neuroendocrine cells [3]. The prostate lies in a hammock of nerves, which can be divided into three zones: proximal neurovascular plate, predominant neurovascular bundles, and accessory distal neural pathways [1].

In the twentieth century, several researched claimed the division of prostate gland into lobes, by analogy with laboratory animals, a concept that became popular even though no distinct lobes can be seen in the human prostate. The current and most widely accepted structural division of prostate encompasses four distinct zones drained by specific ducts and with unique distinguishable features, namely (Figure 2) [4]: the central zone, which occupies about 25 percent of the prostate's volume and is traversed by the ejaculatory ducts; the transition zone, which comprises only 5–10 percent of the glandular tissue; the peripheral zone, corresponding to 70 percent of the prostate volume at the back and to the sides of the gland; and the anterior fibromuscular zone or stroma, the entire anterior surface of the prostate, composed by fibromuscular tissue contiguous with the bladder, without glandular tissue. The susceptibility of these different zones to diseases affecting the prostate are not equal: the peripheral zone is very susceptible to cancer, as it is affected in approximately 85–90 percent of cases; the transition zone is very prone to benign prostatic hyperplasia (BPH); and the central zone is very resistant to BPH and prostate cancer [1] [4] [5].

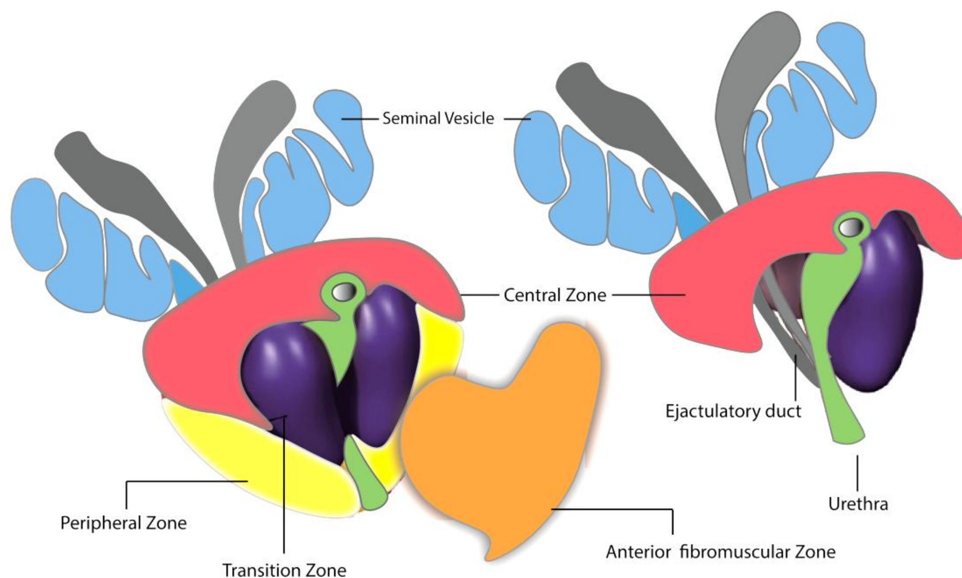


**Figure 2. The zonal anatomy of the prostate gland.** Using the prostatic urethra and ejaculatory ducts as reference, the prostate comprises three principal zones and an anterior fibromuscular layer. The central zone (pink) located at the top of prostate near the bladder and the ejaculatory ducts pass through the central zone to the urethra. The transition zone (purple) surrounds the portion of the urethra closest to the bladder. The peripheral zone (yellow) is located at the posterior side of the prostate closest to the rectum. Peripheral and central zones are collectively referred to as outer prostate, whereas the transition zone and anterior fibromuscular layer (orange) are termed inner prostate.

The prostate is an exocrine gland that produces alkaline secretions righ in sugars and proteins, contributing to the seminal fluid (about 30 percent in volume). These are thought to be important in aiding fertilization in several ways: by maintaining the alkaline pH (that protects the sperm from the acidic environment of the female reproductive system), by helping to nourish the sperm in the semen and by affecting the degree of viscosity of the semen, promoting the viability of sperm after ejaculation [1]. Secretion products of the prostate include zinc, citric acid, spermine, prostaglandins, cholesterol, clotting enzyme,

acid phosphatase (AP), prostatic acid phosphatase (PAP), and prostatic specific antigen (PSA). The AP and PAP are both enzymes that have been used for a long time to monitor the course of prostatic cancer disease in advanced stages. The PSA, is only detected in the epithelial cells of the prostatic ductal elements and is used for detection and monitoring of prostate cancer (PCa) [6].

The prostate also has the ability of converting testosterone into 5α-dihydrotestosterone (DHT), by the activity of 5 α-reductase. DHT is the most important prostatic androgen with a higher affinity for the androgen receptor (AR) than testosterone. DHT binding to AR activates the AR that in the nucleus activates transcription, resulting in the production of messenger RNAs, and protein synthesis increasing cellular growth and prostate volume [7].

# 2. Epidemiological notes on prostate cancer

The PCa is the most common cancer affecting men concerning the number of new cases diagnosed, and represents one of the major causes of cancer death in men worldwide [8]. For example, in America, about 1 man in 7 will be diagnosed with PCa during his lifetime and about 1 man in 39 will die of PCa [9]. The *American Cancer Society* estimates that for 2018 there will be about 164,690 new cases of PCa and 29,430 deaths by PCa in the United States [8]. In Portugal, PCa incidence has been increasing since 1998 (1.8%/year), with the exception of the North Region, with a decrease since 2006 (−3.2%/year). If these trends are maintained, 8600 incident cases and 1700 deaths are estimated for 2020 [10].

PCa is mostly common in North America, northwestern Europe, Australia, and the Caribbean islands and less common in Asia, Africa, Central America, and South America [1]. PCa in African American men exhibits a relatively high incidence and mortality (incidence is 1.6-fold higher than in other populations). The risk factors that drive this are unknown and potentially consist of social, environmental and genetic influences. The biological basis for this disparity is unclear in PCa [11] [12].

PCa develops mainly in older men: about 6 cases in 10 are diagnosed in men aged 65 or older and it is rare before the age of 40, so the average age at the time of diagnosis is about 66 [13]. Apart from age, geography and race/ethnicity, the hereditary can be another risk factor for PCa. This human malignancy has the highest degree of genetic transmission. In some families, the hereditary pattern is so strong that mimics an autosomal dominance trait [14]. Single nucleotide polymorphisms (SNPs) are considered to be the primary genetic cause for hereditary PCa and the study of functional non-synonymous single nucleotide polymorphisms (nsSNPs) would give an insight into the exact cause underlying the onset of hereditary PCa and possible methodologies for the cure or early management of the disease. So far it has been proven that the nsSNPs (missense) have a profound damaging effect on the homeobox domain of the HOXB13 gene which might lead to the altered binding patterns of HOXB13 protein with the DNA. This protein encodes a transcription factor that plays a significant role

in the normal prostate development and is also associated with the increased risk of inherited PCa. Subsequent advances in genetic studies have proved that HOXB13 has a significant role in PCa susceptibility, but the exact mechanism behind it remains undiscovered. However, the exact mechanism and pathology of those predicted nsSNPs should further be validated by *in vivo* experiments and population based studies [15].

The lifestyle and diet also have been indicated as risk factors for PCa [16]. Accordingly, several generally widely recommended healthy lifestyle habits such as not smoking, maintaining body weight within limits, and practicing regular vigorous physical exercise appear to counteract PCa development and progression [17]. Concerning obesity, the body's fat content affects the handling of testosterone and this was suggested as the link between fat and PCa. Recently, it was shown that obesity favors the migration of PCa cells and dissemination of disease by an action promoted directly by the periprostatic adipocyte cells over prostate tumor cells [16]. Several dietary factors, flavonoids and other similar antioxidants are important protective agents because oxidation damages complex biological materials, including DNA, and may be one factor causing cancer. Vitamin E, vitamin A, vitamin C, food rich in lycopene (e.g. tomato), cruciferous vegetables, healthy sources of vegetable fats, and coffee, may also have a role in reducing risk of PCa progression [16] [17]. For this reason, vegetarians are said to be at a much lower risk of developing PCa, perhaps by as much as 50 per cent [16].

Others risk factors for PCa have been pointed out, though with less clear effects. This includes industrial exposure, for example to cadmium, which can occur in men working in copper smelting [18]; chemical exposure to agents such as the Agent Orange, that was a commercially manufactured defoliate sprayed extensively during the Vietnam War [19]; sexual behavior, as some analysis reports demonstrated slight increases of risk associated with the number of sexual partners and history of sexually transmitted infection [20]; vasectomy, as some studies have suggested that men who have a vasectomy have a slightly increased risk for PCa; and inflammation of the prostate, hading a specific attention for your study due to inflammation's role in multiple stages of PCa development. The inflammasome associated with PCa remains uncharacterized and studies characterizing the role of inflammation in PCa are at the beginning stage. Nevertheless, there is evidence that the inflammasomes regulate inflammatory cytokines, thereby modulating the course of inflammation. Inflammasomes activation is linked with infection, stress, or danger signals, which are common events within the prostate gland. Since the pro-inflammatory cytokines may serve as fuel for the developing neoplastic cells in the prostate, the tumor promoting effect of pro-inflammatory cytokines/chemokines may be reduced significantly by understanding the mechanistic regulation of inflammasomes [21].

## 3. Prostate cancer diagnosis and treatment: the basis

The tumor marker detection is of great significance to early clinical diagnosis and monitoring of disease recurrence. Despite the limitation it has, PSA still is the currently used diagnostic biomarker for PCa [22]. To date, a series of techniques have been applied for the quantitative detection of PSA, such the enzyme-linked immunosorbent assay, surface plasmon resonance, luminescence energy transfer, electrochemiluminescence, and electrochemistry methods. Among the various detection techniques, photoelectrochemical immunoassay has aroused increasing attention owing to its good stability, acceptable specificity and favorable sensitivity compared with conventional optical and electrochemical methods. When PSA concentration is higher than 4.0 ng/mL, the patient's health is endangered, but PSA levels should be evaluated in line with other diagnostic techniques when managing a patient with PCa. Not all low or high PSA levels will necessarily indicate that a patient has or does not have PCa, as PSA levels are organ specific and not cancer specific. So PCa screening is done in part through the use of the PSA blood tests and is often combined with a digital rectal examination. Making a definitive diagnosis of PCa generally includes blood count and biochemical profile with PSA quantification (% of free/total PSA), transrectal ultrasound, echography, magnetic resonance imaging, PCA3 test and biopsy [22] [23].

Treatment choice in PCa is based on the well-established prognostic factors, namely, initial PSA level, clinical tumor stage and Gleason score, along with general considerations such as baseline urinary function, comorbidities and patient's age [24]. The most important primary treatment for locally advanced or metastatic PCa is the androgen deprivation therapy (ADT). The progression of PCa is dependent on androgens, and treatments reducing the production of androgens, or antagonizing the function of AR, have been the first line option to treat advanced PCa. Other treatments available include radical prostatectomy, prostate brachytherapy, external beam radiation therapy and active surveillance [24] [25]. So far, basal treatment of PCa relies on surgery, chemotherapies and ionizing radiation, with a low efficacy and high toxicity to healthy tissues. New therapeutic approaches of decreasing the PCa associated mortality and minimizing the nonspecific and undesirable side effects are needed [26].

## 4. Development and progression of prostate cancer

In tumor development and progression, the interaction between cancer cells and the microenvironment is crucial. The development of PCa is dependent on the relationship between the levels of cell proliferation and the rate of apoptosis in prostate cells. Androgens are the main regulators of this relationship, stimulating cell proliferation and inhibiting apoptosis. Almost all prostate cancers are adenocarcinomas, cancers which develop from the glandular cells. There are other types of PCa but they are rare, as sarcomas, small cell carcinomas, neuroendocrine tumors and the transitional cell carcinomas. In PCa, both luminal

cells and basal cells have the potential to give rise to tumors with different phenotypic changes [2][3] [27].

As has been shown previously, the prostate is surrounded by a capsule consisting of a glandular epithelium embedded in a fibromuscular stroma and its epithelium consists of two histologically distinct layers: basal layer and luminal layer [1]. It is believed that the basal layer is the proliferative compartment of the prostate and consists of cuboidal epithelial cells that adhere to the basal membrane. Basal cells express proliferation markers like CK5, CK14 and p63. In turn, the luminal cells express high levels of AR, and are androgen-dependent and secretory cells responsible for production and secretion of PSA. The stem cells, present in the basal layer, have unlimited proliferative capacity, are able to self-renew and yet differentiate into cells of the glandular epithelium with more limited proliferative capacity. When disturbances occur in the cellular environment, the process of differentiation of stem cells can be affected and is believed to contribute, in addition to other factors, to the malignant phenotype of prostate [3] [28].

Overall, it has been suggested that the damage of the prostate epithelium, potentially inflicted by diverse environmental exposures, such as infections and trauma, and/or microenvironment stimuli, such as oxidative stress and hypoxia, triggers pre-malignant inflammatory processes to promote tumor development and progression. In this milieu, the damaged epithelium may generate proliferative inflammatory atrophy (PIA) lesions, which may progress through prostatic intraepithelial neoplasia (PIN) to PCa (Figure 3). High-grade prostatic intraepithelial neoplasia preferentially develops in the peripheral zone of the prostate, which is the site of origin for most adenocarcinomas [2] [27] [29].The AR hyperactivity leads to PCa initiation and progression to advanced disease, which can occur by AR overexpression resulting in a transition from an androgen-sensitive disease to the androgen-resistant cancer, the so-called castration-resistant prostate cancer (CRPC). Therefore, the constant activation of distinct growth signaling pathways in PCa cells, including the activation of AR, leads to the development of local and invasive carcinoma and ultimately to the metastization of different organs, including liver, lungs and bones [30].

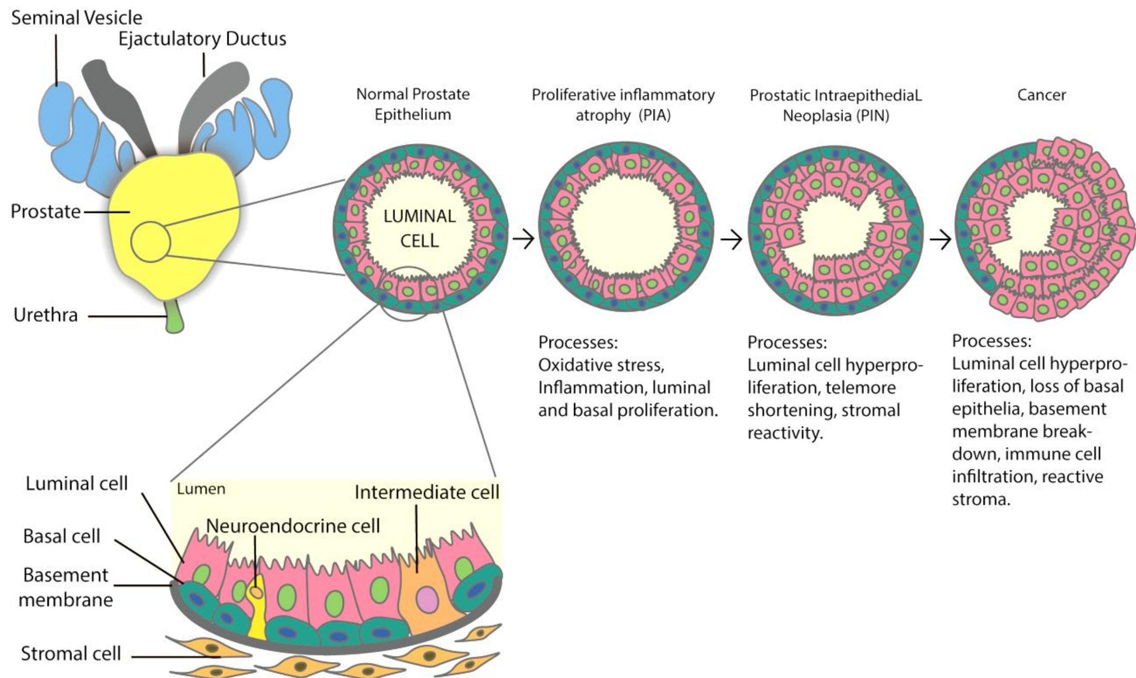


**Figure 3. Development of epithelial prostate cancer.** The pathophysiology of the prostate comprises multiple steps and starts with proliferative inflammatory atrophy (PIA), the appearance of pre-malignant lesions and transformation of prostate epithelial cells by processes like oxidative stress, inflammation, luminal and basal proliferation. In the next step, prostatic intraepithedial neoplasia (PIN), the luminal cell hyperproliferation, telemore shortening and stromal reactivity take place. Finally and consequently the stage of PCa is reached, occuring the luminal cell hyperproliferation, loss of basal epithelia, basement membrane breakdown, immune cell infiltration and stroma reactivity.

## 5. Molecular basis of apoptotic cell death

Apoptosis represents a controlled mechanism of cell death that is altered in cancer cells. Reactivation of apoptosis has been envisaged as a therapeutic tool to eliminate cancer cells. Unfortunately, advanced prostate tumors eventually progress to androgen-insensitive stages, which are resistant to current therapeutic approaches that act by triggering apoptosis [30] [31].

Apoptosis is triggered by signaling events involving a diverse array of protein networks, cellular organelles and macromolecular complexes that converge to the activation of caspases. Apoptotic characteristics include [31]: i) cell shrinkage, ii) membrane blebbing, iii) chromatin condensation, iv) DNA fragmentation caused by internucleosomal DNA cleavage, and v) finally ending with the engulfment by macrophages or neighboring cells, thereby avoiding an inflammatory response in surrounding tissues. Apoptosis execution occurs via two distinct signaling pathways: the intrinsic or mitochondrial-mediated pathway and the extrinsic or death receptor-mediated pathway (Figure 4) [31] [32].

