Saffron extract and its active constituents as preventative and/or treatment agents for prostate cancer using in vitro and in vivo models

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SAFFRON EXTRACT AND ITS ACTIVE CONSTITUENTS AS PREVENTATIVE AND/OR TREATMENT AGENTS FOR PROSTATE CANCER USING IN VITRO AND IN VIVO MODELS

A dissertation
presented in partial fulfillment of requirements
for the degree of Doctor of Philosophy
in the Department of BioMolecular Sciences
The University of Mississippi

by
FAISAL F. ALBAQAMI

May 2016
Saffron extract and its active constituents were evaluated as chemopreventatives and/or treatments for prostate cancer. Different models of prostate cancer (in vitro and in vivo) were used for studying saffron and its constituents’ mechanisms. In normal prostate cells, saffron extract, crocetin, and safranal, in non-toxic concentrations, possessed antioxidant properties against the reactive oxygen species induced by H₂O₂ which further supports saffron constituents as chemopreventative agents. Moreover, saffron and its active constituents produced cytotoxicity in prostate cancer cells through intrinsic apoptosis pathways. Potential efficacy in minimizing prostate cancer metastasis was also examined. Saffron extract, crocetin, and safranal decreased the migration and invasion capability of prostate cancer cells in vitro. Among all tested agents, safranal caused the lowest cytotoxicity in normal prostate cells.

A new HPLC analytical method was developed to simultaneously measure saffron marker compounds (picrocrocin, crocin, safranal, and crocetin), which can be used to assess the quality and to identify adulteration of saffron samples. Our study emphasized the importance of standardizing the solvent systems used during extraction to minimize cytotoxicity in normal cells. The saffron available commercially differed greatly based on their constituents’ concentrations and biological activity, even when the plants came from the same region of cultivation, making it essential to evaluate each batch chemically and biologically.

To overcome the limitations of current mammalian models, a xenograft zebrafish model for prostate cancer was developed as a new alternative. A zebrafish transgenic line was created in
our laboratory which offered unique characteristics, including physical transparency and vasculature system fluorescence. Our study optimized procedures for transplanting cancer cells into zebrafish and was validated using docetaxel as a known chemotherapy for prostate cancer.

In conclusion, the optimized HPLC analytical method is an excellent tool for saffron quality evaluation. Saffron extract and its active constituents are considered good candidates as a chemopreventative and treatment for prostate cancer. The transgenic zebrafish *in vivo* model minimizes the time needed in preclinical evaluations of new medications and offers the potential for personalization of treatment options based on each patient’s case.
DEDICATION

This work is dedicated to my lovely family, friends, the Environmental Toxicology Research Program, and all victims of cancer.
LIST OF ABBREVIATIONS

2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA)

Accumulation of Photosystem One 2 (Apo-2)

Adenosine triphosphate (ATP)

Androgen receptor (AR)

Analysis of variance (ANOVA)

Apoptotic protease activating factor 1 (Apaf-1)

Atmospheric pressure chemical ionization (APCI)

Benign prostatic hyperplasia (BPH)

Benzo(a)pyrene (BaP)

Bovine serum albumin (BSA)

Cardiac troponin T gene (tnnt2)

Caspase enzyme (CASP)

Caspase recruitment domain (CARD)

Catalase (CAT)

Cell Surface Death Receptor (Fas)

Cell Surface Death Receptor Ligand (FasL)

Days post fertilization (dpf)

Days post injection (dpi)
Death effector domain (DED)
Death-inducing signaling complex (DISC)
Dichloromethane (DCM)
Dichromic extended reflector (dcxr)
Digital rectal examination (DRE)
Dihydrotestosterone (DHT)
*Discosoma* species red fluorescent protein (DsRed)
Dulbecco's Modified Eagle Medium (DMEM)
Dulbecco's phosphate buffered saline (DPBS)
Dystrophin gene (DMD)
Electrospray ionization (ESI)
Enhanced green fluorescent protein (EGFP)
Epidermal growth factor receptor (EGFR)
*Escherichia coli* (*E. coli*)
Extracellular matrix (ECM)
Fas-associating protein with death domain (FADD)
Fetal bovine serum (FBS)
Fibroblast growth factor (FGFR)
Geneticin (G418)
Glutathione peroxidase (GPx)
Glutathione S-transferase (GST),
Glycosylphosphatidylinositol (GPI)
Growth factor reduced (GFR)
Heat shock protein 90 alpha (HSP 90α)
High performance liquid chromatography (HPLC)
Hypoxia-inducible factor 1-alpha (HIF-1α)
Institutional Animal Care and Use Committee (IACUC)
Interleukin-6 (IL-6)
International Conference for Harmonization (ICH)
Limit of detection (LOD)
Limit of quantification (LOQ)
Luteinizing hormone-releasing hormone (LHRH)
Mass spectrometry (MS)
Matrix metalloprotease (MMP)
Methyl methanesulfonate (MMS)
Mitogen activated protein kinase (MAPK)
Mouse embryonic fibroblast cell line (STO)
Mouse prostate reconstitution (MPR)
Optical density (OD)
Part per million (ppm)
Phenazine methosulfate (PMS)
Poly ADP-ribose polymerase (PARP)
Polyethersulfone (PES)
Prostate specific antigen (PSA)
Prostatic intraepithelial neoplasia (PIN)
Protein tyrosine phosphatase 1B (PTP1B)
Reactive nitrogen species (RNS)
Reactive oxygen species (ROS)
Real time polymerase chain reaction (RT-PCR)
Relative standard deviation (RSD)
Reverse phase thin layer chromatography (RP-TLC)
Roswell Park Memorial Institute-1640 (RPMI-1640)
Sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium salt (XTT)
Superoxide dismutase (SOD)
The half maximal inhibitory concentration (IC_{50})
Thin Layer Chromatography (TLC)
TNF-related apoptosis-inducing ligand receptor-1 and -2 (TRAIL-R1 and R2)
Transforming growth factor beta-1 (TGF-β1)
Transgenic adenocarcinoma of the mouse prostate (TRAMP)
Tumor necrosis factor (TNF)
Tumor necrosis factor receptor-1 (TNFR1)
Tumor protein p53 (TP53)
Ultra high performance liquid chromatography (UHPLC)

Vascular endothelial growth factors (VEGF)

World Health Organization (WHO)

Zebrafish International Resource Center (ZIRC)
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I would like to thank my amazing advisor, Dr. Kristine Willett, for her strong support and encouragement during many difficult moments. Throughout my studies she taught me a great deal, especially how to critique my work at each stage before the professionals critique my work, in addition to the training and wisdom she shared with me. I learned much from her which will be invaluable for my future scientific career. The friendly and family atmosphere she created in our laboratory produced a great deal of happiness in our scientific life “go team”. It is difficult to find the correct words to express my feelings and level of respect I hold toward her, but as always I am sure she will understand what I mean.

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CHAPTER 1: PROSTATE CANCER

1. Introduction

The prostate is a gland located inferior to the urinary bladder, anterior to the symphysis pubis, and posterior to the rectum. It weighs between 20-25 grams and consists of three different zones: the peripheral, central, and transition zone. The peripheral zone represents \( \approx 70\% \) of the prostate tissue while central zone is 25\%, and the transition zone is 5-10\%. In the reproductive system, the prostate has multi-functions. First, it converts testosterone which is secreted from testes and adrenal glands into dihydrotestosterone (DHT), the most potent form of androgen hormone. Secondly, it produces 37-44\% of seminal fluids which is mandatory for transferring and activating sperm. Finally, it closes the prostate duct during urination and urethra, with help of sphincter muscles, during ejaculation (Myers et al., 1987, Master and Turek, 2001, Mcneal, 1988, Ndovi et al., 2007). Prostate cells consist of three different types: basal, secretory luminal, and neuroendocrine cells. Each cell type can be distinguished according to specific markers. The basal, luminal, and neuroendocrine cells express keratins 5 and keratins 14, keratins 8 and keratins 18, and neuron-specific enolase and neuropeptides, respectively (Van Leenders et al., 2000, Bonkhoff and Remberger, 1996).

Cancer occurs when cells grow without control and do not respond to normal signaling pathways. It can progress to form a tumor and loss of tissue homeostasis. With time, the cancer can continue to grow until it gains the ability to invade the adjacent tissues and enter the blood stream or lymphatic system to metastasize to distant organs (Hanahan and Weinberg, 2011). In
malignant prostate cancer, the luminal cells of prostate tissue provide the origin of adenocarcinoma development (Wang et al., 2014c). Prostate cancer develops most often in the peripheral zone (68%), followed by the transition zone (24%), and 8% in the central zone (Mcneal, 1988).

Prostate cancer is the most diagnosed malignancy in men and the sixth leading cause of cancer deaths in the United States. Despite the improvements with treatment options and earlier diagnosis, in 2016, about 26,120 men died from prostate cancer out of the 180,890 cancer cases diagnosed in the United States (American Cancer Society, 2016). According to World Health Organization (WHO) estimations for 2012, there were 1.095 million new diagnosed cases and 307,000 person deaths from prostate cancer in the world. In developing countries, in particular, the WHO expectation was 353,000 for new diagnosed cases and 165,000 for death cases (Ferlay et al., 2012).

Although the etiology of prostate cancer is still incomplete, some of the greatest predisposing factors of prostate cancer are age, family history, race, diet, hormonal disruption, and environmental pollutants (Gann, 2002, Hu et al., 2012, Clapp et al., 2008). According to 2011-2012 data, ≈ 14% of men have a risk to develop prostate cancer during their lifetime. Usually the diagnosed prostate cancer cases are associated with older age starting over 45 years, and the median age of the diagnosed patients is 66 years old. African Americans have nearly 1.65-times more newly diagnosed and 2.34-times more deaths compared to Caucasians. Generally, the survival rate with prostate cancer is 98.9% at 5 years. The survival rate declines dramatically to 28.2% if the patient is diagnosed with metastatic prostate cancer. Therefore, the early detection in prostate cancer plays an important role in increased survival time, if found early, the majority of tumors are localized which provides more options for patients’ treatments.
While in the USA survival is almost 99% within five years of detection, there have been no significant improvements or new discoveries in the prostate cancer treatments in the last decade. Since the early 1990s, there has been a drastic increase in newly diagnosed patients with prostate cancer due to the development of prostate cancer detection methods. On the other hand, this is not an easy option in developing countries. In the United States there is almost one death corresponding to each 8 newly diagnosed cases, whereas in the whole world this ratio has declined to 1:3.5, and has declined more so in developing countries at 1:2 (Ferlay et al., 2012).

Based on American Cancer Society guidelines for prostate cancer to be diagnosed, there are two categories of exams. The first category is early (preliminary) detection tests; the second category are confirming exams. In the early detection category, there are two options, PSA screening and digital rectal examination (DRE) which should be performed periodically for men over 50 years. In case the results are abnormal, the confirming tests should be conducted. The confirming tests include transrectal ultrasound and a prostate biopsy to determine the stage of prostate cancer and the best treatment options for the patient (Wolf et al., 2010).

Staging the prostate cancer is a critical step needed to design a treatment regimen for each patient and monitor the outcomes of treatment. The prostate cancer staging is based on the TNM staging system (Table 1). There are four factors is needed to use the TNM staging system which include primary tumor location, size, metastasis to nearby lymph node, and metastasis to distant location (Edge and Compton, 2010, Edge et al., 2010).
Table 1: Prostate cancer stages

<table>
<thead>
<tr>
<th>Group</th>
<th>T</th>
<th>N</th>
<th>M</th>
<th>Gleason score</th>
<th>PSA level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>1a-c</td>
<td>0</td>
<td>0</td>
<td>≤ 6</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>2a</td>
<td>0</td>
<td>0</td>
<td>≤ 6</td>
<td>&lt; 10</td>
<td></td>
</tr>
<tr>
<td>Stage IIa</td>
<td>1a-c</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>1a-c or 2a</td>
<td>0</td>
<td>0</td>
<td>≤ 6</td>
<td>10 ≤ PSA &lt; 20</td>
<td></td>
</tr>
<tr>
<td>2a-b</td>
<td>0</td>
<td>0</td>
<td>≤ 7</td>
<td>&lt; 20</td>
<td></td>
</tr>
<tr>
<td>Stage IIb</td>
<td>2c</td>
<td>0</td>
<td>0</td>
<td>Any</td>
<td>Any</td>
</tr>
<tr>
<td>1a-c or 2a-c</td>
<td>0</td>
<td>0</td>
<td>Any</td>
<td>≥ 20</td>
<td></td>
</tr>
<tr>
<td>1a-c or 2a-c</td>
<td>0</td>
<td>0</td>
<td>≥ 8</td>
<td>Any</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>3a-b</td>
<td>0</td>
<td>0</td>
<td>Any</td>
<td>Any</td>
</tr>
<tr>
<td>Stage IV</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>Any</td>
<td>Any</td>
</tr>
<tr>
<td>Any</td>
<td>1</td>
<td>0</td>
<td>Any</td>
<td>Any</td>
<td></td>
</tr>
<tr>
<td>Any</td>
<td>Any</td>
<td>1</td>
<td>Any</td>
<td>Any</td>
<td></td>
</tr>
</tbody>
</table>

TNM system stands to: Tumor site and size (T), Node (N), and Metastases (M). More information about TNM staging system is provided in Appendix 1.

The treatment of prostate cancer is a complicated task and varies depending on the diagnosis and the stage of the cancer. Different types of treatments are available to patients with prostate cancer. In cases of localized prostate cancer, radical prostatectomy and radiation therapy have been used to destroy the cancerous cells (Heidenreich et al., 2011). In almost 30% of patients with metastatic prostate cancer, tumor growth is androgen-dependent (Small and Ryan, 2006). The treatment options for these patients is either orchiectomy or luteinizing hormone-releasing hormone (LHRH) agonists, often in combination with androgen receptor (AR)
antagonists to block the effect of additional available androgens in circulation (See, 2003). In advanced stages of prostate cancer, the patient survival rate has not increased in the last decade (Chu et al., 2003). There are no effective therapies after conventional treatment failure (Shinohara, 2003). Therefore, there have been attempts to explore new approaches and find new treatments. For example, changes in genetics and epigenetics participate in cancer development can disable apoptosis pathways and/or affecting the pathways that control the cell cycle arrest. Cancers have a selective growth advantage and growth becomes out of control. One of the available treatment strategies for cancer focuses on activating apoptosis and cell cycle arrest by interfering with the changes caused through abnormal genetics and epigenetics functions (Lowe and Lin, 2000, Zhou and Elledge, 2000).

1.1. Oxidative stress and prostate cancer

Oxidative stress occurs as a result of disrupted balance between oxidants including reactive oxygen species (ROS) and reactive nitrogen species (RNS) and their elimination by antioxidant mechanisms. The oxidants are normally produced during cellular metabolism especially by the mitochondrial respiratory chain in the mitochondria. Sustained oxidative stress over a long time can cause significant damage to the structure and functions of the cells and result in somatic mutations and neoplastic transformation. Specifically, oxidative stress can induce DNA mutations, genome instability, and chronic inflammation that in turn raises the risk of several tumors including prostate cancer (Reuter et al., 2010). There are relationships between antioxidant enzyme expression and prostate cancer. Superoxide and catalase protein expression were found to be lowered in prostate cancer compared to normal prostate cells (Bostwick et al., 2000).
Generally, the obtained effects on cells from highly acute ROS exposure caused irreversible cell damage and triggered cell death. In the case of prostate cancer, chronic exposure to elevated ROS levels contribute to prostate cancer development by playing important roles as secondary messengers and controlling multiple signal pathways in addition to their ability to damage the DNA. These roles are essential for the activation of different transcription factors such as hypoxia-inducible factor 1-alpha (HIF-1α) and mitogen activated protein kinase (MAPK) that control oncogenes. Furthermore, the high ROS level can drive the cancer to a more aggressive phenotype (Khandrika et al., 2009).

1.2. Migration and invasion

The migratory properties of the cells are required in the development and maintenance of normal physiological conditions and functions of human tissues such as embryogenesis, immune functions, angiogenesis, and wound healing. Furthermore, these mechanisms are involved in the pathophysiology of many medical disorders including inflammation and cancer (Cho and Klemke, 2000). The most life threatening cancers are characterized by their ability for metastasis which represents 90% of the deaths related to cancer. Metastasized cells are aggressive and more resistant to treatments than the original cancer cells (Mehlen and Puisieux, 2006). The primary cancer cells undergo five major steps which are necessary for metastasis to distant areas. These steps include separating of metastatic cells from the primary tumor, invading adjacent tissues, intravasating into circulation, leaving the circulation by extravasation into a distant area, and proliferating to grow at the distant organ while forming a secondary tumor. Each step consists of a series of molecular mechanisms (Chambers et al., 2002). It is often fundamental for the cancer to metastasize to distant sites in order to find suitable microenvironments, similar to the primary
cancer source for successful spreading from the primary location. Normally, motility is necessary for the cancer cells to invade the adjacent tissue, colonize and grow in the area that provides it with an ideal microenvironment (Joyce and Pollard, 2009). Before the cancer cells invade surrounding tissues and/or enter the blood or lymphatic streams, they will move to proliferate using two strategies. These strategies use the organ space in which the tumor originated. When the cells cannot expand anymore because they have taken up the available space and reached the maximum volume, they will secrete proteolytic enzymes such as matrix metalloproteases (MMPs) to degrade the extracellular matrix (ECM) to form de novo space that is required for the metastasizing cancer cells to escape their primary tumor area (Mccawley and Matrisian, 2000). The cancer cells migrate as individuals (amoeboid and mesenchymal behavior) or groups (strands, vascular sprouts, and clusters behavior), which is determined by the cell type and tissue environments. In the majority of the invasion cases, the MMP enzymes are required for the migrated cells as a group to invade distant places, especially in solid tumors. In rare cases, the cells will migrate in a single cell migration pattern which does not require proteolytic enzymes but uses mechanical forces, squeezing through tight junction spaces for invasion (e.g. leukemia) (Friedl, 2004).

MMPs are a major class of zinc-endopeptidase belonging to the metzincin superfamily which exert protease activity to break down different types of ECM proteins including collagens, elastins, matrix glycoproteins, and proteoglycans (Engel et al., 2005, Maskos, 2005). The MMP family of enzymes in humans consists of 23 members which are classified into six categories according to their substrate specificity. The MMP enzyme categories include the collagenases (MMP-1, -8, -13 and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10 and -11), matrilysins (MMP-7 and -26), MT-MMPs includes type I transmembrane proteins (MMP-14, -
15, -16, and -24) and GPI anchored proteins (MMP-17 and -25), and miscellaneous (MMP-12, -19, -20, -21, -23, -27, and -28) (Nagase et al., 2006).

Generally, in normal conditions MMP enzyme activities are low. Their transcription is regulated by inflammatory cytokines, growth factors, hormones, tumor promoters, and the interactions between cells with ECM (Ala-Aho and Kähäri, 2005). Higher levels of MMP enzymes particularly MMP-2 and -9 were detected in prostate cancer patients with metastasis compared to local carcinoma, BPH, and healthy cases. After the patients received therapy, the enzyme levels dropped significantly (Morgia et al., 2005, Zhong et al., 2008). MMP-1 and MMP-9 are characterized by a hemopexin domain or three fibronectin type II motifs in their structures, respectively. MMP-9 cleaves multiple ECM molecules such as type IV collagen and gelatins, whereas, MMP-1 has a broad digestion capability for collagen types I, II and III (Wang et al., 2002, Iyer et al., 2006, Bode et al., 1999). Furthermore, the MMP-1 and MMP-9 degrade the vascular basal membrane to facilitate the migration of endothelial cells for angiogenesis. Angiogenesis is necessary for the cancer to form new vascular systems. The new blood vessels will help to nourish the needs of the growing tumor. In addition, high expression of MMP causes the release of VEGF and growth factors that help cancer growth (Bergers et al., 2000, Tang et al., 2005, Risau, 1997). Therefore, measuring MMP-1 and -9 levels are crucial to monitor prostate cancer metastasis. Furthermore, MMP could represent an important druggable target to get minimize to their activities and lower the invasion risk of a prostate tumor.
1.3. Mechanism of death

There are two mechanisms for cell death namely: apoptosis and necrosis. They are characterized by different biochemical sequences and morphological shapes. In addition, apoptosis requires ATP in order to commit suicide; however, in contrast, necrosis is associated with ATP depletion (Wyllie, 1981).

1.3.1. Necrosis

After cells are exposed to a strong stressor or toxic agent that leads to ATP depletion, cells may undergo necrosis. As a result, the necrotic cells will swell and burst, in turn, causing the cells’ contents including their enzymes, proteins, and nucleic acids to be spilled out onto the surrounding cells and tissues. Ultimately an inflammatory response will be triggered which causes additional damage to neighboring cells and tissues. One of the major differences between apoptosis and necrosis, in terms of saving the neighboring cells and tissues from inflammation, is removing the contents of the dead cells by phagocytosis which is not achieved in necrosis. The cytoplasmic swelling and cell membrane rupture are considered as microscopic markers of necrotic cells (Savill, 1997, Haslett, 1992, Gores et al., 1990).

1.3.2. Apoptosis

Apoptosis is programmed cell death, which is characterized by nuclear membrane degradation, DNA fragmentation, chromatin condensation, cell shrinkage, and membrane blebbing to make the membrane segments that bind cell fragments and form separated vesicles called apoptosis bodies. These changes are triggered by the proteolytic activities of caspase enzymes. Later, cellular fragments undergo phagocytosis to remove them by neighboring cells or
macrophages (Kerr et al., 1972, Thornberry and Lazebnik, 1998). The major caspase enzymes involved in different pathways of apoptosis induction are caspase-3, 7, 8, 9, and 10 (Kuida et al., 1998, Varfolomeev et al., 1998, Fernandes-Alnemri et al., 1996). Apoptosis mechanisms have crucial roles in normal, as well as in disease conditions. An example of apoptosis’ role under normal conditions is what happens during fetal limb development, in which the interdigital space between fingers is separated by inducing apoptosis pathways to remove the cells that connect an embryo’s fingers together (Mori et al., 1995). Also, when a somatic cell becomes abnormal, for example due to a DNA damage, the tumor protein p53 (TP53) gene will be activated to repair the damage. If the process of repair is incomplete, TP53 gene will turn on apoptosis mechanisms to remove this cell. The removed cell will be replaced by a new daughter cell from the neighboring cell. Therefore, there will be a balance between dead and newly formed cells in normal situations (Delong, 1998).

There is a strong association between the genes controlling the apoptosis process, dysfunction, and disease. In the case of Alzheimer’s disease, which is a neurodegenerative disorder, abnormal induction of caspase enzymes is responsible for neuron cell death due to a high level of amyloid-β peptide that upregulates the TP53 gene (Ohyagi et al., 2005). On the other hand, when the damaged cells are not removed and the caspase enzymes are down-regulated, the cells will continue dividing and the daughter cells will inherit the mutated genome. Finally, the abnormal cells under these conditions will be able to form a tumor (Moll and Schramm, 1998).

There are two main pathways that activate caspase cascades, particularly the intrinsic pathway that involves the mitochondria, and the extrinsic pathway which is initiated by cell surface death receptors. Downstream of these pathways is the activation of caspase-3/7 enzymes.
These enzymes are considered the endpoint in the caspase cascade pathway. After their activation, the degradation of the cell components will be started in a programmed way to form apoptotic bodies. The last component to be degraded in the apoptotic cells will be the mitochondria which is an essential part of cells and is needed to complete the apoptosis process because they provide the necessary ATP (Cohen, 1997, Zou et al., 1999, Saleh et al., 1999, Earnshaw et al., 1999, Thornberry and Lazebnik, 1998).

### 1.3.2.1. Intrinsic pathway

Normally, cytochrome c is present in the mitochondrial intermembrane space, whereas the Apaf-1 and procaspase-9 are present in the cytoplasm. The intrinsic pathway depends on mitochondrial permeability which can be induced when exposure activates proapoptotic and/or inhibits antiapoptotic genes to produce the corresponding proteins. In the case of the activation of antiapoptotic genes such as Bcl-2 and Bcl-xL, cytochrome c will not be released from the mitochondria (Vander Heiden et al., 1997, Kluck et al., 1997). On the other hand, when proapoptotic genes such as Bax and Bid are expressed, mitochondrial permeability will occur. As a result, cytochrome c will be released from the mitochondrial intermembrane space into the cytoplasm (Luo et al., 1998). Three essential proteins are necessary to initiate the intrinsic apoptosis pathway including cytochrome c, Apaf-1, and procaspase-9. For example, when an agent activates proapoptotic genes, it will produce proapoptotic proteins which bind to the outer mitochondrial membrane to facilitate cytochrome c leakage from the mitochondrial intermembrane space into the cytoplasm. The cytochrome c with Apaf-1 will form an apoptosome. The apoptosomes have caspase activation and recruitment domains (CARD) which facilitate their binding to the CARD domain of procaspase-9 to activate the caspase-9 initiator.
enzyme. Caspase-9 in turn, converts procaspase-3 and 7 into the caspase-3 and 7 active forms (Hu et al., 1998, Saelens et al., 2004, Li et al., 1997, Scaffidi et al., 1998, Liu et al., 1996). Finally, the activation of caspase-3/7 will cause cytoskeletal degradation, DNA fragmentation, and apoptotic body production (Martinvalet et al., 2005).

1.3.2.2. Extrinsic pathway

The extrinsic apoptosis pathway is activated by the initiator caspase enzymes (caspase-8 and caspase-10). Usually, these caspases are activated after a specific death receptor on the cell surface is activated by their specific ligand such as FasL binds to the Fas receptor, TNF binds to the TNFR1, and Apo-2 binds to TRAIL-R1 and TRAIL-R2 which affects intercellular signals and activates caspase effector enzymes (Ashkenazi and Dixit, 1998). As a general rule, different death receptors upon activation, behave a similar way to activate the executioner caspase. Fas receptor pathway can be used as an example to summarize the activation processes of the extrinsic pathway. The activation process works according to the following steps: first, FasL binds to Fas receptor on the cell surface which activates the death domain in the cytoplasmic region of the receptor. Second, the death domain of the Fas receptor will bind to the death domain of the FADD protein which forms a complex called DISC. Third, the DISC complex contains DED in the FADD proteins that allow for the DED in procaspase-8 to bind to the DISC complex through FADD. Fourth, procaspase-8 is hydrolyzed to an active form called caspase-8. Finally, caspase-8 will hydrolyze procaspase-3 and 7 into the caspase-3 and 7 active forms. Following the activation of the caspase effector enzymes, the cell degradation process will start to form apoptotic bodies. In the case of TNFR1, the activated initiator caspase enzyme will be caspase-10 (Varfolomeev et al., 1998, Muzio et al., Boldin et al., Sprick et al., 2002).
1.4. *In vitro* cell lines models

*In vitro* studies are important to determine the efficacy and potential mechanisms of any new cancer drug lead against prostate cancer disease. In this study, saffron and its active constituents will be specifically tested *in vitro*. Choosing the proper *in vitro* model is considered a critical step, accordingly three different cell lines were chosen to represent different scenarios of prostate tissue cancer. The cell lines include normal prostate (PNT1A), early stages of prostate cancer (22Rv1), and late stages of prostate cancer (PC-3). The results can then suggest the selectivity and effectiveness of saffron treatments among different stages of prostate cancer. All the previous cell lines were derived from human prostate tissue, each with special characteristics to provide an opportunity to test saffron extracts in different conditions.

1.4.1. PNT1A

PNT1A is a cell derived from the normal human prostate tissue of a 35-year-old post mortem Caucasian male. The cells were then immortalized by transfecting them with a plasmid containing a defective replication origin of a SV40 genome to establish a normal cell line. They express cytokeratin 8 and 18 markers of luminal prostatic cells (Cussenot *et al.*, 1991). The cells express wild type androgen receptor, respond to testosterone and DHT and their growth is stimulated by EGF (Avances *et al.*, 2001). PNT1A lacks the ability to invade due to normal cell behaviors, therefore, these cells were non-tumorigenic in nude mice. The doubling time of this cell line is 30 hours (Berthon *et al.*, 1995).
1.4.2. 22Rv1

In 1999, 22Rv1, a new prostate cancer cell line, was introduced into the prostate cancer research field as a primary prostate carcinoma. It was derived from xenografted CWR22R cells (Sramkoski et al., 1999). Originally CWR22, which was established from the primary prostate tumor, was taken from a prostate cancer patient with a Gleason tumor grade 9, stage D and bone metastasis (Wainstein et al., 1994). Then, it was propagated in mice with androgen ablation for 3-10 months to establish the new subline called CWR22R. The major difference between CWR22 and CWR22R is androgen dependency. CWR22 cells are androgen-dependent prostate cancer cells that will not grow in female or castrated male mice. Whereas, CWR22R cells are androgen-independent and can grow in an androgen free environment such as in female or castrated male mice (Nagabhushan et al., 1996). The CWR22R cells were cultured in a pretreated flask with irradiated STO cells as a feeder layer, after a few passages the new cell line was able to grow in a cultured flask without pretreatment. The new cell line, 22Rv1 is androgen sensitive and has similar characteristics to primary prostate cancer in the early stages of development. For example, AR mRNA and protein are expressed, but PSA is expressed as mRNA only. 22Rv1 growth can be stimulated by epidermal growth factor and weakly stimulated by DHT, but is not inhibited by TGF-β1. The doubling time of this cell line is 40 hours (Sramkoski et al., 1999).

1.4.3. PC-3

The last cell line is PC-3, a traditionally studied prostate cancer cell line that is the most aggressive type, and was derived from a 62-year-old Caucasian patient with metastatic prostate cancer cells in his bones (Kaighn et al., 1979). PC3 has similar characteristics to late stage
prostate cancer characterized by androgen independency, lack of androgen receptors, inability to express PSA at both mRNA and protein levels, and a mutation in the TP53 gene which contributes to their survival (Van Bokhoven et al., 2003). Their growth is not affected by stimulatory hormones such as insulin and epidermal growth factor or by inhibitory hormones such as TGF-β1. The PC-3 cell line is able to grow in androgen free environments such as female mice. The doubling time of this cell line is 33 hours (Kaighn et al., 1979).

1.5. Animal models for prostate cancer

Under controlled conditions, in vitro cancer models offer important details about cancer development, the mechanism of action of drugs, and reproducibility (Cekanova and Rathore, 2014). One of the main reasons for failure of preclinical trials in the drug development processes is direct translation of in vitro studies into clinical applications, because the in vitro models lack the microenvironments and show inadequate similarity to human cancer conditions (Sharpless and Depinho, 2006). Therefore, pairing the in vivo with in vitro experiments greatly improves our knowledge about cancer growth and metastatic cascades which are predicted to reveal new biomarkers, drug mechanisms, and new innovation of cancer therapies (Hoelder et al., 2012).

