A STUDY ON THE EFFECTS OF TRITERPENOIDS ON PROSTATE CANCER PREVENTION

by

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ABSTRACT OF THE THESIS

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Prostate cancer is the one of the most common cancers in the world, and the incidence of this disease increases annually. Many phytochemicals have been shown to be potent inhibitors in preventing prostate cancer disease initiation, progression or inhibit metastasis. Cancer chemoprevention by relatively non-toxic commonly consumed dietary phytochemicals or synthetic compounds would be a logical approach in reducing cancer incidence, and progression to advanced-resistant cancer cells. Synthetic oleanane triterpenoids have been shown to be effective compounds in prostate cancer chemoprevention. The nuclear E2-factor related factor 2 (Nrf2) is the master regulator of many detoxifying and antioxidant enzymes. It is considered the most important chemopreventive signaling pathway in inhibiting cancer initiation and progression. In this study, two synthetic triterpenoids CDDO-Im and CDDO-EA were used to investigate their effects on the activation of Nrf2 and Nrf2-mediated downstream target genes. These compounds were first tested on HepG2-C8 cells stably transfected with ARE-luciferase reporter gene, for cell toxicity and their potential to induce the Nrf2/ARE pathway. CDDO-Im and CDDO-EA were found to could induce ARE-luciferase activities at relatively low and non-toxic concentrations. Using quantitative PCR (qPCR) and western blotting, the transcription of Nrf2 downstream genes HO-1 and SOD1 were significantly induced by drug treatments, corresponding with the increase in their protein levels in

human prostate LNCaP cells. These results suggest that CDDO-Im and CDDO-EA could elicit their chemopreventive effect via the activation of Nrf2 pathway. Emerging evidence suggests epigenetic mechanism such as DNA methylation and histone modifications could potentially be involved in the regulation of Nrf2 pathway. In this study, the effects of CDDO-Im and CDDO-EA on histone deacetylases (HDAC) were investigated by using qPCR. Both compounds show inhibitory effects on HDAC1, HDAC2 and HDAC3 which are considered the highly expressed type of HDACs in prostate cancer. Both compounds can also inhibit HDAC6 at low concentrations. In summary, this study shows that CDDO-Im and CDDO-EA are potentially interesting compounds for prostate cancer chemoprevention via activating Nrf2/ARE signaling pathway as well as epigenetic regulation.

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DEDICATION

To my Father (Abid A. Al-Mahmood)

To my Mother (Eehan A. Al-Sharefee)

To my lovely kids (Sarah and Mustafa)

To My sisters and brothers

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CHAPTER 1

1.1 Background and Significance

Prostate Cancer (PCa) is considered the most commonly diagnosed disease among men. Development of PCa is highly associated with the oxidative stress. Oxidative stress usually results from excessive production of the reactive oxygen species (ROS) or inability of the cells to be protected by using their antioxidant defense system. ROS include hydroxyl, peroxide and superoxide radicals that are produced from endogenous or exogenous sources. High production of ROS can result in tissue injury and DNA damage [1]. The inability of the host defense system in protecting the cells appears to be one of the major outcomes of excessive oxidative stress. In addition to DNA damage, ROS can also induce/activate oncogenes, stimulate epigenetic alterations or inhibit tumor suppressor genes. DNA damage can include deletions, strand breakage, base alterations or chromosomal rearrangements that result in the production of aberrant DNA. The antioxidant enzymes include glutathione-s-transferase P1 (GSTP1) and other enzymes can scavenge the produced ROS. The absence of GSTP1 has been considered one of the factors associated with initiating prostate cancer [2]. These antioxidant defense enzymes can effectively scavenge the ROS resulted from electrophiles and other sources [2].

1.2 Nrf2, The master regulator of Cancer Chemoprevention

The interest in cancer prevention has recently been enhanced due the incidence and rate of cancer mortality keeps going up. The costs of managing cancer are also expensive. Understanding of the important targets of carcinogenesis and the molecular biological aspects of cancers have created important opportunity to promote the research in this chemoprevention area [3]. PCa prevention can play an important role in decreasing the

mortality from this disease. The environment and genotype can affect the development of PCa and accelerate the emergence of new cases. So modifications of the diet and life style can be utilized in the prevention of PCa development. Such modifications include eating food rich in natural and healthy ingredients and taking the required supplements [4]. PCa is highly prevalent and develops over many years and this makes the idea of chemoprevention highly applicable. Chemoprevention includes using relatively low-toxicity natural phytochemicals and synthetic drugs to suppress precancerous changes and decrease the morbidity rates [5]. Therefore, chemoprevention provides cell protection from becoming malignant and suppresses metastasis (transforming from premalignant to malignant cells). The nuclear factor erythroid (NF-E2) - related factor 2 (Nrf2) is the master regulator of cancer chemoprevention mechanism. It can activate the ARE signaling pathway. This event will result in the induction of several antioxidant/ detoxifying enzymes resulting in the detoxification of carcinogens and ROS [6].

Many natural compounds, such as isothiocyanates, curcumin and ginseng, have the ability of inducing the ARE-mediated genes. Induction of phase I, II and III drug metabolizing enzymes/transporters arises in response to the presence of oxidative stress. Phase I enzymes catalyze the oxidation and reduction process and resulting in products that undergo conjugation. The conjugation step ends in more water soluble products and this step is catalyzed by phase II enzymes, for example, glutathione S-transferase (GST). Other phase II/antioxidant enzymes include heme oxygenase 1 (HO-1) and uridine-diphospho glucuronosyltransferases (UGT). Activating ARE-mediated genes is the most significant target for Nrf2 transcription factor in cancer prevention [7]. Nrf2 activates the transcription of these genes by binding to the Maf proteins and form heterodimer, which then binds to the promoter ARE and activates transcription of these cytoprotective genes.

All these events occur in response to oxidative stress and electrophiles. Kelch-like ECH-associated protein (Keap1) plays as a regulator of Nrf2 action. It prevents Nrf2 from entering the nucleus by forming a complex with it [8].

The transcription of Nrf2 and downstream genes are attenuated when Nrf2 binds to its inhibitor Keap1 in the cytosol preventing Nrf2 from entering the nucleus. Some dietary phytochemicals have cytoprotective characteristics by inducing the expression of phase II detoxifying and antioxidant genes responsible for cell protection. This action occurs through modifying sulfhydryl groups on Keap1 and Nrf2. In this context, some phytochemicals, analogous to oxidative stress, can activate the Nrf2 signaling pathway when they are achieved certain concentrations in the cells (above the threshold) [9]. The important role of Nrf2 in cancer prevention has been investigated using wild type mice and *Nrf2* null mice. The *Nrf2* null mice were more sensitive to exposure of carcinogens compared with the wild type mice who can activate antioxidant and detoxifying enzymes and be protected from carcinogens [10].

1.3 Epigenetic Alterations in Prostate Cancer

Prostate cancer progression appears to be closely associated with several epigenetic alterations that contribute to the initiation of this disease. Epigenetics can be defined as changes in the gene expression and function without alteration in the DNA sequence [11]. Such epigenetic modifications facilitate the entry of transcription factors to the promoter regions due to alterations in the conformation of the DNA double helix [12]. Access of the transcriptional factors is also regulated by foods and results in alterations in packaging of the chromatin and ultimately the gene expression. Epigenetic modifications are found during PCa development such as changes in DNA methylation. Induced or

suppressed gene expression and chromatin packaging are determined by studying epigenetic regulations [13].

Epigenetic regulations include: DNA methylation, histone modification and miRNA. DNA methylation includes the addition of a methyl group at the 5′ position of cytosine residues within CpG dinucleotides. This addition of methyl group is performed by DNA methyltransferrases (DNMT). DNA methylation is the most important epigenetic alterations observed in cancer and it is also responsible for the development of many cancers. The transcription of tumor suppressor genes can be affected by DNA methylation. On the other hand, hypomethylation can also have an impact on gene transcription. It can lead to activation of oncogenes and genomic instability. Histone modifications of amino acids at the terminal tails, part of the posttranslational changes could lead to gene transcription or silencing. Such changes include open and condensed chromatin state. In condensed chromatin state, the acetyl group of histone is removed and is catalyzed to coenzyme A. This action is mediated by histone deacetylases (HDACs) enzymes and result in gene silencing. Histone acetyltransferase (HAT) catalyzes the removal of the acetyl group to the ε-amino group of the lysine residue [14]. HDAC results in changing the gene transcription due to tight wrapping of DNA around histone. In prostate cancer, there is a high expression of HDAC1, HDAC2 and HDAC3. HDAC1 plays a role in cancer differentiation. HDAC2 expression is considered as a marker of invasive tumor. It can be related with increased cell growth. HDAC3 is also an interesting target for cancer therapy [15]. Metastatic PCa is characterized by increased angiogenesis, and changes in cell migration. These events appear to be closely coupled to the high expression of HDAC enzymes in prostate cancer, but further study would be needed to confirm this [15].

1.4 Summary

Prostate cancer is still a global health problem in the world. New cases are diagnosed annually and this makes the need for the use of effective management method to decrease the mortality rates associated with this disease is urgently needed. Nrf2/ARE signaling pathway is a promising pathway in cancer prevention. Many natural products can be used to reduce the incidence of prostate cancer by activating the antioxidant and detoxifying enzymes. Nrf2 is an important regulator of these enzymes that can help in fighting oxidative stress and electrophiles. Many phytochemicals can activate Nrf2/ARE signaling and can be considered as promising agents in cancer chemoprevention. Regulation of epigenetic changes in prostate cancer may be effective approach in inhibiting metastasis.

CHAPTER 2

Prostate Cancer and Chemoprevention by natural dietary phytochemicals[1, 2, 3]

2.1 Abstract

Prostate Cancer (PCa) is the second leading cancer among men in the United States. Several studies correlated the development of prostate cancer with diet and life style. Balanced diet and an improved life style could successfully inhibit prostate cancer progression. Cancer chemoprevention emerges as an important factor to control cancer development via natural or synthetic compounds. Oxidative stress is among the various factors contributing to prostate cancer development. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is transcriptional factor that controls the expression of detoxifying /antioxidant enzymes. This action is found when Nrf2 bind to the antioxidant response element (ARE) in the promoter of these genes and ends in their expression. Many natural products can fight oxidative stress and protects cells from DNA damage by activating the Nrf2/ARE pathway. High consumption of fruits and vegetables can reduce the disease incidence and invasive tumor. In this review, the role of natural phytochemicals found abundantly in fruits and vegetables in regulating prostate cancer progression and tumor growth will be discussed.

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[2] **Keywords:** Prostate cancer, phytochemicals, chemoprevention, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), oxidative stress, antioxidant response element (ARE)

[3] **Abbreviations:** AIF, apoptosis induced factor ;ALA, alpha-linolenic acid; ARE, antioxidant response element; Bax, BCL2-associated X protein; Bcl-2, B-cell lymphoma 2; BPH, benign prostate hyperplasia; C3G, cyanidin-3-glucoside;CCRC2, cc chemokine receptor 2; COX-2, Cyclogenase-2; CX43,

2.2 Introduction

Prostate cancer is the most frequent type of cancer among males [16, 17] Approximately, one million cases are diagnosed every year across the world [16] Several internal and external factors play a role in the initiation of carcinogenesis in the prostate. Prostate Cancer can start as a symptomatic or a latent period and turns into a more destructive stage. This change in prostate cancer is triggered by external factors ranging from diet and exercise to lifestyle. The most effective way to prevent Prostate cancer is the screening for early markers of the disease [17]. There is a direct link between western diet and obesity due to the high caloric and unsaturated fat content. Furthermore, western diet is poor in natural components including fruits and vegetables. All these factors can support prostate cancer tumorigenesis [16]. It is observed that Asian people have a lower rate of prostate cancer and may be attributed to the nature of their diet which is rich in natural plant products and poor in fat and calories.

CX43, connexin43;DIM, diindolylmethane; DHA, docoshexanoic acid; DMH, 1, 2- dimethylhydrazine; DR, death receptor; EGCG, epigallocatechin gallate; EGFR, epidermal growth factor receptor; EPA, eicosapentanoic acid; ERK, extracellular signal-regulated kinases; GSH, Glutathione; GSK-3, Glycogen synthase kinase 3; GSSG, total glutathione and oxidized glutathione; GSTA2, glutathione S- transferase A2;GSTm2, glutathione S-transferase Mu 2; H_2O_2, hydrogen peroxide; H2DCFDA, 2′, 7′-dichlorofluorescin diacetate; HER2, human epidermal growth factor receptor; HGPIN, high-grade prostatic intraepithelial neoplasia; HO-1, hemeoxygenase-1; I3C, indole-3-carbinol; IGF, insulin growth factor; IKK, IκB kinase; IL-1β, interleukin-1 beta; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; Keap1, Kelch-like ECH-associated protein 1; LNCaP, lymph node carcinoma of the prostate; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinase; mtDNA, mitochondria DNA; mTOR, mammalian target of rapamycin; NF-κB, Nuclear factor-kappa-B; NO, nitric oxide; NQO1, NAD[P]H:quinone oxidoreductase 1; Nrf2, nuclear factor-erythroid 2-related factor 2; PARP, poly (ADP-ribose) polymerase; PC, Protocatechuic acid; P3G, peonidin -3- glucoside; PCa, prostate cancer; PEITC, phenyl isothiocyanate; Pg3G, pelargonidin-3- Glucoside; PhIP, 2-amino-1-methyl-6-phenylimidazo [4, 4-b] pyridine; PI3K, phosphatidylinositol 3-kinase; PNT2, normal prostate epithelial cells; PPC, purple corn color; q-PCR, quantitative real time-polymerase chain reaction; ROS, reactive oxygen species; SFN, sulforaphane; SOD1, superoxide dismutase 1; STAT, signal transducer and activator of transcription; tBHP, tert-butyl hydroperoxide; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor alpha; TRAIL, TNFα -related apoptosis ligand; TRAMP, transgenic adenocarcinoma of mouse prostate; UGT1A1, UDP Glucuronosyltransferase 1 family , polypeptide A1; VEGF, vascular endothelial growth factor.

So, the environment and life style could play an important role in initiation of aggressive tumor in the prostate [18]. Development of prostate cancer usually starts after the age of fifty years old with no clinical signs of disease. Patients in the latent period of prostate cancer are not taking medications and may progress to other diseases of heart, lung or other cancers [19] Several options are available to treat or manage prostate cancer. This includes surgery, radiation and chemotherapy [5, 19] Hormone therapy, proton beam therapy , cryosurgery and high intensity focused ultrasound (HIFU) are also available for prostate cancer management [19] The emergence of using new products to manage and prevent prostate cancer progression appeared after the inability of current methods to treat progressive prostate cancer. Suppression of precancerous stage and inhibition of cancer development could be achieved by using natural inexpensive components. As a result, a significant decline in mortality related to prostate cancer is observed [5].

In this review, we summarize the preventive and therapeutic role of some natural compounds on patients diagnosed with prostate cancer. These compounds include various types of natural products of the plants such as lycopene from tomato [20], curcumin from turmeric [21, 22], sulforaphane, erucin [23], 3.3′- diindolymethane (DIM) [24] and phenethyl isothiocyanate (PEITC) from cruciferous vegetables [25], tocopherols from walnuts [26], anthocyanins from purple corn [27] and Epigallocatechin-3-gallate (EGCG) from Green tea[28], chrysin from honey [29], tocopherols from honey [30] and from vitamin E [31].