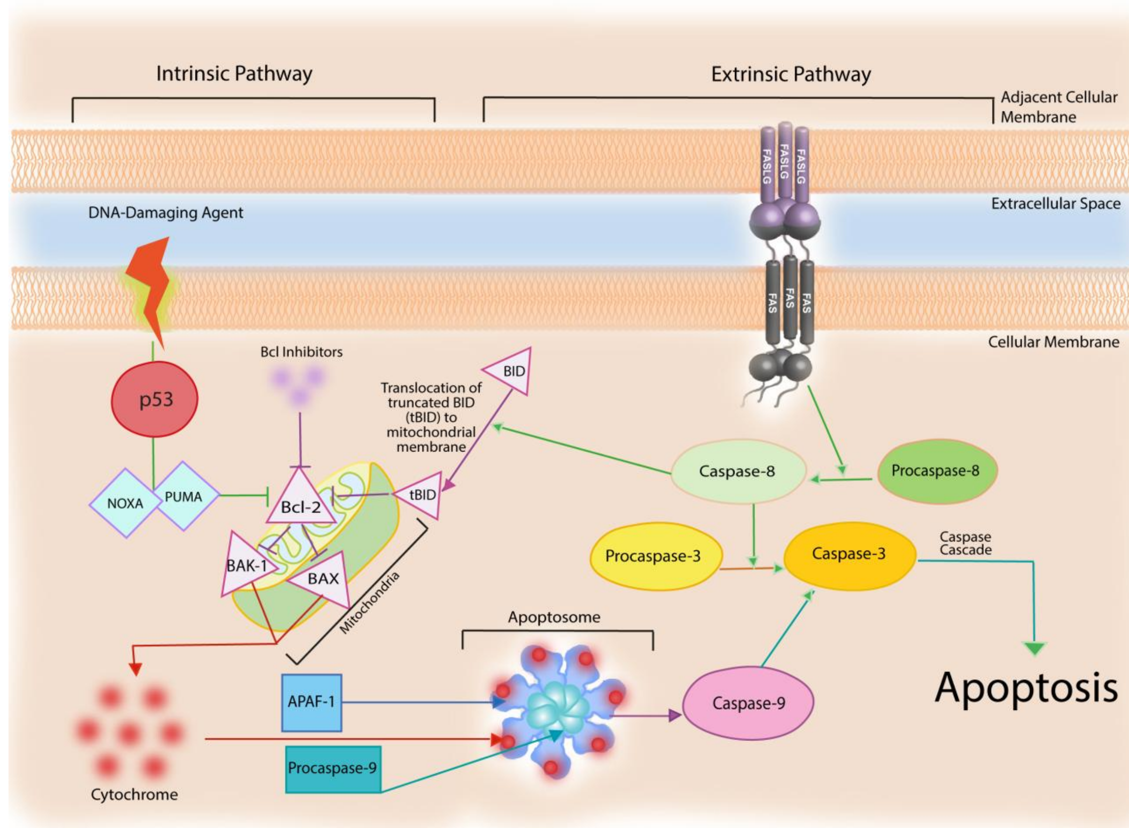


**Figure 4. The intrinsic and extrinsic apoptotic pathways.** The intrinsic pathway is usually activated by p53 that controls the activity of several mediators culminating in the activation of caspases 9 and 3, resulting in apoptosis. External death stimuli bind death receptors at cell membrane (e.g FAS) triggering caspase-8 and -3 activation resulting in apoptosis (Extrinsic pathway).

The intrinsic pathway is usually activated in response to many different damaging influences, for example, DNA damage, oxidative stress, hypoxia, or chemotherapeutic drugs, or by the absence of growth factor signals [32]. In conditions like these, p53 causes cell cycle arrest, primarily by activating the transcription of a cyclin-dependent kinase inhibitor, p21/waf1, and induces apoptosis via transcriptional activation of the pro-apoptotic PUMA, NOXA, and B-cell lymphoma 2 (Bcl-2) family genes (Figure 4). This pathway is mainly regulated by proteins of the Bcl-2 family, which control the release of pro-apoptotic factors from the mitochondrial intermembrane space. More than 20 members of this family have been identified to date in humans, including suppressors (Bcl-2, Bcl-xL, Mcl-1, Bfl-1/A1, Bcl-W, and Bcl-G) and promoters (Bax, Bak, Bok, Bad, Bid, Bik, Bim, Bcl-Xs, Krk, Mtd, Nip3, Nix, Nora, and Bcl-B) of apoptosis. These apoptosis regulators, especially Bax and BAK, will change their conformation, oligomerize, and be attracted to the mitochondria inducing the creation of apoptotic pores in the 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. When cytochrome c is released into the cytoplasm the apoptotic protease-1 (APAF-1) and procaspase-9 are recruited, which in turn activates a series of nuclear and cytoskeletal proteins, and signaling molecules. These

proteins form a functional apoptosome that activates a caspase-9 and -3, resulting in programmed cell destruction [30] [32] [33].

An alternative and complementary signaling pathway that leads to programmed cell death includes the extrinsic death receptor pathway (Figure 4). The extrinsic pathway is initiated by the binding of apoptosis-inducing ligands such as FAS or TRAIL, tumor necrosis factor (TNF) receptors. Activation of death receptors by binding their natural ligands induces receptor clustering and formation of a death-inducing signaling complex. The complex recruits procaspase-8 via the adaptor molecule Fas-associated death domain protein (FADD), resulting in the activation of caspase-8.Procaspase-8 an inactive 'initiator' caspase is proteolytically cleaved becoming activated, and further activating downstream effectors' proteins such as caspases-8 and -3, which induces the degradation of cytosolic, cytoskeletal, nuclear proteins, and DNA, causing inevitable apoptosis [30] [32].

The extrinsic and intrinsic apoptosis pathways are connected by the caspase-8-mediated cleavage of the pro-apoptotic Bcl-2 family member, BID (Figure 4). Therefore, the caspase-8 is also responsible for cleaving BID, a pro-apoptotic member of the Bcl-2 family that forms the principal link between the extrinsic and intrinsic pathways by stimulating the release of cytochrome c from the mitochondrion, followed by induction of apoptosis [33].

More than 50% of human cancers, including PCa, exhibit loss of normal p53 functions and/or defects in the p53 signaling pathway and these molecular alterations are associated with resistance to apoptosis [34]. The p53, tumor suppressor gene product, is a transcription factor that enhances the transcription of several genes known to play a critical role in transducing signals from DNA damage. Its expression and activity are elevated in response to ionizing radiation, UV light, or certain genotoxic chemicals and mediate DNA repair, cell cycle arrest, and apoptosis. The functional activity of p53 is regulated through transcription, translation, protein turnover, cellular compartmentalization as well as it is association with other proteins, such as MDM2 [35] [36].

For the maintenance of prostate growth, the complex equilibrium between cell growth, proliferation factors, and apoptosis-inducing factors is essential. Fluctuations in this balance cause overexpression of factors causing cell survival, cell proliferation and loss of apoptosis leading to tumorigenesis and cancer. The deregulation of cell growth in PCa is notable by apoptotic evasion, loss of differentiation, and uncontrolled proliferation [32].

## 6. Metabolism of cancer cells: the glycolytic pathway

Cancer cells display unique and exquisite features from which the most emblematic is their preference on glycolytic metabolism for obtaining energy. Glycolysis is the cytoplasmic pathway that converts glucose into two molecules of pyruvate, with only a modest releasing of energy is being captured in two substrate-level phosphorylations and one oxidation

reaction. In normal conditions, with functional mitochondria and oxygen supply, glycolysis is aerobic. If either mitochondria or oxygen is lacking, glycolysis may occur anaerobically although some of the available energy is lost, which results in the conversion of pyruvate to lactate [37].

In healthy cells the glycolytic process is tightly regulated and consists of three stages. Firstly, glucose is uptake from the extracellular space via glucose transporters (GLUTs) at cell membrane. These families of proteins allow the energy independent transport of glucose across the hydrophobic cell membrane, down its concentration gradient [38] [39]. GLUT1 and GLUT3 isoforms in particular are present across all mammalian cells and are responsible for a considerable amount of the basal glucose uptake [40]. Once inside the cell, glucose is converted into pyruvate through a chain of reactions. The first stage is phosphorylation of glucose into glucose-6-phosphate, consuming one molecule of ATP, through the action of hexokinase. Glucose-6-phosphate is then converted, in a reversible reaction, into fructose-6-phosphate through the action of the phosphoglucose isomerase (PGI). Fructose-6-phosphate in turn is phosphorylated into fructose 1,6-biphosphatase through the activity of phosphofructokinase-1 (PFK-1). The PFK-1 is a key enzyme in this process since it catalyzes the first irreversible reaction of glycolysis, being a rate-limiting step in glucose metabolism [39] [40].

The second stage of glycolytic process 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 triose phosphate isomerase will then convert the DHAP molecule into a second GADP molecule, thus meaning the final glycolytic stage will occur twice [40].

In the third and final glycolytic step, one GADP molecule will be oxidized 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 produce the process first molecule of ATP and one molecule of 3-phosphoglycerate (3PG). 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, with one final ATP molecule, by the action of the enzyme pyruvate kinase [40].

In normal cells and aerobic glycolysis pyruvate will be converted into acetyl-CoA to its complete oxidation, through the mitochondrion-localized tricarboxylic acid (TCA) cycle and oxidative phosphorylation to $CO_2$ and $H_2O$, which generates 38 ATP molecules per molecule of glucose (Figure 5). In tumor cells, even in the presence of oxygen, glycolysis tends to be

aborted at either of two steps. First, aerobic glycolysis in tumor cells implies conversion of glucose into pyruvate, which generates only two ATP molecules per molecule of glucose, and subsequently into the lactate by lactate dehydrogenase (LDH). Second, in tumor cells, acetyl-CoA tends to be introduced into a truncated TCA cycle, with the result that acetyl-CoA is exported into the cytosol and serves as a building block for cell growth and proliferation [41]. A simplified representation model is observable in Figure 5.

Therefore under anaerobic conditions, or as an alternative used by cancer cells, the end-product of glycolysis, pyruvate, is metabolized by the LDH enzyme, which catalyzes the interconversion of pyruvate to lactate. The lactate produced is then exported to the extracellular medium by specific monocarboxylate transporters, namely by the MCT4, contributing to the acidification of tumor microenvironment. The lactate in the tumor microenvironment, favors cell migration and invasion, also suppressing the anticancer immune defenses [39]. So it has been reported that most cancer cells express high levels of MCTs to ensure the rapid efflux of the lactate produced. During PCa progression, the expression profile of MCT isoforms changes. MCT1 is expressed in normal and malignant prostate, whereas MCT2 expression increases from normal gland to PIN and in situ carcinoma [42]. On the other hand, MCT4 is only expressed in malignancy and highly in more advanced stages of disease [43]. While MCT1 and MCT2 transport a wider range of substrates, MCT4 is specifically associated with the export of lactate in cells with a high glycolytic rate [42]. Furthermore, it has been shown that lactate may enter in stroma or cancer cells following oxidative phosphorylation or alternatively regenerating pyruvate that, in turn, may be used as energetic substrate by tumor cells. This process generates complementary metabolic pathways, which lead to survival and progression of cancer cells. Moreover, MCTs have been shown to be overexpressed in different human cancers and for this reason identified as attractive targets for cancer therapy [42] [43].

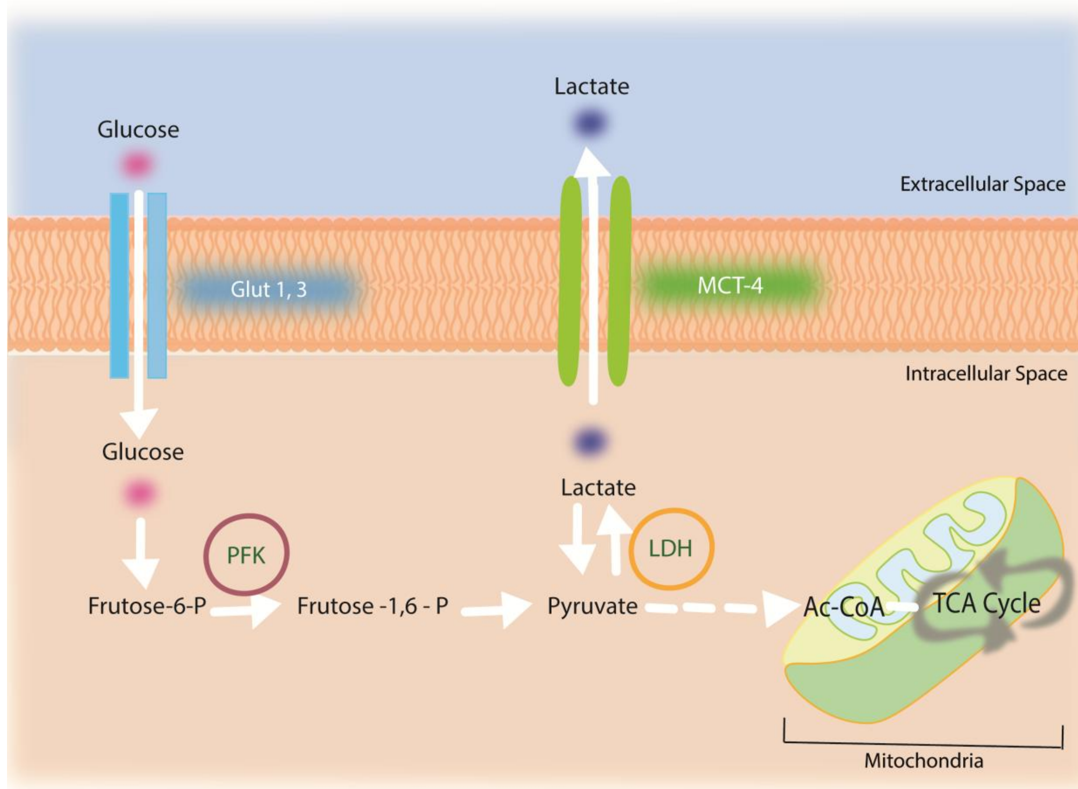


**Figure 5. Glycolysis and the two main possible routes of pyruvate.** In normal cells under aerobiosis pyruvate is converted to Ac-CoA and enters the d tricarboxylic acid (TCA) cycle. In anaerobiosis, or typically as a feature of cancer cells, pyruvate is converted to lactate by the activity of lactate dehydrogenase (LDH).

As previously described, progression of PCa is associated with tumor enrichment in androgen-insensitive proliferating cells, as well as the emergence of new cell phenotypes, leading to a disease stage that frequently displays a fatal behavior. Moreover, PCa has been considered to have unique metabolic features, related with the high levels of citrate, alanine and lactate [44] [45]. Also, the increased glucose consumption seems to be required for rapid proliferation of androgen-insensitive PCa cells [44], which raises the curiosity about the identification of factors that could hamper the glycolytic metabolism of cancer cells.

## 7. Plant extracts, polyphenols and cancer

Polyphenols are a large class of chemicals which are found in plants, have attracted much attention in the last decades due to their properties and the hope that they will show beneficial health effects. Phenolic compounds constitute one of the most extensive groups of chemicals in the plant kingdom. It is estimated that more than 8000 compounds have been isolated and described [46]. Polyphenols occur primarily in conjugated form, linked to sugars moieties, but also to other compounds, such as carboxylic and organic acids, amines, lipids and even to other polyphenols [47]. They can be subdivided in five main subclasses: flavonoids, phenolic acids, stilbenoids, lignans and ellagic acids [48]. Flavonoids, represent the most common and widely distributed group of plant phenolics, and can be further divided into classes including flavones, flavonols, flavanones, anthocyanins and isoflavonoids [49]. They are characterized as containing two or more aromatic rings, each bearing one or more

phenolic hydroxyl groups and connected by a carbon bridge. Phenolic acids are usually divided in two main groups derive from benzoic acids, containing seven carbon atoms, or from cinnamic acids, comprising nine carbon atoms. The smaller subclass of stilbenoids comprises polyhydroxylated stilbenes, the main representative being resveratrol. So, all these polyphenols are found in plants, etherified with glucose and other carbohydrates (glycosides) or as free aglycones [46].

The interest of plant polyphenols derives from the evidence from several studies of their hight antioxidant activity. They are able to scavenge free radicals and their wide range of pharmacologic effects including anti-inflammatory, anti-allergic, anti-proliferative, anti-tumour, apoptosis-inducing, antioxidant and antibacterial activities, provide important health benefits related to metabolic syndrome, cancer, brain health and immune system [47] [49]. The antioxidant properties of phenolic acids and flavonoids have been related to their redox properties and chemical structures, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. In addition, some of them display a metal chelation activity, which hinders transition metals from acting as oxidation promoters [49].The antioxidant capacity in human plasma results in increased antioxidant protection of lipids and proteins [46].

*Crataegus monogyna* and *Arbutus unedo* are plants found in the region of Beira Interior and their composition in phenolic compounds is widely described. This point and the fact the PCa is the second most common cancer, hence the interest in studying this plants extracts effects on non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) prostate cells.

# 8. Characterization of Crataegus monogyna

The genus *Crataegus* is of the class *Mognoliopsida*, order *Rosales* and family *Rosaceae*. This genus has approximately 280 species [50], being one of the most common *C. monogyna*, hawthorn [51]. These plants grow spontaneously in temperate zones, in Europe, Africa and Asia. In the Iberian Peninsula, it is widely distributed along the whole territory, Majorca and Minorca islands. The species *C. monogyna* is the most abundant species in Portugal and is popularly known as a “Pirliteiro” [52]. It is a prickly shrub / small tree 5-10 m tall that produces white flowers in spring, red fruits about 1 cm in size in autumn [53] [54]. Flowers, leaves and fruits are used as dried products for infusions or included as plant extracts in capsules because of their wide pharmacological effects and low toxicity, especially for cardiac and nervous system symptoms as well as for their antioxidant activities [55]. *C. monogyna* was mentioned for the first time in the first century by Dioscorides as a ‘cardiotonic’ remedy. The use of hawthorn in therapeutics was also documented in America since the nineteenth century to treat different heart diseases, angina pectoris, to support the effect of Digitalis, or in cases of irregular heartbeat when Digitalis was not tolerated. Nowadays, traditional use in the European Union recognizes *C. monogyna* for two different therapeutic indications: to relieve symptoms of temporary nervous cardiac complaints (e.g.

palpitations, perceived extra heart beat due to mild anxiety) and for the relief of mild symptoms of mental stress and to aid sleep [56]. Many of these properties are attributed to the presence of flavanols and proanthocyanidins, chlorogenic acids (CGA) and other phenolic compounds. CGA has showed anti-bacterial, anti-mutagenic, anti-oxidant and many other biological activities [57]. In aqueous or alcoholic extracts of aerial parts of hawthorn were identified more than 150 components, including triterpenoids, monoterpenoids, sesquiterpenoids, flavonoids, steroids, lignans, hydroxycinnamic acids, organic acids and nitrogen compounds [55]. So the *C. monogyna* is rich in polyphenols and the geographic origin of the wild plant influences its amount and composition [56].

A study about the effects of *C. monogyna* buds and fruits phenolic extracts in some cancers, showed high antiproliferative activity, indicated by the lowest GI50 values obtained in all the tested cell lines: MCF-7, breast adenocarcinoma; NCI-H460, non-small cell lung cancer; HeLa, cervical carcinoma; HepG2, hepatocellular carcinoma [52]. In another study about antiproliferative activities of several plant extracts from Turkey on rat brain tumor and human cervix carcinoma cell lines, *C. monogyna* extract exhibited the highest antiproliferative activity with the highest performance with its lowest IC50 value among other extracts in human cervix carcinoma cell lines: HeLa and C6 cells [58]. In a different and recent study about the effectiveness of extracts from hawberry *C. monogyna* to inhibit lipid oxidation and odor deterioration during processing of ready-to-eat, the hawberry extract displayed potential usage as an ingredient with antioxidant properties for the manufacture of high quality ready-to-eat meat products [59].