There are various animal models used as in vivo prostate cancer models, but the histology and physiology of each species are both different among each other and from humans. The most common animal models are canines, rats, and mice. Canines, like humans, have a single prostatic gland. The prostate gland has the ability to develop prostatic intraepithelial neoplasia (PIN) and adenocarcinoma when they become older, which mimics humans regarding age as a risk factor. Dogs’ natural spontaneous development of prostate cancer without any intervention is considered an advantage for use in prevention studies. Some rat strains also have the ability to
develop prostate cancer spontaneously induced by using a chemical such as androgenic hormones and carcinogenics. The size of prostate tissue in rats is larger than in mice. Like mice, transgenic rats are available but with limited options compared with mice. All of the previous models are costly and not easy to maintain in labs, which has made mice the most commonly used animal in prostate cancer research for decades (Navone et al., 1998, Ittmann et al., 2013).

1.5.1. Mice prostate cancer model

Normally, it is rare for wild type mice to develop spontaneous cases of prostate cancer during their lifespan (Suwa et al., 2002). Therefore, there are two major options used to induce prostate cancer in the mouse models. These options are either inserting viral oncogenes into the prostate tissue or using genetically modified mice that make the animal more susceptible to develop prostate cancer through specific pathways. Using the viral oncogene insertion strategy, one of the oldest mouse models for prostate cancer is known as the mouse prostate reconstitution (MPR) model. The MPR is usually developed with a higher rate of occurrence > 90% using inbred C57BL/6 mice to originate poorly differentiated prostate cancer when the ras and myc oncogenes are delivered by using a recombinant retrovirus into each of the mesenchymal and epithelial compartments of the urogenital sinus. The genetically engineered mouse is another option that aims to upregulate or dysregulate specific genes that are responsible for particular pathways that are involved in prostate cancer development. For example, the transgenic adenocarcinoma of the mouse prostate (TRAMP) model is the earliest successful transgenic mouse model created and uses the -426/+28 bp probasin promoter expressing the PB-SV40 T to exclusively target the dorsolateral and ventral lobes of prostatic epithelial cells. Male mice are raised with progressive prostatic neoplasia, and by 10 weeks the mice will have prostatic
hyperplasia, and by 18 weeks they develop a prostate adenocarcinoma with the possibility of metastatic spread to distant locations (Gingrich et al., 1996, Thompson et al., 1992).

Despite the convenience provided by the previous models, there are several significant drawbacks. Humans and mice do not share the same histological and physiological characteristics of the prostate. Thus, using the mouse as a model to mimic the prostate cancer development in humans and evaluate the treatment options is more difficult and an unreliable option. There are many examples for the uniqueness of mice prostate tissues’ structures and functions. For example, the mouse prostate tissue consists of four lobes which are the anterior, ventral, dorsal, and lateral lobes. Whereas the human prostate consists of transitional, central, and peripheral zones. Therefore, there is little correlation between the lobes and zones of mouse and human prostate tissues, respectively (Huss et al., 2001, Mcneal, 1988). Also, the mouse prostate does not secrete PSA in response to abnormal circumstances, unlike humans (Diamandis et al., 2004). The pharmacokinetic data obtained from a mouse model is not able to be directly used to predict human pharmacokinetic behaviors because of variations between species in both weight and lifespan (Demetrius, 2005). In mice, the cancer originates from mesenchymal cells whereas in humans it originates from epithelial cells. Human prostate cancer metastasizes to bone, which is not typical in mouse prostate cancer (Depinho, 2000). In conclusion, the mouse prostate lacks PSA, does not have the same zones as human, and has different origin and metastasis patterns. Therefore, the need for xenograft models has been raised as an alternative to the classic model of cancer.
1.5.2. Human prostate cancer cells xenografted mice

The introduction of human cancers in xenograft models using mice completely transformed cancer research to more advance level by covering many of the disadvantages previously described (Sausville and Burger, 2006). Therefore, the xenograft model of prostate cancer is now considered the best available choice. Human prostate cancer cells are xenografted into immunodeficient mice, because these mice’ bodies will provide the prostate cancer cells with environments that mimic the human body, including hormones and nutrition. Immunodeficient mice were chosen because of their ability to host external tumors without rejection due to a lack of immunity (Gray et al., 2004).

There are many advantages of using xenograft models such as monitoring different aspects of the transplanted tumor including its three dimensional shape, angiogenesis, and paracrine with hormonal components, tumor-stroma interactions, and metastasis (Van Der Pluijm et al., 2001, Cheung et al., 2005). Just like human prostate cancer, each of the xenografted human prostate samples release PSA and the proportion of free PSA is similar to human patient values time of tumor harvest (Buhler et al., 1998). In addition, the effectiveness of certain treatment options can easily be assessed as the result of transplantation of the patient samples simultaneously into multiple mice groups. This helps to customize the therapy plan that is suitable for each human patient case (Troiani et al., 2008).

The most widely used immunocompromised mice used as a host in xenografted models are SCID (severe combined immunodeficiency), Foxn1nu (nude mouse), NOD (non-obese diabetic), and RAG1 and RAG2 (recombination activating gene) knockout mice strains. Each of these strains has special characteristics (Table 2). The available strains have been crossed to maximize the advantages and minimize the drawbacks of using them such as SCID x NOD
crossed strain (Belizário, 2009). There still remain limitations of using mice as a host in xenograft models including that the working area should be pathogen free because the mouse lacks immune system, the number of cells needed for the transplantation processes is large, the experiment duration to complete the studies is long, and the experiments are costly.

Table 2: Mice strains used as hosts in xenograft models (Belizário, 2009)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCID</td>
<td>These mice have a mutation in the Prkdc (protein kinase, DNA activated, catalytic polypeptide) protein required for linking non-homologous ends of double-stranded DNA. As they grow older, mice will naturally produce T and B lymphocytes (immunoglobulin leakiness) so they are very sensitive to irradiation.</td>
</tr>
<tr>
<td>Foxn1nu</td>
<td>These mice have a mutation in the Foxn1 gene that inhibits thymus derived T cells. As a result, they are athymic, but they possess activated natural killer cells.</td>
</tr>
<tr>
<td>NOD</td>
<td>NOD mice do not express the crucial histocompatibility complex (MHC) haplotype known as H-2g7 and SNP in CTLA-4 gene, that are necessary for diabetes-induced autoimmunity.</td>
</tr>
<tr>
<td>RAG1 and RAG2</td>
<td>These mice have deleted Rag1 or Rag2 resulting in the arrest of B and T cell receptor rearrangement and lacks differentiation of T and B cells. They have high levels of natural killer cells and are less sensitive to radiation.</td>
</tr>
</tbody>
</table>
1.5.3. Human prostate cancer cells xenografted zebrafish

In recent years, zebrafish (*Danio rerio*) have become an attractive animal model for many different scientific research projects (Chakraborty *et al.*, 2009). As shown in Figure 1, 70% of the genes of this fish species are similar to the human genome, which make the zebrafish an excellent model to assist and aid in the studying of various human diseases which are related to genetic disorders. Examples of the diseases include dilated cardiomyopathies, and Duchenne muscular dystrophy which is caused by a mutation of the cardiac troponin T gene (*tnnt2*) and dystrophin gene (*DMD*), respectively (Howe *et al.*, 2013, Santoriello and Zon, 2012). Moreover, humans and zebrafish have similar tumor suppressor genes and oncogenes which are involved in critical pathways such as proliferation, migration, and apoptosis. As a consequence, the zebrafish is considered a valuable vertebrate animal model in cancer research (Feitsma and Cuppen, 2008). In addition, this animal also shares basic characteristics and structures of human cardiovascular, kidney, and optic systems (Briggs, 2002, Gestri *et al.*, 2012).
Figure 1: The similarity between human, zebrafish, and mouse genome. The diagram shows the number of shared genes among the species’ genomes (Howe et al., 2013).

While genetic similarities are critical for the zebrafish to be used in such scientific experiments, there are also immense practical and logistical advantages to using zebrafish. They are capable of producing a large number of easily-harvested fertilized eggs in a laboratory environment using simple and affordable methods. In addition, using fish is immensely practical as their embryos develop externally, so larvae/embryos can readily be placed, individually, in a single well of 96-multiwell plate (Mathias et al., 2012, Vander Heiden et al., 1997, Williams, 2010). It is also important to note that there are two ways to deliver the tested compounds to zebrafish, either through injecting a compound into the zebrafish directly, or dissolving a compound in water or a delivery solvent such as DMSO and ethanol, after which each well can be individually dosed with any miscible or solubilized drug load (Maes et al., 2012, Stewart et al., 2011, Ali et al., 2011). Additionally, one of the best sites to transplant cancer cells is in the yolk sac of an embryo because it is easy to inject with low mortality rate (less than 20%), is less
susceptible to the embryo tissues signaling, and is rich in nutrition and elements that provide the proper microenvironments for cancer cells (Geiger et al., 2008).

The xenotransplantation of cancer cells into zebrafish embryos does not require immune suppression because the zebrafish do not develop their immune system completely until 28 dpf. Lack of an immune response in zebrafish provides a great opportunity to use them as an animal host for transplanted human cancer cells (Trede et al., 2004, Taylor and Zon, 2009). Accordingly, it is possible to use zebrafish embryos and larvae in high throughput anticancer screens for rapid and easy drug administration when compared to an alternative, such as injecting mice.

In this latter situation however, immunocompromised mice lack specific genes that are responsible for immune system maturation, plus those genes also have different roles in many pathways. This may interfere with the normal behavior of transplanted cancer cells. For example, in the nude mice model, the Foxn1 gene is mutated to prevent immunity maturation (Macor et al., 2008). The Foxn1 gene, plays an important role in the regulation and activation of fibroblast growth factor (FGFR) family genes. Hence, a mutation in Foxn1 gene causes a downregulation of FGFR protein expression as a consequence of its downstream cascades (Turner and Grose, 2010). However, high expression of FGFR protein is usually associated with the growth of different cancer types, including breast and prostate cancer (Eswarakumar et al., 2005, Nakamoto et al., 1992, Toi et al., 1994). Therefore, using these mice for xenograft procedures especially for prostate and breast cancer should be reconsidered because it does not provide the optimum microenvironment condition compared to zebrafish, that may produce a response in the transplanted cancer independently of treatment.
The xenotransplantation of human cells into the available xenograft animal models, such as mice, requires a large number of prostate cancer cells to graft successfully (e.g. 1-5 million cells) (Workman et al., 2010). This creates the problem of having to obtain sufficient cells to do multi-groups and becomes more challenging if the cells are from a primary patient sample. As a result, the zebrafish provide an ideal alternative because the fish can be used for the same type of experiment but require the availability of far fewer cancer cells (hundreds to thousands of cells) (Nicoli et al., 2007, Taylor and Zon, 2009). When using zebrafish, it is possible to determine the maximum tolerated compound dose before the xenograft experiment is conducted (Eimon and Rubinstein, 2009). Establishing a suitable dose will help avoid misinterpretation due to the effects of drug toxicity. Thus, the value of the zebrafish as a xenograft model is increased as it can provide more-detailed results and valuable data where in vivo studies are concerned. In addition, the results of these studies can be obtained within a short time frame (2 days – 1 month) while other animal models, such as the nude mice xenograft model takes appreciably longer (2 – 12 months) (Stoletov and Klemke, 2008, Siolas and Hannon, 2013). Table 3 highlights more comparisons between mice and zebrafish as a xenograft models.
Table 3: The differences between mice- and zebrafish-based xenografted models.

<table>
<thead>
<tr>
<th></th>
<th>Mice-based models</th>
<th>Zebrafish-based models</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>At least 2 months</td>
<td>At least 3 days</td>
</tr>
<tr>
<td><strong>Experiment duration</strong></td>
<td>2-4 months</td>
<td>3-6 days</td>
</tr>
<tr>
<td><strong>Animal cost</strong></td>
<td>$45-60</td>
<td>$1-2</td>
</tr>
<tr>
<td><strong>Maintenance cost</strong></td>
<td>$1-3 per mouse</td>
<td>Cents per tank</td>
</tr>
<tr>
<td><strong>Cancer cell tracking</strong></td>
<td>Tumor mass</td>
<td>Single cells</td>
</tr>
<tr>
<td><strong>Visualizing cancer cells</strong></td>
<td>End Point of the experiment</td>
<td>Daily basis</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td>Each organ must be sectioned separately</td>
<td>A whole fish can be sectioned per slice</td>
</tr>
</tbody>
</table>

1.5.3.1. Tg(fli1a:EGFP) and Casper

Aside from wild-type zebrafish, there are various transgenic and mutant zebrafish lines. These fish lines are used in cancer research fields and one advantage of certain lines is their benefit for visualization. Another reason for the popularity and suitability of zebrafish relates to their transparency facilitating the straightforward ability to observe cancer development in living zebrafish. Transgenic zebrafish present a rare opportunity to observe the cancer development process and the effects of drugs on, for example, prostate cancer in real-time under a fluorescent microscope (Feitsma and Cuppen, 2008). In this dissertation, two different types of transgenic
zebrafish, Tg(fli1a:EGFP) and Casper, were used to develop a new in vivo model for human prostate cancer. The endothelial cells of Tg(fli1a:EGFP) transgenic zebrafish are stably green fluorescently labeled, thus giving researchers the opportunity to examine zebrafish blood vessels under a fluorescent microscope (Lawson and Weinstein, 2002). The Casper zebrafish are characterized by their transparency because they do not form pigmentation during growth, which allows for tracking their body and organ development with a bright field microscope, and the cancer cells under a fluorescent microscope. Casper fish lack melanocytes and iridophores due to mutations in the nacre and roy genes. Because these mutant genes are recessive, the fish must be homozygous to be a Casper fish (White et al., 2008). One of the biggest advantages is that we can image cancer cells in the same fish with mild anesthesia over the course of the experiment time. Importantly, this advantage will decrease any variability caused by monitoring different animals during a series of experiments (Taylor and Zon, 2009, Amatruda et al., 2002).

1.5.3.2. Crossed Tg(fli1a:EGFP) and Casper

Crossing the Tg(fli1a:EGFP) with Casper zebrafish lines produces a unique and ideal model as an animal host for the xenograft procedure. The uniqueness of this cross-bred fish line allows for clear monitoring of the angiogenesis and invasiveness of the transplanted labeled prostate cancer cells without interference from zebrafish pigmentation that develops after 48 hpf in wild-type and Tg(fli1a:EGFP) zebrafish larvae. This advantage allows the researcher to measure the cancer’s fluorescence intensity accurately, which is proportional to the growth of cancer cells. Therefore, we can explore the in vivo transplanted cancer cells’ behavior and metastasis. Furthermore, by assessing prospective chemotherapeutics compounds ability to
decrease tumor volume, the model can be used in new drug screening. So, this line will provide a great model with more sensitive data, a shorter time for the \textit{in vivo} results at a far lower cost.

Because there is no ideal animal model, the pros and cons of each animal model must be weighted and optimized accordingly. For example, in the cases of prostate and breast cancers, when comparing the animal-originated cancer (specific animal organ becomes cancerous through extrinsic intervention) with the xenograft model (transplanted human cancer cells in animal body), the xenograft model is preferred because the animal breast and prostate organs have little correlation to human organs. Moreover, when comparing mice and zebrafish for use in a xenograft model, the zebrafish is preferred because no genetic modification or chemicals to immunocompromise their systems are required in order to conduct any cancer transplantation procedure. Without the need for genetic modification or chemicals for immunocompromising, plus cost-effectiveness and speed of obtaining study results, the benefits of using the zebrafish are often far greater than those offered by mice.

\textbf{1.6. Natural products for prostate cancer}

Currently, natural products are a promising source for developing new treatment modalities. Finding compounds that work as chemo-preventative agents that prevent prostate cancer development, at the same time as cytotoxic agents to treat existing cancer cells is considered a valuable achievement. Saffron extract and its active constituents possess antioxidant properties and additionally have tumoricidal activity. Now, there is a critical need to study the actual mechanisms of saffron against the development, progression, and metastasis of prostate cancer.
1.6.1. Saffron

Saffron consists of the dried stigmas and tops of the styles of *Crocus sativus* (Iridaceae). Saffron has reddish-brown stigmas, a sweet aromatic odor, and a bitter taste. Saffron content analyses identified different chemical compounds which include water, proteins, fats, minerals, reduced and free sugars, and pigments. Vitamins such as thiamine and riboflavin were also found in saffron, in addition to trace amounts of different volatile oils (Sampathu *et al.*, 1984). As a result of the agricultural conditions and geographical origin, the chemical composition of saffron can be different (Maggi *et al.*, 2011). Generally, saffron contains over 150 components, and the major components are crocin (24 - 34%) and picrocrocin (5 - 21%). In the case of hydrolysis processes, crocin produces gentiobiase and crocetin (6 - 11%), while picrocrocin yields glucose and safranal. In saffron, crocin is responsible for color, safranal (0.11 – 1.8% is present in saffron as a free component and represents up to 72% of their total volatile oil) is responsible for its odor, and picrocrocin is responsible for its bitter taste (Figure 2) (Winterhalter and Straubinger, 2000, Schmidt *et al.*, 2007, Lautenschlager *et al.*, 2014, Alonso *et al.*, 1996, Lage and Cantrell, 2009). Saffron plants are used in food for added coloring, flavoring, and aroma. In folk medicine, saffron has been used to treat different disorders and as an antispasmodic, expectorant, sedative, and analgesic natural product (Ríos *et al.*, 1996). Recently, many studies have suggested, that saffron extracts can be used in the treatment of depression (Noorbala *et al.*, 2005), anxiety (Hosseinzadeh and Noraei, 2009), memory impairment (Zhang *et al.*, 1994), hypertension (Imenshahidi *et al.*, 2010), inflammatory diseases (Hosseinzadeh and Younesi, 2002), and cancers. Saffron extracts induce apoptosis and cell cycle arrest, but inhibit cellular proliferation and tumor progression in various cancer cell lines (Dhar *et al.*, 2009, Aung *et al.*, 2007,
Saffron is a good candidate to prevent prostate cancer through its antioxidant effects because it is rich in carotenoids. Dietary carotenoids show the ability to decrease the occurrence of prostate cancer due to their potent antioxidant activity (Willis and Wians, 2003). In clinical trials, it was found that daily intake of nutrients rich in carotenoids such as α-carotene, β-carotene, and lycopene were associated with a decreasing risk of hormone related neoplasms such as prostate cancer (McCann et al., 2005). A different study emphasized the inverse relationship between tomatoes (lycopene) intake and prostate cancer development risk (Giovannucci et al., 2002). Moreover, a large consumption of lycopene provided more protective effects against prostate cancer development in individuals with a family history compared to those who do not have family history of prostate cancer (Kirsh et al., 2006). We will study the potential chemopreventative effects of saffron, especially as it relates to prostate cancer. Recognized active constituents of saffron are crocetin and safranal.
1.6.2. Active constituents

Numerous studies, highlighted below, on saffron have been performed in order to define the important pharmacological active compounds which include crocin (glycosyl ester of crocetin), crocetin (dicarboxylic acid), picrocrocin (monoterpene glycoside), and safranal. Those compounds make contributions for the physical, as well as the medical properties of saffron.
1.6.2.1. Crocin

Crocin is a natural water-soluble carotenoid found in saffron in different derivatives and isomers. The multiple crocin derivatives were given names from crocin-1 to crocin-7 (Figure 3). The molecular weight of crocin varies dependent on its specific derivative which ranges from 434.44 to 1301.25 g/mole. Its structure is composed of dicarboxylic acid, crocetin and D-gentiobioside moieties. It is characterized by its yellow-reddish color which is responsible for saffron’s color. It has multiple unique properties as a glycoside carotenoid, which include hydrophilicity, abundant availability in a single source (saffron plant), and surfactant character due to its amphiphilicity as a result of the presence of highly unsaturated and conjugated sugars (Naess et al., 2006).

Crocin-1 is the derivative that is most extensively studied because it is available in large amounts in saffron and its readily commercially available as a pure compound. A literature review showed that the protective effects of crocin against cancer are suggested via two pathways. First, crocin contains scavenger properties in which the crocin donates a hydrogen bond to a free radical. In a previous study, 500 and 1000 µg/ml of crocin decreased the available free radicals by 50 and 65%, respectively (Assimopoulou et al., 2005). Moreover, crocin demonstrated its ability to activate the antioxidative defense mechanisms of cells by inducing SOD, CAT, GST, and GSH enzymes (Hemshekhar et al., 2012, El-Beshbishy et al., 2012, Zheng et al., 2007). Second, crocin inhibited inflammation by decreasing the specific gene expression of proinflammatory cytokines and inducible inflammatory enzymes such as interleukin-6 (IL-6), interferon γ, cyclooxygenase-2, and nitric oxide synthase. Therefore, using crocin as a dietary supplement significantly prevented the conversion of inflammation induced by azoxymethane and dextran sodium sulfate into colonic adenocarcinomas in mice (Kawabata et al., 2012).
In breast cancer, the estrogen-independent cells (MDA-MB-231) were more sensitive to crocin treatment compared to the estrogen-dependent cells (MCF-7). The IC\textsubscript{50} concentrations were 700 and >1000 µM, respectively. This study showed that crocin is a good candidate to treat the advanced stages of breast cancer (Chryssanthi et al., 2007). Exposure of gastric adenocarcinoma (AGS) cell lines to crocin also showed anticancer activity. This activity was selective to cancer cells whereas no toxic effect was detected in normal cells. The cytotoxic effect was induced by an intrinsic apoptosis pathway (Hoshyar et al., 2013). The pancreatic cancer cell line (BxPC-3) was exposed to 10 µg/ml for 72 hours and resulted in cell viability reduction by activating the apoptosis pathway. This activity was dependent on the dose and time of treatment (Bakshi et al., 2010). The SW-480 human colon cancer cell line growth was inhibited by 52% when dosed with 1 mM crocin (Aung et al., 2007). Similarly, an IC\textsubscript{50} of 3 mM was found in cervical cancers (Escribano et al., 1996).

In an \textit{in vitro} study, crocin was used at 60 µg/ml and caused a reduction of dalton’s lymphoma ascites cell line (DLA) by 50% in a dose- and time-dependent manner. However, \textit{in vivo} xenograft mice revealed the ability of crocin to protect the mice from developing cancer when it was consumed before cancer induction. Crocin exposure increased the lifespan by 44% compared to the control group and lowered the tumor volume by 95.6% after 31 days of treatment in comparison to the untreated group (Bakshi et al., 2009).
Figure 3: Structures of different crocin derivatives
1.6.2.2. Crocetin

Crocetin is one of the carotenoids available in saffron with a molecular weight of 328 g/mole and is chemically known as 8,8’-diapocarotene-8,8’-dioic acid. It is a compound that is responsible for the reddish color of saffron along with crocin. Crocetin is available in different isomers where the ‘trans’ isomer is more intensely colored than the ‘cis’ isomer. In general, crocetin is present in conjugated form with carbohydrates moieties (Tsimidou and Biliaderis, 1997, Tarantilis and Polissiou, 2004). Storage conditions such as temperature, light, and humidity have an effect on the crocetin activity (Tsimidou and Tsatsaroni, 1993).

Crocetin exhibited scavenger activity and decreased free radicals by 40% and 80% in burn-induced intestinal injury model, when the crocetin was added at concentrations of 10 and 100 µM, respectively. Furthermore, crocetin activates endogenous antioxidant enzymes including SOD, CAT, and GPx and reduced lipid peroxidation levels protecting the cardiac cells from oxidative stress injuries induced by norepinephrine. In addition to the antioxidant activities, crocetin reduced inflammatory reactions by significantly decreasing the levels of proinflammatory cytokines such as IL-6 and TNF-α (Zhou et al., 2015, Shen and Qian, 2006). Crocetin has the ability to protect the normal cells from carcinogenic substances such as benzo(a)pyrene (BaP). When cells were pretreated with crocetin the covalent binding of BaP-diol-epoxide with DNA was significantly decreased and inhibited the genotoxicity inhibited (Chang et al., 1996).

Crocetin inhibited the proliferation of the estrogen-dependent breast cancer cell line (MCF-7) at 25µM, whereas the activity against estrogen-independent breast cancer cells (MDA-MB-231) was weak and the IC₅₀ was above 1000 µM. These results indicate that crocetin is the most potent saffron components demonstrated cytotoxicity against MCF-7, so it was
recommended to use this as a chemopreventive agent for breast cancer (Chryssanthi et al., 2007). The mechanism of cell death was through the intrinsic apoptosis pathway by inducing the expression of Bax protein (Mousavi et al., 2009). In another study, incubating 2 μM of crocetin with a promyelocytic leukemia cell line (HL60) for 48 hours resulted in a 50% inhibition of the leukemia cell growth. Interestingly, this study demonstrated that using crocetin at high doses had a less toxic effect compared to retinoids because it is not a provitamin A precursor (Tarantilis et al., 1994). Researchers in a different study found that crocetin displayed antitumor activity against different pancreatic cancer cell lines, including Mia-PaCa-2, as well as pancreatic cancer in an in vivo model. The study showed that the crocetin significantly decreased the expression of the epidermal growth factor receptor (EGFR) and activated the intrinsic apoptosis pathway through inducing Bax and decreasing Bcl-2 proteins (Dhar et al., 2009).

1.6.2.3. Picrocrocin

Picrocrocin is naturally found in saffron and represents one of the major constituents of saffron that is responsible for its bitter taste. It is a colorless glycoside with a molecular weight of 330.37 g/mole and is known as 4-(β-D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (Iborra et al., 1992). Among saffron’s major components, picrocrocin does not possess antioxidant properties (Ochiai et al., 2007).

In a previous study, researchers concluded that picrocrocin had the ability to work as a competitive inhibitor for heat shock protein 90 alpha (HSP 90α) by forming hydrogen bonds in the ATPase catalytic site of HSP 90α (Bhattacharjee et al., 2012). It is known that HSP 90 expression activates multiple pathways that induce cell survival and inhibit apoptosis, by the
activation of STATs, Akt, Bcl-2, and JNK 1/2 (Kim and Kim, 2011). During inactive androgen receptor (AR) status, AR is bound to HSP 90 to prevent its degradation (Vanaja et al., 2002). In early stages of prostate cancer, tumor growth is usually associated with a high expression of AR with HSP 90 (Solit et al., 2002, Abe et al., 2004). It could be hypothesized that prostate cancer cell survival would be reduced because the picrocrocin inhibits HSP 90, leading to a decrease in AR availability which is required for prostate cancer growth in the early stage of its development.

Despite the fact that picrocrocin lacks antioxidant activity, it demonstrated strong antiproliferative effects on different human cancer cell types, which included adenocarcinoma and hepatocarcinoma cells (Kyriakoudi et al., 2015). In addition, picrocrocin exhibited antitumor activity against cervical cancer cells (HeLa) with an IC$_{50}$ corresponding 3 mM (Escribano et al., 1996). Incubating the mouse lung cancer cell line (TC-1) and non-cancerous monkey kidney fibroblast-like cell line (COS-7) with different concentrations of picrocrocin resulted in selective cytotoxic effects on malignant cells in comparison to normal cells which were dependent on dose and time of exposure. The IC$_{50}$s of picrocrocin for TC-1 cells were 4 and 3 mM at 24 and 48 hours after treatment, respectively. The cytotoxicity was achieved through an apoptosis mechanism (Khavari et al., 2015).
1.6.2.4. Safranal

Safranal is a monocyclical terpenic aldehyde which is chemically known as 2, 6, 6-trimethyl-1, 3-cyclohexadiene-1-carboxaldehyde. It is a small molecule with a molecular weight of 150.21 g/mole that can be obtained naturally as a free compound or bound form (picrocrocin) in saffron, or synthetically with a high purity and yield (Könst et al., 1974, Giaccio, 2004).

Chemically, safranal possesses free radical scavenger properties and 500 µg/ml scavenged 34% of free radicals in DPPH assay through donating the hydrogen atom to the free radical (Assimopoulou et al., 2005). In an in vivo study, safranal demonstrated antioxidant activity. Briefly, safranal was administrated to rats prior to inducing oxidative stress by the ischemia-reperfusion injury method which causes damage to the hippocampus. Safranal protected the hippocampal tissue by increasing the antioxidant capacity of the hippocampus (Hosseinzadeh and Sadeghnia, 2005). In another study, safranal restored antioxidant enzymes including SOD and GST which were decreased in rat brains as a result of aging (Samarghandian et al., 2015). Similarly, safranal protected stomachs from developing ulcers caused by using non-steroidal anti-inflammatory drugs. The protective activity was related to the ability of safranal to decrease gastric ulcer lesions and lipid peroxidation as well as elevate the gastric tissue glutathione levels in a dose-dependent manner (Plants and Karaj, 2009).

Providing the mice with different concentrations of safranal prior to DNA damage induction by methyl methanesulfonate (MMS) exposure caused a reduction between ≈ 25 – 31% of DNA damage in different organs including the liver, kidney, spleen, and lung. This study revealed a dose-dependent protective activity of safranal against genotoxic substances (Hosseinzadeh and Sadeghnia, 2007). A different study showed the ability of safranal to reduce the hematological toxicity that was caused by organophosphate insecticides such as diazinon, a
cholinesterase inhibitor. Diazinon reduced hemoglobin, hematocrit, and red blood cells and these adverse outcomes were reversed and restored to normal levels by safranal. The safranal did not show any activity on cholinesterase (Hariri et al., 2011).

In a previous study, safranal demonstrated its ability to block histamine (H₁) receptors. Briefly, isolated tracheal chains of guinea pigs were exposed to 1.4 μM of indomethacin that resulted in histamine release. The histamine induced contractions of the tracheal chains. This activity was significantly inhibited by safranal in a concentration-dependent manner by blocking H₁ receptors (Boskabady et al., 2011). It is known that histamine plays an important role in cancer biology by stimulating tumor growth, progression, and metastasis (Blaya et al., 2010). One of the predominant sites of H₁ receptor expression is prostate tissue (Wang et al., 2014a). Using an H₁ receptor antagonist such as terfenadine against prostate cancer cell lines inhibited cell proliferation and activated an apoptosis intrinsic pathway (Wang et al., 2014b). These observations indicate the ability of safranal to be used as an anticancer therapeutic targeting histamine receptors.

Safranal also showed activity in treating type 2 diabetes mellitus by producing antidiabetic effects after an oral administration of 20 mg/kg on a daily basis in mice. The safranal worked using two different mechanisms to lower the blood glucose level. It significantly induced the translocation of glucose transporter type 4 from muscle and fat cells’ intracellular vesicles to the plasma membrane that increased glucose uptake. Moreover, safranal is a protein tyrosine phosphatase 1B (PTP1B) inhibitor which decreased insulin resistance by inhibiting the tyrosine dephosphorylation of insulin receptors (Maeda et al., 2014). A high expression of PTP1B which is under the control of androgen receptor activity was associated with a high risk of prostate cancer. PTP1B was required for the growth of androgen-dependent prostate cancer. It was also
required for cell migration and the invasion of androgen-independent prostate cancers. Therefore, a PTP1B inhibitor is considered a new target for prostate cancer drug development (Lessard et al., 2012).