2.3 Prostate Cancer: Risk Factors and Epidemiology

Prostate cancer is considered the second leading cause of death among men in USA and it is the most common cancer among men in USA. The precise cause of prostate cancer is not yet established although many studies have been performed to determine the

pathogenesis of prostate cancer. Prevalence and fatality rates from prostate cancer progress in ascending rate in males over fifty years old. With increasing age, the frequency of both clinical and histologic disease is highly built up. With advanced ages, severity of prostate cancer also increases. A wide spread of prostate cancer is shown compared with other cancer [32]. The initiation of prostate cancer can be found at early stages. Studies had shown that presence of foci related to prostate cancer in specimens taken from the prostate of men between twenty and forty and they are not diagnosed with the prostate cancer. This can propose the ability of developing cancer in the early age even though prostate cancer is continuously increased in older people [33]. The family history and environment play a role in developing prostate cancer. About 10% of early prostate cancer is linked with heritable cause. Prostate cancer is extremely diagnosed in USA compared with Asian countries [34]. There is a high fatality rate (about 2.4) in African American men compared with Caucasian men [35]. A family history with a prostate cancer increases the risk of developing the disease. There is a nine folds high risk of diagnosing prostate cancer in men with a father and a brother affected with prostate cancer. Furthermore, the risk of prostate cancer increases to two folds and three folds in men having only a father or a brother diseased with prostate cancer respectively [35]. The precise role of dietary fat in carcinogenesis is not clearly understood. There is a direct relationship between the growth of prostate cell line and high fat in diet in vivo and ex vivo studies. The development of prostate cancer is affected by activated signaling pathways like cc chemokine receptor 2 (CCRC2) and serum monocyte chemoattractant protein-1 (MCP-1) in addition to the role of high fat in prostate cancer development [36]. Relationship between prostate cancer and doing physical activity which is considered a variable factor had been studied. Role of physical activity in protection from prostate

cancer is accomplished by the International Agency For Research on cancer (IARC) during the working group on the role of physical activity in preventing prostate cancer. A group of phytochemicals that have a significant protective role against prostate cancer in animals shown by several studies is called (Phytoestrogen). These phytochemicals have estrogen like properties and include lignans originated from flax seed and isoflavones which are abundantly found in soybean. These phytoestrogen are considered one of the most significant phytochemicals found in plant food [37].

2.4 Prostate Cancer Chemoprevention

Cancer chemoprevention is an efficient approach that implies using useful natural and synthetic compounds to suppress, hamper or inverse cancer development at early stages [38]. Chemoprevention of a disease addresses the reduction of mortality rates, unwanted effects of therapeutic agents and low prevalence of the disease by targeting characteristic pathways leading to decrease disease extent. Disease prevention can take three approaches primary, secondary and tertiary. Primary prevention targets disease prevalence in healthy persons. Suppression of the disease development and management in persons with nonmalignant stage is addressed by the secondary prevention. The tertiary prevention focuses on the reoccurrence of disease in already managed persons from a tumor [39].

Inflammation developed by reactive oxygen species (ROS) is the main mechanism for developing many types of cancers including prostate cancer. ROS are mainly developed by oxidative stress and lead to DNA damage and initiates cancer in human including the prostate [40]. Reduced production of ROS would be the efficient treatment approach for prostate cancer and other cancers. ROS can be produced endogenously or resulted from exogenous sources. Inflammation, metabolism and action of the mitochondria are the

main endogenous sources for ROS. Mutation in the mitochondria can damage the oxidative phosphorylation and electron transport. The last process resulted in generating ROS. With aging, oxidative stress is built up and more ROS will be produced. The prevalence of a prostate cancer is detected by the presence of mutation or deletion in mitochondria DNA (mtDNA) that becomes not only a sign for aging [1]. Development of prostate cancer starts by initiating high-grade prostatic intraepithelial neoplasia (HGPIN). At this stage, primary prevention can be highly useful for patient with premalignant stage .The prolonged multistage pathogenesis of prostate cancer makes targeting specific pathways as the more suitable way for cancer chemoprevention [39].

Antioxidant response element (ARE) is a *cis*-acting element responsible for controlling protective response of certain enzymes to oxidative stress at transcriptional level. ARE can be presented in the promoter area of certain enzymes like NADPH: quinone oxidoreductase 1(NQO1) and glutathione S- transferase A2 (GSTA2) that are essential detoxifying enzymes [41]. Nrf2/ARE signaling pathway could control several protective mechanisms. Over 250 of phase II genes termed as "prolife genes" could be activated through this signaling pathway and leading to protecting cells from death [42]. Phase II detoxifying enzymes are the main regulator elements of cellular detoxification and they include UDP-glucuronyl transferase, glutathione S- transferase (GST) and microsomal epoxide hydrolase [43].

Nuclear factor (erythroid-derived 2) – like 2 (Nrf2 or NFE2L2) is the main transcriptional factor in the cap'n'collar family of basic leucine zipper (bZIP) transcription factors that have a major role in stimulating antioxidant genes through controlling ARE. Stimulation of Nrf2/ARE related genes is the first chemopreventive pathway for most phytochemical that inhibit cancer progression [44]. In the absence of oxidative stress, Nrf2 is sequestered in the cytoplasm by binding to kelch-like- ECH –associated protein 1 (Keap

1). Nuclear translocation of Nrf2 occurs when cysteine residues in keap1 is irritated by oxidants or electrophiles. Nrf2 starts transcription of antioxidant genes by heterodimerizing with small Maf proteins and attaching to ARE in the promoter area of the antioxidant genes [45]. Several chemopreventive compounds can promote activated signaling pathway of Nrf2 leading to stimulated production of phase II enzymes such as heme oxygenase (HO-1). The cellular protective role of HO-1 had been established in cancers, inflammation, atherosclerosis and neurodegenerative disorders. It mediates breaking down of heme to iron, carbon monoxide and biliverdin. The current mechanism implies the nuclear translocation of Nrf2 [7] and it is shown in (Fig.1). Several studies in rodents had been reviewed the activation of the protective genes controlled by ARE signaling pathway by many natural and synthetic compounds found in their diets [46]. Cell response to oxidative stress is accomplished by how Nrf2 is translocated to the nucleus and escapes degradation. During basal conditions, degradation mechanism maintains Nrf2 level in the cell. How to control stress response is directly related to understanding molecular pathway of Nrf2 signaling activation by reactive oxidative species [47]. Many studies revealed the effects of natural products on cancer chemoprevention including the prostate. We will review some of the natural phytochemicals illustrating their effects and molecular mechanisms.

2.5 Natural phytochemicals And Prostate Cancer

2.5.1 Lycopene from tomatoes

Lycopene is one of the carotenoid families and it is abundantly found in tomato [48, 49]. Other sources of lycopene can be papaya, pink grape fruit, pink guava, water melon, and red carrot [50]. Lycopene lacks the action of vitamin A [51]. Lycopene has the ability to decrease the prostate specific antigen and minimizes the scope of DNA oxidation as

shown by several clinical trials. It had been confirmed that aggressive prostate cancer can be minimized with high consumption of tomato based products that leads to high lycopene level [52]. Lycopene can decrease the hazard of breast cancer, lung cancer and pancreatic cancer progression [53]. Lycopene can impede the cancer progression through its antioxidant activity. The antioxidant activity implies cellular protection of proteins and lipids from damage. Several studies had shown the inhibiting role of lycopene for DNA production and cell growth in different cell lines of prostate cancer such as: LNCaP, DU145 and PC3 [48].

Lycopene inhibits prostate cancer progression through lowering insulin growth factor (IGF) level. IGF has two receptors (IGF-1) and (IGF-2) on the cell surface and they have akin sequence like insulin. IGF-1 is the main activating agent for AKT signaling pathway. This pathway is responsible for impeding apoptosis or programmed cell death and inducing cell reproduction. Lycopene exerts the antioxidant function that can scavenge free radicals and remove them. As a result, transcription of phase II detoxifying gene will be stimulated. Antioxidant activity of lycopene is due to eleven bound bonds and absence of betaionone ring in its structure [54].

Induced phase II enzymes by lycopene and other carotenoids include NQO1 and GCS. The transcription factor Nrf2 is the main regulator for the expression of antioxidant enzymes. Induction of these enzymes had been shown in liver cancer cells and other mammary cells. Another mechanism of lycopene is affecting gap junction communication. Retinoids and carotenoids including lycopene can stimulate the expression of connexin 43 (CX43) and increase the gap junctional communication. Ions, nutrients and low molecular weight molecules enter between cells through protein channels or gab junctions located in the cell membrane [55]. Cx43 is the main protein

responsible for cell growth among connexin group including Cx43,Cx50,Cx40 and Cx33. Studies had shown that activated Cx43 expression can lead to decrease fibroblast formation, but decreased Cx43 expression increases cell growth [54].

A study by Mariani S. et al. had been conducted to estimate lycopene concentration in patients with HGPIN. Lycopene level was determined in prostate and the plasma and study its level on prostate cancer development. Those patients were administered a high lycopene diet to study the lycopene effect on prostate cancer progression [56]. The study was conducted for six months and the patients were divided into three groups (prostatitis, prostate cancer and HGPIN) depending on the biopsy sample taken. The cut- off value of lycopene level in the prostate was 1ng/ml. This study concluded that lycopene cumulated in the prostate can reduce apoptosis, oxidative injury and prostate specific antigen. These specific markers are found in many cancers including the prostate cancer. This study also suggested the important role of cumulated lycopene in the prostate. In this study, patients with prostate cancer had a low lycopene concentration in their prostates while they had a high lycopene level in the plasma. Compared with HGPIN group, six month follow up study revealed the presence of low lycopene in their prostate [56].

During cancer development, uncontrolled cell growth is highly characterized. This can be due to the imbalance in cell cycle. Down regulation of first G1 phase is initiated. Studies using DU145 cells were conducted to investigate the lycopene and apo-12-lycopene which is one of the lycopene metabolites on the cell cycle. These studies had shown that cell distribution during the cell cycle was switched. At S phase, cell number was decreased while at G1 phase and G2/M phase, cell number was increased [57]. Lycopene can affect a protein that is related to G0/G1 phase (Cyclin D1). Reduced Cyclin D1 was observed following 24 hours of treatment with lycopene. On the other side, remarkable

increments in cyclin kinase inhibitor: p53, p27 and p21 were shown. Lycopene effect on the cell cycle is attributed to its effect on NF-kB signaling pathway [57].

Lycopene effect on the cell cycle and apoptosis was also investigated by Soares et al.

The cell lines used were the cells from benign prostate hyperplasia (BPH) and prostate cancer cells (PC3 and DU-145). BPH cells are hormone independent and did not respond to lycopene. On the other hand, a significant response to low amount of lycopene (25 μM) is observed in prostate cancer cells (PC3 and DU-145) that are hormone dependent. At this concentration, a high percentage of cell growth was suppressed. Saturated inhibitory result was shown after 96 hours of lycopene treatment even though with increased extracellular level. Lycopene treatment lead to higher apoptotic effect on DU-145 cells than on PC-3 cells. After 96 hours, DU-145 had five fold of apoptosis while PC3 had (2.2 fold) higher apoptotic effect than after 48 hours at 10 μM concentration [58]. Expression of the genes responsible for tumor growth include Bax, Bcl-2 and

CK18 are characterized in prostate cancer. Induced expression of Bax and CK18 genes were shown in both PC3 and DU-145 while impeded expression on Bcl-2 gene in the same cell lines. A remarkable long G0/G1 phase and slow G2/M phase was shown. Blocking G2/M results in serious effects on the cell growth as the cells cannot finish the division process, while blockade in G0/G1 phase can be reversible. Higher blockade in G2/M phase is responsible for high apoptosis in DU-145 compared with PC3 cells. Apoptotic effect on the BPH cells was due to regulating expression of Bcl2 and Bax (high Bax and CK18 and low Bcl-2) [58].

Lycopene can influence the migration and adhesion property of prostate cancer at low concentration (0.01 μM). A study had been shown that lycopene can selectively inhibited the highly activated integrins in prostate cancer ($\alpha v\beta_3$ and $\alpha v\beta_{5)}$ [57]. Integrins are closely

associated with adhesion and migration of cancer cells [57, 59]. These integrins are highly found in developed prostate cancer. Reduced integrin expression was shown in 22RV-1, PC3 and LNCaP cell lines [57]. Elgass et al. investigated the effect of lycopene on the adhesion and migration of prostate cancer cells. Cells used in the study were cells of normal prostate epithelial (PNT2), PC3 and DU-145. Inhibited migration was shown in both PC3 and DU-145 by lycopene. Higher reducing effect was observed in DU-145 than in PC3. Non inhibited migration effect was observed in PNT2 cells except at high concentration of lycopene, an inhibiting effect was shown [59]. Lycopene concentration at (1.15 μM/L) was effective to reduce motility rate by 40% in PC3. Higher inhibiting effect of adhesion is observed in PC3 while a higher inhibiting effect of migration was found in DU-145. This was the first study that demonstrated the lycopene effect on adhesion and migration properties of prostate cancer cell lines at (1-2) μM, which are considered physiologically achieved amount [59].

2.5.2 Anthocyanins from purple corn color

Anthocyanins are natural compounds belong to the flavonoid family and exist as glycosides. These glycosides include galactose, glucose, rhamnose, xylose or arabinose connected with aglycone unit [60, 61]. Several sources of anthocyanins are available such as purple corn, grape, berries, apple in addition to the purple cabbage [61]. Anthocyanins have multiple healthy benefits including antioxidant and anti-inflammatory activities [62, 63]. They can also improve neuronal and heart function as proven by a study on mice. Cancer progression could be suppressed with anthocyanins. Hyperglycemia and obesity could also be adjusted with cyaniding-3-glucoside, one of anthocyanins present in purple corn color [63]. Anthocyanins usually have red, purple or blue color. These colors are found due to the presence of metal ion bound and pH of the

medium. Anthocyanins are soluble in water, in the acidic solvent, they have a positive charge compared with other flavonoids [61]. Anthocyanins are highly found in the extract of seeds of purple corn Zea mays L. The extract is called purple corn color maize morado color and it is commonly used as a drinking solution chichi Morado in South America, specifically in Peru. Anthocyanins are responsible for the purple color of corn seeds Zea mays L. [60, 64].

Purple corn colors have three anthocyanins: cyanidin-3-glucoside (C3G) , pelargonidin-3-glucoside (Pg3G) and peonidin -3- glucoside (P3G) [27]. Anthocyanins have the ability of Scavenging free radicals and fight oxidative stress. A study using HepG2 cells and adenocarcinoma cells Caco-2, cyaniding-3-glucoside had a significant role in inhibiting the synthesis of ROS. C3G can also decrease the production of DNA and protein of malignant cells in addition to scavenging the ROS. C3G had also a protective role of DNA damage in human colon cells [65].

The powerful antioxidant property of anthocyanin is related to the phenolic structure of these compounds. Hydroxyl radicals, hydrogen peroxide and singlet oxygen are the main reactive oxygen species that are taken by anthocyanins. They can also bind to the proteins and exhibit a defensive action through chelating metals. Anthocyanins had been reported to induce apoptosis in vitro. A proapoptotic response had been induced by anthocyanins and anthocyanidins. This response follows two pathways: extrinsic and intrinsic pathway. Regulation of FAS and FAS ligand occurs during the extrinsic pathway, while upregulation of caspase dependent pathway occurs through the intrinsic pathway. Release of cytochrome c is regulated “potential of mitochondrial membrane. Anthocyanins can suppress cancer progression through inhibiting angiogenesis. Decreasing vascular endothelial growth factors (VEGF) and its receptor is a mechanism by which

anthocyanins inhibit angiogenesis in the endothelial cells. VEGF expression can also be inhibited by suppressing factors stimulating VEGF expression such as tumor necrosis factor alpha (TNF- α). Suppression of H_2O_2 can also include within the last category [61]. Anthocyanins could inhibit colon tumorigenesis and this effect was investigated by Hagiwara et al. PPC was used a source for anthocyanins. In this study, tumorigenesis in colon was stimulated by treatment with 2-amino-1-methyl-6-phenylimidazo [4, 4-b] pyridine (PhIP). Studies on rats revealed the tumorigenic role of PhIP in inducing cancer in large intestine and breast cancer. Anthocyanins could significantly inhibit carcinogenesis in the colon induced by PhIP. The percentage of purple corn color in the rat diet was only 5%. The presence of neoplasm was considered the as the end result of tumor formation. The PhIP concentration used was only 0.02% and that was enough to induce carcinogenesis. In this study, 1, 2- dimethylhydrazine (DMH) was used as an indicator to identify the effects of anthocyanins on colon tumorigenesis. Azoxymethane is another inhibitor and can be used for the same purpose. The study concluded that chemoprevention of colon cancer by anthocyanins is achieved and further research is needed to investigate lower amount of anthocyanins on colon cancer [64].