# 9. Characterization of Arbutus unedo

The *Arbutus unedo*, "medronheiro", is a species of the class *Mognoliopsida*, order *Ericales* and family *Rosaceae*. It is an evergreen shrub that has a circum-Mediterranean distribution, being found in western, central and southern Europe, north-eastern Africa (excluding Egypt and Libya), the Canary Islands and western Asia, where frost is not very usual and summer dryness is not very intense [60]. In Portugal, this specie is found mainly in the south (Algarve region), although it can be found in sparse distribution throughout the country and is popularly known as "Medronheiro". This species have importance for community because is drought tolerant and able to regenerate following forestry fires making it quite interesting for forestation programs in Mediterranean regions and is important role in the economy of the regions where it occurs [61].

This shrub have a dark green leaf glossy, long and broad, with a serrated margin, a white hermaphrodite flowers and spherical berries covered with conical spikes, about 2–3 cm in diameter, dark red, soft yellow pulp and tasty only when fully ripe in the autumn [62]. *A. unedo* berries are rarely eaten as fresh fruits, it is more likely used for the production of alcoholic drinks, such as liqueurs, and especially brandies, representing an important income for local agricultural communities [63]. They could be applied in the preparation of jellies

and marmalades, and in the confectionary of pies and pastry fillings [63]. Both leaves and fruits are also used in folk medicine to treat several diseases due to their recognized phytopharmaceutical properties [62] [63].

The leaves of *A. unedo* are used in traditional medicine to treat several diseases as antidiabetic, diuretic, urinary antiseptic, antidiarrheal, astringent, depurative, antiaggregant in human platelets, antioxidant, anti-inflammatory, antihypertensive, antiatherosclerotic, antithrombotic and against blenorrhagia [64] [65] [66] [67] [68] [69]. In oriental Morocco, the leaves are used to treat hypertensive diabetics and in Turkey are used by their diuretic activities [64]. The use of this plant in the prevention or treatment of platelet aggregation linked to arterial hypertension was supported by the results of some research works, like a study in hypertensive rats showing that chronic treatment with *A. unedo* extract regress the development of hypertension and ameliorate cardiovascular and renal functions [70]. The *A. unedo* berries use in folk medicine is claimed in treatment of kidney diseases, cardiovascular, gastrointestinal, dermatologic and urological disorders [62]. These berries are also used in folk medicine as antiseptics, diuretics and laxatives [71]. However, the biological significance of these in vitro-detected properties remains to be determined.

Many of these properties are attributed to several phenolic compounds existing in leaf extracts, like flavonoids, among others, as well as α-tocopherol [72]. The most important phytochemical compounds are presented in figure 6. A study about antioxidant activity of *A. unedo* leaves, demonstrate that the ethanol and methanol extracts of *A. unedo* leaves have antioxidant activity and in phytochemical analysis showed that the extracts contained flavonol glycosides and tannins [65]. The amount of α-tocopherol present in leaf extracts varies, depending on the time of harvesting the sample, the highest amount is found when the leaves are harvesting in March [72]. *A. unedo* berries are already known as a very good dietary source of antioxidants, including phenolic compounds (e.g. anthocyanins and other flavonoids, gallic acid derivatives and tannins), vitamins C and E, and carotenoids [73]. Many studies have attempted to show a possible protective effect of vitamin E against cardiovascular diseases [64].

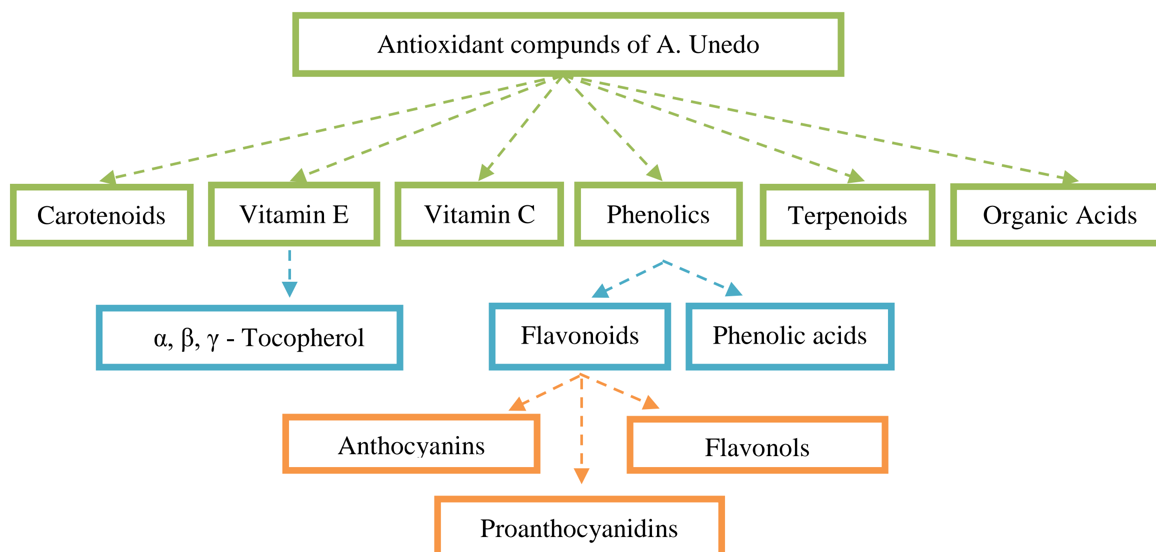


**Figure 6. Antioxidant compounds present in the *Arbutus unedo* trees** [72].

A study about antioxidante activity of *A. unedo* extract demonstrate the high reducing power and high ability for scavenging the free radicals ABTS, DPPH and superoxide, suggesting therefore its antioxidant capacity may be attributed to these mechanisms [71]. Concerning anti-inflammatory activity, a study showed that the aqueous extract of *A. unedo's* leaves presents inhibitory action on interferon-γ (IFN-γ)-elicited activation of STAT1, both in human breast cancer cell line MDA-MB-231 and in human fibroblasts [74]. These results suggest that the employment of the *A. unedo* aqueous extract is promising, at least, as an auxiliary anti-inflammatory treatment of diseases in which STAT1 plays a critical role [74]. In another study it was demonstrate the high capacity of *A. unedo* phenolic extract to inhibit lipid peroxidation in animal brain homogenates, as also a high antitumor potential against NCI-H460 human cell line (non-small lung cancer) [75]. One study about antiaggregant effects of *A. unedo* extracts in human platelets showed antiaggregant actions due to attenuation of $Ca^{2+}$ mobilization, ROS production and protein tyrosine phosphorylation and might be used for the treatment and/or prevention of cardiovascular diseases [76].

Overall, results from literature suggest that *A. unedo* leaves are a promising source of natural antioxidants with potential application in diseases mediated by free radicals [63]. The bioactive compounds present in plants, polyphenols and particularly flavonoids are widely appreciated for their potential beneficial health effects, like antioxidant, antimicrobial and anticarcinogenic activities [77].

It is therefore of considerable interest to validate the bioactivities of *A. unedo* and *C. monogyna* in cell/organism-based assays to assess their potential therapeutic effect against a wide range of human diseases.

# Chapter II - Aim

PCa is the second-leading cause of cancer-related mortality in men in Western societies. Therefore it is of great interest not only to improve the effectiveness of routine PCa screening for the detection of subclinical disease, but also to develop novel therapeutic approaches for this disease. A large of studies has suggested that plants polyphenols have potent anti-inflammatory, anti-allergic, anti-proliferative, anti-tumor, apoptosis inducing and the antioxidant activity. So, it is considerable to validate the bioactivities of these plants in cell/organism-based assays to assess their potential therapeutic effect against a wide range of human diseases. The aim of this dissertation will be to study the activity of two different leafs extracts rich in polyphenols, the *C. monogyna* and *A. unedo*, controlling human non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) prostate cells on the proliferation / cell viability, apoptosis and glycolytic metabolism.

# Chapter III - Material and Methods

## 1. Preparation of plants extracts

The leaves of both plants, *C. monogyna* and *A. unedo*, were collected in Covilhã, September 2016, and dried at room temperature for a month before extraction. Afterward, the dry leaves from both plants were crushed and then submitted to methanolic extraction assisted by ultrasounds, using a power of 37 KHz, for 4 h, with occasional agitation, always under 40 ºC (Branson 5800 2 ½-gallon ultrasonic cleaner). Twenty g of dry leaves and 400 ml of solvent were used. After extraction, the solution was filtered and the samples were centrifuged at 7 000 RCF for 20 minutes at 4°C. The supernatant 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) with water bath temperature at 40 ºC, Pset 340-270. The resulting *C. monogyna* and *A. unedo* extracts were stored at 4°C until use.

The extract solutions for cell treatment were prepared by dissolving 0.01 g of plant extract in 1mL $H_2O$ Mili-Q, resulting in a 100 mg/mL extract solution. The working solutions were obtained from the 100 mg/mL or 1:10 diluted extracts by serial dilutions in culture medium, namely, 10, 50,100, 200, 500 and 1000 µg/mL.

## 2. Cell Culture and treatment

Human non-neoplastic (PNT1A) and neoplastic prostate cells (LNCaP and PC3), were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were maintained in RPMI 1640 medium (Invitrogen, New York, NY) supplemented with 10% FBS (Biochrom, Berlin, Germany) and 1% antibiotic, maintained at 37°C in a 5% $CO_2$ equilibrated atmosphere and passed every 2-3 days.

Prostate cells were seeded into 96-well plastic plates, at a density of 10 000 (PNT1A), 15 000 cells (LNCaP), or 500 cells (PC3) per well in 100 µl culture medium. After allowing cells to attach for 24 h, proliferating cells were incubated with culture medium containing a range of exponentially increasing concentrations of *C. monogyna* or *A. unedo* extracts, 10-1000 µg/ml, over a period of 24h, 48h and 72h.

## 3. Cell viability assay (MTT)

The viability of PNT1A, LNCaP and PC3 cells under treatment with *C. monogyna* and *A. unedo* extracts, for 24, 48 and 72h, was evaluated by the MTT assay. This is a colorimetric test used to assess cell viability as a function of redox potential. The principle behind MTT assay relies on the activity of mitochondrial dehydrogenases, present only in metabolically viable cells. These enzymes cleave the tetrazolium ring, transforming this yellow-colored compound in a dark blue color compound, called formazan, which crystals are insoluble in aqueous solutions. Thus, the production of formazan reflects the functional state of the respiratory chain, that

is, the actively respiring cells convert the water-soluble MTT to an insoluble purple formazan. The formazan is then solubilized and its concentration determined by optical density.

In brief, plates were incubated in the dark for 4h with 10 µl of MTT solution (Sigma-Aldrich, St. Louis, Missouri, USA). After that, the medium plus the MTT solution were carefully removed. The remaining formazan crystals were dissolved in 100 µL DMSO. The plate was wrapped in aluminum foil, agitated for 5-10 minutes for homogenization, and the conversion of the MTT compound to the colored formazan product was detected at 570 nm in a Microplate Reader (Biochrom, Anthos 2020). The relative number of viable cells in each experimental condition was calculated by normalizing the absorbance to that of the corresponding control. All experiments were repeated at least three times.

## 4. Protein quantification

Prostate cells were homogenized using the radio immunoprecipitation 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% phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). The cell lysates were homogenized, centrifuged at 14000 rpm, 4°C for 20 minutes, in a Hettich Mikro 200R centrifuge. The protein-containing supernatant was recovered and stored in a clean separate tube. The protein concentration was determined by the Bradford method 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. The proteins were stored at -80 ºC.

## 5. Quantification of glucose and lactate

The concentration of glucose and lactate in the culture medium of extract-treated and non-treated prostate cells was determined by spectrophotometric analysis using commercial kits (Spinreact, Girona, Spain). All measurements followed the manufacturer's instructions and were normalized to the total number of cells in each experimental condition. For glucose quantification, R1 buffer (92 mmol/L TRIS, pH 7.4 and 0.3 mmol/L phenol) was used to dissolve a determined, sealed measurement 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 of each sample was mixed with 100 µl of the previously mixed working reagent. Simultaneously, a blank with the working reagent and a standard with 1 µl of the aqueous glucose calibration solution was prepared alongside the samples. The plate was mixed and incubated for 10 minutes at 37 °C, and then the absorbance values were read at 505 nm. The blanks were subtracted from the other results and the amount of glucose in each sample was calculated proportionality to the standard.

The principle behind this assay relies 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,

in turn, will react with phenol and aminophenazone, in the presence of phenol oxidase, to form quinone, which gives the wells a red/violet colour, that is the measured parameter.

Quantification of lactate followed a similar methodology. 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 the R2 enzyme powder (800 u/L lactate oxidase, 2000 u/L peroxidase, 0.4 mmol/L 4-aminophenazone). A 100 µl of this working reagent were mixed with 1 µl of each sample, 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 °C s for 5 minutes, and the absorbance values were read at 505 nm. The blank was subtracted from all values and the amount of lactate in each sample was calculated based on the ratio between said sample and the lactate standard.

The principle behind this assay is very similar to the previously described for glucose. The lactate in each sample will be oxidized by the lactate oxidase present in the working reagent, resulting in pyruvate and peroxide. The latter will be transformed 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 associated with the lactate content.

The glucose consumption and lactate production by PNT1A, LNCaP and PC3 cells in each experimental condition with the different concentrations of *C. monogyna* and *A. unedo* extracts were calculated.

## 6. LDH enzymatic activity

The LDH activity assay was performed in 96-well plates. The working reagent was prepared by mixing the assay buffer (imidazole, 65 mmol/L and pyruvate, 0,6mmol/L) with NADH (0,18 mmol/L) in a ratio of 4:1. In each well, 1 µl of prostate cells protein were added to 150 µl of working reagent, and the plate was incubated for 1 minute at 37 °C 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 °C. All readings were taken at 340 nm.

## 7. Caspase-3-like colorimetric activity assay

The activity of caspase-3 was assessed by determining the cleavage of a colorimetric substrate. Briefly, 5 µl prostate cells total protein extracts were incubated with 85 µl assay buffer (25 mM HEPES, pH 7.5, 0.1% CHAPS, 10% sucrose and 10 mM DTT) and 10 µl of caspase-3 substrate (Ac-DEVD-pNA), along with a sample-less blank (with 90 µl assay buffer and 10 µl caspase-3 substrate) at 37º C. The caspase-3-like activity was determined after cleavage of the labeled substrate by detection of the chromophore p-nitroaniline (pNA), measured spectrophotometrically at 405 nm for 70 minutes, 90 minutes and 22h. The method was

calibrated with known concentrations of the yellow product pNA after cleavage of Ac-DEVD-pNA by caspase-3. The amount of generated pNA was obtained by extrapolation with a standard curve of free pNA at different concentrations, and is directly proportional to the activity of caspase-3.

## 8. Western Blot

This procedure was used to determine the expression of GLUT1, GLUT2, GLUT3, MCT-4, PFK-1, Bax, Bcl-2, LDH and ß-actin proteins, in the non-neoplastic cells, PNT1A, and neoplastic cells, LNCaP and PC3 prostate cells.

In an eppendorf 50 µg of each cell line protein extract were mixed with 5 µl of loading buffer (with 10% 2-Mercaptoethanol) and de-natured at 100 º C for 5 minutes. Proteins were 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 was performed at 200 V for 50 minutes.

Proteins were then electro-transferred to activated PVDF membranes (Bio-Rad, Hercules, California) at 1.20 mA for a period of 80 minutes. Membranes were blocked for 1 hour under agitation with a solution containing 5% dried skimmed milk (Regilait), and incubated overnight with one of the following antibodies: anti-rabbit Bax (1:1000, #2772, Cell Signalling Technology), anti-rabbit Bcl-2 (1:1000, 2876, Cell Signalling Technology), anti-rabbit GLUT1 (1:1000, CBL242, Millipore), anti-rabbit GLUT3 (1:1000, H-50, SC-30107, Santa Cruz Biotechnology), anti-rabbit PFK-1 (1:1000, sc-67028, Santa Cruz Biotechnology), anti-rabbit LDH (1:5000, EP15664, Abcam), anti-rabbit PFK-1 (1:1000, H-55, SC-67028, Santa Cruz Biotechnology) and anti-rabbit MCT4 (1:1000, H-90, SC-50329, Santa Cruz Biotechnology).

Each membrane was also incubated with anti-mouse ß-actin (1:10000, A5441, Sigma-Aldrich), in order to serve as a protein loading control to be normalized with each individual antibody's band density.

After the primary antibody incubation and washing, membranes were incubated for 1 hour with 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) secondary antibodies. Then, membranes were incubated with the enhanced chemiluminescence (ECL) substrate (BioRad) for 5 minutes, and immune-reactive proteins were scanned and detected with the ChemiDoc™ MP Imaging System (BioRad). This raw data was posteriorly visualized with, and the band densities processed by volumetric analysis tool of Bio-Rad's Image Lab 5.1 software. This data was then normalized with each membrane's respective ß-actin values.

## 9. Statistical Analysis

The statistical analysis of all data obtained was performed using the GraphPad Prism v6.01 software (GraphPad Software, San Diego, California, USA). Statistically significant differences between the tested groups were obtained by Unpaired Student's T-Test and one-way ANOVA followed by Bonferroni multiple comparison test as applicable. The differences were considered significant when $P<0.05$ ($p<0.05$ = (*), $p<0.01$= (**), $p<0.001$ = (***) and $p<0.0001$ = (****)). Experimental data are shown as mean ± SEM.

# Chapter IV - Results

## 1. *Crataegus monogyna* and *Arbutus unedo* extracts decrease 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 (10, 50, 100, 200, 500 and 1000 µg/mL) of *C. monogyna* and *A. unedo* extracts was determined by the MTT assay for 24, 48 and 72 h of exposure.

*C. monogyna was* the first plant under study with 10, 50, 100 and 1000 µg/mL concentrations tested for 24, 48 and 72 h of exposure. Preliminary results showed that cell viability decreased significantly only after 72 h, at the concentration of 1000 µg/mL. Thus, two other concentrations, 200 and 500 µg/mL, were added in the 72 h experiments. In the case of A. *unedo* extracts, all concentrations previous described was studied at 24, 48 and 72 h. This allowed confirming that ***C. monogyna*** extracts at concentrations above 500 µg/ml for 72 h of treatment significantly reduced the viability of prostate non-neoplastic PNT1A cells (Figure 7).