Safranal showed a cytotoxic effect against the neuroblastoma cell line (N2A) with an IC_{50} of 11.1 μg/ml after 48 hours of incubation by inducing apoptotic cell death (Samarghandian et al., 2014). It showed a stronger antiproliferative activity by treating cervical cancer cells (HeLa) at lower concentrations (IC_{50} of 120 μg/ml after 24 hours) compared to saffron extract, crocin, and picrocrocin with an (Escribano et al., 1996). In breast cancer, safranal induced cell toxicity in both estrogen dependent and independent cancer cells at 75 μg/ml (48 hours) a similar concentrations (Chryssanthi et al., 2007).
CHAPTER 2: HYPOTHESIS AND SPECIFIC AIMS

Based on existing literature, we hypothesize that saffron and its active components could both prevent prostate cancer development and treat existing cancer cells. Our project aims to systematically verify saffron’s mechanisms of action for the first time using different models of prostate cancer. Both in vitro and in vivo studies will provide better and more specific interventions to prevent and/or treat prostate cancer. Therefore, this project will determine whether or not these natural products can be used as a natural supplement for antioxidant and anti-cancerous benefits. Regardless of saffron’s efficacy, a new in vivo model using zebrafish for prostate cancer drug discovery will be developed. Research in this dissertation addresses five main aims.


Hypothesis: Saffron extract and/or its active components will prevent prostate cancer cell proliferation by activating cell apoptosis.

Approach: Use the XTT cytotoxicity assay to determine in vitro IC₅₀s following exposure and the Caspase-Glo kit to quantitate apoptosis.

Aim 2: Investigate the antioxidant potential of saffron’s active constituents in normal prostate cells.

Hypothesis: Saffron’s active components will reduce free radicals by acting as a scavenger and/or by activating antioxidant enzymes. Therefore, these natural components will work as chemo-preventative agents against prostate cancer development.
**Approach:** After the *in vitro* cell lines are exposed to saffron constituents, ROS products will be measured by using H$_2$DCFDA.

**Aim 3:** Investigate the ability of saffron and its active constituents to inhibit migration and/or invasion via decreased matrix metalloproteinase enzymes (MMP) resulting in the inhibition of prostate cancer metastasis.

**Hypothesis:** Saffron and its active constituents inhibit prostate cancer metastasis by decreasing the migration and invasion as a result of decreased MMP 1/9 production.

**Approach:** *In vitro* migration and invasion will be assessed with a wound healing assay and modified Boyden (invasion) chambers, respectively. RT-PCR will be used to measure the gene expression of MMP1/9.

**Aim 4:** Establish and validate the zebrafish xenograft model as a new alternative to mammalian xenograft models for prostate cancer.

**Hypothesis:** The xenografted zebrafish model for prostate cancer cells is an accurate, faster, easier, and cheaper *in vivo* model to host human prostate cancer cells.

**Approach:** Use known drug, docetaxel, as a positive control (used for chemotherapy to treat prostate cancer) to validate the model.

**Aim 5:** Determine the *in vivo* effects of saffron’s active components on human prostate cancer cells in a successful xenografted zebrafish model.

**Hypothesis:** Human prostate cancer cells xenografted in zebrafish will undergo cytotoxicity when exposed to safranal.

**Approach:** Following exposure, the fluorescence intensity of transplanted fluorescein-labeled prostate cells will be used to determine cytotoxicity. Tracking transplanted human prostate cells
in different types of zebrafish will be performed to detect the proliferation and invasion behavior of cancerous cells.
CHAPTER 3: MATERIAL AND METHODS

3.1. Materials

22Rv1 and PC-3 cell lines were purchased from ATCC and PNT1A cell line from Sigma. Matrigel matrix growth factor reduced without phenol red (356231) was from BD Biosciences. Fetal Bovine Serum (FBS) – Advantage (S11050) was from Atlanta Biologicals. Sterile cell culture inserts 8 µm pore size for 24 well plates (662638) were from Greiner Bio-One. LB Broth, Miller (BP1426), Formic acid (10775711), and Kanamycin monosulfate (BP906) were from Fisher Scientific. Xfect single shots transfection reagent (631366), NucleoSpin® RNA Plus (740984), NucleoBond® Xtra Maxi (740414), G418 (631307), and pCMV DsRed-Express2 vector (632539) were from Clontech laboratories. Crocetin (193543) and Hydrogen peroxide (194057) from were MP Biomedicals. H2DCFDA (D399) and DH5α E. coli competent cells were from Invitrogen. Accumax® (AM105) was from Innovative Cell Technologies. Picrocrocin (CS-T-60315) was from Clearsynth. Caspase-Glo® 3/7 Assay (G8091) was from Promega. RaqMan® reverse transcription reagents (N8080234) and Sybr® green PCR master mix (4309155) were from Applied Biosystems. Sodium Bicarbonate (7412) was from Mallinckrodt. QIAEX® II gel extraction kit (20051) was from Qiagen. OmniPur® Agarose (2081) was from EMD. Docetaxel (D-1000) was from LC laboratories. Ethanol 200 proof (2701) was from Decon laboratories. Methanol HPLC grade (A452), water HPLC grade (W5), and Acetonitrile HPLC grade (A21) were from Fisher chemicals. RPMI-1640 medium (R8755), F-12 Ham Kaighn’s Modified medium (N3520), DMEM medium (D2902), DPBS (D5652), D-Glucose (G5400), DMSO (D4540), Ethyl 3-aminobenzoate methanesulfonate salt (A5040), Crocin-1 (17304),
Safranal (W338907), Giemsa Stain (32884), HEPES (H4034), Sodium pyruvate (P2256), Ethidium bromide solution (E1510), Bovine Serum Albumin (A7906), Diethyl pyrocarbonate (159220), Trypsin Solution 10X (59427C), Antibiotic Antimycotic Solution (100×) (A5955), Trypsin inhibitor (T6414), Phenol red sodium salt (P4758), formaldehyde solution (F1635), XTT sodium salt (X4626), and PMS (P9625) were all purchased from Sigma.

3.2. Methods

3.2.1. Saffron identification, extraction, and fractionation

3.2.1.1. Saffron extraction

Saffron stigmas were extracted using 95% ethanol unless stated otherwise at ratio of 1 gram: 100 ml for 24 hours in dark at room temperature using a shaker at speed of 150 rpm. After 24 hours, the extract was filtered using a polyethersulfone (PES) membrane filter with a 0.22 μM pore size. The ethanol was evaporated using a rotary evaporator (Buchi rotavapor, model # R-114) equipped with water chiller system at a medium rotation speed and 40°C for water bath temperature. The whole extract obtained from this procedure was saved at 4°C in the dark for future work.

3.2.1.2. Commercial samples of saffron

A total of 11 different commercial samples were obtained from multiple growing regions including Iran, Spain, and Afghanistan and were extracted using a 95% ethanol solvent (Table 4). All extracts were tested for their biological activities. All saffron stigmas were acquired
commercially and had botanical identified *Crocus sativus* stigmas and were coded by National Center for Natural Products Research (NCNPR). We conducted HPLC analysis on all samples and the marker compounds, such as safranal, picrocrocin, crocin, and crocetin were present in every extract, although the concentrations of constituents differed significantly among each other. The presence of crocin, picrocrocin, safranal, and crocetin indicated that the samples were correctly labeled with their genus and species of the plant material (see Appendix for HPLC graphs).

**Table 4: The summary of the growing regions of commercial saffron samples with their given codes**

<table>
<thead>
<tr>
<th>Code</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>18248</td>
<td>Iran</td>
</tr>
<tr>
<td>18249</td>
<td>Afghanistan</td>
</tr>
<tr>
<td>18250</td>
<td>Spain</td>
</tr>
<tr>
<td>18251</td>
<td>Spain</td>
</tr>
<tr>
<td>18252</td>
<td>Spain</td>
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<td>Spain</td>
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<tr>
<td>18255</td>
<td>Spain</td>
</tr>
<tr>
<td>18256</td>
<td>Iran</td>
</tr>
<tr>
<td>18257</td>
<td>Spain</td>
</tr>
<tr>
<td>18258</td>
<td>Iran (Reference)</td>
</tr>
</tbody>
</table>
3.2.1.3. Fractionation of saffron extract using the centrifugal thin layer chromatography (Chromatotron)

A Chromatotron is a preparative Thin Layer Chromatography (TLC) instrument that produces a centrifugal force through spinning, which makes the mobile phase solvent move from the center area to the side of the device (Figure 4) (Ilias et al., 2014). This movement will make the compounds separate between the mobile and the stationary phases. At the end, the mobile phase eluates the compounds depending on their polarities. There are multiple factors controlling the performance of the Chromatotron regarding separation including types of stationary and mobile phases used, rotation speed, and mobile phase flow rate. This method of fractionation provides many advantages which include the ability to separate large amounts, quick, and less damage to sensitive compounds.

Figure 4: Chromatotron instrument used in fractionation of the saffron extract
The saffron #18256 extract was fractionated using Chromatotron. The stationary phase used in Chromatotron was amino-bonded silica normal phase. A gradient mobile phase consisted of dichloromethane (DCM) and methanol was used. Whole extract (113 mg) was dissolved in a mixture of DCM and methanol in a ratio of 8:2, respectively. The stationary phase was saturated and equilibrated with DCM before adding the sample. The gradient mobile phase started with 100% DCM and ended with 100% methanol by increasing the methanol solvent concentration in 5% increments. The final step was adding the methanol containing 1% of acetic acid. Each increment volume was 10 ml of the solvent mixture. The highest separation and resolution of fractionation was achieved when the flow rate of the mobile phase was 5 ml/minute and the rotation speed was 1000 rpm. The eluents were collected based on the volume and the compound’s bands detected by using the UV detector at 254 and 365 nm wavelengths. The fractions were collected in 63 test tubes held in a tube rack. After collection, the obtained fractions were analyzed using reverse phase TLC (as described in 3.2.1.4) with standard references (crocin, crocetin, and safranal) to combine similar compounds that yielded 13 fractions after combination (Figure 5). The fractions were dried using a speed vacuum concentrator (Speedvac SC210A, Thermo) to keep them stable and avoid sample cross-contamination with regular methods. The total fraction weight was 101.2 mg which was equal to 90% of the starting weight. All samples were then kept in the dark at 4°C for further work. The purity of each fraction was measured using HPLC.
Figure 5: RP-TLC of Chromatotron fractions of the saffron extract compared to standard references (crocetin, crocin, safranal, whole extract). Sixty-three fractions were combined into 13 prior to RP-TLC.

3.2.1.4. Analysis of fractions using RP-TLC

Thin-layer chromatography was performed on a reverse phase silica gel plate containing UV indicator (F254s). After spotting each fraction on the plate, the TLC plate was transferred into a developing chamber. Then, 20 ml of mobile phase consisting of 90% acetonitrile and 10% water was placed into the chamber. When the mobile phase migrated and covered 90% of the TLC plate, the plate was dried, viewed under UV light, and photographed.
3.2.2. HPLC and UHPLC-MS analysis

3.2.2.1. Chromatographic parameters

Quantitative HPLC analysis was conducted using an Agilent 1100 HPLC system equipped with a degasser (G1379A), quaternary pump (G13311A), auto sampler (G1313A), column oven (G1316A), and UV-Diode detector (G1315B) controlled by Chemstation software. The separation of analytes was carried out on RP-C18 column (3×250 mm; particle size 4 µm; Phenomenex, Synergi Hydro-RP) with column oven temperature set at 25°C and using the gradient system of eluent water (A) and methanol (B) for the separation of target compounds. The gradient condition was as follows: 0-2 min (20% B), 2-47 min (100% B), 47-52 min (100% B), 52-52.25 min (20% B), 52.25-60 min (20% B). The flow rate of the solvent was 0.3 ml/min and the injection volume was 5 µl. All the analysis was carried out at wavelengths of 257, 325, and 440 nm with a run time 60 minutes.

3.2.2.2. Mobile phase system

HPLC grade acetonitrile and water solvents were used. Formic acid was added as a modifier to achieve a final concentration of 0.1% in each solvent. Using the ultrasonic bath, the resulting mixture solution was sonicated for 5 minutes.

3.2.2.3. Preparation of stock solution of standard compounds

A stock of standard solutions of four major high-purity compounds present in saffron was prepared in appropriate solvents to ensure complete solubility (picrocrocin and crocin in water, safranal in methanol, and crocetin in DMSO (1%) then in water) with a concentration of 4 mg/ml of crocin, 0.4 mg/ml of safranal, 0.3 mg/ml of crocetin, and 2.5 mg/ml for picrocrocin. Serial
dilutions were then prepared from the stock solutions to create a calibration curve. These were stored in darkness at 4°C until analysis was conducted.

3.2.2.4. Preparation of sample solution

A sample solution of 10 mg/ml of fresh saffron extract was prepared for HPLC analysis. Briefly, the saffron extract sample was prepared by dissolving 10 mg of fine ground powder of fresh saffron stigma in 1 ml of the specified solvent (HPLC grade solvent) and left overnight. It was then centrifuged at a speed 4000 rpm for 5 minutes at a room temperature of at 25°C. An aliquot of the top layer was filtered through a 0.45 µm PTFE filter, then transferred into vials for HPLC analysis. The extract sample was kept in darkness at 4°C until analysis was carried out.

3.2.2.5. Validation parameters of the developed method

3.2.2.5.1. Linearity

The linearity-developed HPLC analytical method was validated using four marker compounds. Two of these were found to be in abundant quantity in saffron which were picrocrocin and crocin, whereas the remaining two were available in small quantities in the plant. Standard solutions ranging from 33.33-2500 µg/ml of picrocrocin, 250-4000 µg/ml of crocin, 10-300 µg/ml crocetin, and 1.25-400 µg/ml of safranal were prepared. Five µl of each prepared standard solution was injected and analyzed using the developed HPLC method. Each concentration of each compound’s standard solution was injected in triplicate. Using the analysis data from the HPLC analysis method of each compound, it was possible to produce calibration curves of marker compounds through the linear regression analysis. Calibration curves were created by plotting the peak area on the y-axis versus concentration of each pure compound in
the x-axis. The linear regression equation \((y=mx+b)\) was calculated by the least-square regression method to determine the linearity. Using the developed analytical HPLC method, the calibration curves of all standard compounds showed good linearity regression, as presented in Figures 6-9.

Figure 6: The calibration curve of picrocrocin ranging from 33.33-2500 µg/ml (three injections for each concentration).
Figure 7: The calibration curve of crocin ranging from 250-4000 µg/ml (three injections for each concentration).

Figure 8: The calibration curve of safranal ranging from 1.25-400 µg/ml (three injections for each concentration).
Figure 9: The calibration curve of crocetin ranging from 10-300 µg/ml (three injections for each concentration).

3.2.2.5.2. Specificity

A blank control (consisting of methanol and water, which is similar to the used gradient system), standard solutions, and saffron extract samples were injected separately in a fixed volume to analyze them for the specificity validation of the developed analytical HPLC method.

3.2.2.5.3. Accuracy

For accuracy determination, the recovery studies of the developed HPLC method were used. A specific amount of each of the four major compounds was accurately prepared and analyzed. The percentage of the recovered amount was calculated using the following formula:

\[
\text{recovered amount (\%)} = \frac{\text{amount spiked} - \text{amount present}}{\text{amount spiked}} \times 100
\]
3.2.2.5.4. Precision

The precision of the developed analytical method was examined using the intraday precision (repeatability) as well as the interday precision (intermediate precision) analysis which was conducted on six replicates for saffron extract samples on the same day for intraday precision and after one week for interday precision. The results were expressed as \( \% \text{RSD} = (\text{SD/mean}) \times 100\% \).

3.2.2.5.5. Sensitivity

Under the conditions of the developed HPLC method, the limit of detection is the lowest compound’s concentration that can be detected distinguished from the baseline noise, whereas the limit of quantification is the lowest compound’s concentration that can be precisely and accurately quantified. The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the following equation formula 3.3 \( \sigma/S \) and \( \text{LOQ} = 10 \sigma/s \), respectively. \( S \) and \( \sigma \) stand for the slope of the calibration curve and the standard deviation of response, respectively.

3.2.3. Cell culture and maintenance

All cell lines were grown in RPMI-1640 phenol red free growth medium supplemented by 2.5 g of D-Glucose (4.5 g/l as a final concentration of D-Glucose), 110 mg of sodium pyruvate, 1\% of antibiotic-antimycotic solution (10,000 units’ penicillin, 10 mg streptomycin, and 25 µg amphotericin), 2.383 g of HEPES, 1.5 g sodium bicarbonate, and 10\% fetal bovine serum (FBS) in a humidified incubator with 5\% CO₂ in the air at 37°C. Cells were grown in 75 cm² tissue culture flasks and passaged when they became 80\% confluent by treating them with
0.25% trypsin reagent, after cells detached an equal amount of trypsin inhibitor was added to stop trypsin activity. Then, the cells were transferred into conical tubes and centrifuged at 1000 rpm for 5 minutes using a Sorvall® RT7 centrifuge. Finally, the supernatant was discarded, replaced with fresh growth medium, subdivided into 1:5 ratios, and dispensed into a new culture flask.

3.2.4. Cytotoxicity assay

A cytotoxicity assay was done to determine the *in vitro* viability IC$_{50}$ of saffron extract and its active constituents in each cell line. The second generation tetrazolium salt, XTT reagent (sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium salt) measures mitochondrial activity which reflects live cell percentage (Scudiero *et al.*, 1988). To seed cells (100 µL) of growth media with 10,000 cells was added in each well of 96 well plate. After 24 hours, 50 µL of growth media was added containing the desired concentration of test compounds or vehicle solvent control. After 24, 48, and 72 hours the cells were incubated with 75 µL of XTT reagent (that contained 1 mg/ml of XTT and 7.66 µg/ml of PMS activator). After 4 hours of incubation, the absorbance at 488 nm was taken using a HTS 7000 microplate reader (PerkinElmer® connected to an HP computer and HT 2.0 software). This method was modified for saffron extract, crocin, and crocetin cytotoxicity measurements. There were two reasons for these modifications. First, these substances are colored and they absorb at 420 - 460 nm which interferes with the XTT reagent absorption. Second, the usual OD background for these substances can reach 2.2 and the OD for converted XTT tetrazolium salt by cells can reach 2, so the total OD will be over 3, and the maximum OD range measured by this model of microplate reader is 3. Therefore, the following steps have been followed after the specific
incubation time of the cells with treatments; the microplate was centrifuged at a speed of 1000 rpm for 5 minutes, then the growth media containing the treatment was aspirated and 150 µL of DPBS was used to wash wells and remove the treatment residuals, centrifugation and aspiration steps were repeated. Then, 150 µL of growth media containing 50 µL of XTT reagent (that contains 1 mg/ml of XTT and 7.66 µg/ml of PMS activator) were added. After 4 hours, the microplate was read using the microplate reader. Each treatment concentration group and their background had at least 3 replicates. For data analysis, the mean of the blank replicates was subtracted from each replicate of treatment concentration group, then was normalized to a control group that represented 100% cell survival according to the equation shown below. The normalized treatment concentration groups were analyzed using a Graphpad Prism 5 to determine IC$_{50}$ and statistics.

\[
\% \text{ Survival (relative to control)} = \left( \frac{\text{absorbance of treatment group} - \text{absorbance of blank group}}{\text{average absorbance of control group} - \text{absorbance of blank group}} \right) \times 100
\]

3.2.5. Apoptosis assay

To measure the apoptotic effects of saffron and its active components, caspase-3/7 enzymes were measured using a Caspase-Glo kit for in vitro studies. This assay is based on the fact that the effector caspase-3/7 enzymes are members of the cysteine aspartic acid-specific protease group. In the presence of the poly ADP-ribose polymerase (PARP) protein, the effector caspase-3/7 will remove the caspase cleaved domain (cleavage site) to deactivate PARP which decreases ATP production and initiates programmed cell death. This assay consists of two components which are the luminogenic caspase-3/7 substrate and cell lysis buffer. The substrate has tetrapeptide sequence Asp-Glu-Val-Asp (DEVD) which is recognized by caspase enzymes to free the bond recombinant luciferase. This thermally stable luciferase releases a stable
luminescent signal that directly correlates with the caspase-3/7 levels available inside the cells which is quantitated after cell lysis.

Prior to conducting the assay (24 hours before), a fresh mixture of the Caspase-Glo® 3/7 Reagents were prepared by mixing and thoroughly dissolving the lyophilized substrate component in the provided buffer, and this was stored at 4°C until use. Cells (10,000) were seeded in each well of the 96 well white plates. After 24 hours, two different concentrations of each compound were added in addition to a vehicle only control. After 6 additional hours of incubation with the treatment, the microplate was centrifuged and the growth media containing the treatments was replaced with warm Dulbecco’s phosphate buffered saline (DPBS) for wash. Then 100 µL of DPBS was added, followed by the addition of 100 µL of Caspase-Glo 3/7 reagent in each well to make the total volume 200 µL and achieve a 1:1 ratio of the reagent to the sample volume. The plates were then covered with their lids and the reagents were mixed using the plate shaker at speed of 150 rpm for 1 minute. The plate was removed from the shaker and covered with aluminum foil to protect it from light and was then incubated at room temperature for 30 minutes to allow for development of the signals. The readings were taken using the Synergy microplate reader (BioTek®) at the luminescent wavelength of 528/20 nm.

\[
\text{Caspase 3/7 level fold (relative to control)} = \frac{\text{luminance of treatment group} - \text{luminance of blank}}{\text{average luminance of control group} - \text{luminance of blank}}
\]

3.2.6. Oxidative stress

A 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) probe was used to determine the radical scavenging capacity of saffron and its active constituents by measuring the reactive oxygen species (ROS) levels after H2O2 exposure. This probe is non-fluorescent until the reagent
is oxidized by ROS present inside the cells. Briefly, when the cells absorb the H$_2$DCFDA, the intracellular esterases cleave the diacetate group which is a lipophilic blocking group. After esterification a charged form of the dye is created which prevents H$_2$DCF from leaving the cells and retains them inside. When the cells generate free radicals upon H$_2$O$_2$ exposure, the free radicals will oxidize the H$_2$DCF which is non-fluorescent into DCF which is fluorescent. Therefore, the fluorescence intensity level directly correlates to the cellular oxidative stress level (Lebel et al., 1992).

PNT1A, normal prostate cells were seeded at density of 2.5 × 10$^4$ cells in 400 µL in each well of a 24-well plate. After 24 hours of incubation, the cells were treated with two different nontoxic concentrations of saffron extract, safranal, and crocetin for 4 hours. The plate was then centrifuged at a speed of 1,000 rpm for 5 minutes and the medium containing the treatment was removed carefully and washed with warm DPBS. Then, a final working concentration of 10 µM H$_2$DCFDA prepared in DPBS was added in the dark and incubated at 37°C to allow for the cells to load the probe. After 30 minutes, the loading buffer was removed and the cells were washed twice with warm DPBS to ensure all of the extracellular probe was removed, which prevents result errors produced from the interaction of extracellular H$_2$DCFDA and the added H$_2$O$_2$. After the cells were washed, 50 µM of H$_2$O$_2$ in 400 µL DPBS was added to each well and was further incubated for 30 minutes to produce free radicals and induce cellular oxidative stress. The fluorescent intensity was measured using a HTS 7000 microplate reader (PerkinElmer® connected to an HP computer and HT 2.0 software). The measurements were taken from the well bottom and the parameters were set up at 492 nm for excitation, 535 nm for emission, and 129 for gain.

% Oxidative level (relative to control) = \( \frac{\text{fluorescence of treatment group} - \text{fluorescence of blank}}{\text{average fluorescence of control group} - \text{Fluorescence of blank}} \times 100 \)
3.2.7. Migration assay (wound healing assay)

A wound healing assay is considered a traditional method to measure the ability of cells to migrate (move) from a condensed area to a less condensed area. Therefore, we used it to detect the ability of saffron and its active components to inhibit migration behavior of prostate cancer cells. Briefly, one million cells were seeded in each well of a 6 well plate. After 24 hours, in each well a scratch line was made using a 10 µL micropipette tip. Then, the growth media was aspirated and washed with DPBS. The growth media containing the treatment was added. Each treatment group had 3 replicates. After 24 hours, the cells were assessed for their ability to migrate toward the scratched area that was previously cell-free. Pictures were taken using an inverted microscope with 4X objective (Nikon TS 200). ImageJ 1.49 software (NIH) to measure the area covered by the cells. Normalized data to the control group that represented 100% of migrated cells according to the equation mentioned below. The normalized data was analyzed using a Graphpad Prism 5 to determine IC50 for migration.

\[
\% \text{ Closed gap (relative to control)} = \left( \frac{\text{uncovered area by treated cells at } 0 \text{ hours} - \text{uncovered area by treated cells at } 24 \text{ hours}}{\text{uncovered area by untreated cells at } 0 \text{ hours} - \text{uncovered area by untreated cells at } 24 \text{ hours}} \right) \times 100
\]

3.2.8. Migration and invasion assay using Boyden (transwell) chamber

The Boyden chamber assay is a valuable tool used to determine the ability of treatments to inhibit or reduce the migration and/or invasion behaviors of cells. The advantage of this method over a wound healing assay is it is more relevant as an in vitro tool to in vivo conditions (Kramer et al., 2013). The Boyden chamber involves a three dimensional condition compared to two dimensions in a wound healing assay. It also mimics in vivo conditions where cancer cells are attracted to a reach area which leads to upregulation of the genes responsible for secretion of matrix metalloprotease enzymes needed to degrade basement membranes to facilitate metastases.
to distant locations. Therefore, we used Matrigel growth factor reduced (GFR) to coat the transwell chamber membrane in order to mimic the basement for the invasion assay. Whereas, in a migration assay, the transwell is used without a Matrigel coating (Figure 10). To aliquot matrigel for assay, the matrigel was transferred from -20°C to 4°C for overnight. On the second day, all materials were chilled at 4°C for 6 hours such as the micropipette tips, the 24 well plate, and the transwell before contact with Matrigel. In the fume hood, the Matrigel was diluted to 1 mg/ml using cold DMEM without FBS, then 35 µL of diluted matrigel was transferred to each transwells and spread evenly without introducing bubbles. After that, the 24 well plates containing coated transwells were incubated overnight at 37°C to gel. On the third day, when the cells in the culture flask were 80% confluent, the regular growth media was replaced with FBS free growth media contains 0.1% bovine serum albumin (BSA) to starve the cells. After four hours, the FBS free growth media was removed and the cells detached using Accumax® for 45 minutes to form a single cell suspension and avoid cluster cells which interfere with the assay’s accuracy. Cells were then transferred from the flask into the incubator for 10 minutes to deactivate the reagent. The cells were transferred from the culture flask into a conical tube and centrifuged at 1,000 rpm for 5 min at room temperature. The supernatant was removed, the cells were suspended in FBS free growth media, and the cell number was counted using a hemocytometer. The cell concentration was adjusted to be 1.5x10^6/ml and the desired treatment concentration was added. The treated cell suspension (200 µL) was added to each transwell, then the transwell was transferred into the lower chamber of a 24 well plate containing 800 µL of growth media with 10% FBS which was used as a chemoattractant for cell migration and invasion. Cells were then incubated for 36 hours. After the incubation period, the media was carefully removed from the insert and washed twice with DPBS. The cells were fixed with 4%
formaldehyde diluted with DPBS for 10 minutes, the formaldehyde was then removed and washed twice with DPBS. Methanol (100%) was used to permeabilize the cells for 20 min, methanol was then removed and washed twice with DPBS. The cells were stained for 20 min using a modified Giemsa stain, afterwards the stain was removed and the cells washed three times with DPBS. The non-migrating and invading cells which stayed in the upper side of the insert membrane were removed by scraping them using a cotton swab. Finally, the migrating and invading cells were counted using an inverted Nikon microscope (TS-100) with 40X objective and the picture was taken with the AmScope camera (MD130).

Figure 10: The differences between migration and invasion assays using Boyden chambers (transwells)
3.2.9. Preparation and optimization of pCMV-DsRed Express2 plasmid for transfection:

Originally, the DsRed Express vector was generated from Discosoma species red fluorescent protein (DsRed) by substituting nine amino acids to accelerate the protein maturation, increase the solubility, and decrease the green emission. It lacks the tendency to aggregate compared to the wild type DsRed (Bevis and Glick, 2002). Later, DsRed Express 2 vectors were engineered to use in sensitive cells and in vivo applications. The engineering of DsRed Express 2 from its original version of DsRed Express improved the maturation rate, solubility, and cell viability with high photostability. The cytotoxicity and phototoxicity effects were negligible when the bacterial and mammalian cells were transfected with the DsRed Express 2 vector. The toxicity was reduced as a result of minimizing protein aggregation which is responsible for toxicity, although the DsRed Express 2 protein is expressed in the same level as the older version but with a higher level of fluorescence (Strack et al., 2008).

The pCMV-DsRed Express2 plasmid is used for whole cell labeling purposes, so it becomes easy to monitor the cells after transplanting in animals for in vivo study. As shown in Figure 11, the plasmid contains the active human cytomegalovirus immediate early promoter (PCMV IE) to drive the DsRed Express2 gene expression, which make the transfected cells constitutively express the red fluorescent protein. The red fluorescence of DsRed Express2 in transfected live cells is easily detected and quantified using a fluorescent microscope and flow cytometry. This plasmid is characterized by the presence of two antibiotic resistant genes which include the G418 resistant gene for a stable transfection in mammalian cells and the kanamycin resistant gene for E. coli propagation (Clontech-Laboratories, 2008).
3.2.9.1. Amplification of DsRED protein plasmid

In the bottom of a culture tube, 5 μl (1 μg/μl) of DsRed Express 2 stock was gently mixed with 100 μl of a DH5α strain of *Escherichia coli* (*E. coli*) cells and chilled on ice for 30 minutes. To insert the plasmid into the DH5α, the heat shock technique was used by incubating the mixture at 37°C for 1 minute, followed by chilling on ice for 2 minutes. SOC medium (1 ml) was added into the tube and incubated at 37°C at a speed of 220 rpm for one hour in a shaking incubator. After incubation, the *E. coli* was pelleted using a centrifuge at a speed of 3,000X g for 5 minutes and 1 ml of the supernatant was removed. The remaining 100 μl of transformed *E. coli* was spread on prewarmed selective LB agar plate containing 50 μg/ml of kanamycin antibiotic to select the cells containing the plasmid. The plate was then incubated upside down at 37°C for 16 hours and the plate was stored at 4°C until extraction and purification. The plasmid was
extracted from the transformed *E. coli* and purified according to the company protocol using a NucleoBond® Xtra Maxi kit. The obtained plasmids were run in 1% agarose gel containing ethidium bromide to get rid of residual RNA contamination for further purification purposes (Figure 12). The plasmid bands were cut out and extracted using a QIAEX II Gel Extraction Kit following the manufacturer’s instructions.

