

Investigation of the Anti-Proliferative and Pro-Apoptotic Effects of Rosemary  
(*Rosmarinus officinalis*) Extract on Androgen Independent Prostate Cancer Cells

Deborah Termini, BSc (Honours)

Submitted in partial fulfillment of the requirements for the degree of:

Master of Science in Applied Health Sciences  
(Health Sciences)

Faculty of Applied Health Sciences

Brock University

St. Catharines, ON.

© Deborah Termini 2022

**Acknowledgments:**

I would like to express my sincerest gratitude to my supervisor, Dr. Litsa Tsiani for the incredible opportunity provided to me the moment she decided to welcome me to her lab with open arms. The excellent guidance I have received from her, paired with a healthy dose of constructive criticism and motherly love, have allowed me to grow as a researcher, and as a person. I will fondly remember all the achievements we have obtained together, from our first published manuscript to my first oral presentation at an international conference. Her unwavering commitment to my success and her incredible patience have been a driving force towards the completion of my program and my thesis.

To my lab colleagues, and friends- Filip, Danja, Eric, and Amanda: it would not have been the same without you. You have given me an incredible amount of emotional support and helped me during the many tumultuous times we have had to face together. I share with you some of the happiest moments of the past two years. Thank you for being the best support group I could ask for.

I would like to extend my thanks to my committee members, Dr. Paul LeBlanc and Dr. Adam MacNeil. Your kindness and incredible knowledge have been invaluable for the successful completion of my program.

Last, but not least, I dedicate part of my success to my family and Jon. Thank you for your continuous support, patience, and excitement. And thank you, mom and dad, for continuously motivating me and shaping me into the person I am today.

## **Abstract:**

Prostatic carcinoma is established as the third most prevalent cancer type in the worldwide population and accounts for 21% of new cancer cases in Canadian men. Prostate cancer can be categorized as androgen dependent or androgen independent, indicative of the tumor's ability to respond to testosterone stimulation. Currently available treatments include prostatectomy, radiation therapy, androgen deprivation therapy, immunotherapy, and chemotherapy. Despite all of these treatment options, biochemical recurrence, and progression into more advanced stages (castration-resistant prostate cancer- CRPC) is often seen, indicating a need for novel therapeutics that specifically and efficiently target the dysregulated mechanisms in prostate cancer. In some studies, rosemary extract and its polyphenolic constituents have been shown to have anticancer properties, but the exact effects and mechanisms of action are not known. The purpose of the present study was to investigate the potential pro-apoptotic and anti-proliferative effects of rosemary (*Rosmarinus officinalis*) extract (RE) on prostate cancer cells. PC-3 and 22Rv1 prostate cancer cells, representative *in vitro* models of androgen independent prostate cancer, as well as the PNT1A non-cancerous prostate epithelial cells were treated with RE and docetaxel (established prostate cancer chemotherapeutic drug) for the purpose of assessing the extent of survival and proliferation, and to investigate changes in expression of key proteins involved in apoptotic and survival signalling cascades. In our studies, RE inhibited the proliferation (IC<sub>50</sub>: 26 µg/mL; 70 µg/mL) and colony formation efficiency (IC<sub>50</sub>: 2.8 µg/mL; 4.8 µg/mL) of PC-3 and 22Rv1 prostate cancer cells, respectively, and enhanced cell death by stimulating apoptosis as shown by the increased levels of cleaved caspases 9, 7, 3, and PARP. Enhanced phosphorylation of ERK 1/2, paired with a notable increase in reactive oxygen species (ROS) were also observed in RE- treated PC-3 cells. In contrast, RE had no effect on the proliferation and survival of PNT1A normal epithelial cells, suggesting

an action of RE promoting inhibition of prostate cancer cells while sparing non-cancerous epithelial cells.

## Table of Contents:

<b>1. Introduction:</b> .....	1
<b>1.1 Cancer</b> .....	1
<b>1.2 The hallmarks of cancer</b> .....	1
<b>1.2.1 Two enabling traits: genome instability and tumor-enabled inflammation</b> ....	2
<b>1.2.2 Over-stimulation of growth:</b> .....	3
<b>1.2.3 Evasion of programmed cell death:</b> .....	4
<b>1.2.4 Down-regulation of inhibitory pathways:</b> .....	5
<b>1.2.5 Stimulation of angiogenesis:</b> .....	6
<b>1.2.6 Indefinite multiplicative capability:</b> .....	7
<b>1.2.7 Metastatic activity:</b> .....	8
<b>1.3 Oncogenes and Tumor Suppressor Genes:</b> .....	10
<b>1.3.2 Oncogenes:</b> .....	10
<b>Ras protein:</b> .....	10
<b>1.3.1 Tumor suppressor genes:</b> .....	11
<b>Tumor Protein 53:</b> .....	11
<b>PTEN Tumor Suppressor:</b> .....	13
<b>1.4 Signaling pathways involved in cancer progression:</b> .....	14
<b>1.4.1 PI3K/Akt/mTOR pathway:</b> .....	14
<b>1.4.2 Ras/MAPK pathway:</b> .....	16
<b>1.4.3 TNFR signaling and NF-kB:</b> .....	18
<b>1.4.4 Intrinsic and extrinsic apoptotic pathways:</b> .....	20
<b>1.5 Prostate characteristics and prostate cancer:</b> .....	23
<b>1.5.1 General characteristics and epidemiology of prostate cancer</b> .....	23
<b>1.5.2 Prostate cancer and the androgen receptor:</b> .....	25
<b>1.5.3 Prostate cancer treatments:</b> .....	27
<b>1.6 Phytochemicals in cancer prevention:</b> .....	29
<b>1.6.1 Rosmarinus officinalis:</b> .....	30
<b>1.6.2 In vitro studies of the effects of RE in prostate cancer cell lines:</b> .....	31
<b>1.6.3 In vivo studies of the effects of RE on prostate cancer:</b> .....	39
<b>2. Rationale, objectives, and hypothesis:</b> .....	41

2.1	<i>Rationale and Aims:</i> .....	41
2.2	<i>Hypotheses:</i> .....	42
3.	<b>Methodology:</b> .....	43
3.1	<b>Materials:</b> .....	47
3.2	<b>Cell culture:</b> .....	48
3.3	<b>Rosemary extract preparation:</b> .....	48
3.4	<b>Crystal violet assay:</b> .....	49
3.5	<b>Clonogenic survival assay:</b> .....	49
3.6	<b>Preparation of whole cell lysates for western blotting:</b> .....	50
3.7	<b>Protein assay:</b> .....	50
3.8	<b>Western blot:</b> .....	51
3.9	<b>Assessment of cell morphology</b> .....	51
3.10	<b>MTT assay:</b> .....	52
3.11	<b>CellRox Green Assay:</b> .....	52
3.12	<b>Statistical analysis:</b> .....	53
4.	<b>Results:</b> .....	53
4.1	<b>Rosemary extract (RE) inhibits proliferation of PC-3 androgen independent prostate cancer cells</b> .....	53
4.2	<b>Rosemary extract (RE) inhibits colony formation efficiency of PC-3 prostate cancer cells</b> .....	56
4.3	<b>Rosemary extract (RE) promotes apoptosis in PC-3 cells</b> .....	59
4.4	<b>Rosemary extract (RE) activates ERK 1/2 and inhibits Akt in PC-3 cells</b> .....	62
4.5	<b>Rosemary extract (RE) treatment increases the levels of reactive oxygen species (ROS) in PC-3 cells</b> .....	64
4.6	<b>Rosemary extract (RE) inhibits proliferation and colony formation efficiency of 22Rv1 androgen independent prostate cancer cells</b> .....	65
4.7	<b>Rosemary extract (RE) promotes apoptosis in 22Rv1 cells</b> .....	67
4.8	<b>Rosemary extract (RE) inhibits ERK 1/2 phosphorylation in 22Rv1 cells</b> .....	71
4.9	<b>Rosemary extract (RE) does not inhibit the proliferation of PNT1A healthy prostate epithelial cells</b> .....	72
4.10	<b>Rosemary extract (RE) does not affect ERK and Akt in noncancerous PNT1A epithelial cell lines</b> .....	74
5.	<b>Discussion:</b> .....	77

<b>5.1 Rosemary extract (RE) inhibits cell proliferation and colony formation efficiency in PC-3 and 22Rv1 androgen independent prostate cancer cells.....</b>	<b>77</b>
<b>5.2 Rosemary extract (RE) effects on prostate cancer signaling: apoptosis .....</b>	<b>81</b>
<b>5.3 Rosemary extract (RE) effects on prostate cancer signaling: PI3K/Akt and Ras/MAPK pathways, and oxidative stress.....</b>	<b>83</b>
<b>6. Conclusions:.....</b>	<b>89</b>
<b>8. Limitations and Future Directions: .....</b>	<b>89</b>
<b>9. References:.....</b>	<b>92</b>
<b>Appendix:.....</b>	<b>131</b>

**List of Figures:**

<b>Figure 1: The hallmarks and enabling characteristics of cancer</b> .....	2
<b>Figure 2: The PI3k/Akt, ras/MAPK, and NF-kB pathways, and the intrinsic and extrinsic apoptotic pathways</b> .....	15
<b>Figure 3: Regulation of apoptosis by the Bcl-2 family of proteins</b> .....	21
<b>Figure 4: Androgen Receptor Domains</b> .....	26
<b>Figure 5: Major available treatments for prostate cancer</b> .....	27
<b>Figure 6: Chemical structure of carnosic acid and carnosol</b> .....	31
<b>Figure 7: Rosemary extract (RE) inhibits PC-3 prostate cancer cell proliferation</b> .....	54
<b>Figure 8: Rosemary extract (RE) and docetaxel (DTX) inhibit PC-3 prostate cancer cell proliferation</b> .....	55
<b>Figure 9: Rosemary extract (RE) inhibits PC-3 cell viability as shown by MTT assay data</b> .....	56
<b>Figure 10: Rosemary extract (RE) inhibits PC-3 cell colony formation efficiency</b> .....	57
<b>Figure 11: Effects of rosemary extract (RE) and docetaxel (DTX) on PC-3 cell morphology</b> .....	58
<b>Figure 12: Effects of Rosemary extract (RE) on Caspases 3, 7, 8, and 9 in PC-3 cells</b> .....	60
<b>Figure 13: Effects of Rosemary extract (RE) on PARP in PC-3 cells</b> .....	61
<b>Figure 14: Effects of Rosemary extract (RE) on Erk 1/2 and Akt in PC-3 cells</b> .....	63

<b>Figure 15: Rosemary extract (RE) treatment increased intracellular ROS levels in PC-3 cells.....</b>	<b>64</b>
<b>Figure 16: Rosemary extract (RE) inhibits 22Rv1 prostate cancer cell proliferation.....</b>	<b>65</b>
<b>Figure 17: Rosemary extract (RE) inhibits 22Rv1 cell colony formation efficiency... ..</b>	<b>66</b>
<b>Figure 18: Effects of rosemary extract (RE) on 22Rv1 cell morphology.....</b>	<b>67</b>
<b>Figure 19: Effects of Rosemary extract (RE) on Caspases 3, 7, 8, and 9 in 22Rv1 cells.....</b>	<b>69</b>
<b>Figure 20: Effects of Rosemary extract (RE) on PARP in 22Rv1 cells.....</b>	<b>70</b>
<b>Figure 21: Effects of Rosemary extract (RE) on ERK 1/2 in 22Rv1 cells.....</b>	<b>71</b>
<b>Figure 22: Rosemary extract (RE) does not inhibit PNT1A prostate cancer cell proliferation.....</b>	<b>72</b>
<b>Figure 23: Effects of rosemary extract (RE) on PNT1A cell morphology.....</b>	<b>73</b>
<b>Figure 24: Effects of Rosemary extract (RE) on Akt, Erk 1/2, and PARP in non-cancerous PNT1A prostate epithelial cell lines.....</b>	<b>75</b>
<b>Figure 25: Summary of the effects of Rosemary extract (RE) in A) PC-3 and B) 22Rv1 prostate cancer cells.....</b>	<b>87</b>

**List of Tables:**

<b>Table 1.1: Current in vitro studies on the effects of RE on prostate cancer cells.....</b>	<b>33</b>
<b>Table 1.2: Current in vitro studies on the Effects of RE polyphenols on prostate cancer cells.....</b>	<b>37</b>
<b>Table 2: Current in vivo studies on the effects of RE or its polyphenolic components on prostate cancer.....</b>	<b>40</b>
<b>Table 3: Prostate cancer cell lines and their respective characteristics .....</b>	<b>45</b>
<b>Table 4: Summary of the effects of rosemary extract (RE) in prostate cancer cells and PNT1A healthy epithelial cells.....</b>	<b>76</b>

## **1. Introduction:**

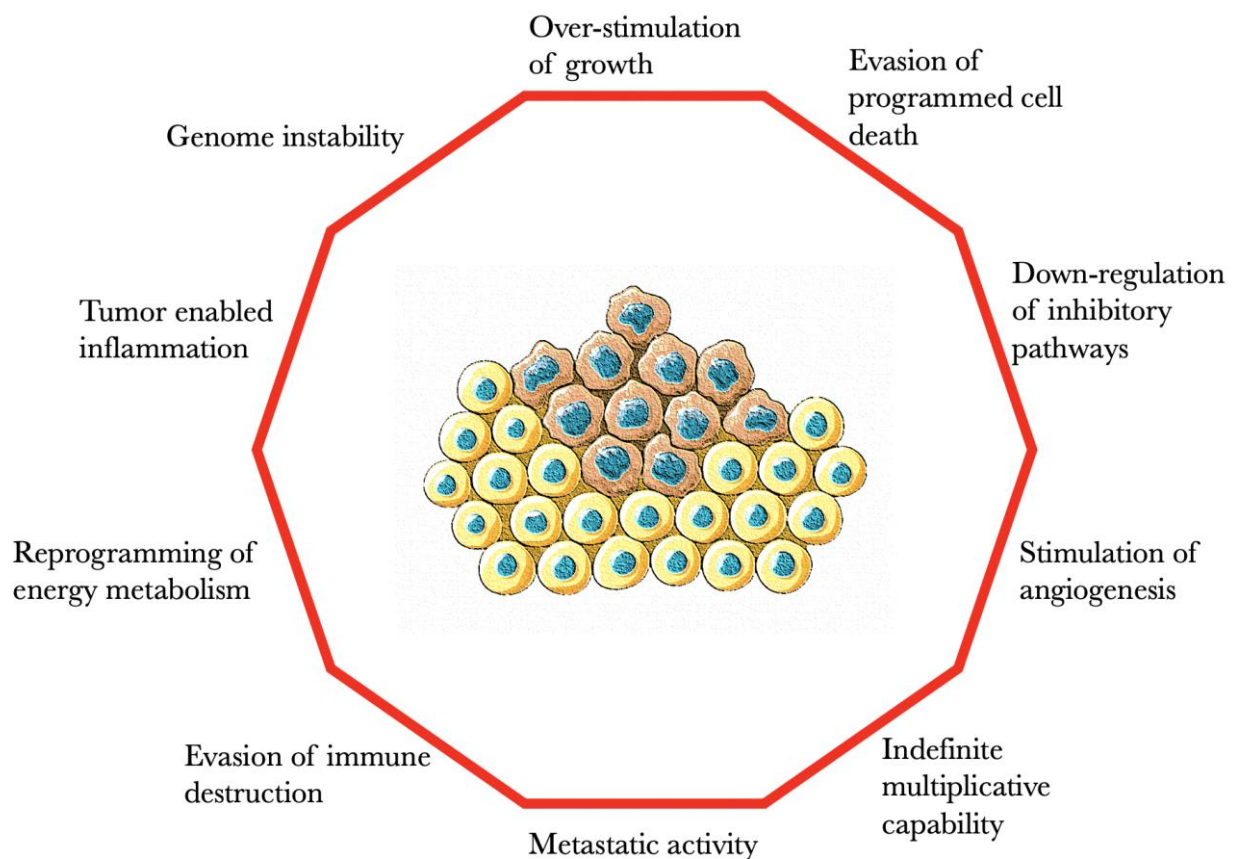
### **1.1 Cancer**

Cancer is the second leading cause of death across the globe, with an estimated 17 million new cases, as well as 9.5 million deaths worldwide in 2018 alone (Bray et al., 2018). Worldwide, about 1 in 6 deaths is due to cancer, while in Canada, 1 in 2 Canadians is expected to develop cancer at some point in their lifetime (Brenner et al., 2020), with a national incidence of 206,200 new cases and 80,800 deaths in 2017 (Canadian Cancer Society's Advisory Committee on Cancer Statistics, 2017). Cancer is characterized by the accumulation of mutations in the DNA contained within the nucleus of a somatic cell, ultimately altering normal metabolic and cell signaling pathways in cells of different body tissues, ranging from epithelial tissues to connective tissues to bone marrow (Bertram, 2000; Wishart, 2015). DNA mutations occur either spontaneously as a result of DNA replication, or due to environmental factors. Once a proliferative cell possesses a mutated gene, it will subsequently create more cells carrying the mutation as a result of cell division, promoting the formation of a tumor (Griffiths et al., 2000). Cancer cells have the peculiarity of possessing abnormally enhanced proliferation rates, as well as a substantial decrease in their rate of programmed cell death; termed apoptosis. Enhanced proliferation and evasion of apoptosis are independent events which become equally necessary for cancer progression, and one or both may occur in cancer development (Evan et al., 2001).

### **1.2 The hallmarks of cancer**

Although the characteristics of cancer cell development and growth are numerous, complex, and varying in specific processes among different types of carcinomas, they could be summarized by a few important hallmarks. These hallmarks of cancer, according to Hanahan and Weinberg, include over-stimulation of growth, evasion of apoptosis, down-

regulation of tumor suppressors/ inhibitory pathways aiming to slow down growth, stimulation of angiogenesis, indefinite multiplicative capability, invasion, and metastasis (Hanahan & Weinberg, 2000). Two emerging hallmarks: reprogramming of energy metabolism and evasion of immune destruction, as well as two enabling traits: genome instability, and tumor-enabled inflammation have later been identified and are still being established (Figure 1) (Fouad & Aanei, 2017; Hanahan & Weinberg, 2011).



**Figure 1: The hallmarks and enabling characteristics of cancer**

### **1.2.1 Two enabling traits: genome instability and tumor-enabled inflammation**

The genomic alteration of neoplastic cells plays a major role in the acquisition of the established cancer hallmarks. Mutant genotypes are transferable from the original cell developing a mutation to cell subclones upon DNA replication and cell division, enabling the exponential growth and dominance of mutant cells (Hanahan & Weinberg, 2011).

Heritable phenotypes may be acquired from other processes as well, including DNA methylation and histone modifications, dysregulating gene expression (Esteller, 2007). In order to achieve the mutations necessary for tumor progression, cancer cells may increase their mutational rate by becoming increasingly sensitive to mutagenic agents through disruption of the DNA-maintenance machinery, or by interfering with the “surveillance system” (including tumor suppressor genes) responsible for maintaining DNA integrity by favouring cell senescence and death (Jackson & Bartek, 2009; Kastan, 2008). Genomic instability may be further promoted by the loss of telomeric DNA rendering the affected cells susceptible to chromosomal mutations (Artandi & DePinho, 2010).

Tumor cells are infiltrated by cells belonging to both innate and adaptive immune systems (Pagès et al., 2010). This mimics an inflammatory microenvironment thought to promote antitumoral responses in the attempt of eliminating and limiting cancer progression. Paradoxically, inflammation may enhance tumorigenesis and acquisition of other cancer hallmarks by providing growth and survival factors, proangiogenic factors, and enzymes to facilitate tumor invasion, and promoting EMT signaling (DeNardo et al., 2010; Grivennikov et al., 2010). Immune cells may also release reactive oxygen species, which promote the development of mutations through oxidative stress, accelerating the tumorigenic process (Grivennikov et al., 2010).

### ***1.2.2 Over-stimulation of growth:***

One of the most common traits in cancer development is the ability to promote and sustain proliferation rates above homeostatic conditions. Growth factors are normally released in a tightly regulated manner from cells into their extracellular environment affecting themselves and neighbouring cells by triggering a variety of processes promoting maintenance of tissue structure and function (Hanahan & Weinberg, 2011). These processes

are carried out by growth factor binding to membrane-spanning receptors on the surface of cells. These receptors possess an intracellular tyrosine kinase domain, which upon activation leads to initiation of a branched signalling cascade ultimately leading to regulation of cell metabolism, proliferation, and survival (Witsch et al., 2010). Cancer cells overproduce growth factor ligands, which binding to cognate receptors in an autocrine way and can trigger signalling cascades (Cheng et al., 2008), as well as the ability to send signals within tumor stroma to normal cells which, in turn, provide growth factors to cancer cells (Bhowmick et al., 2004). Another mechanism involves promoting enhanced expression of receptor proteins within the plasma membrane, leading to increased sensitivity to ligands. The same results can be obtained by morphologically altering the structure of the receptor in order to render it more responsive to growth factor binding (Lindsey & Langhans, 2016). The problem with overstimulation of proliferation resides in eventual cell senescence. It is hypothesized that an increase in intensity of proliferative signalling is only relative to the extent of activation of antiproliferative defense leading to senescence (Collado & Serrano, 2010); Alternatively, cancer cell adaptation to elevated oncogenic signalling could lead to inactivation of pathways promoting senescence and apoptosis (Sever & Brugge, 2015).

### ***1.2.3 Evasion of programmed cell death:***

Programmed cell death/apoptotic pathways are normally activated for the purpose of replacing old or damaged cells without the accumulation of tissue. These apoptotic pathways are driven by the action of tumor suppressor genes, such as the TP53 and retinoblastoma (RB) proteins (Elmore, 2007). In tumorigenesis, these pathways must be circumvented in addition to increasing the proliferative pathways (McGill & Fisher, 1997). The retinoblastoma-associated protein (RB), which primarily receives growth-inhibitory signals originating from outside of the cell, and then decides whether that particular cell should be

undergoing proliferative pathways, is often defective in cancer cells, thus allowing the progression of uncontrolled cell cycle (Burkhart & Sage, 2008). TP53, on the other hand, receives intracellular stress and cellular/genetic damage signals; levels of damage in a cell's genome beyond what can be repaired, as well as low levels of glucose and oxygen, trigger the action of TP53, which can either deter progression in the cell cycle until homeostasis is re-established, or promote apoptosis altogether (Biegging, 2014). TP53 and RB can operate within a larger, repetitive functional network, which aims to limit proliferative abnormality when loss of function in certain tumor suppressor genes is present (Rivlin et al., 2011; Yamasaki, 2003).

#### ***1.2.4 Down-regulation of inhibitory pathways:***

The inhibitory and apoptotic pathways in normal epithelial cells consist of upstream and downstream effectors. These regulators are normally divided into extrinsic and intrinsic apoptotic programs (Adams & Cory, 2007). The extrinsic program receives extracellular apoptosis-inducing signals and processes them, whereas the intrinsic program senses and integrates intracellular signals. Both pathways ultimately lead to the activation of the proteases caspase 8 and 9, which actuate apoptosis by initiating a signalling cascade and activating effector caspases through proteolytic cleavage, leading to gradual degradation and phagocytosis through specialized cells (Hanahan & Weinberg, 2011). Signals that are carried out between regulators and effectors are modulated by a family of pro and anti-apoptotic regulatory proteins, including Bcl-2. Bcl-2 is an apoptosis inhibitor, and it acts by binding to the pro-apoptotic proteins Bax and Bak and suppressing their activity (Hata et al., 2015). When they are not inhibited, Bax and Bak promote the disintegration of the outer mitochondrial membrane and the subsequent release of other pro-apoptotic signalling proteins, including cytochrome c. After its release, in the mid phase of apoptosis,

cytochrome c activates more caspases involved in cellular changes through proteolysis (Figure 3) (Cai & Jones, 1998).

To avoid programmed cell death, cancer cells develop many strategies, and amongst these the most common is TP53 gene mutation, which allows removal of critical damage sensors inducing apoptosis. Additionally, tumors may overexpress anti-apoptotic Bcl-2 proteins by decreasing the expression of pro-apoptotic proteins, such as Bax and Bim (Junttila & Evan, 2009; Lowe et al., 2004).

### ***1.2.5 Stimulation of angiogenesis:***

Tumor cells must obtain a continuous supply of nutrients and oxygen, as well as expel carbon dioxide and other waste, in order to grow and metastasize (Cooper, 2000). Angiogenesis represents the process in which new blood vasculature is generated, and although it is only activated in specific tasks including wound healing and female reproduction cycles in adults, in tumorigenesis it is induced in the early stages of cancer development in a highly dysregulated manner, causing uninterrupted sprouting of new vessels from already existing vasculature (Raica et al., 2009). Some of the factors that regulate angiogenesis are signalling proteins that interact with vascular endothelial cells by binding at the inhibitory or stimulatory receptors. Amongst these, we can find the fibroblast growth factors (FGF), as well as vascular endothelial growth factors (VEGF-A), which initiate angiogenic signalling by binding to the vascular endothelial growth factor tyrosine kinase receptors 1-3 (Carmeliet, 2005). The expression of the VEGF gene can be upregulated by either oncogene signaling or hypoxia, but the proteins could also be moved to the extracellular matrix in inactivated forms until further activation of matrix-degrading proteolytic enzymes (Kessenbrock et al., 2010). On the other hand, proteins like thrombospondin-1 (TSP-1) have the opposite effect on this process and normally act to

suppress angiogenic stimuli by also binding to transmembrane receptors (Kazerounian et al., 2008). The new vasculature that originates from angiogenic processes in tumors is highly abnormal; the vessels sprout precociously and are excessively branched, distorted and much more enlarged than normal, causing leaks and minor hemorrhages (Nagy et al., 2010).

#### ***1.2.6 Indefinite multiplicative capability:***

It has been established that in order for a tumor to grow, a transformation providing cell immortality is required (Hanahan & Weinberg, 2011). Immortalization provides a cell with the capacity for unlimited cell division cycles while avoiding senescence. When cells are left to grow in a culture and are allowed to propagate, they will eventually reach a stage of senescence, or biological aging, causing a high percentage of death. Cells that escape senescence or cell death will have potential for unlimited cell division. Cell lines created in a laboratory are a great example of immortal cells, as they are able to proliferate without showing signs of senescence (Shay & Wright, 2005). One of the reasons behind immortalization is elevated telomerase activity in comparison to non-immortalized cells (Blasco, 2005). Telomerase is a DNA polymerase that allows insertion of nucleotide tandem repeats (telomere) at the end of a DNA strand, and it serves as protection against erosion in subsequent DNA replication processes, favouring maintenance of a physically intact genome and subsequent inhibition of cell apoptosis due to damage. Telomeres shorten in size as cell division occurs, until they are no longer present, thus triggering cell senescence (Chan et al., 2004). Other inducers of cell senescence include intracellular ROS, DNA replication stress, or activation of oncogenes. Suppression of apoptosis and senescence are two crucially important processes in the development of tumorigenic cells, and they are attributed to abnormal increase in telomerase expression, leading to the creation of sufficiently long telomeres (Gaspar et al., 2018; Hanahan & Weinberg, 2011).

### ***1.2.7 Metastatic activity:***

A metastatic tumor arises due to the ability of cancer cells to spread from the tissue of origin and invade other tissues. One of the processes that enables cancer cells to alter their attachment to the extracellular matrix is the loss of E-cadherin, which is a cell-to-cell adhesion molecule that normally functions to form adherens junctions between cells and thus drives formation of epithelial cell sheets (Berx & Van Roy, 2009). Furthermore, genes coding for other cell-to-cell adhesion molecules are altered in invasive carcinomas, whereas adhesion molecules associated with cell migration are upregulated (e.g. N-cadherin)(Cavallaro & Christofori, 2004). Overall, the metastatic cascade involves several biological steps, beginning with invasion of local tissues, continuing with cancer cell invasion of blood and lymphatic vessels, and culminating in the formation of small nodules from cells escaping these vessels in more distant tissues, which will grow forming visible, substantial tumors (Fidler, 2003).

### ***1.2.8 Reprogramming of energy metabolism:***

The Warburg effect is a process in which cancer cells, under aerobic conditions, have a tendency of reprogramming and diverting their metabolism from complete oxidative phosphorylation to glycolysis, a process normally favoured for energy production under anaerobic conditions (Hanahan & Weinberg, 2011). To account for the lower ATP yield, cancer cells may upregulate the expression of glucose transporters (including GLUT1), thus improving glucose transport within a cell's cytoplasm (Jones & Thompson, 2009). Hypoxic tumor microenvironments, paired with the mutation and overactivation of oncoproteins such as Ras, contribute to the enhanced expression of hypoxia inducible factors (HIF1 $\alpha$  and HIF2 $\alpha$ ), which in turn act to upregulate the glycolytic pathway (Kroemer & Pouyssegur,

2008; Semenza, 2010). Increased rates of glycolysis are implicated in the increased formation and diversion of glycolytic intermediates to other cellular pathways, facilitating the formation of nucleosides and amino acids leading to enhanced proliferation (Vander Heiden et al., 2009).

### ***1.2.9 Evasion of immune destruction:***

The immune system may play a role in hindering the formation and progression of cancer, although the mechanism involved is still largely unknown (Hanahan & Weinberg, 2011). Studies have shown that immunocompromised mice had a higher chance of tumor development and growth when compared to the immunocompetent group. Mice deficient in CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs), CD4<sup>+</sup> Th1 helper T cells, and natural killer (NK) cells were more susceptible to cancer development, indicating the participation of both innate and adaptive immune systems in the suppression of cancer (Kim et al., 2007; Teng et al., 2008). Further data indicates that tumors originating in immunodeficient mice could not form secondary tumors in immunocompetent mice following transplantation, whereas tumors originating in immunocompetent mice were able to grow in either immunosuppressed or immunocompetent mice (Kim et al., 2007; Teng et al., 2008). The hypothesis behind this phenomenon is attributed to immunoediting, or the elimination of immunogenic cancer cells in immunocompetent host, with a few weakly immunogenic cancer cells surviving and forming a tumor, whereas immunogenic tumors derived from immunosuppressed mice may not proliferate within an immunocompetent environment as they would be eliminated (Hanahan & Weinberg, 2011). Ultimately, it was reported that ovarian and colon cancer patients with higher levels of CTL and NK cells had a better prognosis than patients with lower levels (Nelson, 2008; Pagès et al., 2010). On the other hand, there is a possibility that highly immunogenic cancer cells may evade immune

responses by paralyzing CTL and NK cells through the secretion of TGF- $\beta$  or other factors, or through the enhanced recruitment of immunosuppressive cells including regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (Yang et al., 2010; Mougiakakos et al., 2010; Ostrand-Rosenberg & Sinha, 2009).

### **1.3 Oncogenes and Tumor Suppressor Genes:**

Mutations in two specific groups of genes namely the oncogenes and tumor suppressor genes, within the DNA are what largely causes disruption of homeostasis. Oncogenes, mutated form of a proto-oncogenes, code for proteins that working in an abnormal way promote cancer advancement (Zhu et al., 2015). Tumor suppressor genes, also known as anti-oncogenes, code for proteins that modulate normal cell behaviour and thus protect them from an eventual transformation into a cancer cell.

#### **1.3.2 Oncogenes:**

Proto-oncogenes code for proteins that normally carry out basic functions in a cell and are specifically correlated to cell division regulation. Once a mutation is introduced in a proto-oncogene, there is a great chance that cellular machinery for DNA repair will solve the issue. The few instances in which cells can escape DNA repair could lead to the formation of an oncogene, potentially leading to the development of a metastatic tumor (Griffiths et al., 2000). Mutations in proto-oncogenes coding for key proteins associated with cellular mechanisms modulating proliferation and apoptosis, allow for a disruption in normal cell homeostasis and subsequent aberrant behaviour as a result, typical of cancer progression (Pierotti et al., 2003).

#### **Ras protein:**

The superfamily of Ras proto-oncogenes encodes GTP-binding proteins involved in the modulation of several, important processes in a cell, including, but not limited to, cell proliferation, differentiation, and protein trafficking (Adjei, 2001). It directly participates in the Ras/MAPK signalling pathway, which promotes downstream activation of transcription factors activating genes coding for proliferation and growth of cells. The Ras proteins that promote tumorigenesis are H-Ras, K-Ras and N-Ras (Barbacid, 1987). In the cell membrane, Ras cycles between GTP (guanosine triphosphate) and GDP (guanosine diphosphate) binding, thus allowing for activation of signalling cascades (Liebmann, 2001). The mutation of the Ras proto-oncogene into its oncogenic form leads to a steep decrease in Ras interaction with the GTPase activator, a process that normally promotes GTP hydrolysis into GDP (Avruch, 2001). As a consequence, after initiation of cascade, instead of reverting back to the inactive form bound to GDP, Ras will remain in its active GTP-bound conformation, which promotes signalling to downstream effectors even when growth factor stimulation is absent, leading to increased proliferation (Molina & Adjei, 2006).

### ***1.3.1 Tumor suppressor genes:***

The inactivation of tumor suppressor genes is one of the two necessary alterations in cells, which are required for tumor formation. Tumor suppressor genes generally act to inhibit unnecessary or altered cell proliferation leading to the development of a tumor. When they are mutated or lost in the replicative process, the negative regulation of cell growth is suppressed, thus favouring cancer progression (Cooper, 2000).

### **Tumor Protein 53:**

p53 is one of the best-studied tumor suppressor genes, and the most common target of genetic mutations in malignant tumors (~ 50% of cancers possess it) including, but not limited to, carcinomas, leukemias, and brain tumors. p53 mutations have the potential of being transmitted from a parent to their offspring, causing a hereditary cancer syndrome, in which genetic mutations that are inherited predispose an affected individual to the development different types of cancer (Cooper, 2000; Muller & Vousden, 2014).

p53 is a nuclear transcription factor whose active structure is composed of a homotetramer and is encoded by the TP53 gene (Ozaki & Nakagawara, 2011). It possesses three functional domains, including a DNA-binding domain (residues 102 through 292), an acidic transactivation domain (NH<sub>2</sub>-terminal, residues 1 through 45), and a carboxyl terminal oligomerization domain (residues 319 through 359) (Ozaki & Nakagawara, 2011). It is normally expressed in very low levels mainly due to proteasomal degradation triggered by the RING-finger type E3 ubiquitin protein ligase MDM2 (Kubbutat et al., 1997). But as DNA damage occurs, post-translational modifications such as phosphorylation and acetylation allow p53 to convert from latent to active form and to accumulate in the nucleus (Sionov & Haupt, 1999). Nuclear p53 binds to the p53 response element, composed of a tandem repeat, upstream of the promoter region of the gene of interest (El-Deiry et al. 1992). By transactivation of genes through this process, p53 directly allows for cell cycle arrest or initiation of apoptosis depending on the degree of DNA damage that has occurred, hindering the transmission of defective genome to daughter cells and their subsequent accumulation in tissues (Ozaki & Nakagawara, 2011). In cancer, the residues in p53 mostly targeted for mutation are Arg-175, Gly-245, Arg-248, Arg-249, Arg-273 and Arg-282 (Joerger & Fersht, 2007), found in the DNA binding domain of the protein. These mutations impair proper interaction with the p53 response element, thus the transcription of genes coding for pro-apoptotic proteins including those involved in cell cycle arrest, such as p21 and Bax

(Selvakumaran et al., 1994; Ozaki & Nakagawara, 2011) is hindered. p53 induces apoptosis in undifferentiated cancer cells by promoting the transcriptional activation of the proapoptotic proteins PUMA and NOXA, which signal downstream activation of intrinsic apoptosis (Figure 2), mediated by the mitochondrial release of cytochrome C (Aubrey et al., 2018). In prostate cancer, p53 mutations are estimated to occur at a higher frequency in CRPC (~50-70%), though primary tumors and androgen responsive tumors may also present high mutation rates (~6-37%) (Teroerde et al., 2021).