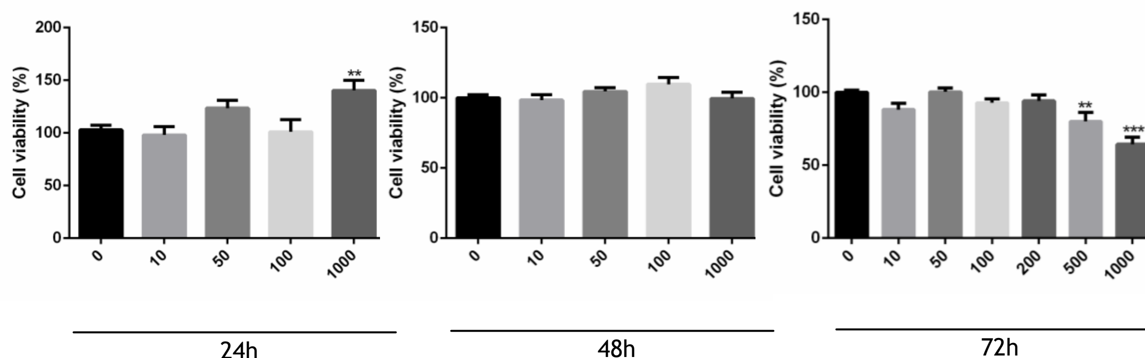


**Figure 7 - Percentage of viable non-neoplastic human prostate epithelial PNT1A cells after exposure to different concentrations of *C. monogyna* extracts (10, 50, 100, 200, 500 and 1000 µg/mL) for 24, 48 and 72 h determined by the MTT assay.** Results are expressed as % relatively of control. Error bars indicate mean ± S.E.M (n≥5). ** P<0.01; *** P<0.001 when compared with the control group.

Relatively to the viability of neoplastic prostate cells LNCaP (Figure 8), which mimic the initial stage of metastatic prostate cancer, it was significantly decreased for 24 h of treatment with 1000 µg/ml of *C. monogyna* extracts. For 48 h of exposure, LNCaP cells viability diminished with the 10 and 100 µg/ml concentrations, and noticeably when using 1000 µg/mL of extracts. The *C. monogyna* extracts significantly reduced LNCaP cells proliferation for 72 h of exposure from 100 to 1000 µg/ml on concentration dependent manner (100-500 µg/ml).

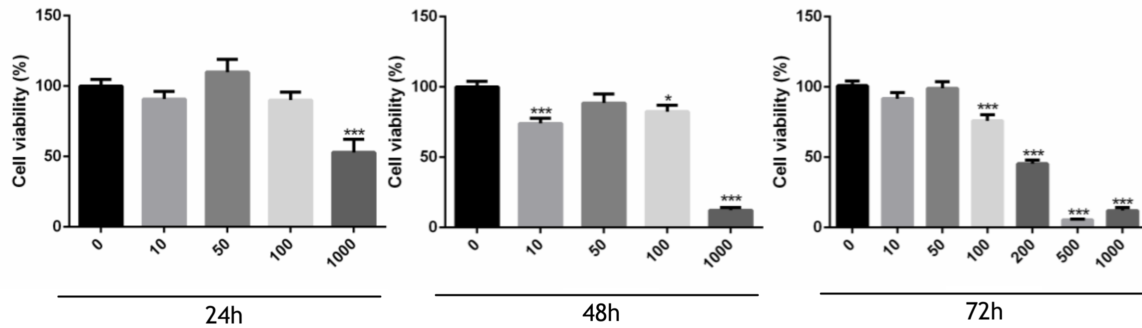


**Figure 8 - Percentage of viable neoplastic human prostate LNCaP cells after exposure to different concentrations of *C. monogyna* extracts (10, 50, 100, 200, 500 and 1000 µg/mL) for 24, 48 and 72 h determined by the MTT assay.** Results are expressed as % relatively of control. Error bars indicate mean ± S.E.M (n≥5). * P<0.05; *** P<0.001 when compared with the control group.

Exposure of the neoplastic prostate cell line PC3, representative of an aggressive stage of metastatic prostate cancer, to *C. monogyna* extracts for 24 and 48 h, had no significant effect on cell viability comparatively with the control group, independently of the concentration (Figure 9). However, for 72 h of treatment, concentrations between 100 and 1000 µg/ml significantly reduced PC3 cells proliferation.

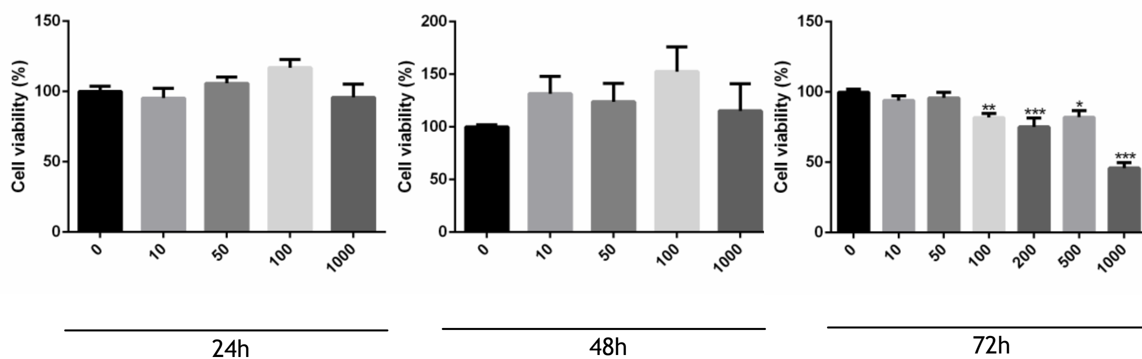


**Figure 9 - Percentage of viable neoplastic human prostate PC3 cells after exposure to different concentrations of *C. monogyna* extracts (10, 50, 100, 200, 500 and 1000 µg/mL) for 24, 48 and 72 h determined by the MTT assay.** Results are expressed as % relatively of control. Error bars indicate mean ± S.E.M (n≥5). * P<0.05; ** P<0.01; *** P<0.001 when compared with the control group.

For the study of ***A. unedo*** effects on human prostate cells viability 10, 50, 100, 200, 500 and 1000 µg/mL concentrations were used for 24 h, 48 h and 72 h of exposure. In the case of PNT1A cells (Figure 10), 24 h of treatment with *A. unedo* extracts significantly decreased cell viability at 10, 100, 200 and 500 µg/mL. At the same exposure time, a significant increase in viability was observed with 1000 µg/mL, when compared to the control group. For 48 h of treatment, all tested concentrations significantly decreased PNT1A cell viability, except in 50 µg/mL. The same pattern of PNT1A cells viability was shown at 72 h.



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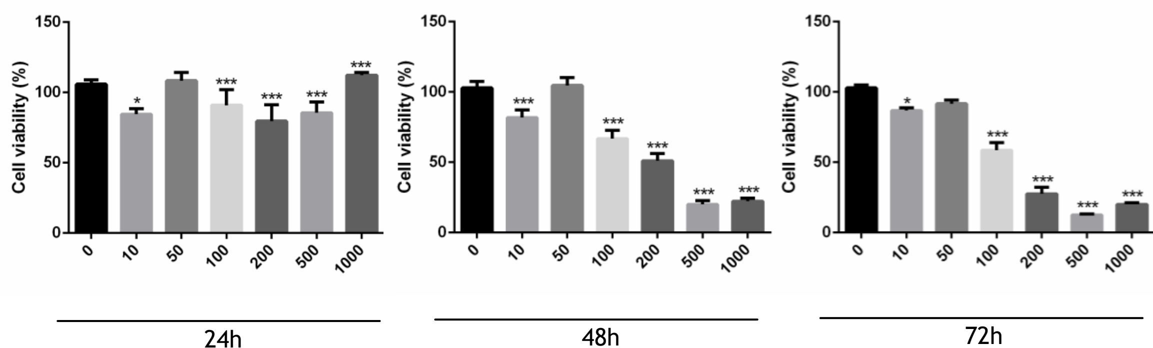
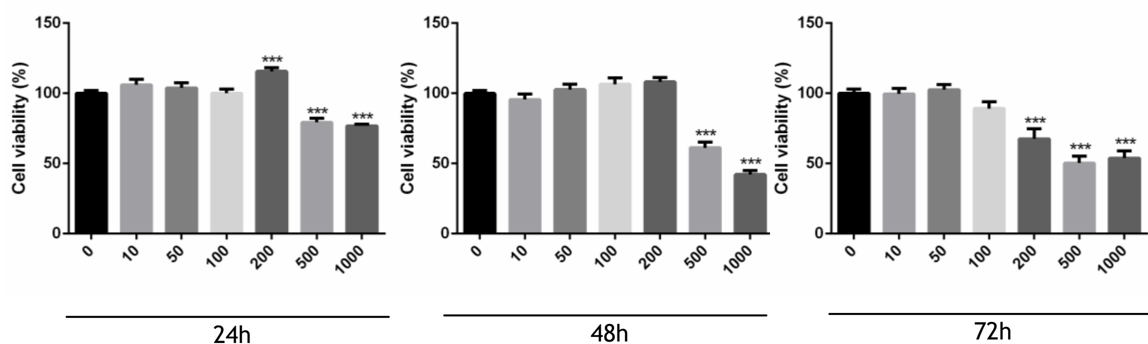



24h 48h 72h

**Figure 10 - Percentage of viable non-neoplastic human prostate epithelial PNT1A cells after exposure to different concentrations of *A. unedo* extracts (10, 50, 100, 200, 500 and 1000 μg/mL) for 24, 48 and 72 h determined by the MTT assay.** Results are expressed as % relatively of control. Error bars indicate mean ± S.E.M (n≥5). * $P<0.05$; *** $P<0.001$ when compared with the control group.

Relatively to the neoplastic prostate cell line, LNCaP (Figure 11), treatment for 24 h with *A. unedo* extracts at 10-100 μg/ml concentrations did not significantly influenced cell viability when compared to the control group, being that an increase in cell viability was visible at a 200 μg/ml concentration. However, when using higher concentrations (500-1000 μg/ml), LNCaP cells viability significantly decreased. For 48 h of exposure, a significant reduction in cell viability was observed for 500 and 1000 μg/ml concentration. Cell viability after 72 h of culture was significantly reduced cell from 200 to 1000 μg/ml of extract.




24h 48h 72h

**Figure 11 - Percentage of viable neoplastic human prostate LNCaP cells after exposure to different concentrations of *A. unedo* extracts (10, 50, 100, 200, 500 and 1000 μg/mL) for 24, 48 and 72 h determined by the MTT assay.** Results are expressed as % relatively of control. Error bars indicate mean ± S.E.M (n≥5). *** $P<0.001$ when compared with the control group.

Finally, concerning the neoplastic prostate cell line PC3 (Figure 12), no significant differences were observed in cell viability for 24 h of treatment comparatively with the control group. Reduced PC3 cells viability was observed for 48 h of treatment with 500 and 1000 μg/ml of *A. unedo* extracts. In 100 to 1000 μg/ml was the effective concentration range, showing significantly reducing PC3 cells proliferation for 72 h of treatment.

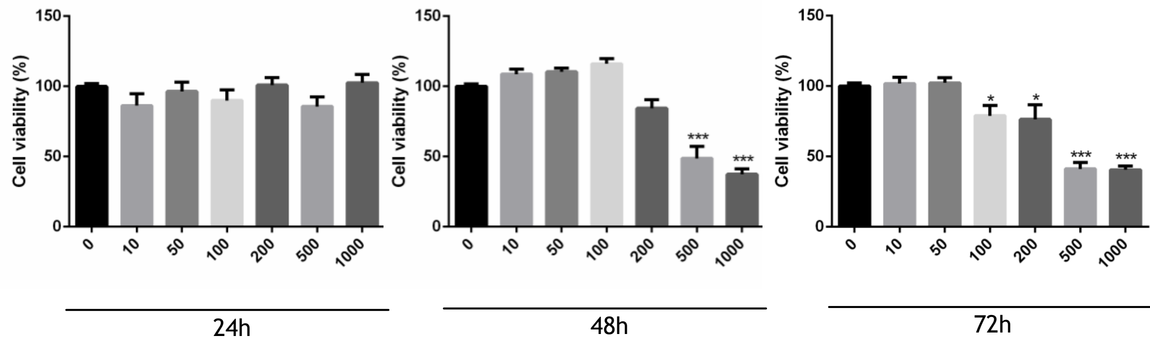



**Figure 12 - Percentage of viable neoplastic human prostate PC3 cells after exposure to different concentrations of *A. unedo* extracts (10, 50, 100, 200, 500 and 1000 µg/mL) for 24, 48 and 72 h determined by the MTT assay.** Results are expressed as % relatively of control. Error bars indicate mean ± S.E.M (n≥5). * $P<0.05$; *** $P<0.001$ when compared with the control group.

Integrating the results of cell viability obtained for the three prostate cell lines under study in the presence of plant extracts, the 200 µg/ml concentration and the incubation time of 72 h were selected for the subsequent analysis of apoptosis and metabolism.

## 2. Effect of *Crataegus monogyna* and *Arbutus unedo* extracts modulating apoptosis of human prostate cells

Apoptosis was evaluated by the biochemical assay measuring activity of caspase-3. Western blot analysis was used to determine the relative expression of apoptosis regulators Bax and Bcl-2.

### 2.1 Differential expression of apoptosis regulators in the non-neoplastic PNT1A cell in the presence of *Crataegus monogyna* and *Arbutus unedo* extracts

In the intrinsic pathway of apoptosis, the outer mitochondrial membrane permeabilization and the consequent release of cytochrome c is considered a "point of no return", since it leads to the activation of the executioner of apoptosis, caspase-3. The balance between Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) regulators is extremely important in the regulation of the apoptotic process by promoting or inhibiting permeabilization of mitochondria membrane [78].

In PNT1A cells, ***C. monogyna*** extract exposure during 72 h, at a concentration of 200 µg/ml, significantly increased the expression of pro-apoptotic protein Bax (Figure 13A) comparatively with the control (1.71 ± 0.21 fold variation, P=0.0390). The same trend was observed in the activity of caspase-3 (Figure 14), a major indicator of apoptosis activated by both the intrinsic and extrinsic pathways of apoptosis, but non-significantly.

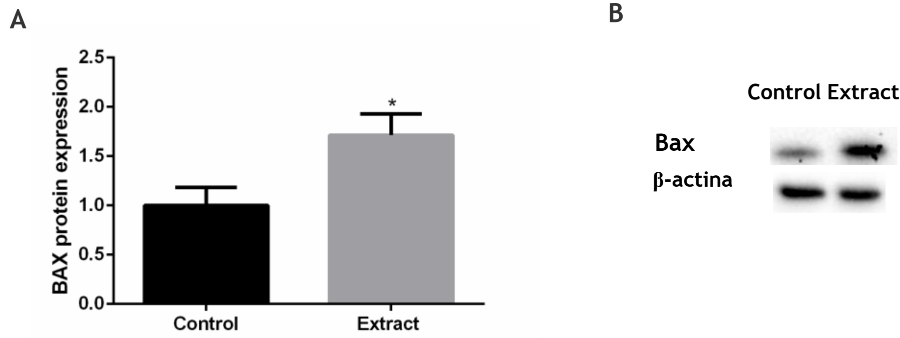



**Figure 13 - Expression of the apoptosis regulator, Bax, in non-neoplastic human prostate epithelial PNT1A cells after treatment with 200 µg/ml of *C. monogyna* extracts for 72 h, determined by Western blot analysis after normalization with ß-actin.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n≥4). Representative blots are shown in panel B.

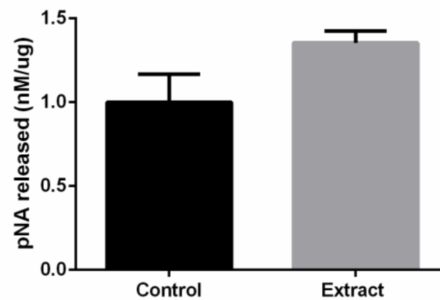



**Figure 14 - Activity of Caspase-3 in non-neoplastic human prostate epithelial PNT1A cells after treatment with 200 µg/ml of *C. monogyna* extracts for 72 h, determined by a colorimetric assay.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n=6).

Upon exposure to ***A. unedo*** extracts, the non-neoplastic PNT1A cells displayed a significantly diminished expression of both pro-apoptotic protein Bax (0.61 ± 0.02 fold variation, P=0.0300, Figure 15A), and the anti-apoptotic protein Bcl-2 (0.39 ± 0.07 fold variation, P=0.0001, Figure 15B), comparatively with the control. Despite these marked changes on Bax and Bcl-2 expression, the activity of caspase-3 (Figure 16) was significantly increased in PNT1A treated cells in comparison with the control values ( 0.52 ± 0.10 fold variation, P=0.0374).

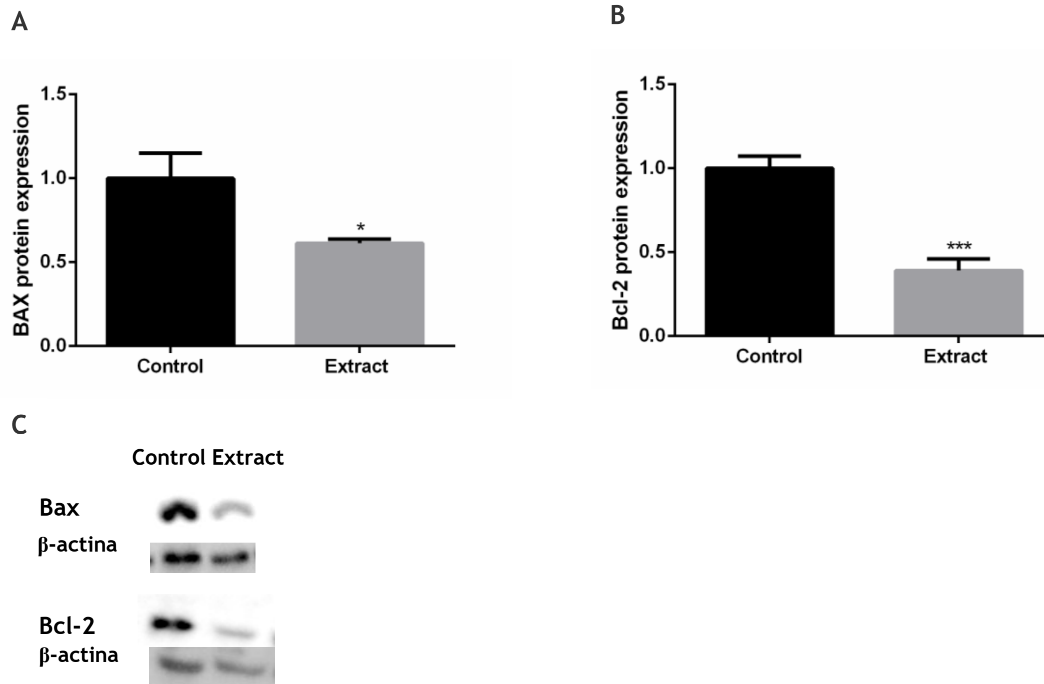



**Figure 15 - Expression of the apoptosis regulators (A-B) in non-neoplastic human prostate epithelial PNT1A cells after treatment with 200 µg/ml of *A. unedo* extracts for 72 h, determined by Western blot analysis after normalization with ß-actin.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n=6). * P<0.05; *** P<0.001 when compared with the control group. Representative blots are shown in panel C.