![Figure 12: Residual RNA from *E. coli* were removed by extracting the plasmids only](image-url)
3.2.9.2. Measurement of G418 cytotoxicity using clonogenic assay

A clonogenic assay was used to determine the survival cells rate in which it measures the ability of a single cell in the population to form a colony. A fraction of the cells when seeded can generate colonies (Franken et al., 2006). Our study used a clonogenic assay to determine the minimum concentration of geneticin (G418) required to induce the selection pressure on transfected cells. The advantages of using a clonogenic assay over regular cytotoxicity assays, such as XTT and MTT, are the incubation time and cultural maintenance are more relevant to the selection process, because the selection process usually takes place for one month with a high concentration and that can be decreased later to a lower concentration as a maintenance procedure to indefinitely keep the selection pressure on transfected cells.

Prostate cancer cell lines (22Rv1 and PC3) were grown on 6-well culture plates for 10 days in which 500 cells of each type were seeded in each well in triplicate. After 24 hours, two concentrations of the G418 antibiotic were added to the cells in addition to the control group. Every three days, the growth media was replaced with fresh growth media containing the exact concentration of the antibiotic. On day 10, the formed colonies were stained using ethidium bromide (Guda et al., 2007). Briefly, the growth media was removed and a freshly prepared solution of 0.05% of 10 mg/ml ethidium bromide in 50% ethanol was added to cover the colonies in each well. After 15 seconds, the ethidium bromide solution was aspirated and the colonies were immediately visualized and pictured using a VersaDoc 3000 imaging system (BioRad®) interfaced with PDQuest software under the control of the connected computer (HP®). The colonies were counted using ImageJ software and analyzed using GraphPad software.
3.2.10. Cell line stable transfection with DsRED protein

Prostate cancer cells (22Rv1 and PC3) were seeded with $3 \times 10^5$ into each well of 6 well plates. After 24 hours, 5 µg of purified DsRed Express 2 plasmid was suspended in 100 µL of autoclaved water. The diluted plasmid was mixed with xfect single shots and vortexed for 10 seconds. After 10 min at room temperature, the suspension was added to 1 ml of the complete regular growth medium. Then, the old growth media was removed from the well and replaced with the new prepared growth media containing the plasmid. After 72 hours, each well containing the cells was subcultured as usual and seeded in a 75 cm$^2$ culture flask containing the selection concentration of G418 antibiotic (1000 µg/ml) for stable transfection. The growth media that contained the selective concentration of G418 was changed twice weekly. Enough cells expressing the DeRed Express 2 protein were obtained after one month of cell growth and maintenance. The protein expression was confirmed using a fluorescent microscope. The G418 were decreased to the maintenance concentration (250 µg/ml) for regular cell maintenance during the study period.

3.2.11. Doubling time

To determine the cells doubling time after 48 hours of incubation in the specified conditions (37°C vs. 35°C and transfected vs. non-transfected). Two 96-well plates were prepared by seeding the cells in six replicates of each plate. After 24 hours of seeding the cells, the XTT along with its activator PMS agents were added in the first plate as previously described in the cytotoxicity method. Three hours later, the absorbance of the first plate was measured. Then, the second plate was measured after 48 hours from first plate absorbance measurement (72
hours after seeding). The population doubling time was calculated according to the following formula (Atcc, 2014):

\[
\text{Doubling Time} = T \times \frac{\ln 2}{\ln \left( \frac{A}{B} \right)}
\]

T: is the incubation time in hours.

A: is the cell absorbance at the beginning of the incubation time.

B: is the cell absorbance at the end of the incubation time.

3.2.12. Zebrafish culture

Tg(fli1a:EGFP)y1 zebrafish was a generous gift from Dr. Robert Tanguay (Oregon State University) and roy9; mitfa\(^9\) zebrafish “Casper” was purchased from ZIRC, catalog ID: ZL1714 (Eugene, OR). Zebrafish have been raised according to IACUC protocol #14-020 and kept in restricted access clean facilities. The light cycle in the zebrafish facilities was 14 hours of light and 10 hours of dark (9 pm - 7 am) to mimic natural breeding conditions. The zebrafish water parameters were 26-30°C for water temperature, 7.4-7.6 for pH, and 60 ppm for conductivity which adjusted by Instant Ocean salt. Adult fish were fed twice daily with tropical flake fish food and live brine shrimp which is enough to be consumed in five minutes to avoid overfeeding problems. Larvae at age 5 dpf were fed twice daily with 20-80 micron size ArteMac-0 powered (Bio-Marine), at 11-13 dpf with ArteMac-0 powered in the morning and live brine shrimp in the evening, at 14-30 dpf, live brine shrimp twice daily. When the larvae become older than 30 dpf, they were fed as adults. Prior to 9/10/2015, zebrafish were fed as described above. After 9/10/2015, the culture colonies were transitioned to Gemma food (75 micro for 5-30 dpf, 150 micro 30-70 dpf, and 300 micro for > 70 dpf). For egg collection, 1:1 male to female ratio of matured zebrafish were spawned twice weekly in a spawning trap tank. Two hours after the
onset of the light cycle, the fertilized eggs were collected from the breeding zebrafish. Then, the fertilized eggs were washed, dead removed, and transferred into a petri dish that contained embryo zebrafish water with 0.5 ppm methylene blue and kept in a zebrafish incubator (28°C temperature and 14:10 hour light cycle). Dead zebrafish embryos were removed from the petri dish and the water was changed on a daily basis. When the larvae reached the age of 5 dpf, they were transferred to a regular tank in the zebrafish system.

3.2.13. Crossbreeding zebrafish

The Casper zebrafish genome should possess homozygous mutated royα9 and mitfaω2 genes to be transparent and lack pigmentation on their body. These mutated genes are recessive alleles. On the other hand, Tg(fli1a:EGFP) transgenic zebrafish express the enhanced green fluorescent protein permanently in their endothelial cells under the control of the fli1a promoter during their whole life. Therefore, Tg(fli1a:EGFP) transgenic with Casper lines were crossed to produce a transparent Tg(fli1a:EGFP) zebrafish that would make it easier to monitor their labeled vasculature system under the fluorescent microscope without pigmentation interfering with imaging. The Casper and Tg(fli1a:EGFP) transgenic lines were maintained, bred separately, and then crossed together in order to generate the hybrid line and stock them for experiments according to the approval # 14-020 obtained from the Institutional Animal Care and Use Committee (IACUC) of the University of Mississippi at University, MS.

The crossing was conducted in multiple steps and took almost one year to complete, for more clarification see Figure 13. Adult Tg(fli1a:EGFP) male and Casper female zebrafish were crossed at a ratio of 1 male to 1 female in the breeding tanks. The fertilized eggs were collected from the lower chamber of the breeding tanks which is separated by a plastic mesh. These
embryos represented the first generation (F1) of the crossed lines. All F1 generation embryos have heterozygous dominant wildtype and recessive mutated versions of roy\textsuperscript{a9} and mitfa\textsuperscript{w2} genes as a result of equal distributions of the parent’s alleles. Therefore, the appearance of F1 zebrafish are similar to Tg(fli1a:EGFP) transgenic line as a result of the dominant allele. Furthermore, all embryos gained Tg(fli1a:EGFP) which makes their vasculature fluorescent. After 48 hpf, all embryos were examined under a fluorescent microscope to keep the strongest and exclude the weakest expressing EGFP, the selected embryos were then raised to adulthood. After 4 months, the F1 generation reached sexual maturation and were incrossed to produce the second generation (F2). Besides the ability of all F2 generation to express EGFP, they are variable in their appearances due to differences in inheritance patterns. The probability of getting a Casper with two homozygous mutated genes is 1/16 (Tables 5-6). The obtained Casper Tg(fli1a:EGFP) transgenic were raised to adulthood and bred to obtain the third generation (F3). All experiments related to the zebrafish were conducted using the embryos of the founded colonies from F3. The hybrid line of crossing the Tg(fli1a:EGFP) with Casper did not reveal any defects in animal development.

Table 5: Crossing of F-1 zebrafish, AaBb x AaBb whereas (A) and (B) letters represent roy\textsuperscript{a9} and mitfa\textsuperscript{w2}, respectively. The uppercase and lowercase letter represent the dominant wildtype allele and recessive mutated allele, respectively.

<table>
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<tr>
<th></th>
<th>AB</th>
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Figure 13: Description of zebrafish crossing steps
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<th>Inheritance pattern 3</th>
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<td>Homozygous of mutated mitfa^w2 and Heterozygous wildtype mitfa^w2</td>
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</table>

Table 6: Results of the inheritance patterns of crossing Casper and Tg(fli1a:EGFP)
3.2.14. Zebrafish optimization for transplantation

3.2.14.1. Determination of the maximum tolerated temperature by zebrafish

The optimum temperature for human cultured cells is 37°C whereas it is 28°C for zebrafish larvae. Multiple temperature points were used to find the maximum tolerated temperature without affecting zebrafish survival rates. Three temperature points: 28°C, 32°C, 35°C, and 37°C were selected for evaluation. Afterwards, each fertilized egg was transferred into each well of a 96-well uncoated plate and incubated at the specified temperature point for up to six days with daily monitoring and changing their water.

3.2.14.2. Optimizing the microscopic fluorescent filters setting

It is known that the enhanced green fluorescent protein (EGFP) emission wavelength is broad. Because it has an autofluorescence background which can cause an interference with the red wavelength emission, the fluorescent filter combination should be carefully selected for the DsRed Express 2 protein to obtain the specific spectrum with high efficiency rather than the EGFP autofluorescence effects (Figure 14). Overestimating the cancer cell’s signal intensity is possible as a result of using the crossed zebrafish which express EGFP with transfected cancer cells expressing DsRed Express 2 protein. Therefore, it is necessary to find the proper filter set combination to overcome this problem especially when a quantitative fluorescence intensity is required. Narrowing the excitation and emission filter bands by using the proper dichromic extended reflector (dcxr) with the correct cut-off between the excitation and emission wavelengths minimizes the interference between wavelengths and will help to exclusively monitor the DsRed Express 2 while removing the EGFP emission in DsRed Express 2 protein wavelength emission range (Table 7). It is very important to swiftly obtain pictures of both
proteins after the fluorescent light path is opened to avoid photobleaching effects which may produce inaccurate results of their intensity. Therefore, the bright field was used when each individual zebrafish larvae was transferred from its plate and prepared for imaging.

![Fluorescent Proteins Spectra](image)

Figure 14: The excitation and emission wavelength spectrums of EGFP and DsRed Express 2 proteins. (University of Arizona, www.spectra.arizona.edu).

<table>
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<tr>
<th>Filter</th>
<th>Ex (nm)</th>
<th>DCXR (nm)</th>
<th>Em (nm)</th>
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<tr>
<td>DsRed Express 2</td>
<td>555/11</td>
<td>587</td>
<td>589/10</td>
</tr>
</tbody>
</table>
3.2.15. Xenografted of human transfected prostate cell into crossed zebrafish and treatment

3.2.15.1. Preparation of cells for transplantation

On the injection day, the growth media that contains G418 antibiotic was removed and replaced with DPBS as a wash. After washing the cells, the DPBS was removed and replaced with an Accumax® solution to detach the cells from the culture flask. After 45 minutes of incubation at room temperature to form a single cell suspension, the suspension was transferred to a centrifugation tube and then it was centrifuged for 5 minutes at 1000 rpm and 4°C and then the cells’ debris and solution was aspirated. The cells pellet was suspended in cold FBS-free DMEM which contained 1.5% of matrigel and 0.04% phenol red, then it was placed on ice until injection time. The cell concentration was 20x10^6 cells/ml.

3.2.15.2. Preparation of zebrafish for transplantation

Zebrafish embryos (1dpf) were dechorionated by placing them in a petri dish containing 0.5 mg/ml of protease and swirling them occasionally for 4 minutes, then removing the protease and washing the embryos twice with water. After that they were transferred into a new petri dish containing fresh culture water.

3.2.15.3. Xenograft procedure

The zebrafish larvae were anaesthetized with 0.02% tricaine methanesulfonate: 0.04% sodium bicarbonate until they became inactive. Under a dissecting microscope (Carl Zeiss), the injections were conducted. The transfected prostate cancer cells were loaded into a pulled glass micropipette which was attached to an air-driven microinjector (Narishige IM 300). The needle was inserted into the yolk sac of each zebrafish larvae and cancer cells were delivered in a single
injection. The injection volume ranged from 15 nl to 20 nl of cell suspension. The number of the injected cells was optimized to between 300-400 cells in each injection used using a 25 ms pulse time and 14 psi pressure. After injection, the zebrafish larvae were transferred immediately into embryo water for recovery and placed in an incubator at (35°C).

3.2.15.4. Visualizing the zebrafish

The endothelial cells of the Tg(fli1a:EGFP) zebrafish express a green fluorescent protein and the cancer cells express red fluorescent proteins. After 24 hours of transplantation (2dpf), non-successful cancer transplantation into zebrafish larvae and dead larvae were removed. The successful xenografted zebrafish larvae were anesthetized and examined under a fluorescent microscope (Nikon Eclipse E600) for a visible prostate cancer cell mass in the yolk to exclude non-successful transplanted zebrafish. Then the images were captured using a CCD camera (Nikon) using NIS elements software (Nikon). Each active xenografted zebrafish was transferred into a single well of a 48-well plate and labelled.

3.2.15.5. Treating the xenografted model

The experiment was conducted using these zebrafish which were examined daily through to 4 dpf. Each well had 200 µl of water that contained a specified treatment concentration (maximum DMSO concentration was 0.05%). After 24 hours of treatment, the prostate cancer fluorescent intensity zebrafish were measured again using a fluorescent microscope. At 4 dpf and after visualizing the zebrafish larvae, they were euthanized using a tricaine methanesulfonate overdose (2%) to terminate the experiment. The red fluorescence images were analyzed using ImageJ software and quantified as described in the following equations:
(1) Corrected intensity = intensity – background intensity

(2) \% intensity (normalized to 24 hpi) = \frac{\text{corrected intensity at 2 or 3 dpi}}{\text{corrected intensity at 1 dpi}} \times 100

3.2.16. Gene expression using Real Time PCR

Cells (2x10^5) were seeded in a 6-well plate and after 24 hours, the specified treatment was added in triplicate for each concentration. After 72 hours of incubating with the treatment, the cells were washed with DPBS twice and the cells were detached from the plate by using the cell scraper. They were then transferred into micro-tubes and centrifuged at 1000 rpm for 5 minutes. The DPBS was replaced by the lysis buffer provided by the kit for homogenization. Afterward, the total RNA was isolated using the NucleoSpin® RNA Plus (Clontech). The RNA quantity was determined by the NanoDrop 2000 spectrophotometer (Thermo Scientific). Using small PCR tubes, 250 ng of total RNA was mixed with the reaction buffer (Random Hexamers, RNase Inhibitor, and Multiscribe RT) to form a final concentration of 10 ng/µl of RNA. The mixture incubated in the thermocycler at: 25°C for 10 minutes, 37°C for 60 minutes, and 95°C for 5 minutes to inactivate the reverse transcriptase. Then it was stored at -80°C until analysis. A mixture of 2 µl of cDNA, 6.5 µl of water, 1 µl of each primer pair (5 µM) and 12.5 µl of SYBR Green PCR master mix were added in triplicate to measure the gene expression using an Applied Biosystems 7500. Table 8 demonstrates the primer sequences which were used in the measurements of six genes. The expression of all genes was normalized using the reference gene (18S). The relative gene expression was calculated according to the Pfaffl formula; the obtained results were then analyzed for any significant differences in their expression using GraphPad (ANOVA, n=3) (Pfaffl, 2001).
The relative expression ratio = \( \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}(\text{control} - \text{sample})}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}(\text{control} - \text{sample})}} \)

E: the PCR efficiency for the gene’s primers

\( \Delta CP \): the difference of crossing point

### Table 8: Genes’ primer sequences

<table>
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<th>Gene</th>
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<th>Reverse primer</th>
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<td>5'-CGCCACTTGTCCCTCTAAGAA-3'</td>
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<td>CASP7</td>
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<td>CASP9</td>
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<td>5'-CAGCATTAGCGACCCTAAGCAG-3'</td>
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<tr>
<td>Migration and Invasion</td>
<td>MMP1</td>
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<td>5'-ATTGTTGTCACACTTCATCTTC-3'</td>
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<td>MMP9</td>
<td>5'-CTTCCAGTACGGAGGAAGCC-3'</td>
<td>5'-CCACCTGGTCACACTCC-3'</td>
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</table>

### 3.2.17. Statistical analysis

All results were presented as mean ± S.E. and analyzed using GraphPad Prism 5.04 software (La Jolla, CA). Statistical differences between treatment groups were determined by one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc test or student t-test. Statistical significance between treatment and corresponding control groups was accepted at \( p \leq 0.05 \).
CHAPTER 4: DEVELOPMENT OF EXTRACTION AND ANALYSIS METHODS FOR SAFFRON AND ASSESSMENT OF COMMERCIALY AVAILABLE SAFFRON

4.1. Results

4.1.1. The effect of different solvent systems used for saffron extraction on its cytotoxic activity

The cytotoxic effects of different concentrations of methanolic and ethanolic saffron extract #18258 on normal human and prostate cancer cell lines were evaluated with the XTT assay, as described in Chapter 3. All cell lines were exposed to multiple concentrations (0.125–2 mg/mL) of saffron extracts for 72 hours. All the extracts inhibited cell proliferation in all cell lines in a concentration-dependent manner. There was a direct relationship between the concentration of ethanol used in saffron extraction and the cytotoxic effects. On the other hand, when methanol solvent was used, the relationship also applied to 22Rv1, but PNT1A and PC3 cytotoxicity did not directly correlate when methanol concentration was lower than 70%. The study showed that the 95% ethanol extraction was least cytotoxic on normal cells and more cytotoxic to PC3 compared to 95% methanol. Generally, ethanolic extracts comparably demonstrated stronger growth inhibition on all prostate cancer cell lines than the methanolic extracts. This suggests that the anticancer compounds were primarily concentrated in the ethanol and the compounds responsible for normal prostate cell toxicity were mostly concentrated in the methanol.
4.1.1.1. The cytotoxicity of different ethanolic concentrations of saffron extracts

Different concentrations of ethanol solvent were used to extract the saffron. The cytotoxic response differed significantly as the solvent / water ratio was changed. The weakest cytotoxic effects against the prostate cancer cell lines was obtained using 50% ethanolic saffron extract, but the cytotoxic effect increased when a higher percentage ethanolic saffron extract was used. Incubation of 50% ethanolic extract with the cells produced a significant decrease in cell viability of PNT1A cells starting at 0.125 mg/ml with an IC$_{50}$ equal to 0.73 ± 0.16 mg/ml, while at the same time cell viability of 22Rv1 decreased at 0.5 mg/ml with an IC$_{50}$ equivalent to 2.27 ± 0.72. The survival of PC3 decreased at 1 mg/ml with an IC$_{50}$ corresponding to 3.23 ± 0.83 mg/ml (Figure 15). When 60% ethanolic extract was used, it led to decreases in the required extract concentration to produce cytotoxic activity on all cells. A significant reduction in cell viability of PNT1A cells started at 0.125 mg/ml with an IC$_{50}$ of 0.66 ± 0.15 mg/ml, while cell viability of 22Rv1 and PC3 declined at 0.5 mg/ml with an IC$_{50}$ matching 2.09 ± 0.5 and 2.77 ± 0.65 mg/ml, respectively (Figure 16). Using 70% ethanolic saffron extract significantly increased the cytotoxicity of PNT1A cells which was induced at 0.25 mg/ml with an IC$_{50}$ equal to 0.63 ± 0.13 mg/ml. Cytotoxicity in 22Rv1 was detectable at 0.25 mg/ml with an IC$_{50}$ corresponding to 1.71 ± 0.33, while in PC3 cytotoxicity started at 1 mg/ml with an IC$_{50}$ equivalent to 2.35 ± 0.63 mg/ml (Figure 17). A significant decrease in cell viability of PNT1A, 22Rv1, and PC3 cells was found with 0.125, 0.25, and 0.5 mg/ml respectively when 80% ethanolic saffron extract was used. The IC$_{50}$s were 0.49 ± 0.10, 1.55 ± 0.26, and 2.2 ± 0.88 mg/ml for PNT1A, 22Rv1, and PC3, respectively (Figure 18). The maximum inhibitory effects were found with 95% ethanolic saffron extract. A significant growth inhibition of PNT1A cells began at 0.125 mg/ml with an IC$_{50}$ of 0.38 ± 0.07 mg/ml, whereas cell viability of 22Rv1 was reduced at 0.5 mg/ml with an IC$_{50}$
matching 1.32 ± 0.19. PC3 viability started to decrease at 0.25 mg/ml with an IC50 equal to 1.77 ± 0.23 mg/ml (Figure 19). Therefore, the strongest cytotoxic effects were achieved using 95% ethanolic saffron extract. As a general rule, the cytotoxic effects toward normal cells were higher than early and advanced stages of prostate cancer cells, respectively, with all ethanol solvent concentrations used.

Figure 15: The cytotoxicity of prostate cell lines after 72 hours using 95% ethanolic saffron extract # 18258. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)
Figure 16: The cytotoxicity of prostate cell lines after 72 hours using 80% ethanolic saffron extract # 18258. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)

Figure 17: The cytotoxicity of prostate cell lines after 72 hours using 70% ethanolic saffron extract # 18258. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)
Figure 18: The cytotoxicity of prostate cell lines after 72 hours using 60% ethanolic saffron extract # 18258. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)

Figure 19: The cytotoxicity of prostate cell lines after 72 hours using 50% ethanolic saffron extract # 18258. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)
4.1.1.2. The cytotoxicity of different methanolic concentrations of saffron extracts

With all methanolic solvent concentrations used to extract the saffron, a significant inhibitory effect of saffron extracts on the early stages of prostate cancer cells (22Rv1) started at 0.5 mg/ml. The cytotoxic activities produced by all concentrations of methanolic solvent system, except 95%, against the advanced stages of prostate cancer (PC3) were triggered at 1 mg/ml of saffron extract, whereas for 95% the methanol extract at 0.5 mg/ml induced cytotoxicity. The IC\textsubscript{50}s of 50% methanolic extracts were 2.17 ± 0.64 and 2.98 ± 0.67 mg/ml for 22Rv1 and PC3, respectively (Figure 20). Incubation of 60% methanolic saffron extract with the cancerous cells produced a significant decrease in cell viability of 22Rv1 and PC3 with IC\textsubscript{50}s equal to 2.27 ± 0.6 and 2.95 ± 0.74 mg/ml, respectively (Figure 21). Methanolic saffron extract (70%) significantly increased the cytotoxicity of the cells with an IC\textsubscript{50}s corresponding to 1.91 ± 0.38 for 22Rv1 and 2.99 ± 0.66 mg/ml for PC3 (Figure 22). Regarding the activity of methanolic saffron extracts on normal prostate cells (PNT1A), our study found that all methanolic extracts began to exert their cytotoxic effects at concentrations as low as 0.125 mg/ml. When 50-70% methanolic extracts were used, they produced similar activity and their IC\textsubscript{50} was 0.4 ± 0.07. The responses of PNT1A and PC3 cells were unchanged when the concentration of methanol used in saffron extraction was increased from 50% to 70%. In contrast, 22Rv1 cell line growth was declined when the concentration of methanol used in extraction process was increased. In addition, when 80% methanolic saffron extract was used, the IC\textsubscript{50}s were 0.36 ± 0.06, 1.53 ± 0.32, and 2.44 ± 0.6 mg/ml for PNT1A, 22Rv1, and PC3, respectively (Figure 23). Finally, the maximum inhibitory effects on cellular proliferation of all cell types was gained with 95% methanolic extract. The IC\textsubscript{50} on PNT1A, 22Rv1, and PC3 was 0.32 ± 0.05, 1.26 ± 0.25, and 1.98 ± 0.39 mg/ml, respectively (Figure 24).
Figure 20: The cytotoxicity of prostate cell lines after 72 hours using 95% methanolic saffron extract # 18258. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)

Figure 21: The cytotoxicity effects of prostate cell lines 72 hours using 80% methanolic saffron extract # 18258. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)
Figure 22: The cytotoxicity of prostate cell lines after 72 hours using 70% methanolic saffron extract # 18258. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)

Figure 23: The cytotoxicity of prostate cell lines after 72 hours using 60% methanolic saffron extract # 18258. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)
Figure 24: The cytotoxicity of prostate cell lines after 72 hours using 50% methanolic saffron extract # 18258. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)

4.1.2. The cytotoxicity effects of different commercial saffron samples on prostate cells

Our study measured the differences in the cytotoxicity and selectivity of 10 commercially available saffron (*Crocus sativus*) samples, grown abroad and all available in the USA, on prostate cancer cells. All samples were identically prepared using 95% ethanol solvent with the exact steps of the extraction process and the cytotoxicity evaluated in normal (PNT1A) and prostate cancer (22Rv1 and PC3) cell lines after 72 hours of exposure using an XTT assay as described in Chapter 3. Our study revealed that all concentrations of the obtained saffron extracts differed significantly in their cytotoxic effects on the normal as well as the prostate cancer cell lines (Figures 25-34). Also, two out of the ten tested samples (#18250 (Spain) and #18252 (Spain)) produced significant cell cytotoxicity against prostate cancer cells at a concentration lower than 2 mg/ml.
Of particular interest, the PNT1A cells exposed to saffron extracts #18250 showed a slight decrease in viability compared to other commercial samples that were tested in this study. The IC\textsubscript{50} after incubation was 1.69 ± 0.5, 1.78 ± 0.27, and 1.97 ± 0.34 mg/ml for PNT1A, 22Rv1, and PC3, respectively. However, using 2 mg/ml of saffron extract #18252 resulted in an only 26\% increase in cytotoxicity in PNT1A cells indicating that this saffron sample had the lowest toxicity against normal cells compared to other extract samples. The IC\textsubscript{50}s using the extract #18252 on PNT1A, 22Rv1, and PC3 corresponded to 1.15 ± 0.19, 1.67 ± 0.49, and 1.97 ± 0.35 mg/ml, respectively.

In contrast, saffron extract #18253 (Spain) demonstrated weak activity against cancerous cells (22Rv1 and PC3) where the IC\textsubscript{50}s were 3.08 ± 0.56 and 5.01 ± 1.25 mg/ml for 22Rv1 and PC3, respectively. In addition, saffron extract #18257 (Spain) had similar cytotoxicity in 22Rv1 and PC3 cells with an IC\textsubscript{50} equal to 8.19 ± 2.36 and 10.22 ± 2.38 mg/ml, respectively, but only 0.76 ± 0.12 mg/ml was needed in PNT1A cells to produce a similar effect. Therefore, our study showed this commercial sample extract (# 18257) was highly cytotoxic in normal cells and only had weak activity against cancer cells. Moreover, saffron extracts #18248 and 18249, from Iran and Afghanistan respectively, exhibited no significant effect against the PC3 cell line, and the predicted IC\textsubscript{50}s of 8.22 ± 2.56 mg/ml and ≥ 34.47 mg/ml were projected to be above the dose range tested.
Figure 25: The cytotoxicity of prostate cell lines of saffron commercial sample # 18248 after 72 hours using 95% ethanolic solvent. The IC₅₀s were 1.37 ± 0.33, 2.56 ± 0.46, and 8.22 ± 2.56 mg/ml for PNT1A, 22Rv1, and PC3, respectively. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)

Figure 26: The cytotoxicity of prostate cell lines of saffron commercial sample #15249 after 72 hours using 95% ethanolic solvent. The IC₅₀s were 1.78 ± 0.39, 2.85 ± 0.67, and ≥ 34.47 mg/ml for PNT1A, 22Rv1, and PC3, respectively. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)
Figure 27: The cytotoxicity of prostate cell lines of saffron commercial sample #15250 after 72 hours using 95% ethanolic solvent. The IC\textsubscript{50}s were 1.69 ± 0.5, 1.78 ± 0.27, and 1.97 ± 0.34 mg/ml for PNT1A, 22Rv1, and PC3, respectively. Bars with * symbol are statistically significant from control group (ANOVA; N = 4).

Figure 28: The cytotoxicity of prostate cell lines of saffron commercial sample #15251 after 72 hours using 95% ethanolic solvent. The IC\textsubscript{50}s were 1.64 ± 0.42, 3.05 ± 0.65, and 3.91 ± 1.19 mg/ml for PNT1A, 22Rv1, and PC3, respectively. Bars with * symbol are statistically significant from control group (ANOVA; N = 4).
Figure 29: The cytotoxicity of prostate cell lines of saffron commercial sample #18252 after 72 hours using 95% ethanolic solvent. The IC50s were 1.15 ± 0.19, 1.67 ± 0.49, and 1.97 ± 0.35 mg/ml for PNT1A, 22Rv1, and PC3, respectively. Bars with * symbol are statistically significant from control group (ANOVA; N = 4).

Figure 30: The cytotoxicity of prostate cell lines of saffron commercial sample #15253 after 72 hours using 95% ethanolic solvent. The IC50s were 1.42 ± 0.28, 3.08 ± 0.56, and 5.01 ± 1.25 mg/ml for PNT1A, 22Rv1, and PC3, respectively. Bars with * symbol are statistically significant from control group (ANOVA; N = 4).
Figure 31: The cytotoxicity of prostate cell lines of saffron commercial sample #15254 after 72 hours using 95% ethanolic solvent. The IC₅₀s were 1.09 ± 0.18, 2.39 ± 0.55, and 3.85 ± 1.02 mg/ml for PNT1A, 22Rv1, and PC3, respectively. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)

Figure 32: The cytotoxicity of prostate cell lines of saffron commercial sample #15255 after 72 hours using 95% ethanolic solvent. The IC₅₀s were 1.06 ± 0.17, 1.88 ± 0.34, and 3.76 ± 0.99 mg/ml for PNT1A, 22Rv1, and PC3, respectively. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)
Figure 33: The cytotoxicity of prostate cell lines of saffron commercial sample #18256 after 72 hours using 95% ethanolic solvent. The IC$_{50}$s were 0.79 ± 0.12, 4.2 ± 0.45, and 4.77 ± 0.9 mg/ml for PNT1A, 22Rv1, and PC3, respectively. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)

Figure 34: The cytotoxicity of prostate cell lines of saffron commercial sample #18257 after 72 hours using 95% ethanolic solvent. The IC$_{50}$s were 0.76 ± 0.12, 8.19 ± 2.36, and 10.2 ± 2.38 mg/ml for PNT1A, 22Rv1, and PC3, respectively. Bars with * symbol are statistically significant from control group (ANOVA; N = 4)
4.1.3. HPLC analysis

4.1.3.1. HPLC analytical method development and optimization

Development of a new analytical HPLC method that has the capability to measure, simultaneously, multiple compounds in complex extracts is considered a challenging task that relies on obtaining high resolution peaks for each compound’s quantification. The selection of appropriate HPLC parameters such as column, temperature, flow rate, mobile phase, gradient system, and wavelength are crucial optimization steps. That parameters’ selection of the developed method was targeted to produce a high resolution chromatogram with low baseline noise when it is used to separate the major saffron compounds including the four marker compounds (picrocrocin, crocin, safranal, and crocetin).