Anthocyanins also showed a significant suppressing effect on prostate carcinogenesis *in vitro* and *in vivo*. A study by Long et al was started using anthocyanins on prostate cancer cells lines (LNCaP) and transgenic rat for adenocarcinoma of prostate (TRAMP) model. For LNCaP cells, anthocyanins successfully suppressed their growth after 72 hours of treatment. A significant reduction in cyclin D1 was showed by using qPCR and western blotting techniques, while there was a significant increase in the number of cells in G_0/G_1 phase of the cell cycle. For TRAMP model, the promoter of probasin gene is the controlling element of simian virus 40T antigen. Androgen receptor regulates the

expression of this antigen. This study reported the clinical findings of anthocyanin treatment on prostate carcinogenesis. Development of prostate cancer in (TRAMP) model was indicated by development of adenocarcinoma and high grade prostatic intraepithelial neoplasia (HGPIN) [27].

Carcinogenesis in the prostate was critically slowed by adding anthocyanins P3G, C3G and Pg3G. These anthocyanins are tested individually on LNCaP cells to find the most effective component in purple corn color. P3G did not inhibit the growth of LNCaP cells while C3G and Pg3G significantly suppress the growth of these cells. This result indicated C3G and Pg3G are the most potent compounds in PCC. The latter two compounds reduced the level of cyclin D1 protein and increase the number of cells in G_0/G_1 phase. This can also due to the presence of hydroxyl radicals in their structure that could have a specific response on prostate cancer suppression. The study concluded that PCC successfully inhibited prostate cancer progression in vitro and in vivo. Mixture of PCC and its active components showed the significant inhibiting result on LNCaP cells growth and suppressing effect on prostate tumorigenesis in vivo [27].

Anthocyanins exhibit their benefits through various mechanisms. Hou D. explained in his review mechanism of action of anthocyanins. He illustrated the ability of anthocyanins in inhibiting the nitric oxide (NO) production. Infection with bacteria or a virus induces NO production that can trigger oxidative stress and cancer. Prevention from cardiovascular disease and inflammation could be accomplished with utilizing a food rich in anthocyanins content in berries. The whole mechanism is through obstructing oxidative stress resulted from high levels of NO. Anthocyanin can also work through influencing the epidermal growth factor receptor (EGFR). Cyanidin and delphinidin can impede the action of EGFR through suppressing tyrosine kinase [66].

Anthocyanins can also affect cancer metastasis or progression of cancer. Metastasis involves controlled movement of chemotactic compounds by autocrine motility factor that include NDP-kinase, cell adhesion and secretion of specific enzymes like metalloproteinase (MMP). Suppressing effect of MMPs was shown by delphinidin with absent effect on cell adhesion and hepatotactic movement [66].

The phenolic structure of anthocyanins is responsible for their antioxidant property. They have a significant scavenging activity of the free radicals. This is effectively due to the presence of hydroxyl chains and a double bonded structure. This antioxidant activity of anthocyanins is responsible in part for their anticancer activity. Anthocyanins have the ability of stimulating apoptosis in cancer cells though accumulating ROS in these cells. They can also hamper cancer progression through actively transcribing of detoxifying phase II enzymes. It should be noted that their phenolic structure is responsible for their ability to chelate to metals and connect to proteins [67]. The protective role of anthocyanins had been investigated using breast cancer cell lines. A study by Fukamachi et al was conducted to determine the therapeutic effect on carcinogenesis in the breast of rats. Breast cancer was induced in rats by using 7, 12-dimethylbenz[a]anthracene (DMBA). Both transgenic and non-transgenic rats were used in this study. Transgenic rats are rats having a copy of human c-Ha-ras proto oncogene and PCC was used as a source of anthocyanins. C3G is found to be the most potent one. Protocatechuic acid (PC) is one of the metabolites following C3G administration. This compound has a powerful antioxidant activity. The study concluded that tumor volume was reduced in rats fed with PCC. Although the number of tumors large size only were effectively reduced but cancer progression could not inhibited by PCC. The concentration of PCC used was 1% and that was enough to suppress tumor progression in the transgenic rats. In non-transgenic rats,

1% PCC could significantly inhibit the production of massive tumors and minimize the number of mammary tumors [60].

In this study, the specific molecular mechanism of anthocyanin is inhibiting Ras signaling pathway and inducing apoptosis through caspase activation. Activated Ras signaling is associated with development of many tumors like colon, breast, and thyroid and lung adenocarcinoma. Abnormal stimulation of Ras signaling is found with 80% of tumors. So, direct inhibition of Ras signaling can protect cells from oxidative stress and DNA damage. Apoptosis will be the end result. Apoptosis was activated through caspase -3 activation by PCC. The study concluded the important therapeutic effect of PCC including C3G and PC on mammary tumors by inactivating ERK and reducing Ras protein without altering Ras gene expression [60].

2.5.3 Nutrients from Walnuts

A walnut (Juglans regia L.) is a natural plant found mainly in the climate areas around the world. Several uses of walnuts are available mainly as food. Leaves, shells, husks (epicarps) of walnut plant could imply in medical uses and cosmetics. Walnut plant was also associated with folk medicine [68]. Several beneficial effects of walnuts had been demonstrated. It had reported that walnut could improve many diseases including cancer. Disease related to the life style such as hypertriglyceridemia, diabetes, arteriosclerosis, hypercholesterolemia and cardiovascular disease could also be ameliorated with walnuts [69]. Consumption of walnuts can significantly impede systemic inflammation. High levels of omega-6 could also be minimized with walnuts as proved by a studies done on human and animals. These studies indicated the role of walnuts in improving endothelial dependent vasodilatation [70].

Walnuts contain several nutrients such as tocopherols such as alpha tocopherols [68] and gamma tocopherols [68, 71]. Walnuts contain phytosterol mainly β-Sitosterol, melatonin, fibers and polyphenols mainly ellagitannin. Carotenoids are also found in walnuts. Alpha-linolenic acid (ALA, 18 C) is abundantly found in Walnuts [71]. B-sitosterol is a sitosterol compound and it is considered one of the plant sterols commonly found in the western diet. Other plant sterols may also be found in the diet such as campesterol and stigmasterol. B- Sitosterol belongs to the phytosterol family that has a similar structure of cholesterol. Phytosterol are commonly found in the fatty part of nuts. Phytosterols have the ability of suppressing the inflammation leading to decreased cholesterol levels Cholesterol retention in the body will also be hampered [72].

Phytosterols possess several health benefits including cancer. They can activate "ceramide" cascades and induce apoptosis in cancer cell lines. B- Sitosterol can induce apoptosis in cancer cells. Apoptosis induced by B -Sitosterol was found in hormone and non-hormone dependent prostate cancer cells (LNCaP and PC3) respectively, also in colon adenocarcinoma (HT-29) and breast cancer cell line (MDA-MB-23) which are non hormone dependent [73]. Walnuts have antioxidant properties resulting from the presence of melatonin and other ingredients. Melatonin is a potent antioxidant compound. Studies on rats demonstrated the protective role of melatonin against oxidative stress. In a controlled trial, total glutathione and oxidized glutathione (GSSG) and catalase action were significantly improved in people consumed walnut meal (WM) [74].

The fiber content in walnut has a significant preventive role in cancer. Walnut fiber content could suppress cancer progression. The effect of fiber content of diet on cancer in women consumed nuts and seeds were demonstrated in European Prospective

Investigation into Cancer and Nutrition (EPIC) study. EPIC study demonstrated that consumption of fiber rich food could decrease the risk of colorectal cancer by 21% and high fiber content from fruits lead to increase the risk of prostate cancer. On the other hand, a French study showed that risk of prostate cancer was not affected by fiber content taken from fruits and vegetables (insoluble), but PCa risk decreases with fiber content from legumes (soluble). Walnuts had been reported to inhibit prostate cancer using several PCa cells lines PC3 and LNCaP and also in TRAMP model [26, 68, 70].

A study led by Alshatwi et al. investigated the effect of walnut on prostate cancer. The prostate cancer cell line used was PC3.In this study, three organic extracts of walnut was used. These include n-hexane, chloroform and methanol extracts of the green bark of walnut. Walnuts are used as the air dried green husk (WNGH). The growth of PC-3 cells was significantly inhibited with the three organic extracts of walnuts. Apoptosis is the mechanism of inhibiting the growth of PC-3 cells lines. Higher inhibiting effect was observed with n-hexane than other extracts [68].

Induction of apoptosis was explained by examining the expression of the proapoptotic and antiapoptotic genes. A significant increment in mRNA level of p53, caspase 3 and Bax was observed. A remarkable decline in mRNA level of Bcl-2 resulted. All organic extracts induced apoptosis but in a variable degree. N-hexane showed the highest inhibiting activity on PC-3. This can be due to the presence of diverse quantity of polyphenols in the organic extract of WNGH and this variation could be attributed to the type of solvent used. Bcl-2 family has a role in controlling the apoptosis. Apoptosis mechanism is regulated by P53. P53 inhibited the transcription of antiapoptotic genes while it activated the proapoptotic gene transcription. Transcription of these genes occurred through a pathway regulated by the mitochondria. Mitochondrial permeability

of Bax is induced by P53 interaction with Bcl-2. P53 could activate Bax through activating caspase pathway. The ratio of Bax: Bcl-2 was enhanced by p53 and this result in inducing apoptosis. Apoptosis was activated through caspase and cytochrome c activation. In this study, P53 induced apoptosis through inhibiting Bcl-2, activating Bax and caspase signaling pathway [68].

A study by Davis et al was conducted to investigate the effects of whole walnut taken as a diet on prostate cancer. In this study, TRAMP is used as a model for prostate cancer. High and low fat food was used as control diet. The levels of α-tocopherol and γ-tocopherols were the same in three types of food. The study concluded that the high fat content did not affect prostate tumorigenesis. A significant reduction in genitourinary intact tract (GUI) weight was observed following the intake of whole walnuts, in addition to the rate of tumor growth was also suppressed [26]. High IGF-1 is associated with increasing the risk of prostate cancer. High insulin and IGF-1 can stimulate cell growth and induce carcinogenesis. It is important to target IGF-1 as the initial strategy in controlling tumor growth as reported by several studies. A significant increase in tumor rate was obtained when IGF-1 was not targeted. Following fifteen weeks of consuming whole walnut diet, the rate of tumor growth was hampered and a significant decrease in tumor volume was observed. High resistin level was associated with increasing tumor growth. Suppression of resistin level was observed in this study. The whole walnut diet resulted in higher suppressing effect of resistin than high fat diet. The level of LDL was also reduced with whole walnut diet compared with high fat diet. In this study, factors related to the tumor growth were suppressed following the whole walnut diet and no specific fatty acid or individual tocopherol could responsible for the whole walnut effects [26].

Walnut consumption could effectively suppress tumorigenesis in breast cancer in mice [75, 76]. The diverse content of walnuts helped in fighting cancer progression. As mentioned previously, walnuts have multiple components that have a role in inhibiting carcinogenesis. Mechanism of action of walnuts needs to be addressed carefully and determine the main component responsible for cancer preventive role. An early study by Hardman et al was started to investigate the role of α-Linolenic acid on breast cancer progression. α- Linolenic is one of the walnut components and it is reported to decrease the growth rate of cancer. Transgenic mice (C3) Tag were used as a cancer model. These mice carried SV40 T antigen and could express cancer in the mammary glands. 10% corn oil was used as a negative control. High level of ALA is present in the corn oil and this amount is close to its amount in the western food [76].

A higher level of ALA was is associated with walnut consumption compared with other nuts [75, 76]. In this study, the transgenic mice and their progeny were given walnut diet, canola oil and corn oil. ALA is also found in the canola oil. The risk of breast cancer in the mice generation was effectively decreased following canola oil consumption during gestation and lactation. Reduced cancer progression was observed with α-linolenic but α-linoleic acid could induce tumorigenesis. The study found that walnut consumption resulted in suppressing tumor progression. A decline in the tumor growth was also found due to walnut consumption by mice and their offspring. Both walnut and canola oil have α-linolenic acid. Both of them could suppress cancer progression compared with consuming corn oil diet. There are equal quantities of omega-3-fatty an acid in both walnut and canola oil, but a higher inhibiting effect was found following walnut consumption [76].

The effect of walnut consumption on breast cancer was examined in other study by Hardman et al. In this study, nude mice were used and breast cancer was induced through injecting MDA-MB-231 human breast cancer cells to these mice. Corn oil was also used as a negative control. The breast carcinogenesis was critically reduced following walnut consumption. Omega-3-fatty acid underwent elongation into saturated fatty acids 20 or 22C. These elongated omega-3-fatty acids include eicosapentanoic acid (EPA) and docoshexanoic acid (DHA). These fatty acids are associated with inhibiting carcinogenesis or reducing tumor volume. These metabolic fatty acids are responsible for the protective and preventive role of alpha-linolenic acid. Walnut consumption effectively provided a high level of α- linolenic acid. As a conclusion, there is no need to add EPA or DHA to the diet as walnut diet can provide these important metabolic fatty acids [75]. Walnut consumption could effectively slow breast cancer growth as shown by several studies on mice [75, 76]. The significant inhibiting role of walnut may not be due to a specific component as walnuts have many ingredients and all of them exert a protective role against cancer. The α-linolenic acid in walnut could suppress the growth rate of breast cancer. Because the diverse content of walnut, no defined component of walnut could be responsible for its preventive effects [76]. Diet rich in walnut could suppress breast carcinogenesis and production of malignant cells. The proliferation of metastatic cells could also be inhibited that will lead to decrease mortality related to cancer [75].

2.5.4 Sulforaphane from cruciferous vegetables

Sulforaphane is an organosulfur phytochemical found in most cruciferous vegetables including cabbage, Brussels sprout and broccoli. Many studies proved the anticancer, antioxidant role of SFN. SFN exhibit antioxidant action through stimulating the

expression of Keap1/Nrf2 pathway. SFN also had a beneficial role in diabetes. It can protect kidney from damage induced by diabetes through its antioxidant action. This effect had been shown after four months treatment with SFN in mice with diabetes [77]. SFN is a one member of the isothiocyanate family that has a role in inhibiting prostate cancer. Cruciferous vegetables have the precursors of isothiocyanates known as glucosinolates [38]. SFN is released following hydrolysis of glucoraphanin, a compound highly found in cruciferous vegetables particularly in broccoli [78, 79].]. Hydrolysis of glucoraphanin is achieved by either thioglucosidase in colon or thioglucosidase myrosinase in the plant [78].