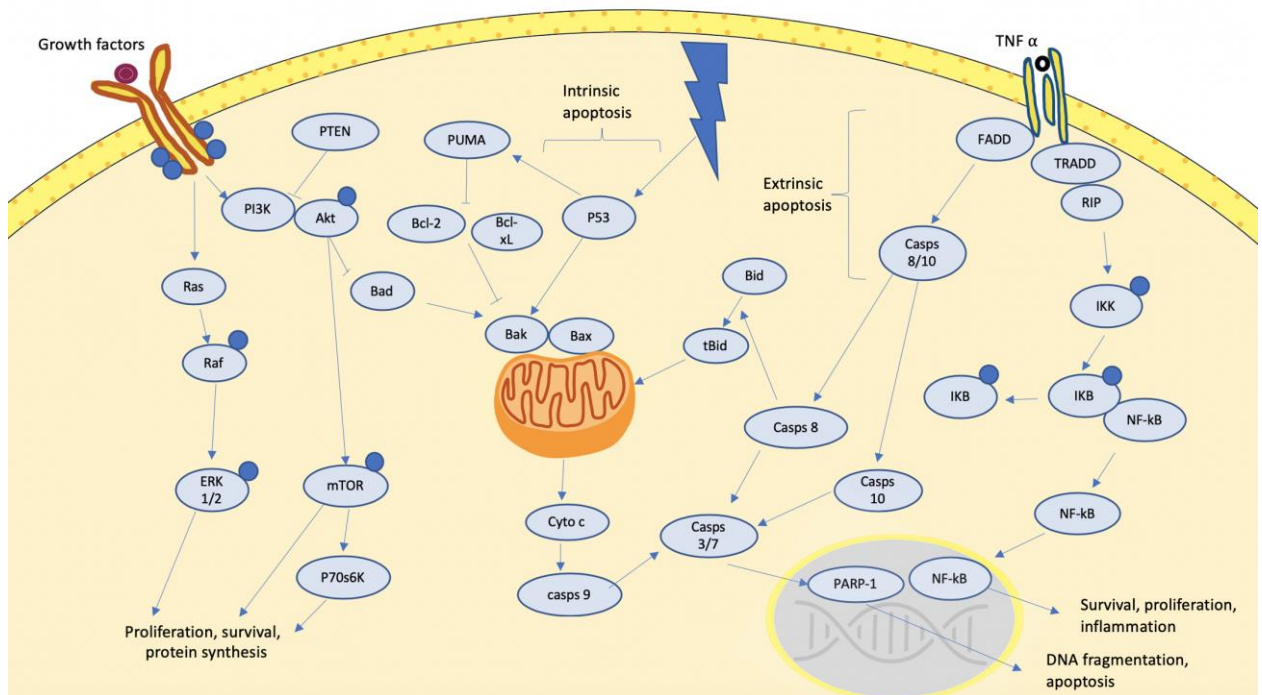
### **PTEN Tumor Suppressor:**

PTEN (phosphatase and tensin homologue), another tumor suppressor gene, acts as a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase. It contains a tensin-like domain as well as a catalytic (phosphatase) domain, which dephosphorylates phosphoinositide substrates. It negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and functions as a tumor suppressor by negatively regulating the Akt/PKB signaling pathway (Jiang & Liu, 2009). PTEN, in addition, is able to hinder cellular motility through a different type of mechanism that does not seem to involve phosphatase action (Leslie et al., 2005). It is another targeted molecule for genetic alteration, which normally involves the complete deletion of its gene on chromosome 10 (Leslie & Downes, 2004), or loss of function through promoter methylation (Yuan & Cantley, 2008), although smaller mutations inhibiting its action are also possible but are relatively rare (Leslie & Downes, 2004). Non-functional PTEN cannot act as a phosphatase to reduce the 3-phosphoinositide levels thus resulting in activation of Akt/PKB and amplified survival and proliferative signalling which is essential for cancer progression (Yuan & Cantley, 2008). PTEN mutations have been identified in about 20% of all primary prostate tumors as well as 50% in castration-resistant tumors (Jamaspishvili et al., 2018).

## **1.4 Signaling pathways involved in cancer progression:**

### **1.4.1 *PI3K/Akt/mTOR pathway:***

The PI3k/Akt /mTOR pathway is a signaling cascade involved in the regulation of cell cycle, normal cell proliferation, and survival. This signaling cascade is activated in response to growth factors binding to their tyrosine kinase receptors (Martini et al., 2014) as well as other ligands including hormones and extracellular matrix components (Nicholson & Anderson, 2002). Activation of this pathway leads to downstream activation of phosphoinositide-3-kinase (PI3K), ultimately resulting in the phosphorylation/activation of Akt, a serine/threonine protein kinase responsible for the activation of the mammalian target of rapamycin (mTOR), a serine/threonine kinase that functions as a regulator of cell motility, cell survival and proliferation, protein synthesis, and autophagy (Figure 2) (Lipton & Sahin, 2014).



**Figure 2: The PI3k/Akt, Ras/MAPK, and NF-kB pathways, and the intrinsic and extrinsic apoptotic pathways.**

There are three classes of PI3K, class I being the most commonly involved one in cancer disease. Class I is subsequently divided into IA and IB, making it a heterodimeric protein (Kaplan et al.,1987). This in turn is comprised of a p110 catalytic subunit and a p85 regulatory subunit (Kaplan et al., 1987). PI3K is able to carry out signal transduction initiated from receptor tyrosine kinases (RTK) or G protein coupled receptors (GPCR), as well as Ras (Zhao & Vogt, 2008). Following stimulation by growth factors and other ligands, the regulatory p85 subunit of PI3K interacts through its Src-homology 2 (SH2) domains, with the phosphorylated tyrosine residues of the intracellular section of the receptor allowing activation of the p110 catalytic subunit. The activated p110 catalytic subunit subsequently interacts with phospholipids in the membrane to transform 3-phosphoinositides into phosphatidylinositol-3-phosphate by phosphorylating its 3' hydroxy group (Zhao & Vogt,

2008). PIP3 is an important second messenger as it represents the mediator of PI3K activity (Martini et al., 2014). PIP3 activation leads to the activation of protein dependent kinase (PDK1), which targets and phosphorylates Akt following its translocation in the inner side of the plasma membrane through the pleckstrin homology domain (Wick et al., 2000). Akt phosphorylation/activation leads to the downstream activation of the complex 1 of mammalian target of rapamycin (mTORC1). mTORC1 activation leads to increased protein synthesis, as well as cell proliferation and survival (Nave et al., 1999). On the other hand, activation of mTORC2 by PI3K fully activates Akt by phosphorylating its Ser473 residue, triggering other phosphorylation events including inhibition of FOXO, a family of pro-apoptotic proteins (Martini et al., 2014), as well as Bad (which activates Bax inducing cytochrome c release from the mitochondria), Raf and procaspase-9 (Chang et al., 2003). The PI3K/Akt signalling is downregulated by the action of PTEN, a tumor suppressor gene, which acts by removing a phosphate group from PIP3 preventing the downstream effect of PI3K on Akt phosphorylation and activation (Figure 2) (Jiang & Liu, 2009).

It has been reported that amplifications in the PI3KCA gene, responsible for the expression of the catalytic subunit (p110) of PI3K, can be found in prostate cancer and contribute to invasiveness and poor prognosis in patients (Pearson et al., 2018). Higher levels of p-Akt, paired with PTEN inactivation, have also been identified in prostate cancer (Shukla et al., 2007), and it has been reported that the levels of mTOR and phosphorylated mTOR are twice as great in prostate cancer tissue when compared to normal prostate epithelium (Kremer et al., 2006). Furthermore, over-activation of the PI3K/Akt/mTOR pathway has been correlated to prostate cancer resistance to docetaxel treatment (Qian et al., 2010).

#### **1.4.2 Ras/MAPK pathway:**

The primary function of the Ras/MAPK pathway is to transduce signals from extracellular environments to the cell's nucleus to activate specific genes responsible for proliferation and differentiation of cells, as well as cell cycle modulation, tissue repair, angiogenesis, and more (Molina & Adjei, 2006). The pathway is activated when growth factors such as epidermal growth factor or other signalling molecules bind to plasma membrane tyrosine kinase receptors (Molina & Adjei, 2006). Following that, oligomerization of the receptor occurs, favouring combination of both the catalytic and cytosolic domains in a way that allows activation of kinase activity for transphosphorylation and subsequently phosphorylation of downstream targets (Figure 2). The sequence homology 2 domains (SH2) in growth factor receptor binding protein 2 (Grb2) recognize the phosphorylated tyrosine residues in the intracellular domain of the receptor. Grb2 is associated, via the SH3 domain, with the guanine nucleotide exchange factor sons-of-sevenless (SOS). Therefore, the binding of Grb2 to tyrosine phosphorylated receptor brings/recruits SOS to the inner side of the plasma membrane and in the proximity of Ras (Schlessinger, 2000). SOS interacts with Ras to favour a conformational change exchanging GDP bound in inactivated Ras to GTP, activating it. Following this, the Raf molecule, a serine/threonine kinase, is recruited to the lipid bilayer by binding to the switch I domain in Ras (Marais et al., 1995). Raf phosphorylates and activates mitogen-activated protein kinase kinases (MEK1, MEK2), initiating a signalling cascade consisting of their subsequent phosphorylation of extracellular signal regulated kinases (ERK 1/2 1 and ERK 1/2 2) (Figure 2), which in turn activate through phosphorylation a variety of downstream kinases and transcription factors, such as Ets-1, Bcl-2, p90RSK1, and MNK1/2, responsible for modulation of cell proliferation, angiogenesis, and more (Molina & Adjei, 2006). In cancer cells, the Ras/MAPK pathway is often dysregulated, mainly due to mutations in the Ras proto-oncogene, most likely to occur in codons 12 and 61 (Mascaux et al., 2006; Dergham

et al., 1997). Studies have demonstrated that a variety of other factors, including oxidative stress and DNA damage, may contribute to the downstream phosphorylation and activation of Erk 1/2 (Rezatabar et al., 2019).

### ***1.4.3 TNFR signaling and NF- $\kappa$ B:***

Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) is a proinflammatory cytokine belonging to the TNF superfamily and is secreted by inflammatory cells (Chu, 2013). Upon binding TNF receptor (TNFR) of target cells, it plays an important role in the regulation of cell survival, proliferation, apoptosis, and differentiation (Hayden & Ghosh, 2014). It has been established that TNF $\alpha$  signaling is of pleiotropic nature, as it may either activate caspase 8, leading to initiation of apoptosis, or NF- $\kappa$ B, leading to cell survival (Oberst & Green, 2011)(Figure 2). When the ligand initially binds to TNFR, receptor-interacting serine/threonine-protein kinase 1 (RIPK1) is recruited to complex I of the TNFR, composed of TNFR, the adaptor protein TNFR-associated death domain (TRADD), the receptor-interacting serine/threonine-protein kinase 1 (RIP1), and TNF receptor-associated factor 2 (TRAF2), favouring a rapid initial activation of NF- $\kappa$ B, which in turn upregulates the expression of the caspase 8 inhibitor FLIP (FLICE-inhibitory protein) (Micheau et al., 2001; Micheau & Tschopp, 2003). Subsequently, TRADD and RIP1 from complex I associate with Fas associated death domain (FADD) protein and caspase 8 forming complex II within the cytoplasm (Micheau & Tschopp, 2003). RIPK1 ubiquitination favours its recruitment to this complex, and its subsequent recruitment of either a caspase 8 and FLIP heterodimer, or a caspase 8 homodimer predicts how the pathway will progress (Oberst & Green, 2011). If FLIP is present due to its upregulation by NF- $\kappa$ B, the signaling cascade will promote cell survival due to caspase downregulation, whereas low or no FLIP levels will promote the formation of a caspase 8 homodimer, leading to interdomain cleavage, cleavage of

executioner caspases and Bid, and apoptosis (Oberst & Green, 2011; Oberst et al., 2010) (Figure 2).

NF- $\kappa$ B comprises a family of 5 transcription factors which bind to promoter regions of specific genes to favour a variety of processes, including inflammatory and immunological responses, cell growth, and other cellular activities (Xia et al., 2014). Its activation depends on different sources of upstream regulators including growth factors, viral and bacterial products, ionizing radiation, DNA damage, and oncogenic stress. Upon ligand binding to the TNFR, the inhibitor of I $\kappa$ B kinase (IKK) is activated (Xia et al., 2014). IKK phosphorylates I $\kappa$ B causing its disassociation from NF- $\kappa$ B and marking it for proteasomal degradation. Once NF- $\kappa$ B is free from I $\kappa$ B, it dimerizes and translocates to the nucleus, subsequently binding to promoter regions to regulate transcription (Figure 2) (Liu et al., 2012; Li & Verma, 2002). In cancer, NF- $\kappa$ B may favour the activation of anti-apoptotic proteins including the caspase 8 inhibitor FLIP, and the inhibitors of apoptosis c-IAP1/2 and XIAP (Xia et al., 2014). NF- $\kappa$ B activation is involved in additional tumorigenic processes including the regulation of epithelial-to-mesenchymal transition (EMT), tumor metabolism, angiogenesis, and metastasis (Kawauchi et al., 2008; Li et al., 2012). Mutations within the IKK family of proteins have been reported in prostate cancers, leading to the over-activation of NF- $\kappa$ B (Greenman et al., 2007; Boehm et al., 2007). Dysregulation of the NF- $\kappa$ B pathway has been associated with the development and progression of numerous other cancers including leukemia, glioblastoma, colorectal, and breast cancers (Wang & Lin, 2009; Xia et al., 2014). In addition, mutations in the KRAS proto-oncogene paired with p53 deficiency may contribute to cancer progression through NF- $\kappa$ B overactivation (Meylan et al., 2009). NF- $\kappa$ B activity is reportedly higher in androgen-independent prostate cancer cells as opposed to androgen-dependent, and in highly metastatic cancers as opposed to localized (Chen & Sawyers, 2002; Ismail et al., 2004). p53, often mutated in cancer, is involved in the

NF- $\kappa$ B modulation of tumor metabolism. It was shown that p53-mediated regulation of cytochrome-c oxidase by NF- $\kappa$ B is abolished in p53 null tumors, and that in such instance NF- $\kappa$ B enhances the Warburg Effect while preventing oxidative phosphorylation (Mauro et al., 2011).

#### ***1.4.4 Intrinsic and extrinsic apoptotic pathways:***

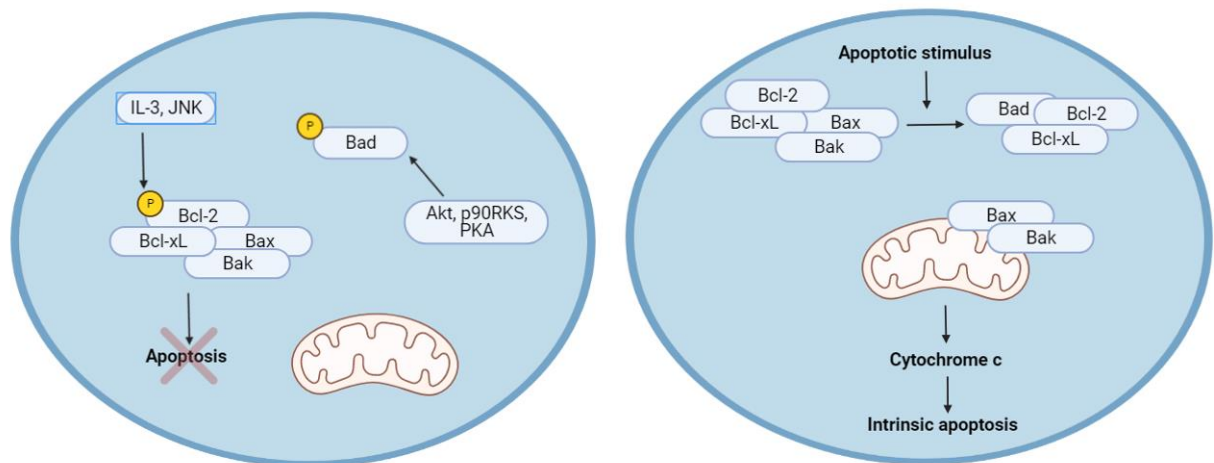
Apoptosis, or programmed cell death, is a cellular mechanism that exists to balance other signaling pathways involved in cell proliferation or as a response to a variety of physiological or pathological conditions which ultimately result in targeted cell destruction (Wong, 2011). Apoptosis can be initiated by an intrinsic or an extrinsic pathway (Figure 2). The extrinsic pathway is triggered through ligand binding to a death receptor, including the tumor necrosis factor receptor-1 (TNFR1) and Fas (CD95) (Hengartner, 2001). Upon binding of TNF and FasL, respectively, the intracellular domain recruits adaptor proteins (e.g. TRADD and FADD) forming the ligand-receptor-adaptor protein complex known as DISC (death-inducing signaling complex) which ultimately recruits and activates pro-caspase 8, an initiator caspase. Activated caspase 8 downstream activates executioner caspases including caspase 3 (O'Brien et al., 2008; Karp, 2008).

The intrinsic pathway, on the other hand, is initiated following internal stress in the form of hypoxia, oxidative damage, DNA damage, and more, leading to increased mitochondrial permeability and the release of cytochrome c in the cytoplasmic environment (Karp, 2008). This process is modulated by the BCL2 family of proteins, including the pro-apoptotic proteins Bax, Bim, Bad, and Bak, and the anti-apoptotic proteins Bcl-2 and Bcl-xL (Tsujimoto et al., 1984).

Bcl-2 and Bcl-xL tightly regulate apoptosis by binding to proteins such as Bax and Bak thus preventing the release of cytochrome c from mitochondria (Figure 3) (Dluogsz et

al., 2006). The phosphorylation of Bcl-2 at the S70 residue by interleukin-3 and JNK has also been associated with increased anti-apoptotic activity though the exact mechanism of action remains unclear. Several reports observed that S70 phosphorylation of Bcl-2 may protect against drug-induced apoptosis and prevent oxidative-stress induced DNA damage (Chong et al., 2020; Correia, 2015; Deng et al., 2001).

Bad, in its unphosphorylated form, binds to both Bcl-2 and Bcl-xL to prevent their association with Bax (Elmore, 2007)(Figure 3). Unbound Bax, as well as Bak, act as proapoptotic proteins by binding to the mitochondrial outer membrane and permeabilizing it, favouring the release of cytochrome c in the cytosol. When Bad is phosphorylated at various residues (S112, S136) by signaling molecules such as Akt, p90RSK, and PKA, its association with Bcl-2 and Bcl-xL is disrupted, and Bcl-2 and Bcl-xL remain bound to Bax preventing it from binding to the mitochondria to promote apoptosis (Figure 3) (Elmore, 2007).



**Figure 3: Regulation of apoptosis by the Bcl-2 family of proteins. Image created with biorender.com**

The formation of an apoptosome, which includes cytochrome c, Apaf-1 and caspase-9, activates the downstream executioner caspase-3. Other mitochondrial proteins that are

released by the mitochondria, including SMAC and DIABLO, induce apoptosis by binding to apoptosis inhibitors (IAP) and preventing their interaction with caspases 3 and 9 (Kroemer et al., 2007). The intrinsic and extrinsic apoptotic pathways eventually merge to the execution phase of apoptosis, in which a series of caspases are activated through proteolytic cleavage, resulting in two or more cleaved products. Caspases have been recognized as major contributors to apoptosis. Each caspase is activated through proteolytic processing by upstream caspases that yields cleaved proteins of lower molecular weight.

Caspase-9 is a protein of 47 MW kDa that when activated is cleaved yielding proteins of 35 and 37 kDa. Caspase-8 is a protein of 57 MW kDa that when activated is cleaved yielding proteins of 18 and 43 kDa. Caspase-7 is a protein of 35 MW kDa that is activated through proteolytic processing by upstream caspases that yields cleaved proteins of 20 and 30kDa. Caspase-3 is a protein of 35 MW kDa that is activated through proteolytic processing by upstream caspases that yields cleaved proteins of 17 and 19 kDa.

Full length as well as cleaved products of caspase can be selectively recognized using specific antibodies. Caspase 9 and 8 activate caspase 3 and 7 (Figure 2). Caspase 7 and caspase 3, in turn, cleave the inhibitor of caspase-activated deoxyribonuclease (ICAD) effectively initiating nuclear apoptosis. Caspases activate other targets to favour apoptosis through inhibition of cell cycle, DNA repair proteins, and certain signaling cascades (Ghobrial et al., 2005). Poly (ADP-ribose) polymerase (PARP), a nuclear protein which is normally involved in DNA repair and maintenance, is cleaved and degraded by caspase 3, favouring irreparable damage and cell death (Herceg & Wang, 2001).

In cancer, evasion of programmed cell death may occur through dysregulations of the balance between anti-apoptotic and pro-apoptotic proteins within the family of Bcl-2 proteins, in which either anti-apoptotic proteins are upregulated or pro-apoptotic proteins

are downregulated, leading to enhanced survival (Wong, 2011). It has been reported that overexpression in the Bcl-2 protein leads to inhibition of apoptosis in prostate and breast cancers, as well as neuroblastomas and glioblastomas (Raffo et al., 1995; Fulda et al., 2002) whereas Bax mutations, more frequent in colon cancer, may lead to increased resistance to cancer treatments (Miquel et al., 2005). Other mechanisms which contribute to decreased apoptotic processes include mutations within the p53 tumor suppressor (Bai & Zhu, 2006; Vikhanskaya et al., 2007), the downregulation of caspases, including caspase 9 and 3 (Shen et al., 2010; Devarajan et al., 2002), as well as defects within the death signaling pathway which initiates the extrinsic apoptotic pathway, including lower membrane expression of death receptors (TNFR1, Fas) and loss of receptor function (Fulda, 2010; Reesink-Peters et al., 2005).

## **1.5 Prostate characteristics and prostate cancer:**

### ***1.5.1 General characteristics and epidemiology of prostate cancer***

The prostate is an exocrine gland surrounding the male urethra, whose function is to secrete an alkaline fluid constituting 30% of the overall volume of male semen (Huggins et al., 1942). The alkaline fluid allows degradation of sperm coagulum in the vagina as well as survivability in its acidic environments, in addition to contributing to better sperm motility (Suarez & Pacey, 2006). The male prostate is of the size of a walnut generally weighing no more than 16 grams and majorly composed of luminal and basal epithelial cells surrounded by stroma (Aaron et al., 2016). Whereas luminal cells are columnar and highly express androgen receptors (AR), the basal epithelium possess low AR levels, and is further characterized by the presence of neuroendocrine cells, secreting both neuropeptides and growth factors necessary for growth of the luminal epithelium (Knudsen & Vasioukhin,

2010). The peripheral zone of the prostate, surrounding the urethra's distal section, accounts for the area in which 70-80% of prostate cancers originate (Oh et al., 2003).

Prostatic neoplasia, according to recently collected statistical data, is the second most diagnosed cancer in males and the sixth cause of death from cancer worldwide (Jemal et al, 2011). It has been observed that global mortality from prostate cancer seems to have increased over the past two decades; around 156,000 deaths were confirmed in 1990 as opposed to 256,000 in 2010 (Lozano et al., 2012). In 2018, the mortality rate further increased, with 1,276,106 new cases and 358,989 deaths (Bray et al., 2018). The incidence rate greatly varies across different countries, being greater across Europe, Australia, New Zealand and North America and more uncommon in East and South Asia (National Collaborating Centre for Cancer, 2014). It is also more prevalent amongst African Americans than Asian and white men (Powell, 2007). In Canada, it has been established that 1 in 7 males will develop prostate cancer, with risk increasing in individuals of 65 years of age and older (Fradet et al., 2009).

As male individuals get older, benign hyperplasia, although not a precursor to prostate cancer, is extremely more likely to happen, and detectable in ~80% of men older than 80 years old. On the other hand, localized adenocarcinoma (high grade prostate intraepithelial neoplasia) does progress to prostate cancer, characterized by dysplasia of the luminal epithelial cells as well as an overall decrease in number of basal cells (Schrecengost & Knudsen, 2014).

It has been recognized that aberrant androgen receptor expression, as well as impaired activity of certain tumor suppressor genes and activation of proto-oncogenes play a significant role in prostate cancer initiation and metastatic activity (Oh et al., 2003). Specifically, the inactivation of p53, p27 and PTEN tumor suppressor genes seems to greatly

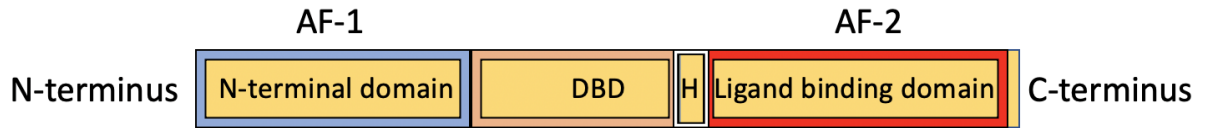
contribute to the development of prostatic tumors (Osman et al., 1999; Cordon-cardo et al., 1998; Whang et al., 1998).

The use of prostate cancer cell lines has contributed significantly to better understanding of prostate cancer at the molecular and cellular level. The human prostate cancer cell lines available and commonly used in *in vitro* studies are PC-3, 22Rv1, DU-145 (androgen independent) and LNCaP (androgen dependent). Furthermore, injection of human prostate cancer cells to animals and establishment of prostate xenografts provides knowledge of the behaviour of prostate cancer *in vivo*.

### ***1.5.2 Prostate cancer and the androgen receptor:***

Androgens play a major role in the development and progression of prostate cancer, as they regulate processes involved in proliferation, differentiation, angiogenesis, metastasis, and apoptosis (Imamoto et al., 2008). The biosynthesis of testosterone is driven by the hypothalamic-pituitary-gonadal axis (Kluth et al, 2014), in which gonadotropin-releasing hormone (GnRH), periodically secreted by the hypothalamus, is released into the hypophyseal portal circulation to activate G-protein coupled receptors of the anterior pituitary gonadotropic cells (Orlowsky & Sarao, 2020). This controls their production and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Once LH reaches the testes, it promotes testosterone synthesis, and once testosterone concentrations rise, they in turn inhibit further release of GnRH and LH in a negative feedback loop (Kluth et al., 2014). Testosterone is produced in the Leydig cells of the testes, and while majority of it binds to sex hormone binding globulin while in the serum, the remaining is converted to dihydrotestosterone (DHT) by 5-alpha-reductase in the prostate cells (Fujita & Nonomura, 2019). Testosterone and DHT are the primary ligands of the androgen receptor (AR). The AR is a steroid receptor and transcription factor composed of four main domains:

the N-terminal domain, DNA-binding domain, hinge region, and ligand-binding domain (Davey & Grossmann, 2016) (Figure 4).



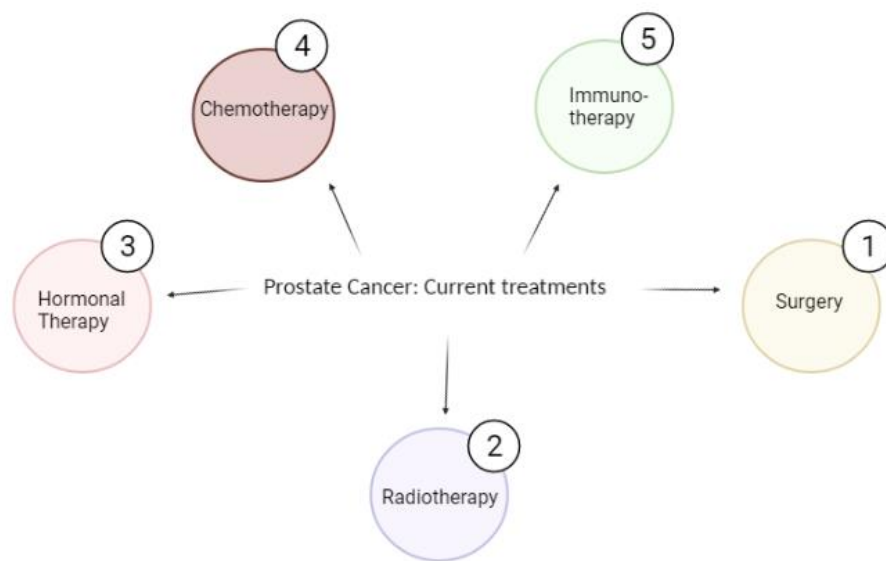
**Figure 4: Androgen receptor domains**

Androgens bind to the ligand-binding domain of the AR, thus favouring its activation and translocation to the nucleus, where it dimerizes and acts as a transcription factor for the regulation of many proteins involved in growth, PSA secretion, cell cycle regulation, as well as processes involved in the maintenance of the reproductive, musculoskeletal, and immune systems amongst others (Davey & Grossman, 2016; Eder et al., 200; Peterziel et al., 1999; Takayama & Inoue, 2013). The androgen/AR complex presents other functions that are “non-canonical” and independent of DNA binding, including the rapid activation of ERK 1/2, Akt, and MAPK.

AR activation is indispensable for the initiation and progression of early-stage prostate cancer as it stimulates growth and lipid metabolism, and its effects may persist in CRPC as the receptor adapts to the extremely low levels of androgens as a result of mutations within the AR gene (Fujita & Nonomura, 2019; Huggins & Hodges, 2002; Shafi et al., 2013). Up to 62.7% of all CRPC present AR mutations (Robinson et al., 2015), which include but are not limited to, point mutations and amplification, leading to either loss of specificity towards androgens or AR overexpression to promote cell survival in low levels of androgen (Fujita & Nonomura, 2019). Splicing variants of the AR that are functionally active without the need for androgen binding may be expressed in some prostate cancer subtypes (Bryce &

Antonarakis, 2016; Fujita & Nonomura, 2019). Furthermore, *in vitro* studies on prostate cancer cells have shown that phosphorylation of Akt (often enhanced in prostate cancer) promotes the phosphorylation of the AR at Ser210 and Ser790, suppressing p21 and inhibiting apoptosis (Lin et al., 2001).

### 1.5.3 Prostate cancer treatments:



**Figure 5: Major available treatments for prostate cancer. Image created with biorender.com**

Current treatments available for prostate cancer are surgery, radiation therapy, chemotherapy, and hormonal therapy (Figure 5). Surgery is most often performed in high-risk cases with advanced *in situ* carcinoma, and although the reduction in metastatic progression as well as localized tumor progression is remarkably reduced, it is associated with a relatively low reduction in mortality risk after 10 years (Bill-Axelsson et al., 2005; Lawrentschuk et al., 2010). Another method for high-risk localized tumors is radiotherapy, and specifically external-beam radiotherapy and brachytherapy (Hayden et al., 2010). Low-dose brachytherapy methods generally involve the long-term insertion of radioactive seeds

with a half-life of approximately 60 days, whereas high-dose rate brachytherapy is carried put through exposure to higher doses of radiation in a relatively shorter time span (Law & McLaren, 2010). The utilization of external-beam radiotherapy and high dose rate brachytherapy is recommended for patients with greater life expectancy without metastatic progression to distant tissues (Law & McLaren, 2010; Pinkawa, 2010)

Hormonal therapy typically involves dihydrotestosterone deprivation, as androgens occupy an important position in prostate tumor advancement (Huggins & Hodges, 2002). AR's mechanism of action represents one of the processes which promote initial prostate cancer formation through development of surrogate pathways, which ultimately allow amplification of AR signalling without the need for androgen stimulation (Ramsay & Leung, 2009). For this reason, androgen deprivation is regarded as one of the most aggressive and successful initial treatments for malign prostate cancer (Perlmutter & Lepor, 2007). Androgen deprivation therapy can also be used in combination with surgery and radiation therapy, and it is often performed in palliative care patients as well (Chen & Zhao, 2013). In order to achieve androgen deprivation, medications like luteinizing hormone releasing hormone, estrogen, gonadotropin releasing hormone agonists, AR blockers, and other inhibitors, are used (Johnson et al., 2010). Some controversy is attached with androgen deprivation therapy as it seems to be associated with adverse events including, but not limited to, erectile dysfunction, hot flashes, anemia, and depression (Perlmutter & Lepor, 2007).

Chemotherapy is sometimes not effective against prostate cancer progression, although it has been shown to provide successful results in patients with hormone-refractive prostate cancer (hormone resistant) (Picard et al., 2012). Common drugs for chemotherapeutic treatment are docetaxel, paclitaxel, mitoxantrone and doxorubicin which are often used in combination to produce greater effects (Tannock et al., 2004).

Immunotherapy for prostate cancer, though highly debated, showed modest efficacy in past years, recently re-emerging as a viable treatment option in CRPC (Cha et al., 2020; Khalili et al., 2019). Due to its ability to exert significant chronic inflammatory responses, prostate cancer has been an ideal candidate for immunotherapy. This generally involves either active or passive approaches. Passive immunotherapy relies on the delivery of pro-inflammatory cytokines and monoclonal antibodies. Examples in prostate cancer treatment include chimeric antigen receptor (CAR) T-cell therapy and radiolabelled monoclonal antibodies targeting the prostate specific membrane antigen (PSMA) and the prostate stem cell antigen (PSCA) due to their relatively high rate of expression in tumorigenic prostate tissue. In active immunotherapy, the patient's own immune response is stimulated, leading to the recruitment of immune cells, natural killer cells, and the eventual production of tumor-specific antibodies. This is done to promote adaptive immune response and memory T cell formation against cancer-specific antigens (Cha et al., 2020).

## **1.6 Phytochemicals in cancer prevention:**

Today, it is estimated that around 25% of modern medicines are directly or indirectly derived from plants (Samuelsson, 2004). It is well-established that phytochemicals, non-nutritive compounds produced by plants to promote their survival and interaction with the environment, exert protective roles as antioxidants, radical scavengers, and against certain microorganisms such as bacteria and viruses (Kennedy & Wightman., 2011). Phytochemicals have also been utilized in cancer therapy, for example taxanes such as docetaxel and paclitaxel, derived from the European (*Taxus baccata*) and Pacific yew (*Taxus brevifolia*), are two of the most utilized chemotherapeutics in prostate cancer treatment. Taxanes act by hindering mitosis through microtubule binding, leading to inhibition of

depolymerization and formation of mitotic spindles (Abal et al., 2003; Lyseng-Williamson & Fenton, 2005).

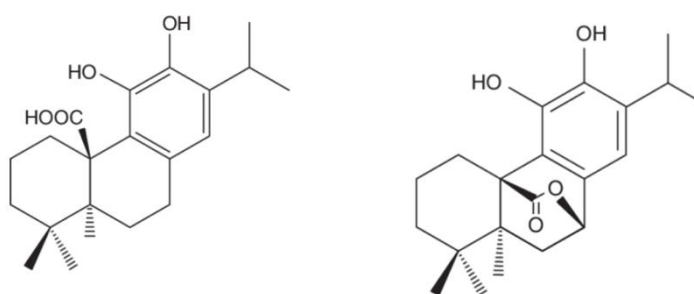
A continuous increase in cancer cases worldwide, paired with excessive toxicity and the development of resistance of available chemotherapeutics indicate a need for alternative strategies to prevent cancer. The remarkable potential of plants, and their medicinal properties, have driven extensive research in the attempt to identify natural compounds which may exert anti-cancer effects.

### **1.6.1 *Rosmarinus officinalis*:**

*Rosmarinus officinalis*, known as rosemary, is a plant native to the Mediterranean region and has been traditionally utilized across the world in the preparation of foods, fragrances, as well as medicinal compounds. Rosemary extract (RE) has been approved recently by the European Union as a natural antioxidant sold in health food stores and as an additive to increase the shelf life of certain perishable foods (Habtemariam, 2016).

Rosemary has been shown to have antioxidant, antibacterial, anti-inflammatory, anti-diabetic, and anti-carcinogenic properties (Habtemariam, 2016; Moore et al., 2016; Naimi et al., 2017). These properties have been attributed to the chemical components within rosemary, ranging from substances such as camphene and borneol in essential oils which possess antioxidant and antimicrobial properties (Estévez et al., 2007; Bozin et al., 2007), to phenolic acid derivatives such as rosmarinic acid, to polyphenolic diterpenes, which represent the compounds receiving the highest attention within the past few years in terms of newly developed therapeutics (Habtemariam, 2016). The more abundant polyphenolic diterpenes in rosemary are carnosic acid and carnosol (Figure 6), accounting for 90% of the antioxidant effects of rosemary (Santos-Gomes et al., 2003). Other polyphenolic diterpenes present in much smaller quantities, include rosmanol, epirosmanol, isorosnamol, and

rosmadial (Habtemariam, 2016). Carnosic acid levels in rosemary are influenced by environmental and genetic factors and represent approximately ~2% of total weight of dried rosemary leaves (Wenkert et al., 1965). Carnosic acid it is not a relatively stable compound and following extraction it may oxidize to carnosol or rosmanol via free radicals or enzymatic dehydrogenation (Schwarz & Ternes, 1992).