During the development process different solvents were tried, including methanol, acetonitrile, and water. A mobile phase composed of gradient methanol and water was found to be the most effective system to separate the saffron extract constituents. Moreover, adding the formic and acetic acids to the mobile phase was evaluated. Adding 0.1% formic acid enhanced peak’s shape by increasing its sharpness and decreasing tailing. Multiple run times with flow rates were examined and 60 minutes with 0.3 ml/minute was found to be the best combination to produce a good separation of different compounds. After the solvents with their additives were chosen, the gradient system selection was made based on the obtained results of various tests of the starting organic percentage such as 10, 20, and 30% of methanol at the beginning of the run. The best composition of the mobile phase at the beginning of the run was 20% methanol:80% water. The gradient system comprises water (A) and methanol (B) as: 0-2 min, 20% B; 2-47 min, 100% B; 47-52 min, 100% B; 52-52.25 min 20% B; 52.25-60 min, 20% B (Figure 35).
Maximum wavelength absorbance for each of marker compounds was optimized and found to be 257 nm for picrocrocin, 315 nm for safranal, and 428 nm as well as 458 nm for carotenoids (crocin and crocetin) as presented in Figure 36. Although the maximum absorbance wavelengths of safranal and carotenoids were determined, shifting from these wavelength was employed for several reasons. For safranal there were noticeable interfering compounds that had higher absorbance at 315 nm which affected the peak baseline and thus the detection sensitivity. Therefore, the safranal absorbance was shifted to 325 nm to minimize the interference peaks. The 440 nm rather than double peaks (428 and 458 nm) was used for detecting the absorbance of carotenoids (crocin and crocetin) because the single 440 nm wavelength was between the two maximum wavelengths.

Figure 35: Gradient system used in the developed HPLC method.
4.1.3.2. Validation parameters of the developed analytical HPLC method

4.1.3.2.1. Linearity

The linearity of the developed analytical HPLC method was validated using multiple concentrations of each of the pure compounds ranging between 1.25-400 µg/ml of safranal, 10-300 µg/ml crocetin, 33.3-2500 µg/ml of picrocrocin, and 250-4000 µg/ml of crocin. The concentration range of each pure compound was chosen based on the expected concentration to be found in saffron extract in addition to the maximum linearity that could be achieved. Serial dilution of the pure stock solution was prepared for the purpose. The analysis of standard solutions was carried out in triplicate for each concentration. As shown in Table 9, the obtained standard calibration curves depicted linearity in the tested range for each compound with good correlation coefficients for all marker compounds, ranging between 0.9992 and 0.9996.
Table 9: The linearity and regression equations of saffron’s marker compounds.

<table>
<thead>
<tr>
<th>Marker compound</th>
<th>Calibration curve *</th>
<th>(R^2)</th>
<th>Linear range (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Picrocrocin</td>
<td>(y = 11.745x - 75.563)</td>
<td>0.9992</td>
<td>33.33-2500</td>
</tr>
<tr>
<td>Crocin</td>
<td>(y = 8.5293x + 380.17)</td>
<td>0.9992</td>
<td>250-4000</td>
</tr>
<tr>
<td>Safranal</td>
<td>(y = 48.006x - 35.122)</td>
<td>0.9993</td>
<td>1.25-400</td>
</tr>
<tr>
<td>Crocetin</td>
<td>(y = 6.003x + 20.291)</td>
<td>0.9996</td>
<td>10-300</td>
</tr>
</tbody>
</table>

* The calibration curves were generated by plotting the peak areas on the y-axis versus the concentration of each pure compound on the x-axis.

4.1.3.2.2. Specificity

The elution of the four marker compounds with well resolved and symmetric peaks were found to be at 21.48 ± 0.84 min for picrocrocin, 31.33 ± 0.58 min for crocin, 41.87 ± 0.42 min for safranal, and 49.55 ± 0.39 min for crocetin. In Figure 37, the saffron extract chromatograms at different wavelengths show all markers compounds with a good separation of their peaks. Monitoring each peak retention time and peak shape for each marker compounds, including start, apex, and end of spectral peak revealed that each chromatogram was well resolved with highly symmetrical peak shape. Therefore, the developed HPLC analytical method showed acceptable specificity toward the saffron constituents with high sensitivity and without interference peaks.
Figure 37: HPLC analysis of saffron #18258 extract using 95% ethanol at wavelengths (A) 257 nm, (B) 325 nm, and (C) 440 nm.
4.1.3.2.3. Accuracy

The accuracy evaluation of the developed analytical HPLC method was done using known amounts of each pure compounds to spike a pre-measured extract. Then, the ability to recover the added amount was assessed, as shown in Table 10. The recovery of the added amount ranged from 97.9% to 101% for picrocrocin, 98.8 to 99.9% for crocin, 99.1 to 101% for safranal, and 98.5 to 102.7% for crocetin and relative standard deviations ranged from 0.45 to 6.09%. The recovery results demonstrated the high accuracy of the developed method.

Table 10: Recovery percentage of saffron marker compounds.

<table>
<thead>
<tr>
<th>Spiked sample</th>
<th>Concentration (µg/ml)</th>
<th>Amount recovered ± SD</th>
<th>% Average recovery</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Picrocrocin</td>
<td>2200</td>
<td>2195 ± 32.7</td>
<td>99.8</td>
<td>1.49</td>
</tr>
<tr>
<td></td>
<td>1100</td>
<td>1111 ± 18.6</td>
<td>101.1</td>
<td>1.68</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>293 ± 8.21</td>
<td>97.9</td>
<td>2.8</td>
</tr>
<tr>
<td>Crocin</td>
<td>3600</td>
<td>3596 ± 16.3</td>
<td>99.9</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>2400</td>
<td>2395 ± 23.8</td>
<td>99.8</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>1200</td>
<td>1185 ± 16.1</td>
<td>98.8</td>
<td>1.36</td>
</tr>
<tr>
<td>Safranal</td>
<td>300</td>
<td>300 ± 4.2</td>
<td>100</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>202 ± 3.46</td>
<td>101</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>19.8 ± 0.86</td>
<td>99.1</td>
<td>4.34</td>
</tr>
<tr>
<td>Crocetin</td>
<td>240</td>
<td>241 ± 4.19</td>
<td>100.5</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>118 ± 3.01</td>
<td>98.5</td>
<td>2.55</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>30.8 ± 1.88</td>
<td>102.7</td>
<td>6.09</td>
</tr>
</tbody>
</table>
4.1.3.2.4. Precision

The % relative standard deviation (RSD) for both inter- and intra-day precision was below 3.15%, which indicated a good precision of the analytical method (Table 11). The repeatability of the analytical method was examined by RSD values of peak areas which was < 1.93%.

Table 11: Intra- and inter-day precision of saffron marker compounds.

<table>
<thead>
<tr>
<th>Sample (µg/ml)</th>
<th>Intra-day precision</th>
<th>Inter-day precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount recovered + SD</td>
<td>% RSD</td>
</tr>
<tr>
<td>Picrocrocin (1300)</td>
<td>1303 ± 19.4</td>
<td>1.49</td>
</tr>
<tr>
<td>Crocin (2200)</td>
<td>2195 ± 23.8</td>
<td>1.08</td>
</tr>
<tr>
<td>Safranal (250)</td>
<td>251 ± 4.84</td>
<td>1.93</td>
</tr>
<tr>
<td>Crocetin (180)</td>
<td>180 ± 3.25</td>
<td>1.81</td>
</tr>
</tbody>
</table>
4.1.3.2.5. Sensitivity

The quantification parameters including limit of detection (LOD) and limit of quantification (LOQ) were calculated and are shown in Table 12, which indicated the high sensitivity of the developed method.

Table 12: LOD and LOQ of saffron’s marker compounds

<table>
<thead>
<tr>
<th>Marker compound</th>
<th>LOD (µg/ml)</th>
<th>LOQ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Picrocrocin</td>
<td>44.2</td>
<td>134</td>
</tr>
<tr>
<td>Crocin</td>
<td>71.7</td>
<td>217</td>
</tr>
<tr>
<td>Safranal</td>
<td>1.98</td>
<td>5.99</td>
</tr>
<tr>
<td>Crocetin</td>
<td>4.84</td>
<td>14.7</td>
</tr>
</tbody>
</table>
4.1.4. Identification of saffron chemical composition using HPLC-MS

One of the developed HPLC method’s advantages is the capability to link it with mass spectrometry for detection. Mass spectrophotometry provides information regarding the compounds presented in the saffron extract which facilitates the detection of adulterated samples. The electrospray ionization (ESI) mode was used as the ionization method in mass spectrometry and revealed the compounds evident in saffron as shown in Appendix #3. Most of saffron constituents were identified by matching the fragmentation patterns obtained from HPLC-MS to the known molecular weight of saffron compounds (Table 13). For example, picrocrocin at 3.93 min was detected using negative ESI mode and spectrum of [M-H+HCOOH]⁻ ions (m/z 375.2) as shown in Figure 38.

Table 13: Identified compounds present in the saffron extract using UHPLC-MS.

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>Formula</th>
<th>Mode of detection</th>
<th>m/z</th>
<th>Structural elucidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.93</td>
<td>260</td>
<td>C₁₆H₂₆O₇</td>
<td>negative ESI</td>
<td>375.2 [M-H+HCOOH]⁻</td>
<td>Picrocrocin</td>
</tr>
<tr>
<td>5.32</td>
<td>440</td>
<td>C₄₄H₆₄O₂₄</td>
<td>negative ESI</td>
<td>975.2 [M-H]⁻</td>
<td>Crocin-1</td>
</tr>
<tr>
<td>5.84</td>
<td>440</td>
<td>C₃₈H₇₄O₁₉</td>
<td>negative ESI</td>
<td>859.2 [M-H+HCOOH]⁻</td>
<td>Crocin-2</td>
</tr>
<tr>
<td>7.80</td>
<td>440</td>
<td>C₃₂H₄₄O₁₄</td>
<td>negative ESI</td>
<td>697.2 [M-H+HCOOH]⁻</td>
<td>Crocin-3</td>
</tr>
<tr>
<td>11.44</td>
<td>325</td>
<td>C₁₀H₁₄O</td>
<td>positive ESI</td>
<td>151.2 [M+H]⁺</td>
<td>Safranal</td>
</tr>
<tr>
<td>11.80</td>
<td>440</td>
<td>C₂₀H₂₄O₄</td>
<td>positive ESI</td>
<td>329.2 [M+H]⁺</td>
<td>Crocetin</td>
</tr>
</tbody>
</table>
Figure 38: Fragmentation patterns of picrocrocin detected using negative ESI mode of UHPLC-MS
4.1.5. HPLC quantification of saffron constituents produced using different solvent systems

Our study investigated the amount of saffron constituents present in saffron extract after using different solvent systems. It was found that each solvent system had a different affinity to extract specific compounds which became obvious when the concentration of the aqueous portion was increased. Table 14 shows the ability of different organic solvents as well as their concentration to extract each marker compound in saffron. The concentrations of picrocrocin and crocin increased when the aqueous portion in the used solvents (ethanol and methanol) was increased. This observation was expected because the picrocrocin and crocin are hydrophilic compounds which are more soluble in water than organic solvents. However, regardless of extraction solvent there were no significant changes in the concentrations of safranal and crocetin likely due to their low presence in saffron extracts. Specifically, safranal and crocetin made up only approximately 0.4 and 0.8% of the dry weight of the extract. In contrast picrocrocin and crocin were maximally 20 and 40% dry weight of the same extract (Table 14).
Table 14: The constituent concentrations in saffron extract #18258 produced by using different solvent systems.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Concentration (µg/ml)</th>
<th>% Dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Picrocrocin</td>
<td>Crocin</td>
</tr>
<tr>
<td>EtOH</td>
<td>95%</td>
<td>1022 ± 36.4</td>
</tr>
<tr>
<td></td>
<td>80%</td>
<td>1667 ± 27.4</td>
</tr>
<tr>
<td></td>
<td>70%</td>
<td>1643 ± 31.3</td>
</tr>
<tr>
<td></td>
<td>60%</td>
<td>1838 ± 30.8</td>
</tr>
<tr>
<td></td>
<td>50%</td>
<td>1922 ± 31.8</td>
</tr>
<tr>
<td>MeOH</td>
<td>95%</td>
<td>1367 ± 66.2</td>
</tr>
<tr>
<td></td>
<td>80%</td>
<td>1793 ± 62.1</td>
</tr>
<tr>
<td></td>
<td>70%</td>
<td>1794 ± 33.4</td>
</tr>
<tr>
<td></td>
<td>60%</td>
<td>1875 ± 17.8</td>
</tr>
<tr>
<td></td>
<td>50%</td>
<td>2029 ± 45.6</td>
</tr>
</tbody>
</table>
4.1.6. Relationship between the solvent used in saffron extraction and their cytotoxicity effects

This study investigated the potential relationship between the solvent system used in saffron extraction and the observed cytotoxicity in prostate cell lines. The biological evaluation revealed that using different solvent systems resulted in variations in cytotoxicity effects (previous Figures 15-24). Moreover, HPLC analysis demonstrated the constituents’ concentrations varied in saffron extracts due to the use of different solvent systems for saffron extraction. Therefore, it was possible to use regression analysis to investigate the potential relationship between the solvent composition versus the mean of cytotoxicity response in each cell line produced by saffron extract #18258. As demonstrated in Table 15 and Figure 39, a higher organic solvent percentage was negatively correlated to the observed cytotoxicity in all prostate cell types. Therefore, when the aqueous portion in the extraction solvent increased, the cytotoxicity was decreased. With increasing hydrophilicity, the concentrations of picrocrocin and crocin were increased. Therefore, picrocrocin and crocin are not expected to strongly contribute to saffron extract cytotoxicity because there was a decline in cytotoxicity in both cancer cell line when picrocrocin and crocin concentrations increased (HPLC analysis).
Table 15: $R^2$ and $p$ value for the regression organic solvent composition used in saffron extraction versus cytotoxicity in each of prostate cell line

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Methanol</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNT1A</td>
<td>0.8322 ($p &lt; 0.0308$)</td>
<td>0.9683 ($p &lt; 0.0024$)</td>
</tr>
<tr>
<td>22Rv1</td>
<td>0.9076 ($p &lt; 0.0123$)</td>
<td>0.9683 ($p &lt; 0.0024$)</td>
</tr>
<tr>
<td>PC3</td>
<td>0.8285 ($p &lt; 0.0319$)</td>
<td>0.9695 ($p &lt; 0.0023$)</td>
</tr>
</tbody>
</table>

Figure 39: The correlation between the solvent used in saffron extraction and cytotoxicity effects
4.1.7. HPLC quantification of the constituents of commercial saffron samples

This study determined the chemical composition of commercial saffron samples obtained from the USA market (described in Chapter 3). The saffron extracts were subjected to analysis using the analytical HPLC method. As shown in Table 16, the saffron samples demonstrated variations in their constituents, even in the samples that claimed to have been cultivated in the same country. High concentrations of picrocrocin was detected in saffron extracts #18248, 18253, and 18254, respectively. Moreover, crocin was at higher concentrations in saffron extract #18255, 18251, 18253, and 18248.
Table 16: The constituent concentrations in commercial saffron samples.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Concentration (µg/ml)</th>
<th>% Dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Picrocrocin</td>
<td>Crocin</td>
</tr>
<tr>
<td>18248</td>
<td>1819 ± 36.6</td>
<td>2542 ± 51.7</td>
</tr>
<tr>
<td>18249</td>
<td>1205 ± 41.8</td>
<td>906 ± 9.44</td>
</tr>
<tr>
<td>18250</td>
<td>495 ± 9.81</td>
<td>1309 ± 79.8</td>
</tr>
<tr>
<td>18251</td>
<td>1039 ± 40.7</td>
<td>2842 ± 43.8</td>
</tr>
<tr>
<td>18252</td>
<td>1039 ± 22.3</td>
<td>1681 ± 28.8</td>
</tr>
<tr>
<td>18253</td>
<td>1358 ± 24.5</td>
<td>2695 ± 58.5</td>
</tr>
<tr>
<td>18254</td>
<td>1170 ± 2.86</td>
<td>1824 ± 49.5</td>
</tr>
<tr>
<td>18255</td>
<td>739 ± 38.5</td>
<td>3037 ± 30.6</td>
</tr>
<tr>
<td>18256</td>
<td>831 ± 29.8</td>
<td>1665 ± 35</td>
</tr>
<tr>
<td>18257</td>
<td>388 ± 8.56</td>
<td>1359 ± 25.8</td>
</tr>
</tbody>
</table>
4.1.8. Exportation and importation data for saffron

Our analysis of available raw data from the Nations Commodity Trade Statistics Database provided a good example of misleading marketing strategies by changing the country of origin of saffron. The production of saffron in Iran slightly changed from 205 tons in 2004 to 210 tons in 2011 (Hosseini, 2012), whereas its exportation declined from 178 tons in 2004 to 96 tons in 2011. On the other hand, some countries have with no known history of saffron agriculture (e.g. UAE) and other countries (France and Italy) import more saffron than export it then potentially re-export it as home-grown. This relationship becomes more obvious by comparing 2004 and 2011 data when those countries like Spain, France, and Italy decrease exportation alongside the Iranian saffron exportation declines as shown in Table 17 (United-Nations, 2015).

Table 17: Exported and imported saffron by country

<table>
<thead>
<tr>
<th>Parameters</th>
<th>2004</th>
<th>2011</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Import (tons)</td>
<td>Export (tons)</td>
</tr>
<tr>
<td>Iran</td>
<td>0</td>
<td>178</td>
</tr>
<tr>
<td>Spain</td>
<td>64.1</td>
<td>123</td>
</tr>
<tr>
<td>France</td>
<td>40</td>
<td>24.1</td>
</tr>
<tr>
<td>Italy</td>
<td>15.3</td>
<td>7.4</td>
</tr>
</tbody>
</table>
4.2. Discussion

In recent years, saffron extract used as a natural product supplement has gained popularity and numerous different brands of this saffron supplements became available on the market. Unlike pharmaceutical drug marketing in the USA, there is no regulatory oversight for natural supplements regarding their effectiveness, quality, or safety. There are many methods used for adulteration purposes in natural products. This is true especially with saffron stigmas because it is the most expensive spice in the world. The adulteration methods include: 1) addition of cheap bulking agents to increase the weight, such as dyed-red wood filaments and pomegranate fibers, 2) adding of synthetic components to increase the quality of saffron such as safranal, 3) mixing low quality with high quality of saffron to increase revenue (Gohari et al., 2013). The relabeling of the origin country is intended to increase the price of saffron or to overcome logistics problems such as economic sanctions, as implicated in Table 17 (Hosseini, 2012, Gresta et al., 2009). Therefore, finding and developing an accurate systemic evaluation method to authenticate saffron has become a mandatory step toward insuring the maximum benefits with minimum health risks that could arise from using unassessed and adulterated natural product supplements like saffron. Any developed evaluation methods should consider both chemical and biological aspects, because saffron has hundreds of constituents which it is impossible to detect all of using standard basic laboratory methods. By combining biological and chemical analysis, a more accurate and simplified saffron evaluation is possible.

This study has developed RP-HPLC analytical method which is a simple, repeatable, and reproducible procedure. This analytical method is specifically designed to analyze the saffron stigma as well as the supplemented products by measuring their ‘fingerprint’ including picrocrocin, crocin, safranal, and crocetin. The developed method was validated according to
International Conference for Harmonization (ICH) guideline. The new method could possibly be used in quality control analysis of saffron and its supplemental derivatives, because it provides accurate analysis results with a simple and reproducible approach. It has the ability to simultaneously quantify the most important saffron constituents in a single process. Using the gradient chromatography system enhances the ability to separate the complex combination of compounds that are the constituents of saffron, at very low concentrations. It can separate the most hydrophilic compounds such as picrocrocin in addition to the most hydrophobic compounds like crocetin.

As demonstrated in this dissertation, the chromatographic peaks presented in saffron extract were identified using the retention times of known standards as well as HPLC-MS. The mobile phase of the developed method is compatible for use with mass spectrometry because it contains 0.1% formic acid which facilitates the protonation of the available compounds in saffron extract. The HPLC-MS is useful and provides an easier way to identify the presence of unusual peaks that may indicate a potential adulteration in commercially available saffron and supplemental products.

The anti-cancer properties of saffron extracts were determined and summarized in Table 18. The IC$_{50}$ of the ethanolic-extracted saffron for normal, early, and advanced stages of cancer ranged from 0.38 to 0.73 mg/ml, 1.32 to 2.27 mg/ml, and 1.77 to 3.23 mg/ml, respectively. On other hand, the IC$_{50}$ of the methanolic-extracted saffron for normal, early, and advanced stages of cancer ranged from 0.32 to 0.4 mg/ml, 1.26 to 2.27 mg/ml, and 1.98 to 2.99 mg/ml, respectively. It is clear that there are differences between the cytotoxicity effects achieved by using different solvent types which could be observed when we compared, side by side, each solvent concentration. Those differences could exist due to various reasons, such as the chemical
interaction between the solvents and the plant components (Wang and Weller, 2006) which may decompose plant constituents (Tønnesen et al., 2002) or convert them to toxic compounds; the ability of the solvents to extract, exclusively, some components that produced pharmacological effects, the solvent polarity that extracts more or less amounts of hydrophilic or lipophilic compounds (Maltese et al., 2009), and the required temperature for solvent evaporation to dry the extracts (Munin and Edwards-Lévy, 2011).

Table 18: Summary of the cytotoxicity effects of solvent systems on the cell lines. All concentration units are mg/ml.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Concentration</th>
<th>PNT1A</th>
<th>22Rv1</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>95%</td>
<td>0.38 ± 0.07</td>
<td>1.32 ± 0.19</td>
<td>1.77 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>80%</td>
<td>0.49 ± 0.10</td>
<td>1.55 ± 0.26</td>
<td>2.2 ± 0.88*</td>
</tr>
<tr>
<td></td>
<td>70%</td>
<td>0.63 ± 0.13</td>
<td>1.71 ± 0.33</td>
<td>2.35 ± 0.63*</td>
</tr>
<tr>
<td></td>
<td>60%</td>
<td>0.66 ± 0.15</td>
<td>2.09 ± 0.5*</td>
<td>2.77 ± 0.65*</td>
</tr>
<tr>
<td></td>
<td>50%</td>
<td>0.73 ± 0.16</td>
<td>2.27 ± 0.72*</td>
<td>3.23 ± 0.83*</td>
</tr>
<tr>
<td>MeOH</td>
<td>95%</td>
<td>0.32 ± 0.05</td>
<td>1.26 ± 0.25</td>
<td>1.98 ± 0.39</td>
</tr>
<tr>
<td></td>
<td>80%</td>
<td>0.36 ± 0.06</td>
<td>1.53 ± 0.32</td>
<td>2.44 ± 0.6*</td>
</tr>
<tr>
<td></td>
<td>70%</td>
<td>0.4 ± 0.07</td>
<td>1.91 ± 0.38</td>
<td>2.99 ± 0.66*</td>
</tr>
<tr>
<td></td>
<td>60%</td>
<td>0.4 ± 0.07</td>
<td>2.27 ± 0.6*</td>
<td>2.95 ± 0.74*</td>
</tr>
<tr>
<td></td>
<td>50%</td>
<td>0.4 ± 0.07</td>
<td>2.17 ± 0.64*</td>
<td>2.98 ± 0.67*</td>
</tr>
</tbody>
</table>

* Expected IC$_{50}$ using GraphPad Prism 5 (IC$_{50}$ higher than 2 mg/ml, the maximal tested concentration)
The extraction of saffron constituents using a methanolic solvent regardless of its concentrations produced more cytotoxic effects on normal cells compared to cancerous cells. This can clearly be seen with the higher concentration of the methanol solvent. In addition, there is an obvious relationship of the toxic effects between normal and advanced-stage prostate cancer cells. When the concentration of the organic solvent decreased from 95% to 80% and from 80% to 70%, the cytotoxicity in both cell types decreased, though when the methanol concentration decreased under 70%, there was almost no change in both cell responses. This demonstrates the capability of high methanol concentration to extract unique components or convert constituents to toxic compounds which exert their cytotoxic effects against those cells; the capability for the extraction or the conversion is decreased with low methanol concentration.

In ethanolic-extracted saffron, there was a noticeable difference regarding the extract activity between different concentration groups. Among all groups, the lowest amount required to produce cytotoxicity effects for both prostate cancer stages was obtained by using 95% ethanolic saffron extract and closely followed by 80%. Without an exception, the cytotoxicity response of all cell types to the whole saffron extract declined when using lower concentrations of the ethanolic solvent. It is possible the high concentration of ethanol is able to extract compounds that are responsible for activation of the cytotoxicity mechanisms which are present in all cell types. In both solvents, saffron extracts were strongly dependent on the solvents’ organic composition.

Previous studies found that using different solvents had a significant difference on the phenolic and flavonoid content of saffron extracts. The methanolic saffron extract has stronger antioxidants and scavenger activity compared to ethanolic saffron extract. These differences are interpreted by the ability of methanol to extract two times the amount of total flavonoids than
ethanol solvent (Karimi et al., 2010). Moreover, multiple studies have shown a variation in the in vitro activities of saffron extract. The response of cervical cancer (HeLa) and hepatocellular carcinoma (HepG2) cell lines to saffron extract as anti-cancer therapy represent the best examples of the controversial activity results (Table 19). Three studies conducted experiments using a HeLa cell line. Two of them used the same solvent but with different solvent concentrations, the IC50 after 24 hours of exposure was 2 and 2.3 mg/ml for the 96% and 75% ethanolic solvent, respectively (Escribano et al., 1996, Tavakkol-Afshari et al., 2008). A different study used 80% methanolic solvent and the IC50 was 653 µg/ml, whereas the IC50 of the 96% ethanolic solvent was 700 µg/ml after 72 hours of exposure (Rahimi Fard et al., 2011, Tavakkol-Afshari et al., 2008). Moreover, two studies were conducted using different solvents for saffron extraction. After 72 hours of saffron extract incubation with the HepG2 cell line, the IC50 for the aqueous extract was 400 µg/ml whereas it was 650 µg/ml for 96% ethanolic extract (Parizadeh et al., 2011, Tavakkol-Afshari et al., 2008). Indeed, the variation of the IC50 obtained from the earlier studies is in agreement with our finding regarding the variation of the observed activity due to the differences in the extraction solvent system. This study addresses the importance of the solvent selection regarding to its pharmacological effectiveness in saffron. Our study used the advantage of the variation in saffron constituents as well as biological activity which resulted in using different solvent systems for saffron extraction to investigate the most likely compounds that are responsible on saffron extract activity. From the correlation study, it is clearly picrocrocin and crocin which are the most likely compounds that are not responsible for exerted saffron’s cytotoxicity toward prostate cancer.
Table 19: The variation in IC\textsubscript{50} values reported in literature using different solvents for extraction

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HeLa</th>
<th>HepG2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation time</td>
<td>24 hours</td>
<td>72 hours</td>
</tr>
<tr>
<td>Solvents used for saffron extraction</td>
<td>75% ethanol</td>
<td>96% ethanol</td>
</tr>
<tr>
<td>IC\textsubscript{50}</td>
<td>2.3 mg/ml</td>
<td>2 mg/ml</td>
</tr>
</tbody>
</table>

(Escribano et al., 1996, Parizadeh et al., 2011, Rahimi Fard et al., 2011, Tavakkol-Afshari et al., 2008)

A total of 11 different commercial samples were obtained from multiple regions including Iran, Spain, and Afghanistan and were acquired commercially and botanical identified as Crocus sativus stigmas. Saffron samples were extracted using 95% ethanol solvent and tested for biological activities (Table 20). We conducted HPLC analysis for all samples and the marker compounds such as safranal, picrocrocin, crocin, and crocetin were present in all of them although their concentrations differed significantly among each sample (see appendix for HPLC chromatograms). Although we cannot confirm the geographical origins of the commercial saffron samples, there were differences in their compositions which were revealed by the HPLC profiling results. These results confirm the finding of another study that concluded that the changes in the environments of saffron agriculture will cause variations in the saffron component concentration. However, even when there are variations, there are no specific compounds which could be used as a fingerprint to authenticate the saffron’s origins (Maggi et al., 2011).
Table 20: Summary of the cytotoxicity effects of commercial samples of saffron on the cell
lines. All concentration units are mg/ml.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>PNT1A</th>
<th>22Rv1</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>18258 (Reference)</td>
<td>0.38 ± 0.07</td>
<td>1.32 ± 0.19</td>
<td>1.77 ± 0.23</td>
</tr>
<tr>
<td>18248</td>
<td>1.37 ± 0.33</td>
<td>2.56 ± 0.46*</td>
<td>8.22 ± 2.56*</td>
</tr>
<tr>
<td>18249</td>
<td>1.78 ± 0.39</td>
<td>2.85 ± 0.67*</td>
<td>≥ 34.47*</td>
</tr>
<tr>
<td>18250</td>
<td>1.69 ± 0.5</td>
<td>1.78 ± 0.27</td>
<td>1.97 ± 0.34</td>
</tr>
<tr>
<td>18251</td>
<td>1.64 ± 0.42</td>
<td>3.05 ± 0.65*</td>
<td>3.91 ± 1.19*</td>
</tr>
<tr>
<td>18252</td>
<td>1.15 ± 0.19</td>
<td>1.67 ± 0.49</td>
<td>1.97 ± 0.35</td>
</tr>
<tr>
<td>18253</td>
<td>1.42 ± 0.28</td>
<td>3.08 ± 0.56*</td>
<td>5.01 ± 1.25*</td>
</tr>
<tr>
<td>18254</td>
<td>1.09 ± 0.18</td>
<td>2.39 ± 0.55*</td>
<td>3.85 ± 1.02*</td>
</tr>
<tr>
<td>18255</td>
<td>1.06 ± 0.17</td>
<td>1.88 ± 0.34</td>
<td>3.76 ± 0.99*</td>
</tr>
<tr>
<td>18256</td>
<td>0.79 ± 0.12</td>
<td>4.20 ± 0.45*</td>
<td>4.77 ± 0.90*</td>
</tr>
<tr>
<td>18257</td>
<td>0.76 ± 0.12</td>
<td>8.19 ± 2.36*</td>
<td>10.22 ± 2.38*</td>
</tr>
</tbody>
</table>

* Expected IC$_{50}$ using GraphPad Prism 5 (IC$_{50}$ higher than 2 mg/ml)

The majority of saffron extracts exhibited cytotoxic effects against all three human cell
lines, include normal and cancerous cells, in a dose-dependent manner. The viability assay
carried out using different concentrations of saffron extracts ranged from 0.125 – 2 mg/ml. In
general, all evaluated saffron extracts displayed stronger cytotoxic effects against normal as
opposed to early prostate cancer cells and the lowest effects were noticed in advanced stages of
cancer (Table 20). Among all 11 tested saffron extracts, three of them #18250, 18252, and 18258
showed antiproliferative effects against the cancer cell lines but cytotoxicity was in each case
still higher in normal prostate cells. The IC₅₀ for cancer cells was less than 2 mg/ml and from 0.38 to 1.69 mg/ml for normal cells. The weakest commercial saffron which exerted cytotoxic effects on normal prostate cells was sample #18249 whereas sample #18258 had the strongest cytotoxicity. These variations in the biological responses matched with the findings of other studies. The sources and the geographical origins of saffron have a strong impact on the stigma physical characteristics as well as the chemical composition (Zarinkamar et al., 2011).