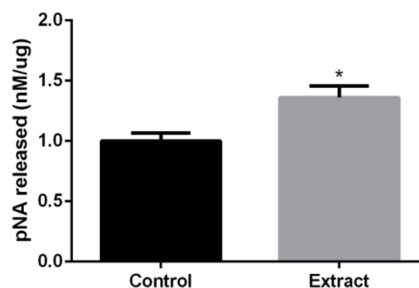



**Figure 16 - Activity of Caspase-3 in non-neoplastic human prostate epithelial PNT1A cells after treatment with 200 µg/ml of *A. unedo* extracts for 72 h, determined by a colorimetric assay.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n=6). * P<0.05 when compared with the control group.

## 2.2 Expression of pro-apoptotic genes and the activity of caspase-3 are unaltered in LNCaP cells by exposure to *Crataegus monogyna* extract and decreased after exposure to *Arbutus unedo*

In LNCaP cells treated with 200 µg/ml ***C. monogyna*** extracts treatment for 72 h, no significant differences were observed in the expression of Bax pro-apoptotic (Figure 17A) and Bcl-2 anti-apoptotic (Figure 17B) proteins. Also, the activity of caspase-3 was unaltered between treated and non-treated LNCaP cells (Figure 18).

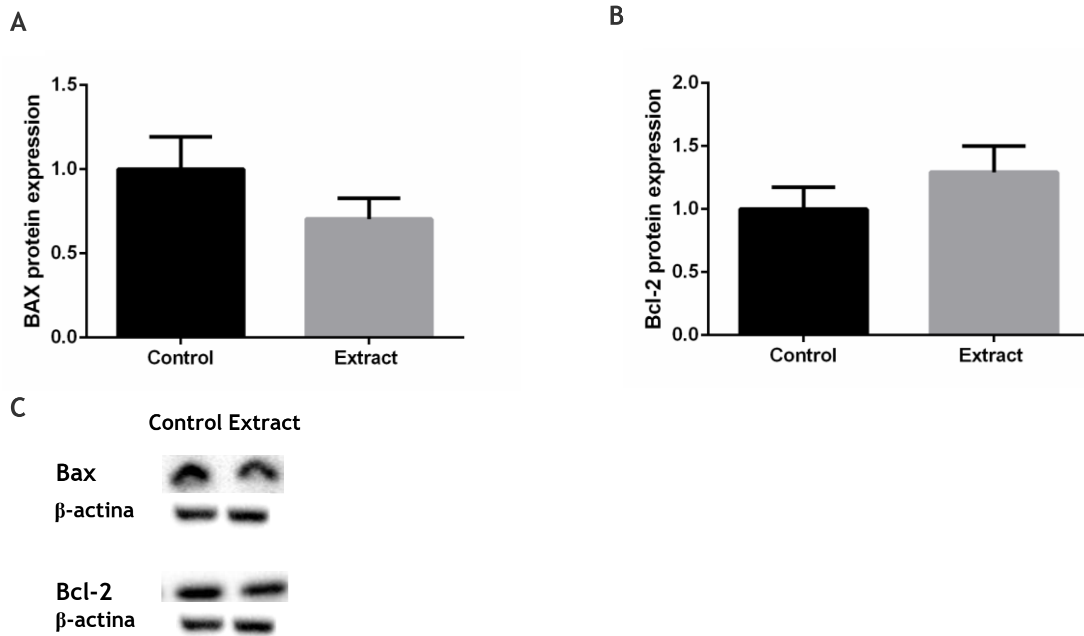



**Figure 17 - Expression of the apoptosis regulators (A-B) in neoplastic human prostate epithelial LNCaP cells after treatment with to 200 µg/ml of *C. monogyna* extracts for 72 h, determined by Western blot analysis after normalization with ß-actin.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n=6). Representative blots are shown in panel C.

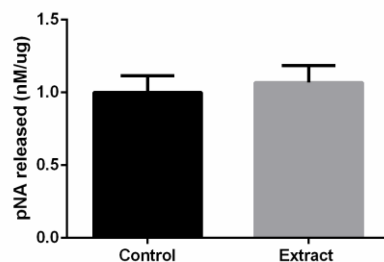



**Figure 18 - Activity of Caspase-3 in neoplastic human prostate epithelial LNCaP cells after treatment with 200 µg/ml of *C. monogyna* extracts for 72 h, determined by a colorimetric assay.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n=6).

In case of ***A. unedo***, LNCaP treated cells demonstrated to have a significantly increased expression of pro-apoptotic protein Bax (Figure 19A), comparatively with the control (2,79 ± 0,60 fold variation, P=0.0157). However, the expression of the anti-apoptotic protein Bcl-2 (Figure 19B), as well as the activity of caspase-3 (Figure 16) were significantly diminished in the presence of *A. unedo* extracts), comparatively with the control (0.60 ± 0.08 fold variation, P=0.0034 and 0.70 ± 0.10 fold variation, P=0.0486, respectively).

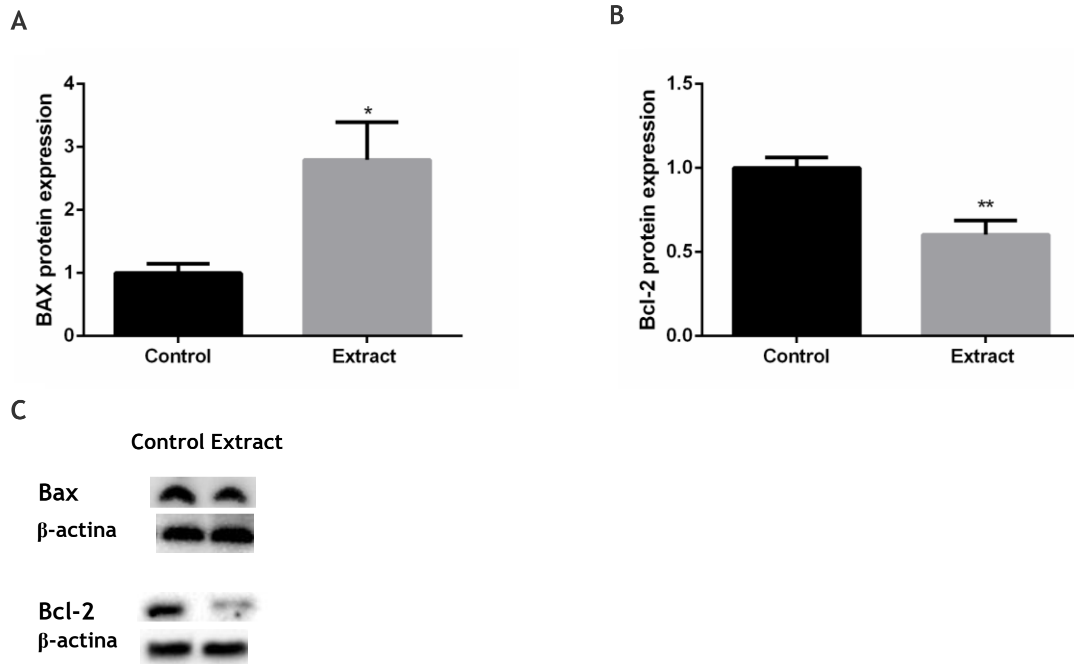



**Figure 19 - Expression of apoptosis regulators (A-B) in neoplastic human prostate epithelial LNCaP cells after treatment with 200 µg/ml of *A. unedo* extracts for 72 h, determined by Western blot analysis after normalization with ß-actin.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n=6). * P<0.05 and ** P<0.01 when compared with the control group. Representative blots are shown in panel C.

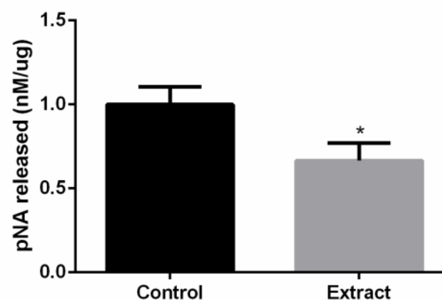



**Figure 20 - Activity of Caspase-3 in neoplastic human prostate epithelial LNCaP cells after treatment with 200 µg/ml of *A. unedo* extracts for 72 h, determined by a colorimetric assay.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n=6). * P<0.05 when compared with the control group.

## 2.3 Expression of pro-apoptotic genes and activity of caspase-3 are unaltered or down-regulated in PC3 cells after exposure to *Arbutus unedo* extract

The neoplastic PC3 cells treated with 200 µg/ml ***C. monogyna*** extract, displayed no significant changes in the expression of pro-apoptotic protein Bax (Figure 21A), and activity of caspase-3 (Figure 22), comparatively with the control.

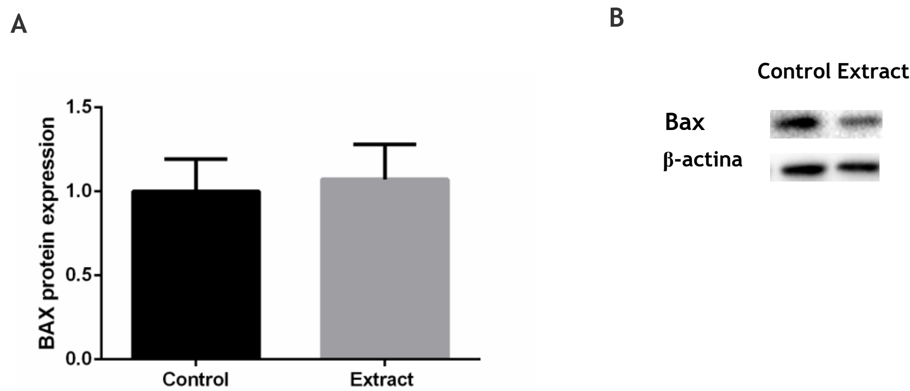



**Figure 21 - Expression of the apoptosis regulator, Bax (A), in neoplastic human prostate epithelial PC3 cells after treatment with 200 µg/ml of *C. monogyna* extracts for 72 h, determined by Western blot analysis after normalization with ß-actin.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n=6). Representative blots are shown in panel B.

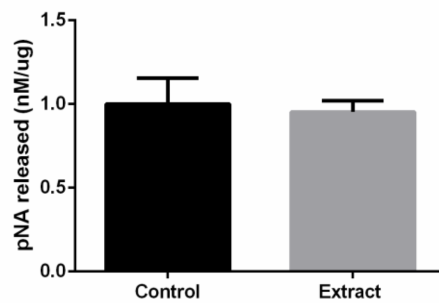



**Figure 22 - Activity of Caspase-3 in neoplastic human prostate epithelial PC3 cells after treatment with 200 µg/ml of *C. monogyna* extracts for 72 h, determined by a colorimetric assay.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n=6).

In case of exposure to ***A. unedo***, no significant changes were observed on the expression of pro-apoptotic Bax (Figure 23A) and anti-apoptotic Bcl-2 (Figure 23B) proteins in PC3 cells. However the activity of caspase-3 (Figure 24) was significantly diminished in PC3-treated cells in comparison with the control (0.52 ± 0.10 fold variation, P=0.0374).

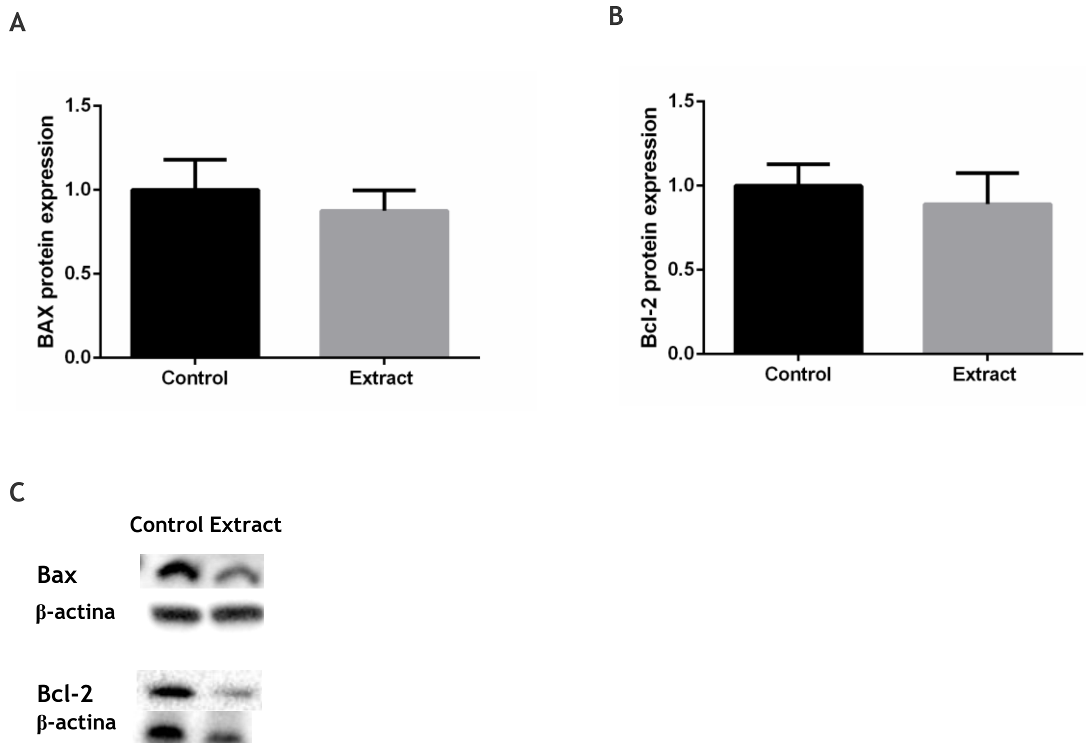



**Figure 23 - Expression of the apoptosis regulators (A-B) in neoplastic human prostate epithelial PC3 cells after treatment with 200 µg/ml of *A. unedo* extracts for 72 h, determined by Western blot analysis after normalization with ß-actin.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n=6). Representative blots are shown in panel C.

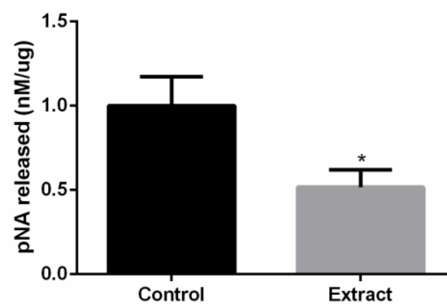



**Figure 24 - Activity of Caspase-3 in neoplastic human prostate epithelial PC3 cells after treatment with 200 µg/ml of *A. unedo* extracts for 72 h, determined by a colorimetric assay.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n=6). * $P<0.05$ when compared with the control group.

## 3. Analysis of human prostate cells glycolytic metabolism in response to *Crataegus monogyna* and *Arbutus unedo* extracts

The glucose consumption, lactate production and LDH activity were determined by biochemical assays. Western blot analysis was used to analyze the protein expression of GLUT1, GLUT2, GLUT3, PFK1, LDH and MCT4.

### 3.1 The glycolytic metabolism of non-neoplastic PNT1A cell is suppressed in the presence of *Arbutus unedo* extracts

The non-neoplastic PNT1A prostate cells treated with 200 µg/ml of ***C. monogyna*** extract for 72 h showed non-significant changes in glucose consumption and lactate production (Figure 25).

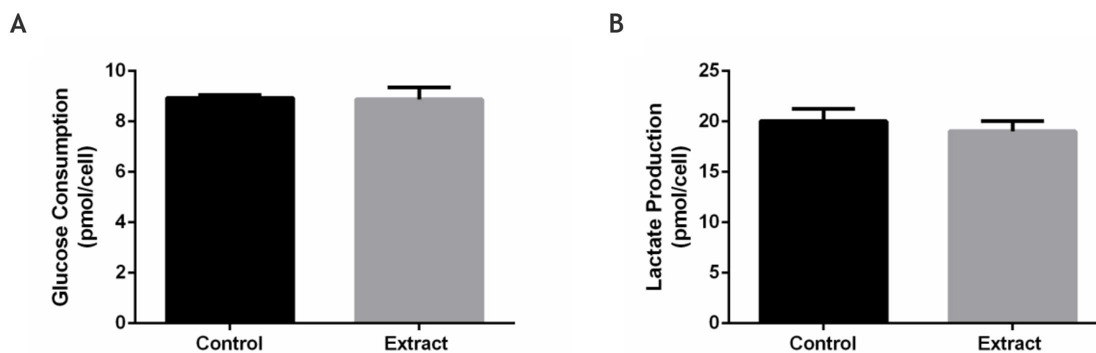



**Figure 25 - Glucose consumption (A) and lactate production (B) in non-neoplastic human prostate epithelial PNT1A cells in response to 200 µg/ml treatment of *C. monogyna* extracts for 72 h.** Error bars indicate mean ± S.E.M (n=6).

Accordingly with the unaltered glucose consumption, no significant changes were found on the expression of GLUTs, GLUT1 (Figure 26A) and GLUT3 (Figure 26B), as well as on the expression of PFK-1 (Figure 26C), the enzyme that limits the glycolytic flux, in PNT1A cells upon exposure to *C. monogyna* extract.

Concerning lactate production, the expression of LDH (Figure 26D), the enzyme responsible for the conversion of pyruvate into lactate, was not significantly altered (P=0.5898). The lactate can be exported to the extracellular space by the activity of MCTs, specifically the MCT4, whose expression was significantly decreased in PNT1A cells exposed to *C.monogyna* (0.52 ± 0.05 fold variation, P=0.0074, Figure 26E). Finally, despite no changes were perceived on lactate production, the activity of the LDH (Figure 27) was significantly increased in PNT1A cells in response to the administration of *C. monogyna* extract (1.97 ± 0,35 fold variation relativey to the control., p= 0,0286).