SFN can stimulate the nuclear translocation of Nrf2 and ARE gene expression by affecting on the Keap1-Nrf2 complex. SFN can interact with the cysteine thiols on keap1 C151, C489, C583 and lead to Nrf2 escape from the keap1 [80]. Nuclear translocation of Nrf2 enables binding of Nrf2 to the promoters of phase II enzymes and stimulates their release. Stimulating phase II enzymes by SFN helps in inhibiting cancer initiation. Expression of phase II enzymes helps the cell in getting rid of reactive molecules by combining “moieties” with them and making them more soluble to increase their elimination. Prevention of cell death can is achieved following upregulation of phase II enzymes such as NQO1 and HO-1 by SFN. SFN can also decrease the activity of electrophiles by neutralizing them. It can bind glutathione to electrophiles and reduce their activity. SFN is a potent stimulator of glutathione S-transferase and neutralize electrophiles [81]. SFN can also induce apoptosis or programmed cell death and inhibit cell cycle in different cell lines. Apoptosis was shown in human colon cancer cells and prostate cancer cells (DU145) induced by SFN. SFN can also induce cytochrome C release from the mitochondria and/or activation of caspase 7 and 9 to activate apoptosis

pathway. SFN can inhibit the cell cycle in some cancer cell lines like prostate cancer cell lines (PC3 and LNCaP) and human colon cancer cells (HCT116). Treatment of prostate cancer cells LNCaP with 10 μM of SFN could significantly inhibit cell cycle at the G1/S phase. Inhibition of cell cycle by SFN was shown in colon cancer cells HT-29 at G1 phase accompanied with reduction of cyclin D1 and activation for p21 [79]. SFN is a promising chemopreventive agent that induces the expression of phase II enzymes. Toxic substances will be converted to more excreted products via these enzymes. Induction of phase II enzymes by SFN is proposed to occur by two mechanisms. First mechanism is by inducing transcription of Nrf2 and the second mechanism is by modulating mitogen-activated protein kinase (MAPK) and inducing ARE-genes transcription. Important signaling proteins the c-jun N-terminal kinase (JNK) and the p38 kinase are regulatory proteins of MAPK. These proteins can modulate the transduction of intracellular functions induced by extracellular signals [82]. SFN is highly studied for its role in cancer. Inhibition of cancer growth and apoptosis are induced by SFN. Cancer progression is also significantly inhibited by SFN at the post initiation stage. In prostate cancer, SFN can induce apoptosis through inhibiting cell growth and the cell cycle. SFN can inhibit the protein synthesis in PC3 cells which is crucial for cell growth and their viability. Translation process is usually controlled by mTOR kinase. S6 kinase1 is one of the targets of mTOR kinase and it regulates several processes in protein synthesis such as starting and extending the translation in addition to ribosome biogenesis. SFN treatment resulted in phosphorylation of S6K1 and leads to block transduction between mTOR and S6 kinase1. As a result, early inhibition of translation is achieved [83]. SFN can also induce apoptosis through inhibiting STAT3 activation. STAT3 is a signal transducer and activator of transcription 3 and it is important transcription factor in cancer development.

SFN could inhibit interleukin 6(IL-6) that is associated with STAT3 stimulation. This study revealed that SFN could inhibit several genes associated with STAT3 activation such as cyclin D1 and surviving in prostate cancer cells [84].

SFN has been found to inhibit NFκB transcription. This transcription factor has two subunits p50 and p56. NFκB stimulation is found in several cancers like colon and prostate cancer and it is correlated with stimulated gene expression of inflammation and cell growth and antiapoptotic events. SFN can down regulate the action of NFκB and inhibits the nuclear translocation of p65-NFκB following treatment. This effect was shown after treating PC3 cell with 20 μM of SFN for one hour. Another target for cancer prevention is induction of apoptosis via induced production of ROS. The production of ROS in the mitochondria requires high doses of SFN. A study showed that a temporary increase in ROS following treatment of DU145 cells with 10 μM of SFN. Several events are associated with stimulated production of ROS in the mitochondria. "Disruption" in membrane potential, cytochrome C release resulting in apoptosis [85]. A study using PC3 and DU154 cells, apoptosis caused by SFN was investigated. The study revealed that ROS were responsible for stating cell death and both intrinsic and extrinsic pathways took apart in cell death. Release of cytochrome C and disruption of the membrane potential of mitochondria was also shown. Apoptosis induced by SFN was reduced by the effect of BcL-xL. BcL-x is one of the BcL-2 family regulators of cell death and it acts as inhibiting protein of apoptosis [86]. SFN has a protective role of the skin cells from inflammation induced by UV. This effect was shown in a study using C57BL/6J and C57BL/6J/Nrf2 (-/-) mice. This study concluded that the protective role of SFN is resulted through Nrf2 activation and the damage was removed within 8 days but this effect was not shown in knockout mice [45]. In a study using C57BL/6 TRAMP mice,

SFN was able to inhibit tumor growth via activating apoptosis in mitochondria and activating Nrf2/ARE signaling pathway. This resulted in inducing expression of apoptosis related genes like Bax protein while a decrease in BcL-xL was observed. Also, activation of Nrf2/ARE signaling resulted in induced expression of Nrf2 and its related genes HO-1 while decreased expression of Keap1 [87]. SFN is a promising chemopreventive agent in prostate cancer and other cancers for its high efficacy. SFN has several molecular targets and each proved its efficiency in protecting body cells.

2.5.5 Curcumin from turmeric

Curcumin or diferuloylmethane is the most important ingredient of turmeric spice, *Curcuma longa L.* [45, 88]. Curcumin is usually extracted from the rhizomes of the plant *Curcuma longa Linn* [22, 88] that is one of the Zingiberaceae family [88]. Curcumin had been reported to have anti-inflammatory and antioxidant characteristics [22, 89], anticancer effects. Several clinical trials had used curcumin as anticancer agent due to its safety and tolerability. It is found that curcumin is safe for up to 12 gm/day when it is administered orally. It can be related the powerful antioxidant property of curcumin with its ability in protecting the normal cells and killing the cancer cells. That means curcumin is not toxic to the normal body cells [90].

Curcumin was used to treat and prevent several cancers such as prostate, lung, colorectal, breast, pancreatic, head and neck squamous cancers. Other cancers such as multiple myeloma are also included. Curcumin has the ability of inducing autophagy in metastatic cells. In a study using curcumin on CML K562 human chronic myeloid leukemia, curcumin was able to induce autophagy and stimulate apoptosis that result in cell death. The mitochondrial membrane potential was decreased accompanied with activating cascade 3 signaling pathway. There was a dose and time dependent decrease in the cell

viability due to inducing apoptosis. Curcumin has an inhibiting effect of the cell cycle at the G2/M phase. This effect was shown in a study using (U373-MG and U87 MG) glioma cells [91]. It is known that the oxidative stress is the important cause of inflammation that leads to cancer. In oxidative stress, activated macrophages or neutrophils produce reactive oxygen and nitrogen species. Both ROS and RNS lead to cancer initiation in the cells. Curcumin can scavenge the free radicals including nitric oxide. In a study using RAW 264.7 cells (mouse macrophages cell lines), curcumin can reduce the level of inducible nitric oxide synthase (iNOS) in the cells that resulted from gamma interferon and lipopolysaccharide (LPS) treatments. Curcumin can significantly affect the Nrf2 signaling and NF- κB to enhance HO-1 transcription. As a result oxidative stress will be prevented [92].

In a study using renal epithelial cells, curcumin could upregulated the expression of HO-1 and the expression of phase II genes via enhancing Nrf2 escape from keap1 complex. This induced the nuclear translocation of Nrf2 and activation and enhanced expression of detoxifying genes [92, 93]. Curcumin has a significant effect on prostate cancer cells in vivo studies. In a study using nude mice, in situ cell death assay was used to measure apoptosis in the tumor in the animals. The test showed that a significant increase in apoptosis was resulted induced by curcumin. The cell growth was also inhibited. In another study, curcumin was able to induce TNFα related apoptosis ligand (TRAIL) to activate apoptosis in LNCaP xenograft in nude mice. Tumor growth was also inhibited by curcumin. Many studies investigated the combination between curcumin and phenethyl isothiocyanate (PEITC) on prostate cancer. In a study using PC3 25 μM of curcumin and 10 μM of PEITC highly inhibited phosphorylation of Akt pathway and of epidermal growth factor. The combination significantly induced apoptosis via stimulating cleavage

of cascade 3 and poly (ADP-ribose) polymerase (PARP) protein. Activation of phosphatidylinositol 3-kinase (PI3K) was also observed in this study [94]. Metastasis in prostate cancer can be prevented by inhibiting cell motility. A study using nude mice, the growth of prostate nodules had been reduced due to apparent inhibition of metalloproteinase (MMP2 and MMP-9). Cancer metastasis results when growth of cancer cells is highly expanded and starts to spread to other tissues. Metastasis significantly depends on the role of MMPs in enhancing cell motility. Curcumin has an important role in inhibiting inflammation in prostate cancer. It is found that COX-2 expression is highly activated in prostate cancer. Curcumin can inhibit its expression as a result of inhibiting several signaling pathways. These include dephosphorylating of c jun N-terminal kinase (JNK) and protein kinase p38. This effect resulted from activating the mitogen-activated protein kinase-5 (MKP5). As a result, Inhibition of expression of proinflammatory cytokines such as IL-6 is achieved. Inactivation of NF-κB pathway is also achieved. IL-6 is one of the proinflammatory cytokines and it is autocrine growth factor in prostate cancer. Curcumin is able to inhibit angiogenesis in prostate cancer cells, in addition to its effect on arresting cell cycle and inhibiting growth factors. As a result curcumin can significantly inhibit metastasis in prostate cancer. These effects had been shown in several studies using LNCaP xenograft, androgen sensitive and insensitive prostate cancer cells [22].

Curcumin can inhibit cell proliferation through inhibiting the expression of multiple growth factors such as VEGF and HER2. VEGF such as Avastin has a crucial role in angiogenesis. A significant inhibition of VEGF by curcumin was shown in vivo. As a result, angiogenesis can also be inhibited. Activation of HER2 is observed in prostate cancer and leads to over growth of tumor cells. HER2 activity is inhibited by curcumin

and leads to its degradation. Curcumin can also prevent its tyrosine kinase activity [95]. Curcumin is important regulator of androgen receptor in prostate cancer. In prostate cancer, abnormal expression of androgen receptor (AR) has been found. It is found that β-catenin can directly interact with AR pathway. β-catenin is one of the important genes that resulted from activating Wnt signaling pathway that can be found in many cancers including prostate. In a study using LNCaP cells by Choi H. et al, curcumin significantly inhibited androgen receptor activity. This effect was dose dependent fashion. Degradation of β-catenin was also resulted due to enhanced phosphorylation of β-catenin. It is found that GSK-3β and PI3K/Akt are up regulated in prostate cancer and they are related with increased activity of β-catenin. Curcumin could significantly inhibit these signaling pathways and activate degradation of β-catenin [96]. Many clinical trials started starting investigation of using curcumin in controlling cancer due to the many targets that curcumin can control. These targets include inhibiting the angiogenesis, cancer progression and tumor growth. These studies are done *in vitro, in vivo or ex vivo* and many attempts were presented to introduce the appropriate formula of curcumin [88].

2.5.6 Triterpenoids from various plants and wax of fruit surface

Triterpenoids are the metabolic products of isopentenyl pyrophosphate oligomers that include many types of natural compounds [97, 98]. Triterpenoids can be found in various plants such as oregano, thyme, rosemary, mistletoe, olives, cranberries, apples, blueberries, lavender and figs. They are also found in the wax in fruit peel or in other plants such as sea-weeds. More than 20,000 types of Triterpenoids are found [97]. Triterpenoids are a group of compounds that are synthesized by the cyclization of squalene [45, 99, 100], which is a triterpene compound and acts as a precursor of many steroids [45]. In Asian countries, Triterpenoids are widely used as therapeutic agents. Natural triterpenoids had shown several beneficial effects

against cancer and inflammation such as ursolic acid (UA) and oleanolic acid, but these effects were relatively insignificant [99, 100]. Synthetic oleanane triterpenoids (SO) are resulted from modifications in triterpenoids structure. Several studies had shown the significant effects of SO in controlling cancer and inflammation. Several studies reported the anti-inflammatory, antiproliferation effects of SO in cancer. SO induce apoptosis in cancer cell that are resistant to chemotherapy. They are cytoprotective and enhance differentiation in cancer cells. Several SO are known such as 2-cyano-3, 12-dioxooleana-1, 9[11]-dien-28-oic acid (CDDO) and its methyl ester (CDDO-Me). Further derivatives of CDDO were synthesized to improve their efficacy such as amides include ethyl amide (CDDO-EA) and methyl amide (CDDO-Me), dinitrile (Di-CDDO) and the imidazolides (CDDO-Im). SO are multitarget agents and that means they have several molecular mechanisms. SO can target STAT signaling, keap1 (the suppressor of Nrf2), transforming growth factor-β and IκB kinase (IKK) [101]. Synthesis of SO is important in controlling inflammation and oxidative stress, that are responsible for starting many diseases including cancer. Many studies had reported the ability of SO in suppressing the nitric oxide production that can be induced by LPS, or by interferon γ or TNFα and IL-1β. SO can activate Nrf2 signaling pathway and induce the transcription of many cytoprotective genes such as HO-1, superoxide dismutase, catalase, glutahthione-1, UDP-glucuronosyltransferases and other protective enzymes. In the resting state, Nrf2 is tolerable for degradation by proteosome. SO can directly interact with Keap1 and allows Nrf2 escape from degradation. This will induce transcription of protective genes via ARE. Many studies done *in vitro* and *in vivo* showed the ability of SO in inducing Nrf2 signaling pathway. So are linked directly between reducing oxidative stress and induced Nrf2 transcription and its related protective genes. Several studies reported that the role of SO in inhibiting the carcinogens is related to its ability in stimulating Nrf2/ARE signaling pathway [102]. Treatment with CDDO and CDDO-Im could significantly induced HO-1 expression via stimulating Nrf2/ARE signaling pathway *in vitro* and *in vivo*. In this study, breast cancer cell lines (T-47D and MCF10), lung carcinoma cells (A549) and leukemia

cells (THP-1 and U937) were used. A significant elevation in HO-1expression was shown following CDDO-Im treatment. This compound is shown to be stronger than CDDO in inducing HO-1 expression. CDDO-Im successfully could reduce the oxidative stress in U937 at different concentrations. This assay included the addition of 2′, 7′-dichlorofluorescin diacetate (H2DCFDA), and then the cells will be treated with tert-butyl hydroperoxide (tBHP). The resultant compound is 2′, 7′-dichlorofluorescein which is measured by flow cytometry. Oxidation triggered by tBHP was decreased following CDDO-Im treatment. In this study, CDDO-Im 100 nM/L showed a strong inhibition of oxidation (about 53%).This study also showed that stimulating Nrf2/ARE signaling pathway is necessary for CDDO-Im inhibition of oxidative stress. In Nrf2 deficient mice, CDDO-Im could not show the antioxidant activity. Increased expression of HO-1 is also dependent on activating Nrf2/ARE pathway [103]. CDDO-Im significantly reduced LPS-induced inflammation by activating Nrf2/ARE pathway. CDDO-Im decreased the inflammation in macrophages and neutrophils in these studies [104, 105]. LPS is an endotoxin that activates the TLR4-NF-κB pathway in macrophages and neutrophils. This will induce the expression of proinflammatory proteins that ends in cell death [105]. In these studies, treatment with CDDO-Im increased Nrf2 transcription and its related cytoprotective genes such as HO-1, NQO-1 and others. Production of ROS and inflammatory mediators were reduced by CDDO-Im. CDDO-Im significantly decreased the inflammation stimulated by LPS [104, 105]. The anti-inflammatory effect of CDDO-Im towards LPS was investigated using Nrf2 deficient mice *nrf2 -/-* and wild type animals *nrf2 +/+*. Higher release of inflammatory cytokines and chemokines was shown in nrf2 -/- neutrophils. This will suggest a remarkable inflammatory effect in these cells. Expression of many antioxidant genes is controlled by the transcription factor, Nrf2.