Also, our results emphasized an important fact, which is the safety of saffron extracts on normal cells. Saffron extracts are available on the market in the form of capsulated supplements which are used for weight loss, as antioxidants, for mood improvements, and so on. The FDA neither requires safety studies prior to these supplements’ marketing nor need mention the origin of the saffron. Our study suggests more caution should be followed by evaluating each saffron batch on human normal cells before marketing them as health supplements.
4.3. Conclusion

According to our knowledge this is the first study on the comparison of the chemical constituents’ quantification and bio-evaluation of saffron after extraction using different solvent systems, as well as the analysis of available commercial samples in US market. The validation results of the developed analytical HPLC method demonstrated simplicity, accuracy, selectivity, sensitivity, and repeatability. This RP-HPLC used important marker compounds that differentiate saffron characteristics which can be effective for rapid analysis of saffron stigma purity as well as herbal formulation and analysis of supplemental products that contain saffron. The method can be used as an alternative to the ISO method to evaluate the quality of saffron. The purity of the saffron stigmas which were used in this study was verified to be high by analyzing saffron extracts using HPLC. The solvent systems used in saffron extraction clearly affected their anti-cancer capability. The results obtained for the anti-cancer activity of extracts differed depending on the type, concentration of solvent, and saffron source. Regardless of the saffron’s origin, the most efficient solvents for saffron extraction were 95% and 80% ethanol, respectively. Both whole extracts exerted similar activities regarding the ratio of normal to cancer cytotoxicity effects, although the 95% ethanol is preferred due to its stronger activity on advanced- and early-stage cancer, respectively. Our study concluded that methanol used for saffron extraction was toxic to normal cells with low anti-cancer activity, so it is not recommended to use this as a solvent. The study outcomes revealed that the solvent systems with various polarity have major effects and play important roles in determining the anti-cancer activity of saffron extracts. The concentrations of saffron constituents in the commercial samples varied, which affected the biological responses. Therefore, this study highlights multiple points, such as the importance of evaluating the saffron for its activity and safety on normal cells. In addition, when a study
investigates the activity of natural products, it is best to consider using multiple samples from different geographic locations. Because there is no simple method available to detect the chemical composition of natural products which could reach hundreds of compounds, it is best to combine biological with chemical evaluations.
CHAPTER 5: SAFFRON EXTRACT AND ITS CONSTITUENTS AS PREVENTATIVE AND/OR TREATMENT AGENTS FOR PROSTATE CANCER

5.1. Results

5.1.1. Isolation and measurement of picrocrocin compound purity

Using the chromatotron method to isolate pure compounds from saffron extract provides excellent results including: it can quickly recover a large amount of starting material by weight (90%) and it isolates compounds with high purity. Sixty-three fractions were obtained from the saffron #18258 extract. These fractions were initially analyzed using reverse phase thin layer chromatography (RP-TLC), that facilitated the combination fractions with similar band patterns. After combination a total of 13 fractions were obtained. Three of fractions contained picrocrocin, and one of these fraction (27-34) represented 13.36% of the recovered weight. The purity of this fraction (27-34) was measured by HPLC and determined its purity percentage by using the calibration curve of pure picrocrocin as described in Chapter 3 (Figure 40). Fraction 27-34 was the purest picrocrocin fraction obtained with 93.49% of purity.
5.1.2. Antioxidant activity of saffron extract and its active constituents

The capacity of saffron extract and its active components to activate anti-oxidative mechanisms in normal cells against oxidants was assessed using an H$_2$DCFDA probe. The normal prostate cells (PNT1A) were pre-incubated with two different nontoxic concentrations of saffron extract (100 and 200 µg/ml), safranal (6 and 12 µg/ml), and crocetin (15 and 30 µg/ml) for 4 hours as shown in Figure 41. After removing the treatment, oxidative stress was induced when 50 µM of H$_2$O$_2$ was added. In untreated groups, the oxidative stress was 60% higher in exposed normal cells with H$_2$O$_2$ (positive control) compared to unexposed cells (negative control). The oxidative stress significantly dropped when the cells were pre-incubated with both concentrations of safranal. The level of oxidative stress decreased by ≈ 50% compared to positive control group, but it was still higher by almost 30% compared to negative control group. When the cells were pre-incubated with 200 µg/ml of saffron extract or 30 µg/ml of crocetin, similar results for both were obtained. The oxidative stress level significant declined to ≈ 46%
compared to positive control group, but was not returned to normal level (∼ 27% higher compared to negative control group). In conclusion, the results demonstrated that saffron extract (200 µg/ml), safranal (6 and 12 µg/ml), and crocetin (30 µg/ml) had antioxidant effects.

![Graph showing antioxidant activity of saffron extract and its active constituents](image)

**Figure 41:** Antioxidant activity of saffron extract and its active constituents against oxidative stress induced by H₂O₂ in PNT1A cells. Bars with # symbol are statistically significant from the control group whereas * symbol are statistically significant from the group exposed with H₂O₂ only (ANOVA; n = 3 replicates/treatment group, p<0.05).
5.1.3. Cytotoxicity of saffron extract and its constituents on different prostate cells

5.1.3.1. Cytotoxicity of saffron extract #18258

We evaluated the viability of normal and prostate cancer cells treated with increasing concentrations of saffron extract which was obtained by using 95% ethanol for extraction (0, 0.125, 0.25, 0.5, 1, 2 mg/ml) for 24, 48 or 72 hours using the XTT assay, as explained in Chapter 3, to find whether the saffron extract is effective in treating prostate cancer. A significant inhibition in malignant prostate cell growth was achieved using saffron extract in dose- and time-dependent manners, the effects of saffron extract were more clear after 48 and 72 hours of incubation, as shown in Figures 42-44. Only 42.41% of 22Rv1 and 43.68% of PC3 cells survived after 72 hours of 2 mg/ml extract exposure. In addition, after 72 hours of exposure, the IC\textsubscript{50} for both cancer cells was reached. The IC\textsubscript{50} concentrations were calculated at 24, 48, 72 hours post incubation, and were 7.38 ± 2.16, 2.77 ± 0.67, and 1.32 ± 0.19 mg/ml for 22Rv1, and 10.42 ± 4.21, 5.85 ± 1.59, and 1.77 ± 0.23 mg/ml for PC3, respectively (\(p < 0.05\)). We also studied the effects of saffron extract on normal prostate cells (PNT1A). Saffron extract induced the cytotoxicity in normal cells starting at 0.5 mg/ml after 24 and 48 hours of incubation. The cytotoxicity was induced with increasing the incubation time, which made the minimum concentration necessary for cytotoxicity lower (0.125 mg/ml). Incubating the PNT1A cells with 2 mg/ml for 72 hours lead to only 27.68% survival of cells compared 100% in the control. The IC\textsubscript{50} concentrations for PNT1A were determined at 24, 48, 72 hours after incubation, which were 4.46 ± 1.09, 1.53 ± 0.29, and 0.38 ± 0.07 mg/ml, respectively (\(p < 0.05\)).
Figure 42: Cytotoxicity of saffron extract against PNT1A, 22Rv1, and PC3 cells after 24 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 4 replicates/treatment group, p<0.05).

Figure 43: Cytotoxicity of saffron extract against PNT1A, 22Rv1, and PC3 cells after 48 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 4 replicates/treatment group, p<0.05).
Figure 44: Cytotoxicity of saffron extract against PNT1A, 22Rv1, and PC3 cells after 72 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 4 replicates/treatment group, p<0.05).

5.1.3.2. Cytotoxicity of picrocrocin

A cytotoxicity assay was conducted using XTT reagent as described previously in Chapter 3, to investigate the picrocrocin effects at different concentrations ranging from 0.125-2 mg/ml in normal and tumor prostate cell lines as shown in Figure 45. It was expected that picrocrocin would show cytotoxicity in prostate cancer similar to other cancer types and because its chemical structure contains the safranal moiety. The results indicated that picrocrocin exhibited weak cytotoxic effects in all cell lines after 72 hours of incubation. The toxic effect was significant at a concentration of 2 mg/ml. The obtained IC50s were 4.03 ± 1.44, 4.48 ± 1.51, and 4.52 ± 1.55 mg/ml in PNT1A, 22Rv1, and PC3 cell lines, respectively. The activity of this constituent lacked the cell type selectivity because it induced cytotoxicity in normal cells more
than cancer cells. Also, compared to other saffron components such as safranal and crocetin it showed lower cytotoxicity in prostate cancer cells.

![Graph showing cell survival percentage](image)

**Figure 45**: Cytotoxicity of picrocrocin against PNT1A, 22Rv1, and PC3 cells after 72 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).

### 5.1.3.3. Cytotoxicity of crocin

The anti-proliferative effects of pure crocin on normal (PNT1A) and prostate cancer (22Rv1 and PC3) cell lines are illustrated in Figure 46. The saffron extract showed cytotoxicity effect, so we investigated the crocin cytotoxicity alone because it is present in saffron in relatively high concentrations. In addition, its structure the contains crocetin moiety, so when crocin is hydrolyzed crocetin will be produced. Crocin had a concentration-dependent effect. After 72 hours of incubating all cell types with crocin, the IC\textsubscript{50}s were 1.18 ± 0.3, 1.43 ± 0.32, and
1.9 ± 0.48 mg/ml for the PNT1A, 22Rv1, and PC3 cell lines, respectively. At 2 mg/ml, the percentage of PNT1A surviving cells was 32%. However, the prostate cancer cells 22Rv1 and PC3 did not show as much cytotoxicity as in the PNT1A cells where 39 and 45% of cells survived, respectively. Crocin had a greater toxic effect on normal cells than cancerous prostate cells. Although a significant decrease in the cell viability of all cell types was observed at high concentrations, other saffron components (crocetin and safranal) showed stronger activity.

Figure 46: Cytotoxicity of crocin against PNT1A, 22Rv1, and PC3 cells after 72 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).
5.1.3.4. Cytotoxicity of safranal

Safranal was investigated for its cytotoxicity at different concentrations ranging from 6.25-100 μg/ml in both normal and cancerous prostate cell lines. In the literature, different types of cancer respond to safranal treatment. Therefore, safranal cytotoxicity was studied to determine if safranal had activity against prostate cancer cells. Safranal induced a cytotoxic response in time- and concentration-dependent manners for all cell lines. The effect of different concentrations and exposure times is shown in Figures 47-49. After 24 hours of safranal incubation, the cytotoxicity was initiated at concentrations of 12.5 and 25 μg/ml for 22Rv1 and PC3 cells, respectively. A significant inhibitory effect in 22Rv1 at 48 hours started at a safranal concentration of 6.25 μg/ml. There was a slightly stronger activity of safranal against PC3 at 48 hours post incubation, but the minimum concentration required to produce cytotoxicity was not changed compared to 24 hours of incubation. The maximum reduction in cellular proliferation for both cancer cell lines was achieved after 72 hours post incubation at a concentration of 6.25 μg/ml and higher. Using 50 and 100 μg/ml for 72 hours caused a reduction in survival rate of 22Rv1 by 83.25 ± 1.48 and 86.64 ± 3.66%, respectively whereas it was 68.24 ± 7.81 and 84.05 ± 4.35% for PC3 cell line, respectively. The IC$_{50}$ of safranal concentrations were determined at 24, 48, 72 hours post incubation, which were 22.31 ± 6.97, 14.92 ± 3.28, and 10.91 ± 1.71 μg/ml for 22Rv1 whereas they were 44.4 ± 14.26, 30.22 ± 7.12, and 19.44 ± 4.09 μg/ml for PC3, respectively ($p < 0.05$). The safranal was also used against a normal prostate cell line (PNT1A). Incubating the PNT1A cell with 50 and 100 μg/ml of safranal for 72 hours decreased the survival of cells by 85.39 ± 4.19 and 88.11 ± 3.52%, respectively. The IC$_{50}$ concentrations for PNT1A were determined at 24, 48, 72 hours after incubation, which were 41.16 ± 12.4, 21.87 ± 3.53, and 13.9 ± 3.04 μg/ml, respectively ($p < 0.05$). These results indicated that unlike early prostate
cancer cells (22Rv1), all used safranal concentrations were less cytotoxic towards the PNT1A cell line after 24 - 72 hours of incubation. In contrast to advanced stage of prostate cancer cells (PC3), the safranal had stronger cytotoxic effects on PNT1A compared to PC3.

Figure 47: Cytotoxicity of safranal against PNT1A, 22Rv1, and PC3 cells after 24 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).
Figure 48: Cytotoxicity of safranal against PNT1A, 22Rv1, and PC3 cells after 48 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).

Figure 49: Cytotoxicity of safranal against PNT1A, 22Rv1, and PC3 cells after 72 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).
5.1.3.5. Cytotoxicity of crocetin

Multiple studies found that the crocetin was the strongest saffron constituent to produce anti-cancer activity in different cancer types. Therefore, we investigated crocetin cytotoxicity against prostate cancer. The crocetin cytotoxicity at different time points was determined using concentrations ranging from 25 - 200 µg/ml for 24, 48, and 72 hours as depicted in Figures 50-52. After 24 hours of crocetin exposure, the cytotoxicity was significantly increased in all prostate cell lines at a concentration of 100 µg/ml. After 24 hours of treatment, the IC₅₀s were 93.52 ± 30.72, 172.2 ± 49.31, and 165 ± 39.33 µg/ml for the PNT1A, 22Rv1, and PC3 cell lines, respectively. When PC3, 22Rv1, and PNT1A cells were incubated with 200 µg/ml of crocetin, survival was 47.29%, 48.82%, and 37.56%, respectively. Furthermore, after 48 hours of treatment, increased cytotoxicity was observed. The IC₅₀s were 69.75 ± 19.91, 86.3 ± 23.75, and 90.31 ± 26.12 µg/ml for the PNT1A, 22Rv1, and PC3 cell lines, respectively. At an exposure of 200 µg/ml, PNT1A, 22Rv1, and PC3 cell viability was significantly reduced to 29.87, 33.26, and 36.73%, respectively. Finally, when the cells were incubated with the treatment for 72 hours, the low concentration of crocetin (25 µg/ml) exerted a toxic effect in all cell types. Moreover, the IC₅₀ dropped to 47.51 ± 11.14, 62.85 ± 15.60, and 59.33 ± 13.18 µg/ml for the PNT1A, 22Rv1, and PC3 cell lines. Incubating the cells with 200 µg/ml of crocetin for 72 hours resulted in a reduction of cell viability of PNT1A to 21.8%, 22Rv1 to 29.16%, and PC3 to 29.08%.
Figure 50: Cytotoxicity of crocetin against PNT1A, 22Rv1, and PC3 cells after 24 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).

Figure 51: Cytotoxicity of crocetin against PNT1A, 22Rv1, and PC3 cells after 48 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).
Figure 52: Cytotoxicity of crocetin against PNT1A, 22Rv1, and PC3 cells after 72 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).

5.1.4. Determination the mechanism of cell death caused by saffron extract and its active constituents

This study investigated whether saffron and its active constituents caused cell death in PNT1A, 22Rv1, and PC3 cells through the apoptosis pathway. For that purpose, the apoptotic enzymes caspase 3 and 7 (CASP3 and 7) were measured after 6 hours of treatment as described in Chapter 3. Generally, the apoptotic cells were increased in a concentration-dependent manner in all of the different treatment groups. In the normal prostate cells (PNT1A), saffron extract at different concentrations (0.75 and 1.5 mg/ml) significantly induced the CASP3/7 enzyme levels by 3.19- and 4.57-fold, respectively. When the cells were treated with 100 and 200 µg/ml of crocetin, the enzymes levels were significantly induced by 3.01-and 4.22-fold, respectively.
Also, at concentrations of 25 and 50 µg/ml of safranal, the CASP3/7 significantly increased by 2.65- and 3.45-fold (Figure 53).

On the other hand, different responses were observed in prostate cancer cells. As observed in Figure 54, when the 22Rv1 cell line was exposed to 0.75 and 1.5 mg/ml of saffron extract there was no significant change obtained in CASP 3/7 level. A significant induction by 3.9-fold in CASP 3/7 was achieved when the cells were treated with 200 µg/ml of crocetin. In addition, 25 and 50 µg/ml of safranal resulted in a significant enhancement in CASP 3/7 enzymes levels by 4.77- and 6.68-fold, respectively in 22Rv1 cell line.

Moreover, the CASP 3/7 enzyme levels in the PC3 cells were not significantly different from the control group when treated with saffron extract at concentrations of 0.75 and 1.5 mg/ml as depicted in Figure 55. There was marked induction in CASP 3/7 enzymes by 3.73- and 4.76-fold at 100 and 200 µg/ml of crocetin exposure, respectively. Furthermore, a significant induction by 3.7- and 5.91-fold in CASP 3/7 was measured in cells exposed to safranal at concentrations of 25 and 50 µg/ml of safranal, respectively. Thus the primary cell death mechanism by saffron and its active components was by inducing apoptosis, which was activated through an upstream caspase cascade (intrinsic or extrinsic).
Figure 53: The effect of saffron and its active constituents on caspase 3/7 enzyme levels in PNT1A cells after 6 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).

Figure 54: The effect of saffron and its active constituents on caspase 3/7 enzyme levels in 22Rv1 cells after 6 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).
Figure 55: The effect of saffron and its active constituents on caspase 3/7 enzyme levels in PC3 cells after 6 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).

5.1.5. Identification of apoptosis pathway genes using RT-PCR

Our research used a normal prostate cell line (PNT1A) to mechanistically investigate the apoptosis induction produced by saffron extract and its active components either through intrinsic or extrinsic pathways. Three genes CASP3, 7, and 9 were measured using real time PCR as shown in Figures 56-58. RT-PCR results revealed that at 72 hours safranal dose-dependently increased the expression of CASP3, 7, and 9 mRNA. Specifically, the CASP3 expression was induced by 1.8- and 2.7-fold upon treatment with safranal at concentrations of 25 and 50 µg/ml, respectively. The expression of the CASP7 gene was increased by 2.6- and 3.4-fold when treated with 25 and 50 µg/ml of safranal, respectively. An induction of 3.1- and 4.7-fold in CASP9
mRNA was achieved with exposure to 25 and 50 µg/ml of safranal, respectively. When the cells were treated with 100 µg/ml of crocetin, high gene expression of CASP3, 7, and 9 was obtained with respective 3.1-, 4.8-, and 5-fold inductions. High concentrations of crocetin as well as the saffron extract did not significantly induce caspase gene expression.

![Figure 56: The effect of saffron and its active constituents on caspase 3 mRNA expression level in PNT1A cells after 72 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).](image)
Figure 57: The effect of saffron and its active constituents on caspase 7 mRNA expression level in PNT1A cells after 72 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).

Figure 58: The effect of saffron and its active constituents on caspase 9 mRNA expression level in PNT1A cells after 72 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).
5.1.6. Wound healing assay

Saffron extract, safranal, and crocetin showed cytotoxicity against prostate cancer, so we studied the ability of saffron extract and these active components to prevent prostate cancer metastasis development by inhibiting the prostate cancer migration and invasion characteristics without affecting normal cell functions. We assessed the migration of normal and prostate cancer cells to fill the gap that was created in a wound healing assay after treating them with saffron extract and its active constituents. An important consideration of this assay is that the concentrations tested do not cause cytotoxicity so that migratory suppression is assessed. Our results indicate that 200 µg/ml of saffron inhibited the migration of both prostate cancer types (22Rv1 and PC3) and high concentrations of crocetin induced the migration rate of both prostate cancer cells. Low concentrations of saffron extract induced migration in normal cells only. The high concentration of safranal had opposite effects in normal and prostate cancer cells. It enhanced the migration rate in normal cells whereas it decreased the rate in cancer cells.

5.1.6.1. Wound healing assay in normal cell line (PNT1A)

Two non-cytotoxic concentrations of saffron, safranal, and crocetin were used to determine their effects on migration activities in normal prostate cells (PNT1A). The wound healing assay was used with the following concentrations: 6 and 12 µg/ml of safranal, 15 and 30 µg/ml of crocetin, and 0.1 and 0.2 mg/ml of saffron extract as shown in Figures 59-60. After 24 hours of incubation, the control group of PNT1A cells slightly closed the gap of the scratched area, whereas the cells treated with 12 µg/ml of safranal and 0.1 mg/ml of saffron extract were found to migrate significantly faster. The remaining treatment groups had similar results to control group and did not affect the migratory behavior of normal cells. In contrast, the wound
Closure rate was almost two times more than the control group with 12 µg/ml of safranal and 0.1 mg/ml of saffron extract. Generally, this result showed there is no interfering between the migratory behavior of normal cells when the saffron extract and its active constituents used in non-toxic dose.

![Graph showing the effect of saffron and its active constituents on cell migration]

**Figure 59:** The effect of saffron and its active constituents after 24 hours of exposure on the ability of migrating PNT1A cells to close the scratched wound. Bars with * symbol are statistically significant from control group. Area was measured using ImageJ software, (ANOVA; n = 3 replicates/treatment group, p<0.05).
<table>
<thead>
<tr>
<th>Group</th>
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<th>24 hours</th>
</tr>
</thead>
<tbody>
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<tr>
<td>Crocetin</td>
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</table>

Figure 60: Representative images of PNT1A cells in wound healing assay at 0 and 24 hours after treatment exposures. Pictures were taken with 4X microscopic magnification. Area of gap was measured using ImageJ software.
5.1.6.2. Wound healing assay in androgen sensitive prostate cancer cell line (22Rv1)

The effect of saffron extracts (0.1 and 0.2 mg/ml), safranal (6 and 12 µg/ml), and crocetin (15 and 30 µg/ml) on the migratory function of 22Rv1 was evaluated using a wound healing assay as presented in Figure 61. Our results demonstrated that 12 µg/ml of safranal significantly suppressed the migration of cells into the wounded area by 39% in comparison to the untreated control group. A stronger suppression was obtained when saffron extract was used at a concentration of 0.2 mg/ml and resulted in 71% decline in migration rate in 22Rv1. On the other hand, 30 µg/ml of crocetin significantly stimulated the 22Rv1 cells migration which consequently closed the gap of the scratched area at a faster rate (Figure 62).

![Figure 61: The effect of saffron and its active constituents after 24 hours of exposure on the ability of migrating 22Rv1 cells to close the scratched wound. Bars with star symbol are statistically significant from control group. Area was measured using ImageJ software, (ANOVA; n = 3 replicates/treatment group, p<0.05).]
<table>
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<tr>
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</table>

Figure 62: Representative images of 22Rv1 cells in wound healing assay at 0 and 24 hours after treatment exposures. Pictures were taken with 4X microscopic magnification. Area of gap was measured using ImageJ software.
5.1.6.3. Wound healing assay in androgen independent prostate cancer cell line (PC3)

The effect of 6 and 12 µg/ml of safranal, 15 and 30 µg/ml of crocetin, and 0.1 and 0.2 mg/ml of saffron extract on PC3 cell migration was measured by the wound healing assay as shown in Figures 63-64. PC3 cells that were exposed to high concentrations of saffron extract (0.2 mg/ml) and safranal (12 µg/ml) showed a reduction in the migration of the cells to fill the gap area as compared to the control cells. In contrast, the high concentration of crocetin (30 µg/ml) promoted the cell migration by 60% compared to the control group. Lower concentrations of the compounds did not produce any significant changes in the PC3 cell migration behaviors.

Figure 63: The effect of saffron and its active constituents after 24 hours of exposure on the ability of migrating PC3 cells to close the scratched wound. Bars with * symbol are statistically significant from control group. Area was measured using ImageJ software, (ANOVA; n = 3 replicates/treatment group, p<0.05).
<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (µg/ml)</th>
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<th>24 hours</th>
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</thead>
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<td>Control</td>
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<tr>
<td>Safranal</td>
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Figure 64: Representative images of PC3 cells in wound healing assay at 0 and 24 hours after treatment exposures. Pictures were taken with 4X microscopic magnification. Area of gap was measured using ImageJ software.
5.1.7. The effects of saffron extract and its active constituents on prostate cell migration using a transwell (Boyden chamber) assay

A transwell assay was conducted to further confirm the observations obtained from the wound healing assay. The effects of two concentrations of saffron extract, crocetin, and safranal on cell migration were examined on all prostate cell lines. In summary, the results showed that a high concentration of safranal (12 µg/ml) inhibited migration on both types of prostate cancer cells (22Rv1 and PC3). A high concentration of crocetin (30 µg/ml) induced the migration activity of PC3 only. A low concentration of saffron extract (100 µg/ml) promoted the migration of the normal prostate cells (PNT1A), whereas it suppressed the migration of 22Rv1. In addition, 200 µg/ml of saffron extract inhibited only the migration of 22Rv1 cells.

5.1.7.1. The effects of saffron extract and its active constituents on normal prostate cell migration using a transwell assay

As shown in Figures 65-66, after 36 hours of incubating the normal prostate cells (PNT1A) with 100 µg/ml of saffron extract, a significant induction in cell migration took place, whereas the other investigated compounds did not exert any effects on the cell migration. When 100 µg/ml of saffron extract was used, the migrated cells increased by ≈ 36%. There was no change in the migratory cells achieved when a higher concentration of saffron extract (200 µg/ml) was used, so the activity obtained from saffron extract did not follow a concentration dependent manner. In addition, 12 µg/ml of safranal promoted the migration by ≈ 19%, but that induction was not significantly different from the control group.
Figure 65: The effect of saffron and its active constituents on migrated PNT1A cells toward chemoattractant using 3D Boyden chambers after 36 hours of exposure. Bars with * symbol are statistically different from control group. The cells number were counted using ImageJ software (ANOVA; n = 3 replicates/treatment group, p<0.05).
<table>
<thead>
<tr>
<th>Group</th>
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Figure 66: Representative images of PNT1A cells in migration assay using Boyden chambers at 36 hours after treatment exposures. The pictures were taken using 40X microscopic magnification. The cells number were counted using ImageJ software.
5.1.7.2. The effects of saffron extract and its active constituents on the 22Rv1 prostate cancer cell migration using a transwell assay

The effects of two nontoxic concentrations of saffron extract, safranal, and crocetin on the migration of 22Rv1 cells was monitored by a transwell assay, as shown in Figures 67-68. The result revealed that both concentrations of saffron extract (0.1 and 0.2 mg/ml), as well as 12 µg/ml of safranal significantly inhibited the migration of 22Rv1 cells activated by an FBS stimuli (p < 0.05) after 36 hours of incubation. All these treatments had similar efficacy in which ≈ 30% of reduction in the migration of 22Rv1 cells was achieved. On the other hand, the crocetin did not show any effect on the migration behavior of 22Rv1 cells when compared to the control group.

![Graph showing the effects of saffron, safranal, and crocetin on 22Rv1 cell migration](image)

*Figure 67: The effect of saffron and its active constituents on migrated 22Rv1 cells toward chemoattractant using 3D Boyden chambers after 36 hours of exposure. Bars with * symbol are statistically different from control group. The cells number were counted using ImageJ software (ANOVA; n = 3 replicates/treatment group, p<0.05).
<table>
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<th>Group</th>
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</tbody>
</table>

Figure 68: Representative images of 22Rv1 cells in migration assay using Boyden chambers at 36 hours after treatment exposures. The pictures were taken using 40X microscopic magnification. The cells number were counted using ImageJ software.
5.1.7.3. The effects of saffron extract and its active constituents on the PC3 prostate cancer cell migration using a transwell assay

The effect of saffron extract, crocetin, and safranal on the migratory potential of prostate cancer cells (PC3) was determined by using a transwell cell migration assay as demonstrated in Figures 69-70. After incubating 12 µg/ml of safranal with the PC3 cells, the migrated cells were significantly decreased compared to the control group by 31%. In contrast, 30 µg/ml of crocetin exerted a stimulatory effect on the migration behavior of the PC3. That concentration of crocetin resulted in a 23% induction of the migratory cells. There was no inhibition in cell migration observed when the saffron extract was used at either concentration for this assay.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
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</table>

![Graph showing the effect of saffron and its active constituents on migrated PC3 cells](image)

**Figure 69:** The effect of saffron and its active constituents on migrated PC3 cells toward chemoattractant using 3D Boyden chambers after 36 hours of exposure. Bars with * symbol are statistically different from control group. The cells number were counted using ImageJ software (ANOVA; n = 3 replicates/treatment group, p<0.05).
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<tr>
<th>Group</th>
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</table>

Figure 70: Representative images of PC3 cells in migration assay using Boyden chambers at 36 hours after treatment exposures. The pictures were taken using 40X microscopic magnification. The cells number were counted using ImageJ software.
5.1.8. The anti-invasiveness effects of saffron and its active constituents on prostate cell lines

An invasion assay using Boyden (Transwell) inserts which were coated with Matrigel was run to assess whether saffron and its active constituents could prevent prostate cancer cell invasion. The secretion of MMP enzymes are required from the cells to degrade the matrigel to allow movement through the membrane toward the lower chamber. Three cell lines were used in this experiment: normal prostate cells (PNT1A) and prostate cancer cells (22Rv1 and PC3). The assay was conducted using two sub-toxic concentrations of saffron extract, crocetin, and safranal. In summary, the high concentration of safranal inhibited the invasion of both cancer cell types and activated invasion in normal cells. The lower concentration of crocetin promoted the invasion of PNT1A where an opposite effect was observed in 22Rv1. The saffron extract at low concentrations enhanced the invasion of PNT1A and decreased it in 22Rv1, whereas at the higher concentration it inhibited the invasion of both types of cancer cells.