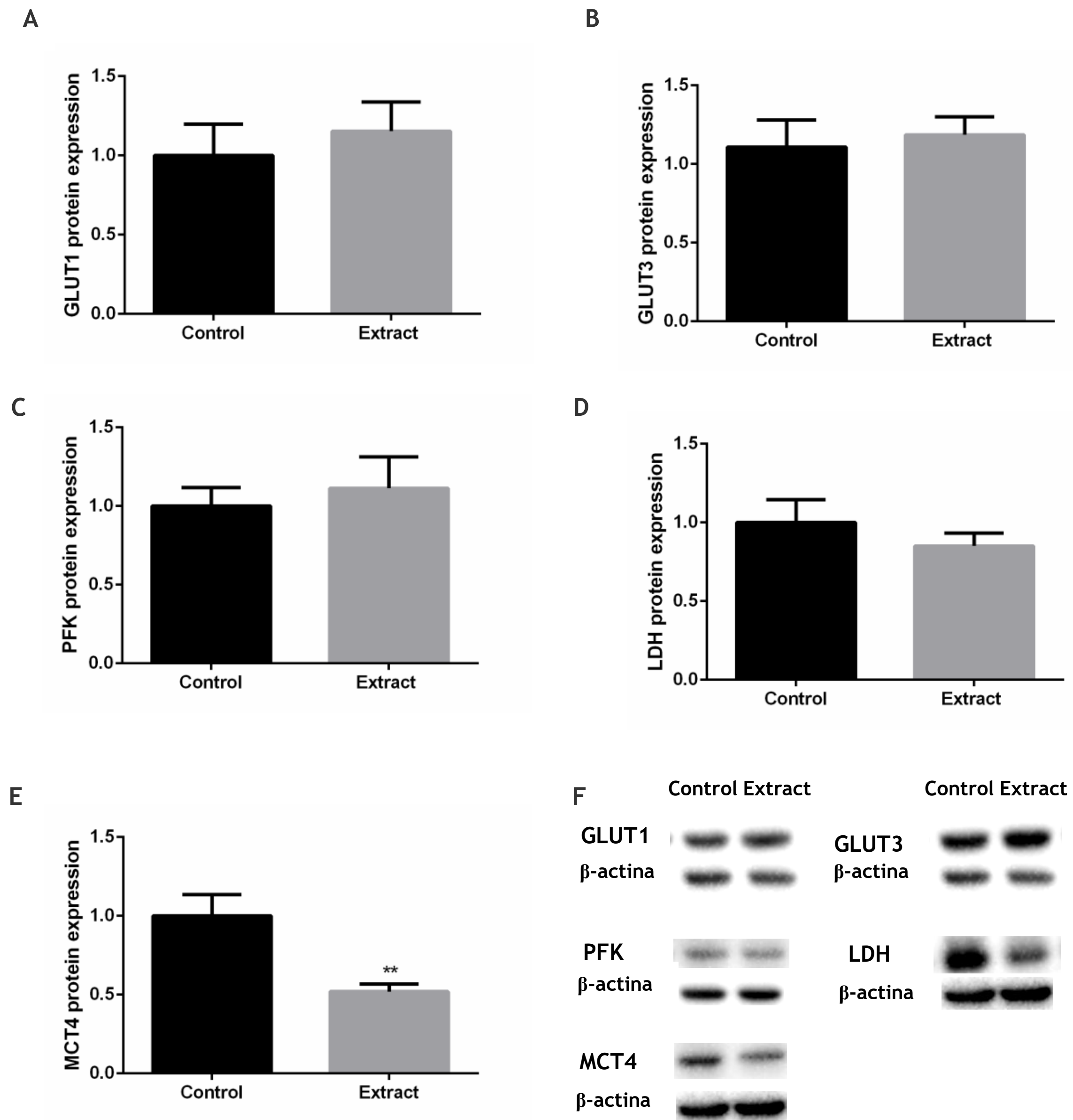



**Figure 26 – Expression of metabolism-associated proteins (A-E) in non-neoplastic human prostate epithelial PNT1A cells in response to 200 µg/ml treatment of *C. monogyna* extracts for 72 h, determined by Western blot analysis after normalization with β-actin.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n=6). ** P<0.01 when compared with the control group. Representative blots are shown in panel F.

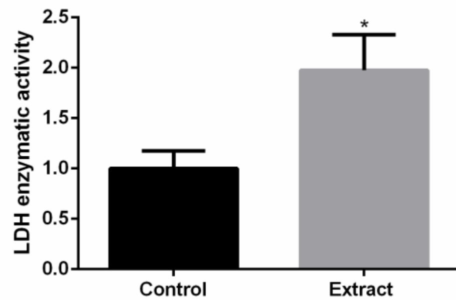



**Figure 27 – LDH enzymatic activity in non-neoplastic human prostate epithelial PNT1A cells in response to 200 µg/ml treatment of *C. monogyna* extracts for 72 h, determined by spectrophotometric assays.** Results are expressed as U/L relatively of control. Error bars indicate mean ± S.E.M (n=6). * P<0.05 when compared with the control group.

In the case of ***A. unedo*** treatment, the glycolytic profile of PNT1A cells was different. For 72 h of treatment, a concentration of 200 µg/ml extract significantly diminished glucose consumption (0.60 ± 0.38 Vs. 6.83 ± 0.25 pmol/cell in the control group, P<0.0001), whereas no significant alteration was observed in lactate production (P=0.7149) (Figure28).

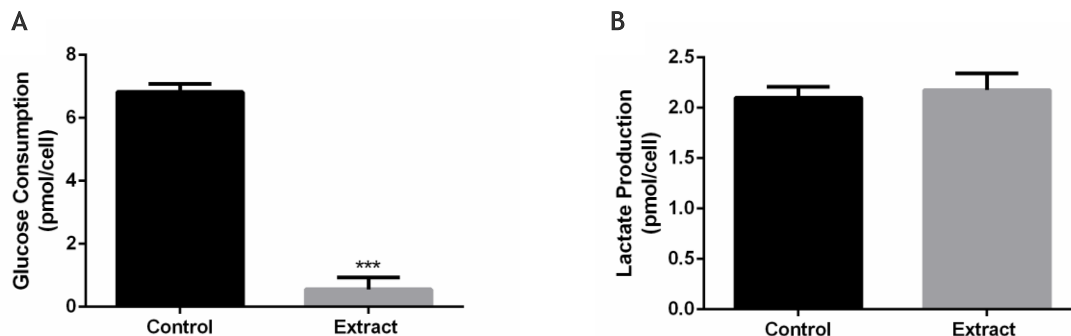



**Figure 28 - Glucose consumption (A) and lactate production (B) in non-neoplastic human prostate epithelial PNT1A cells in response to 200 µg/ml treatment of *A. unedo* extracts for 72 h.** Results are expressed as pmol/cell of control. Error bars indicate mean ± S.E.M (n=6). *** P<0.001 when compared with the control group.

The lower glucose consumption in PNT1A cells treated with ***A. unedo*** extract was accompanied by the diminished expression of GLUT3 (0.65 ± 0.11 fold variation relatively to the control, P=0.0183, Figure 29B). GLUT1 (Figure29A), and PFK1 (Figure 29C) expression were not significantly altered upon ***A. unedo*** treatment.

Although lactate production (Fig, 28B), and LDH (Figure 29D) and MCT4 (Figure 29E) expression were not altered in the presence of extract, the activity of the LDH (Figure 30) was significantly decrease in PNT1A-treated cells (0.23 ± 0.16 fold variation in comparison with the control, P=0.0471).

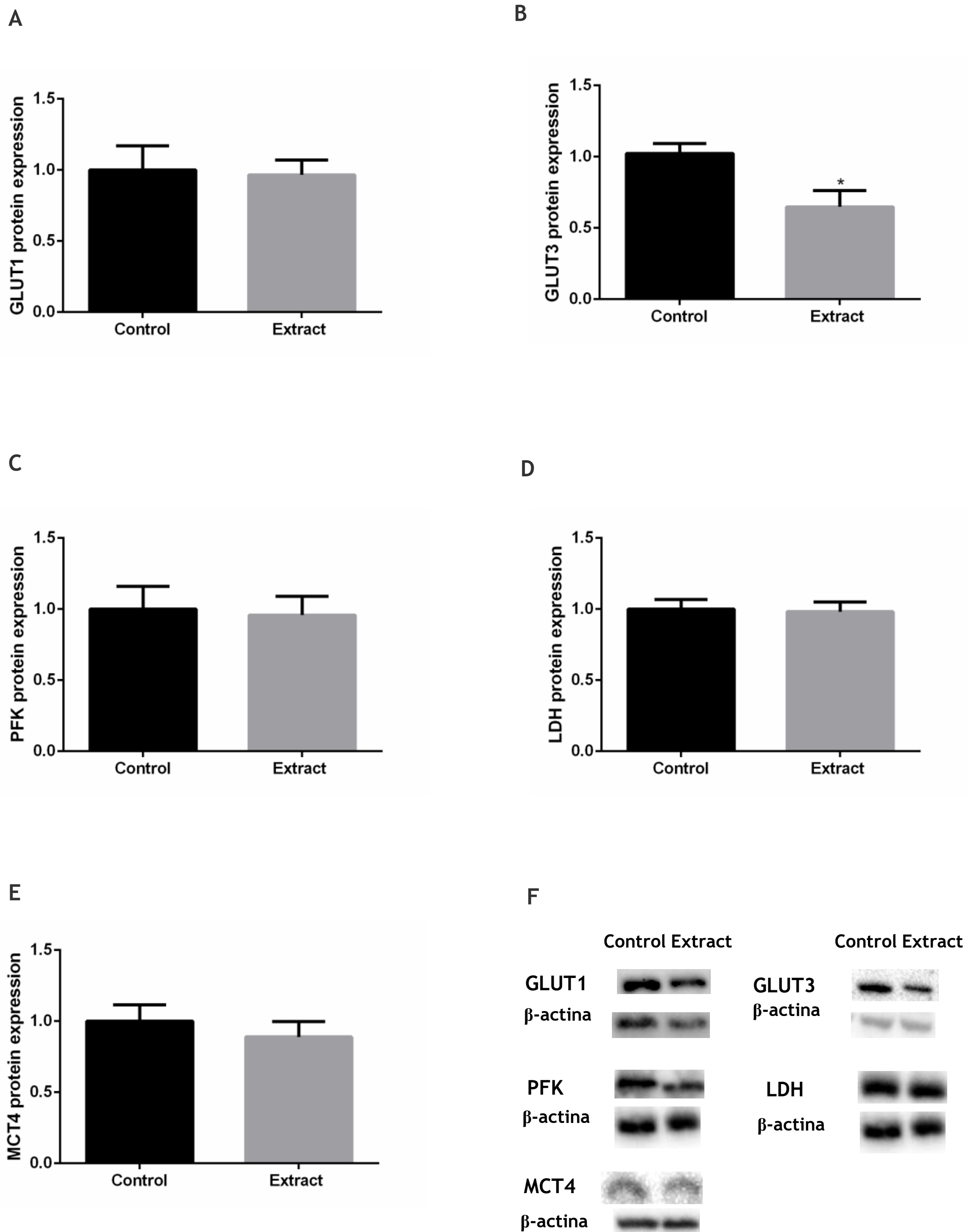



**Figure 29 - Expression of metabolism-associated proteins (A-E) in neoplastic PNT1A cells in response to 200 µg/ml treatment of *A. unedo* extracts for 72 h, determined by Western blot analysis after normalization with ß-actin.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n≥4). * P<0.05; ** P<0.01; *** P<0.001 when compared with the control group. Representative blots are shown in panel F.

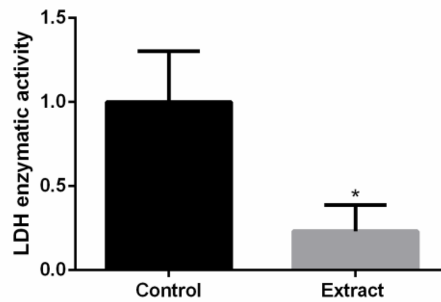



**Figure 30 – LDH enzymatic activity in non-neoplastic human prostate epithelial PNT1A cells in response to 200 μg/ml treatment of *A. unedo* extracts for 72 h, determined by spectrophotometric assays.** Results are expressed as U/L relatively of control. Error bars indicate mean ± S.E.M (n≥5). * P<0.05 when compared with the control group. P=0.0477.

## 3.2 Both *Crataegus monogyna* and *Arbutus unedo* extracts stimulate glucose consumption in the neoplastic LNCaP cells differentially affecting lactate

In LNCaP cells treated with ***C. monogyna*,** extract (200 μg/ml), glucose consumption significantly increased (55.57 ± 1.06 Vs. 42.52 ± 0.20 pmol/cell in the control group, P<0.0001) whereas lactate production was decreased (35.43 ± 2.51 Vs 43.08 ± 1.65 pmol/cell in the control group, P=0.0291, Figure31).

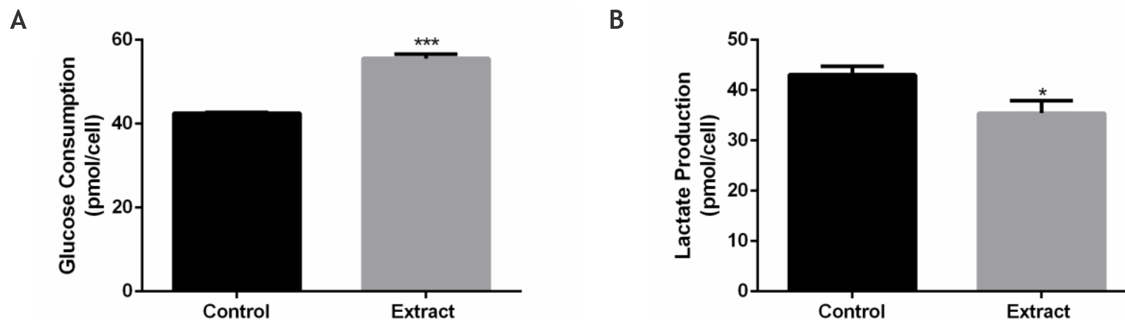



**Figure 31 - Glucose consumption (A) and lactate production (B) in neoplastic human prostate LNCaP cells in response to 200 μg/ml treatment of *C. monogyna* extracts for 72 h.** Results are expressed as pmol/cell of control. Error bars indicate mean ± S.E.M (n≥4). * P<0.05; *** P<0.001 when compared with the control group.

Despite the marked change on glucose consumption, the expression of GLUT1 (Figure 32A) and GLUT3 (Figure 32B) was not affected by the presence of extracts. However, PFK-1 expression was significantly increased (3.72 ± 1.24 fold variation relatively to control, P=0.0271, Figure 32C).

The expression (Figure 32D) of LDH was not significantly altered, but its activity was significantly decreased in LNCaP cells in response to the administration of *C. monogyna* extract (0.31 ± 0.07 fold variation relatively to control, P>0.0468, Figure 33).

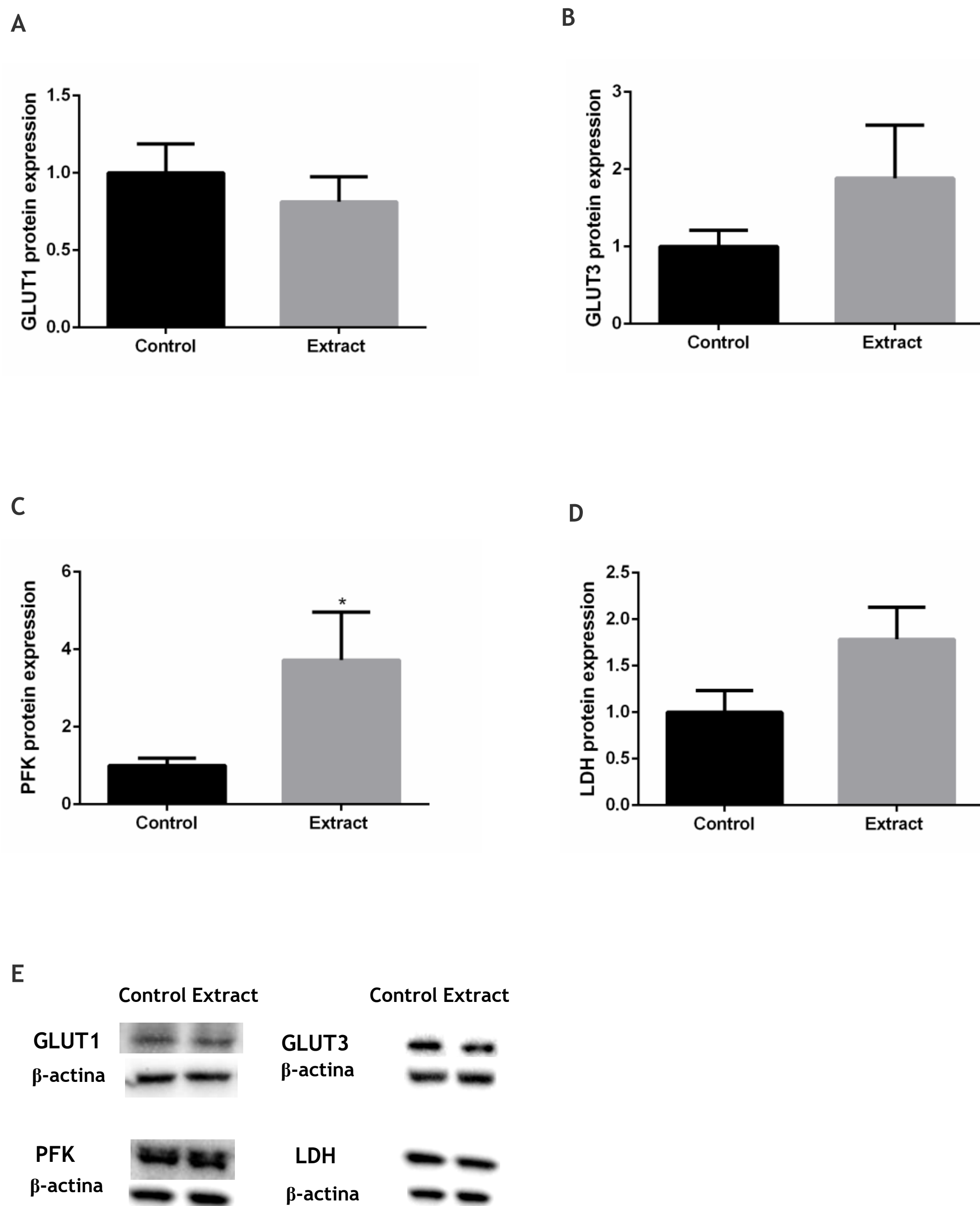



**Figure 32 – Expression of metabolism-associated proteins (A-E) in neoplastic human prostate LNCaP cells in response to 200 µg/ml treatment of *C. monogyna* extracts for 72 h, determined by Western blot analysis after normalization with β-actin.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n≥4). * P<0.05 when compared with the control group. Representative blots are shown in panel E.

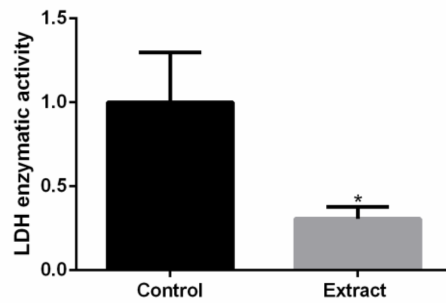



**Figure 33 – LDH enzymatic activity in neoplastic human prostate LNCaP cells in response to 200 µg/ml treatment of *C. monogyna* extracts for 72 h, determined by spectrophotometric assays.** Results are expressed as U/L relatively of control. Error bars indicate mean ± S.E.M (n=6). * P<0.05 when compared with the control group.

Concerning ***A. unedo***, treatment at concentration 200 µg/ml extract for 72 h, significantly increased glucose consumption (33.76 ± 1.40 Vs. 27.95 ± 0.91 fold variation, P=0.0059, Figure 34A) in LNCaP cells (Figure 34B).