**Figure 6: Chemical structure of carnosic acid and carnosol (Makris & Boscou, 2014)**

*Rosmarinus officinalis* has been shown to possess chemotherapeutic effects in different cancer cell lines, including lung, colorectal, and breast (Moore et al., 2016). Its benefits may be attributable to its polyphenolic compounds carnosol, carnosic acid and rosmarinic acid. Despite some evidence pointing to anticancer properties of rosemary, the available literature is limited, indicating the need for more research to help elucidating the cellular mechanisms of action of RE and RE polyphenols (REP) in prostate cancer. Below is a summary of *in vitro* cell culture, and *in vivo* animal studies on the effects of RE and REP on prostate cancer. The studies are presented in chronological order, older to more recent ones (Table 1).

### **1.6.2 *In vitro* studies of the effects of RE in prostate cancer cell lines:**

The effects of RE on prostate cancer have been investigated in a limited number of *in vitro* studies. The cell lines used, the concentration and time of exposure to RE, and the findings of these studies are summarized in (Table 1.1) and presented below to give the reader an update of the existing literature up to now. Rosemary infusion (0.2-1  $\mu\text{g}/\mu\text{L}$  which contained 3.3  $\mu\text{g}/\text{mL}$  of rosmarinic acid) reduced PC-3 cell growth after 24, 48, and 72h treatments, while inhibiting the expression of the IL-8 protein in TNF $\alpha$ -stimulated cells. Contrarily, the expression of the NF- $\kappa$ B p60 subunit was not significantly altered when compared to the control untreated cells (Kaliora et al., 2014). RE (0-250  $\mu\text{g}/\text{mL}$ ) derived from different Moroccan crops containing carnosol (11.7- 17.3%) and carnosic acid (1.09- 3%) inhibited the androgen receptor positive LNCaP cell proliferation in a dose-response manner with IC50 values ranging from 14.15  $\mu\text{g}/\text{mL}$  to 15.04  $\mu\text{g}/\text{mL}$  (Bourhia et al., 2019). Treatment of LNCaP and 22Rv1 cells with RE (standardized to 43% carnosic acid) led to an increase in expression of the protein stress response, as shown by increased expression of endoplasmic reticulum stress proteins PERK 1/2, IRE1 $\alpha$ , XBP-1, BiP, and CHOP, whereas ER stress seemed to be downregulated in normal prostate epithelial cells treated with RE (Petiwala et al., 2014). Additional results showed an overall inhibition of cell viability and proliferation, paired with increased rates of apoptosis confirmed by the increased expression of Bax and cleaved caspase-3 and -4 (Petiwala et al., 2014). Furthermore, an inhibition of prostate specific antigen (PSA) and androgen receptor expression (which is normally indicative of prostate cancer progression in men) was observed in LNCaP cells, which seemed to be correlated to the modulation of CHOP to favour the activation of the ER stress response, thus leading to AR degradation (Petiwala et al., 2014).

Table 1.1: Current *in vitro* studies on the effects of RE on prostate cancer cells.

Authors	Cell Lines	Experiment	Findings
Yesil-Celiktas et al. (2010)	PC-3, DU-145	6.25-50 µg/mL RE for 48h	↓ Cell viability
Kaliora et al. (2014)	PC-3	0.2-1 µg/µL rosemary herbal infusion for 24-72h	↓ Cell growth ↓ IL-8
Bourhia et al. (2019)	LNCaP	0-250 µg/mL RE from different rosemary crops for 72h	↓ Cell proliferation
Petiwala et al. (2014)	22Rv1, LNCaP	Rosemary extract standardized to 43% carnosic acid (CA) for 24h at concentrations of 0 and 50 µg/mL to assess cell proliferation extent, and for 48 hours at 0-70 µg/mL to assess cell viability.  Protein expression was investigated by treating cells at 0-50 µg/mL for 24h.	↓ Cell viability ↓ Cell proliferation ↑ Apoptosis ↑ Bax ↑ Cleaved caspase-3 ↓ AR ↑ PSA ↑ PERK 1/2 ↑ IRE1α ↑ BiP ↑ CHOP ↑ XBP-1 mRNA splicing ↑ Caspase-4

In addition to the effects of RE, a number of studies have examined the effects of the RE polyphenols carnosol, carnosic acid and rosmarinic acid on prostate cancer cells (Table 1.2). A study published by Johnson et al. in 2008 assessed the effects of carnosol in PC-3 cells. A dose- and time- dependent inhibition in cell viability compared to the control was observed following treatment with carnosol. Additionally, cell cycle arrest at the G2 phase occurred, paired with a downregulation in the PI3K/Akt pathway proteins (PI3K, p-Akt, p-mTOR), p-p70S6K, and an increased activation of AMPK, leading to an overall decrease in m-TOR phosphorylation. P21, an inhibitor of cell cycle progression, was remarkably upregulated in carnosol-treated cells, inhibiting the activity of cyclin A, D1, and D2, as well as CDK 2 and 6. The expression of the anti-apoptotic protein Bcl-2 was suppressed, while the pro apoptotic proteins Bax and Bad, and caspases 8 and 9, were upregulated in carnosol-treated PC-3 cells (Johnson et al., 2008).

A study by Johnson et al. published in 2010 found that carnosol could dose-dependently inhibit cell viability in both LNCaP and 22Rv1 prostate cancer cells. Additionally, carnosol inhibited the mRNA and protein levels of PSA, AR and ER- $\alpha$ . Translocation of AR and ER from the cytoplasm to the nucleus was also inhibited. Caspase-3 activation (cleavage) was significantly upregulated by carnosol in 22Rv1 cells, indicative of apoptosis. Carnosol did not have a significant effect on normal prostate epithelial cell (PrEC) viability even after a 100  $\mu$ M treatment, as shown by the high percentage of viable cells when compared to tumorigenic cell lines (Johnson et al., 2010).

Treatment with 20-100  $\mu$ M of carnosic acid induced both intrinsic and extrinsic apoptotic pathways in PC-3 cells as shown by the enhanced expression of cleaved caspases 8, 9, 3, and 7, and the pro apoptotic protein Bax, while downregulating Bcl-2 and the inhibitor of apoptosis family of proteins (IAP). These processes ultimately lead to the release of cytochrome c from the mitochondrial inner membrane. Additionally, apoptosis was

further stimulated by carnosic acid suppression of the IKK/NF- $\kappa$ B pathway, apparent from a decrease in DNA binding activity, nuclear translocation of p50 and p65 and I $\kappa$ B $\alpha$  phosphorylation. This downregulation in PC-3 cells seemed to be mediated by the inhibition of Akt phosphorylation, paired with phosphatase 2A (PP2A) increased activity. Pharmacological inhibition of the PP2A reversed carnosic acid's effects on apoptosis, indicating a possible mechanism of action which modulates the Akt/IKK/NF- $\kappa$ B pathway through PP2A activation. Ultimately, the intrinsic apoptotic pathway was also enhanced in DU-145 cell lines as shown by PARP and caspase-3 cleavage, followed by an increase in the Bax-Bcl-2 ratio and the release of cytochrome c (Kar et al., 2012).

Another study by Petiwala et al. found that carnosic acid (CA) could inhibit proliferation and decrease viability of 22Rv1 and LNCaP prostate cancer cells. The percentage of apoptotic cells was higher in the CA-treated group as shown by the greater number of green, TUNEL-positive cells, and increased levels of cleaved caspase-3. LNCaP cells were more sensitive to the effects of CA as a greater extent of apoptosis induction could be observed when compared to 22Rv1 cells. CA further inhibited the expression of AR (greater in LNCaP) and PSA, whereas it upregulated proteins involved in the unfolded protein response including BiP, IRE1 $\alpha$ , CHOP, SXPB-1 and ATF-6. The levels of cleaved caspase-4 were also upregulated in both cell lines although greater effects could be noticed in LNCaP cells, indicating that androgen-dependent cell lines may be more sensitive to the effects of this polyphenol (Petiwala et al., 2016).

Guo et al. investigated the effects of the rosemary polyphenol carnosol on androgen-dependent LNCaP and androgen-independent DU-145 cells, and observed that lower doses of the compound (0-16  $\mu$ M) could elicit a negative effect on the extent of cell survival in both cell lines, while downregulating the levels of Gill and SHH, proteins involved in the

hedgehog signaling pathway which promotes cancer invasion and metastasis, and increasing the activity of caspase 3, thus favouring cell death (Guo et al., 2017).

In a study by Jang et al., high concentrations of rosmarinic acid (200-300  $\mu\text{M}$ ) drastically inhibited cell viability in PC-3 and DU-145 cells after a 48h treatment, whereas 25 and 100  $\mu\text{M}$  RA minimally affected cell viability when compared to the control, DMSO-only treated cells. Treatment with RA further inhibited the colony formation efficiency of both cell lines and decreased the size of tumor spheroids when compared to the control group. In DU-145 cells, RA increased the number of cells in the late apoptosis/necrosis stage, whereas a higher number of PC-3 cells was observed in the early apoptosis stage. In both cell lines, apoptosis through DNA fragmentation could be detected. Western blotting results indicated a downregulation of histone deacetylase 2 (HDAC2) and an induction in p53 expression in both cell lines when compared to the DMSO-treated group. Additionally, RA induced the expression of p21 in PC-3 cells only, whereas the expression of genes involved in cell cycle progression including PCNA, cyclin D1, and cyclin E1 were inhibited in both cell lines, indicating inhibition of cell proliferation by induction of cell-cycle arrest. On the other hand, the increased expression of Bax and cleaved PARP, paired with the inhibition of Bcl-2, indicate that RE also induces cell death by upregulating apoptotic processes (Jang et al., 2018).

**Table 1.2:** Current in vitro studies on the Effects of RE polyphenols on prostate cancer cells.

<b>Authors</b>	<b>Cell Lines</b>	<b>Experiment</b>	<b>Findings</b>
Johnson et al. (2008)	PC-3	Carnosol (COH) at concentrations 20-60 $\mu$ M for 48h to determine morphology and protein expression, and 20-70 $\mu$ M for 24, 48 and 72h to determine change in cell viability	<ul style="list-style-type: none"> <li>↓ Cell proliferation</li> <li>↓ p-mTOR</li> <li>↓ p-Akt</li> <li>↓ PI3K</li> <li>↑ p-AMPK</li> <li>↑ Apoptosis</li> <li>↓ Bcl-2</li> <li>↑ Cell cycle arrest at the G2 phase</li> <li>↓ CDK2</li> <li>↓ CDK6</li> </ul>
Johnson et al. (2010)	LNCaP, 22Rv1	10–60 $\mu$ M carnosol (COH) for 24–48h	<ul style="list-style-type: none"> <li>↓ Cell viability</li> <li>↓ AR</li> <li>↓ ER- <math>\alpha</math></li> <li>↓ PSA</li> <li>↑ Cleaved caspase 3</li> </ul>
Kar et al. (2012)	PC-3	Carnosic acid (CA) at concentrations ranging from 20-100 $\mu$ M for 24,48 and 72h to	<ul style="list-style-type: none"> <li>↓ Cell viability</li> <li>↑ Apoptosis</li> <li>↓ Pro-caspase 8 and 9</li> </ul>

		determine viability, and for 6-36h to assess protein expression.	<ul style="list-style-type: none"> <li>↑ Cleaved caspase 8 and 9</li> <li>↑ Cleaved PARP</li> <li>↓ Bcl-2</li> <li>↑ Bax</li> <li>↑ Cytochrome c in cytosol</li> </ul>
Petiwala et al. (2016)	22Rv1, LNCaP	0-45 $\mu$ M carnosic acid for 24h to assess cell proliferation, 0-100 $\mu$ M for 48-72h to assess cell viability, 0-75 $\mu$ M for 24h for other assays	<ul style="list-style-type: none"> <li>↓ Cell viability</li> <li>↓ Cell proliferation</li> <li>↑ Apoptosis</li> <li>↑ Cleaved caspase 3</li> <li>↓ AR</li> <li>↓ PSA</li> <li>↑ BiP</li> <li>↑ IRE1 <math>\alpha</math></li> <li>↑ CHOP</li> <li>↑ SXBP-1</li> <li>↑ ATF-6</li> <li>↑ Cleaved caspase-4</li> </ul>
Gao et al. (2017)	LNCaP, DU-145	0.25-16 $\mu$ M carnosol; 24h to assess the effects on cell survival, 0.5-4 $\mu$ M carnosol; 24h to assess mRNA and protein expression, as	<ul style="list-style-type: none"> <li>↓ Cell survival</li> <li>↓ Gli1 mRNA and protein</li> <li>↓ SHH mRNA and protein</li> <li>↑ Caspase 3 activity</li> </ul>

		well as caspase 3 activity.	
Jang et al. (2018)	PC-3, DU-145	25-300 $\mu$ M rosmarinic acid (RA) for 48h in most experiments (200 $\mu$ M), for 2 weeks to assess colony formation efficiency, and for 1 week in a hanging drop assay	↓ Cell viability ↓ Colony formation efficiency

From the above-mentioned *in vitro* studies, only 4 studies examined the effects of RE on prostate cancer cells. The one study (Petiwala 2014) utilized RE fortified with CA and it is the only study that examined apoptosis. It is clear that no studies exist examining the effects of RE (without any fortification) on apoptosis of prostate cancer cells and there is a lack of understanding of the signaling mechanisms involved.

### ***1.6.3 In vivo studies of the effects of RE on prostate cancer:***

A limited number of studies examined the effects of rosemary extract and its polyphenols on prostate cancer growth *in vivo* (Table 2). Previous studies have demonstrated that athymic mice xenografted with 22Rv1 prostate cancer cells fed 100 mg/kg rosemary extract (Petiwala et al., 2014), 30 mg/kg carnosol (Johnson et al., 2010), or 100 mg/kg carnosic acid (Petiwala et al., 2016) had smaller tumor volumes compared to control mice. Additionally, examination of tumor tissue demonstrated lower AR (Johnson et al., 2010; Petiwala et al., 2014; Petiwala et al., 2016), PSA (Petiwala et al., 2014) and ER- $\alpha$  (Johnson

et al., 2010) expression compared to control, whereas the expression of stress protein CHOP was enhanced (Petiwala et al., 2014; Petiwala et al, 2016).

**Table 2:** Current *in vivo* studies on the effects of RE or its polyphenolic components on prostate cancer

<b>Authors</b>	<b>Experiment</b>	<b>Findings</b>
Petiwala et al. (2014)	Athymic mice xenografted with 22Rv1 cells and fed a RE dose of 100 mg/kg dissolved in olive oil for 22 days	↓ Tumor mass/size ↑ Apoptosis ↓ AR ↓ PSA ↑ CHOP
Johnson et al. (2010)	Athymic mice xenografted with 22Rv1 cells and fed 30 mg/kg carnosol dissolved in cottonseed oil for 28 days	↓ Tumor volume ↓ Tumor growth ↓ AR ↓ ER- $\alpha$
Petiwala et al. (2016)	Athymic mice xenografted with 22Rv1 cells and fed 100 mg/kg of carnosic acid dissolved in 100 uL of cottonseed oil for 25 days	↓ Tumor growth ↓ Tumor mean weight ↑ CHOP ↓ AR

The available literature provides some data that demonstrate anti-cancer properties of rosemary extract and its polyphenolic constituents. Rosemary and rosemary polyphenols favoured cell death, lower proliferative rates, and reduced cell survival in both androgen

independent and dependent prostate cancer cells while promoting apoptosis, cell cycle arrest and ER stress. Most of the available data present the effects of individual polyphenols found within RE, but not RE as a whole. The RE studies available provide limited information regarding the signaling molecules involved. Furthermore, the effects of RE on prostate cancer cell apoptosis are not understood.

## **2. Rationale, objectives, and hypothesis:**

### **2.1 *Rationale and Aims:***

Prostate cancer is the second most diagnosed cancer in males and the sixth leading cause of cancer death worldwide (Jemal et al., 2011). Current treatments, which generally include prostatectomy, androgen deprivation therapy, radiation therapy, and chemotherapy are sometimes insufficient, indicating a need for further development of medications to prevent progression or reoccurrence. Treatment of hormone-sensitive prostate cancer through androgen deprivation therapy, though successful in suppressing proliferation at early-stages, has been proven ineffective in preventing the progression to castration-resistant prostate cancer, observed in 10-20% of patients with metastatic prostate cancer (Karantanos et al., 2013; Kirby et al., 2011). Prognosis at this stage remains poor, with a median survival of only 14 months following diagnosis (Kirby et al., 2011).

At the subcellular level, PTEN inactivation, which normally acts by inhibiting the PI3K/Akt pathway, loss of p53 function and upregulation PI3K/Akt and Ras/MAPK signaling pathways have been reported in prostate cancer leading to suppression of apoptosis and increased proliferation and survival (Martin et al., 2011). Overexpression of the anti-apoptotic protein Bcl-2 was reported in prostate cancer, paired with elevated expression of inhibitor of apoptosis (IAP) proteins and increased expression of Bad, events correlated with evasion of apoptotic processes and increased tumor growth (Ali & Kulik, 2021; Krajewska

et al., 2003; Smith et al., 2009). Additionally, overactivation of the PI3K/Akt pathway has been correlated to increased resistance of prostate cancer to docetaxel, an established chemotherapeutic drug for advanced prostate cancer (Bumbaca & Li, 2018).

Rosemary and rosemary polyphenols have shown potentially therapeutic effects *in vitro* and *in vivo* against different forms of cancer including prostate, breast, and colorectal (Moore et al., 2016), and despite this evidence, substantial data on their apoptotic mechanisms of action in prostate cancer is still not available.

The aim of the present study was to examine the pro-apoptotic and anti-proliferative effects of rosemary extract in prostate cancer and elucidate the signaling mechanisms involved. The effects of RE on cell proliferation, colony formation efficiency, and apoptosis of androgen-independent PC-3 and 22Rv1 prostate cancer cells, and normal prostate epithelial PNT1A cells were examined. Furthermore, changes in protein expression of key signaling pathways were measured through western blotting. The PNT1A cell line was used to examine the effects of RE on normal prostate epithelium, compare the data to the data obtained utilizing the prostate cancer cell lines, and examine whether RE may selectively target dysregulated pathways in cancer cells. Understanding the *in vitro* mechanisms of action of RE will form the base for future *in vivo* research utilizing animal prostate cancer xenografts.

## **2.2 Hypotheses:**

- Rosemary extract inhibits proliferation and survival of PC-3 and 22Rv1 prostate cancer cells but does not affect proliferation of PNT1A non-tumorigenic prostate epithelial cells

- Rosemary extract promotes apoptosis in prostate cancer cells by increasing the levels of cleaved caspases 3, 9, 7, 8, and PARP, proteins involved in the intrinsic and extrinsic apoptotic pathway.
- Rosemary extract inhibits the PI3K/Akt and the Ras/MAPK cell signaling cascades in prostate cancer cells.
- Rosemary extract promotes ROS formation in prostate cancer cells, potentially contributing to decreased cell survival and induction of apoptosis.

### **3. Methodology:**

Immortalized cell lines are widely utilized in *in vitro* research as they have the peculiarity of indefinite multiplicative capability, rendering them fundamentally immortal. They possess most of the characteristics that could be found in normal proliferating cancer cells/ tumors but offer a population of cells that are phenotypically homogeneous. This advantage allows extensive investigation of the behaviour and biological properties of different types of cells (Hanahan & Weinberg, 2011).

The two prostate cancer cell lines, utilized for our experiments, PC-3 and 22RV1 cells are commonly utilized prostate cancer cell lines in research, and they each represent different prostate cancer subtypes. The histology and the known mutations of the cell lines are summarized in the table below (Table 3). PC-3 cells, derived from a 62-year-old Caucasian male's bone metastasis of grade IV prostate cancer, present null mutations in both PTEN and p53 tumor suppressor genes. PC-3 cells do not express AR and referred as androgen independent.

22Rv1 cells, derived from a human prostatic carcinoma xenograft (CWR22R) serially propagated in nude mice, present a p53 heterozygous mutation. 22Rv1 cells weakly express the AR and for this reason are referred as androgen -sensitive in some studies. However, the presence of androgens does not significantly affect their growth rate. They are not dependent on androgens for growth, and they are referred as androgen independent. Examination of the genetic ancestry of prostate cancer cells used in research revealed that the 22Rv1 cells carry mixed ancestry, the main ancestry proportions were 0.41 West African and 0.42 European while PC-3 cells were 0.73 European (Woods-Burnham et al., 2017). The 22Rv1 cells line carries substantial African ancestry and its use in preclinical studies for mechanistic studies is important. Prostate cancer disproportionately affects African men who also have higher mortality. There is a need for scientists to diversify the available

biospecimens and use cells that better represent the African population in order to find potential biological mediators of the existing mortality disparities.

Both PC-3 and 22RV1 prostate cancer cells are androgen-independent but they have different mutations and represent different prostate cancer subtypes. PC-3 cells are representative of highly aggressive prostatic carcinomas, 22Rv1 cells are considered representatives of prostatic tumors with lower invasiveness (Table 3). These 2 cancer cell lines were chosen because they have different mutations and represent different prostate cancer subtypes and were established and used previously in Dr Tsiani’s lab (Rashid et al., 2011). The human prostate PNT1A cells representing healthy (non-cancerous) prostate epithelial cells were also used in order to examine effects of our treatment in non-cancerous cells.

**Table 3:** Prostate cancer and non-cancer cell lines and their respective characteristics

<b>Prostate Cancer Cell Line</b>	<b>Category</b>	<b>Origin</b>	<b>Mutations</b>	<b>Representative prostate cancer subtype</b>
<b>PC-3</b>	Prostatic adenocarcinoma	Derived from a bone metastasis of grade IV prostate cancer extracted from a 62-year-old Caucasian male	PTEN, p53 null (Seim et al., 2017), no PSA or AR expression	Androgen insensitive, highly invasive, rare small-cell prostatic carcinoma (Tai et al., 2011; Gaupel et al., 2013)

		(Kaighn et al., 1979)		
22RV1	Prostatic adenocarcinoma	Derived from a human prostate carcinoma xenograft (CWR22R) serially propagated in nude mice following the castration-induced regression and relapse of androgen dependent CWR22 xenograft (Sramkoski et al., 1999; Pretlow et al., 1993)	P53 heterozygous mutation, expression of AR (Carrol et al., 1993)	Androgen sensitive but androgen independent, low invasiveness, tumors form primarily osteosclerotic lesions (Gaupel et al., 2013; Wang et al., 2008)

PNT1A	Immortalized healthy prostate epithelial cells	Healthy prostate epithelium obtained from a 35 year old male post mortem (Degeorges et al., 1995)	N/A	Non-cancerous
-------	--	---	-----	---------------

### 3.1 Materials:

Materials for cell culturing, including RPMI 1640 medium (Roswell Park Memorial Institute), antibiotic-antimycotic solution, fetal bovine serum (FBS), and trypsin were acquired from GIBCO Life Technologies (Burlington, ON). 75cm<sup>2</sup> flasks, 6 well plates, 96 well plates, pipette tips, microcentrifuge tubes, and syringe filters were purchased from VWR International (Mississauga, ON). Prostate adenocarcinoma PC-3 and 22Rv1 cells were purchased from American Type Culture Collection (ATCC). Chemicals including dimethyl sulfoxide (DMSO), bovine serum albumin, formalin, crystal violet, methylene blue and buffer salts were purchased from Sigma Aldrich (Oakville, ON). Western blotting materials including glass plates, polyvinylidene fluoride (PVDF) membrane, running and transfer apparatus, sodium dodecyl sulfate (SDS), Tris-base, glycine, TEMED, Tween, ammonium persulfate (APS), phenylmethanesulfonylfluoride (PMSF), acrylamide/Bis solution, Bradford reagent for protein assay, peroxide and luminol/enhancer solutions were purchased from Bio-Rad (Mississauga, ON). Primary antibodies utilized in western blotting,

as well as IgG HRP-linked anti-rabbit and anti-mouse secondary antibodies were obtained from Cell Signaling Technologies through New England Biolabs (Mississauga, ON). The CellRox Green reagent (Invitrogen) was purchased from Fisher Scientific, and the MTT assay kit was acquired from Abcam.

### **3.2 Cell culture:**

PC-3, 22RV1, and PNT1A cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and supplemented with 1% antibiotic-antimycotic solution following previously established protocols by our group (Rashid et al., 2011). Media in the flask was replaced every 48h, and upon reaching ~80% confluency, cells were trypsinized and seeded in well plates to carry out the specified assays. Cells were grown in an incubator at 37° C and exposed to 5.0% carbon dioxide (CO<sub>2</sub>). Cell handling was performed in a Forma Class II, A2 Biological Safety Cabinet (BSC).

### **3.3 Rosemary extract preparation:**

A methanol extract of rosemary (RE) was prepared according to a previously established protocol (Moore et al., 2016). Dried *Rosmarinus officinalis* leaves (Mississauga, ON, Canada) were ground, passed through a mesh strainer and steeped overnight (16 hours) in dichloromethane-methanol (1:1) (30mL). The filtrate was collected, followed by methanol extraction for 30 minutes. The solvent was removed by rotary evaporation and the green powder obtained was stored at -20°C. RE stock solution was prepared in dimethyl sulfoxide (DMSO) (100mg/mL) and stored at -20°C. The day of each experiment, a working stock of RE solution was prepared in RPMI 1640 media.

### **3.4 Crystal violet assay:**

The crystal violet assay was used to assess cell proliferation (growth). This assay has been established previously in Dr Tsiani's lab (Jaglanian & Tsiani, 2020; Moore et al., 2016). The crystal violet stain binds DNA and thus measures the ability of cells to grow and divide. Cells were seeded (800-1000; counted using haemocytometer) in triplicates in 100µL RPMI-1640 media in 96 well plates and allowed to adhere overnight. The following day, rosemary extract was added at concentrations ranging from 5 to 150µg/mL (final volume in each well 200µL); cells were subsequently incubated for 72 hours without changing the media. After the three-day course, cells were fixed with 10% formalin, rinsed twice with PBS and stained using crystal violet dye (~50µL/well). Stain was removed after 15 minutes by rinsing the plates thrice with tap water and were then allowed to dry overnight. The following day, solubilizer was added (100µL/well) and absorbance at 570nm was measured using the KC4 microplate reader.

### **3.5 Clonogenic survival assay:**

The clonogenic assay, established previously in Dr Tsiani's lab (Jaglanian & Tsiani, 2020; Moore et al, 2016) was used to assess the colony forming extent of the cells. Colony formation is a peculiar characteristic of cancer cells, able to spread and metastasize thus forming malignant tumors. Cells were seeded at a density of 1000 cells/well (counted using haemocytometer) in 6-well plates, in duplicates, and supplemented with RPMI-1640 media for a final volume of 2mL per well; cells were allowed to adhere overnight. The following day, media was removed and treatments were administered by adding fresh media containing RE (RE working stock) in each well. Cells were incubated for 7 days, and at the end of the time course, media was removed; cells were washed twice with PBS, and subsequently stained with 0.05% w/v methylene blue in deionized water. Cells were stained

for 15 minutes and left to dry overnight at room temperature with a semi-open lid. Colonies possessing more than 50 cells were counted under a bright-field microscope and results were expressed as a percentage of the control (untreated).

### **3.6 Preparation of whole cell lysates for western blotting:**

Preparation of cell lysates were done as previously (Jaglanian & Tsiani, 2020; Moore et al., 2016; Rashid et al., 2011). Cells were seeded in duplicate in 6-well plates and treated upon reaching 70-80% confluence. The RE stock utilized for treatment was dissolved in DMSO at a concentration of 100 mg/mL, and then further diluted in media at a concentration of 400 µg/mL prior to treatment. Cells were exposed to a final concentration of DMSO of 0.05%. A 10 mM docetaxel stock was prepared by dissolving 8.0 mg of DTX powder in 1 mL of DMSO, and a 10 µM working stock was prepared by diluting 5 µL of stock in 5 mL of media prior to treatment at 10 nM DTX. Following treatment, the media was aspirated, 150 µl of cell lysis buffer with PMSF was added per well, and the plates were put on ice for 20 minutes followed by scraping and lysate transfer into 1.5mL Eppendorf tubes. Following protein assay, if necessary, lysates were diluted for blotting with additional cell lysis buffer. Subsequently, 3x SDS was added to the lysates in a 1:3 ratio. Final samples were left to boil for 4 minutes and subsequently stored at -20°C until ready to use.

### **3.7 Protein assay:**

A protein assay was performed to quantify total protein levels in lysates, and to ensure equal amounts of protein/sample are loaded for western blotting. To obtain a standard curve from which unknown concentrations can be obtained, BSA protein standards were prepared. 10µl of 2000, 1000, 500, 250, 125, and 62.5 µg/mL BSA were pipetted in triplicates in a 96-well plate and an appropriate amount of diluted Bradford reagent (1:4

dilution) was added into each well for a total volume of 210  $\mu$ l per well. Lysate samples were also pipetted in triplicates alongside the BSA samples (10  $\mu$ l of protein and 200  $\mu$ l of BSA). Absorbance was measured at 595 nM using the KC4 microplate reader after a programmed plate shaking to ensure proper mixing of BSA with the protein and guarantee colour shift from brown to blue according to determinate amounts of protein present. The absorbance of the BSA standards was utilized to create a standard curve through Excel, and the equation of the curve obtained was utilized to determine the protein content of the lysates given their absorbance values.

### **3.8 Western blot:**

Western blotting was performed to investigate total and phosphorylated levels of a given protein utilizing specific antibodies. Lysate samples containing 25  $\mu$ g of protein were loaded beside a Biorad Kaleidoscope ladder, and separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10-15% polyacrylamide gels for ~60 minutes at 150 V. Subsequently, samples were transferred to a PVDF membrane and blocking buffer was added for 1 hour at room temperature. The PVDF membrane was incubated overnight with primary antibody at a temperature of 4 °C, and the following day, after antibody removal and washing, the membrane was incubated with HRP-linked IgG anti-rabbit secondary antibody for 1 hour at room temperature. Band visualization was carried out using the LI-COR C-Digit software and signals were detected using the Biorad Clarity Max Western Enhanced Chemiluminescence (ECL) Solution. Densitometry of the bands was performed with the Imagej Software.

### **3.9 Assessment of Cell Morphology:**

Treated and untreated PC-3, 22Rv1 and PNT1A cells were imaged to visually assess the effects of RE on cell density and morphology (shape; spherical vs elongated shapes). Cells seeded on a 6-well plate were treated with RE for 24h or 48h. following treatment, the media was discarded, and cells were fixed with 10% formalin for 15 minutes as previously established in our lab (Jaglanian & Tsiani, 2020; Moore et al., 2016). The plates were then visualized with a Cytation 5 cell imaging reader (BioTek), and images were taken from the same area in each well for data consistency.

### **3.10 MTT assay:**

The MTT assay was performed using an MTT assay kit (#ab211091, Abcam) according to manufacturer's instructions to determine changes in cells' metabolic activity. The MTT assay protocol is based on the conversion of water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) compound to an insoluble blue formazan product by NADPH-dependent oxidoreductases in metabolically active cells. Briefly, 5000 cells/well were seeded in a 96-well plate and allowed to adhere overnight. Cells were then treated in triplicates for 72h. Following treatment time, media was carefully removed without disturbing the blue formazan crystals formed, and MTT solvent was added to solubilize the crystals. Absorbance at 570 nm was then measured with a spectrophotometer.

### **3.11 CellRox Green Assay:**

To measure levels of reactive oxygen species (ROS), a ROS assay utilizing the CellRox Green reagent (#C10444, Invitrogen) was carried out as reported by Schattauer et al. (2019) and according to manufacturer instructions. The Cellrox reagent exists as a weakly

fluorescent DNA dye, which, upon oxidation by reactive oxygen species (ROS), binds to DNA. As a result of this, signal is primarily localized in the nucleus and mitochondria and is visualized through fluorescent microscopy. Cells were seeded in 12- or 24-well plates, allowed to reach ~50% confluency and then treated with the desired compounds. Following treatment, cells were exposed to 5  $\mu$ M CellRox reagent for 30 minutes, washed, and fixed with 10% formalin. Intracellular green fluorescence (GFP) in each treatment group was detected with a Cytation 5 cell imaging reader, and rapid imaging of each well was carried out through experimental mode using the Gen5 software

### **3.12 Statistical analysis:**

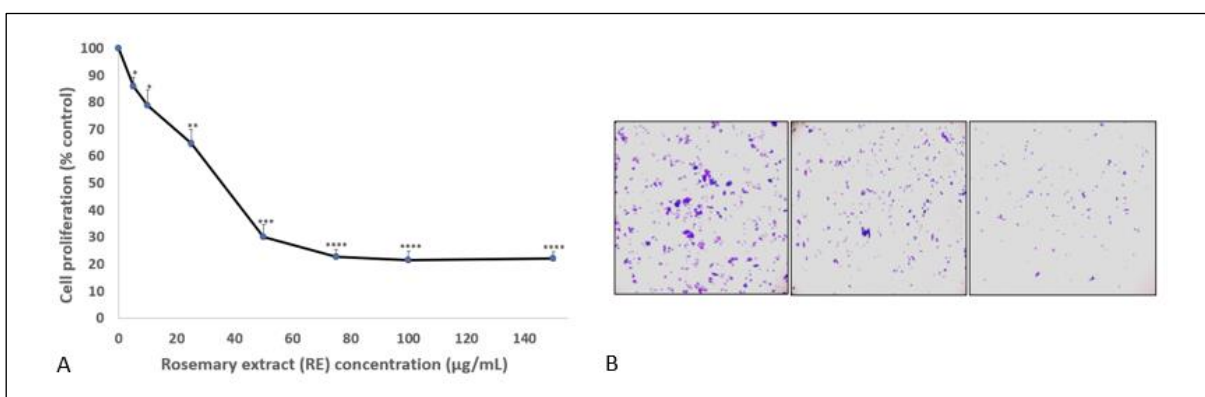
The results are the mean  $\pm$  standard error mean of the indicated number of independent experiments. Variance between groups was assessed through one-way ANOVA for the crystal violet assay, clonogenic survival assay and MTT assay, whereas a two-sample T-test was performed to quantify western blotting images. Statistical significance was assumed at  $P < 0.05$ . Statistical analysis was performed with the Microsoft Excel data analysis tool. We compared the analysis of a subset of data using SPSS and using the Microsoft Excel data analysis tool and found no differences, for this reason we continued the analysis using the Excel data tool.

## **4. Results:**

### **4.1. Rosemary extract (RE) inhibits proliferation of PC-3 androgen independent prostate cancer cells.**

PC-3 cells were treated with 0, 5, 10, 25, 50, 75, 100, or 150  $\mu$ g/mL of RE for 72h prior to assessment of cell proliferation through crystal violet staining. A significant inhibition of proliferation was observed with RE treatment in a dose-dependent fashion (Figure 7).

Maximum inhibition was observed with 75  $\mu\text{g}/\text{mL}$  RE ( $22.93 \pm 2.62$  % of control), with no further inhibition observed in groups treated with higher RE concentrations (100, 150  $\mu\text{g}/\text{mL}$ ). 50  $\mu\text{g}/\text{mL}$  of RE inhibited cell proliferation to  $30.21 \pm 4.42$  % of the control (Figure 7A,B). The concentration of RE required for half max inhibition ( $\text{IC}_{50}$ ) was found to be 26.94  $\mu\text{g}/\text{mL}$ . PC-3 cells were treated at a concentration of 50  $\mu\text{g}/\text{mL}$  RE in further experiments.