Treatment with CDDO-Im, a strong activator of Nrf2 could significantly stimulate expression of the antioxidant genes in *nrf2 +/+* neutrophils, but in *nrf2 -/-* neutrophils, the expression of these genes could not be induced [105]. Synthetic oleanane triterpenoids (SO) activate apoptosis pathway in different cancer cell. In leukemia and myeloma cancers, SO result in apoptosis in cancer cells but not in normal lymphocytes in these patients. Apoptosis results due to the elevated amount of ROS in cancer cells. This suggests that SO are "selective" inducers of apoptosis. Apoptosis induced by SO is found to be at (1-5) µM concentration and via different mechanisms. The main mechanism of inducing apoptosis by CDDO, CDDO-Im and CDDO-Me is through activating extrinsic and intrinsic pathways. The induced apoptosis depends on the cancer cell used. Intrinsic mechanism is mitochondrial pathway while the extrinsic pathway is death receptor pathway. These compounds can induce apoptosis by activating caspase (3, 8, and 9) and activating death receptors (4, 5). They can also stimulate apoptosis induced factor (AIF) that results in cytochrome c release following BAX transfer to the mitochondria [101]. Many studies had shown the ability of CDDO and its derivatives in inducing apoptosis in different cancer cells [106-111]. CDDO induced apoptosis in a study using osteosarcoma cells (Saos-2 and U20S) cells. Treatment with CDDO leads to activation of cascades and induced apoptosis. Release of cytochrome c was also shown. Apoptosis is started by activating caspase 8 that results in caspase 3 activation. Release of cytochrome c and cleavage of Bid was shown after caspase-8 activation [109]. CDDO-Im induced apoptosis in leukemia cells. Activation of cascade 3 is the main mechanism for apoptosis in this study. Expression of proapoptotic proteins Bax was also induced [106]. Disruption of the redox state in cells is another mechanism by which SO induce apoptosis. Activation of the c-JUN N terminal kinase JNK will be resulted and inhibiting

the antioxidant activity in the cells due to reduced level of glutathione in the mitochondria. Several events can happen and can have a role in starting the apoptosis. These events include blocking STAT signaling or NFκB and others [101]. CDDO-Im at nanomolar doses induced apoptosis in a study using pancreatic cancer cells (COLO357, PANC1). CDDO-Im also reduced the total glutathione amount in a dose and time dependent fashion. GSH is an important protein that has a role in blocking apoptosis in addition to scavenging ROS. These ROS are produced from the mitochondria during metabolism process [110]. CDDO also induced apoptosis in a study using myeloid leukemia cells (U-937, HL-60) .The mechanism of apoptosis included activation of caspase 3 cleavage through activation of caspase 8 pathway. Release of cytochrome c was also included [107]. In prostate cancer, the development of cancer was blocked following CDDO treatment in TRAMP model. Also, the growth of prostate cancer cells was inhibited in vitro and in vivo and this inhibition was induced by CDDO and its derivatives in studies using hormone sensitive and insensitive prostate cancer cells. In a study using TRAMP C1 model, CDDO-Me induced apoptosis in TRAMP-C1 cells. CDDO-Me could also inhibit the activated signaling pathways in prostate cancer (Akt, mTOR and NF-κB). CDDO-Me suppressed cancer development in the prostate. It also significantly blocked cancer spread and inhibited the metastasis in TRAMP C1 model [112].

In another study using CDDO compound in TRAMP model, CDDO was effectively inhibiting the cancer metastasis and induced apoptosis. It also inhibited angiogenesis and cell growth and thus limited carcinogenesis in the prostate. CDDO also blocked the regulatory pathways of cell growth and apoptosis (Akt and NF-κB) signaling pathways.

CDDO inhibited the levels of antiapoptotic proteins BCL-2 and BcL-xL and survivin [113].

In a study using multiple prostate cancer cell lines, CDDO and CDDO-Im and CDDO-Me were used to investigate their effects on these cells. This study involved using both hormone sensitive and insensitive prostate cancer cells. Apoptosis was induced by these compounds in all the cell lines of prostate cancer but a higher apoptotic effect was shown by CDDO-Im and CDDO-Me than CDDO. These compounds induced apoptosis through caspase-8 mediated mechanism. For CDDO-Im and CDDO, they induced apoptosis depending on the death receptors (DR4 and DR5). Very small concentrations of CDDO, CDDO-Im and CDDO-Me were toxic to prostate cancer cells, this suggested the high sensitivity of these cells to triterpenoids compounds [114]. In a study using LNCaP and PC3 cells, CDDO-Me was able to induce apoptosis in both cell lines through activating both mitochondrial and death receptor pathways. CDDO-Im blocked the pathways responsible for cell surviving. A significant inhibition of NF-κB, p-AKT and mTOR was induced by CDDO-Me. CDDO-Me also activated the production of reactive oxygen species that have a role in inducing apoptosis [115].

2.6 Summary

Prostate Cancer is the second leading cause of death in the world. Several factors contributed to the initiation and development of prostate cancer. Emergence of this disease is highly associated with type of diet used and life style. Diet rich in fat and sedentary life style can induce prostate tumorigenesis. Many studies found that healthy natural diet could reduce prostate cancer development and inhibit metastasis. Improving life style can also help in reducing incidence of cancer. In general, eating a whole balanced diet can efficiently minimize tumor growth and inhibit the development of

advanced prostate cancer. Triterpenoids specifically synthetic oleanane (SO) are shown to have antiproliferative characteristics and can induce apoptosis in prostate cancer cell lines. Furthers research is needed to examine the effects of using natural compounds on prostate cancer in vivo.

CHAPTER 3
The synthetic triterpenoids, CDDO-Im and CDDO-EA and prostate cancer prevention, involving Nrf2 signaling pathway

3.1 Objectives of the study

Tumorigenesis is associated with oxidative stress and inflammation. Cancer chemopreventive compounds can reduce the oxidative stress and retard cancer development. This biological response appears to be mainly mediated by Nrf2/ARE [nuclear factor (erythroid-derived 2)-like 2 (NFE2L2)/antioxidant response element (ARE)] pathway. Induction of phase II detoxifying/antioxidant enzymes is an important step in controlling oxidative stress and this is mediated by activation of the Nrf2/ARE pathway. The goal of this study is to investigate the effects of synthetic oleanane triterpenoids on the induction of phase II detoxifying /antioxidant enzymes in human hepatocellular carcinoma cells (HepG2-C8) and human prostate cancer cells (LNCaP). Two compounds of the synthetic oleanane triterpenoids (CDDO-Im and CDDO-EA) are used at low nano-molar concentrations. The expression of Nrf2 downstream genes are measured after CDDO-Im and CDDO-EA treatment. Cell viability assay (MTS) was used to measure the toxicity of these compounds in both cell lines. ARE luciferase activity assay was used to measure the induction folds of ARE by both compounds. Reverse-transcription-polymerase chain reaction (RT-PCR) was used to measure the mRNA level of Nrf2 and its downstream genes. Western blotting was used to measure the protein expression of heme oxygenase-1 (HO-1) and superoxide dismutase 1 (SOD1). RT-PCR was also used to measure the mRNA expression of histone deacetylases (HDACs) in LNCaP cells. The expression of HDAC1, HDAC2, HDAC3 and HDAC6 was also quantitated.

3.2 Introduction

Inflammation and oxidative stress are the major factors contributing to cancer initiation. [116]. Phase III transporters and Phase II drug metabolizing enzymes (DME) are the main cellular protecting enzymes responsible for fighting oxidative stress. Human body metabolizes the harmful substances in order to get rid of them. The metabolism process involves oxidation, reduction and conjugation. These processes are catalyzed by Phase I and II drug metabolizing enzymes. The final products are water soluble and can be removed through urine or bile [116]. The nuclear factor erythroid 2 (NF-E2) –related factor 2 (Nrf2) is an important transcriptional factor in regulating the activity of Phase II detoxifying/antioxidant enzymes [6]. Nrf2 is one of the Cap 'N' Collar (CNC) family of transcription factors. It significantly reduces the oxidative stress and induces the expression of many antioxidant genes. Nrf2 can inhibit production of reactive oxygen species and protect the DNA damage by oxidative stress, thus prevent tumorigenesis [117]. Stimulation of antioxidant genes comes through the binding of Nrf2 and Maf protein in antioxidant response element (ARE) in the promoter region in the nucleus. The most common antioxidant genes activated by Nrf2 are heme oxygenase 1 (HO-1), quinine oxidoreductase gene (NQO1), and superoxide dismutase (SOD1 and SOD2) [118].

Triterpenoids are natural compounds found in the plants and they are produced through cyclization of squalene [97, 99-101]. Squalene is a triterpene hydrocarbon and acts as a precursor for many steroids. The main source for triterpenoids are wax coatings of many fruits such as figs, apples, thyme and others. Triterpenoids are also found in the sea weed [97]. Triterpenoids such as oleanolic acid and ursolic acid are used as anticancer and anti-inflammatory agents [100]. For many years, triterpenoids were used as therapeutic

agents in traditional Asian medicine [99-101]. Many derivatives of oleanolic and ursolic acids are introduced to increase the efficacy of these triterpenoids in cancer prevention [103]. Synthetic oleanane triterpenoids (SO) include 2-cyano-3, 12-dioxooleana-1, 9(11)-dien-28-oic acid (CDDO) and its derivatives (Di-CDDO, CDDO-MA, CDDO-EA, CDDO-Im). SO are multitargeted compounds for many diseases. They exhibit many activities such as inhibition of proliferation and inflammation. They are cytoprotective compounds and can stimulate apoptosis and differentiation. These effects are shown in a dose dependent manner [101]. Different molecular targets for SO are found such as KEAP1, NFκB pathway, signal transducer and activator of transcription (STAT) signaling and transforming growth factor-β signaling (TGFβ) [101]. The effects of CDDO-Im and CDDO-EA on the Nrf2 and Nrf2-mediated gene expression are investigated in this study. Their effect on protein expression of Nrf2 mediated genes was also investigated. Their effect on HDAC1, HDAC2, HDAC3 and HDAC6 was also measured in this study.

3.3 Materials and Methods

3.3.1 Chemical Regents

1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im, Fig.2.A) and CDDO-ethyl amide (CDDO-EA, Fig.2.B) are kindly given as a gift from Michael B. Sporn, MD, Professor of Pharmacology and Medicine, Dept. of Pharmacology, Dartmouth Medical School. Sulforaphane (SFN) was purchased from LKT laboratories (catalog # S8044; St.Paul, MN, USA). Dimethysulfoxide (DMSO) was purchased from sigma-Aldrich (St.Louis, MO, USA).

3.3.2 Cell Culture

HepG2-C8 cell line was established in the laboratory of Dr. Ah-Ng Tony Kong. HepG2 cell lines are human hepatoma cells that were purchased from (American Type Culture Collection, Manassas, USA). These cells were transfected with pARE-TI-Luciferase [118]. HepG2-C8 cell lines were cultured in Dulbecco′s Modified Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) , 3.7 gm/L sodium bicarbonate, 100 units/ml penicillin and 100 µg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2. LNCaP Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 gm/L sodium bicarbonate, 100 units/ml penicillin and 100 µg/ml streptomycin at 37˚C in a humidified atmosphere containing 5% CO_2 .

3.3.3 Cell Viability (MTS) Assay

The cytotoxicity of CDDO-Im and CDDO-EA was evaluated using in HepG2-C8 cells using the cellTiter 96® Aqueous One Solution cell proliferation assay Kit [MTS: 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (Promega, Madison, WI, USA). This assay includes production of colored product (formazan) from tetrazolium compound in MTS assay. This change is highly seen in active cells and catalyzed by the NADPH or NADH via dehydrogenase enzymes. HepG2-C8 cells were seeded in 96-well plate at the density of $1x10^4$ cells /well in 100 µL of DMEM medium (1% FBS).The cells incubated overnight to allow cell attachment to the wells. Then, they were treated with different concentrations of CDDO-Im and CDDO-EA compounds for 24 hours using 1% FBS medium. After 24 hours, the medium was discarded and the cells were treated with MTS solution and incubated at 37˚C for one hour in a humidified 5% CO_2 atmosphere. The absorbance of the formazan

product was measured at 490 nm using μQuant Biomolecular Spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT, USA). LNCaP cells were seeded in 96-well plate at the density of $5x10^3$ cells /well in 100 μL using RPM1 medium (10% FBS). The cells were incubated overnight to allow cell attachment to the wells. Then, they were treated with different concentrations of CDDO-Im and CDDO-EA compounds for 3 days using 1% FBS medium and the treatment medium was changed every 2 days. On the third day, cell viability was analyzed. The cell viability was calculated as a percentage using the following equation: (absorbance in treated sample / absorbance in control) x 100 %. Three independent experiments were performed.

3.3.4 ARE reporter gene activity -Luciferase Assay

HepG2 C8 cells were seeded in 6-well plates at the density of 2×10^5 cells/well in 2 mL of medium containing 1% FBS. The cells were allowed to adhere to the wells overnight. On the next day, the cells were treated with different concentrations of CDDO-Im and CDDO-EA. The vehicle control was treated with DMSO 0.1% and the positive control was treated with 10 μM SFN. After 24 hours of treatment, cells were washed twice with ice-cold phosphate-buffered saline (1X PBS, pH 7.4) and immediately harvested with 1X Luciferase Cell Culture Lysis Buffer (Promega, Madison, USA) and kept at -20˚C overnight. The luciferase activity was determined using the Luciferase kit (Promega, Madison, USA) according to the manufacturer's instructions. On the next day, the samples were thawed and centrifuged at 12000 rpm for 5 min at 4˚C. The bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, USA) was used to determine the protein concentration. The luciferase activity was determined by taking the 10 μL of the supernatant of each sample using the SIRUS luminometer (Berthold Detection System

GmbH, Pforzheim, Germany). The results of the luciferase activity were normalized with the determined protein concentration. The luciferase activity was expressed as folds of induction compared with the control treated cells. Three independent experiments were performed.

3.3.5 Western blotting

HepG2-C8 cells were treated in the same way like in MTS and ARE experiments using 1% FBS medium. After 24 hour of treatment, the cells were washed with ice-cold phosphate-buffered saline (1X PBS, pH 7.4) and harvested using radioimmunoprecipitation assay (RIPA) buffer (Sigma–Aldrich, St. Louis, MO, USA) supplemented with protein inhibitor cocktail (Sigma–Aldrich). The homogenate was centrifuged at 4 °C, 12000 rpm for 5min. The supernatants were collected and 15 μg of total protein, as determined by BCA protein assay (Pierce, Rockford, USA), was mixed with 5 μL Laemmli's SDS-sample buffer (Boston Bioproducts Ashland, MA, USA) and denatured at 95 °C, for 5min. The proteins were separated using 4–15% SDS-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA) for 60 min at 130 mA. Then the proteins were transferred to a polyvinylidene difluoride membrane (PVDF) (Millipore, Bedford, MA, USA) followed by blocking with 5% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA, USA) in Tris-buffered saline and Tween 20 buffer (1X TBST) (Boston Bioproducts, Ashland, MA, USA) for 1 h at room temperature. Then, the membranes were sequentially incubated with specific primary antibodies (diluted 1:1000 in 3% BSA in 1X TBST) overnight at 4 C. The primary antibodies against Actin (catalog No. sc-1616), HO-1 (catalog No. sc-7695), and SOD-1 (catalog No. sc-11407) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz,

CA, USA) and were used. After incubation with the primary antibodies, the membrane was washed with 1X TBST four times at room temperature and each time for 15 minutes. Then, the membrane was incubated with the secondary antibody (1:5000 dilution in 3% BSA in 1X TBST; Santa Cruz Biotechnology, Inc., CA, USA) for 1 h at room temperature. Then, 1X TBST was used to wash the membrane four times at room temperature. The blots were visualized using an enhanced chemiluminescence system (Thermo Scientific, Rockford, IL, USA) and Supersignal West Femto chemiluminescent substrate (Pierce, Rockford, IL, USA). The blots were visualized and captured using the BioRad ChemiDox XRS system (Hercules, CA, USA). Densitometry of the bands was analyzed using ImageJ (version 1.44, National Institute of Health, USA). The relative intensity of the protein was determined by calculating the intensity of the protein compared with 0.1% DMSO, and subsequently the band intensity was normalized with the actin intensity for each sample. Three independent experiments were performed.