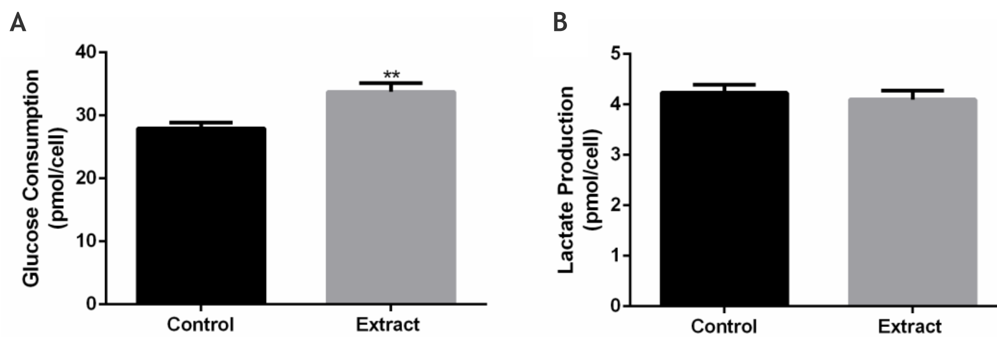



**Figure 34 - Glucose consumption (A) and lactate production (B) in neoplastic human prostate LNCaP cells in response to 200 µg/ml treatment of *A. unedo* extracts for 72 h.** Results are expressed as pmol/cell of control. Error bars indicate mean ± S.E.M (n=6). ** P<0.01 when compared with the control group.

The expression of GLUT3 (0.64 ± 0.06 fold variation, P= 0.0433, Figure 35A), and PFK-1 (0.65 ± 0.05 fold variation, P= 0.0410, Figure 35B) in LNCaP-treated cells was significantly decreased comparatively with the control. Both the expression of LDH (Figure 35C) and its enzymatic activity (Figure 36) were unaltered (P=0.8133) with the administration of *A. unedo* extract.

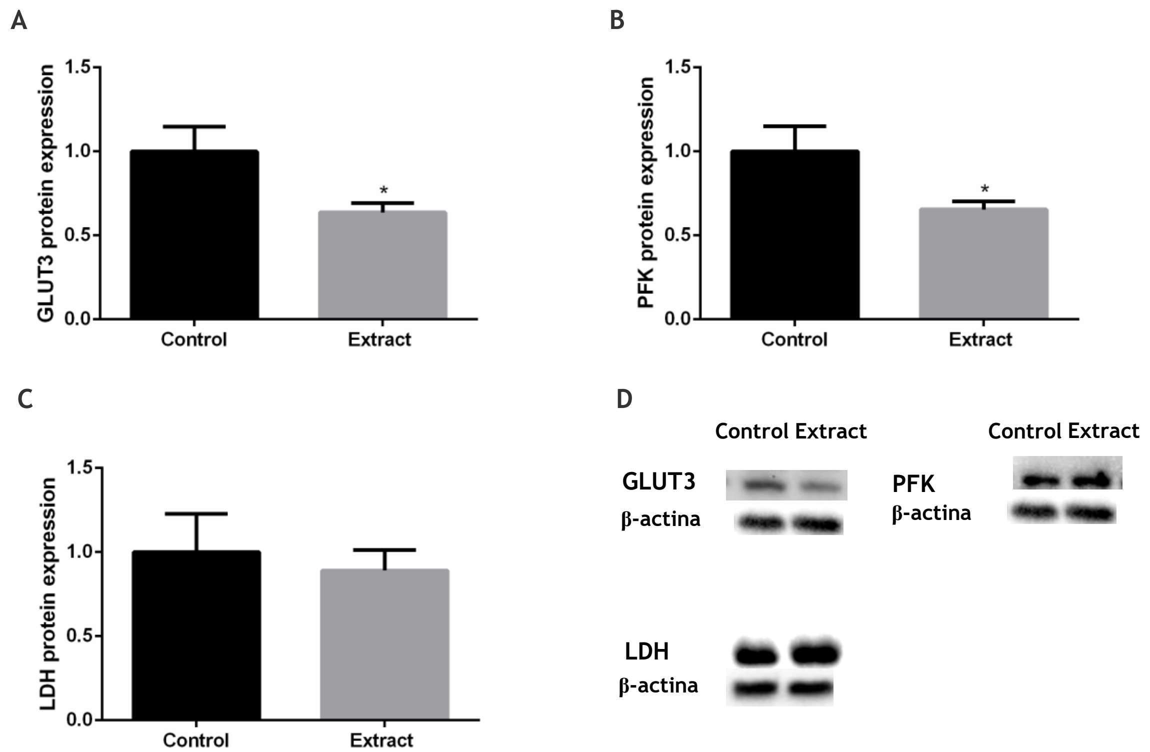



**Figure 35 – Expression of metabolism-associated proteins (A-E) in neoplastic human prostate LNCaP cells in response to 200 µg/ml treatment of *A. unedo* extracts for 72 h, determined by Western blot analysis after normalization with ß-actin.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n≥5). * $P<0.05$; ** $P<0.01$; *** $P<0.001$ when compared with the control group. Representative blots are shown in panel D.

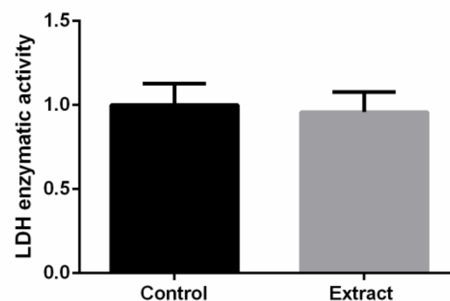



**Figure 36 – LDH enzymatic activity in neoplastic human prostate LNCaP cells in response to 200 µg/ml treatment of *A. unedo* extracts for 72 h, determined by spectrophotometric assays.** Results are expressed as U/L relatively of control. Error bars indicate mean ± S.E.M (n=6).

## 3.3 *Crataegus monogyna* extract enhanced glucose consumption and lactate export in neoplastic PC3 cells

Both glucose consumption (Figure 37A) and lactate production (Figure 37B) were significantly increased in PC3 cells under stimuli of ***C. monogyna*** extract (respectively, 14.39 ± 0.03 Vs. 11.53 ± 0.02 pmol/cell in the control, $P<0.0001$ and 29.10 ± 2.86 Vs. 12.79 ± 3.22 pmol/cell in the control, $P=0.0035$).

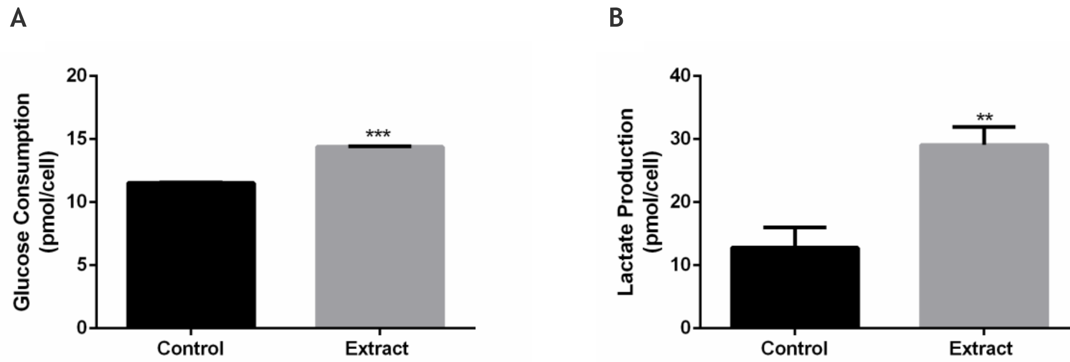



**Figure 37 - Glucose consumption (A) and lactate production (B) in neoplastic human prostate PC3 cells in response to 200 μg/ml treatment of *C. monogyna* extracts for 72 h.** Results are expressed as pmol/cell of control. Error bars indicate mean ± S.E.M (n=6). ** P<0.01; *** P<0.001 when compared with the control group.

The expression of GLUT1 (Figure 38A), GLUT3 (Figure 38B) and PFK-1 (Figure 38C) was non-significantly altered comparatively with the corresponding control groups.

Although LDH expression (Figure 38D) was not significantly altered, the LDH activity (Figure 39) had a significant increase in PC3-treated cells relatively with the control (1.41 ± 0.10 fold variation, P=0.0213). Also the expression of the lactate exporter MCT4 (Figure 38E) was not significantly altered in response to *C. monogyna* extract.

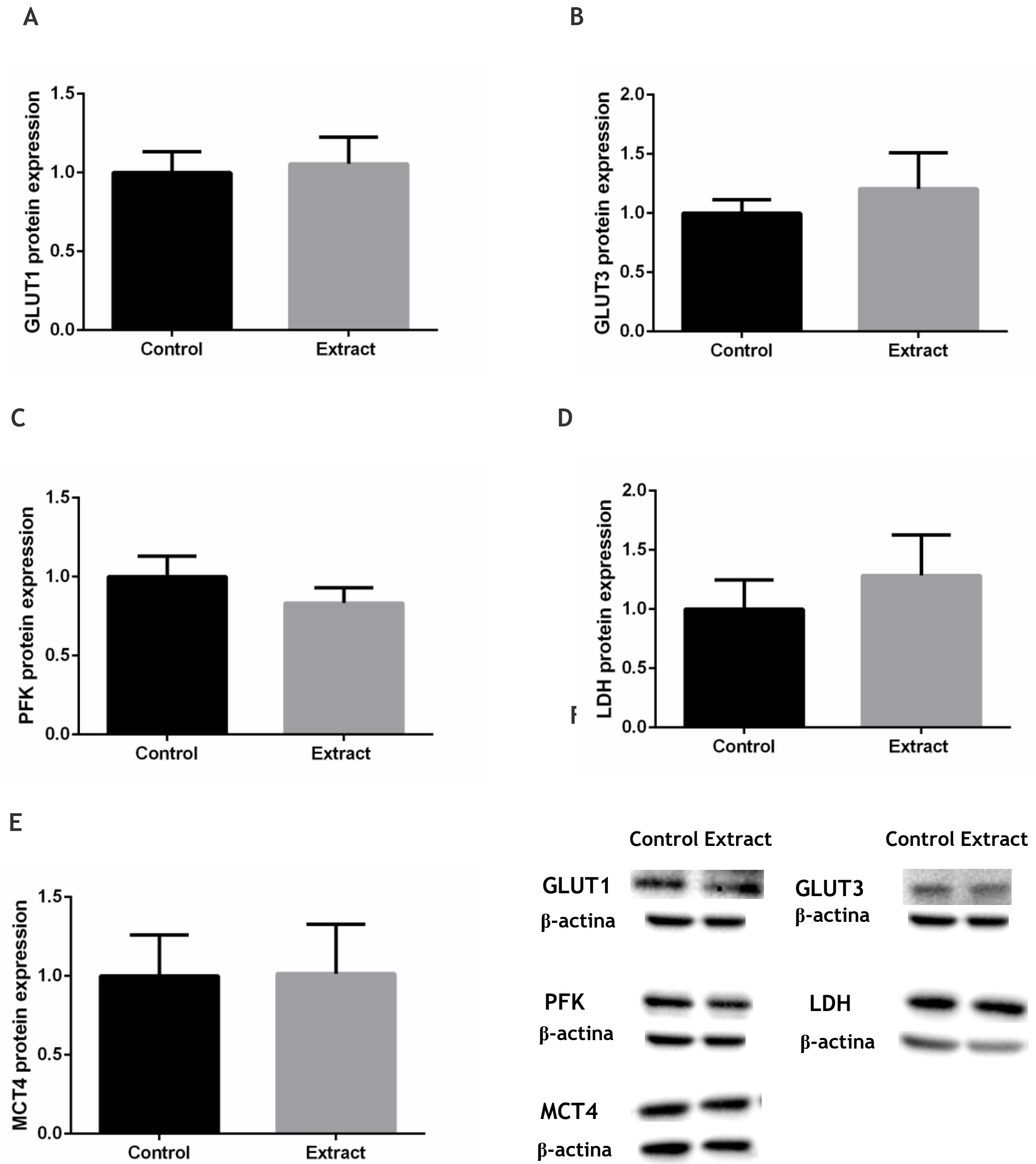



**Figure 38 – Expression of metabolism-associated proteins (A-E) in neoplastic human prostate PC3 cells in response to 200 µg/ml treatment of *C. monogyna* extracts for 72 h, determined by Western blot analysis after normalization with β-actin.** Results are expressed as fold-variation relatively of 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. Representative blots are shown in panel F.

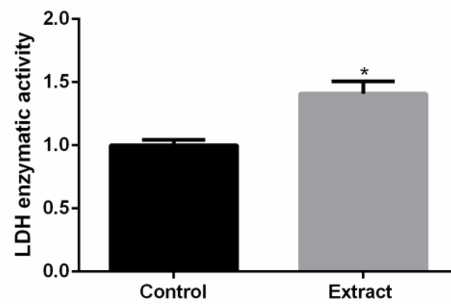



**Figure 39 – LDH enzymatic activity in neoplastic human prostate PC3 cells in response to 200 µg/ml treatment of *C. monogyna* extracts for 72 h, determined by spectrophotometric assays.** Results are expressed as U/L relatively of control. Error bars indicate mean ± S.E.M (n≥4).

The neoplastic PC3 cells treated with 200 µg/ml ***A. unedo*** extrat for 72 h of displayed no significant changes in glucose consumption and lactate production (Figure 40).

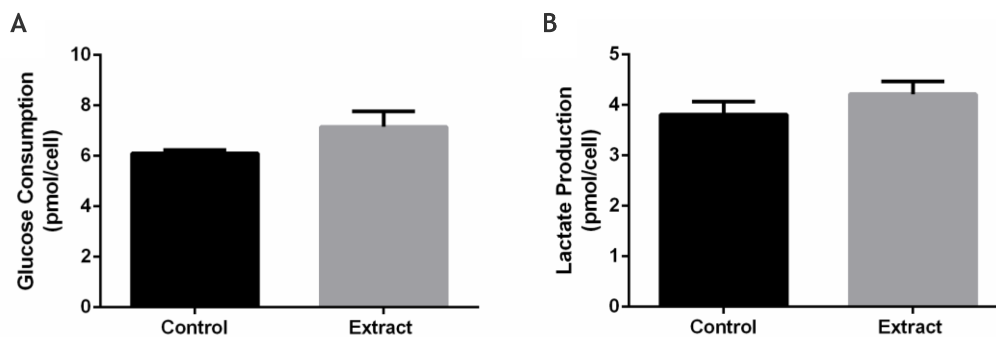



**Figure 40 - Glucose consumption (A) and lactate production (B) in neoplastic human prostate PC3 cells in response to 200 µg/ml treatment of *A. unedo* extracts for 72 h.** Results are expressed as pmol/cell of control. Error bars indicate mean ± S.E.M (n=6).

GLUT3 (Figure 41A) and glycolytic enzyme PFK-1 (Figure 41B) expression also were unchanged upon treatment.

Concerning lactate-associated proteins, both the expression (Figure 41C) and activity (Figure 42) of LDH were unaltered in PC3-treated cells when compared to the control group.

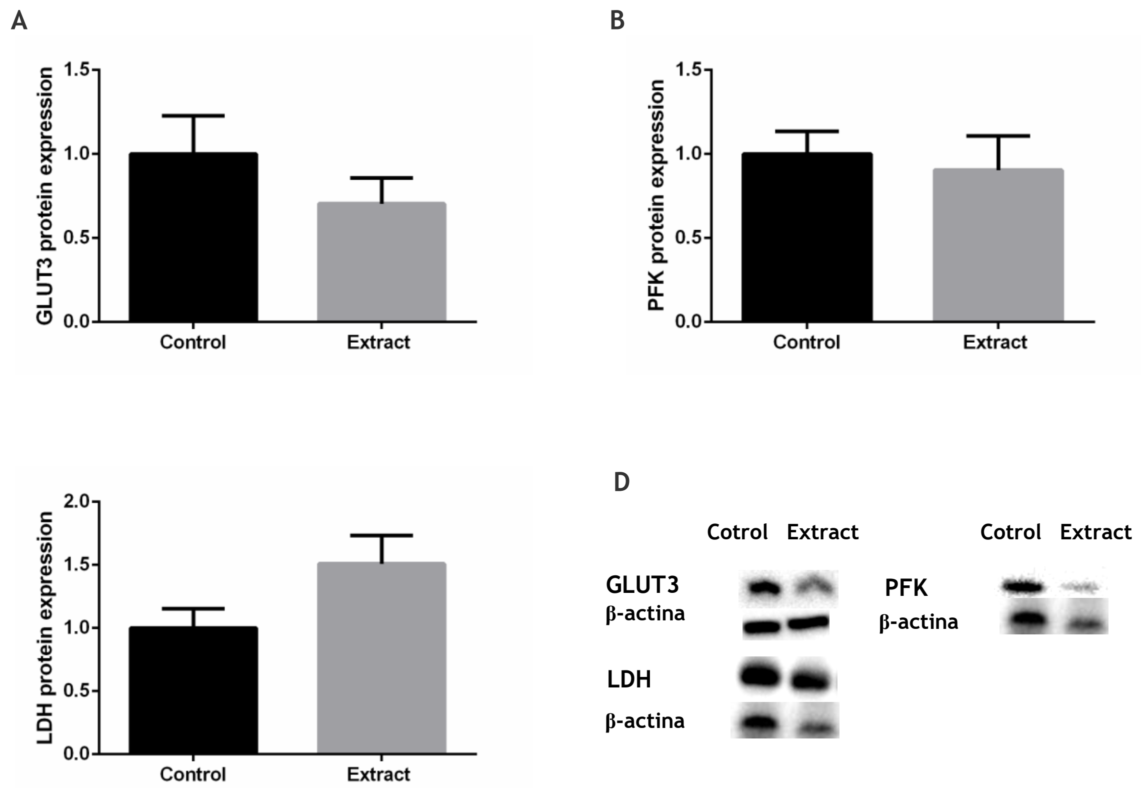



**Figure 41 – Expression of metabolism-associated proteins (A-E) in neoplastic human prostate PC3 cells in response to 200 µg/ml treatment of *A. unedo* extracts for 72 h, determined by Western blot analysis after normalization with β-actin.** Results are expressed as fold-variation relatively of control. Error bars indicate mean ± S.E.M (n=6). * P<0.05; ** P<0.01; *** P<0.001 when compared with the control group. Representative blots are shown in panel D.

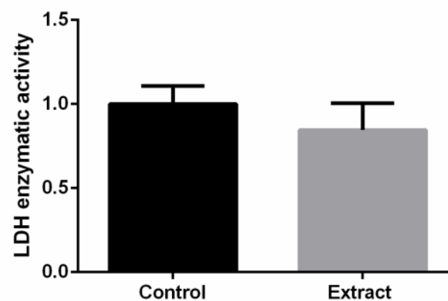



**Figure 42 – LDH enzymatic activity in neoplastic human prostate PC3 cells in response to 200 µg/ml treatment of *A. unedo* extracts for 72 h, determined by spectrophotometric assays.** Results are expressed as U/L relatively of control. Error bars indicate mean ± S.E.M (n=6).