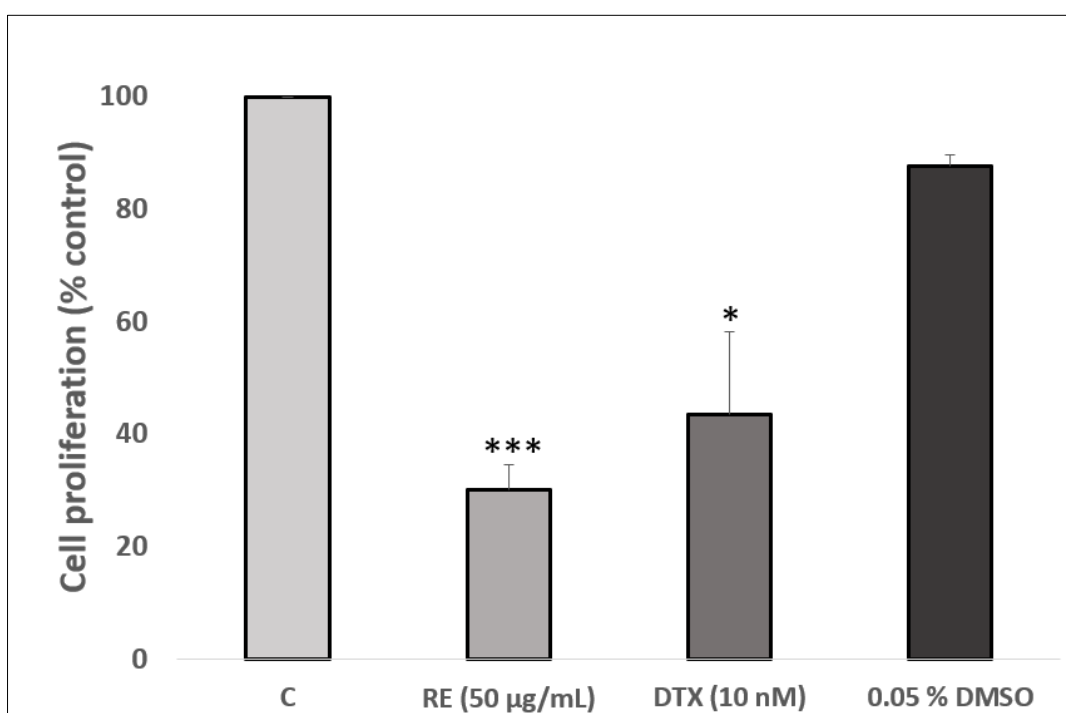


**Figure 7: Rosemary extract (RE) inhibits PC-3 prostate cancer cell proliferation**

PC-3 cells were treated with 0-150  $\mu\text{g}/\text{mL}$  RE for 72h followed by a proliferation assay (A). Crystal violet-stained PC-3 cells after treatment with 0, 50, 100  $\mu\text{g}/\text{mL}$  RE (B) were photographed (10x magnification). The data correspond to the mean  $\pm$  standard error of 5 independent experiments and are expressed as a percentage (%) of the control. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

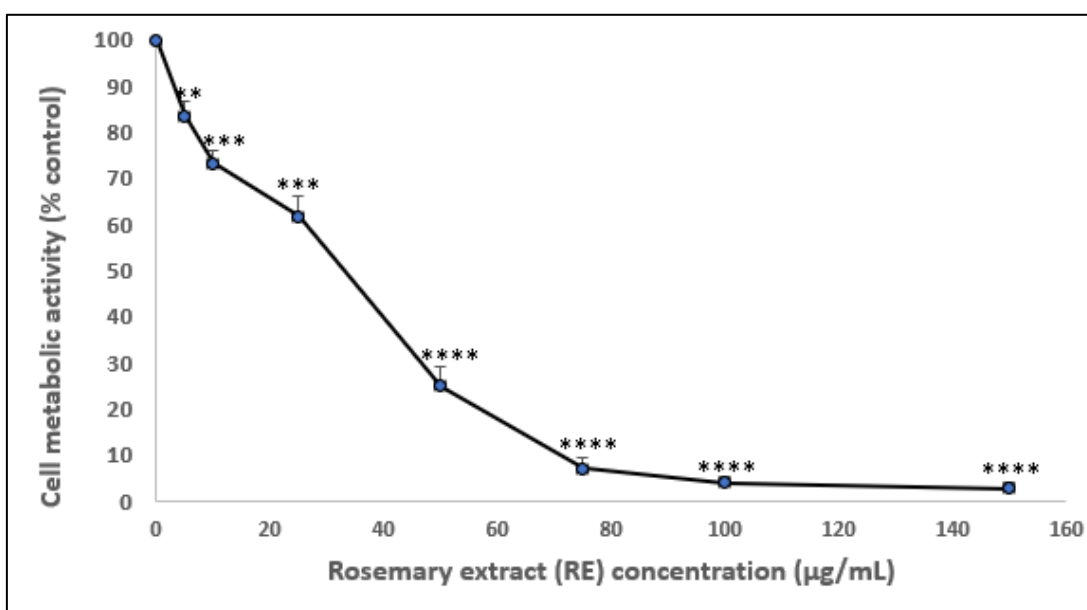
Furthermore, we wished to compare the effects of RE to the effects of docetaxel (DTX), an established chemotherapeutic drug utilized in prostate cancer treatment. A DTX concentration of 10 nM was utilized based on evidence from its use at similar concentrations in other cancer cells, including prostate cancer cells (Banerjee et al., 2017; Cristofani et al., 2018; Jaglanian & Tsiani, 2020; Kim et al., 2010; O'Neill et al., 2011; Xiao et al., 2016).

A significant inhibition of PC-3 cell proliferation was observed with 10 nM DTX treatment ( $43.63 \pm 14.6\%$  of control) and 50  $\mu\text{g}/\text{mL}$  of RE ( $30.21 \pm 4.42\%$  of control), though higher inhibition was seen following treatment with RE (Figure 8). Since RE was dissolved in DMSO, we wished to further analyze the effects of DMSO alone in PC-3 cells. 0.05% DMSO was chosen as it is the amount of DMSO cells are exposed to when treated with 50  $\mu\text{g}/\text{mL}$  of RE for the majority of the experiments. Proliferation of the DMSO-treated cells was not significantly different than the control, untreated cells (Figure 8). It should be noted that the same DMSO levels are used routinely in Dr Tsiani's lab as most of the chemicals/polyphenols studied are prepared in DMSO stock. Previous studies performed by other students tested DMSO using the same prostate cancer cells and found no effects.



**Figure 8: Rosemary extract (RE) and docetaxel (DTX) inhibit PC-3 prostate cancer cell proliferation.** PC-3 cells were treated with 50  $\mu\text{g}/\text{mL}$  RE, 10 nM docetaxel (DTX) or 0.05% DMSO for 72h. The data correspond to the mean  $\pm$  standard error of 5 independent experiments and are expressed as a percentage (%) of the control. \* $p < 0.05$ , \*\*\* $p < 0.001$ .

An MTT assay, which assesses cellular metabolic activity as opposed to extent of cell adherence (as assessed with crystal violet assay), was further conducted to investigate the influence of RE in PC-3 cells. Similarly to the data obtained with the crystal violet assay, RE dose-dependently inhibited PC-3 cell viability, with a maximum inhibitory effect observed after treatment with 75  $\mu\text{g/mL}$  ( $7.16 \pm 2.1$  % of control,  $p < 0.0001$ ). Furthermore, inhibition to  $25.1 \pm 4.01\%$  of control was observed with 50  $\mu\text{g/mL}$  RE ( $p < 0.0001$ ) (Figure 9).

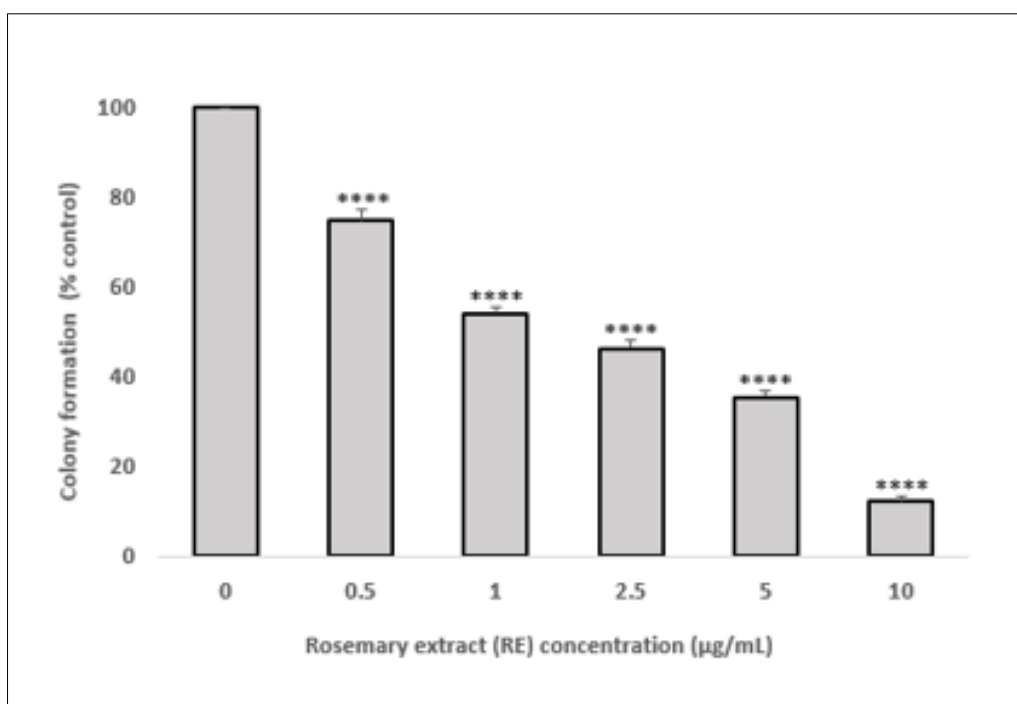


**Figure 9: Rosemary extract (RE) inhibits PC-3 cell viability as shown by MTT assay data.** Cells were treated with RE (0-150  $\mu\text{g/mL}$ ) for 72h prior to assessing NADPH-dependent oxidoreductase activity and formazan production through an MTT assay. The data correspond to the mean  $\pm$  standard error of 5 independent experiments and are expressed as a percentage (%) of the control. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

#### **4.2. Rosemary extract (RE) inhibits colony formation efficiency of PC-3 prostate cancer cells.**

To assess whether RE may influence prostate cancer cell survival, a clonogenic survival assay was conducted. Treatment with RE dose-dependently reduced the colony formation

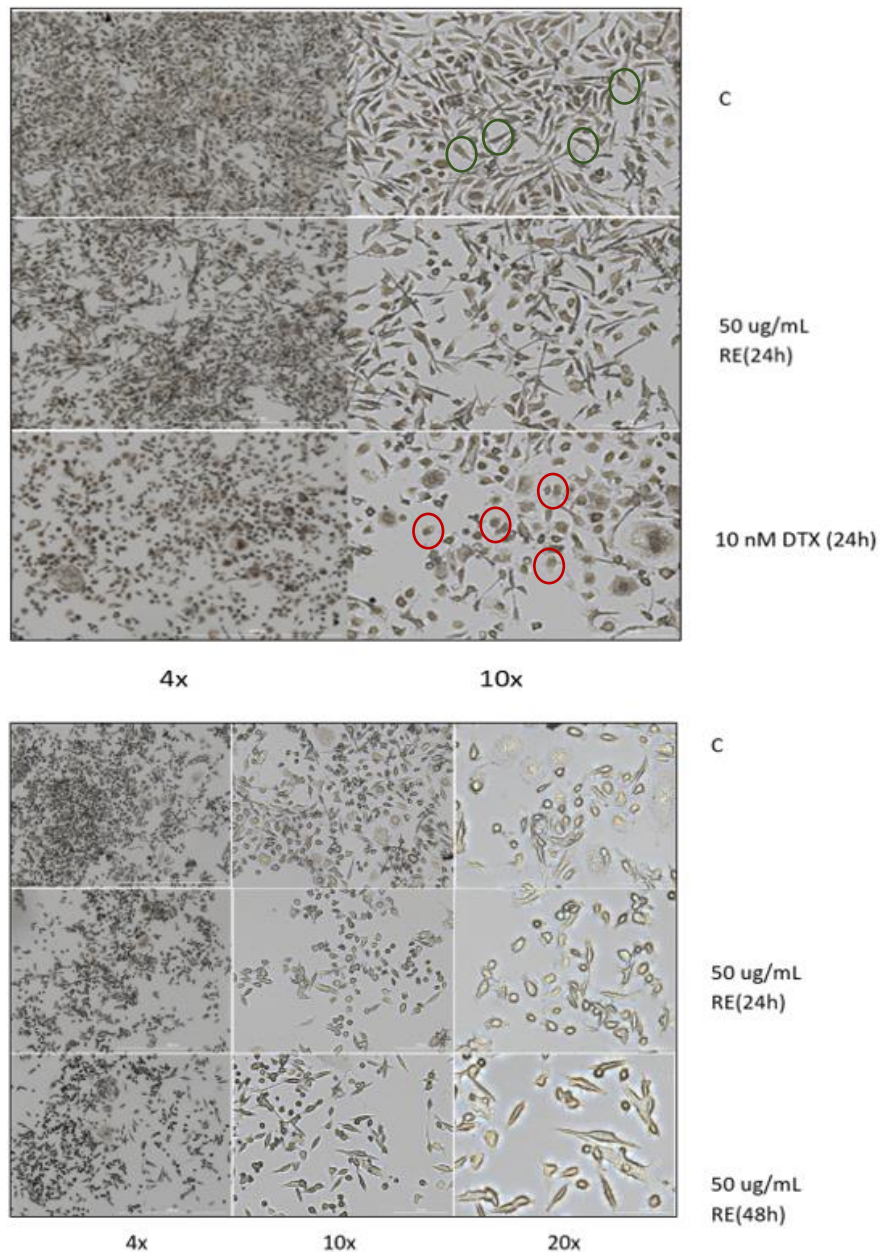
efficiency of PC-3 cells with maximum inhibition observed at 10  $\mu\text{g}/\text{mL}$  ( $12.28 \pm 1.0\%$  of control). Inhibition of colony formation could be observed with lower concentrations of RE ( $74.87 \pm 2.52\%$  of control with 0.5  $\mu\text{g}/\text{mL}$  RE,  $54.22 \pm 1.39\%$  of control with 1  $\mu\text{g}/\text{mL}$  RE) (Figure 10) while concentrations greater than 10  $\mu\text{g}/\text{mL}$  (15 and 20  $\mu\text{g}/\text{mL}$  were used) resulted in complete inhibition of colony formation (no colonies were present to be counted). The IC<sub>50</sub> value for PC-3 cells was 2.76  $\mu\text{g}/\text{mL}$ .



**Figure 10: Rosemary extract (RE) inhibits PC-3 cell colony formation efficiency.** PC-3 cells were treated with RE as indicated for 7 days (IC<sub>50</sub>: 2.757  $\mu\text{g}/\text{mL}$ ). The data are the mean  $\pm$  standard error of 5 independent experiments and is expressed as a percentage (%) of the control. \*\*\*\* $p < 0.0001$ .

Treated and untreated PC-3 cells were imaged utilizing a Cytation 5 machine (BioTek) to assess changes in cell density and morphology. It is important to note that cells were seeded at the same density as the control in each treatment group. Treatment with RE for 24 and 48h decreased overall PC-3 cell density. Lower cell density could be observed

after 48h relative to the 24h treatment group (Figure 11). Lower PC-3 cell density was also observed in the docetaxel (DTX)-treated group. DTX-treated PC-3 cells further appeared rounded (red circles) indicative of cytotoxicity, and not elongated (green circles) as shown in the untreated group (Figure 11).

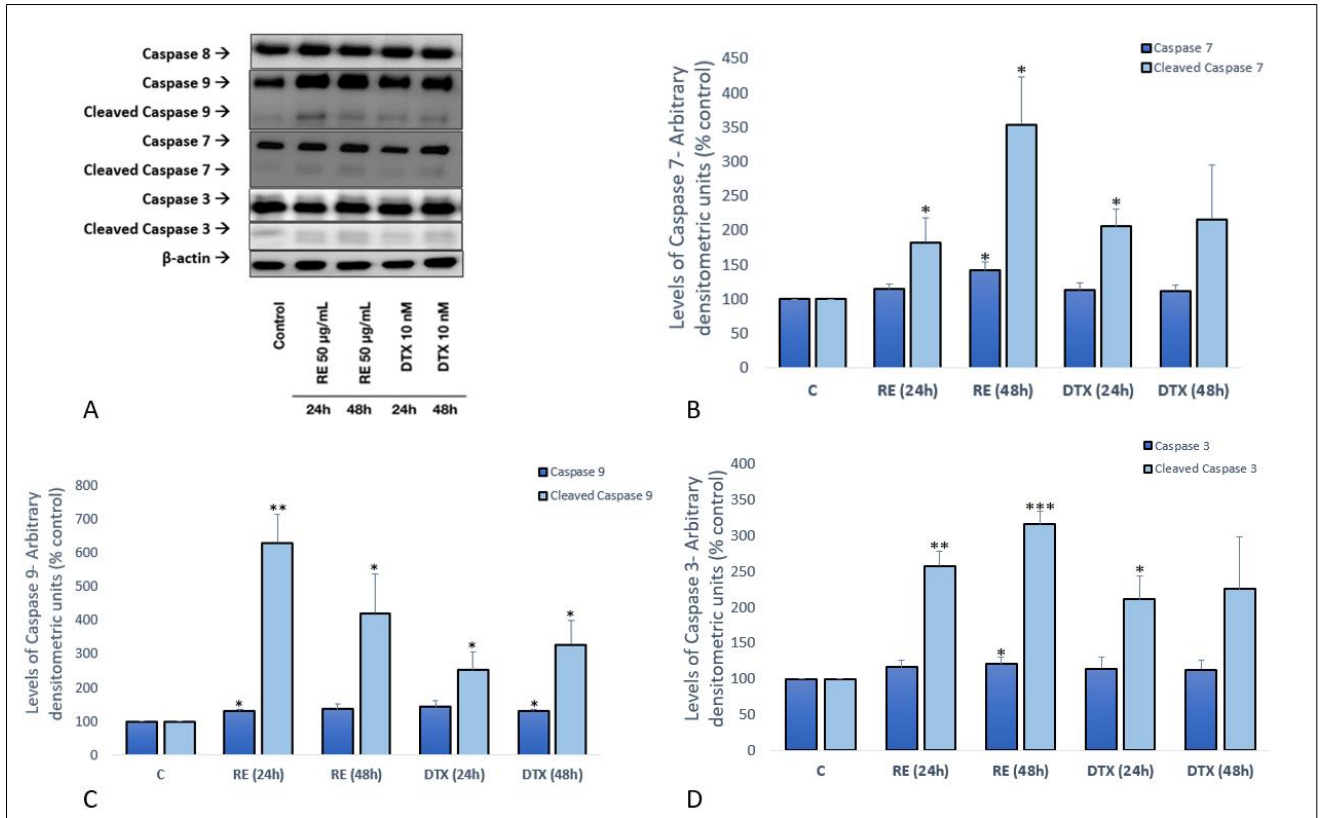


**Figure 11: Effects of rosemary extract (RE) and docetaxel (DTX) on PC-3 cell morphology.** Cells were treated with 50  $\mu\text{g}/\text{mL}$  of RE for 24 and 48h or 10 nM of DTX for 24h. Following treatment, cells were washed twice with PBS and fixed for 15 minutes with

10% formalin. Photographs were obtained using a Cytation 5 imaging system by BioTek Instruments at 4x, 10x, and 20x magnification. Green circles (control) indicate untreated PC-3 cells, whereas red circles indicate cells treated with DTX. Clear differences in cell morphology are noted, with DTX-treated PC-3 cells appearing rounded when compared to the control.

#### **4.3 Rosemary extract (RE) promotes apoptosis in PC-3 cells.**

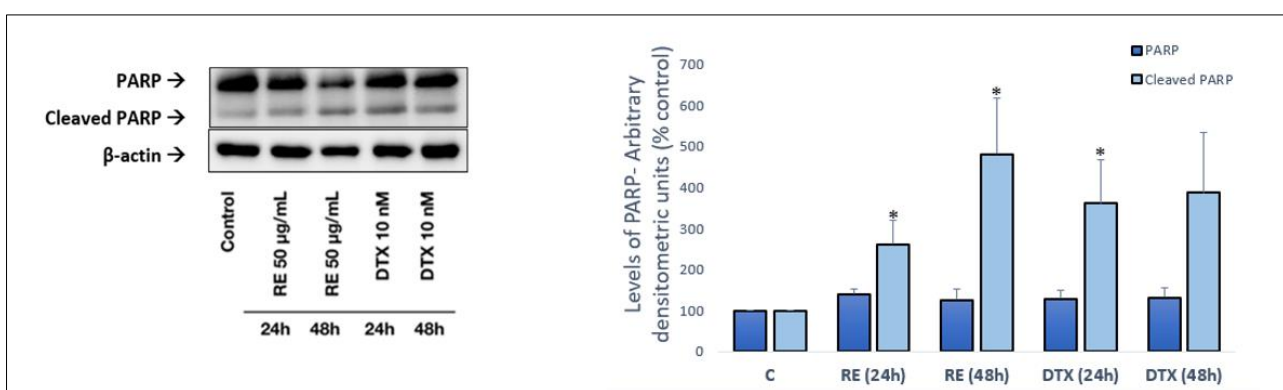
Cleavage/ activation of caspases 9,8,7, and 3 has been established as an indicator of DNA fragmentation and programmed cell death/apoptosis. By utilizing specific antibodies, we examined the levels of total and cleaved caspases. Treatment of PC-3 cells with 50  $\mu\text{g}/\text{mL}$  RE for 24h promoted the cleavage/activation of caspase 9 ( $630.82 \pm 83.59$  % of control,  $p < 0.01$ ) (Figure 12 C), caspase 7 (Figure 12B) ( $182.58 \pm 36.16$  % of control,  $p < 0.05$ ), and caspase 3 ( $258.26 \pm 20.66$  % of control,  $p < 0.01$ ) (Figure 12D), though no activation of caspase 8 was observed due to absence of cleaved fragments (Figure 13A). Treatment with RE for 48h also promoted caspase 9 ( $421.27 \pm 117.8$  % of control,  $p < 0.05$ ), caspase 7 ( $354.61 \pm 68.85$  % of control,  $p < 0.05$ ), and caspase 3 ( $316.35 \pm 18.59$  % of control,  $p < 0.001$ ) cleavage, but no changes in caspase 8 levels were observed. DTX treatment (10 nM) for 24h promoted the activation of cleaved caspase 9 ( $252.46 \pm 53.87$  % of control,  $p < 0.05$ ), cleaved caspase 7 ( $205.66 \pm 26.18$  % of control,  $p < 0.05$ ), and cleaved caspase 3 ( $211.58 \pm 32.27$  % of control,  $p < 0.05$ ). Increased levels of activated caspases 7 ( $216.81 \pm 78.15$  % of control,  $p > 0.05$ ) and 3 ( $225.82 \pm 72.31$  % of control,  $p > 0.05$ ) were also observed in the 48h DTX group, though statistically significant results were only obtained for changes in cleaved caspase 9 ( $328.58 \pm 70.16$  % of control,  $p < 0.05$ ) (Figure 12).



**Figure 12: Effects of Rosemary extract (RE) on Caspases 3, 7, 8, and 9 in PC-3 cells.**

Whole cell lysates were obtained following treatment with 50 µg/mL rosemary extract (RE) or 10 nM docetaxel for 24 or 48 hours and immunoblotted using specific antibodies for Caspase 7 (A, B), 9(A, C), and 3(A, D), or β-actin (A). Densitometric values were obtained with Imagej, corrected to β-actin levels, and expressed as a percentage (%) of the control. The data are the mean ± SEM of 3 independent experiments. \*p<0.05 \*\*p<0.01, \*\*\*p<0.001.

Rosemary extract promoted PARP cleavage, indicative of apoptotic activity, following treatment for 24h ( $262.06 \pm 56.84\%$  of control,  $p < 0.05$ ) and 48h ( $481.21 \pm 136.28\%$  of control,  $p < 0.05$ ), whereas changes in total PARP levels were not observed with any of the treatments. Similarly, PARP cleavage was also observed in the 24h DTX-treated PC-3 cells, (DTX24:  $361.73 \pm 106.7\%$  of control,  $p < 0.05$ ; DTX48:  $387.56 \pm 146.97\%$  of control,  $p > 0.05$ ) (Figure 13).



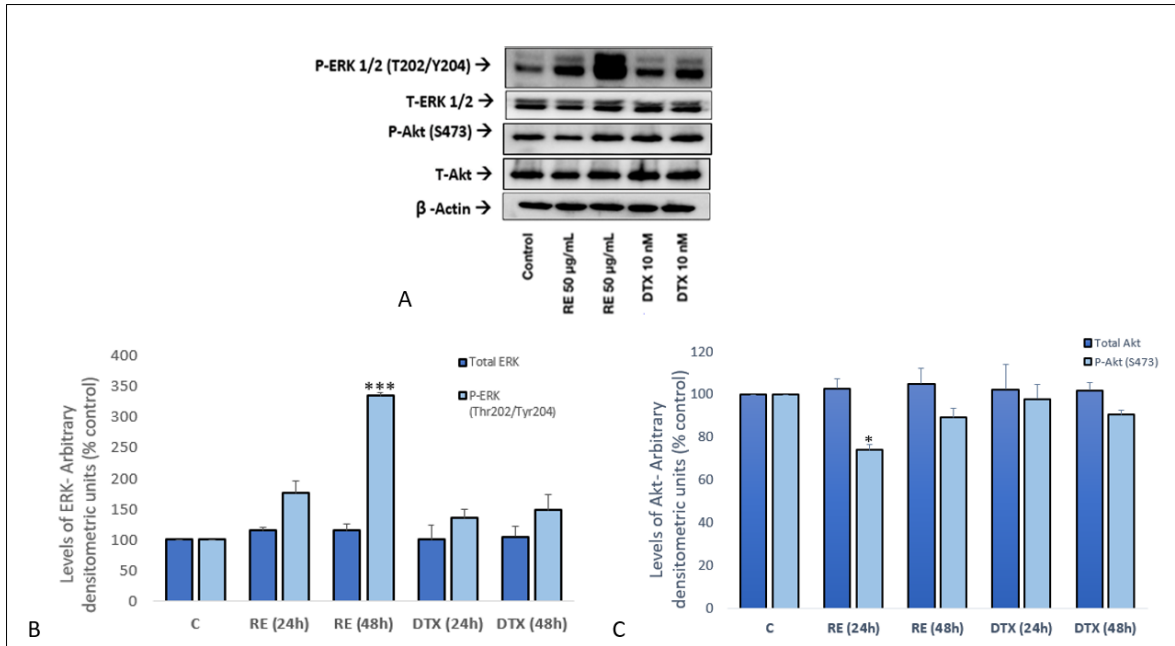
**Figure 13: Effects of Rosemary extract (RE) on PARP in PC-3 cells.** PC-3 cells were treated with 50 µg/mL rosemary extract (RE) or 10 nM docetaxel for 24 or 48 hours and whole cell lysates were immunoblotted using a PARP- specific antibody. Densitometric values were obtained with Imagej, corrected to β-actin levels, and expressed as a percentage (%) of the control. The data are the mean ± SEM of 3 independent experiments. \* $p < 0.05$

#### **4.4. Rosemary extract (RE) activates ERK 1/2 and inhibits Akt in PC-3 cells.**

Changes in the phosphorylated levels of the ERK 1/2 and Akt proteins following treatment with RE were further assessed. The activation of the Ras/MAPK and PI3K/Akt pathways is implicated in many cancers of epithelial origin, and is correlated to increased proliferation and survival, and modulation of apoptosis.

Treatment with RE for 24 or 48h did not have an effect on total levels of ERK 1/2 in PC-3 cells (Figure 14), whereas treatment for 48h resulted in significant phosphorylation of ERK 1/2 (T202/Y204) ( $332.99 \pm 5.21\%$  of control,  $p < 0.001$ ). No statistically significant changes were observed in ERK 1/2 phosphorylation after 24h of treatment with RE ( $175.04 \pm 19.58\%$  of control,  $p > 0.05$ ) or in DTX-treated PC-3 cells (DTX24:  $134.61 \pm 13.84\%$  of control,  $p > 0.05$ ; DTX48:  $148.2 \pm 25.21\%$  of control,  $p > 0.05$ ) (Figure 14).

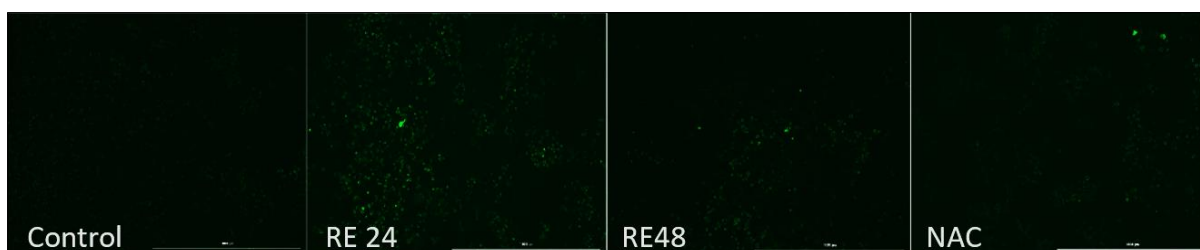
RE did not have a significant effect on total levels of Akt in PC-3 cells, whereas it reduced levels of phosphorylated Akt (S473) after 24h of treatment ( $74.06 \pm 6.76\%$  of control,  $p < 0.05$ ) but not after 48h ( $89.37 \pm 9.15\%$  of control,  $p > 0.05$ ). 10 nM of DTX did not affect levels of phosphorylated Akt at either time point (DTX24:  $97.94 \pm 2.76\%$  of control,  $p > 0.05$ ; DTX48:  $90.54 \pm 9.20\%$  of control,  $p > 0.05$ ) (Figure 14).



**Figure 14: Effects of Rosemary extract (RE) on Erk 1/2 and Akt in PC-3 cells.** PC-3 cells were treated with 50 µg/mL rosemary extract (RE) or 10 nM docetaxel for 24 or 48 hours and whole cell lysates were immunoblotted using specific antibodies for ERK (A,B) Akt (A, C) or β-actin (A). Densitometric values were obtained with Imagej, corrected to β-actin levels, and expressed as a percentage (%) of the control. The data are the mean ± SEM of 3 independent experiments. \* $p < 0.05$  \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

#### 4.5. Rosemary extract (RE) treatment increases the levels of reactive oxygen species (ROS) in PC-3 cells.

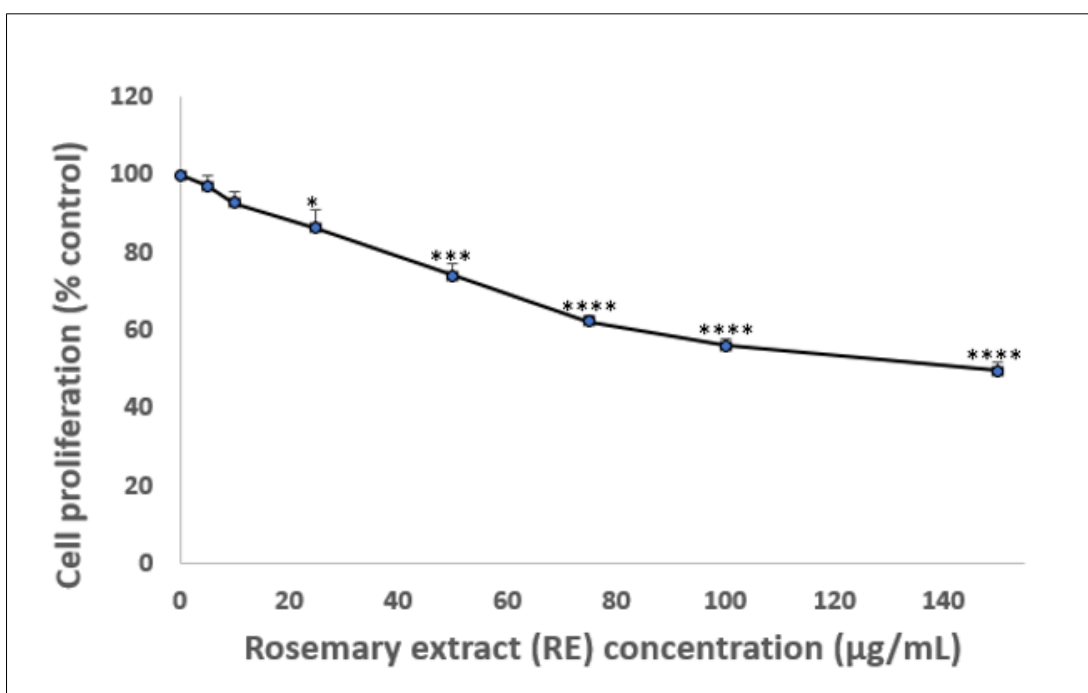
It is well established that many chemicals with anticancer properties, including current chemotherapy agents, increase the intracellular levels of reactive oxygen species (ROS). This increase leads to destabilization of cancer signaling pathways resulting in inhibition of proliferation and apoptosis. A ROS assay utilizing the CellRox Green reagent was used to measure cellular ROS levels following treatment with RE. It is important to note that cells were seeded at the same density in each group prior to treatment and staining, and that representative images were captured from the same area in each well. PC-3 prostate cancer cells treated with RE showed an increase in intracellular ROS levels (Figure 15). Furthermore, PC-3 cells treated with the antioxidant NAC (N-acetylcysteine) presented lower levels of ROS when compared to the RE-treated groups (Figure 15).



**Figure 15: Rosemary extract (RE) treatment increased intracellular ROS levels in PC-3 cells.** PC-3 cells were treated with 50  $\mu\text{g}/\text{mL}$  RE for 24h or 48h, or 5 mM of N-acetylcysteine (NAC) for 24h. CellRox Green reagent was then added to each well, and cells were fixed with 10% formalin. Photographs of cell fluorescence in each group were taken with a Cytation 5 imager.

#### 4.6. Rosemary extract (RE) inhibits proliferation and colony-formation efficiency of 22Rv1 androgen independent prostate cancer cells.

22Rv1 androgen-independent prostate cancer cells were treated with 0, 5, 10, 25, 50, 75, 100, or 150  $\mu\text{g}/\text{mL}$  of RE for 72h prior to assessment of cell proliferation through crystal violet staining. Similarly to what could be observed for PC-3 cells, a significant inhibition of proliferation was observed in a dose-dependent fashion (Figure 16). 150  $\mu\text{g}/\text{mL}$  rosemary extract promoted the highest inhibition ( $49.62 \pm 2.3\%$  of control), whereas 50  $\mu\text{g}/\text{mL}$  RE inhibited proliferation to  $74.04 \pm 3.35\%$  of control (Figure 16). The concentration of RE required for half max inhibition ( $\text{IC}_{50}$ ) was found to  $69.919 \mu\text{g}/\text{mL}$ .

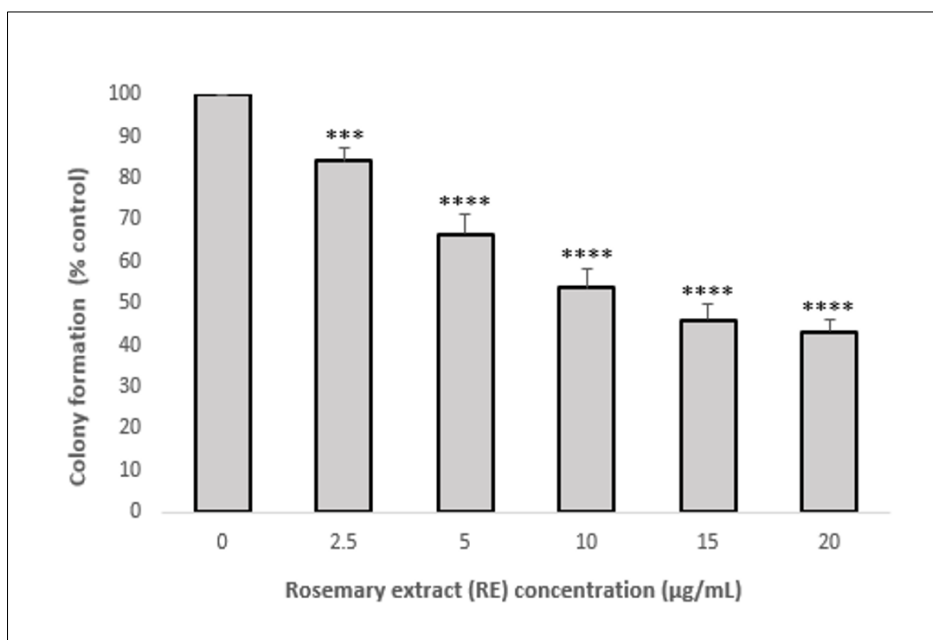


**Figure 16: Rosemary extract (RE) inhibits 22Rv1 prostate cancer cell proliferation.**

22Rv1 cells were treated with 0-150  $\mu\text{g}/\text{mL}$  RE for 72h followed by a proliferation assay.