3.3.6 RNA Extraction and quantitative real-time polymerase chain reaction (qPCR)

HepG2-C8 cells were seeded in the same manner in the luciferase activity experiments. The cells were seeded in 6-well plates at the density of 2 x 10^5 cells/well using 1% FBS medium. The cells were allowed to adhere to the wells during the night. On the next day, cells were treated with different concentrations of CDDO-Im and CDDO-EA for 6 h. The RNA mini Kit (Qiagen, Valencia, CA, USA) was used to extract the RNA samples according to the manufacturer's instructions. The RNA concentration was determined. TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) were used to reverse transcribe RNA to cDNA. The gene expression was determined using

qPCR on an ABI7900HT system (Applied Biosystems) and SYBR Green PCR Master Mix (Applied Biosystems Inc, Foster City, CA). On the other hand, human Prostate Cancer cells (LNCaP) were seeded in 10-cm dish and they were treated on the next day with different concentrations of CDDO-Im and CDDO-EA. The medium was changed every 2 days and on the third day, cells were collected to extract RNA. The gene expression of Nrf2, HO-1, NQO1, SOD1, UDP-Glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1), glutathione S-transferase mu 2 (GSTm2), and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (as an internal standard) were determined. The gene expression of histone deacetylases (HDAC1, 2, 3 and 6) was also determined in LNCaP cells. The primer pairs of (Nrf2, HO-1, NQO-1, SOD1, UGT1A1 and GSTm2) were designed using the Primer Quest Oligo Design and Analysis Tool (Integrated DNA Technologies, Inc., Coralville, IA, USA), and the primer sequences are listed in Table 2. The primer sequences of HDAC1, HDAC2, HDAC3 and HDAC6 are listed in Table 3. Three independent experiments were performed.

3.3.7 Data presentation and statistical analysis

The data are presented as the mean ± standard error of the mean (SEM) and three independent experiments were performed for each analysis. The data were analyzed using student′s t-test. P value less than 0.05 were considered statistically significant and are indicated with *, P values less than 0.01 were considered very significant and are indicated with **.

3.4 Results

3.4.1 Effects of triterpenoids (CDDO-Im and CDDO-EA) treatment on the cell viability

To test the cytotoxicity of CDDO-Im and CDDO-EA compounds, MTS assay was used. Viable cells produce formazan and increase the absorbance at 490 nm. The effect of the compounds on the cell viability was dose dependent. The compounds are relatively non-toxic for HepG2-C8 cells after 24 h of treatment (Fig.3.a). For LNCaP cells, treatment with both compounds at concentration lower than 50 nM was also non toxic after 3 days of treatment whereas higher concentrations of both compounds exhibit toxicity (Fig.5.b). Specifically, CDDO-Im and CDDO-EA are toxic at ≥ 100 nM and ≥ 750 nM in LNCaP respectively. The cell viability was significantly inhibited by CDDO-EA at 750 nM and by CDDO-Im at (250, 750 and 1000) nM concentrations. CDDO-Im and CDDO-EA at concentrations lower than 100 nm were unable to inhibit 50% of the cell viability. Both of CDDO-Im and CDDO-EA exhibit toxicity at ≥100 nM and reduce the cell viability in LNCaP cells (except CDDO-EA at 250 nM, the cell viability was higher than at 100 nM). Two to three non-toxic concentrations were selected for the next experiments.

3.4.2 Induction of ARE-Luciferase activity by CDDO-Im and CDDO-EA is determined by the luciferase reporter gene assay

ARE-Luciferase reporter assay was used to test the ability of compounds in activating transcription of ARE. HepG2-C8 cells were treated with different concentrations of CDDO-Im and CDDO-EA. As shown in (Fig.4), the tested compounds activated ARE in a dose dependent manner. The maximum fold induction was shown with CDDO-EA at 500 nM (fold= 13.43 ±3.08, $p < 0.05$). This ARE induction is higher than the fold induction elicited by CDDO-Im at this concentration. CDDO-Im at 50 nM significantly activated ARE by 9.51± 0.73 fold compared with 0.1% DMSO ($P < 0.01$) indicating that CDDO-Im is very potent in inducing ARE induction folds at 50 nM. At this

concentration, ARE induction fold is higher than the fold induced by CDDO-EA at the same concentration (5.84 ± 0.05). CDDO-Im presented a very significant and similar ARE induction folds at 25 nM and 50 nM concentration (9.33± 0.51) and (9.51 ±0.73) ($P<0.01$) respectively. CDDO-EA significantly activated ARE at 100 nM and 250 nM concentration (7.02± 0.18) and (7.87 ± 1.58) respectively. Based on the result of ARE induction, two to three concentrations were selected to be used in qPCR and western blotting.

3.4.3 Induction of the Nrf2 downstream proteins expression by Western blotting

The effect of the compounds on the expression of the downstream proteins in Nrf2-ARE pathway was determined using Western blotting. Actin was used as the endogenous house keeping control (Fig.5). SOD1 and HO-1 are selected as representative downstream proteins in the Nrf2-ARE pathway and their expression was determined by Western blotting. A significant induction of HO-1 protein expression was shown by both CDDO-Im and CDDO-EA. CDDO-EA at concentration of 500 nM showed an induction of the protein expression of HO-1 (fold = 6). For CDDO-Im, HO-1 expression was significantly increased by 4 fold compared with 0.1% DMSO at 25 nM. CDDO-Im also activated SOD1 protein expression by 2 fold at the concentration of 25 nM and CDDO-EA activated SOD-1 protein expression by 1.26 fold at 500 nM. The low concentration of both compounds also activated the protein expression of HO-1 and SOD1.

3.4.4 Induction of mRNA Expression of Nrf2 and Nrf2-Target Genes as determined by RT-PCR

The ability of these compounds in inducing ARE-Nrf2 mediated mRNA expression was evaluated by using qPCR (Fig.6). Values higher than one were considered positive in

comparison to the cells treated with 0.1% DMSO. Both CDDO-Im and CDDO-EA induced the expression of Nrf2, HO-1, NQO-1 and SOD1 in HepG2-C8 cells. The expression of Nrf2 mRNA by CDDO-Im and CDDO-EA increased in a dose dependent manner. Nrf2 induction was increased by (1.93±0.57) by CDDO-Im at 25 nM. The expression of Nrf2 increased by (2.78 ± 1.16) by CDDO-EA at 500 nM. The induction of mRNA expression of HO-1 by both compounds was statistically significant. The increased HO-1 expression was also in a dose dependent manner. CDDO-Im induced a significant increase in SOD1 expression at 25 nM by (1.29± 0.06). CDDO-EA also induced SOD1 expression in a dose dependent manner. CDDO-EA at 500 nm induced high expression of SOD1 by (1.61±0.59). There was also an increase in NQO-1 expression by both compounds but this increase did not change with increasing compound concentration. Both compounds also increased the expression of UGT1A1and CDDO-Im significantly induced the expression of UGT1A1 at 10 nM. At this concentration the expression was increased by (1.31 ± 0.41). CDDO-Im and CDDO-EA induced GSTm2 expression. The increase in GSTm2 expression was dose dependent manner by CDDO-Im. At 25 nM of CDDO-Im, GSTm2 expression increased by (1.45± 0.48. This difference between the ARE results by luciferase reporter gene assay and the variable induction of the mRNA of Nrf2 downstream genes can be explained by inconsistency in the kinetics of the gene transcription [119]. Human Prostate cancer cell line (LNCaP) cells were treated to investigate the expression of endogenous Nrf2 expression and other antioxidant enzymes including (HO-1 and NQO-1 and SOD1). After 3 days of treatment, the results show that the expression of Nrf2 was increased compared with the control. CDDO-Im activated Nrf2 expression higher than found with CDDO-EA at 25 nM. High expression of both HO-1 and NQO-1 was determined in LNCaP cells.

CDDO-Im increased the expression of HO-1 and NQO-1 higher than found with CDDO-EA. The expression of SOD1 was not changed in LNCaP cells treated with CDDO-Im and CDDO-EA. The expression of HDAC (1-3) and HDAC6 were also determined in LNCaP cells. It is found that both compounds can inhibit HDAC1 expression compared with the control. A significant inhibition was shown by CDDO-Im at 5 nM (0.65±0.23) and by CDDO-EA at 25 nM (0.85 ±0.20). The expression of HDAC2 was significantly inhibited by both compounds. Inhibition of HDAC2 expression was dose dependent. Significant inhibition of HDAC3 was found with CDDO-Im at 5 nM (0.66±0.11) and CDDO-EA at 25 nM (0.72±0.03). HDAC6 expression was also decreased by CDDO-Im and CDDO-EA. A significant decrease in HDAC6 mRNA expression was shown with CDDO-EA at 25 nM.

3.5 Discussion

Activation of Nrf2 signaling pathway is required for anti-inflammatory action of synthetic oleanane triterpenoids in immune cells. These effects could not be found in *Nrf2*$^{-/-}$ fibroblast [101]. During oxidative stress or exposure to toxins, Nrf2 can activate the transcription of many genes that help in detoxifying the effects of chemicals. Protection from inflammation and many diseases including cancer can be regulated by the transcription factor, Nrf2 [117]. A study in our lab demonstrated that Nrf2 expression was reduced in prostate cancer in TRAMP mice and in LNCaP cells. Low expression of Nrf2 could increase DNA damage and ends in tumorigenesis. Epigenetic modification of Nrf2 expression could regulate its expression in prostate cancer. Such modifications include DNA methylation and histone modification. This study also showed that the expression of Nrf2 and Nrf2 –mediated genes such as NQO-1 and HO-1 were also

decreased in LNCaP. Nrf2 expression was restored after treatment with (5-aza/TSA) in TRAMP C1. 5-aza-2′-deoxycytidine (5-aza) is a DNA methyltransferrase (DNMT) inhibitor. Trichostatin (TSA) is an inhibitor of histone deacetylases (HDAC) [120].

Class I HDAC activity was shown to be over expressed in adenocarcinoma in prostate cancer. The HDACs include HDAC1, HDAC2 and HDAC3, are highly expressed in prostate cancer [121]. Uncontrolled cell growth can be attributed to the over-expression of HDAC2. HDAC3 could also responsible for tumor growth. Differentiation in prostate cancer is related with high HDAC1 activity [121]. In our study, human prostate cancer cells were treated with CDDO-Im and CDDO-EA for three days. These compounds appeared to be effective in inhibiting HDAC (1-3) mRNA expression at 5 nM. Similarly the expression of HDAC6 appeared to be also reduced by both compounds, but not statistically significance except CDDO-EA at 25 nM.

Several studies found that the concentration of SO in inducing the expression of cytoprotective genes via Nrf2 signaling pathway was the same concentration used in inhibition of inflammation. These cytoprotective genes include many genes such asHO-1, super dismutase and others [102]. In this study, CDDO-Im and CDDO-EA induced the expression of Nrf2 and Nrf2-downstream genes including HO-1, NQO-1, SOD1, UGT1A1 and GSTm2. Expression of these genes is increased at the mRNA level. The expression of Nrf2, HO-1 and NQO-1 was increased in HepG2 cells and LNCaP cells. The expression of UGT1A1 and GSTm2 was also increased by both compounds. The expression of SOD1 was induced on the mRNA and the protein level in HepG2-C8 cells although this induction cannot be shown in LNCaP cells. A study using CDDO and CDDO-Im on human leukemia cells, found that CDDO-Im is more potent than CDDO in

inducing HO-1 on the mRNA and protein levels. The HO-1 mRNA expression was significantly increased by CDDO-Im at 100 nM concentration. The expression of HO-1 protein was also induced at (10-100) nM of CDDO-Im and at (30-300) nM of CDDO [103]. In our study, both of CDDO-Im and CDDO-EA induced HO-1 expression (Fig.6 and Fig.7). The protein expression of HO-1 increased in a dose-dependent manner (Fig.5). This is in consistent with the previous study of using CDDO-Im in inducing the cytoprotective gene expression (HO-1). Both CDDO and CDDO-Im are powerful compounds in inducing the expression of many cytoprotective genes and inhibit development of many diseases including cancer [103]. In our study, both CDDO-Im and CDDO-EA activated Nrf2/ARE signaling pathway. They induced the expression of the cytoprotective genes such as Nrf2, HO-1, NQO-1 and SOD1. Expression of UGT1A1 and GSTm2 was also induced. Also, in our study, CDDO-Im and CDDO-EA can inhibit HDAC1, HDAC2, HDAC3 and HDAC6 expression at low concentration and this suggests that these compounds can exhibit epigenetic regulation characteristics but further experiments are needed to confirm the relationship between these compounds and HDAC expression.

Chapter 4

Conclusion

Prostate cancer is a very serious problem and new cases are developing annually. The need for efficient pathway is highly increased due to the high mortality rate from this disease. High fat and caloric diet and reduced physical activity can be important factors in developing prostate cancer. These include the major risk factors that help in initiating prostate cancer. Modifications of these factors can help in reducing the incidence of this disease. High consumption of fruits and vegetables can help in combat oxidative stress and cancer. The Nrf2 transcriptional factor is the main regulator of the phase II detoxifying / antioxidant enzymes. Activation of Nrf2 can take place following exposure to toxins, oxidative stress or exposure to phytochemicals. Transcription of many genes will be stimulated following Nrf2 activation. These genes include NQO-1, HO-1 and SOD1 and others. Nuclear translocation of Nrf2 during activation is an important step in fighting oxidative stress and stimulating gene transcription. Prostate cancer chemoprevention includes using natural or synthetic compounds that help in inhibiting cancer development or reducing tumor progression. Many natural phytochemicals are found to be very efficient in prevention from prostate Cancer. These compounds are highly found in the plants, fruits and vegetables. They have different mechanism of action. Synthetic oleanane triterpenoids are very promising compounds in cancer prevention. These compounds are more powerful than natural triterpenoids against inflammation. Synthetic oleanane triterpenoids include CDDO and related derivatives including (CDDO-Im and CDDO-EA). Several studies were done to investigate the mechanism of action of these compounds. These compounds are multitargeted and can

exert various mechanisms in malignant cells. One of the important mechanisms is Nrf2/Keap1 signaling pathway. They activate Nrf2 transcription and its mediated genes by inhibiting the protein Keap1. They are very effective in controlling inflammation and oxidative stress. As discussed earlier in the thesis, CDDO –Im and CDDO-EA can interact with Keap1 and induce the transcription of Nrf2 factor. As a result, the expression of many cytoprotective genes will be stimulated. Examples of these important cytoprotective genes are HO-1 and SOD1. In our study, low nanomolar concentration of CDDO-Im and CDDO-EA were used in HepG2 cells and can activate Nrf2 and Nrf2 mediated genes including HO-1, NQO-1, SOD1, UGT1A1 and GSTm2. The expression of HO-1 and NQO-1 was highly found in these cells. This comes in consistent with other studies that proved the ability of CDDO-Im on inducing Nrf2 and Nrf2downstream genes [104]. The expression of HO-1 was higher than the expression of other genes induced by both compounds. This can also confirms previous study using CDDO-Im in stimulating HO-1 expression [103]. In our study, CDDO-EA can induce the expression of HO-1 in both cell lines. The expression of Nrf2 in human prostate cancer cells is silenced due to dysregulation of Nrf2 expression. This can lead to increase DNA damage and enhance tumorigenesis [120]. It is found that the HDAC expression can be significantly increased in prostate cancer cells [122]. In our study, both of CDDO-Im and CDDO-EA can inhibit HDAC (1-3) and HDAC6. This inhibiting effect suggests potential epigenetic modification properties. Western blotting will be needed to confirm their ability of suppressing HDACs activity. Further research should be done on these compounds to investigate their mechanism on DNA methylation and histone modifications in prostate cancer.