5.1.8.1. The effects of saffron and its active constituents on normal prostate cells invasion behavior

In this study, we investigated the effects of different concentrations of saffron extract, crocetin, and safranal on the invasive ability of a normal prostate cell line (PNT1A) as shown in Figures 71-72. The cells treated with 12 μg/ml of safranal significantly activated the cell invasion compared to the control group. The invasion of PNT1A cells was also increased upon exposure to 15 μg/ml of crocetin, but no change was obtained at higher concentrations. In addition, 0.1 mg/ml of saffron extract significantly stimulated the invasion behavior of PNT1A cells whereas
no significant differences were gained when a higher concentration of saffron extract was used.

The induction in cell invasion of the PNT1A cells were between 117 and 124%.

Figure 71: The effect of saffron and its active constituents after 36 hours of exposure on PNT1A cells invasion. Bars with * symbol are statistically significant from control group. The cells number were counted using ImageJ software (ANOVA; n = 3 replicates/treatment group, p<0.05).
<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (µg/ml)</th>
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</tr>
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<tbody>
<tr>
<td>Control</td>
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<td>Safranal</td>
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<td>Crocetin</td>
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Figure 72: Representative images of PNT1A cells in invasion assay using Boyden chambers at 36 hours after treatment exposures. The pictures were taken using 40X microscopic magnification. The cells number were counted using ImageJ software.
5.1.8.2. The effects of saffron and its active constituents on 22Rv1 prostate cancer cells invasion behavior

The invasion activity of prostate cancer cells (22Rv1) was measured when incubated with different concentrations of saffron extract, crocetin, and safranal as shown in Figures 73-74. 22Rv1 cells, treated with 12 µg/ml of safranal, had inhibited ability to invade. The % of cells that invaded toward the chemoattractant was only 72% compared to the control group. Upon exposure to 15 µg/ml of crocetin, a significant inhibition of invasion was obtained and the % of invaded cells decreased by 22% from control (100%), whereas no significant effects were found when 30 µg/ml of crocetin was used. A stronger inhibitory effect on cell invasion (invaded cells decreased by 33-41%) was achieved when both concentrations of saffron extract were used (0.1 and 0.2 mg/ml).

![Figure 73](image_url)

Figure 73: The effect of saffron and its active constituents after 36 hours of exposure on 22Rv1 cell invasion. Bars with * symbol are statistically significant from control group. The cells number were counted using ImageJ software (ANOVA; n = 3 replicates/treatment group, p<0.05).
<table>
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Figure 74: Representative images of 22Rv1 cells in invasion assay using Boyden chambers at 36 hours after treatment exposures. The pictures were taken using 40X microscopic magnification. The cells number were counted using ImageJ software.
5.1.8.3. The effects of saffron and its active constituents on PC3 prostate cancer cells
invasion behavior

The invasion behavior was analyzed after exposing the prostate cancer cell line (PC3) to saffron extract (0.1 and 0.2 mg/ml), crocetin (15 and 30 μg/ml), and safranal (6 and 12 μg/ml) as shown in Figures 75-76. A reduction in the cell invasion was observed when the cells were exposed to 0.2 mg/ml of saffron extract, the invaded cells decreased to 72% compared to the control group (100%). A stronger inhibitory effect on the invasion ability was observed in PC3 cells with 12 μg/ml of safranal. The invaded cells were decreased to 60%. On the other hand, neither concentration of crocetin affected the invasion activity of prostate cancer.

![Figure 75: The effect of saffron and its active constituents after 36 hours of exposure on PC3 cell invasion. Bars with * symbol are statistically significant from control group. The cells number were counted using ImageJ software (ANOVA; n = 3 replicates/treatment group, p<0.05).](image-url)
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**Figure 76:** Representative images of PC3 cells in invasion assay using Boyden chambers at 36 hours after treatment exposures. The pictures were taken using 40X microscopic magnification. The cells number were counted using ImageJ software.
5.1.9. Effects of saffron extract and its active components on matrix metalloproteases

mRNA expression using RT-PCR

At certain concentrations and cell lines, saffron and its constituents showed inhibitory effects on cancer cells migration and invasion. Therefore, we investigated which MMP genes responded to the various treatments. Two MMP enzymes were chosen to represent two different families of MMP enzymes. Matrix Metalloprotease-1 (MMP1) and Matrix Metalloprotease-9 (MMP9) were picked, and they mainly degrade collagens and gelatins, respectively. Two concentrations of saffron extract, crocetin, and safranal were used to measure MMP1 and MMP9 gene expression. The gene expression was measured by RT-PCR after 72 hours of treatment.

5.1.9.1. MMP1 gene expression

MMP1 mRNA gene expression was dependent on cell types (Figures 77-79). Safranal, at 25 and 50 µg/ml, did not cause any significant changes in MMP1 gene expression in any tumor cell lines (22Rv1 and PC3), whereas in PNT1A cells MMP1 expression was significantly induced by 6.6- and 14-fold, respectively. Crocetin (100 µg/ml) induced the expression of MMP1 in PNT1A and 22Rv1 by 7- and 2.7-fold, respectively, whereas there was no change in PC3. At the higher concentration of crocetin (200 µg/ml), a 1.9- and 5.1-fold increase was achieved in PC3 and 22Rv1. At 0.75 mg/ml of saffron, there was a significant increase by of 1.72- and 14.3-fold in PC3 and PNT1A cells, respectively.
Figure 77: The effect of saffron and its active constituents on MMP1 mRNA expression level in PNT1A cell after 72 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).

Figure 78: The effect of saffron and its active constituents on MMP1 mRNA expression level in 22Rv1 cell after 72 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).
Figure 79: The effect of saffron and its active constituents on MMP1 mRNA expression level in PC3 cell after 72 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).

5.1.9.2. MMP9 gene expression

The MMP9 expression was measured after treating the cells with saffron and its active constituents (Figures 80-82). Our study showed there were no changes in MMP9 mRNA expression in any cell lines when treated with 25 µg/ml of safranal, but when the cells were exposed to 50 µg/ml, a 5-fold induction in normal cells, 40%-inhibition in 22Rv1 cells and no change in PC3 cells was observed. Incubating the cells with crocetin at 100 µg/ml caused an increase in MMP9 gene expression in PNT1A and PC3 cells by 8- and 1.9-fold, respectively, an opposite effect was observed in 22Rv1 when MMP9 gene expression was inhibited by 45% compared to the control group. At 200 µg/ml of crocetin, different responses were obtained from the cancer cell lines. In PC3 cells the MMP9 expression was induced by 1.73-fold whereas the
expression in 22Rv1 was inhibited by 42%. Low concentrations of saffron crude extract (0.75 mg/ml) significantly increased the expression of MMP9 by 26.7-fold in PNT1A cells but caused a 37% inhibition in 22Rv1 cells. In addition, high concentrations of extract (1.5 mg/ml) significantly reduced the MMP9 mRNA expression in both 22Rv1 by 60% and PC3 by 74%.

Figure 80: The effect of saffron and its active constituents on MMP9 mRNA expression level in PNT1A cell after 72 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).
Figure 81: The effect of saffron and its active constituents on MMP9 mRNA expression level in 22Rv1 cell after 72 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, excluded 1500 µg/ml saffron group from statistics, p<0.05).

Figure 82: The effect of saffron and its active constituents on MMP9 mRNA expression level in PC3 cell after 72 hours of exposure. Bars with * symbol are statistically significant from control group (ANOVA; n = 3 replicates/treatment group, p<0.05).
5.2. Discussion

Multiple natural products have been suggested to provide protection and can also be used in the treatment of several different tumor types, including prostate cancer (Anand et al., 2008). Specifically, saffron and its components have proven results as being capable of acting against cervical, lung, breast, and neuroblastoma tumors (Samarghandian et al., 2011, Samarghandian et al., 2014, Escribano et al., 1996, Chryssanthi et al., 2007). Here, we compared the potential anticancer activity of saffron extract and its constituents (crocin, crocetin, picrocrocin, and safranal) in both prostate cancer and normal cell lines. Three prostate cell lines were selected which represent prostate cells in three different scenarios. PNT1A represents normal prostate cells, 22Rv1 cells represents early-stage prostate cancer, and PC3 represents aggressive and advanced prostate cancer.

In this dissertation, our results indicated that saffron and its active constituents possess two important anticancer properties which are effective in cases of prostate cancer by preventing tumor promotion and treating of existing prostate cancer cells. Specifically, lowering free radicals can minimize the contributing factors involved in cancer initiation, promotion, and aggression and which in turn could prevent prostate cancer development (Hecht et al., 2016, Rebillard et al., 2013, Fuchs-Tarlovsky, 2013). Finding a phytochemical agent that possesses antioxidant activity with minimal toxic effects could make an excellent candidate as a cancer chemopreventive agent (Steward and Brown, 2013, Bahmani et al., 2016). Our results found that saffron and its active components have cancer-preventative properties through their scavenger abilities in addition to the activation of cellular antioxidant defense mechanisms which inhibit free radical formation. Pre-incubation of the normal prostate cells with saffron and its active constituents before inducing oxidative stress with H$_2$O$_2$ resulted in a decrease in reactive oxygen
species (ROS) formation. By preventing cell damage, cancer progression is inhibited. In general, the crocetin possesses greater activity as a chemical scavenger property when compared to safranal, but in our cell culture system, safranal displayed stronger antioxidant properties compared to both crocetin and the saffron extract. As a consequence, it is proposed that the ability of safranal to activate the cellular antioxidant mechanisms is greater than that for saffron extract and crocetin. Therefore, safranal removes free radicals more efficiently, even at a low dose (6 µg/ml), where saffron extract and crocetin require at least 200 µg/ml and 30 µg/ml, respectively (Table 21). The possible antioxidant mechanisms of saffron and its constituents involved the activation of catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), and superoxide dismutase (SOD) enzymes (Samarghandian et al., 2013a, Niska et al., 2015, Altinoz et al., 2014). Therefore, using saffron and its active constituents at sub-toxic concentrations is promising as chemopreventive agents.

Our observation has clearly supported other findings regarding the increase of the anticancer activity through the long-term incubation of cancer cells with saffron and its active components (Parizadeh et al., 2011, He et al., 2014, Chen et al., 2015, Feizzadeh et al., 2008). These suggested saffron extract taken in a low dose as a natural supplement and long-term is the optimum way to maximize saffron’s anticancer properties. The ethanolic saffron extract exhibited anticancer activity on prostate cancer cells, especially in androgen-dependent prostate cancer (22Rv1). Unfortunately, this activity lacked selectivity and caused severe cytotoxic effects in normal prostate cells compared to other saffron constituents. Additionally, the measured cytotoxicity from saffron extract in prostate cancer (IC$_{50}$ for 22Rv1 and PC3 was 1.32 and 1.77 mg/ml, respectively) was less than the reported effects achieved in various other cancer types such as breast cancer (MCF-7), lung carcinoma (A549), cervical cancer (HeLa), and
hepatocellular carcinoma (HepG-2) where IC$_{50}$s equaled 400, 650, 800, 950 µg/ml, respectively (Tavakkol-Afshari et al., 2008, Samarghandian et al., 2011, Mousavi et al., 2009). Therefore, saffron extract is not strongly effective in the treatment of prostate cancer compared to its constituents (crocetin and safranal).

Weak cytotoxic potential was also achieved using picrocrocin and crocin against prostate cancer, even with longer incubation periods, of up to 72 hours. Due to limited available studies concerning picrocrocin anticancer activity using in vitro models, we investigated its inhibitory effects against prostate cancer (22Rv1 and PC3) and normal cells (PNT1A). Picrocrocin concentrations of up to 2 mg/ml for 72 hours resulted in approximately a 30% reduction in prostate cancer growth. This reduction is considered to be a minor effect compared to results achieved with other types of cancer, such as cervical cancer (HeLa cell line) in which 991 µg/ml resulted in a 50% reduction in cell growth (Escribano et al., 1996). The cervical cancer study suggested the picrocrocin can be used to treat a variety of cancer types, but we found it was relatively inactive for treatment of prostate cancer.

In the case of crocin, numerous studies have been conducted to determine the effectiveness of crocin in the treatment of various cancer types. Because crocin had demonstrated strong properties as an anticancer agent, we examined its potential as a treatment for prostate cancer. Cytotoxicity IC$_{50}$s were detected in prostate cancer cell lines, but the activities were lower than that reported in other cancer types, including pancreatic adenocarcinoma (BxPC-3) and colorectal adenocarcinoma (Caco-2 and HT-29), where the IC$_{50}$s were of 10 µg/ml, 150 µg/ml, and 390 µg/ml, respectively (Garcia-Olmo et al., 1999, Bakshi et al., 2010, Eid et al., 2012). Similar cytotoxicity was observed in gastric adenocarcinoma (AGS) and hepatocellular carcinoma (HepG2) with a IC$_{50}$s of 2.7 mg/ml and 2.8 mg/ml, respectively (Kim et al., 2014,
Hoshyar et al., 2013). Therefore, crocin’s anticancer properties vary depending on the cancer type, with less efficacy in prostate cancer.

Indeed, crocetin and safranal produced the strongest anticancer results among the tested saffron constituents. Crocetin arrested the growth of prostate cancer cells regardless of the androgen-dependency status of the prostate cancer cells. The IC\textsubscript{50} after 48 hours was 86-90 µg/ml, which was weaker than that required to have a positive effect on gastric adenocarcinoma (AGS) (IC\textsubscript{50} of 32.8 µg/ml), but was stronger than its activity against lung adenocarcinoma (A549) and hepatocellular carcinoma (HepG2) with IC\textsubscript{50}s of 134 and 200 µg/ml, respectively (Bathaie et al., 2013, Kim et al., 2014). The most potent effect of crocetin was achieved after 72 hours of incubation with an IC\textsubscript{50} corresponding to 59-63 µg/ml. This anticancer property of crocetin comes at a price, which is the lack of selectivity toward prostate cancer cells. In fact, crocetin was most cytotoxic when it was incubated with normal prostate cells (PNT1A). The IC\textsubscript{50} was equal to 70 µg/ml and 47 µg/ml after 48 and 72 hours, respectively which is less than the required concentration to inhibit the growth of prostate cancer cells. Most of the available chemotherapeutic agents used today show lack of selectivity toward cancer cells. Despite the fact that saffron and crocetin showed higher toxicity in normal cells than in cancer cells, further safety studies need to be conducted to evaluate the efficacy and safety of using these natural compounds as a chemotherapeutic agent for cancer and as an alternative to traditional therapies for curing different diseases.

Safranal was more potent in androgen-dependent prostate cancer (22Rv1) with an IC\textsubscript{50} of 10.9 µg/ml than with the androgen-independent prostate cancer cell line (PC3) with an IC\textsubscript{50} of 19.4 µg/ml. The statistical differences in the response of the two prostate cancer cell types may have indicated that the safranal activity is dependent to some degree on androgen receptor
cascades. In addition, the activity of safranal on prostate cancer was stronger than on cervical cancer (HeLa) cells with an IC$_{50}$ of 120 µg/ml and breast cancer (MCF-7 and MDA-MB-231) with an IC$_{50}$ of 75 µg/ml (Escribano et al., 1996, Chryssanthi et al., 2007). Hence, safranal could potentially be used specifically to treat prostate cancer. Moreover, our research demonstrated the selectivity of safranal against prostate cancer with minimal toxicity toward normal prostate cells (PNT1A) with an IC$_{50}$ of 13.9 µg/ml. The IC$_{50}$ of cytotoxicity of safranal in normal cells was higher than 22Rv1 but less than PC3. This result suggests the potential to use safranal with a higher safety margin compared to saffron, crocin, picrocrocin and crocetin as a chemotherapeutic agent for treating the early stages of prostate cancer which is usually an androgen-dependent cancer type.

Cancer development is usually associated with abnormal apoptosis signaling pathways which is one of the primary factors that is responsible for treatment failure owing to mutations that suppress apoptosis and decrease treatment sensitivity. Drugs are needed that activate the apoptosis mechanism which can counteract the mutated oncogenes that disrupt the apoptosis cascades in cancer cells (Lowe and Lin, 2000). Therefore, it is an essential step to determine whether saffron and its active constituents exert their cytotoxicity through apoptosis or necrosis.

Our study found that the cytotoxicity exerted by saffron and its active constituents was produced through apoptosis mechanisms. Faster responses in cancer cells were obtained when the effector caspases enzymes 3/7 were induced after 6 hours of incubation during the treatments. This activity was generally stronger in cancer cells compared to normal cells, although the saffron extract triggered the caspase 3/7 enzymes earlier than cancer cells. This result supported other findings about the activation of caspase 3/7 secretions upon the incubation of saffron extract with lung carcinoma cells (Samarghandian et al., 2013b). In prostate cancer
cells, the safranal and crocetin stimulated the caspase 3/7 production in a concentration-dependent manner. Safranal cytotoxicity via apoptosis was also reported in neuroblastomas (Samarghandian et al., 2014). In addition, similar results were observed on cervical cancer (HeLa), lung cancer (A549), and ovarian cancer (SKOV3) cell lines (Zhong et al., 2011). It is clear the cytotoxicity of saffron extract, crocetin, and safranal is primarily dependent on the activation of apoptosis pathways. Moreover, the androgen-sensitive prostate cancer cell line (22Rv1) responded to safranal more than the androgen-independent prostate cancer cell line (PC3), while on the contrary, the PC3 was more sensitive to crocetin than the 22Rv1.

Our study focused on which apoptotic pathway was involved in the actions of saffron and its active constituents (intrinsic, extrinsic, or both). The extrinsic pathway heavily relies on caspase 8 and/or 10 pathways dependent on the activated death receptors such as the Fas receptor on the cell surface, whereas the intrinsic pathway is associated with the activation of caspase 9 (Figure 83). We used real-time PCR to measure caspase 3, 7, and 9 in normal prostate cell lines to determine the major apoptotic pathway. Safranal and crocetin activity was dependent on the activation of the caspase 9 pathway, which means the active saffron components work through the intrinsic apoptosis pathway (Figure 84). The induction of caspase 9 expression may relate to the ability of saffron and its active constituents to upregulate Bax and p53 and suppress Bcl-2 and NF-κB expression (Bathaie et al., 2013, Amin et al., 2011, Mousavi et al., 2009). In contrast, the levels of caspase 3, 7, and 9 were unchanged when incubated with saffron at both concentrations (0.75 mg/ml and 1.5 mg/ml) and for crocetin at a high concentration (200µg/ml). The unchanged gene expression could be explained in two ways.

First, when the cells were incubated with saffron extract (0.75 mg/ml and 1.5 mg/ml) or with the high concentration of crocetin (200µg/ml), the caspase 3/7 enzymes were even higher
after 6 hours than those protein levels induced by safranal and a low crocetin concentration. Because we measured the caspase gene expression after 72 hours, it was possible that the caspase mRNA was higher at earlier points, but became degraded before we conducted the RT-PCR. This explanation is in agreement with the findings of a previous study which investigated and compared the stability of mRNA expression and its related proteins using a quantitative model. A research group studied over 5000 genes of mammalian cells with their corresponding expressed protein and calculated their half-lives. The half-life of mRNA was 9 hours, whereas it was 46 hours for proteins, which makes proteins, on average five times more stable than mRNA. According to that study, there was no correlation between the half-lives of mRNA and protein (Schwanhausser et al., 2011). Accordingly, earlier multi-time points of measuring mRNA will provide a more accurate result that reflects the corresponding mRNA expression level of caspase 3, 7, and 9 genes.

The second reason for the variation found in our study between the caspase’s mRNA and its proteins was the possibility of no direct relationship between the levels of mRNA and protein expression, which was proven in earlier studies. A proteomic study was conducted to investigate the correlation between 425 proteins and their corresponding mRNA. This study found that only 19% had a good correlation between mRNA and protein expression and even 1% had an inverse correlation. Generally, there was no good correlation between all examined genes mRNA and proteins with R= 0.59 (Tian et al., 2004). Similar findings of another study were obtained with R= 0.46 and which additionally highlighted the importance of post-transcriptional regulation and processes which have an effect on the relationship between mRNA and proteins levels (Vogel et al., 2010). Therefore, the obtained mRNA of caspase is not necessary reflected in the caspase
enzymes levels when the cells are treated with saffron extract (0.75 mg/ml and 1.5 mg/ml) or crocetin (200µg/ml).

Figure 83: Cell death mechanisms
The migration of any cell depends on two factors, cell-cell interaction and chemotaxis (Liang et al., 2007). Our research demonstrated that saffron and its active constituents have the ability to inhibit prostate cancer cell migration. The present study measured the cell migration that was dependent on the cell-cell interaction behavior of cells, using a wound healing assay. Safranal at 12 µg/ml inhibited the cell migration of both types of prostate cancer while at the same time activating the migration of normal cells. In a different study, when crocetin at 32 µg/ml was incubated with esophageal squamous carcinoma (KYSE-150), crocetin decreased cell migration by 20% (Li et al., 2015). Conversely, our investigation found that 30 µg/ml of crocetin activated the migration of both prostate cancer cell types while having no effect on normal cells.
Saffron extract at 100 µg/ml activated normal cell migration whereas a higher concentration (200 µg/ml) inhibited the migration of both prostate cancer types.

Furthermore, migration which is dependent on chemotaxis was assessed using a Boyden chamber. The saffron extract (100 µg/ml) activated the migration of normal cell lines and inhibited 22Rv1 cells, while a higher concentration (200 µg/ml) resulted only in an inhibition of 22Rv1 migration. Using 12 µg/ml of safranal inhibited migration of both cancer cell types, whereas 30 µg/ml of crocetin activated migration of PC3 only. Combining data obtained from both these migration experiments that were based on different aspects of migration (cell-cell contact and chemo-attractive stimulation) identified that 12 µg/ml of safranal was the most suitable compound to inhibit cancer cell migration, but it increased the migration of normal cells. The higher concentration of crocetin (30 µg/ml) produced results that demonstrated its ability to induce cancer cell migration. Low concentrations of saffron induced the migration of normal cells, while higher concentrations inhibited cancer cell migration. As mentioned in previous studies, mature organisms depend heavily on physiological cell migration to play an important role in tissue homeostasis, immune response, and wound repair (Trepat et al., 2012). Because the saffron extract and safranal exclusively stimulated the migration behavior of normal cells, that makes them good candidates to use in conditions when a stimulation of the immune system as well as an acceleration of the wound healing process are required, such as in cases of burns to the skin (Khorasani et al., 2008).

Generally, the invasive behavior of cells is dependent on two major factors, cell migration (cell motility) and extracellular matrix (ECM) digestion through secreting proteolytic enzymes (MMPs). Therefore, if cells cannot migrate, it means that the cells will not invade, whereas all invaded cells should be migrated cells. Therefore, we studied the ability of saffron
extract, crocetin, and safranal to inhibit the invasion behavior of prostate cancer cells using Boyden chambers. Safranal at a higher concentration (12 µg/ml) decreased the invasiveness of both prostate cancer cell lines and promoted the activity of normal prostate cells. Crocetin (15 µg/ml) inhibited the invasive behavior of androgen-sensitive prostate cancer cells (22Rv1), whereas this behavior was triggered in the normal cell line. At different concentrations of saffron, the invasion of 22Rv1 was inhibited, while at higher concentrations the PC3 invasion was decreased, whereas at low concentrations invasiveness was stimulated. This data supported conclusions from a different study which confirmed the ability of saffron extract and crocetin to prevent the invasion of various cancer cells through down-regulation of MMP2 and 9 (Chryssanthi et al., 2011, Mousavi et al., 2014). In our study, RT-PCR analysis of MMP1 and MMP9 demonstrated that MMP9 expression was related more closely to the anti-invasion activity of saffron and its active constituents, whereas the MMP1 was closely associated with migration behaviors (Tables 21-22). The correlation between MMP9 and invasion activity can be explained by the fact that the barrier used in the Boyden chamber consisted of matrigel, which is digested effectively with gelatinase enzymes such as MMP9 and less effectively with collagenase enzymes such as MMP1. It was proven in a previous study, when the MMP1 was knocked down, lung, breast, and prostate cancer cells lost their migration ability (Ho et al., 2009, Liu et al., 2012, Li and Tai, 2012, Casimiro et al., 2013).

The MMP’s activity stimulates vascular endothelial growth factor (VEGF) expression which plays important roles in the angiogenesis process of a tumor. Because saffron and its active constituents decreased MMP1 and MMP9, it is possible that these natural products are capable of decreasing VEGF expression as they may be angiogenesis inhibitors (Kim et al., 2010). Our results concluded that saffron and its active components inhibit migration as well as
the invasion of prostate cancer, and by doing this they could prevent cancer from metastasizing to distant sites. These inhibitory effects are not observed in normal prostate cells, however, but in certain situations saffron and its active constituents enhanced the migration activity, which may be considered to be an advantage when migration is required to treat other diseases and conditions.

Table 21: Summary of antioxidant, migration, and invasion activity of prostate cells after treatments

<table>
<thead>
<tr>
<th>Measured effects</th>
<th>Cell line</th>
<th>Saffron extract (µg/ml)</th>
<th>Crocetin (µg/ml)</th>
<th>Safranal (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>200</td>
<td>15</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>PNT1A</td>
<td>-</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Migration activity</td>
<td>PNT1A</td>
<td>↑</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>22Rv1</td>
<td>-</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PC3</td>
<td>-</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Wound healing</td>
<td>PNT1A</td>
<td>↑</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>22Rv1</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PC3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Boyden chamber</td>
<td>PNT1A</td>
<td>↑</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>22Rv1</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PC3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Invasion activity</td>
<td>PNT1A</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>22Rv1</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>PC3</td>
<td>-</td>
<td>↓</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 22: Summary of MMP1 and MMP9 gene expression of prostate cells after treatments

<table>
<thead>
<tr>
<th>Measured effects</th>
<th>Cell line</th>
<th>Saffron extract (µg/ml)</th>
<th>Crocetin (µg/ml)</th>
<th>Safranal (µg/ml)</th>
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<tr>
<td></td>
<td></td>
<td>750</td>
<td>1500</td>
<td>90</td>
</tr>
<tr>
<td>MMP1</td>
<td>PNT1A</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>22Rv1</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>PC3</td>
<td>↑</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MMP9</td>
<td>PNT1A</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>22Rv1</td>
<td>↓</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>PC3</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
</tbody>
</table>
5.3. Conclusion

Saffron and its active constituents were used to assess their capability as chemo-preventive and chemotherapeutic agents for the treatment of prostate cancer. Saffron extract, picrocrocin, crocin, crocetin, and safranal were used to treat two different human prostate cancer cell lines that differed in their androgen-dependency status (22Rv1 and PC3) and normal prostate cell line (PNT1A). Picrocrocin and crocin had weaker cytotoxicity compared to saffron extract, crocetin, and safranal. Viability of the prostate cancer cells was dependent on their androgen sensitivity status. The cytotoxic effects were exerted through an intrinsic apoptosis pathway. In addition, saffron extract, crocetin, and safranal demonstrated the ability to prevent metastasis through inhibiting prostate cancer cell migration, as well as their invasion, by decreasing matrix metalloproteases enzymes. In regards to the chemo-preventive ability of saffron extract, crocetin, and safranal, all of them decreased oxidative stress which plays a major role in the initiation and progression of cancer. Among these treatments, safranal had the lowest toxic effect on normal cell lines. Ultimately, the conclusion to this study is that the saffron extract, crocetin, and safranal have all clearly demonstrated properties that allow us to conclude they have potential as chemo-preventive and chemotherapeutic agents for prostate cancer.
6.1. Results

6.1.1. Creation of transfected prostate cancer cell lines with DsRed Express 2 protein

First, the transfection process included determining the proper selective and maintenance concentrations of G418 antibiotic to exert a selective pressure to obtain stable transfected cell lines, followed by lowering the G418 antibiotic to the maintenance concentration so as to keep the transfection status unchanged. Second, evaluating the transfection procedure effects was carried out by measuring the cells’ doubling time to ensure the transfection did not cause any major changes in cell replication. Finally, the cytotoxicity of a known drug in both transfected and non-transfected cell lines was performed to ensure there was no significant impact of the treatment and the transfection process.

6.1.1.1. Determination of selective and maintenance concentration of G418 for cell transfection

The plasmid used for DsRed Express 2 protein (Figure 11, Chapter 3) contains the G418 resistance gene which is activated in mammalian cells once the transfection process has been successful whereas human cells (non-transfected) are sensitive to G418. This study evaluated the ability of prostate cancer cells to grow and form colonies by performing a clonogenic assay in the presence of geneticin (G418) antibiotic. After determining the required G418 concentration that caused the cell growth inhibition, this concentration was used to select the successfully
transfected cells and exclude the non-transfected cells. As shown in Figure 85, treatment of 22Rv1 and PC3 prostate cancer cell lines with G418 at a concentration of 500 and 1000 µg/ml for 9 days caused a significant decrease in cell growth and the ability of a single cancer cell to form a colony. Figure 86 illustrates that a concentration of 500 µg/ml of G418 achieved stronger inhibitory effects in colony formation of 22Rv1 cells with a reduction of 94.29 ± 8.08% in formed colonies, whereas 500 µg/ml in PC3 cells caused an 89.19 ± 10.11% reduction in their ability to form colonies. The higher 1000 µg/ml concentration of G418 significantly suppressed the growth and colony formation ability in 22Rv1 and PC3 cells where a reduction in formed colonies was achieved by 100% and 97.3 ± 3.82%, respectively.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control</th>
<th>G418 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>500</td>
</tr>
<tr>
<td>22Rv1</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>PC3</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
</tr>
</tbody>
</table>

*Figure 85: Representative pictures demonstrating the ability of non-transfected prostate cancer cell lines to form colonies after 9 days of G418 antibiotic exposure.*
Figure 86: The effects of G418 on the colony formation in prostate cancer cell lines after 9 days of incubation. Bars with a * symbol are statistically significant from the untreated control group (ANOVA, n = 3 replicates/group, p<0.05).

6.1.1.2. Transfection of prostate cancer cell lines with DsRed Express 2 plasmid

Our goal in using DsRed Express 2 plasmid was to provide the cells with a tracking protein that would facilitate visualizing the cells with a fluorescent microscope after they were transplanted into a zebrafish embryo. After one month of using the selection concentration (1000 µg/ml) of the G418 antibiotic, stable transfected cells were obtained for both cancer cell lines. Selected cells were maintained with the maintenance concentration (250 µg/ml) of the G418 antibiotic. As depicted in Figure 87, both transfected cell lines (22Rv1 and PC3) expressed the DsRed Express 2 protein, which was visualized under the fluorescent microscope, using the proper filter combinations. The merging of a bright field with red filter pictures for each cell line indicated that all cells were capable of expressing the transfected protein.
Figure 87: The expression of DsRed Express 2 protein in 22Rv1 (A for bright field and B for red fluorescent filter) and PC3 (C for bright field and D for red fluorescent filter) of prostate cancer cell lines after 48 hours of incubation. The pictures were taken with 20X microscopic objective magnification.
6.1.1.3. The effects of DsRed Express 2 plasmid transfection on the proliferation rate of prostate cancer cell lines

This study investigated the possibility of the influence of the transfection process on the growth rate of prostate cancer cell lines by measuring the doubling time. After 48 hours of incubation, the doubling time was calculated and, as demonstrated in Figure 88, it was determined that no significant changes were found in either prostate cancer cell line. Doubling time was 42.48 ± 3.44 and 35.02 ± 2.6 hours for non-transfected 22Rv1 and PC3 cell lines, respectively, whereas it was 44.27 ± 3.24 and 36.49 ± 2.98 hours for transfected 22Rv1 and PC3 cells. The results showed that the transfection of DsRed Express 2 plasmid did not cause any significant effects on the growth rate of prostate cancer cell lines.