The data correspond to the mean  $\pm$  standard error of 5 independent experiments and are expressed as a percentage (%) of the control. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

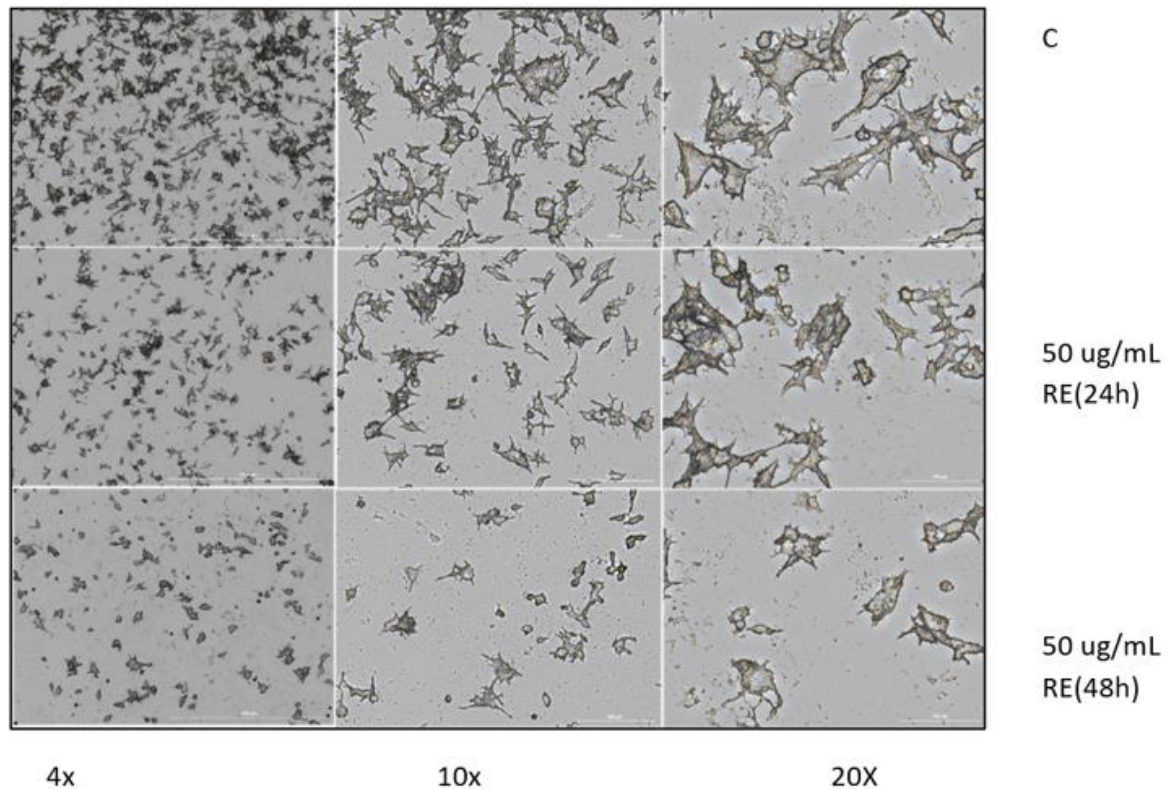
RE inhibited colony formation efficiency in 22Rv1 cells in a dose-dependent manner, with maximum inhibition seen with 20  $\mu\text{g}/\text{mL}$  of RE ( $43.12 \pm 2.84\%$  of the control) (Figure 17). The IC<sub>50</sub> value for 22Rv1 cells was 4.81  $\mu\text{g}/\text{mL}$ .



**Figure 17: Rosemary extract (RE) inhibits 22Rv1 cell colony formation efficiency.**

22Rv1 cells were treated with RE as indicated for 7 days (IC<sub>50</sub>: 2.757  $\mu\text{g}/\text{mL}$ ). The data are the mean  $\pm$  standard error of 5 independent experiments and is expressed as a percentage (%) of the control. \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

Treated and untreated 22Rv1 cells were imaged utilizing a Cytation 5 machine (BioTek) to assess changes in cell density and morphology. Cells were seeded at the same density as the control in each treatment group. Treatment with RE for 24 and 48h decreased overall 22Rv1 cell density, and lower cell density could be observed after 48h relative to the 24h treatment group (Figure 18). No significant changes in cell morphology were observed.



**Figure 18: Effects of rosemary extract (RE) on 22Rv1 cell morphology.** Cells were treated with 50  $\mu\text{g/mL}$  of RE for 24 or 48h, washed twice with PBS, and fixed for 15 minutes with 10% formalin. Photographs were obtained using a Cytation 5 imaging system by BioTek Instruments at 4x, 10x, and 20x magnification.

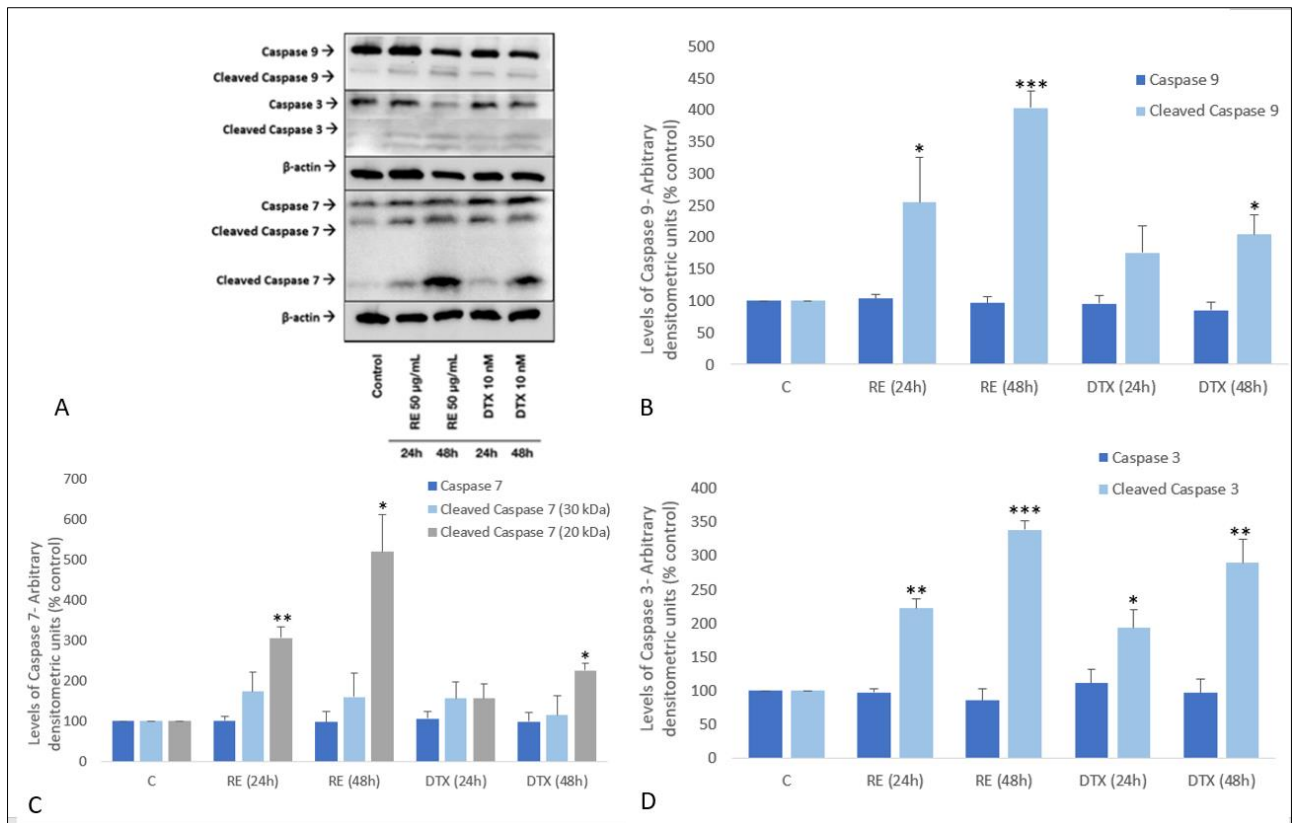
#### **4.7. Rosemary extract (RE) promotes apoptosis in 22Rv1 cells.**

Next, we aimed to investigate the effects of RE on caspases and PARP cleavage in 22Rv1 prostate cancer cells to evaluate similarities and differences with observed data in PC-3 cells. Rosemary extract promoted caspase 9 (Figure 19B), caspase 7 (Figure 19C), and caspase 3 cleavage (Figure 19D) after treatment for 24h and 48h. Higher levels of cleaved caspases were observed in the 48h group (cleaved C-9:  $403.31 \pm 26.49\%$  of control,  $p < 0.001$ ; cleaved C-7 (20 kDa):  $519.82 \pm 91.96\%$  of control  $p < 0.05$ ; cleaved C-3:  $338.05 \pm$

14.01% of control,  $p < 0.001$ ) when compared to the 24h group (cleaved C-9:  $255.26 \pm 70.53\%$  of control,  $p < 0.05$ ; cleaved C-7 (20 kDa):  $305.98 \pm 27.73\%$  of control  $p < 0.01$ ; cleaved C-3:  $222.23 \pm 14.71\%$  of control,  $p < 0.01$ ) (Figure 19). It is important to note that both the 30 kDa and the 20 kDa cleaved fragments of caspase 7 could be seen in 22Rv1 cells (Figure 19C), whereas only the 30 kDa cleaved fragment was visible in blots from PC-3 cells. The presence of either 30 kDa or 20 kDa fragment is indicative of caspase 7 activation.

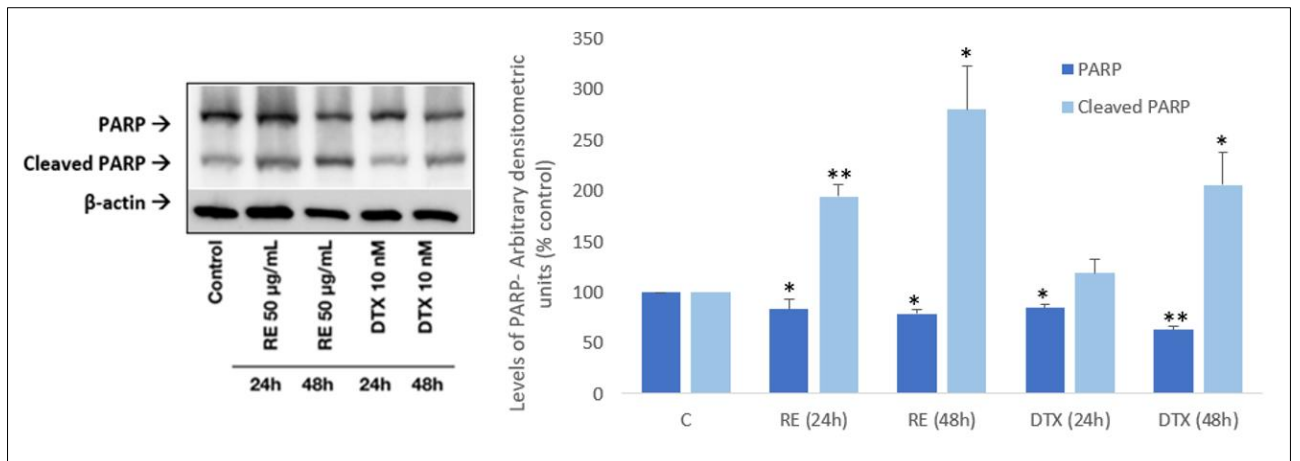
Treatment of 22Rv1 cells with DTX for 48h increased the levels of cleaved caspases 9 ( $204.8 \pm 30.61\%$  of control,  $p < 0.05$ ), cleaved caspase 7 (20 kDa):  $225.99 \pm 18.47$ ,  $p < 0.05$ ), and cleaved caspase 3 ( $289.39 \pm 35.66$ ,  $p < 0.01$ ) (Figure 19). Statistically significant changes after DTX treatment for 24h were only observed in cleaved caspase 3 ( $193.62 \pm 27.2\%$  of control,  $p < 0.05$ ) (Figure 19D), and not in cleaved caspase 9 ( $176.19 \pm 42.43\%$  of control,  $p > 0.05$ ) (Figure 19B) or cleaved caspase 7 ( $156.21 \pm 36.82\%$  of control,  $p > 0.05$ ) (Figure 19C). Overall, RE-treated 22Rv1 cells presented greater caspase cleavage compared to DTX-treated 22Rv1 cells at either time point.

Similarly, the levels of cleaved PARP in 22Rv1 cells were significantly increased after treatment with RE for 24h ( $194.56 \pm 11.75$ ,  $p < 0.01$ ) and 48h ( $280.55 \pm 43.31$ ,  $p < 0.05$ ), and after treatment with DTX for 48h ( $206.16 \pm 32.59$ ), but not for 24h ( $119.07 \pm 13.40$ ,  $p < 0.05$ ) (Figure 20).



**Figure 19: Effects of Rosemary extract (RE) on Caspases 3, 7, and 9 in 22Rv1 cells.**

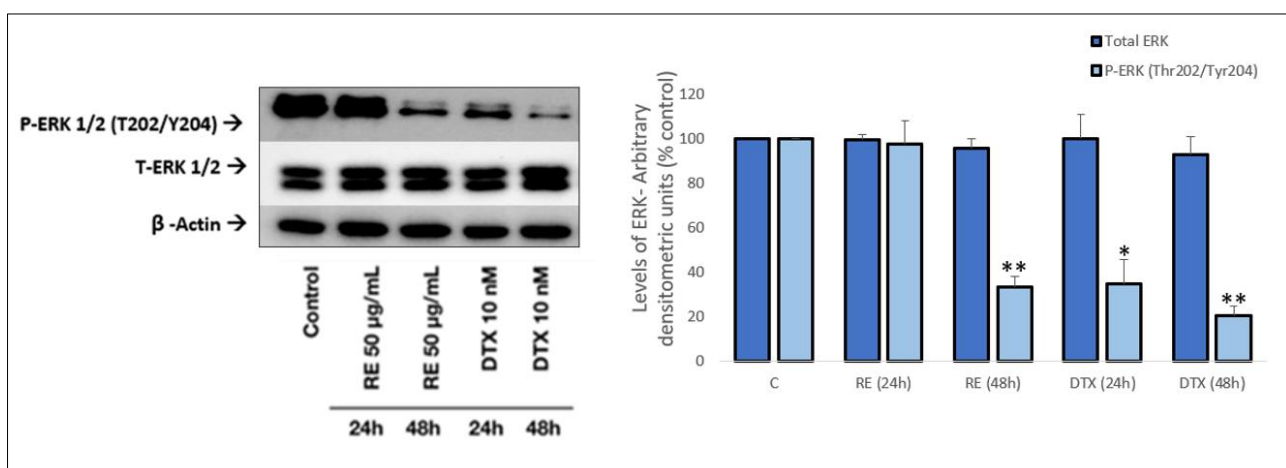
22Rv1 cells were treated with 50 µg/mL rosemary extract (RE) or 10 nM docetaxel for 24 or 48 hours and whole cell lysates were immunoblotted using specific antibodies for Caspase 9 (A, B), 7(A, C), and 3(A, D), or β-actin (A). Densitometric values were obtained with Imagej, corrected to β-actin levels, and expressed as a percentage (%) of the control. The data are the mean ± SEM of 3 independent experiments. \*p<0.05 \*\*p<0.01, \*\*\*p<0.001.



**Figure 20: Effects of Rosemary extract (RE) on PARP in 22Rv1 cells.** 22Rv1 cells were treated with 50 µg/mL rosemary extract (RE) or 10 nM docetaxel for 24 or 48 hours and whole cell lysates were immunoblotted using specific antibodies for PARP. Densitometric values were obtained with Imagej, corrected to β-actin levels, and expressed as a percentage (%) of the control. The data are the mean ± SEM of 3 independent experiments. \*p<0.05 \*\*p<0.01.

#### 4.8. Rosemary extract (RE) inhibits ERK 1/2 phosphorylation in 22Rv1 cells.

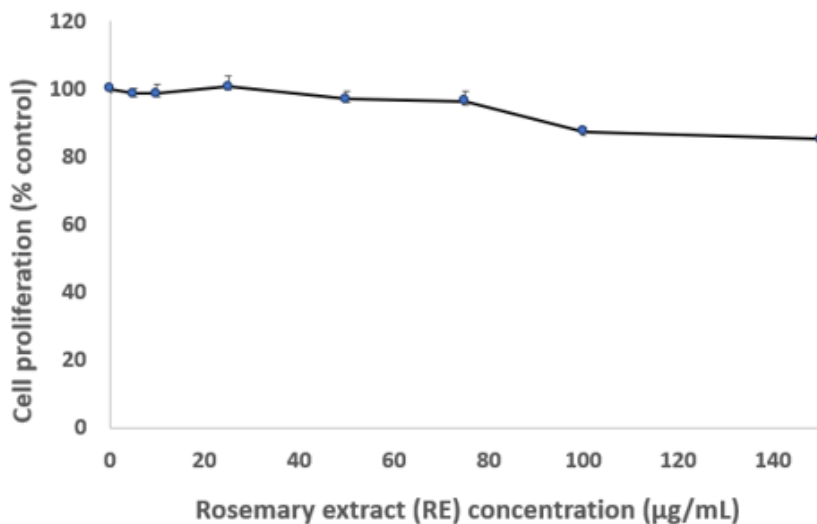
RE treatment of 22Rv1 cells did not affect the total levels of ERK 1/2 (Figure 21). Conversely to what was observed in PC-3 cells, RE significantly inhibited ERK phosphorylation (T202/Y204) after 48h of treatment ( $33.21 \pm 4.92\%$  of control,  $P < 0.01$ ) though statistically significant changes could not be observed after 24h ( $97.57 \pm 10.51\%$  of control,  $p > 0.05$ ). Similarly, levels of phosphorylated ERK (T202/Y204) were significantly decreased in DTX-treated 22Rv1 cells (DTX24:  $34.77 \pm 10.82\%$  of control,  $p < 0.05$ ; DTX48:  $20.61 \pm 4.19\%$  of control,  $p < 0.01$ ) (Figure 21).



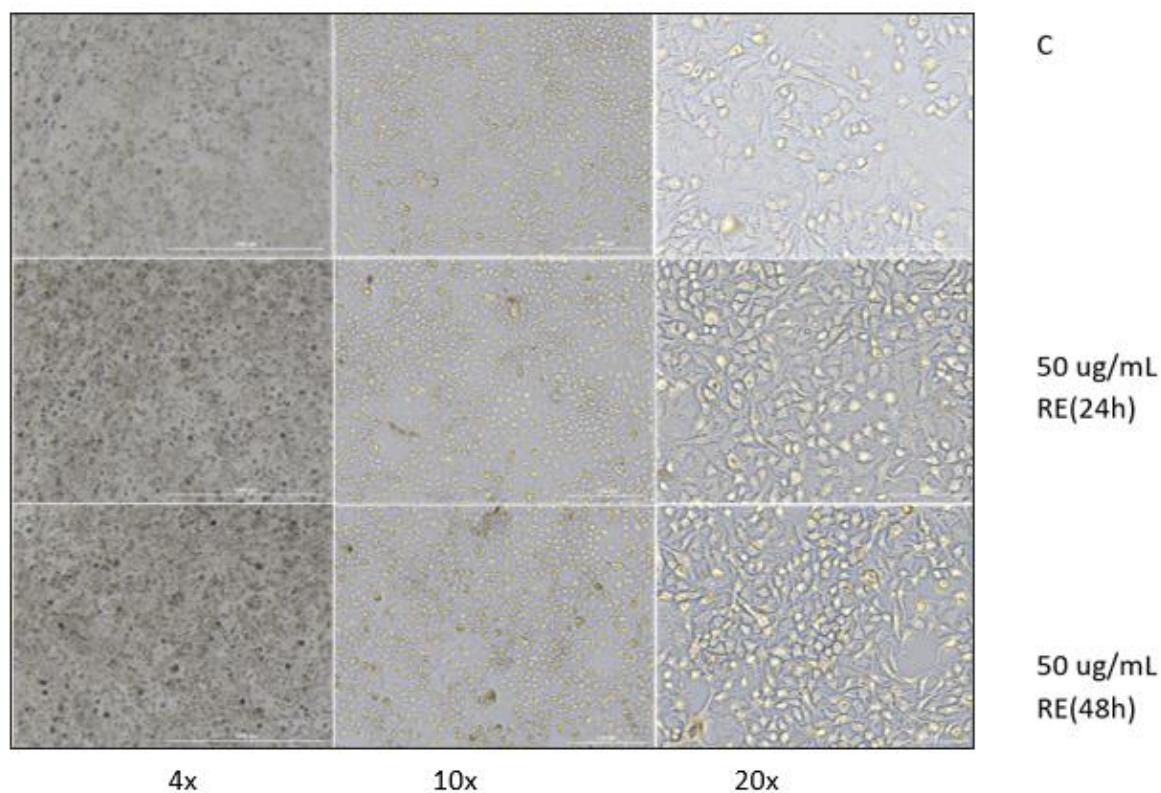
**Figure 21: Effects of Rosemary extract (RE) on ERK 1/2 in 22Rv1 cells.** 22Rv1 cells were treated with 50 µg/mL rosemary extract (RE) or 10 nM docetaxel for 24 or 48 hours and whole cell lysates were immunoblotted using specific antibodies for ERK 1/2 and P-ERK 1/2 (T202/Y204). Densitometric values were obtained with Imagej, corrected to β-actin levels, and expressed as a percentage (%) of the control. The data are the mean ± SEM of 3 independent experiments. \* $p < 0.05$  \*\* $p < 0.01$ .

#### 4.9. Rosemary extract (RE) does not inhibit the proliferation of PNT1A healthy prostate epithelial cells.

To assess whether RE could alter cell proliferation in non-cancerous prostate cells, the crystal violet assay was repeated in PNT1A healthy prostate epithelial cells treated with the same concentrations of RE utilized in PC-3 and 22Rv1 prostate cancer cells. RE did not significantly affect the proliferation of PNT1A non-cancerous epithelial cells, even at higher concentrations (100-150  $\mu\text{g}/\text{mL}$ ) (Figure 22). Furthermore, treated and untreated PNT1A cells were imaged utilizing a Cytation 5 machine (BioTek) to assess changes in cell density and morphology. It is important to note that cells were seeded at the same density as the control in each treatment group. Compared to PC-3 and 22Rv1 cells, 50  $\mu\text{g}/\text{mL}$  of RE did not affect cell density, nor cell morphology of PNT1A cells after treatment for either 24h or 48h (Figure 23).



**Figure 22: Rosemary extract (RE) did not inhibit proliferation of PNT1A normal epithelial cells.** PNT1A cells were treated with 0-150  $\mu\text{g}/\text{mL}$  RE for 72h followed by a proliferation assay. The data correspond to the mean  $\pm$  standard error of 5 independent experiments and are expressed as a percentage (%) of the control.

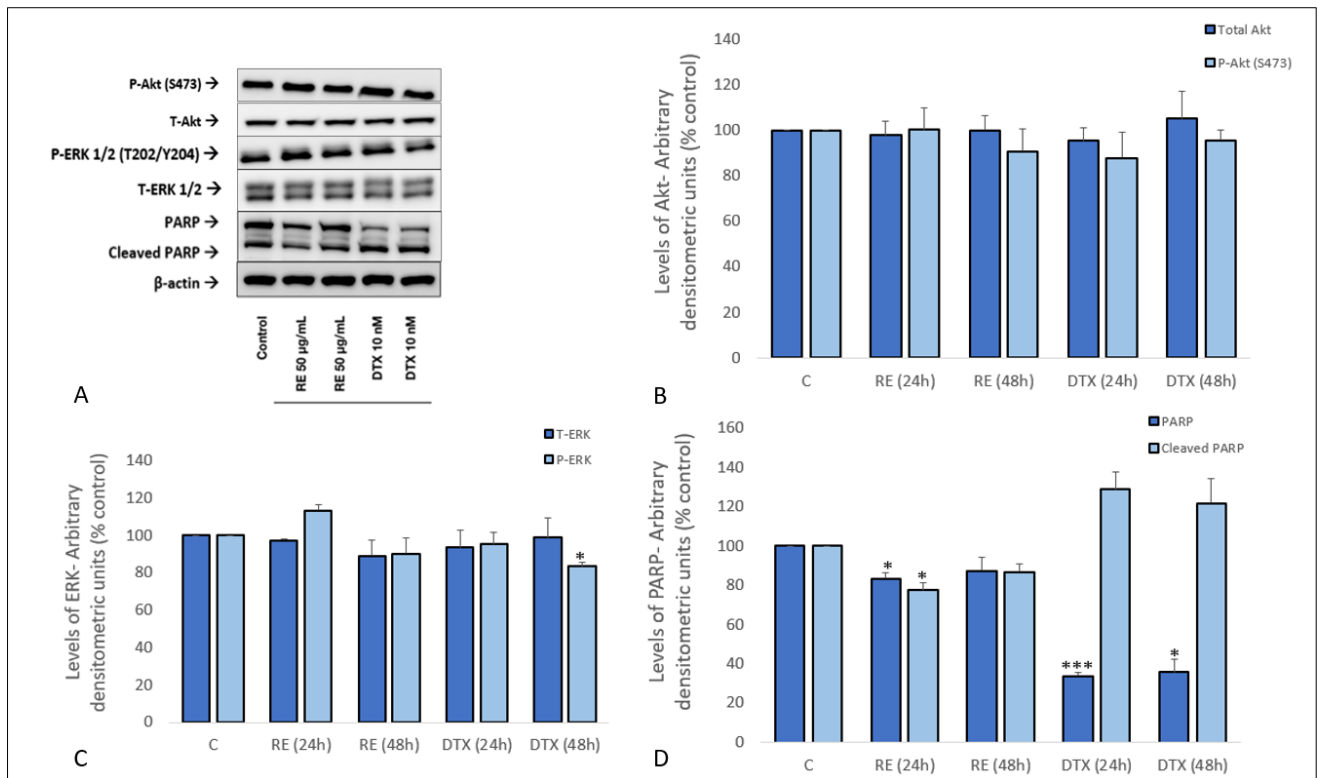


**Figure 23: Effects of rosemary extract (RE) on PNT1A cell morphology.** Cells were treated with 50  $\mu\text{g}/\text{mL}$  of RE for 24 or 48h, washed twice with PBS, and fixed for 15 minutes with 10% formalin. Photographs were obtained using a Cytation 5 imaging system by BioTek Instruments at 4x, 10x, and 20x magnification.

#### **4.10. Rosemary extract (RE) does not affect ERK and Akt in noncancerous PNT1A epithelial cell lines.**

Since RE did not impact the proliferation of healthy prostate epithelial PNT1A cells, we also looked at its effects on ERK and Akt as well as cleaved PARP (Figure 24). The data obtained indicated that after either 24h or 48h of treatment with RE, there were no significant changes in total Akt (RE24:  $97.82 \pm 6.22$  % of control; RE48:  $100.04 \pm 6.22$ % of control,  $p > 0.05$ ) (Figure 24B), total ERK 1/2 (RE24:  $97.4 \pm 0.51$ % of control; RE48:  $89.0 \pm 8.24$ % of control,  $p > 0.05$ ) (Figure 24C), or phosphorylated Akt (RE24:  $100.22 \pm 9.73$ % of control; RE48:  $90.6 \pm 9.83$ % of control,  $p > 0.05$ ) (Figure 24B) and ERK 1/2 (RE24:  $113.24 \pm 3.39$ % of control; RE48:  $90.27 \pm 8.61$ % of control,  $p > 0.05$ ) (Figure 24C). Similarly, phosphorylated Akt levels in DTX-treated PNT1A cells were not significantly different from the control untreated group (DTX24:  $87.81 \pm 11.20$ % of control; DTX48:  $95.58 \pm 4.71$ % of control,  $p > 0.05$ ) (Figure 24B). However, inhibition of phosphorylated ERK 1/2 could be observed in PNT1A cells treated with DTX for 48h (DTX48:  $83.53 \pm 2.05$ % of control,  $p < 0.05$ ) (Figure 24C).

The levels of cleaved and total PARP were reduced in PNT1A cells treated with RE for 24h (total:  $83.46 \pm 2.95$ % of control; cleaved:  $77.46 \pm 3.57$ % of control,  $p < 0.05$ ) but not for 48h (total:  $87.27 \pm 7.04$ % of control; cleaved:  $86.83 \pm 4.07$ % of control,  $p > 0.05$ ). Interestingly, the levels of total PARP were remarkably reduced in DTX-treated cells after 24h ( $33.71 \pm 1.90$ % of control,  $p < 0.001$ ) and 48h ( $35.66 \pm 6.87$ % of control,  $p < 0.01$ ), whereas levels of cleaved PARP remained unchanged (DTX24:  $128.69 \pm 8.88$ % of control; DTX48:  $121.78 \pm 12.45$ % of control,  $p > 0.05$ ) (Figure 24D).



**Figure 24: Effects of Rosemary extract (RE) on Akt, Erk 1/2, and PARP in non-cancerous PNT1A prostate epithelial cell lines.** PNT1A cells were treated with 50 µg/mL rosemary extract (RE) or 10 nM docetaxel for 24 or 48 hours and whole cell lysates were immunoblotted using specific antibodies for Akt, P-Akt (S473) (A, B), ERK 1/2, P-ERK 1/2 (T202/Y204) (A, C), or PARP (A, D). Densitometric values were obtained with Imagej, corrected to β-actin levels, and expressed as a percentage (%) of the control. The data are the mean ± SEM of 3 independent experiments. \*p<0.05 \*\*p<0.01, \*\*\*p<0.001.

**Table 4: Summary of the effects of rosemary extract (RE) in prostate cancer cells and PNT1A healthy epithelial cells.**

Cell Line	Mutations and representative PC subtype	RE Effects on Proliferation	RE Effects on Cell Signaling
PC-3	P53, PTEN null  Castration resistant  High invasiveness	↓ Cell proliferation/ Viability  ↓ Cell density  ↓ Colony formation efficiency	↑ Cleaved caspases 3,7,9  ≈ Cleaved caspase 8  ↑ Cleaved PARP  ≈ P-Akt (S473)  ↑ P-ERK (T202/Y204)  ↑ Intracellular ROS
22Rv1	P53 heterozygous mutation  Castration resistant  Low invasiveness	↓ Cell proliferation  ↓ Cell density  ↓ Colony formation efficiency	↑ Cleaved caspases 3,7,9  ↑ Cleaved PARP  ↓ P-ERK (T202/Y204)
PNT1A	N/A	≈ Cell proliferation  ≈ Cell density	≈ P-Akt (s473)  ≈ P-ERK (T202/Y204)  ↓ Total PARP  ↓ Cleaved PARP

## 5. Discussion:

### 5.1. Rosemary extract (RE) inhibits cell proliferation and cell survival/colony formation efficiency in PC-3 and 22Rv1 androgen independent prostate cancer cells

Prostate cancer is the second most frequent malignancy in men worldwide, accounting for 4% of all male cancer-related deaths. The prognosis for individuals diagnosed with the aggressive form of the disease, or castration-resistant prostate cancer, is generally very poor, with a mean survival of only 9-36 months depending on symptoms and site of metastasis (Heidenreich et al., 2014). Aggressive prostate cancer is also associated with high levels of chemotherapeutic resistance and poor response in affected individuals (Lohiya et al. 2016), indicating a need for better treatment strategies.

Plant-derived compounds are commonly utilized for the development of pharmaceuticals and chemotherapeutic agents, and it is estimated that around 25% of modern medicines may be directly or indirectly derived from plants (Samuelsson, 2004). Polyphenolic compounds found in herbs and foods have received attention in recent years due to their anti-cancer properties seen in *in vitro* and *in vivo* studies, as well as other health benefits (Cory et al., 2018). Rosemary is rich in polyphenols and was shown to possess anti-bacterial and antioxidant properties, but despite the available data, limited evidence exists on its anti-cancer properties in prostate cancer as well as the signaling pathways involved in its mechanism of action. Our study investigated the anti-cancer properties of rosemary extract (RE) in PC-3 and 22Rv1 prostate cancer cells, both representative androgen-independent models of prostate cancer characterized by different mutations and different genetic ancestry. Overall, although both cells lines are androgen-independent they are not the same and they represent different prostate cancers seen in patients. Intense investigations

of the subtypes of androgen-independent prostate cancer are required in order to identify the most effective therapeutic approaches.

The effects of RE on PNT1A healthy prostate epithelial cells were also assessed to identify potential cytotoxicity of the extract in non-tumorigenic tissue. Our study has shown that 50  $\mu\text{g}/\text{mL}$  of rosemary extract (RE) inhibited the proliferation of both PC-3 and 22Rv1 androgen-independent prostate cancer cells (Figure 7, Figure 16). The IC<sub>50</sub> values obtained were 26.94  $\mu\text{g}/\text{mL}$  for PC-3 cells and 69.919  $\mu\text{g}/\text{mL}$  for 22Rv1 cells. The MTT assay data also demonstrated that RE could dose-dependently inhibit PC-3 cell viability (Figure 9). The IC<sub>50</sub> value obtained for this assay was 35.141  $\mu\text{g}/\text{mL}$ , which is close to the 26.94  $\mu\text{g}/\text{mL}$  value obtained in the crystal violet assay. Interestingly, RE treatment even at high concentrations (100-150  $\mu\text{g}/\text{mL}$ ) did not affect the proliferation of PNT1A healthy prostate epithelial cells (Figure 22). These data indicate that RE inhibits prostate cancer cells, known to possess high proliferative rates, while it has no significant inhibitory effect on non-tumorigenic cells. Since the RE used in our study was dissolved in DMSO, we wanted to examine whether the anti-cancer effects observed were possibly due to DMSO and not due to RE alone. PC-3 cells treated with 0.05% DMSO (vehicle control) showed no significant difference in proliferation when compared to the untreated control (Figure 8). These data clearly indicate that the anticancer effects observed with RE treatment are due to RE components and not due to DMSO. It should be noted that DMSO is used routinely in Dr Tsiani's lab as most of the chemicals/polyphenols studied are prepared in DMSO stock. Previous studies performed by other students tested the same levels of DMSO using the same prostate cancer cells and found no effects (unpublished).

In the current studies we utilized the chemotherapeutic docetaxel (DTX) as a positive control since it is an established anticancer agent used in the treatment of prostate cancer. Our data indicate that treatment with RE has similar anticancer effects as treatment with DTX. It is known that chemotherapeutic drugs utilized in prostate cancer, though effective, exert a degree of toxicity in healthy tissue as well. Docetaxel, specifically, is known to cause side effects such as cutaneous and gastrointestinal toxicity, neuropathy, and pneumonitis (Ho et al., 2014). Our data indicated no inhibitory effects of RE on normal prostate epithelial PNT1A cells suggesting a lack of toxicity in healthy tissues. Treatment with RE may be a novel promising anti-cancer approach and future in vivo studies in animals and humans are warranted.

RE components, through unknown mechanisms, may promote anti-proliferative effects in prostate cancer cells through potential interaction with dysregulated signaling within the tumor environment. Yesil-Celiktas et al. (2010) found a decline in PC-3 cell viability following treatment with up to 50  $\mu\text{g}/\text{mL}$  of rosemary for 48h. Moreover, rosemary herbal infusions (0.2-1  $\mu\text{g}/\text{mL}$ ) also negatively impacted PC-3 cell growth (Kaliora et al., 2014). Inhibition of proliferation and decreased cell viability by RE in 22Rv1 (androgen independent) and LNCaP (androgen dependent) prostate cancer cells were previously associated with apoptosis as shown by increased levels of Bax and cleaved caspase-3, and with endoplasmic reticulum (ER) stress as increased levels of BiP and CHOP, key proteins in the unfolded protein response, were also observed (Petiwala et al., 2014). Similarly, prostate cancer cell viability and proliferation was reduced upon treatment with carnosic acid, a rosemary polyphenol, which also significantly increased apoptosis as shown by higher levels of cleaved caspases 8, 9, and PARP in PC-3 cells (Kar et al., 2012) and higher levels of caspase-3 in 22Rv1 and LNCaP cells (Petiwala et al., 2016). In addition, treatment of LNCaP prostate cancer cells with carnosol also reduced cell viability while inducing

apoptosis as shown by higher levels of cleaved caspase-3 (Jang et al., 2018; Johnson et al., 2010). This indicates that reduction of viability and cell proliferation exerted by treatment with RE or RE components may be related to the induction of apoptosis, and that similar effects may translate to androgen dependent prostate cancer cells as shown by increased apoptosis in LNCaP cells.

In a similar fashion to inhibition of proliferation, RE dose-dependently inhibited colony formation in PC-3 and 22Rv1 cells, with IC<sub>50</sub> values of 2.757 µg/mL and 4.8071 µg/mL, respectively (Figure 10, Figure 17). No other studies examining the effects of rosemary extract on prostate cancer cell survival have been published, though early evidence demonstrated that 20-40 µg/mL of rosemary extract could prevent colony formation in HT-29, SW480, and HGUE-C-1 colon cancer cells by promoting apoptosis and intracellular ROS generation (Pérez-Sánchez et al., 2019), indicating once again the possible relationship between RE treatment and activation of programmed cell death.