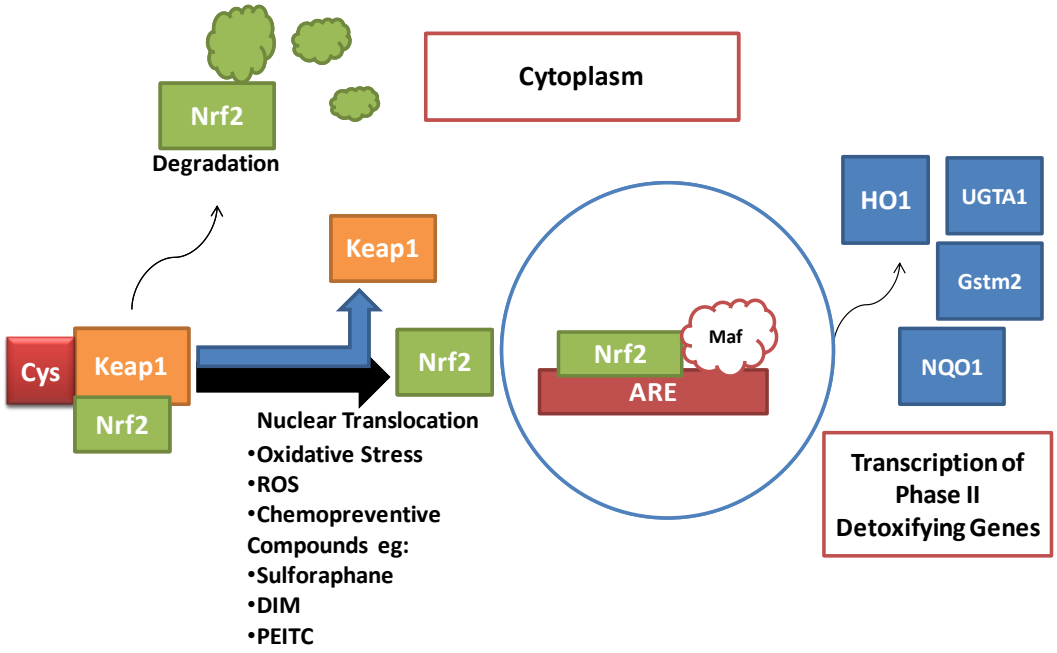


Figure.1 Nrf2-ARE Transcription Pathway. Under basal conditions, Nrf2 is sequestered in the cytoplasm by binding with keap1 and forming a complex. Nuclear translocation of Nrf2 occurs when cysteine residues in keap1 is irritated by oxidants or electrophiles. Nrf2 starts transcription of antioxidant genes by heterodimerizing with small Maf proteins and attaching to ARE in the promoter area of the antioxidant genes such as HO-1, NQO1, Gstm2 and UGT1A1. Exposure to certain phytochemicals can also induce expression of phase II detoxifying/antioxidant enzymes such as SFN, PEITC and DIM

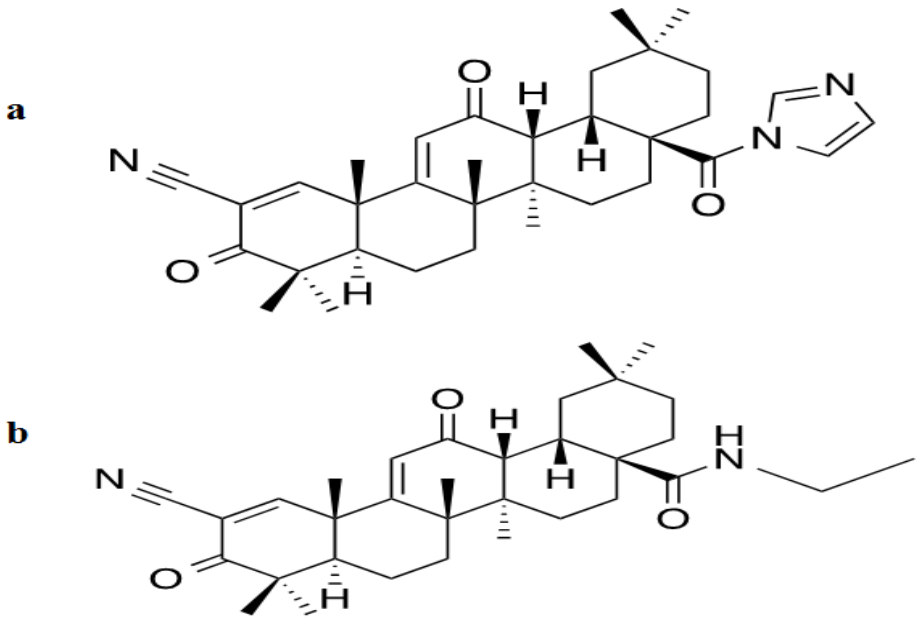


Figure.2 Chemical structures of triterpenoids used in the study. **(a)** CDDO-Im **(b)** CDDO-EA

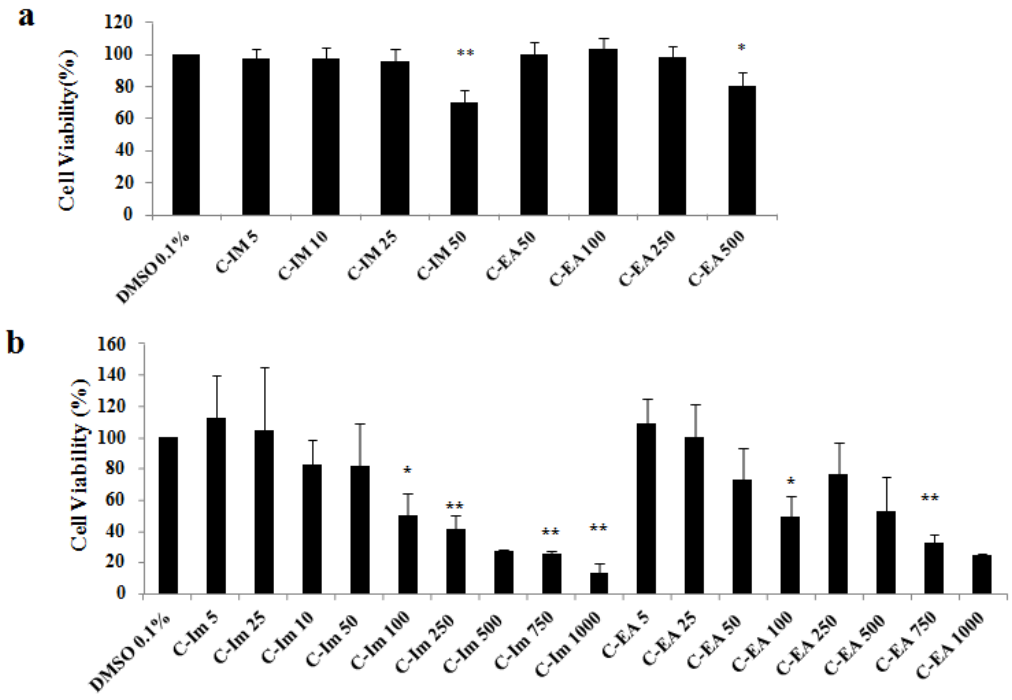


Figure.3 The effect of CDDO-Im and CDDO-EA on the cell viability as determined by MTS assay. **(a)** HepG2-C8 cells were treated with different concentrations of the compounds for 24 h. **(b)** LNCaP cells were treated with different concentrations of the compounds for 3 days. The results are expressed as the mean ± SEM (n=3). * $P < 0.05$, ** $P < 0.01$ compared with cells treated with 0.1% DMSO (negative control). All concentrations are in nM. C-Im: CDDO-Im, C-ea: CDDO-EA

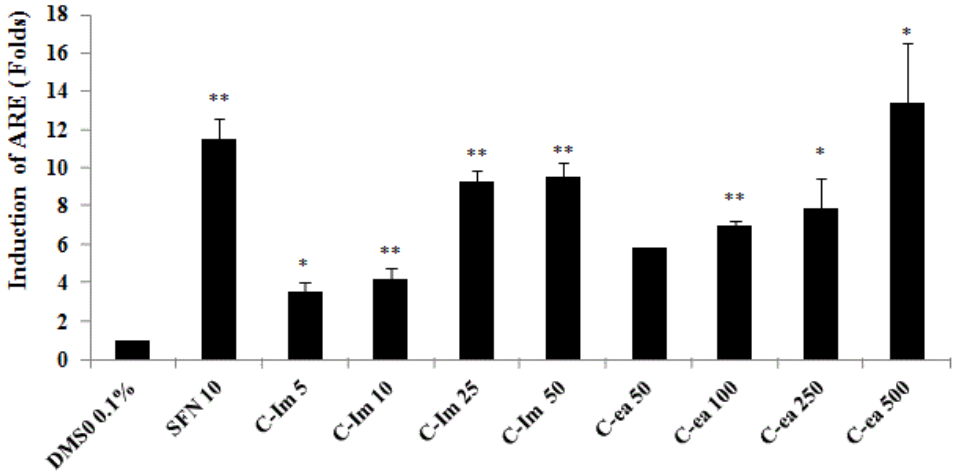


Figure.4 ARE Induction by CDDO-Im and CDDO-EA is measured by Luciferase reporter assay. HepG2-C8 cells were treated with compounds for 24 h. All samples were normalized to protein concentration. Fold induction was obtained by normalizing each sample with the vehicle control.0.1% DMSO was used as the vehicle control and 10 μM SFN was used as the positive control. The data are presented as mean ± SEM (n=3). * P< 0.05, ** P< 0.01 compared with the cells treated with 0.1% DMSO. C-Im: CDDO-Im, C-ea: CDDO-EA. All concentrations are in nM except SFN is μM.

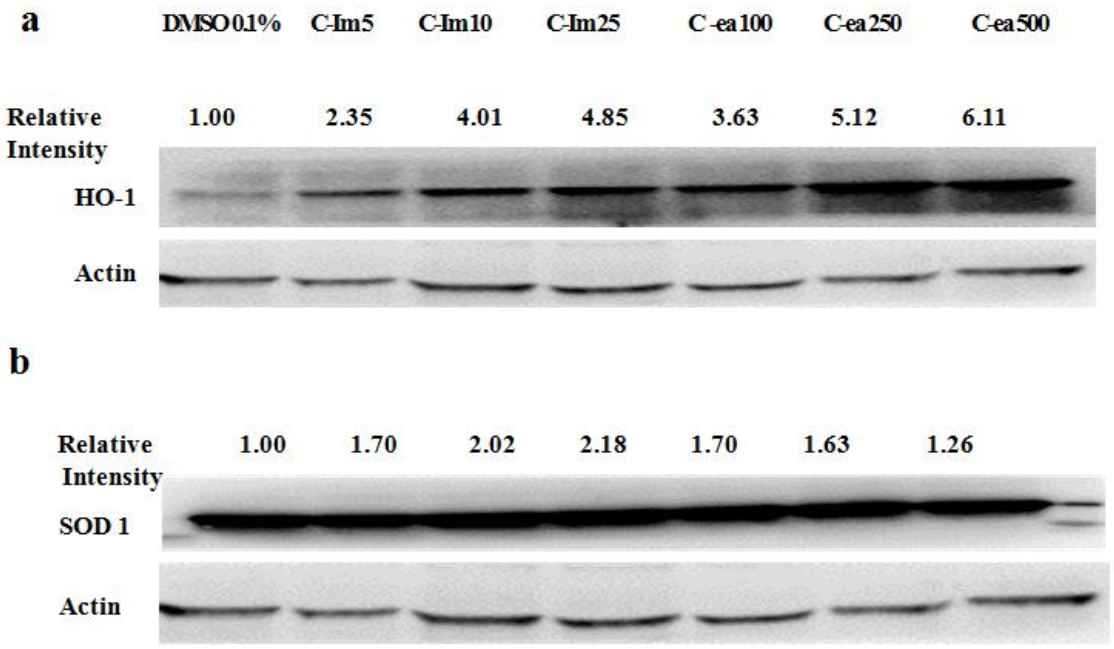


Figure.5 Protein Expression in HepG2-Ce8 cells by Western Blotting after 24 h of treatment. Actin was used as endogenous housekeeping protein. (a) HO-1 protein expression. (b) SOD1 protein expression. 15 µg of protein was analyzed and relative intensity was determined by dividing the intensity of each sample by that of 0.1% DMSO and normalizing to the intensity of actin using image J software. Representative images of three independent experiments are presented. C-Im: CDDO-Im, C-ea :CDDO-EA. All concentrations are in nM.

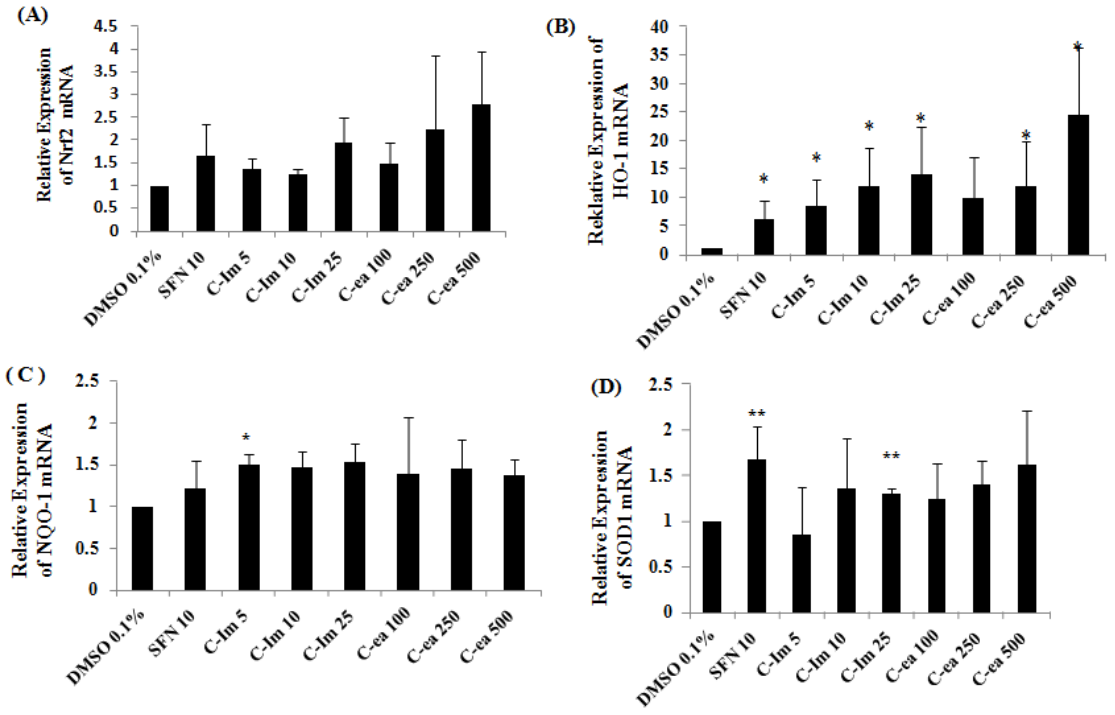


Figure.6 Induction of mRNA expression of Nrf2 and downstream genes in HepG2-C8 cells. HepG2-C8 cells were treated with CDDO-Im and CDDO-EA for 6 h. The induction of Nrf2 (A), HO-1 (B), NQO-1 (C), SOD1 (D) was normalized to the vehicle control (0.1% DMSO) and expressed as the fold change using GAPDH as the endogenous housekeeping gene. The data are presented as the mean ± SEM (n=3). 0.1% DMSO was used as the negative control and 10 µM SFN was used as the positive control * $P < 0.05$, ** $P < 0.01$ compared with cells treated with 0.1% DMSO. C-Im: CDDO-Im, C-ea: CDDO-EA. All concentrations are in nM except SFN in µM.