# Chapter V – Discussion

Natural products or phytochemicals have been gaining increasing interest in treatment of cancer due to remarkable advantages, such as safety and lower drug resistance [29]. Their anticancer effects can occur through several mechanisms, namely, inhibition of cell proliferation, induction of apoptosis, and modulation of intra cellular signaling pathways and oxidative stress [79]. Plants polyphenols have been identified as one of the main bioactive compounds responsible for these effects, and have been widely used to study the behavior of cancer cells [34] [80] [81] [82] [83] [84] [85] [86] [87]. *C. monogyna* and *A. Unedo* are both plants highly-enriched in polyphenols, which have been used in the treatment of different diseases [52] [58] [74] [75] [76].

The present thesis investigated the effect of *C. monogyna* and *A. Unedo* extracts modulating proliferation, apoptosis and glycolytic metabolism of neoplastic (PNT1A) and non-neoplastic (LNCaP and PC3) prostate cells. LNCaP and PC3 cells are representative of different stages of prostate cancer with moderate metastatic potential (androgen-sensitive) and elevated metastatic potential (androgen-insensitive), respectively [88]. These cell line models have been widely used to study the behavior of PCa cells in multiple aspects, including the evaluation of the glycolytic metabolism [39] [43] [44].

As a starting point, we first evaluated effects of *C. monogyna* and *A. Unedo* extracts in prostate cells viability/proliferation, as this one of the key hallmarks of cancer. Indeed, proliferative activity is the most fundamental trait of cancer cells involving their ability to sustain chronic growth [89].

Overall, MTT proliferation assays allowed firstly concluding that *C. monogyna* and *A. Unedo* extracts diminished viability/proliferation of both neoplastic and non-neoplastic prostate cells. The three prostate cell types behaved consistently with what was observed in the literature in similar cases. For example, in the presence of *C. monogyna* extracts, proliferation of MCF-7 breast adenocarcinoma, cells was shown to decrease [52], and diminished the proliferation of NCI-H460 non-small lung cancer cells [75]. In presence of *A. unedo* extracts, can induce cell growth inhibition in HCT 116 cell Line human colon carcinoma [90]. Despite both plant extracts diminished the proliferation of all prostate cell lines used in this study, there were noticeable differences between them. Nevertheless, the treatment duration that had more significance as anti-proliferation effect was 72 h.

Concerning PNT1A cells, the effective decrease in cell proliferation were when treated with *A. Unedo* extracts (Figure 10). In LNCaP cells, the *C. monogyna* extracts were the greater decreasing cell proliferation (Figure 8). For PC3 cells, a similar decrease in proliferation occurred with both plants extracts treatment (Figure 9 and 12). Also it is noteworthy that the

anti-proliferative effects of plants extracts were more pronounced in PNT1A and LNCaP cells treated with *A. Unedo* or *C. monogyna*, respectively. Aggressive cancer cells usually have higher proliferation rates and stimulated metabolic activity, thus it would be expected that plant extracts have enhanced effects in PC3 cells dependently of their naturally higher division rates and faster metabolization. But this did not happened, and no definitive explanation can be advanced at the moment. However, it cannot be excluded from the discussion the fact that plants extracts did not undergo any purification process, as well as the possibility that some of the bioactive compounds, such as polyphenols, could have been degraded into other compounds (including sugars), which were unaccounted for, and could possibly contribute to the findings obtained. In addition, some difficulties were experienced while carrying out the MTT proliferation assays in LNCaP cells, which also may be underpinning the lower consistency of results, obtained in this cell line.

Gathering all the information from the proliferation assays in the non-neoplastic cells, PNT1A, and in neoplastic, LNCaP and PC3, the concentration of 200 µg/mL and an exposure time of 72 h were selected for the subsequent stimuli and analysis of apoptosis and glycolytic metabolism.

Another recognizes hallmark of cancer is the resistance of cancer cells to apoptotic death. In the beginning of the last decade, the apoptotic machinery and the detailed intracellular pathways, as well as, the strategies used by cancer cells to evade apoptosis started to be disclosed [89]. Therefore, the apoptotic activity also is a relevant process to be evaluated when studying the behavior of cancer cells in response to bioactive compounds.

The diminished viability of PNT1A, LNCaP and PC3-treated cells determined by the MTT assays, was accompanied by the altered expression and activity of target regulators of apoptosis, which generally sustained the anti-proliferative effects of plant extracts.

In PNT1A cells, the upregulated expression of pro-apoptotic protein BAX (Figure 13) would be indicative of an increased apoptotic activity in these cells after exposure to *C. monogyna* extracts. However, no significance increase was observed in the activity of caspase-3 (Figure 14), which hampers concluding that *C. monogyna* extracts stimulated apoptosis in PNT1A cells. These results are in agreement with the decreased proliferation. Similarly, a study using a plant polyphenol, Japanese quince fruit, demonstrated that it did not decrease the number of PNT1A cells, and that Bax/Bcl-2 ratio decreased which indicates increased resistance to apoptosis [91]. In case of treatment with *A. Unedo*, the despite the down-regulation of pro-apoptotic protein BAX and of anti-apoptotic Bcl-2 proteins (Figure 15), would be indicative of a decreased apoptotic activity in these cells after exposure to 200 µg/mL of *A. Unedo* extract for 72 h. However, an increase was observed in the activity of caspase-3 (Figure 16). Considering, the caspase-3 is a major indicator of apoptosis modulation by both the intrinsic and extrinsic pathways, this result suggests that the extrinsic pathway of

apoptosis was activated but the intrinsic did not after exposure to 200 µg/mL of *A. Unedo* extract for 72 h in PNT1A cells.

Non alterations were perceived on the expression of apoptosis regulators in LNCaP cells treated with *C. monogyna* extracts. Contrastingly, *A. Unedo* treatment upregulated the expression of the pro-apoptotic protein BAX were as down-regulating the anti-apoptotic Bcl-2 protein (Figure 19). The subsequent increase in the BAX/Bcl-2 ratio would be indicative of an increased apoptotic activity in LNCaP cells after exposure to 200 µg/mL of *A. Unedo* extracts. However, caspase-3 activity was decreased in LNCaP-treated cells (Figure 20), being plausible to believe that apoptosis is being inhibited at some point. Many mechanisms would be involved in this inhibition, namely a family of proteins called inhibitors of apoptosis proteins (IAPs) that, after caspases activation, can bind to them avoiding cleavage [92]. Caspase-3 is activated at the crossroad of both intrinsic and extrinsic pathways of apoptosis, and maybe inhibition of these proteins involved in the extrinsic pathway, also is involved, which cannot be confirmed since this pathway was not evaluated.

In the case of PC3 cells, as occurred in LNCaP, no alteration in apoptosis was observed after exposure to *C. monogyna* extracts. In case of *A. Unedo* extracts treatment, BAX and Bcl-2 (Figure 23) protein expression remained unaltered but caspase-3 activity (Figure 24) was diminished. Thus, the both plant extracts did not demonstrate to have influence in PC3 cells apoptosis induction. These results do not agree with diminished proliferation. This cell line, PC3, has been widely used to study the behavior of prostate cancer cells in what concerns to apoptosis or survival mechanisms, and it was found that the polyphenol compound in plants can suppress cell growth and induce apoptosis in PC3 cells [80] [87]. There is no definitive explanation for these occurrences, maybe it can be because the choice of concentration for study, the 200 µg/mL.

In the last years, the reprogramming of cell metabolism has been recognized as another important hallmark of cancer and thus, an interesting point of intervention for treatment [89] [93]. 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 [89]. Most importantly, cancer cells prioritize the usually anaerobic pathway, which results in the increased glucose uptake and production of very large amounts of lactate without the usual requirement of a hypoxic environment [89] [94]. The subsequent accumulation of lactate in solid tumors is a pivotal and early event in the development of malignancies, and the determination of lactate has been considered entering clinical trials to confirm its relevance in cancer biology [89] [94] [95]. This phenomenon of glycolysis dependency has also been documented in the particular case of PCa, particularly in the metastatic and aggressive phases of disease [39]. Previous findings from our research group have shown that PC3 and LNCaP cells display a distinct glycolytic metabolism comparatively with PNT1A cells, and that androgens are important regulators of this metabolic pathway [39] [44].

So in order to get a more complete picture of the reasons behind the decreased proliferation observed in PNT1A, LNCaP and PC3 cells under plant extracts-stimulation, the glycolytic metabolism was also studied.

PNT1A cells exposed to *C. monogyna* extracts did not presented alterations in glycolytic metabolism, either in glucose consumption (Figure 25A) or lactate production (Figure 25B), which was consistent with the unaltered expression of GLUT1, GLUT3, PFK1 and LDH (Figure 26). However, MCT4 expression was shown to be decreased (Figure 26E), whereas LDH enzymatic activity (figure 27) was considerably augmented in response to *C. monogyna* extracts treatment, which do not, sustains the unaltered lactate production relatively to control. It is not possible to drive a definitive explanation for this fact at the moment, but it could suggest that LDH is not actively producing lactate. LDH is the enzyme that catalyzes the reversible conversion of the final product of glycolysis pyruvate into lactate, and maybe PNT1A cells are using lactate to produce pyruvate, which is then redirected to the mitochondria.

When PNT1A cells were exposed to *Arbutus Unedo* extracts no significant differences were found in GLUT1 protein expression levels whereas GLUT3 expression (Figure 29) was significantly decreased in the presence of *Arbutus Unedo* extracts, which followed the observed diminished glucose consumption (Figure 28A). The LDH enzymatic activity (Figure 30) was considerably diminished in response to *A. Unedo* extracts treatment, which sustains the downregulation in glucose consumption, but did not supports the unaltered lactate production relatively to control (Figure 28B). These results demonstrated a positive effect of *A. Unedo* extracts in PNT1A cells inactivating the glycolytic metabolism.

Regarding the glycolytic metabolism of LNCaP cells, protein expression levels of PFK1 (Figure 32C) were significantly increased in the presence of *C. monogyna* extracts, which followed the observed higher glucose consumption (Figure 31A). However, no significant differences were found in GLUT1 and GLUT3 (Figure 32) expression. Cell glucose intake cell is ensured by specific GLUTs, of which GLUT1 and GLUT3 are the most well-characterized and frequently-studied in prostate cells [96]. 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 [97] [98] [99]. So, it cannot be excluded the possibility that other GLUTs, namely GLUT12, may be involved in the augmented uptake of glucose by LNCaP cells.

PFK-1 is one of the most important enzymes in the glycolytic process catalyzing the limiting step of, the conversion of fructose 6-phospate into fructose 1-6 biphosphate. Thus, increased expression of PFK-1 would signify a cell increased capacity to produce pyruvate. However, in LNCaP-treated cells the pyruvate does not appear to be used for lactate production, since these observations are not in accordance with the results of LDH enzymatic activity and

lactate production. The LDH enzymatic activity (Figure 33) was considerably diminished in response to *C. monogyna* extracts treatment, which sustains the downregulation in lactate production relatively to control (Figure 31B). Analyzing the diminished proliferation alongside with no alteration in apoptosis rates, the increased glucose consumption and decreased lactate production, it leads to believe that in the LNCaP, with administration *C. monogyna* extracts, the aerobic glycolysis occurs. In another words, the results with treatment C. monogyna extracts in LNCaP cells, induce to believe that occurs the conversion of glucose via pyruvate into acetyl-CoA. After this is complete oxidation, through the mitochondrion-localized TCA cycle and the oxidative phosphorylation to $CO_2$ and $H_2O$, likewise in normal cells occurs. Thus, the decrease in LDH enzymatic activity and lactate production together with the diminished proliferation observed in LNCaP-treated cells can be viewed as a positive effect of *C. monogyna* extracts, which raises the curiosity whether these compounds would ameliorate the response to anti-cancer therapies.

*A. Unedo* extracts treatment in LNCaP cells had no significant effect were found in GLUT1 (Figure 35A) protein expression levels whereas GLUT3 and PFK-1 expression (Figure 35) were significantly decreased. Contradictorily increased glucose consumption was observed (Figure 34A) in LNCaP-treated cells. As already discussed, it cannot be excluded the possibility that other GLUTs, namely GLUT12, may be involved in the augmented uptake of glucose by LNCaP cells in response to *A. Unedo* extracts. The LDH protein expression (Figure 35C) and LDH enzymatic activity (Figure 36) did not presented alterations in response to *A. Unedo* extracts treatment, which sustains the unaltered lactate production relatively to control (Figure 34B). The diminished proliferation in LNCaP cells was contradictory with the decrease in apoptosis rates and increase glucose consumption, but this does not lead to increased lactate production.

The results pertaining to the glycolytic metabolism of PC3 cells, showed their distinct behavior comparatively with LNCaP. Administration of *C. monogyna* extracts significantly increased glucose consumption by PC3 cells, despite the unaltered expression levels of GLUTs (both GLUT1 and 3) and PFK1. As already discussed for LNCaP 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 unaltered LDH and MCT4 expression. However, most importantly, the LDH activity was increased in PC3-treated cells, which sustains the higher lactate production relatively to control (Figure 34B). In this context, it should be considered that the 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 [42]. Therefore, administration of *C. monogyna* extracts to PC3 cells, induced the glycolysis anaerobic pathway, the common pathway in cancer cells. In this case, the end-product of glycolysis, pyruvate, is metabolized by LDH-enzyme, which catalyzes the interconversion of pyruvate to lactate, as indicated by the augmented lactate production.

Overall, the results obtained in the present thesis allowed concluding that:

In response to *C. monogyna* and *A. Unedo* extracts exposure, distinct responses were observed in non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) prostate cells. These observations are summarized in Tables 1 and 2.

Globally, these results pointed to an overall decrease in proliferation for all three cell lines after treatment with with *C. monogyna* and *A. Unedo* extracts.

Interestingly, the effects in viability of PCa cells under stimulation with *C. monogyna* extracts were underpinned by positive alterations in the glycolytic metabolism, in the neoplastic cells, LNCaP. Since, LNCaP cells are a mimic initial stage of metastatic PCa, induce to affirm that extract can be used in initial stage of PCa.

In case of *A. Unedo* extracts, the effects on viability of PCa cells were not supported, with non-alterations in the glycolytic metabolism, in neoplastic prostate cells, LNCaP and PC3.

Table 1 - Synthesis of the effects of *C. monogyna* extracts in proliferation, apoptosis and glycolytic metabolism of non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) prostate cells.

| | ***Crataegus monogyna*** | | | | |
|---|---|---|---|---|---|
| | | Apoptose | Glycolytic Metabolism | | |
| | Proliferation | Caspase-3 Act. | LDH Act. | Glucose Cons. | Lactate Exp. |
| PNT1A | ↓ | — | ↑ | — | — |
| LNCaP | ↓ | — | ↓ | ↑ | ↓ |
| PC3 | ↓ | — | ↑ | ↑ | ↑ |

↓ Downregulated ↑ Upregulated — Unaltered

Table 2 - Synthesis of the effects of *A. Unedo* extracts in proliferation, apoptosis and glycolytic metabolism of non-neoplastic (PNT1A) and neoplastic (LNCaP and PC3) prostate cells.

| | ***Arbutus Unedo*** | | | | |
|---|---|---|---|---|---|
| | | Apoptose | Glycolytic Metabolism | | |
| | Proliferation | Caspase-3 Act. | LDH Act. | Glucose Cons. | Lactate Exp. |
| PNT1A | ↓ | ↑ | ↓ | ↓ | — |
| LNCaP | ↓ | — | — | ↑ | — |
| PC3 | ↓ | — | — | — | — |

↓ Downregulated ↑ Upregulated — Unaltered

# Chapter VI – Conclusions and future perspectives

The present dissertation demonstrated that polyphenols-rich *C. monogyna* and *A. Unedo* extracts effectively decreased the proliferation of both neoplastic (LNCaP and PC3) and non-neoplastic (PNT1A) prostate epithelial cells. However, only the activity of *A. unedo* extracts increased the apoptotic rate of PNT1A, which was underpinned by the altered expression of key regulators apoptosis. The apoptotic rate of the neoplastic LNCaP and PC3 cells did not increased in the presence of *C. monogyna* or *A. Unedo* extracts, which suggested that the extracts weren't particularly cytotoxic to unhealthy cells. Although it wasn't possible to identify these precises mechanisms, which caused the number of viable cells to decrease.

Interestingly, *C. monogyna* extracts revealed to be more effective in LNCaP cells. In this cell type, the diminished proliferation was followed by increased apoptotic rates and a heavy down-regulation of the Warburg effect evidenced by the suppression of LDH activity and diminished lactate production, which was accompanied by the altered expression/activity of glycolytic transporters and enzymes.

Curiously, PC3 cells had an overall down-regulation of apoptosis and glycolytic metabolism in the presence of both *C. monogyna* and *A. unedo* extracts, despite the decreased proliferation. However the effect of *C. monogyna* 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, *C. monogyna* extracts maintained the resistance to apoptosis and enhanced the glycolytic metabolism typical of cancer cells. Overall, the effect of *C. monogyna* extracts on these highly aggressive, highly-metastatic PCa cells seemed to be beneficial based on the MTT assays alone, but considering the apoptosis and metabolism results no definitive idea about the benefits could be discerned.

The present findings indicate that polyphenols-rich *C. monogyna* and *A. unedo* extracts may have beneficial effects on prostate cells, both neoplastic and non-neoplastic, diminishing proliferation and controlling the apoptotic and glycolytic pathways. Demonstrated to be cytotoxic to prostate cancer cells with moderate metastatic potential (LNCaP cells) while being less harmful to the healthy epithelial cells, which raises the curiosity whether the consumption of *C. monogyna* and *A. unedo* extracts could prove beneficial to prostate cancer patients.

Before any definitive statement can be made on the benefits of *C. monogyna* and *A. Unedo* extracts regarding their preventive or attenuating effects on the progression of PCa, there are still certain parameters that must be analyzed. It will be crucial to analyze the expression

of other apoptosis-related proteins and other metabolic routes, also known to be enhanced in cancer cells. This includes the analysis of other glucose and lactate transporters to ascertain how the glycolytic processes are altered by *C. monogyna* and *A. Unedo* extracts. It wil be essential to repeat the tests done during this dissertation not only with 200 μg/ml concentration but also with other concentrations and other incubation times for the subsequent analysis of apoptosis and metabolism on all studied cell lines. In order to definitively prove that *C. monogyna* and *A. Unedo* extracts are beneficial for PCa prevention and attenuation, further studies are needed.

Also, further investigation on the effects of fresh leaves of *C. monogyna* and *A. Unedo* extracts in prostate cells should be considered.

# Chapter VII – References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