Interestingly, a more potent inhibitory effect of RE was detected in PC-3 cells (Proliferation assay IC<sub>50</sub>: 26.94 µg/mL; Clonogenic survival assay IC<sub>50</sub>: 2.757 µg/mL), considered representative of highly invasive small cell prostatic carcinoma, when compared to 22Rv1 androgen-sensitive cells (Proliferation assay IC<sub>50</sub>: 69.919 µg/mL; Survival assay IC<sub>50</sub>: 4.8071 µg/mL), representative of tumors with lower invasiveness. PC-3 cells present mutations in both PTEN and p53 tumor suppressor genes, whereas 22Rv1 cells are p53 heterozygous and fully express PTEN. In prostate cancer, loss of function of tumor suppressor genes PTEN and p53 are common and occur in around 70% and 6-36% of all human prostate cancers, respectively (Sircar et al., 2009; Teroerde et al., 2021). Both mutations are implicated in the formation and metastasis of aggressive prostate cancer as processes of apoptosis in DNA-damaged cells are derailed, promoting the accumulation of

additional genetic mutations (Cox, 1997). The differences in PTEN and p53 (considered a “master regulator” of biological processes) may, to an extent, explain the difference in RE-induced inhibition of cell proliferation and survival in the cancer cells examined. The lower levels of mutations in tumor suppressor genes in 22Rv1 cells may promote a relatively more homeostatic stable intracellular environment, while PC-3 cells have more mutations presenting higher genomic instability. RE did not affect the PNT1A normal epithelial cells. Overall, the data show that RE is more effective/targets cells with more dysregulated pathways and more aggressive phenotype.

## **5.2. Rosemary extract (RE) effects on prostate cancer signaling apoptosis**

Cleavage of caspase 9, and subsequent activation of caspases 3 and 7 are events associated with intrinsic programmed cell death, or mitochondria-mediated apoptosis, a process which is activated following an apoptosis stimulus such as DNA damage and oxidative stress (Elmore, 2007) (Figure 2). Evasion of apoptosis frequently occurs in cancer to promote cell survival and proliferation leading to cancer metastasis.

In our study, 50 µg/mL of rosemary extract (RE) was shown to promote apoptosis in androgen-independent, p53 and PTEN null PC-3 cells as shown by increased levels of cleaved caspase-9, -7 and -3 (Figure 12). Similarly, the levels of caspases were significantly enhanced in RE-treated 22Rv1 cells when compared to the untreated control group (Figure 19). Our data demonstrate cleavage of caspase 7 resulting in two cleaved products of 30 and 20 kDa in 22Rv1 cells (Figure 19) and one cleaved product of 30 kDa in PC-3 cells (Figure 12). This difference may be cell specific. As it is mentioned in the introduction of this thesis (page 32) Caspase-7 is a protein of 35 kDa that is activated through proteolytic processing that yields cleaved proteins of 20 and 30 kDa. The presence of either cleaved

product is established as a marker/indicator of caspase 7 activation. Overall, our data clearly demonstrate cleavage /activation of caspase 7 by RE treatment. Generally, cleaved caspase levels were higher in the 48h RE treatment groups when compared to the 24h RE treatment groups. In a similar fashion, the chemotherapeutic docetaxel promoted apoptosis in PC-3 and 22Rv1 cells as shown by increased levels of cleaved caspases (Figure 12, Figure 19). Interestingly, the data show a more robust caspase cleavage with RE treatment suggesting more potent pro-apoptotic effects in these cell lines when compared to DTX treatment (10 nM). RE did not promote cleavage of caspase-8 in PC-3 cells. Cleavage of caspase-8 is associated with activation of extrinsic apoptosis (Figure 12), and therefore these data suggest that RE-induced cell death in PC-3 cells may be promoted through activation of mitochondrial-dependent apoptosis.

In previous studies, caspase 3 was activated in 22Rv1 prostate cancer cells following treatment with RE (Petiwala et al., 2014). It is possible that the effects of RE on caspase cleavage is due to polyphenol components of RE. As mentioned previously, RE contains the polyphenols carnosic acid, and rosmarinic acid which have been shown to induce caspase cleavage. Caspase-9 cleavage was observed in PC-3 prostate adenocarcinoma cells, treated with carnosic acid (Kar et al., 2010). Furthermore, carnosic acid treatment promoted caspase-3 cleavage in LNCaP and PC-3 prostate adenocarcinoma cells (Kar et al., 2012; Petiwala et al., 2014). Caspase 7 activation was observed in PC-3 cells treated with carnosic acid for 0-72h (Kar et al., 2012).

The downstream target of effector caspase-3, poly (ADP-ribose) polymerase (PARP), functions as a DNA-repair protein in its uncleaved form (Herceg & Wang, 2001). When cleaved in late apoptosis, PARP loses the ability to perform base excision repair (Chaitanya et al., 2010). In our study, significant PARP cleavage was observed in both PC-3 and 22Rv1 cells treated with 50 µg/mL of RE (Figure 13, Figure 20). Similarly, previous studies by our

group have shown that rosemary extract and the rosemary polyphenols CA and RA could induce PARP cleavage in lung cancer cells (Moore et al., 2016). Interestingly, in the present studies, RE-treated PNT1A cells did not show a significant increase in PARP cleavage, if anything, PARP cleavage was reduced with 24h RE treatment (Figure 24). These data indicate that RE does not induce significant apoptosis in non-cancerous cells. We also observed a robust reduction in total PARP in DTX-treated cells and these data indicate that DTX may interfere with DNA repair in healthy prostate epithelial cells. The data suggest that RE may be better suited for targeted therapy and this justifies the need for more studies to investigate the anti-cancer effects of RE in vivo in animals and in humans.

### **5.3. Rosemary extract (RE) effects on prostate cancer signaling: PI3K/Akt and Ras/MAPK pathways, and oxidative stress.**

To determine whether RE may also target growth-factor activated signaling cascades, we investigated its effects on the activation of ERK 1/2, a downstream effector of the Ras/MAPK pathway, and Akt, a key member of the PI3k/Akt pathway. Activation of ERK 1/2 is generally stimulated by growth factors, hormones, and oxidative stress, and it is implicated in many cancers of epithelial origin, including prostate cancer. ERK 1/2 phosphorylation and activation at residues T202 and Y204 by MEK 1/2 is correlated, in most cases, to enhanced proliferation and survival, as well as cell differentiation and migration (Olea-Flores et al., 2019). Similarly, the PI3K/Akt pathway is often dysregulated in prostate cancer, promoting tumor growth and resistance to drug therapies. Akt phosphorylation at the S473 residue by mTORC2 is associated with increased cell survival, growth, and proliferation (Hart & Vogt, 2011; Shorning et al., 2020). Because of this, it was hypothesized that ERK 1/2 and Akt phosphorylation and activation in PC-3 and 22Rv1

prostate cancer cells would be reduced by RE treatment. A decrease in Akt phosphorylation was seen after treatment with RE for 24h but not 48h (Figure 14). Previous studies by our group found inhibition of Akt phosphorylation/ activation in RE-treated lung (Moore et al., 2016) and breast (Jaglanian & Tsiani, 2020) cancer cells. It is possible that RE inhibits Akt phosphorylation/activation and this leads to activation of compensatory mechanisms that tend to bring Akt activity back to elevated levels and that is the reason that we did not see an inhibition at 48h. A time course experiment should be performed in future studies to examine this possibility.

Our data further revealed that RE enhanced the levels of p-ERK 1/2 (T202/Y204) in PC-3 cells after 48h of treatment (Figure 14), whereas in 22Rv1 cells, p-ERK 1/2 levels were significantly reduced after 48h (Figure 21). The exact role of ERK 1/2 activation on cancer cell function is not clear. Generally, increased activation of the Ras/MAPK pathway, mainly due to Ras mutations/overactivation, is correlated to invasive prostate cancer, resistance to androgen deprivation therapy and poor prognosis (McCubrey et al., 2007; Rodríguez-Berriguete et al., 2012). It has been reported, though, that increased ERK phosphorylation/activation may be mediated by cellular stress and the presence of reactive oxygen species leading to cell death, senescence, intrinsic and extrinsic apoptosis, cell cycle arrest and autophagy (Cagnol & Chambard, 2010). In agreement with our data showing increased ERK 1/2 phosphorylation in RE-treated PC-3 cells, increased ERK 1/2 phosphorylation and apoptosis was observed in p53-null HeLa and HL-60 cancer cell lines following treatment with the anti-cancer drug cisplatin (Sheridan et al., 2010). Similarly, rosemary extract was shown to increase the levels of P-ERK 1/2 and induce apoptosis in HCT116 colorectal carcinoma cells (Yan et al., 2015). Furthermore, our data showed increased ROS after 24h of treatment but no significant increase in phosphorylated ERK levels, which were instead shown after 48h of treatment. This may be due to a time-

dependent effect, in which ROS activation may occur prior to ERK 1/2 phosphorylation. Performing time-course experiments to determine when, effectively, ERK 1/2 phosphorylation occurs, and when ROS levels start increasing, would contribute in elucidating this phenomenon further.

This data suggests that ERK inhibition by RE in 22Rv1 cells but not in PC-3 cells may be promoted by direct or indirect interaction with tumor suppressors PTEN and p53. PTEN is a known regulator of the PI3k/Akt pathway, but its role in the inhibition of ERK 1/2 signaling in cancer is not well established. Constitutively active ERK1/2 was observed in 22Rv1 cells in a previous study (Kharaziha et al., 2012), indicating that, despite the presence of wild type PTEN and heterozygous p53, increased survival and proliferation promoted by ERK 1/2 is still occurring.

Previous studies have reported that PTEN reconstitution in PTEN-null PC-3 and LNCaP prostate cancer cells reduced ERK 1/2 and Akt activity (Bouali et al., 2009; Chetram et al., 2011), whereas inhibition of PTEN by ROS-mediated inactivation increased phosphorylation of both kinases (Chetram et al., 2011). PTEN silencing was further shown to accelerate disease progression and MAPK signaling in HER2/neu breast carcinomas, whereas its restoration downregulated its activity (Ebbesen et al., 2016).

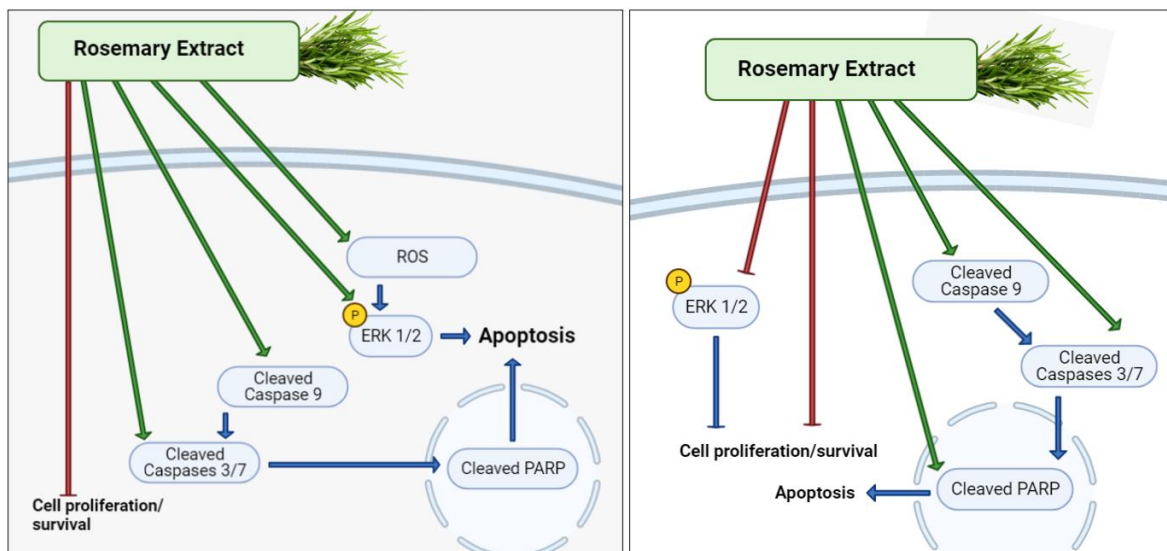
Other studies have shown that expression of the Ras/MAPK cascade was significantly higher in prostate cancer cells presenting only heterozygous mutations in the p53 gene (22Rv1) when compared to highly aggressive PC-3 cells, which overexpress Akt as a result to null mutations in p53 and PTEN. Akt overexpression is correlated with the downstream inactivation of Raf, which activates ERK 1/2, thus inhibiting cellular differentiation and promoting continuous proliferation in advanced prostate cancer (McCubrey et al., 2007).

This evidence may suggest a possible interaction of RE with either PTEN or p53 in 22Rv1 prostate cancer cells to promote ERK 1/2 inactivation and induction of apoptosis in

22Rv1 cells. Such interaction could further explain why decreased levels of phosphorylated ERK 1/2 were not observed in p53 and PTEN null PC-3 cells, but do not provide insight as to why ERK 1/2 activity was instead significantly enhanced in this highly aggressive representative subtype of prostate cancer.

Polyphenols and other plant-derived chemicals are known to elicit either pro-oxidant or anti-oxidant activity depending on the different intracellular environments encountered (Fernando et al., 2019; Guardado et al., 2012; Halliwell, 2007). Many studies observed increased ERK 1/2 phosphorylation, oxidative stress and apoptosis in cancer cells treated with plant-derived compounds. To investigate whether RE can promote ROS formation in PC-3 cells, we conducted a ROS assay utilizing the CellRox Green reagent. The data obtained showed increased levels of intracellular ROS following RE treatment for 24h and 48h in RE-treated PC-3 cells (Figure 15), providing early evidence of a possible relationship between oxidative stress and apoptosis in RE-treated PC-3 cells. Similarly to our data, triptolide, a diterpenoid found in the thunder god vine (*Tripterygium wilfordii*) was shown to induce apoptosis in MDA-MB-231 breast carcinoma cells that was correlated to phosphorylation of ERK 1/2, ER stress and increased reactive oxygen species levels (Tan & Tan, 2013). Thymoquinone, a phytochemical found in black seed oil, inhibited cell proliferation, induced apoptosis via ROS formation and activated the ERK 1/2 and JNK signaling cascades in DLD-1 osteosarcoma (El-Najjar et al., 2010). Etoposide, a chemotherapeutic drug derived from the podophyllotoxin in American Mayapple plants, promoted DNA damage through ROS generation, activation of ERK 1/2, and apoptosis in HK-2 human kidney proximal tubule cells (Shin et al., 2016). We found increased ROS production, increased ERK 1/2 and increased PC-3 prostate cancer cell apoptosis in the present studies. Based on our data and the evidence from triptolide (Tan & Tan, 2013), a chemical with structural similarities with polyphenols found in RE (all polyphenols have a

common phenol ring), we hypothesize that polyphenols or other phytochemicals within RE may activate a ROS-ERK-apoptosis cascade. Oxidative stress indicated by increased ROS levels in RE-treated PC-3 cells could lead to increased phosphorylation/activation of ERK 1/2 that leads to increased apoptosis. High levels of ROS after 24h of treatment further indicate that ROS induction may occur prior to increased ERK 1/2 phosphorylation. These findings are novel, and further studies are necessary to verify the pro-oxidant effects of RE in different prostate cancer cell lines. Furthermore, RE decreased ERK 1/2 activity in 22rv1 cells but we do not know if an increase in intracellular ROS may still occur, which remains subject of further investigation. The mechanism by which RE-induced apoptosis occurs in 22Rv1 cells may be different, driven by oxidative stress independently of ERK 1/2 activation, or driven by processes which do not involve increased oxidative stress at all.



**Figure 25: Summary of the effects of Rosemary extract (RE) in PC-3 (left) and 22Rv1 (right) prostate cancer cells.** The arrows indicate effects on the specific molecules examined. The effects observed could be direct or indirect. Green arrows indicate stimulation/activation, red arrows indicate inhibition, blue arrows indicate established signaling events. Images created with biorender.com

There is no available data on the effects of whole RE (and not individual polyphenols) on 22Rv1 and PC-3 prostate cancer cell proliferation and survival, thus making the findings novel. Rosemary extract is rich in polyphenols such as carnosol, carnosic acid, rosmarinic acid, ursolic acid and chlorogenic acid (de Oliveira et al., 2019).

In a previous study, it was shown that 200  $\mu$ M of rosmarinic acid (RA), a rosemary polyphenol, suppressed colony formation in PC-3 and DU-145 cells (Jang et al., 2018), thus providing early evidence on the possible role of rosemary as a chemopreventive agent. Additional studies conducted on PC-3 prostate cancer cells demonstrated that the rosemary polyphenols carnosic acid and carnosol could decrease cell viability (Johnson et al., 2008; Kar et al., 2012).

Future studies should focus on examining the components of RE responsible for its anticancer effects. It is possible that components of RE act synergistically to exert anticancer properties.

Conventional therapy in cancer treatment poses great challenges pertaining to high toxicity, severe detrimental effects, high costs and mechanisms of drug resistance, and the relatively novel role of phytochemicals in cancer treatment due to their potent anti-proliferative and pro-apoptotic properties has attracted scientists to investigate combination therapies to stimulate higher inhibitory effects or potential sensitization to conventional drugs. Combination treatment of phytochemicals such as resveratrol, EGCG, curcumin, and quercetin with established drugs including oxaliplatin, paclitaxel, cisplatin and doxorubicin promoted synergistic effects that were higher than either compound utilized alone, indicating the beneficial role of plant-derived compounds in the treatment of cancer and in sensitization of cancer to established drugs (Rizeq et al., 2020). Similarly, combination

treatment of rosemary extract with cisplatin, doxorubicin, paclitaxel, tamoxifen, and vinblastine exerted great anti-cancer effects in *in vitro* and *in vivo* (González-Vallinas et al., 2015). Rosemary extract and its components may enhance the effects of current drugs used in prostate cancer treatment (e.g. docetaxel, doxorubicin, paclitaxel, and more), therefore future studies should examine this possibility.

## **6. Conclusions:**

The data of this thesis demonstrate anti-cancer effects of RE in prostate cancer cells and no effects on normal epithelial cells. Table 4 and Figure 25 summarize the findings. Treatment of prostate cancer cells resulted in significant inhibition of proliferation and survival, and induction of apoptosis. In PC-3 cells, we found increased ROS levels and increased ERK 1/2 phosphorylation, as well as increased caspase and PARP cleavage with RE treatment. This indicates that RE may stimulate a ROS-ERK1/2-apoptosis pathway. The data warrants further investigation to support this hypothesis. In 22Rv1 cells, we found increased caspase and PARP cleavage with RE treatment, as well as inhibition of ERK 1/2 phosphorylation. This indicates that RE may act differently to promote apoptosis in 22Rv1 cells compared to PC-3 cells, and further investigation is required to better understand the signaling mechanisms involved.

## **7: Limitations and future directions:**

The study conducted presents some limitations that must be acknowledged. First, anti-cancer effects of a novel compound in cancer cell lines cannot necessarily be translated to more complex models of the disease, though *in vitro* experimental designs are helpful in establishing proof-of-principle. Cell lines represent useful, relatively inexpensive models that have been well established over the years, but they poorly mimic the behaviour

of primary tumors as they do not grow under physiologically relevant conditions. For example, the physioxic environment in which cancer cells proliferate and thrive in living organisms (1-6% O<sub>2</sub>) is absent when in vitro culturing is performed, unless appropriate hypoxia chambers are utilized to maintain such conditions (Stuart et al., 2018). Additionally, it has been well-established that commercial media utilized in cell culture contains a significantly higher concentration of glucose compared to what can be found within the tumor microenvironment (Chang et al., 2015; Ishida et al., 2016). Nonetheless, some of the advantages of cell culture include consistency and fast data reproducibility, helpful in establishing dose-responses and dose toxicity.

In this study, rosemary extract was shown to induce caspase cleavage in both PC-3 and 22Rv1 androgen-independent prostate cancer cells. Despite this, further confirmation of apoptosis through Annexin/PI staining and flow cytometric analysis should be attained, and other signaling molecules upstream of the apoptotic signaling pathway such as IAPs, FOXO, PUMA and NOXA should be investigated in the attempt to further elucidate the mechanism behind RE-induced apoptosis. It is also important to assess whether RE, other than in PC-3 cells, may also increase ROS levels in 22Rv1 cells or in other prostate cancer cell lines to determine whether oxidative stress may play a role in RE-induced apoptosis. Additionally, the effects seen with increased ROS after 24h of RE treatment but no significant increase in P-ERK 1/2 until 48h post-treatment may indicate a time-dependent effect (or delayed response). Performing a time-course experiment to determine when, effectively, ERK phosphorylation occurs, and when ROS levels start increasing, should be the focus of future studies.

Our studies showed induction of apoptosis and inhibition of cell proliferation in PC-3 and 22Rv1 cell lines. Despite this, further studies indicating whether RE truly affects cell proliferation and does not, instead, simply promote cytotoxicity should be conducted.

Besides assessing overall metabolic activity, measuring BrdU (a thymidine analog) incorporation in the DNA, or the proliferating cell nuclear antigen (PCNA) marker may provide a more accurate indication of the extent of cell proliferation. Additionally, exploring changes in other signaling molecules involved in the regulation of cell proliferation and survival would be beneficial.

It is noted that the anti-proliferative and pro-apoptotic effects of RE in this study were only assessed in *in vitro* models of androgen-independent prostate cancer, therefore they are currently unapplicable to other representative subtypes such as androgen-dependent LNCaP prostate cancer cells. Gaining a better understanding of how RE may affect apoptosis and inhibit proliferation within different prostate cancer cells is required for future *in vivo* studies examining its effects in more complex models of the disease to determine effective doses, toxicity, and address issues such as bioavailability.

The specific compounds within RE that elicit increased apoptosis and decreased cell proliferation in prostate cancer cells are not known, and based on the evidence showing anticancer effects of RE polyphenols (Moore et al., 2016), we can speculate that these effects may be attributable to polyphenols found within the extract. Levels of carnosic acid and rosmarinic acid in RE were previously measured in our lab, and it was found that it contained up to  $2.12 \pm 0.22\%$  carnosic acid and  $13.39 \pm 0.23\%$  rosmarinic acid (Naimi et al., 2017; Vlavecski et al., 2017). It is possible that carnosic acid and/or rosmarinic acid and/or other compounds such as ursolic acid, carnosol, and quercetin found in rosemary may alone or in combination (synergistically) contribute to its anticancer properties. Future studies should explore this issue.

## 7. References:

- Aaron, L., Franco, O., & Hayward, S. W. (2016). Review of Prostate Anatomy and Embryology and the Etiology of BPH. *The Urologic Clinics of North America*, 43(3), 279–288. <https://doi.org/10.1016/j.ucl.2016.04.012>
- Abal M, Andreu JM, Barasoain I. (2003). Taxanes: microtubule and centrosome targets, and cell cycle dependent mechanisms of action. *Curr Cancer Drug Targets*. 3(3):193-203. doi: 10.2174/1568009033481967. PMID: 12769688.
- Adams, J. M., & Cory, S. (2007). The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene*, 26(9), 1324–1337. <https://doi.org/10.1038/sj.onc.1210220>
- Adjei, A. A. (2001). Blocking oncogenic Ras signaling for cancer therapy. *Journal of the National Cancer Institute*, 93(14), 1062–1074. <https://doi.org/10.1093/jnci/93.14.1062>
- Ali, A., & Kulik, G. (2021). Signaling Pathways That Control Apoptosis in Prostate Cancer. *Cancers*, 13(5), 937. <https://doi.org/10.3390/cancers13050937>
- Artandi, S. E., & DePinho, R. A. (2010). Telomeres and telomerase in cancer. *Carcinogenesis*, 31(1), 9–18. <https://doi.org/10.1093/carcin/bgp268>
- Aubrey, B. J., Kelly, G. L., Janic, A., Herold, M. J., & Strasser, A. (2018). How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression? *Cell Death & Differentiation*, 25(1), 104–113. <https://doi.org/10.1038/cdd.2017.169>
- Avruch, J., Khokhlatchev, A., Kyriakis, J. M., Luo, Z., Tzivion, G., Vavvas, D., & Zhang, X. F. (2001). Ras activation of the Raf kinase: Tyrosine kinase recruitment of the MAP kinase cascade. *Recent Progress in Hormone Research*, 56, 127–155. <https://doi.org/10.1210/rp.56.1.127>
- Bai, L., & Zhu, W. (n.d.). p53: Structure, function and therapeutic applications. *J Cancer Molecules*. 2006;2:141–153. 367TCTP in ovarian cancer cell lines ©Polish Society for Histochemistry and Cytochemistry *Folia Histochem Cytobiol*.

- Banerjee, S., Singh, S. K., Chowdhury, I., Lillard, J. W., Jr, & Singh, R. (2017). Combinatorial effect of curcumin with docetaxel modulates apoptotic and cell survival molecules in prostate cancer. *Frontiers in bioscience (Elite edition)*, 9, 235–245. <https://doi.org/10.2741/e798>
- Barbacid, M. (1987). Ras genes. *Annual Review of Biochemistry*, 56, 779–827. <https://doi.org/10.1146/annurev.bi.56.070187.004023>
- Bertram, J. S. (2000). The molecular biology of cancer. *Molecular Aspects of Medicine*, 21(6), 167–223. [https://doi.org/10.1016/s0098-2997\(00\)00007-8](https://doi.org/10.1016/s0098-2997(00)00007-8)
- Berx, G., & van Roy, F. (2009). Involvement of Members of the Cadherin Superfamily in Cancer. *Cold Spring Harbor Perspectives in Biology*, 1(6). <https://doi.org/10.1101/cshperspect.a003129>
- Bhowmick, N. A., Neilson, E. G., & Moses, H. L. (2004). Stromal fibroblasts in cancer initiation and progression. *Nature*, 432(7015), 332–337. <https://doi.org/10.1038/nature03096>
- Biegging, K. T., Mello, S. S., & Attardi, L. D. (2014). Unravelling mechanisms of p53-mediated tumour suppression. *Nature Reviews. Cancer*, 14(5), 359–370. <https://doi.org/10.1038/nrc3711>
- Bill-Axelsson, A., Holmberg, L., Garmo, H., Rider, J. R., Taari, K., Busch, C., Nordling, S., Häggman, M., Andersson, S.-O., Spångberg, A., Andrén, O., Palmgren, J., Steineck, G., Adami, H.-O., & Johansson, J.-E. (2014). Radical Prostatectomy or Watchful Waiting in Early Prostate Cancer. *New England Journal of Medicine*, 370(10), 932–942. <https://doi.org/10.1056/NEJMoa1311593>
- Blasco, M. A. (2005). Telomeres and human disease: Ageing, cancer and beyond. *Nature Reviews. Genetics*, 6(8), 611–622. <https://doi.org/10.1038/nrg1656>

- Boehm, J. S., Zhao, J. J., Yao, J., Kim, S. Y., Firestein, R., Dunn, I. F., Sjostrom, S. K., Garraway, L. A., Weremowicz, S., Richardson, A. L., Greulich, H., Stewart, C. J., Mulvey, L. A., Shen, R. R., Ambrogio, L., Hirozane-Kishikawa, T., Hill, D. E., Vidal, M., Meyerson, M., ... Hahn, W. C. (2007). Integrative genomic approaches identify IKBKE as a breast cancer oncogene. *Cell*, *129*(6), 1065–1079. <https://doi.org/10.1016/j.cell.2007.03.052>
- Bordeira Gaspar, T., Sá, A., Lopes, J. M., Sobrinho-Simões, M., Soares, P., & Vinagre, J. (2018). Telomere Maintenance Mechanisms in Cancer. *Genes*, *9*(5). <https://doi.org/10.3390/genes9050241>
- Bouali S, Chrétien AS, Ramacci C, Rouyer M, Becuwe P, Merlin JL. (2009). PTEN expression controls cellular response to cetuximab by mediating PI3K/AKT and RAS/RAF/MAPK downstream signaling in KRAS wild-type, hormone refractory prostate cancer cells. *Oncol Rep.* Mar;21(3):731-5. PMID: 19212633.
- Bourhia, M., Laasri, F. E., Aourik, H., Boukhris, A., Ullah, R., Bari, A., Ali, S. S., El Mzibri, M., Benbacer, L., & Gmouh, S. (2019). Antioxidant and Antiproliferative Activities of Bioactive Compounds Contained in Rosmarinus officinalis Used in the Mediterranean Diet. *Evidence-Based Complementary and Alternative Medicine: ECAM*, 2019, 7623830. <https://doi.org/10.1155/2019/7623830>
- Bozin, B., Mimica-Dukic, N., Samojlik, I., & Jovin, E. (2007). Antimicrobial and antioxidant properties of rosemary and sage (*Rosmarinus officinalis* L. and *Salvia officinalis* L., Lamiaceae) essential oils. *Journal of Agricultural and Food Chemistry*, *55*(19), 7879–7885. <https://doi.org/10.1021/jf0715323>
- Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide

- for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, 68(6), 394–424.  
<https://doi.org/10.3322/caac.21492>
- Brenner, D. R., Weir, H. K., Demers, A. A., Ellison, L. F., Louzado, C., Shaw, A., Turner, D., Woods, R. R., & Smith, L. M. (2020). Projected estimates of cancer in Canada in 2020. *CMAJ: Canadian Medical Association Journal*, 192(9), E199–E205.  
<https://doi.org/10.1503/cmaj.191292>
- Bryce, A. H., & Antonarakis, E. S. (2016). Androgen receptor splice variant 7 in castration-resistant prostate cancer: Clinical considerations. *International Journal of Urology: Official Journal of the Japanese Urological Association*, 23(8), 646–653.  
<https://doi.org/10.1111/iju.13134>
- Bumbaca, B., & Li, W. (2018). Taxane resistance in castration-resistant prostate cancer: Mechanisms and therapeutic strategies. *Acta Pharmaceutica Sinica B*, 8(4), 518–529.
- Burkhardt, D. L., & Sage, J. (2008). Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nature Reviews. Cancer*, 8(9), 671–682.  
<https://doi.org/10.1038/nrc2399>
- Cai, J., Yang, J., & Jones, D. P. (1998). Mitochondrial control of apoptosis: The role of cytochrome c. *Biochimica Et Biophysica Acta*, 1366(1–2), 139–149.  
[https://doi.org/10.1016/s0005-2728\(98\)00109-1](https://doi.org/10.1016/s0005-2728(98)00109-1)
- Cagnol, S., & Chambard, J.-C. (2010). ERK and cell death: Mechanisms of ERK-induced cell death - apoptosis, autophagy and senescence: ERK and cell death. *FEBS Journal*, 277(1), 2–21. <https://doi.org/10.1111/j.1742-4658.2009.07366.x>
- Canadian-Cancer-Statistics-2017-EN.pdf*. (n.d.). Retrieved June 24, 2020, from <https://www.cancer.ca/~media/cancer.ca/CW/cancer%20information/cancer%20101/Canadian%20cancer%20statistics/Canadian-Cancer-Statistics-2017-EN.pdf>

- Carmeliet, P. (2005). VEGF as a key mediator of angiogenesis in cancer. *Oncology*, *69 Suppl* 3, 4–10. <https://doi.org/10.1159/000088478>
- Carroll, A. G., Voeller, H. J., Sugars, L., & Gelmann, E. P. (1993). P53 oncogene mutations in three human prostate cancer cell lines. *The Prostate*, *23*(2), 123–134. <https://doi.org/10.1002/pros.2990230206>
- Cavallaro, U., & Christofori, G. (2004). Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nature Reviews. Cancer*, *4*(2), 118–132. <https://doi.org/10.1038/nrc1276>
- Cha, H. R., Lee, J. H., & Ponnazhagan, S. (2020). Revisiting Immunotherapy: A Focus on Prostate Cancer. *Cancer research*, *80*(8), 1615–1623. <https://doi.org/10.1158/0008-5472.CAN-19-2948>
- Chaitanya, G. V., Steven, A. J., & Babu, P. P. (2010). PARP-1 cleavage fragments: signatures of cell-death proteases in neurodegeneration. *Cell communication and signaling : CCS*, *8*, 31. <https://doi.org/10.1186/1478-811X-8-31>
- Chan, S. R. W. L., & Blackburn, E. H. (2004). Telomeres and telomerase. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *359*(1441), 109–121.
- Chang, C. H., Qiu, J., O'Sullivan, D., Buck, M. D., Noguchi, T., Curtis, J. D., Chen, Q., Gindin, M., Gubin, M. M., van der Windt, G. J., Tonc, E., Schreiber, R. D., Pearce, E. J., & Pearce, E. L. (2015). Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. *Cell*, *162*(6), 1229–1241. <https://doi.org/10.1016/j.cell.2015.08.016>
- Chang, F., Lee, J. T., Navolanic, P. M., Steelman, L. S., Shelton, J. G., Blalock, W. L., Franklin, R. A., & McCubrey, J. A. (2003). Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: A target for cancer chemotherapy. *Leukemia*, *17*(3), 590–603. <https://doi.org/10.1038/sj.leu.2402824>

- Chen, C. D., & Sawyers, C. L. (2002). NF-kappa B activates prostate-specific antigen expression and is upregulated in androgen-independent prostate cancer. *Molecular and Cellular Biology*, 22(8), 2862–2870. <https://doi.org/10.1128/mcb.22.8.2862-2870.2002>
- Chen, F., & Zhao, X. (2013). Prostate Cancer: Current Treatment and Prevention Strategies. *Iranian Red Crescent Medical Journal*, 15(4), 279–284. <https://doi.org/10.5812/ircmj.6499>
- Chen J, Jin S, Abraham V, Huang X, Liu B, Mitten MJ, Nimmer P, Lin X, Smith M, Shen Y, Shoemaker AR, Tahir SK, Zhang H, Ackler SL, Rosenberg SH, Maecker H, Sampath D, Levenson JD, Tse C, Elmore SW. (2011). The Bcl-2/Bcl-X(L)/Bcl-w inhibitor, navitoclax, enhances the activity of chemotherapeutic agents in vitro and in vivo. *Mol Cancer Ther*. 10(12):2340-9. doi: 10.1158/1535-7163.MCT-11-0415. Epub 2011 Sep 13. PMID: 21914853
- Cheng, N., Chytil, A., Shyr, Y., Joly, A., & Moses, H. L. (2008). Transforming growth factor-beta signaling-deficient fibroblasts enhance hepatocyte growth factor signaling in mammary carcinoma cells to promote scattering and invasion. *Molecular Cancer Research: MCR*, 6(10), 1521–1533. <https://doi.org/10.1158/1541-7786.MCR-07-2203>
- Chetram MA, Odero-Marrah V, Hinton CV. (2011). Loss of PTEN permits CXCR4-mediated tumorigenesis through ERK1/2 in prostate cancer cells. *Mol Cancer Res*. Jan;9(1):90-102. doi: 10.1158/1541-7786.MCR-10-0235. Epub 2010 Nov 12. PMID: 21076047; PMCID: PMC3443870.
- Chetram MA, Don-Salu-Hewage AS, Hinton CV. (2011). ROS enhances CXCR4-mediated functions through inactivation of PTEN in prostate cancer cells. *Biochem Biophys Res*

- Commun.* Jul 1;410(2):195-200. doi: 10.1016/j.bbrc.2011.05.074. Epub 2011 May 24.  
PMID: 21627959; PMCID: PMC3163383.
- Chetram, M. A., & Hinton, C. V. (2012). PTEN regulation of ERK1/2 signaling in cancer. *Journal of receptor and signal transduction research*, 32(4), 190–195.  
<https://doi.org/10.3109/10799893.2012.695798>
- Chirumbolo, S., Bjørklund, G., Lysiuk, R., Vella, A., Lenchyk, L., & Upyr, T. (2018). Targeting Cancer with Phytochemicals via Their Fine Tuning of the Cell Survival Signaling Pathways. *International journal of molecular sciences*, 19(11), 3568.  
<https://doi.org/10.3390/ijms19113568><https://doi.org/10.1016/j.apsb.2018.04.007>
- Chong, S. J. F., Iskandar, K., Lai, J. X. H., Qu, J., Raman, D., Valentin, R., Herbaux, C., Collins, M., Low, I. C. C., Loh, T., Davids, M., & Pervaiz, S. (2020). Serine-70 phosphorylated Bcl-2 prevents oxidative stress-induced DNA damage by modulating the mitochondrial redox metabolism. *Nucleic Acids Research*, 48(22), 12727–12745.  
<https://doi.org/10.1093/nar/gkaa1110>
- Chu, W.-M. (2013). Tumor necrosis factor. *Cancer Letters*, 328(2), 222–225.  
<https://doi.org/10.1016/j.canlet.2012.10.014>
- Collado, M., & Serrano, M. (2010). Senescence in tumours: Evidence from mice and humans. *Nature Reviews. Cancer*, 10(1), 51–57. <https://doi.org/10.1038/nrc2772>
- Cooper, G. M., & Cooper, G. M. (2000). *The Cell* (2nd ed.). Sinauer Associates.
- Cordon-Cardo, C., Koff, A., Drobnjak, M., Capodiecici, P., Osman, I., Millard, S. S., Gaudin, P. B., Fazzari, M., Zhang, Z. F., Massague, J., & Scher, H. I. (1998). Distinct altered patterns of p27KIP1 gene expression in benign prostatic hyperplasia and prostatic

- carcinoma. *Journal of the National Cancer Institute*, 90(17), 1284–1291.  
<https://doi.org/10.1093/jnci/90.17.1284>
- Correia, C., Lee, S. H., Meng, X. W., Vincelette, N. D., Knorr, K. L., Ding, H., Nowakowski, G. S., Dai, H., & Kaufmann, S. H. (2015). Emerging understanding of Bcl-2 biology: Implications for neoplastic progression and treatment. *Biochimica et biophysica acta*, 1853(7), 1658–1671. <https://doi.org/10.1016/j.bbamcr.2015.03.012>
- Cory, H., Passarelli, S., Szeto, J., Tamez, M., & Mattei, J. (2018). The Role of Polyphenols in Human Health and Food Systems: A Mini-Review. *Frontiers in nutrition*, 5, 87.  
<https://doi.org/10.3389/fnut.2018.00087>
- Cox, L. S. (1997). Multiple pathways control cell growth and transformation: Overlapping and independent activities of p53 and p21Cip1/WAF1/Sdi1. *The Journal of Pathology*, 183(2), 134–140. [https://doi.org/10.1002/\(SICI\)1096-9896\(199710\)183:2<134::AID-PATH960>3.0.CO;2-D](https://doi.org/10.1002/(SICI)1096-9896(199710)183:2<134::AID-PATH960>3.0.CO;2-D)
- Cristofani, R., Montagnani Marelli, M., Cicardi, M. E., Fontana, F., Marzagalli, M., Limonta, P., Poletti, A., & Moretti, R. M. (2018). Dual role of autophagy on docetaxel-sensitivity in prostate cancer cells. *Cell Death & Disease*, 9(9), 889.  
<https://doi.org/10.1038/s41419-018-0866-5>
- Davey, R. A., & Grossmann, M. (2016). Androgen Receptor Structure, Function and Biology: From Bench to Bedside. *The Clinical Biochemist Reviews*, 37(1), 3–15.
- Degeorges A, Hoffschir F, Cussenot O, Gauville C, Le Duc A, Dutrillaux B, Calvo F. (1995). Recurrent cytogenetic alterations of prostate carcinoma and amplification of c-myc or epidermal growth factor receptor in subclones of immortalized PNT1 human prostate epithelial cell line. *Int J Cancer*. Sep 15;62(6):724-31. doi: 10.1002/ijc.2910620613. PMID: 7558421.