Figure 88: The effects of DsRed Express 2 plasmid transfection on the proliferation rate of prostate cancer cell lines after 48 hours of incubation. Non and transfected cells within cell type were not significant different (t-test, n = 6 replicates/group, p<0.05).
6.1.1.4. The \textit{in vitro} cytotoxicity of docetaxel in both non-transfected prostate cancer cell lines

As previously mentioned, the aim of the transfection of the cells with the DsRed Express 2 protein plasmid was tracking of the prostate cells after they were transplanted into zebrafish. Docetaxel is a known effective chemotherapy for prostate cancer. Hence, it was used to validate the xenograft model. We measured the cytotoxicity response of docetaxel in transfected cells to compare it with the cytotoxicity obtained in non-transfected cell lines so as to ensure there were no changes in the drug response caused by the plasmid transfection process.
6.1.1.4.1. The *in vitro* cytotoxicity of docetaxel in both non-transfected prostate cancer cell lines

Because the incubation window available for evaluating the efficacy of any treatment using the *in vivo* zebrafish xenograft model is 48 hours, the cytotoxicity was determined for both non-transfected 22Rv1 and PC3 cell lines after 48 hours of treatment incubation. As shown in Figure 89, the docetaxel concentrations, ranging from 2.5-20 ng/ml, were used. After 48 hours of treatment exposure, it was found that the IC₅₀ were 3.94 ± 0.87 and 11.47 ± 2.7 ng/ml for 22Rv1 and PC3, respectively.

![Figure 89: The cytotoxicity of non-transfected prostate cell lines after 48 hours using docetaxel. (ANOVA; N = 3)](image-url)
6.1.1.4.2. The *in vitro* cytotoxicity of docetaxel in both transfected prostate cancer cell lines

After 48 hours of incubating the transfected cell lines with docetaxel, as demonstrated in Figure 90, the IC$_{50}$ for 22Rv1 was 4.11 ± 1.03 and 11.83 ± 3.43 ng/ml for PC3. There were no significant changes found in the toxicity of the cells between transfected and non-transfected prostate cancer cell lines caused by docetaxel.

![Graph showing cell survival after 48 hours of incubation with docetaxel](image)

Figure 90: The cytotoxicity of non-transfected prostate cell lines after 48 hours using docetaxel. (ANOVA; N = 3)
6.1.2. Determination of a common temperature point

An experimental temperature point compatible with both human prostate cell growth and zebrafish larvae survival was optimized. Both maximal thermal tolerance of zebrafish and effects of that temperature on human prostate cell doubling time were assessed.

6.1.2.1. The effects of various temperatures on zebrafish larvae survival

Normally, zebrafish are kept at 28°C. Hence, our study focused on finding the maximum temperature that zebrafish larvae could tolerate without affecting their survival. Whether the zebrafish larvae could grow within or close to the human normal temperature range (37°C) was investigated. Therefore, 28, 32, 35, and 37°C were chosen for assessment. After 6 days of incubation at each specified temperature, the total surviving zebrafish larvae were determined and compared to the control group (the zebrafish larvae incubated at 28°C). As shown in Figure 91, there was a significant decrease in zebrafish larvae survival for those that were incubated at 37°C, whereas no significant change was found between 32°C or 35°C and the control group (28°C). The 6th day survival of zebrafish larvae compared to the control group was 101.95 ± 4.23%, 94.85 ± 5.36% and 65.94 ± 5.98% for 32, 35 and 37°C, respectively.
Figure 91: The effects of multiple temperatures on zebrafish larvae survival after 6 days of incubation. Bars with a * symbol are statistically significant from the control group. (ANOVA, n = 3 replicates/group, each replicate consisted of 96-128 zebrafish, p<0.05).

6.1.2.2. The effects of various temperatures on the proliferation rate of prostate cancer cell lines

Human cell lines are usually incubated at 37°C for optimal cell growth. Because the maximum tolerated temperature to zebrafish larvae was determined to be 35°C, the effects of incubating the human prostate cancer cell lines at 35°C on their growth rate were studied to determine whether the temperature changes have any significant effect. As described in Chapter 3, the proliferation rate was determined by measuring the cell doubling time after 48 hours of incubation. Although the doubling time slightly increased in both cell lines, no significant changes were found (Figure 92). The doubling time at 37°C was 42.48 ± 3.44 hours for 22Rv1 and 35.02 ± 2.6 hours, whereas the doubling time at 35°C was 46.67 ± 4.61 hours for 22Rv1 and 38.77 ± 3.7 hours. These results revealed the possibility of incubating both prostate cancer cell lines at 35°C without any significant changes in their growth behavior.
Figure 92: The effects of multiple temperatures on the proliferation rate of prostate cancer cell lines after 48 hours of incubation. In neither cell line did temperature have a significant effect on cell doubling time. (t-test, n = 6 replicates/group, p<0.05).

6.1.3. Xenograft of prostate cancer cells in Tg(fli1a:EGFP) and crossbred zebrafish comparison

We studied the transplantation of transfected prostate cancer cells in different transgenic zebrafish lines. As shown in Figure 93, Tg(fli1a:EGFP) interfered with the transfected prostate cancer cells’ fluorescent emission due to the presence of larvae pigmentation. Using the transgenic line (Tg(fli1a:EGFP) x Casper) which we created in our laboratory, there was no interference found between the fluorescent emission of transplanted prostate cancer cells and the zebrafish larvae body. Actually, the embedded zebrafish endothelial cells in the tumor mass were easily observed 24 hours into the transplantation process (Figure 94). This benefit was only marginally evident using Tg(fli1a:EGFP). Therefore, our developed model using crossbred zebrafish larvae provide a great tool not only to study transplanted cancer proliferation and
migration, but also for use in studying the angiogenesis process involved in cancer generally, and prostate cancer specifically. This model could also be leveraged for angiogenesis inhibitor drugs discovery.

Figure 93: The differences between Tg(fli1a:EGFP) and crossbred zebrafish after transplanting prostate cancer cells. A & B: PC3 xenografted into Tg(fli1a:EGFP) zebrafish and C & D: 22Rv1 xenografted into crossbred Tg(fli1a:EGFP) x Casper zebrafish.
Figure 94: The vascular endothelial cells of zebrafish recruited into prostate cell mass (angiogenesis). A: 24 hours after transplanting PC3 into zebrafish (2 dpf), B: 72 hours after transplanting 22Rv1 into zebrafish (4 dpf), and C: 72 hours after transplanting PC3 into zebrafish
6.1.4. Determination the time frame for the treatment study using the xenografted zebrafish model

We investigated the duration that cells could be xenografted before cell viability was artificially compromised. The transplanted prostate cancer cells in zebrafish continued to grow and migrate until fourth day post injection (dpi). At 4 dpi, the transplanted prostate cancer cell number started to decline, which indicated the possibility of interference between the zebrafish immune system and the transplanted cancer cells. Figure 95, shows that the transplanted cells stopped growing and declined at 4 dpi. Therefore, our results suggest that the maximum time frame for this model is 3 dpi (4 dpf) to avoid overestimation of the screened drug activity.
Figure 95: The growth of PC3 xenografted in zebrafish up to 5 dpf (4 dpi). A: 2 dpf, B: 3 dpf, C: 4 dpf, and D: 5 dpf

6.1.5. Drug toxicity screening using zebrafish

Docetaxel and safranal toxicity was assessed in zebrafish to determine the maximum tolerated concentrations of these agents without causing any significant changes in zebrafish survival compared to controls.
6.1.5.1. Docetaxel toxicity in zebrafish

Zebrafish at 48 hpf were exposed to multiple docetaxel concentrations (200, 400, 2000 ng/ml) daily. After 48 hours of exposure (96 hpf), the 200 and 400 ng/ml concentrations of docetaxel did not show any significant changes in zebrafish survival compared to the control group and their survival was 97.06 ± 4.41% and 93.38 ± 6.74%, respectively (Figure 96). However, the concentration of 2000 ng/ml significantly reduced zebrafish survival to 79.41 ± 10.11%. The results indicated there was no observed toxicity when 48 hpf zebrafish are exposed to a docetaxel concentration of up to 400 ng/ml.

Figure 96: The toxicity of multiple concentrations of docetaxel in zebrafish after 48 hours of exposure. Bars with a * symbol are statistically significant from the control group. (ANOVA, n = 3 replicates/group, each replicate consisted of 48 zebrafish, 1 larva per well of 48 well plate in 200 µl of solution, p<0.05).
6.1.5.2. Safranal toxicity in zebrafish

Zebrafish larvae (48 hpf) were exposed daily to 7.5, 15, or 30 µg/ml of safranal. The 7.5 and 15 µg/ml concentrations of safranal did not significantly decrease zebrafish survival (98.53 ± 5.55% and 94.12 ± 7.75%, respectively) (Figure 97). On the other hand, 30 µg/ml caused a significant reduction in zebrafish survival, which dropped to 57.35 ± 14.47%. Thus, safranal up to 15 µg/ml was safe to use in zebrafish without decreasing their survival.

Figure 97: The toxicity of multiple concentrations of safranal in zebrafish after 48 hours of exposure. Bars with a * symbol are statistically significant from the control group. (ANOVA, n = 3 replicates/group, each replicate consisted of 48 zebrafish, p<0.05).
6.1.6. The activity of docetaxel on the transplanted prostate cancer cells

Crossed transgenic zebrafish larvae (Tg(fli1a:EGFP) x Casper) were injected with 300-400 cells at 48 hpf. To validate the developed model, the xenografted prostate cancer cell models were treated with two different concentrations of docetaxel (200 ng/ml and 400 ng/ml) which is an established chemotherapy drug used in treating prostate cancer. Each treatment group consisted of 3-7 zebrafish larvae. As the concentration of docetaxel increased, the transplanted prostate cancer growth declined (Figures 98-99). At three dpi, the growth of the 22Rv1 untreated control group was 136.74 ± 18.06% compared to the 1st dpi, whereas PC3 control group reached 138.28 ± 16.05%. The docetaxel at 200 ng/ml decreased the growth of 22Rv1 and PC3 cells by 36.77 ± 4.01% and 30.32 ± 4.7%, respectively from the untreated controls at 3 dpi. The growth of 22Rv1 and PC3 decreased by 47.63 ± 12.13% and 34.58% ± 5.48%, respectively, when a higher concentration of docetaxel (400 ng/ml) was used. The projected IC$_{50}$s were 393.8 ± 153.54 and 620.8 ± 254.35 ng/ml for 22Rv1 and PC3 cells, respectively. The docetaxel growth inhibition was stronger in 22Rv1 than in PC3 cells, which was in accord with the in vitro results determined in this study where IC$_{50}$ were ≈ 4 and 12 ng/ml, respectively. Therefore, these results support that the developed xenograft model is a reliable way to investigate, in vivo, candidate drugs quickly.
Figure 98: Docetaxel activity in xenograft zebrafish model transplanted with 22Rv1 cells. Bars with a * symbol are statistically significant from the untreated control group at 3 dpi (ANOVA, n= 3)

Figure 99: Docetaxel activity in xenograft zebrafish model transplanted with PC3 cells. Bars with a * symbol are statistically significant from the untreated control group at 3 dpi (ANOVA, n= 3-7)
6.1.7. The activity of safranal on transplanted prostate cancer cells

The *in vitro* results showed that safranal was cytotoxic for both prostate cancer cell lines with lower cytotoxicity in the normal cell line. Based on our findings, we investigated the activity of safranal using our developed xenograft model. Each treatment group consisted of 3 zebrafish larvae. Two concentrations of safranal (7.5 µg/ml and 15 µg/ml) were used and 15 µg/ml of safranal inhibited the growth of transplanted 22Rv1 cells significantly (Figure 100). The growth of 22Rv1 cells was decreased from 136.74 ± 18.06% to 105.51 ± 3.77% and 86.57 ± 2.15% when the larvae were incubated with 7.5 µg/ml and 15 µg/ml of safranal, respectively. The expected IC₅₀ for safranal was 25.68 ± 8.14 µg/ml. Therefore, our results showed that safranal was effective *in vitro* as well as *in vivo*, which make it an excellent candidate for further studies.

Figure 100: Safranal activity in xenograft zebrafish model transplanted with 22Rv1 cells. Bars with a * symbol are statistically significant from the untreated control group at 3 dpi (ANOVA, n= 3)
6.2. Discussion

Zebrafish is a popular vertebrate animal model for various human diseases (Lieschke and Currie, 2007). For example, zebrafish and human hepatocellular carcinoma share the same molecular pathway characteristics which highlights the utility and relevance of zebrafish as an alternative to mammalian models (Lam and Gong, 2006). Here, we developed a zebrafish-based xenograft model for human prostate cancer. Using two different cell lines, we optimized the conditions and procedures for a reproducible xenograft zebrafish model for human prostate cancer cells (22Rv1 and PC3). The studied parameters included zebrafish and human prostate cells’ incubation temperature, zebrafish age, time frame for experiment, injection site, and injection method.

The importance of using crossbred transparent zebrafish (Casper) with the Tg(fli1a:EGFP) zebrafish can be viewed from two perspectives. First, in wild type as well as Tg(fli1a:EGFP) zebrafish, pigment develops gradually and is easily visualized at 1 dpf and is more obvious at 2 dpf. The pigmentation would decrease the quantification sensitivity of the transplanted prostate cancer cells because it blocks the emission of the cancer cell fluorescent protein marker. Thus, cell visualization and quantification is adversely impacted. This disadvantage is not present in the Casper zebrafish because the genes responsible for pigmentation are mutated, so when the prostate cancer cells are transplanted, they can be accurately quantified. At the same time, Tg(fli1a:EGFP) zebrafish provide a great advantage over Casper zebrafish. The unique advantage is the green fluorescence of the zebrafish vasculatory system allows for tracking the interaction between the zebrafish blood vessels and the prostate cancer cells after transplantation. Therefore, visualization of the route used by the cancer cells to migrate and invade distant sites, as well vascular tract recruitment are possible.
with Tg(fli1a:EGFP) but not Casper fish. Therefore, using the crossbred zebrafish larvae provided a great opportunity to visualize the relationship between the endothelial cells of zebrafish embedded in human prostate cancer masses and enable an accurate quantification of transplanted cancer cells.

The cancer cells were quantified by measuring the red fluorescent intensity of DsRed Express 2 protein. Because the crossed zebrafish larvae (Tg(fli1a:EGFP) x Casper) are transparent, the interaction of labeled human prostate cells and zebrafish endothelial cells was easily visualized using a fluorescent microscope. The tumorigenesis of human prostate cancer cells was fast and easily assessed using the xenograft zebrafish model. Furthermore, zebrafish endothelial cell recruitment to the human prostate cancer masses was apparent.

Our study optimized the appropriate temperature to accommodate both human cell proliferation and zebrafish larvae survival. We found that 35°C represents the best incubation temperature without affecting human cells’ proliferation or zebrafish survival.

The best time frame to conduct the experiment using the developed xenograft model was also optimized. Multiple studies have used different time frames of up to 10 dpf for their xenograft zebrafish larvae models without stating their reasons for the time frame choice (Huiting et al., 2015, Geiger et al., 2008, Teng et al., 2013). Based on that, our study screened the time frame window ranging from 1-9 dpf. We noticed that the cells started to decline in some zebrafish when their age reached 5 dpf, although in some zebrafish the transplanted cells continued to survive and grew up to 9 dpf. Decreased cell viability could be explained by the fact that T cell progenitors are fully colonized in thymus at 5 dpf and the immune system is totally mature when the zebrafish larvae reach 4-6 wpf (Trede et al., 2004). Accordingly, our study
suggested terminating the experiment at 4 dpf (3 dpi) to avoid misleading results produced by the developed zebrafish immune system that could be responsible for the transplanted cells’ decline.

We tried multiple time points to inject zebrafish ranging from 1-4 dpf and found that prostate cancer cells transplanted into the zebrafish yolk sac at 1 dpf was best so that the prostate cancer cells grew, formed masses, and migrated to distant site locations in zebrafish faster compared to other time points of transplantation. These results could be explained based on the fact that zebrafish embryos form a complete and functional vascular system by 30 hpf (Ellertsdóttir et al., 2010). Moreover, similar to humans, the formation of zebrafish blood vessels is triggered by the high expression of VEGF (Goishi and Klagsbrun, 2004). In addition, prostate cancer is known to be express high VEGF (Botelho et al., 2010). By transplanting the cancer cells before 30 hpf, the prostate cells can embed and recruit zebrafish blood vessels into a tumor mass which in turn, carries blood and necessary nutrition to prostate cancer cells. Therefore, our results suggested that the best time for xenografting human cancer cells into zebrafish is 1 dpf to optimism optimum microenvironment for cancer cell proliferation.

Both prostate cancer cell lines (control groups) proliferated, formed a tumor mass and migrated to a distant site when xenografted into zebrafish larvae. This was slightly more obvious in the aggressive type of prostate cancer cell line (PC3), especially regarding the migration behavior in comparison to the 22Rv1 cell line (data not shown). These results were similar with the normal in vitro behaviors of the different cell lines as well as in vivo studies when these cell lines were transplanted in a xenografted mice model (Drake et al., 2009). Our finding highlighted the suitability of using the xenografted zebrafish model because it does not interfere with functions and behaviors of the transplanted prostate cancer cells.
Docetaxel is a known as a chemotherapy drug used in the treatment of multiple cancer types such as prostate, breast, and gastric cancer. In 2004, the FDA approved the use of docetaxel for treating prostate cancer patients by administrating it intravenously (75 mg/m$^2$) every three weeks with 5 mg oral prednisone twice daily for 10 cycles (Fda, 2014). In a previous human study, it was found that the plasma concentrations of docetaxel were 3.54-4.06 µg/ml, when the patients were treated with 75 mg/m$^2$ for various cancer types, including prostate cancer (Ten Tije et al., 2005).

As a positive control, we first tested docetaxel to validate the developed in vivo model. The higher concentrations of docetaxel (200 and 400 ng/ml) which were added daily to expose xenografted zebrafish larvae produced growth inhibition effects, and the projected IC$_{50}$s ranged between 394-621 ng/ml. The in vivo IC$_{50}$ were higher compared to in vitro studies which ranged from 4-12 ng/ml. Because, in vitro results showed suggested efficacy of safranal in treating prostate cancer, we also screened its efficacy using the xenografted zebrafish model. It was clear that safranal showed anticancer activity in vivo with a projected IC$_{50}$ of 25.7 µg/ml, which makes it a good candidate for further investigation.

One important consideration highlighted in Table 23 from our preliminary studies, is that the calculated in vivo IC$_{50}$ both exceeded our dose range and may reach concentrations acutely toxic to the zebrafish larvae. It will be important to further establish the predicted therapeutic relevance of cancer cell growth inhibition. For example, we must determine what percent inhibition is therapeutically meaningful. Furthermore, to complement even easier in vitro testing paradigms, the zebrafish model fundamentally must provide enhanced in vivo relevance (e.g. associated absorption, distribution, and metabolism) across species. Future studies can
investigate prodrug positive controls to further validate metabolic drug activation by larval zebrafish.

The variation between the data obtained from *in vitro* and *in vivo* was expected for various reasons. First, the cells in *in vitro* studies obtained the drug directly on exposure, whereas in the *in vivo* model, the drug will undergo multiple pharmacokinetic processes, including absorbance, metabolism, distribution, plasma binding, and excretion, which will affect the availability of the drug to the transplanted cells. Second, because the *in vitro* studies are conducted with a controlled system (specific growth media compositions), it may disable or activate survival or apoptotic pathways as a result of the absence of specific hormones or molecules. Therefore, it is possible for the cells to gain these activities again when they are transplanted into an *in vivo* model due to the presence of the proper microenvironment. Finally, the docetaxel has low stability in water at room temperature (25°C), which is 4 hours (Hart and Acott, 2010). Because we incubated the xenografted zebrafish at 35°C, there is a possibility that the docetaxel was degraded during our exposures. Other drug delivery mechanisms including microinjection of larvae could be used for water insoluble or unstable drug leads.

### Table 23: Comparison between the calculated IC$_{50}$ concentrations between the different tested model systems

<table>
<thead>
<tr>
<th>Treatment</th>
<th><em>In vitro</em> study</th>
<th><em>In vivo</em> study</th>
<th>Reported serum human concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>22Rx1 PC3</td>
<td>22Rx1 PC3</td>
<td></td>
</tr>
<tr>
<td>Docetaxel</td>
<td>4 ng/ml 12 ng/ml</td>
<td>394 ng/ml 621 ng/ml</td>
<td>3.5 - 4.1 µg/ml</td>
</tr>
<tr>
<td>Safranal</td>
<td>15 µg/ml 30 µg/ml</td>
<td>25.7 µg/ml -</td>
<td>-</td>
</tr>
</tbody>
</table>

(Ten Tije *et al.*, 2005)
Zebrafish larvae provided a relatively high-throughput approach for conducting preclinical studies (Huiting et al., 2015). Because the whole procedure can be completed within one week, new drug leads can be screened quickly. Using this alternative to mammalian models will shorten the required time to move the candidate drug from an *in vitro* investigation into clinical human trails.

Another advantage of the model is the need for only small numbers of cancer cells (hundreds). This advantage presents the opportunity to take a biopsy from a cancer patient, dissociate the cells then transplant it in numerous zebrafish that can be divided in multiple treatment groups. From this type of study, a best treatment option could be identified within a single week. Therefore, this model could help personalize cancer treatments to ensure the maximum chance for effective treatment and help avoid chemotherapy failure. This advantage is not possible using mammalian models because of the need for high number of tumor cells (millions) required per animal. Experience shows that it is impossible to obtain sufficient numbers of cells from a biopsy to transplant them into mammals in multiple groups. Furthermore, mammalian experiments take appreciably longer to complete, which is not in the best interest of the patient. Therefore, greater emphasis should be placed on increasing the zebrafish xenotransplantation research to advance the potential of the methods investigated to provide an effective anti-cancer treatment and to therefore increase cancer patients’ survival rates.
6.3. Conclusion

The xenograft zebrafish model is an excellent in vivo model and especially for prostate cancer cells. We optimized multiple factors that may have an effect on the integrity and validity of this model, including the time frame for the experiment (transplantation and termination time points), the imaging procedure, drug toxicity screening time, temperature effects in zebrafish and human prostate cancer cells, and transfection procedure effects. The model was validated by using docetaxel which was found to be effective in treating the transplanted prostate cancer cells. Therefore, we are confident that the xenografted zebrafish model will be a highly useful and relevant addition in cancer drug discovery.
CHAPTER 7: FUTURE STUDIES

Saffron extract, crocin, crocetin, and picrocrocin produced stronger cytotoxicity in normal human prostate cells as opposed to prostate cancer cells. It is possible this cytotoxicity is unique because the normal cell line (PNT1A) used in this study is more sensitive as result of genetic variation in population, or because prostate cells are generally more sensitive compared to other organs’ cells. Therefore, studying the cytotoxicity effects of previously mentioned agents in various of normal prostate cell lines as well as other organ cell lines will confirm whether the observed cytotoxicity is specific to this cell line, cancerous and noncancerous prostate cells, or all normal cells.

Saffron extract showed excellent anti-cancer activity (1.32-1.77 mg/ml), but its activity on cancer cells and the cytotoxicity in normal cells was dependent on the method of saffron extraction used. Because there is a great deal of saffron extract available on the market as a natural health supplement, it is necessary to investigate activity of these supplements on cancer cells and their cytotoxicity in normal cells. The importance of such a study is because saffron extract supplements depend on saffron quality, and different saffron batches tested herein produced different cytotoxicity in normal cells. Furthermore, the \textit{in vivo} stability and metabolic fate of saffron constituents in human is largely unknown. Concentrations that can realistically be achieved in the prostate must be investigated.

Additional studies on saffron extract and its active constituents in the apoptosis assay at different time points (<72 hours), will be important to correlate the observed caspase protein
expression with its mRNA. In addition, studying the extrinsic apoptosis mechanisms will provide valuable information about the cytotoxicity effects produced by saffron and its active constituents. Further insight into cell death mechanisms will reveal if saffron and its active constituents work exclusively through activation of intrinsic pathways as our studies suggest.

Saffron is an expensive spice which enhances the probability of its adulteration before being sold. Developing a rapid and comprehensive new method to help in identification of the original region of cultivation and will enable its acquisition in its pure and not adulterated form. In addition, it will authenticate and prevent the variations in chemical composition that can impact biological activity when using saffron from differing batches.

Finally, our study successfully developed new a xenograft zebrafish model. Using this model with different cancer types, we have aimed to establish this model’s efficacy as an alternative to mammalian models. We believe that validating the use of xenograft models for different cancer types will greatly facilitate effective cancer research and facilitate discovering new and successful treatment options.


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Appendix 1: TNM Staging System
The TNM Staging System is based on the extent of the tumor (T), the extent of spread to the lymph nodes (N), and the presence of metastasis (M) (Edge et al., 2010).

**TNM categories**

<table>
<thead>
<tr>
<th>The T category describes the original tumor</th>
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<tbody>
<tr>
<td>TX</td>
</tr>
<tr>
<td>T0</td>
</tr>
<tr>
<td>Tis</td>
</tr>
<tr>
<td>T1 - T4</td>
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</table>

<table>
<thead>
<tr>
<th>The N category describes whether or not the cancer has reached nearby lymph nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX</td>
</tr>
<tr>
<td>N0</td>
</tr>
<tr>
<td>N1 – N3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>The M category describes whether there are spread of cancer to other parts of the body</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
</tr>
<tr>
<td>M1</td>
</tr>
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Appendix 2: HPLC chromatograms
HPLC chromatogram of standard picrocrocin at wavelength 257 nm.

HPLC chromatogram of standard crocin at wavelength 440 nm.
HPLC chromatogram of standard safranal at wavelength 325 nm.

HPLC chromatogram of standard crocetin at wavelength 440 nm.
HPLC chromatogram of saffron #18258 extract used 95% ethanol at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of saffron #18258 extract used 80% ethanol at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of saffron #18258 extract used 70% ethanol at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of saffron #18258 extract used 60% ethanol at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of saffron #18258 extract used 50% ethanol at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of saffron #18258 extract used 95% methanol at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of saffron #18258 extract used 80% methanol at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of saffron #18258 extract used 70% methanol at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of saffron #18258 extract used 60% methanol at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of saffron #18258 extract used 50% methanol at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of 95% ethanolic extract of saffron commercial sample #18248 at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of 95% ethanolic extract of saffron commercial sample #18249 at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of 95% ethanolic extract of saffron commercial sample #18250 at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of 95% ethanolic extract of saffron commercial sample #18251 at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of 95% ethanolic extract of saffron commercial sample #18252 at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of 95% ethanolic extract of saffron commercial sample #18253 at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of 95% ethanolic extract of saffron commercial sample #18254 at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of 95% ethanolic extract of saffron commercial sample #18255 at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of 95% ethanolic extract of saffron commercial sample #18256 at wavelengths (A) 257, (B) 325, and (C) 440 nm.
HPLC chromatogram of 95% ethanolic extract of saffron commercial sample #18257 at wavelengths (A) 257, (B) 325, and (C) 440 nm.
Appendix 3: UHPLC-MS
UHPLC-MS chromatogram of 95% ethanolic extract of saffron sample #18258 at wavelengths (A) 260, (B) 325, and (C) 440 nm
UHPLC-MS chromatogram of 95% ethanolic extract of saffron sample #18258 at different ionization methods. A: Positive ESI and B: Negative ESI
Appendix 3: Fluorescent microscope filters selection
Microscopic pictures of zebrafish using wide range fluorescence filters show A) bright field, B) broad fluorescence filter for EGFP, C) broad fluorescence filter for DeRed Express2. Proper filter selection is a crucial step to get an accurate quantitative results and avoid interfering bandwidth between multiple fluorescence wavelengths.
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Education

Jan 2010 – Present: Ph.D. student in Pharmacology & Toxicology department, University of Mississippi, University – USA


Aug 2003 – Feb 2009: Bachelor’s degree of Pharmaceutical Sciences, King Saud University, Riyadh – Saudi Arabia

Membership in professional association

Member of Society of Toxicology (SOT)
Member of the Saudi Pharmaceutical Society (SPS)
Member of American Chemical Society (ACS)
Member of the American Society for Pharmacology and Experimental Therapeutic (ASPET)

Experience

Feb 28 2009 – Present: Demonstrator of Pharmacology, College of Pharmacy, King Saud University

Oct 9 2008 - Feb 9 2009: Pharmacist trainee in Kingdom Hospital


May 1 2007 - Aug 31 2007: Pharmacist trainee in Kingdom Hospital
**Attended scientific meeting and continuing education**

1- Attended “the 54th Annual Meeting of the Society of Toxicology”, in San Diego, CA – USA on 22-26 Mar 2015.

2- Attended “the 32nd Annual Meeting of the South Central Chapter of the Society of Toxicology”, in University, MS – USA on 23-24 Oct 2014.

3- Attended The Mary Frances Picciano Dietary supplement Research Practicum at NIH, in Bethesda, MD on 3-6 Jun 2014.

4- Attended “the 53rd Annual Meeting of the Society of Toxicology”, in Phoenix, AZ – USA on 24-27 Mar 2014.

5- Attended “the 52nd Annual Meeting of the Society of Toxicology”, in San Antonio, TX – USA on 10-14 Mar 2013.

6- Attended “the 30th Annual Meeting of the South Central Chapter of the Society of Toxicology”, in Little Rock, AR – USA on 1-2 Nov 2012.

7- Attended “the 1st International Conference in Biotechnology”, in Riyadh – Saudi Arabia on 16-18 Feb 2009, accredited by SCHS with a total of 18 CME units

8- Attended “the 7th International Saudi Pharmaceutical Conference”, in Riyadh - Saudi Arabia on 19-21 Mar 2007, accredited by SCHS with a total of 30 CME units

9- Attended “the 9th International Pharmaceutical Sciences Conference & Exhibition”, in Riyadh – Saudi Arabia on 17-21 Dec 2005, accredited by SCHS with a total of 24 CME units

**Publication**