- DeNardo, D. G., Andreu, P., & Coussens, L. M. (2010). Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Reviews*, 29(2), 309–316. <https://doi.org/10.1007/s10555-010-9223-6>
- Deng, X., Xiao, L., Lang, W., Gao, F., Ruvolo, P., & May, W. S. (2001). Novel Role for JNK as a Stress-activated Bcl2 Kinase. *Journal of Biological Chemistry*, 276(26), 23681–23688. <https://doi.org/10.1074/jbc.M100279200>
- Devarajan, E., Sahin, A. A., Chen, J. S., Krishnamurthy, R. R., Aggarwal, N., Brun, A.-M., Sapino, A., Zhang, F., Sharma, D., Yang, X.-H., Tora, A. D., & Mehta, K. (2002). Down-regulation of caspase 3 in breast cancer: A possible mechanism for chemoresistance. *Oncogene*, 21(57), 8843–8851. <https://doi.org/10.1038/sj.onc.1206044>
- Ebbesen, S. H., Scaltriti, M., Bialucha, C. U., Morse, N., Kasthuber, E. R., Wen, H. Y., Dow, L. E., Baselga, J., & Lowe, S. W. (2016). Pten loss promotes MAPK pathway dependency in HER2/neu breast carcinomas. *Proceedings of the National Academy of Sciences*, 113(11), 3030–3035. <https://doi.org/10.1073/pnas.1523693113>
- Eder, I. E., Culig, Z., Putz, T., Nessler-Menardi, C., Bartsch, G., & Klocker, H. (2001). Molecular biology of the androgen receptor: From molecular understanding to the clinic. *European Urology*, 40(3), 241–251. <https://doi.org/10.1159/000049782>
- El-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., & Vogelstein, B. (1992). Definition of a consensus binding site for p53. *Nature Genetics*, 1(1), 45–49. <https://doi.org/10.1038/ng0492-45>
- El-Najjar, N., Chatila, M., Moukadem, H., Vuorela, H., Ocker, M., Gandesiri, M., Schneider-Stock, R., & Gali-Muhtasib, H. (2010). Reactive oxygen species mediate thymoquinone-induced apoptosis and activate ERK and JNK signaling. *Apoptosis*, 15(2), 183–195. <https://doi.org/10.1007/s10495-009-0421-z>

- Elmore, S. (2007). Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology*, 35(4), 495–516. <https://doi.org/10.1080/01926230701320337>
- Esteller, M. (2007). Cancer epigenomics: DNA methylomes and histone-modification maps. *Nature Reviews. Genetics*, 8(4), 286–298. <https://doi.org/10.1038/nrg2005>
- Estévez, M., Ramírez, R., Ventanas, S., & Cava, R. (2007). Sage and rosemary essential oils versus BHT for the inhibition of lipid oxidative reactions in liver pâté. *Food Science and Technology*. <https://agris.fao.org/agris-search/search.do?recordID=US201300803821>
- Estrada, M., Espinosa, A., Müller, M., & Jaimovich, E. (2003). Testosterone stimulates intracellular calcium release and mitogen-activated protein kinases via a G protein-coupled receptor in skeletal muscle cells. *Endocrinology*, 144(8), 3586–3597. <https://doi.org/10.1210/en.2002-0164>
- Evan, G. I., & Vousden, K. H. (2001). Proliferation, cell cycle and apoptosis in cancer. *Nature*, 411(6835), 342–348. <https://doi.org/10.1038/35077213>
- Fernando, W., Rupasinghe, H. P. V., & Hoskin, D. W. (2019). Dietary phytochemicals with anti-oxidant and pro-oxidant activities: A double-edged sword in relation to adjuvant chemotherapy and radiotherapy? *Cancer Letters*, 452, 168–177. <https://doi.org/10.1016/j.canlet.2019.03.022>
- Fidler, I. J. (2003). The pathogenesis of cancer metastasis: The “seed and soil” hypothesis revisited. *Nature Reviews. Cancer*, 3(6), 453–458. <https://doi.org/10.1038/nrc1098>
- Fouad, Y. A., & Aanei, C. (2017). Revisiting the hallmarks of cancer. *American Journal of Cancer Research*, 7(5), 1016–1036.
- Fradet, Y., Klotz, L., Trachtenberg, J., & Zlotta, A. (2009). The burden of prostate cancer in Canada. *Canadian Urological Association Journal*, 3(3 Suppl 2), S92–S100.

- Fujita, K., & Nonomura, N. (2019). Role of Androgen Receptor in Prostate Cancer: A Review. *The World Journal of Men's Health*, 37(3), 288–295. <https://doi.org/10.5534/wjmh.180040>
- Fulda, S. (2010). Evasion of apoptosis as a cellular stress response in cancer. *International Journal of Cell Biology*, 2010, 370835. <https://doi.org/10.1155/2010/370835>
- Fulda, S., Meyer, E., & Debatin, K.-M. (2002). Inhibition of TRAIL-induced apoptosis by Bcl-2 overexpression. *Oncogene*, 21(15), 2283–2294. <https://doi.org/10.1038/sj.onc.1205258>
- Gao H, Song Q, Yang J, Yu S, Zhao J, Yu G. (2017). Carnosol inhibits Hedgehog signaling pathway in both LNCaP and DU145 prostate cancer cell lines. *Cell Mol Biol (Noisy-le-grand)*. 63(8):104-108. doi: 10.14715/cmb/2017.63.8.22. PMID: 28886322.
- Gaupel, A.-C., Wang, W.-L. W., Mordan-McCombs, S., Yu Lee, E. C., & Tenniswood, M. (2013). Chapter 39—Xenograft, Transgenic, and Knockout Models of Prostate Cancer. In P. M. Conn (Ed.), *Animal Models for the Study of Human Disease* (pp. 973–995). Academic Press. <https://doi.org/10.1016/B978-0-12-415894-8.00039-7>
- Ghobrial, I. M., Witzig, T. E., & Adjei, A. A. (2005). Targeting apoptosis pathways in cancer therapy. *CA: A Cancer Journal for Clinicians*, 55(3), 178–194. <https://doi.org/10.3322/canjclin.55.3.178>
- González-Vallinas, M., Reglero, G., & Ramírez de Molina, A. (2015). Rosemary (Rosmarinus officinalis L. ) Extract as a Potential Complementary Agent in Anticancer Therapy. *Nutrition and Cancer*, 67(8), 1223–1231. <https://doi.org/10.1080/01635581.2015.1082110>
- Greenman, C., Stephens, P., Smith, R., Dalgliesh, G. L., Hunter, C., Bignell, G., Davies, H., Teague, J., Butler, A., Stevens, C., Edkins, S., O'Meara, S., Vastrik, I., Schmidt, E. E., Avis, T., Barthorpe, S., Bhamra, G., Buck, G., Choudhury, B., ... Stratton, M. R.

- (2007). Patterns of somatic mutation in human cancer genomes. *Nature*, 446(7132), 153–158. <https://doi.org/10.1038/nature05610>
- Griffiths, A. J., Miller, J. H., Suzuki, D. T., Lewontin, R. C., Gelbart, W. M., Griffiths, A. J., Miller, J. H., Suzuki, D. T., Lewontin, R. C., & Gelbart, W. M. (2000). *An Introduction to Genetic Analysis* (7th ed.). W. H. Freeman.
- Grivennikov, S. I., Greten, F. R., & Karin, M. (2010). Immunity, inflammation, and cancer. *Cell*, 140(6), 883–899. <https://doi.org/10.1016/j.cell.2010.01.025>
- Guardado, E., Molina, E., Joo, M., & Uriarte, E. (2012). Antioxidant and Pro-Oxidant Effects of Polyphenolic Compounds and Structure-Activity Relationship Evidence. In J. Bouayed (Ed.), *Nutrition, Well-Being and Health*. InTech. <https://doi.org/10.5772/29471>
- H, P., S, M., A, S., M, B., H, K., & Ac, C. (1999, April 11). *Rapid Signalling by Androgen Receptor in Prostate Cancer Cells*. *Oncogene*; *Oncogene*. <https://doi.org/10.1038/sj.onc.1203032>
- Habtemariam, S. (2016). The Therapeutic Potential of Rosemary (*Rosmarinus officinalis*) Diterpenes for Alzheimer’s Disease. *Evidence-Based Complementary and Alternative Medicine : ECAM*, 2016. <https://doi.org/10.1155/2016/2680409>
- Halliwell, B. (2007). Dietary polyphenols: Good, bad, or indifferent for your health? *Cardiovascular Research*, 73(2), 341–347. <https://doi.org/10.1016/j.cardiores.2006.10.004>
- Hanahan, D., & Weinberg, R. A. (2000). The Hallmarks of Cancer. *Cell*, 100(1), 57–70. [https://doi.org/10.1016/S0092-8674\(00\)81683-9](https://doi.org/10.1016/S0092-8674(00)81683-9)
- Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*, 144(5), 646–674. <https://doi.org/10.1016/j.cell.2011.02.013>

- Hart, J. R., & Vogt, P. K. (2011). Phosphorylation of AKT: a mutational analysis. *Oncotarget*, 2(6), 467–476. <https://doi.org/10.18632/oncotarget.293>
- Hata, A. N., Engelman, J. A., & Faber, A. C. (2015). The BCL2 Family: Key Mediators of the Apoptotic Response to Targeted Anticancer Therapeutics. *Cancer Discovery*, 5(5), 475–487. <https://doi.org/10.1158/2159-8290.CD-15-0011>
- Hayden, A. J., Catton, C., & Pickles, T. (2010). Radiation therapy in prostate cancer: A risk-adapted strategy. *Current Oncology (Toronto, Ont.)*, 17 Suppl 2, S18-24. <https://doi.org/10.3747/co.v17i0.704>
- Hayden, M. S., & Ghosh, S. (2014). Regulation of NF- $\kappa$ B by TNF Family Cytokines. *Seminars in Immunology*, 26(3), 253–266. <https://doi.org/10.1016/j.smim.2014.05.004>
- Heidenreich, A., Bastian, P. J., Bellmunt, J., Bolla, M., Joniau, S., van der Kwast, T., Mason, M., Matveev, V., Wiegel, T., Zattoni, F., & Mottet, N. (2014). EAU Guidelines on Prostate Cancer. Part II: Treatment of Advanced, Relapsing, and Castration-Resistant Prostate Cancer. *European Urology*, 65(2), 467–479. <https://doi.org/10.1016/j.eururo.2013.11.002>
- Hengartner, M. O. (2001). Apoptosis: Corraling the corpses. *Cell*, 104(3), 325–328. [https://doi.org/10.1016/s0092-8674\(01\)00219-7](https://doi.org/10.1016/s0092-8674(01)00219-7)
- Herceg, Z., & Wang, Z.-Q. (2001). Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 477(1), 97–110. [https://doi.org/10.1016/S0027-5107\(01\)00111-7](https://doi.org/10.1016/S0027-5107(01)00111-7)
- Ho, M. Y., & Mackey, J. R. (2014). Presentation and management of docetaxel-related adverse effects in patients with breast cancer. *Cancer management and research*, 6, 253–259. <https://doi.org/10.2147/CMAR.S40601>

- Horoszewicz, J. S., Leong, S. S., Kawinski, E., Karr, J. P., Rosenthal, H., Chu, T. M., Mirand, E. A., & Murphy, G. P. (1983). LNCaP model of human prostatic carcinoma. *Cancer Research*, *43*(4), 1809–1818.
- Huang, L., Hu, J., Tao, W., Li, Y., Li, G., Xie, P., Liu, X., & Jiang, J. (2010). Gossypol inhibits phosphorylation of Bcl-2 in human leukemia HL-60 cells. *European Journal of Pharmacology*, *645*(1–3), 9–13. <https://doi.org/10.1016/j.ejphar.2010.06.070>
- Huang, S., Pettaway, C. A., Uehara, H., Bucana, C. D., & Fidler, I. J. (2001). Blockade of NF-kappaB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. *Oncogene*, *20*(31), 4188–4197. <https://doi.org/10.1038/sj.onc.1204535>
- Huggins, C., & Hodges, C. V. (2002). Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941. *The Journal of Urology*, *168*(1), 9–12. [https://doi.org/10.1016/s0022-5347\(05\)64820-3](https://doi.org/10.1016/s0022-5347(05)64820-3)
- Huggins, C., Scott, W. W., & Heinen, J. H. (1942). Chemical composition of human semen and of the secretions of the prostate and seminal vesicles. *American Journal of Physiology-Legacy Content*, *136*(3), 467–473. <https://doi.org/10.1152/ajplegacy.1942.136.3.467>
- Ishida, T., Shimamoto, T., Ozaki, N., Takaki, S., Kuchimaru, T., Kizaka-Kondoh, S., & Omata, T. (2016). Investigation of the Influence of Glucose Concentration on Cancer Cells by Using a Microfluidic Gradient Generator without the Induction of Large Shear Stress. *Micromachines*, *7*(9), 155. <https://doi.org/10.3390/mi7090155>
- Ismail, H. A., Lessard, L., Mes-Masson, A.-M., & Saad, F. (2004). Expression of NF-kappaB in prostate cancer lymph node metastases. *The Prostate*, *58*(3), 308–313. <https://doi.org/10.1002/pros.10335>

- Jackson, S. P., & Bartek, J. (2009). The DNA-damage response in human biology and disease. *Nature*, *461*(7267), 1071–1078. <https://doi.org/10.1038/nature08467>
- Jaglanian, A., & Tsiani, E. (2020). Rosemary Extract Inhibits Proliferation, Survival, Akt, and mTOR Signaling in Triple-Negative Breast Cancer Cells. *International journal of molecular sciences*, *21*(3), 810. <https://doi.org/10.3390/ijms21030810>
- Jamaspishvili, T., Berman, D. M., Ross, A. E., Scher, H. I., De Marzo, A. M., Squire, J. A., & Lotan, T. L. (2018). Clinical implications of PTEN loss in prostate cancer. *Nature Reviews. Urology*, *15*(4), 222–234. <https://doi.org/10.1038/nrurol.2018.9>
- Jang, Y.-G., Hwang, K.-A., & Choi, K.-C. (2018). Rosmarinic Acid, a Component of Rosemary Tea, Induced the Cell Cycle Arrest and Apoptosis through Modulation of HDAC2 Expression in Prostate Cancer Cell Lines. *Nutrients*, *10*(11). <https://doi.org/10.3390/nu10111784>
- Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. (2011). Global cancer statistics. *CA: A Cancer Journal for Clinicians*, *61*(2), 69–90. <https://doi.org/10.3322/caac.20107>
- Jiang, B.-H., & Liu, L.-Z. (2009). PI3K/PTEN Signaling in Angiogenesis and Tumorigenesis. *Advances in Cancer Research*, *102*, 19–65. [https://doi.org/10.1016/S0065-230X\(09\)02002-8](https://doi.org/10.1016/S0065-230X(09)02002-8)
- Joerger, A. C., & Fersht, A. R. (2007). Structure–function–rescue: The diverse nature of common p53 cancer mutants. *Oncogene*, *26*(15), 2226–2242. <https://doi.org/10.1038/sj.onc.1210291>
- Johnson, J. J., Syed, D. N., Heren, C. R., Suh, Y., Adhami, V. M., & Mukhtar, H. (2008). Carnosol, a dietary diterpene, displays growth inhibitory effects in human prostate cancer PC-3 cells leading to G2-phase cell cycle arrest and targets the 5'-AMP-

- activated protein kinase (AMPK) pathway. *Pharmaceutical Research*, 25(9), 2125–2134. <https://doi.org/10.1007/s11095-008-9552-0>
- Johnson, J. J., Syed, D. N., Suh, Y., Heren, C. R., Saleem, M., Siddiqui, I. A., & Mukhtar, H. (2010). Disruption of androgen and estrogen receptor activity in prostate cancer by a novel dietary diterpene carnosol: Implications for chemoprevention. *Cancer Prevention Research (Philadelphia, Pa.)*, 3(9), 1112–1123. <https://doi.org/10.1158/1940-6207.CAPR-10-0168>
- Johnson, M. T., Lowe, G. J., & Bahnson, R. R. (2010). Androgen deprivation therapy: A primer on concepts and therapeutic options. *Journal of Men's Health*, 7(4), 358–367. <https://doi.org/10.1016/j.jomh.2010.09.226>
- Jones, R. G., & Thompson, C. B. (2009). Tumor suppressors and cell metabolism: A recipe for cancer growth. *Genes & Development*, 23(5), 537–548. <https://doi.org/10.1101/gad.1756509>
- Jung, K.-J., Min, K., Bae, J. H., & Kwon, T. K. (2015). Carnosic acid sensitized TRAIL-mediated apoptosis through down-regulation of c-FLIP and Bcl-2 expression at the post translational levels and CHOP-dependent up-regulation of DR5, Bim, and PUMA expression in human carcinoma caki cells. *Oncotarget*, 6(3), 1556–1568. <https://doi.org/10.18632/oncotarget.2727>
- Junttila, M. R., & Evan, G. I. (2009). p53—A Jack of all trades but master of none. *Nature Reviews. Cancer*, 9(11), 821–829. <https://doi.org/10.1038/nrc2728>
- Kaighn, M. E., Narayan, K. S., Ohnuki, Y., Lechner, J. F., & Jones, L. W. (1979). Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Investigative Urology*, 17(1), 16–23.
- Kaliora, A. C., Kogiannou, D. A. A., Kefalas, P., Papassideri, I. S., & Kalogeropoulos, N. (2014). Phenolic profiles and antioxidant and anticarcinogenic activities of Greek

- herbal infusions; balancing delight and chemoprevention? *Food Chemistry*, 142, 233–241. <https://doi.org/10.1016/j.foodchem.2013.07.056>
- Kang, H.-Y., Cho, C.-L., Huang, K.-L., Wang, J.-C., Hu, Y.-C., Lin, H.-K., Chang, C., & Huang, K.-E. (2004). Nongenomic androgen activation of phosphatidylinositol 3-kinase/Akt signaling pathway in MC3T3-E1 osteoblasts. *Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research*, 19(7), 1181–1190. <https://doi.org/10.1359/JBMR.040306>
- Kaplan, D. R., Whitman, M., Schaffhausen, B., Pallas, D. C., White, M., Cantley, L., & Roberts, T. M. (1987). Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity. *Cell*, 50(7), 1021–1029. [https://doi.org/10.1016/0092-8674\(87\)90168-1](https://doi.org/10.1016/0092-8674(87)90168-1)
- Kar, S., Palit, S., Ball, W. B., & Das, P. K. (2012). Carnosic acid modulates Akt/IKK/NF- $\kappa$ B signaling by PP2A and induces intrinsic and extrinsic pathway mediated apoptosis in human prostate carcinoma PC-3 cells. *Apoptosis: An International Journal on Programmed Cell Death*, 17(7), 735–747. <https://doi.org/10.1007/s10495-012-0715-4>
- Karantanos T, Corn PG, Thompson TC. (2013). Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches. *Oncogene* 32:5501–5511. <https://doi.org/10.1038/onc.2013.206>
- Karp, G. (2009). *Cell and Molecular Biology: Concepts and Experiments*. John Wiley & Sons.
- Kastan, M. B. (2008). DNA damage responses: Mechanisms and roles in human disease: 2007 G.H.A. Clowes Memorial Award Lecture. *Molecular Cancer Research: MCR*, 6(4), 517–524. <https://doi.org/10.1158/1541-7786.MCR-08-0020>

- Kawauchi, K., Araki, K., Tobiume, K., & Tanaka, N. (2008). P53 regulates glucose metabolism through an IKK-NF-kappaB pathway and inhibits cell transformation. *Nature Cell Biology*, *10*(5), 611–618. <https://doi.org/10.1038/ncb1724>
- Kazerounian, S., Yee, K. O., & Lawler, J. (2008). Thrombospondins in cancer. *Cellular and Molecular Life Sciences: CMLS*, *65*(5), 700–712. <https://doi.org/10.1007/s00018-007-7486-z>
- Kennedy, D.O., Wightman, E.M. (2011). Herbal Extracts and Phytochemicals: Plant Secondary Metabolites and the Enhancement of Human Brain function, *Advances in Nutrition*, *2*(1), 32–50. <https://doi.org/10.3945/an.110.000117>
- Kessenbrock, K., Plaks, V., & Werb, Z. (2010). Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. *Cell*, *141*(1), 52–67. <https://doi.org/10.1016/j.cell.2010.03.015>
- Khalili, N., Keshavarz-Fathi, M., Shahkarami, S., Hirbod-Mobarakeh, A., & Rezaei, N. (2019). Passive-specific immunotherapy with monoclonal antibodies for prostate cancer: A systematic review. *Journal of Oncology Pharmacy Practice*, *25*(4), 903–917. <https://doi.org/10.1177/1078155218808080>
- Kharaziha, P., Rodriguez, P., Li, Q., Rundqvist, H., Björklund, A. C., Augsten, M., Ullén, A., Egevad, L., Wiklund, P., Nilsson, S., Kroemer, G., Grander, D., & Panaretakis, T. (2012). Targeting of distinct signaling cascades and cancer-associated fibroblasts define the efficacy of Sorafenib against prostate cancer cells. *Cell death & disease*, *3*(1), e262. <https://doi.org/10.1038/cddis.2012.1>
- Kim DH, Park KW, Chae IG, Kundu J, Kim EH, Kundu JK, Chun KS. (2016). Carnosic acid inhibits STAT3 signaling and induces apoptosis through generation of ROS in human colon cancer HCT116 cells. *Mol Carcinog.* *55*(6):1096-110. doi: 10.1002/mc.22353.

- Kim, R., Emi, M., & Tanabe, K. (2007). Cancer immunoediting from immune surveillance to immune escape. *Immunology*, *121*(1), 1–14. <https://doi.org/10.1111/j.1365-2567.2007.02587.x>
- Kim, S. M., Lee, S. Y., Cho, J. S., Son, S. M., Choi, S. S., Yun, Y. P., Yoo, H. S., Yoon, D. Y., Oh, K.-W., Han, S. B., & Hong, J. T. (2010). Combination of ginsenoside Rg3 with docetaxel enhances the susceptibility of prostate cancer cells via inhibition of NF- $\kappa$ B. *European Journal of Pharmacology*, *631*(1–3), 1–9. <https://doi.org/10.1016/j.ejphar.2009.12.018>
- Kirby M, Hirst C, Crawford ED. (2011). Characterising the castration-resistant prostate cancer population: a systematic review. *Int J Clin Pract* 65:1180–1192. <https://doi.org/10.1111/j.1742-1241.2011.02799.x>
- Kluth, L. A., Shariat, S. F., Kratzik, C., Tagawa, S., Sonpavde, G., Rieken, M., Scherr, D. S., & Pummer, K. (2014). The hypothalamic-pituitary-gonadal axis and prostate cancer: Implications for androgen deprivation therapy. *World Journal of Urology*, *32*(3), 669–676. <https://doi.org/10.1007/s00345-013-1157-5>
- Knudsen, B. S., & Vasioukhin, V. (2010). Mechanisms of prostate cancer initiation and progression. *Advances in Cancer Research*, *109*, 1–50. <https://doi.org/10.1016/B978-0-12-380890-5.00001-6>
- Kondo, E., Miyake, T., Shibata, M., Kimura, T., Iwagaki, H., Nakamura, S., Tanaka, T., Ohara, N., Ichimura, K., Oka, T., Yanai, H., Shibasaki, F., & Yoshino, T. (2005). Expression of Phosphorylated Ser 70 of Bcl-2 Correlates with Malignancy in Human Colorectal Neoplasms. *Clinical Cancer Research*, *11*(20), 7255–7263. <https://doi.org/10.1158/1078-0432.CCR-05-0274>

- Krajewska M, Krajewski S, Banares S, Huang X, Turner B, Bubendorf L, Kallioniemi OP, Shabaik A, Vitiello A, Peehl D, Gao GJ, Reed JC. (2003). Elevated expression of inhibitor of apoptosis proteins in prostate cancer. *Clin Cancer Res.* 15;9(13):4914-25. PMID: 14581366.
- Kremer, C. L., Klein, R. R., Mendelson, J., Browne, W., Samadzedeh, L. K., Vanpatten, K., Highstrom, L., Pestano, G. A., & Nagle, R. B. (2006). Expression of mTOR signaling pathway markers in prostate cancer progression. *The Prostate*, 66(11), 1203–1212. <https://doi.org/10.1002/pros.20410>
- Kroemer, G., Galluzzi, L., & Brenner, C. (2007). Mitochondrial membrane permeabilization in cell death. *Physiological Reviews*, 87(1), 99–163. <https://doi.org/10.1152/physrev.00013.2006>
- Kroemer, G., & Pouyssegur, J. (2008). Tumor Cell Metabolism: Cancer's Achilles' Heel. *Cancer Cell*, 13(6), 472–482. <https://doi.org/10.1016/j.ccr.2008.05.005>
- Kubbutat, M. H., Jones, S. N., & Vousden, K. H. (1997). Regulation of p53 stability by Mdm2. *Nature*, 387(6630), 299–303. <https://doi.org/10.1038/387299a0>
- Kumar, M., Kaur, V., Kumar, S., & Kaur, S. (2016). Phytoconstituents as apoptosis inducing agents: strategy to combat cancer. *Cytotechnology*, 68(4), 531–563. <https://doi.org/10.1007/s10616-015-9897-2>
- Law, A. B., & McLaren, D. B. (2010). Non-surgical treatment for early prostate cancer. *The Journal of the Royal College of Physicians of Edinburgh*, 40(4), 340–342; quiz 342. <https://doi.org/10.4997/JRCPE.2010.419>
- Lawrentschuk, N., Trottier, G., Kuk, C., & Zlotta, A. R. (2010). Role of surgery in high-risk localized prostate cancer. *Current Oncology*, 17, S25–S32. <https://doi.org/10.3747/co.v17i0.705>

- Leslie, N. R., Yang, X., Downes, C. P., & Weijer, C. J. (2005). The regulation of cell migration by PTEN. *Biochemical Society Transactions*, 33(Pt 6), 1507–1508. <https://doi.org/10.1042/BST20051507>
- Leslie, Nick R., & Downes, C. P. (2004). PTEN function: How normal cells control it and tumour cells lose it. *Biochemical Journal*, 382(Pt 1), 1–11. <https://doi.org/10.1042/BJ20040825>
- Li, C.-W., Xia, W., Huo, L., Lim, S.-O., Wu, Y., Hsu, J. L., Chao, C.-H., Yamaguchi, H., Yang, N.-K., Ding, Q., Wang, Y., Lai, Y.-J., LaBaff, A. M., Wu, T.-J., Lin, B.-R., Yang, M.-H., Hortobagyi, G. N., & Hung, M.-C. (2012). Epithelial-mesenchyme transition induced by TNF- $\alpha$  requires NF- $\kappa$ B-mediated transcriptional upregulation of Twist1. *Cancer Research*, 72(5), 1290–1300. <https://doi.org/10.1158/0008-5472.CAN-11-3123>
- Li, Q., & Verma, I. M. (2002). NF-kappaB regulation in the immune system. *Nature Reviews. Immunology*, 2(10), 725–734. <https://doi.org/10.1038/nri910>
- Liebmann, C. (2001). Regulation of MAP kinase activity by peptide receptor signalling pathway: Paradigms of multiplicity. *Cellular Signalling*, 13(11), 777–785. [https://doi.org/10.1016/s0898-6568\(01\)00192-9](https://doi.org/10.1016/s0898-6568(01)00192-9)
- Lin, H.-K., Yeh, S., Kang, H.-Y., & Chang, C. (2001). Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 98(13), 7200–7205. <https://doi.org/10.1073/pnas.121173298>
- Lindsey, S., & Langhans, S. A. (2015). Epidermal growth factor signaling in transformed cells. *International Review of Cell and Molecular Biology*, 314, 1–41. <https://doi.org/10.1016/bs.ircmb.2014.10.001>

- Lipton, J. O., & Sahin, M. (2014). The Neurology of mTOR. *Neuron*, 84(2), 275–291.  
<https://doi.org/10.1016/j.neuron.2014.09.034>
- Liu, F., Xia, Y., Parker, A. S., & Verma, I. M. (2012). IKK biology. *Immunological Reviews*, 246(1), 239–253. <https://doi.org/10.1111/j.1600-065X.2012.01107.x>
- Liu L, Xie Y, Lou L. (2006). PI3K is required for insulin-stimulated but not EGF-stimulated ERK1/2 activation. *Eur J Cell Biol.* May;85(5):367-74. doi: 10.1016/j.ejcb.2005.11.005. Epub 2006 Jan 10. PMID: 16406609.
- Liu, Y., Sun, S. Y., Owonikoko, T. K., Sica, G. L., Curran, W. J., Khuri, F. R., & Deng, X. (2012). Rapamycin induces Bad phosphorylation in association with its resistance to human lung cancer cells. *Molecular cancer therapeutics*, 11(1), 45–56.  
<https://doi.org/10.1158/1535-7163.MCT-11-0578>
- Lohiya, V., Aragon-Ching, J. B., & Sonpavde, G. (2016). Role of Chemotherapy and Mechanisms of Resistance to Chemotherapy in Metastatic Castration-Resistant Prostate Cancer. *Clinical Medicine Insights. Oncology*, 10(Suppl 1), 57–66.  
<https://doi.org/10.4137/CMO.S34535>
- Lopez-Illasaca M, Crespo P, Pellici PG, Gutkind JS, Wetzker R. (1997). Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase gamma. *Science*. Jan 17;275(5298):394-7. doi: 10.1126/science.275.5298.394. PMID: 8994038.
- Lowe, S. W., Cepero, E., & Evan, G. (2004). Intrinsic tumour suppression. *Nature*, 432(7015), 307–315. <https://doi.org/10.1038/nature03098>
- Lozano, R., Naghavi, M., Foreman, K., Lim, S., Shibuya, K., Aboyans, V., Abraham, J., Adair, T., Aggarwal, R., Ahn, S. Y., AlMazroa, M. A., Alvarado, M., Anderson, H. R., Anderson, L. M., Andrews, K. G., Atkinson, C., Baddour, L. M., Barker-Collo, S., Bartels, D. H., Murray, C. J. (2012). Global and regional mortality from 235 causes of

- death for 20 age groups in 1990 and 2010: A systematic analysis for the Global Burden of Disease Study 2010. *The Lancet*, 380(9859), 2095–2128. [https://doi.org/10.1016/S0140-6736\(12\)61728-0](https://doi.org/10.1016/S0140-6736(12)61728-0)
- Lyseng-Williamson, K. A., & Fenton, C. (2005). Docetaxel: A review of its use in metastatic breast cancer. *Drugs*, 65(17), 2513–2531. <https://doi.org/10.2165/00003495-200565170-00007>
- Makris, D. P., & Boskou, D. (2015). Plant-derived antioxidants as food additives. In N. K. Dubey (Ed.), *Plants as a source of natural antioxidants* (pp. 169–190). CABI. <https://doi.org/10.1079/9781780642666.0169>
- Marais, R., Light, Y., Paterson, H. F., & Marshall, C. J. (1995). Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *The EMBO Journal*, 14(13), 3136–3145.
- Martin, P., Liu, Y. N., Pierce, R., Abou-Kheir, W., Casey, O., Seng, V., Camacho, D., Simpson, R. M., & Kelly, K. (2011). Prostate epithelial Pten/TP53 loss leads to transformation of multipotential progenitors and epithelial to mesenchymal transition. *The American journal of pathology*, 179(1), 422–435. <https://doi.org/10.1016/j.ajpath.2011.03.035>
- Martini, M., De Santis, M. C., Braccini, L., Gulluni, F., & Hirsch, E. (2014). PI3K/AKT signaling pathway and cancer: An updated review. *Annals of Medicine*, 46(6), 372–383. <https://doi.org/10.3109/07853890.2014.912836>
- Mauro, C., Leow, S. C., Anso, E., Rocha, S., Thotakura, A. K., Tornatore, L., Moretti, M., De Smaele, E., Beg, A. A., Tergaonkar, V., Chandel, N. S., & Franzoso, G. (2011). NF- $\kappa$ B controls energy homeostasis and metabolic adaptation by upregulating mitochondrial respiration. *Nature Cell Biology*, 13(10), 1272–1279. <https://doi.org/10.1038/ncb2324>

- McCubrey, J. A., Steelman, L. S., Chappell, W. H., Abrams, S. L., Wong, E. W., Chang, F., Lehmann, B., Terrian, D. M., Milella, M., Tafuri, A., Stivala, F., Libra, M., Basecke, J., Evangelisti, C., Martelli, A. M., & Franklin, R. A. (2007). Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochimica et biophysica acta*, 1773(8), 1263–1284. <https://doi.org/10.1016/j.bbamcr.2006.10.001>
- McGill, G., & Fisher, D. E. (1997). Apoptosis in tumorigenesis and cancer therapy. *Frontiers in Bioscience: A Journal and Virtual Library*, 2, d353-379. <https://doi.org/10.2741/a197>
- Meylan, E., Dooley, A. L., Feldser, D. M., Shen, L., Turk, E., Ouyang, C., & Jacks, T. (2009). Requirement for NF- $\kappa$ B signaling in a mouse model of lung adenocarcinoma. *Nature*, 462(7269), 104–107. <https://doi.org/10.1038/nature08462>
- Micheau, O., Lens, S., Gaide, O., Alevizopoulos, K., & Tschopp, J. (2001). NF-kappaB signals induce the expression of c-FLIP. *Molecular and Cellular Biology*, 21(16), 5299–5305. <https://doi.org/10.1128/MCB.21.16.5299-5305.2001>
- Micheau, Olivier, & Tschopp, J. (2003). Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell*, 114(2), 181–190. [https://doi.org/10.1016/s0092-8674\(03\)00521-x](https://doi.org/10.1016/s0092-8674(03)00521-x)
- Miquel, C., Borrini, F., Grandjouan, S., Aupérin, A., Viguier, J., Velasco, V., Duvillard, P., Praz, F., & Sabourin, J.-C. (2005). Role of bax mutations in apoptosis in colorectal cancers with microsatellite instability. *American Journal of Clinical Pathology*, 123(4), 562–570. <https://doi.org/10.1309/JQ2X-3RV3-L8F9-TGYW>
- Mohamad Anuar, N. N., Nor Hisam, N. S., Liew, S. L., & Ugusman, A. (2020). Clinical Review: Navitoclax as a Pro-Apoptotic and Anti-Fibrotic Agent. *Frontiers in Pharmacology*, 11, 564108. <https://doi.org/10.3389/fphar.2020.564108>

- Molina, J. R., & Adjei, A. A. (2006). The Ras/Raf/MAPK Pathway. *Journal of Thoracic Oncology*, 1(1), 7–9. [https://doi.org/10.1016/S1556-0864\(15\)31506-9](https://doi.org/10.1016/S1556-0864(15)31506-9)
- Montero, J., & Letai, A. (2018). Why do BCL-2 inhibitors work and where should we use them in the clinic?. *Cell Death & Differentiation*, 25(1), 56–64. <https://doi.org/10.1038/cdd.2017.183>
- Moore, J., Yousef, M., & Tsiani, E. (2016). Anticancer Effects of Rosemary (Rosmarinus officinalis L.) Extract and Rosemary Extract Polyphenols. *Nutrients*, 8(11). <https://doi.org/10.3390/nu8110731>
- Moore, J., Megaly, M., MacNeil, A. J., Klentrou, P., & Tsiani, E. (2016). Rosemary extract reduces Akt/mTOR/p70S6K activation and inhibits proliferation and survival of A549 human lung cancer cells. *Biomedicine & Pharmacotherapy*, 83, 725–732. <https://doi.org/10.1016/j.biopha.2016.07.043>
- Mougiakakos, D., Choudhury, A., Lladser, A., Kiessling, R., & Johansson, C. C. (2010). Regulatory T cells in cancer. *Advances in Cancer Research*, 107, 57–117. [https://doi.org/10.1016/S0065-230X\(10\)07003-X](https://doi.org/10.1016/S0065-230X(10)07003-X)
- Muller, P. A. J., & Vousden, K. H. (2014). Mutant p53 in Cancer: New Functions and Therapeutic Opportunities. *Cancer Cell*, 25(3), 304–317. <https://doi.org/10.1016/j.ccr.2014.01.021>
- Nagy, J. A., Chang, S.-H., Shih, S.-C., Dvorak, A. M., & Dvorak, H. F. (2010). Heterogeneity of the tumor vasculature. *Seminars in Thrombosis and Hemostasis*, 36(3), 321–331. <https://doi.org/10.1055/s-0030-1253454>
- Naimi, M.; Vlavcheski, F.; Murphy, B.; Hudlicky, T.; Tsiani, E. (2017). Carnosic acid as a component of rosemary extract stimulates skeletal muscle cell glucose uptake via AMPK activation. *Clin. Exp. Pharmacol. Physiol.* 44, 94–102, doi:10.1111/1440-1681.12674.

- National Collaborating Centre for Cancer (UK). (2014). *Prostate Cancer: Diagnosis and Treatment*. National Collaborating Centre for Cancer (UK). <http://www.ncbi.nlm.nih.gov/books/NBK247469/>
- Navé, B. T., Ouwens, M., Withers, D. J., Alessi, D. R., & Shepherd, P. R. (1999). Mammalian target of rapamycin is a direct target for protein kinase B: Identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochemical Journal*, *344*(Pt 2), 427–431.
- Nelson, B. H. (2008). The impact of T-cell immunity on ovarian cancer outcomes. *Immunological Reviews*, *222*, 101–116. <https://doi.org/10.1111/j.1600-065X.2008.00614.x>
- Nicholson, K. M., & Anderson, N. G. (2002). The protein kinase B/Akt signalling pathway in human malignancy. *Cellular Signalling*, *14*(5), 381–395. [https://doi.org/10.1016/s0898-6568\(01\)00271-6](https://doi.org/10.1016/s0898-6568(01)00271-6)
- Oberst, A., & Green, D. R. (2011). It cuts both ways: Reconciling the dual roles of caspase-8 in cell death and survival. *Nature Reviews. Molecular Cell Biology*, *12*(11), 757–763. <https://doi.org/10.1038/nrm3214>
- Oberst, A., Pop, C., Tremblay, A. G., Blais, V., Denault, J.-B., Salvesen, G. S., & Green, D. R. (2010). Inducible dimerization and inducible cleavage reveal a requirement for both processes in caspase-8 activation. *The Journal of Biological Chemistry*, *285*(22), 16632–16642. <https://doi.org/10.1074/jbc.M109.095083>
- O'Brien, M. A., & Kirby, R. (2008). Apoptosis: A review of pro-apoptotic and anti-apoptotic pathways and dysregulation in disease. *Journal of Veterinary Emergency and Critical Care*, *18*(6), 572–585. <https://doi.org/10.1111/j.1476-4431.2008.00363.x>

- Oh, W. K., Hurwitz, M., D'Amico, A. V., Richie, J. P., & Kantoff, P. W. (2003). Biology of Prostate Cancer. *Holland-Frei Cancer Medicine. 6th Edition*. <https://www.ncbi.nlm.nih.gov/books/NBK13217/>
- Olea-Flores, M., Zuñiga-Eulogio, M. D., Mendoza-Catalán, M. A., Rodríguez-Ruiz, H. A., Castañeda-Saucedo, E., Ortuño-Pineda, C., Padilla-Benavides, T., & Navarro-Tito, N. (2019). Extracellular-Signal Regulated Kinase: A Central Molecule Driving Epithelial-Mesenchymal Transition in Cancer. *International journal of molecular sciences*, 20(12), 2885. <https://doi.org/10.3390/ijms20122885>
- de Oliveira, J. R., Camargo, S., & de Oliveira, L. D. (2019). Rosmarinus officinalis L. (rosemary) as therapeutic and prophylactic agent. *Journal of biomedical science*, 26(1), 5. <https://doi.org/10.1186/s12929-019-0499-8>
- O'Neill, A. J., Prencipe, M., Dowling, C., Fan, Y., Mulrane, L., Gallagher, W. M., O'Connor, D., O'Connor, R., Devery, A., Corcoran, C., Rani, S., O'Driscoll, L., Fitzpatrick, J. M., & Watson, R. W. G. (2011). Characterisation and manipulation of docetaxel resistant prostate cancer cell lines. *Molecular Cancer*, 10(1), 126. <https://doi.org/10.1186/1476-4598-10-126>
- Orlowski, M., & Sarao, M. S. (2020). Physiology, Follicle Stimulating Hormone. In *StatPearls*. StatPearls Publishing. <http://www.ncbi.nlm.nih.gov/books/NBK535442/>
- Osman, I., Drobnjak, M., Fazzari, M., Ferrara, J., Scher, H. I., & Cordon-Cardo, C. (1999). Inactivation of the p53 pathway in prostate cancer: Impact on tumor progression. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, 5(8), 2082–2088.
- Ostrand-Rosenberg, S., & Sinha, P. (2009). Myeloid-derived suppressor cells: Linking inflammation and cancer. *Journal of Immunology (Baltimore, Md.: 1950)*, 182(8), 4499–4506. <https://doi.org/10.4049/jimmunol.0802740>

- Ozaki, T., & Nakagawara, A. (2011). Role of p53 in Cell Death and Human Cancers. *Cancers*, 3(1), 994–1013. <https://doi.org/10.3390/cancers3010994>
- Pagès, F., Galon, J., Dieu-Nosjean, M.-C., Tartour, E., Sautès-Fridman, C., & Fridman, W.-H. (2010). Immune infiltration in human tumors: A prognostic factor that should not be ignored. *Oncogene*, 29(8), 1093–1102. <https://doi.org/10.1038/onc.2009.416>
- Pearson, H. B., Li, J., Meniel, V. S., Fennell, C. M., Waring, P., Montgomery, K. G., Rebello, R. J., Macpherson, A. A., Koushyar, S., Furic, L., Cullinane, C., Clarkson, R. W., Smalley, M. J., Simpson, K. J., Phesse, T. J., Shepherd, P. R., Humbert, P. O., Sansom, O. J., & Phillips, W. A. (2018). Identification of Pik3ca mutation as a genetic driver of prostate cancer that cooperates with Pten loss to accelerate progression and castration-resistant growth. *Cancer Discovery*. <https://doi.org/10.1158/2159-8290.CD-17-0867>
- Pérez-Sánchez, A., Barrajon-Catalán, E., Ruiz-Torres, V., Agulló-Chazarra, L., Herranz-López, M., Valdés, A., Cifuentes, A., & Micol, V. (2019). Rosemary (*Rosmarinus officinalis*) extract causes ROS-induced necrotic cell death and inhibits tumor growth in vivo. *Scientific Reports*, 9(1), 808. <https://doi.org/10.1038/s41598-018-37173-7>
- Perlmutter, M. A., & Lepor, H. (2007). Androgen Deprivation Therapy in the Treatment of Advanced Prostate Cancer. *Reviews in Urology*, 9(Suppl 1), S3–S8.
- Petiwala, S. M., Berhe, S., Li, G., Puthenveetil, A. G., Rahman, O., Nonn, L., & Johnson, J. J. (2014). Rosemary (*Rosmarinus officinalis*) Extract Modulates CHOP/GADD153 to Promote Androgen Receptor Degradation and Decreases Xenograft Tumor Growth. *PLoS ONE*, 9(3). <https://doi.org/10.1371/journal.pone.0089772>
- Petiwala, S. M., Li, G., Bosland, M. C., Lantvit, D. D., Petukhov, P. A., & Johnson, J. J. (2016). Carnosic acid promotes degradation of the androgen receptor and is regulated

- by the unfolded protein response pathway in vitro and in vivo. *Carcinogenesis*, 37(8), 827–838. <https://doi.org/10.1093/carcin/bgw052>
- Picard, J. C., Golshayan, A.-R., Marshall, D. T., Opfermann, K. J., & Keane, T. E. (2012). The multi-disciplinary management of high-risk prostate cancer. *Urologic Oncology*, 30(1), 3–15. <https://doi.org/10.1016/j.urolonc.2009.09.002>
- Pierotti, M. A., Sozzi, G., & Croce, C. M. (2003). Mechanisms of oncogene activation. *Holland-Frei Cancer Medicine. 6th Edition*. <https://www.ncbi.nlm.nih.gov/books/NBK12538/>
- Pinkawa, M. (2010). External beam radiotherapy for prostate cancer. *Panminerva Medica*, 52(3), 195–207.
- Powell, I. J. (2007). Epidemiology and pathophysiology of prostate cancer in African-American men. *The Journal of Urology*, 177(2), 444–449. <https://doi.org/10.1016/j.juro.2006.09.024>
- Pretlow, T. G., Wolman, S. R., Micale, M. A., Pelley, R. J., Kursh, E. D., Resnick, M. I., Bodner, D. R., Jacobberger, J. W., Delmoro, C. M., Giaconia, J. M., & Pretlow, T. P. (1993). Xenografts of Primary Human Prostatic Carcinoma. *JNCI: Journal of the National Cancer Institute*, 85(5), 394–398. <https://doi.org/10.1093/jnci/85.5.394>
- Qian, D. Z., Rademacher, B. L. S., Pittsenbarger, J., Huang, C.-Y., Myrthue, A., Higano, C. S., Garzotto, M., Nelson, P. S., & Beer, T. M. (2010). CCL2 is induced by chemotherapy and protects prostate cancer cells from docetaxel-induced cytotoxicity. *The Prostate*, 70(4), 433–442. <https://doi.org/10.1002/pros.21077>
- Raffo, A. J., Perlman, H., Chen, M. W., Day, M. L., Streitman, J. S., & Buttyan, R. (1995). Overexpression of bcl-2 protects prostate cancer cells from apoptosis in vitro and confers resistance to androgen depletion in vivo. *Cancer Research*, 55(19), 4438–4445.

- Raica, M., Cimpean, A. M., & Ribatti, D. (2009). Angiogenesis in pre-malignant conditions. *European Journal of Cancer (Oxford, England: 1990)*, 45(11), 1924–1934. <https://doi.org/10.1016/j.ejca.2009.04.007>
- Ramsay, A. K., & Leung, H. Y. (2009). Signalling pathways in prostate carcinogenesis: Potentials for molecular-targeted therapy. *Clinical Science (London, England: 1979)*, 117(6), 209–228. <https://doi.org/10.1042/CS20080391>
- Rashid, A., Liu, C., Sanli, T., Tsiani, E., Singh, G., Bristow, R. G., Dayes, I., Lukka, H., Wright, J., & Tsakiridis, T. (2011). Resveratrol enhances prostate cancer cell response to ionizing radiation. Modulation of the AMPK, Akt and mTOR pathways. *Radiation Oncology*, 6(1), 144. <https://doi.org/10.1186/1748-717X-6-144>
- Reesink-Peters, N., Hougardy, B. M. T., van den Heuvel, F. a. J., Ten Hoor, K. A., Hollema, H., Boezen, H. M., de Vries, E. G. E., de Jong, S., & van der Zee, A. G. J. (2005). Death receptors and ligands in cervical carcinogenesis: An immunohistochemical study. *Gynecologic Oncology*, 96(3), 705–713. <https://doi.org/10.1016/j.ygyno.2004.10.046>
- Rezatabar, S., Karimian, A., Rameshknia, V., Parsian, H., Majidinia, M., Kopi, T. A., ... Yousefi, B. (2019). RAS/MAPK signaling functions in oxidative stress, DNA damage response and cancer progression. *Journal of Cellular Physiology*. doi:10.1002/jcp.28334
- Rizeq, B., Gupta, I., Ilesanmi, J., AlSafran, M., Rahman, M. M., & Ouhtit, A. (2020). The Power of Phytochemicals Combination in Cancer Chemoprevention. *Journal of Cancer*, 11(15), 4521–4533. <https://doi.org/10.7150/jca.34374>
- Rivlin, N., Brosh, R., Oren, M., & Rotter, V. (2011). Mutations in the p53 Tumor Suppressor Gene. *Genes & Cancer*, 2(4), 466–474. <https://doi.org/10.1177/1947601911408889>

- Roberts, A. W. (2020). Therapeutic development and current uses of BCL-2 inhibition. *Hematology*, 2020(1), 1–9. <https://doi.org/10.1182/hematology.2020000154>
- Robinson, D., Van Allen, E. M., Wu, Y.-M., Schultz, N., Lonigro, R. J., Mosquera, J.-M., Montgomery, B., Taplin, M.-E., Pritchard, C. C., Attard, G., Beltran, H., Abida, W., Bradley, R. K., Vinson, J., Cao, X., Vats, P., Kunju, L. P., Hussain, M., Feng, F. Y., Chinnaiyan, A. M. (2015). Integrative clinical genomics of advanced prostate cancer. *Cell*, 161(5), 1215–1228. <https://doi.org/10.1016/j.cell.2015.05.001>
- Rodríguez-Berriguete, G., Fraile, B., Martínez-Onsurbe, P., Olmedilla, G., Paniagua, R., & Royuela, M. (2012). MAP Kinases and Prostate Cancer. *Journal of signal transduction*, 2012, 169170. <https://doi.org/10.1155/2012/169170>
- Samuelsson, G. (ed.). (2004). *Drugs of Natural Origin: A Textbook of Pharmacognosy*, 5th Edn. Stockholm: Swedish Pharmaceutical Press.
- Santos-Gomes, P. C., Seabra, R. M., Andrade, P. B., & Fernandes-Ferreira, M. (2003). Determination of phenolic antioxidant compounds produced by calli and cell suspensions of sage (*Salvia officinalis*L.). *Journal of Plant Physiology*, 160(9), 1025–1032. <https://doi.org/10.1078/0176-1617-00831>
- Schattauer, S. S., Bedini, A., Summers, F., Reilly-Treat, A., Andrews, M. M., Land, B. B., & Chavkin, C. (2019). Reactive oxygen species (ROS) generation is stimulated by  $\kappa$  opioid receptor activation through phosphorylated c-Jun N-terminal kinase and inhibited by p38 mitogen-activated protein kinase (MAPK) activation. *The Journal of biological chemistry*, 294(45), 16884–16896. <https://doi.org/10.1074/jbc.RA119.009592>
- Schlessinger, J. (2000). Cell Signaling by Receptor Tyrosine Kinases. *Cell*, 103(2), 211–225. [https://doi.org/10.1016/S0092-8674\(00\)00114-8](https://doi.org/10.1016/S0092-8674(00)00114-8)

- Schrecengost, R. S., & Knudsen, K. E. (2013). Molecular Pathogenesis and Progression of Prostate Cancer. *Seminars in Oncology*, 40(3), 244–258. <https://doi.org/10.1053/j.seminoncol.2013.04.001>
- Schuler, M., Bossy-Wetzel, E., Goldstein, J. C., Fitzgerald, P., & Green, D. R. (2000). P53 induces apoptosis by caspase activation through mitochondrial cytochrome c release. *The Journal of Biological Chemistry*, 275(10), 7337–7342. <https://doi.org/10.1074/jbc.275.10.7337>
- Schwarz, K., & Ternes, W. (1992). Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis*. *Zeitschrift Für Lebensmittel-Untersuchung Und Forschung*, 195(2), 99–103. <https://doi.org/10.1007/BF01201766>
- Seim, I., Jeffery, P. L., Thomas, P. B., Nelson, C. C., & Chopin, L. K. (2017). Whole-Genome Sequence of the Metastatic PC-3 and LNCaP Human Prostate Cancer Cell Lines. *G3 (Bethesda, Md.)*, 7(6), 1731–1741. <https://doi.org/10.1534/g3.117.039909>
- Selvakumaran, M., Lin, H. K., Miyashita, T., Wang, H. G., Krajewski, S., Reed, J. C., Hoffman, B., & Liebermann, D. (1994). Immediate early up-regulation of bax expression by p53 but not TGF beta 1: A paradigm for distinct apoptotic pathways. *Oncogene*, 9(6), 1791–1798.
- Semenza, G. L. (2010). Defining the Role of Hypoxia-Inducible Factor 1 in Cancer Biology and Therapeutics. *Oncogene*, 29(5), 625–634. <https://doi.org/10.1038/onc.2009.441>
- Sever, R., & Brugge, J. S. (2015). Signal Transduction in Cancer. *Cold Spring Harbor Perspectives in Medicine*, 5(4). <https://doi.org/10.1101/cshperspect.a006098>
- Shafi, A. A., Yen, A. E., & Weigel, N. L. (2013). Androgen receptors in hormone-dependent and castration-resistant prostate cancer. *Pharmacology & Therapeutics*, 140(3), 223–238. <https://doi.org/10.1016/j.pharmthera.2013.07.003>

- Shay, J. W., & Wright, W. E. (2005). Senescence and immortalization: Role of telomeres and telomerase. *Carcinogenesis*, 26(5), 867–874. <https://doi.org/10.1093/carcin/bgh296>
- Shen, X.-G., Wang, C., Li, Y., Wang, L., Zhou, B., Xu, B., Jiang, X., Zhou, Z.-G., & Sun, X.-F. (2010). Downregulation of caspase-9 is a frequent event in patients with stage II colorectal cancer and correlates with poor clinical outcome. *Colorectal Disease: The Official Journal of the Association of Coloproctology of Great Britain and Ireland*, 12(12), 1213–1218. <https://doi.org/10.1111/j.1463-1318.2009.02009.x>
- Sheridan C, Brumatti G, Elgendy M, Brunet M, Martin SJ. An ERK-dependent pathway to Noxa expression regulates apoptosis by platinum-based chemotherapeutic drugs. *Oncogene*. 2010 Dec 9;29(49):6428-41. doi: 10.1038/onc.2010.380. Epub 2010 Aug 30. PMID: 20802529.
- Shin, H. J., Kwon, H. K., Lee, J. H., Anwar, M. A., & Choi, S. (2016). Etoposide induced cytotoxicity mediated by ROS and ERK in human kidney proximal tubule cells. *Scientific reports*, 6, 34064. <https://doi.org/10.1038/srep34064>
- Shorning, B. Y., Dass, M. S., Smalley, M. J., & Pearson, H. B. (2020). The PI3K-AKT-mTOR Pathway and Prostate Cancer: At the Crossroads of AR, MAPK, and WNT Signaling. *International journal of molecular sciences*, 21(12), 4507. <https://doi.org/10.3390/ijms21124507>
- Shukla, S., MacLennan, G. T., Hartman, D. J., Fu, P., Resnick, M. I., & Gupta, S. (2007). Activation of PI3K-Akt signaling pathway promotes prostate cancer cell invasion. *International Journal of Cancer*, 121(7), 1424–1432. <https://doi.org/10.1002/ijc.22862>
- Sionov, R. V., & Haupt, Y. (1999). The cellular response to p53: The decision between life and death. *Oncogene*, 18(45), 6145–6157. <https://doi.org/10.1038/sj.onc.1203130>

- Sircar, K., Yoshimoto, M., Monzon, F. A., Koumakpayi, I. H., Katz, R. L., Khanna, A., Alvarez, K., Chen, G., Darnel, A. D., Aprikian, A. G., Saad, F., Bismar, T. A., & Squire, J. A. (2009). PTEN genomic deletion is associated with p-Akt and AR signalling in poorer outcome, hormone refractory prostate cancer. *The Journal of Pathology*, 218(4), 505–513. <https://doi.org/10.1002/path.2559>
- Smith, A. J., Karpova, Y., D'Agostino, R., Jr, Willingham, M., & Kulik, G. (2009). Expression of the Bcl-2 protein BAD promotes prostate cancer growth. *PLoS one*, 4(7), e6224. <https://doi.org/10.1371/journal.pone.0006224>
- Spans, L., Atak, Z. K., Van Nieuwerburgh, F., Deforce, D., Lerut, E., Aerts, S., & Claessens, F. (2012). Variations in the exome of the LNCaP prostate cancer cell line. *The Prostate*, 72(12), 1317–1327. <https://doi.org/10.1002/pros.22480>
- Sramkoski, R. M., Pretlow, T. G., Giaconia, J. M., Pretlow, T. P., Schwartz, S., Sy, M. S., Marengo, S. R., Rhim, J. S., Zhang, D., & Jacobberger, J. W. (1999). A new human prostate carcinoma cell line, 22Rv1. *In Vitro Cellular & Developmental Biology. Animal*, 35(7), 403–409. <https://doi.org/10.1007/s11626-999-0115-4>
- Stuart, J. A., Fonseca, J., Moradi, F., Cunningham, C., Seliman, B., Worsfold, C. R., Dolan, S., Abando, J., & Maddalena, L. A. (2018). How Supraphysiological Oxygen Levels in Standard Cell Culture Affect Oxygen-Consuming Reactions. *Oxidative Medicine and Cellular Longevity*, 2018, 1–13. <https://doi.org/10.1155/2018/8238459>
- Suarez, S. S., & Pacey, A. A. (2006). Sperm transport in the female reproductive tract. *Human Reproduction Update*, 12(1), 23–37. <https://doi.org/10.1093/humupd/dmi047>
- Tai, S., Sun, Y., Squires, J. M., Zhang, H., Oh, W. K., Liang, C.-Z., & Huang, J. (2011). PC-3 is a cell line characteristic of prostatic small cell carcinoma. *The Prostate*, 71(15), 1668–1679. <https://doi.org/10.1002/pros.21383>

- Takayama, K., & Inoue, S. (2013). Transcriptional network of androgen receptor in prostate cancer progression. *International Journal of Urology: Official Journal of the Japanese Urological Association*, 20(8), 756–768. <https://doi.org/10.1111/iju.12146>
- Tan, Y., Demeter, M. R., Ruan, H., & Comb, M. J. (2000). BAD Ser-155 Phosphorylation Regulates BAD/Bcl-XL Interaction and Cell Survival. *Journal of Biological Chemistry*, 275(33), 25865–25869. <https://doi.org/10.1074/jbc.M004199200>
- Tan, Y., Ruan, H., Demeter, M. R., & Comb, M. J. (1999). P90RSK Blocks Bad-mediated Cell Death via a Protein Kinase C-dependent Pathway. *Journal of Biological Chemistry*, 274(49), 34859–34867. <https://doi.org/10.1074/jbc.274.49.34859>
- Tan, B., & Tan, B. (2013). Role of oxidative stress, endoplasmic reticulum stress and ERK activation in triptolide-induced apoptosis. *International Journal of Oncology*, 42, 1605–1612. <https://doi.org/10.3892/ijo.2013.1843>
- Tannock, I. F., de Wit, R., Berry, W. R., Horti, J., Pluzanska, A., Chi, K. N., Oudard, S., Théodore, C., James, N. D., Turesson, I., Rosenthal, M. A., & Eisenberger, M. A. (2004). Docetaxel plus Prednisone or Mitoxantrone plus Prednisone for Advanced Prostate Cancer. *New England Journal of Medicine*, 351(15), 1502–1512. <https://doi.org/10.1056/NEJMoa040720>
- Teng, M. W. L., Swann, J. B., Koebel, C. M., Schreiber, R. D., & Smyth, M. J. (2008). Immune-mediated dormancy: An equilibrium with cancer. *Journal of Leukocyte Biology*, 84(4), 988–993. <https://doi.org/10.1189/jlb.1107774>
- Teroerde, M., Nientiedt, C., Duensing, A., Hohenfellner, M., Stenzinger, A., & Duensing, S. (2021). Revisiting the Role of p53 in Prostate Cancer. In Urology Department, Frimley Park Hospital, Portsmouth Rd, Frimley, Camberley GU16 7UJ, UK, S. R. Bott, & K. Lim Ng (Eds.), *Prostate Cancer* (pp. 113–124). *Exon Publications*. <https://doi.org/10.36255/exonpublications.prostatecancer.p53.2021>

- Tsujimoto, Y., Finger, L. R., Yunis, J., Nowell, P. C., & Croce, C. M. (1984). Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science (New York, N.Y.)*, 226(4678), 1097–1099. <https://doi.org/10.1126/science.6093263>
- Vander Heiden, M. G., Cantley, L. C., & Thompson, C. B. (2009). Understanding the Warburg effect: The metabolic requirements of cell proliferation. *Science (New York, N.Y.)*, 324(5930), 1029–1033. <https://doi.org/10.1126/science.1160809>
- Vikhanskaya, F., Lee, M. K., Mazzeletti, M., Broggin, M., & Sabapathy, K. (2007). Cancer-derived p53 mutants suppress p53-target gene expression—Potential mechanism for gain of function of mutant p53. *Nucleic Acids Research*, 35(6), 2093–2104. <https://doi.org/10.1093/nar/gkm099>
- Vlavcheski, F.; Naimi, M.; Murphy, B.; Hudlicky, T.; Tsiani, E. (2017). Rosmarinic Acid, a Rosemary Extract Polyphenol, Increases Skeletal Muscle Cell Glucose Uptake and Activates AMPK. *Molecules* . 22, doi:10.3390/molecules22101669.
- Wang R, Cong WH, Guo G, Li XX, Chen XL, Yu XN, Li H. (2012). Synergism between carnosic acid and arsenic trioxide on induction of acute myeloid leukemia cell apoptosis is associated with modulation of PTEN/Akt signaling pathway. *Chin J Integr Med*. 18(12):934-41. doi: 10.1007/s11655-012-1297-z. Epub 2012 Dec 13. PMID: 23239002.
- WANG, X., & LIN, Y. (2008). Tumor necrosis factor and cancer, buddies or foes? *Acta Pharmacologica Sinica*, 29(11), 1275–1288.
- Wang, X., Tryndyak, V., Apostolov, E. O., Yin, X., Shah, S. V., Pogribny, I. P., & Basnakian, A. G. (2008). Sensitivity of human prostate cancer cells to chemotherapeutic drugs depends on EndoG expression regulated by promoter

- methylation. *Cancer Letters*, 270(1), 132–143.  
<https://doi.org/10.1016/j.canlet.2008.04.053>
- Wenkert, E., Fuchs, A., & McChesney, J. D. (1965). Chemical Artifacts from the Family Labiatae. *The Journal of Organic Chemistry*, 30(9), 2931–2934.  
<https://doi.org/10.1021/jo01020a012>
- Whang, Y. E., Wu, X., Suzuki, H., Reiter, R. E., Tran, C., Vessella, R. L., Said, J. W., Isaacs, W. B., & Sawyers, C. L. (1998). Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression. *Proceedings of the National Academy of Sciences of the United States of America*, 95(9), 5246–5250.
- Wick, M. J., Dong, L. Q., Riojas, R. A., Ramos, F. J., & Liu, F. (2000). Mechanism of Phosphorylation of Protein Kinase B/Akt by a Constitutively Active 3-Phosphoinositide-dependent Protein Kinase-1. *Journal of Biological Chemistry*, 275(51), 40400–40406. <https://doi.org/10.1074/jbc.M003937200>
- Wishart, D. S. (2015). Is Cancer a Genetic Disease or a Metabolic Disease? *EBioMedicine*, 2(6), 478–479. <https://doi.org/10.1016/j.ebiom.2015.05.022>
- Witsch, E., Sela, M., & Yarden, Y. (2010). Roles for growth factors in cancer progression. *Physiology (Bethesda, Md.)*, 25(2), 85–101.  
<https://doi.org/10.1152/physiol.00045.2009>
- Woods-Burnham, L., Basu, A., Cajigas-Du Ross, C. K., Love, A., Yates, C., De Leon, M., Roy, S., & Casiano, C. A. (2017). The 22Rv1 prostate cancer cell line carries mixed genetic ancestry: Implications for prostate cancer health disparities research using pre-clinical models. *The Prostate*, 77(16), 1601–1608. <https://doi.org/10.1002/pros.23437>

- Wong, R. S. (2011). Apoptosis in cancer: From pathogenesis to treatment. *Journal of Experimental & Clinical Cancer Research: CR*, 30(1), 87. <https://doi.org/10.1186/1756-9966-30-87>
- Xia, Y., Shen, S., & Verma, I. M. (2014). NF- $\kappa$ B, an active player in human cancers. *Cancer Immunology Research*, 2(9), 823–830. <https://doi.org/10.1158/2326-6066.CIR-14-0112>
- Xiao, P., Ma, T., Zhou, C., Xu, Y., Liu, Y., & Zhang, H. (2016). Anticancer effect of docetaxel induces apoptosis of prostate cancer via the cofilin-1 and paxillin signaling pathway. *Molecular Medicine Reports*, 13, 4079-4084. <https://doi.org/10.3892/mmr.2016.5000>
- Yamasaki, L. (2003). Role of the RB tumor suppressor in cancer. *Cancer Treatment and Research*, 115, 209–239. [https://doi.org/10.1007/0-306-48158-8\\_9](https://doi.org/10.1007/0-306-48158-8_9)
- Yan, M., Li, G., Petiwala, S. M., Householter, E., & Johnson, J. J. (2015). Standardized rosemary (*Rosmarinus officinalis*) extract induces Nrf2/sestrin-2 pathway in colon cancer cells. *Journal of Functional Foods*, 13, 137–147. <https://doi.org/10.1016/j.jff.2014.12.038>
- Yesil-Celiktas, O., Sevimli, C., Bedir, E., & Vardar-Sukan, F. (2010). Inhibitory effects of rosemary extracts, carnosic acid and rosmarinic acid on the growth of various human cancer cell lines. *Plant Foods for Human Nutrition (Dordrecht, Netherlands)*, 65(2), 158–163. <https://doi.org/10.1007/s11130-010-0166-4>
- Yip, K. W., & Reed, J. C. (2008). Bcl-2 family proteins and cancer. *Oncogene*, 27(50), 6398–6406. <https://doi.org/10.1038/onc.2008.307>
- Yuan, T., & Cantley, L. (2008). PI3K pathway alterations in cancer: Variations on a theme. *Oncogene*, 27(41), 5497–5510. <https://doi.org/10.1038/onc.2008.245>

- Zhang, H., Kong, Q., Wang, J., Jiang, Y., & Hua, H. (2020). Complex roles of cAMP–PKA–CREB signaling in cancer. *Experimental Hematology & Oncology*, 9(1), 32. <https://doi.org/10.1186/s40164-020-00191-1>
- Zhao, L., & Vogt, P. K. (2008). Class I PI3K in oncogenic cellular transformation. *Oncogene*, 27(41), 5486–5496. <https://doi.org/10.1038/onc.2008.244>
- Zhu, K., Liu, Q., Zhou, Y., Tao, C., Zhao, Z., Sun, J., & Xu, H. (2015). Oncogenes and tumor suppressor genes: Comparative genomics and network perspectives. *BMC Genomics*, 16(7), S8. <https://doi.org/10.1186/1471-2164-16-S7-S8>

## **Appendix:**

### **Solutions:**

All solutions were prepared utilizing deionized (MilliQ) water.

#### **Phosphate buffered saline solution (PBS):**

137mM NaCl, 2.7mM KCl, 1.5mM KH<sub>2</sub>PO<sub>4</sub>, 8.1mM Na<sub>2</sub>HPO<sub>4</sub>, 0.68mM CaCl<sub>2</sub>, 0.49mM MgCl<sub>2</sub> diluted in MilliQ water. The pH is adjusted to 7.4 and the solution is then sterilized through autoclaving in glass bottles before use.

#### **Cell Lysis Buffer**

20mM Tris solution at a pH of 7.5, 1% Triton X-100, 2.5mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 150mM NaCl, 1mM EDTA, 1mM glycerol 2-phosphate, 1mM EGTA, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1µg/mL leupeptin. 1 mM of PMSF is then added to the cell lysis buffer before use.

#### **3X SDS Sample Buffer**

187.5mM Tris solution at a pH of 7.5, 6% SDS, 30% glycerol, 0.03% bromophenol blue, water; add 0.15% β-mercaptoethanol before use.

### **Western blot solutions**

#### **0.5M Tris:**

6g Tris base, 60mL water. Bring final volume to 100 mL and adjust pH to 6.8 with HCl.

#### **1.5M Tris:**

27.23g Tris base, 80mL deionized water. Bring volume to 150 mL and adjust pH to 8.8 with HCl.

Resolving Gel Buffer (10%) – for 4 gels:

12.3mL water, 9.9mL 30% acrylamide solution, 7.5mL 1.5M Tris, 0.3mL 10% SDS. Subsequently add 150µL of APS (0.01g/100µL water) and 30µL TEMED.

Stacking Gel Buffer (4%) – for 4 gels:

18.3mL water, 3.9mL 30% acrylamide, 7.5mL 0.5M Tris, 0.3mL 10% SDS. Subsequently add 150µL of APS (0.01g/100µL water) and 30µL TEMED.

10x Tris-Buffered Saline:

24.2g Tris base, 80g NaCl. Bring volume to 1L with deionized water; adjust pH to 7.6 with HCl. Add 50mL of 10x TBS and 500µL Tween to 450mL deionized water.

10x Running Buffer:

15.15g Tris base, 72g glycine, 5g SDS. Add water to a final volume of 500mL. Add 50mL of stock into 450mL deionized water.

Transfer Buffer:

3.03g Tris base, 15.01g glycine, 20% methanol. Bring final volume to 1L.

Blocking Buffer:

15mL 10xTBS, 135mL deionized water; 7.5g non-fat powdered milk, 0.15mL Tween. Final volume of 150 mL.

Primary Antibody Dilution Buffer:

18mL deionized water, 2mL of 10xTBS, 1.0g BSA, 20 $\mu$ L Tween. Add 10 $\mu$ L of primary antibody before use (1 in 2000 dilution) and freeze at -20°C after use.

Secondary Antibody Solution:

10mL blocking buffer to be mixed with 5 $\mu$ L of secondary antibody. Discard after use.

**Crystal violet assay solutions**

Crystal Violet Stain:

0.5% w/v crystal violet stain diluted in 25% methanol, to be kept away from light

Solubilizer:

0.05M NaH<sub>2</sub>PO<sub>4</sub> in 50% ethanol, to be kept away from light