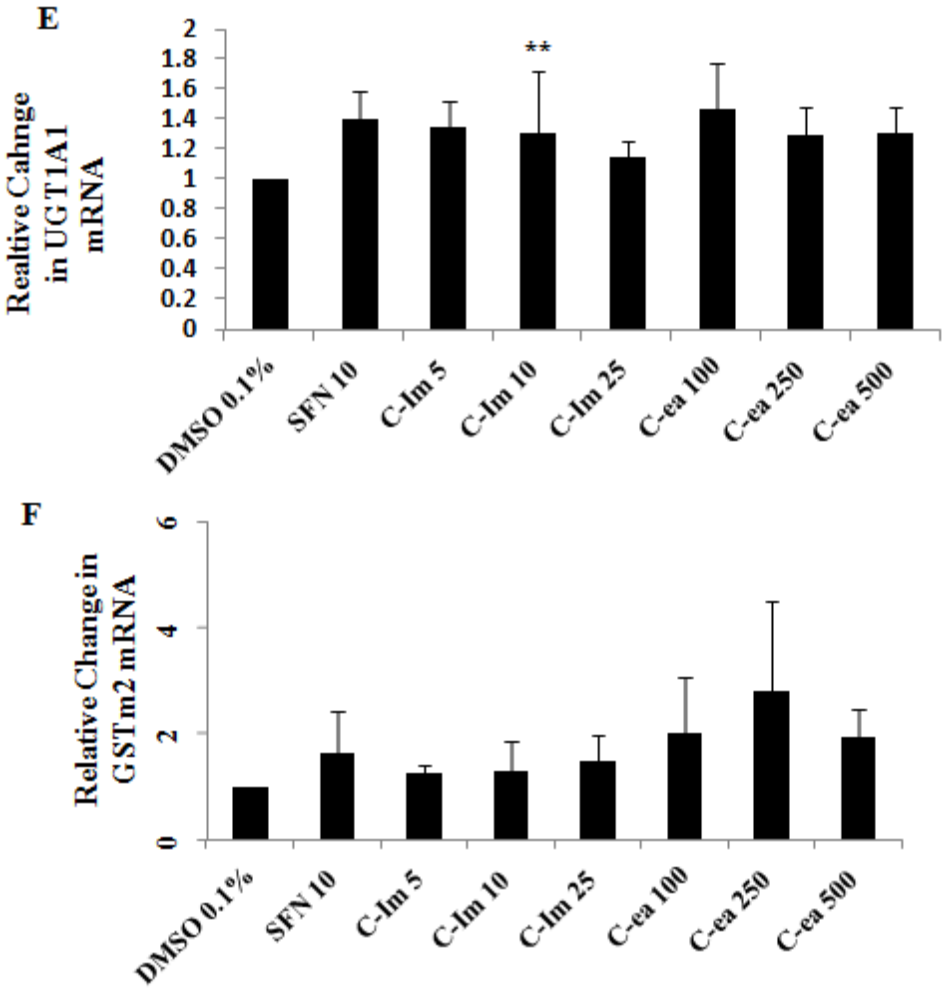


Figure.6 (Cont'd) Induction of mRNA expression of Nrf2 and downstream genes in HepG2-C8 cells. HepG2-C8 cells were treated with CDDO-Im and CDDO-EA for 6 h. The induction of UGT1A1 (E) and GSTm2 (F) was normalized to the vehicle control (0.1% DMSO) and expressed as the fold change using GAPDH as the endogenous housekeeping gene. The data are presented as the mean ± SEM (n=3) 0.1% DMSO was used as the negative control and 10 µM SFN was used as the positive control. * $P < 0.05$, ** $P < 0.01$ compared with cells treated with 0.1% DMSO. C-Im: CDDO-Im, C-ea: CDDO-EA. All concentrations are in nM except SFN in µM.

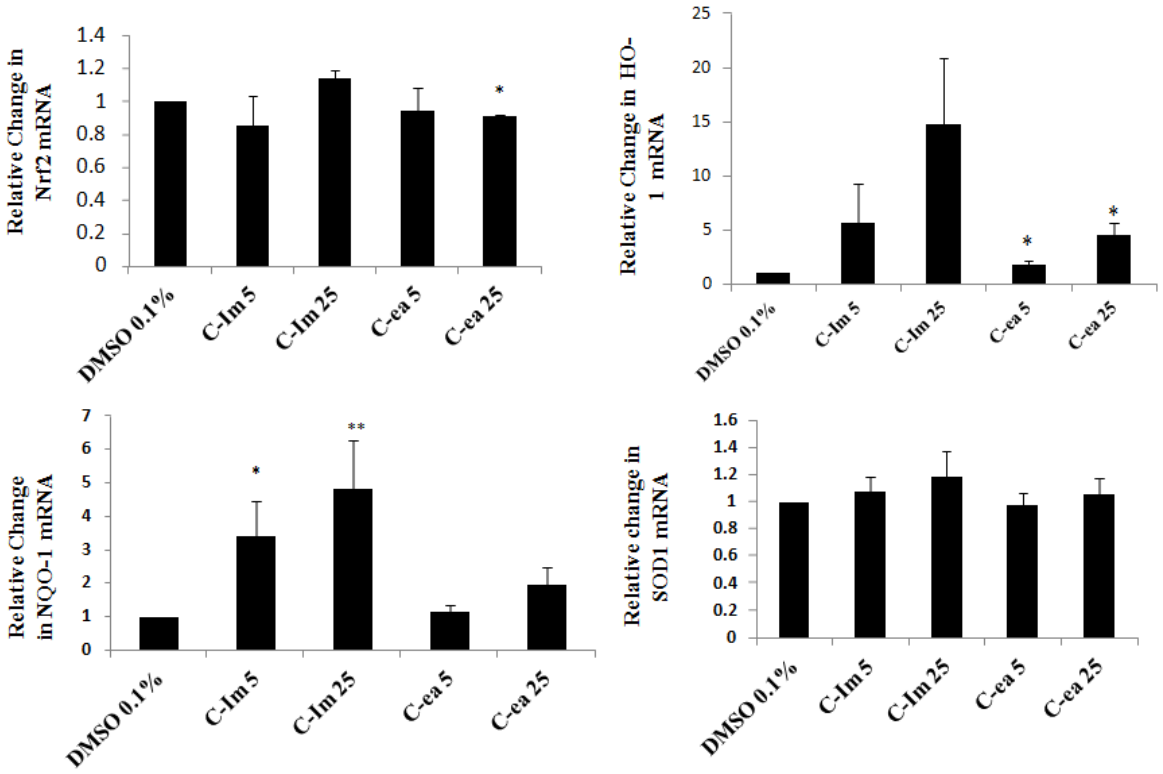


Figure.7 Induction of mRNA expression of Nrf2 and downstream genes in LNCaP cells. LNCaP cells were treated with CDDO-Im and CDDO-EA for 3 days. The induction of Nrf2, HO-1, NQO-1 and SOD1 was normalized to the vehicle control (0.1% DMSO) and expressed as the fold change using GAPDH as the endogenous housekeeping gene. The data are presented as the mean ± SEM (n=3) 0.1% DMSO was used as the negative control. * $P < 0.05$, ** $P < 0.01$ compared with cells treated with 0.1% DMSO. C-Im: CDDO-Im, C-ea: CDDO-EA. All concentrations are in nM.

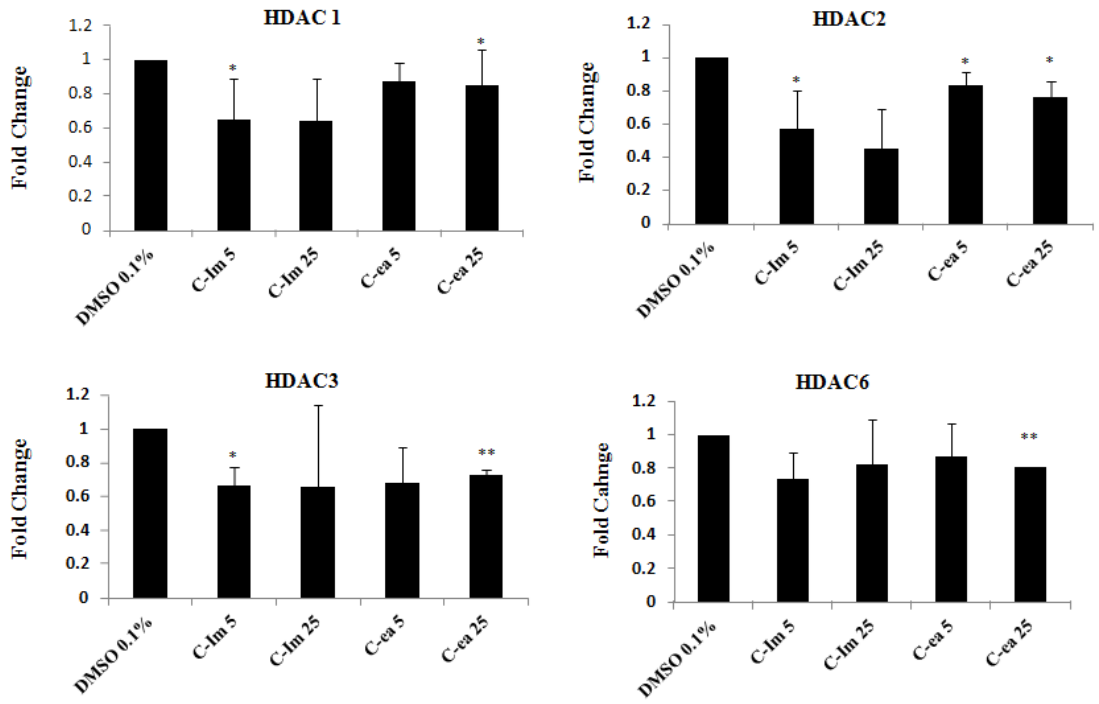
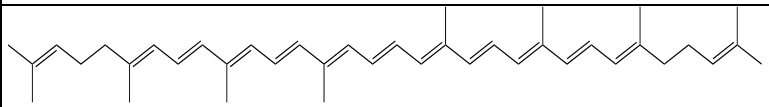
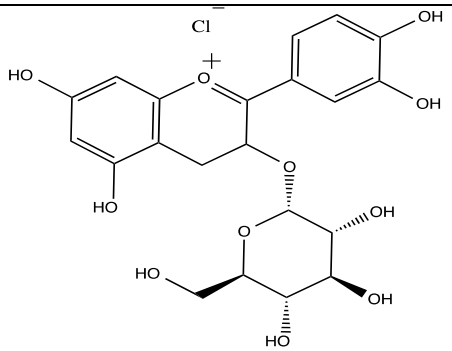
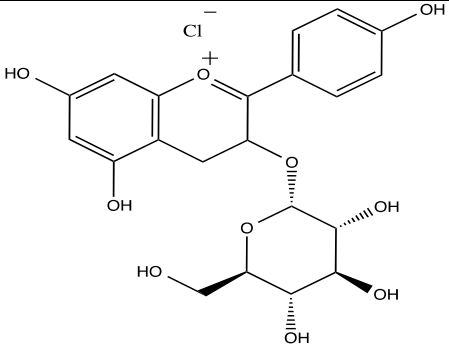
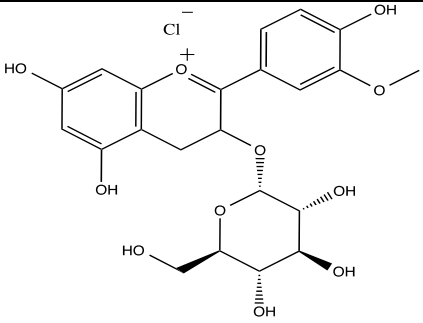
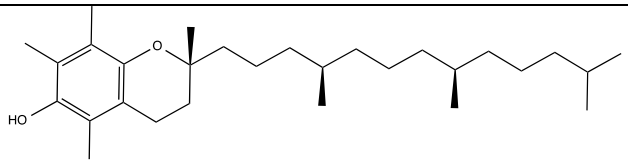
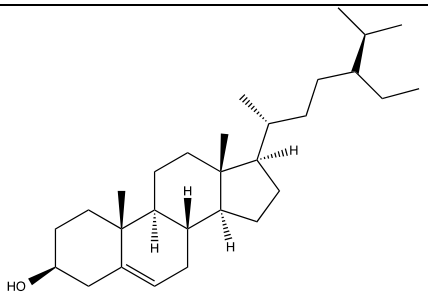
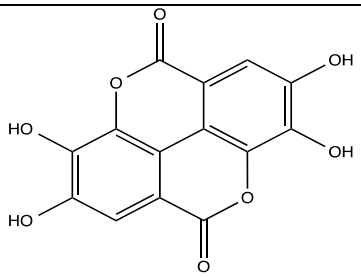
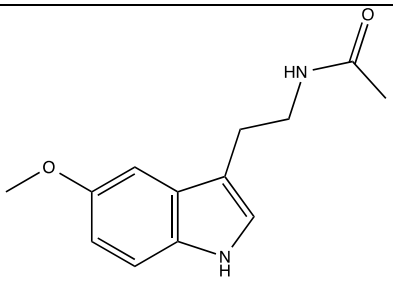
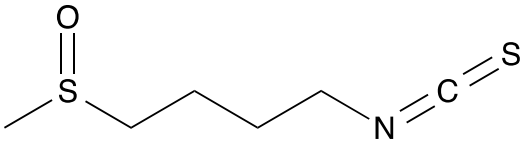
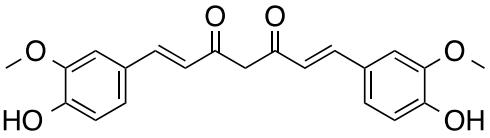
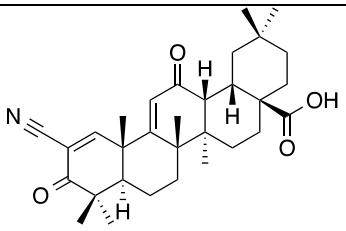
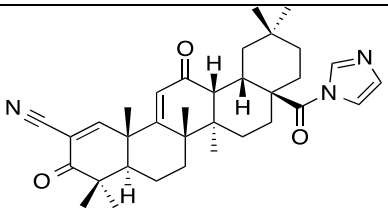


Figure.8 mRNA expression of HDACs (1, 2, 3 and 6) in LNCaP cells. LNCaP cells were treated with CDDO-Im and CDDO-EA for 3 days. The induction of HDACs (1, 2, 3 and 6) was normalized to the vehicle control (0.1% DMSO) and expressed as the fold change using GAPDH as the endogenous housekeeping gene. The data are presented as the mean ± SEM (n=3). 0.1% DMSO was used as the negative control * $P < 0.05$, ** $P < 0.01$ compared with cells treated with 0.1% DMSO. C-Im: CDDO-Im, C-ea: CDDO-EA. All concentrations are in nM.

Table. 1

Chemical structures of the phytochemicals

Compound	Chemical Structure
Lycopene	
Cyannidin-3-glucoside (C3G)	
Pelargonidin-3-glucoside (Pg3G)	
Peonidin-3-glucoside (P3G)	
Alpha-Tocopherol	

Beta – Sitosterol	
Ellagic acid	
Melatonin	
Sulforaphane (SFN)	
Curcumin	
CDDO	
CDDO-Im	

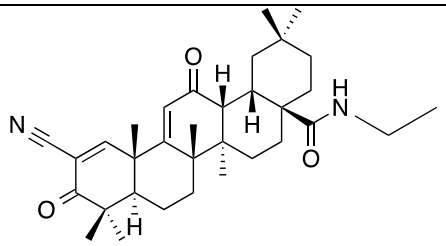
CDDO-EA	

Table .2

Human oligonucleotide primers used for qPCR

Gene	Forward (5') primer	Reverse (3') primer
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	5'-TCG ACA GTC AGC CGC ATC TTC TTT-3'	5'-ACC AAA TCC GTT GAC TCC GAC CTT-3'
NAD(P)H dehydrogenase, quinone 1 (NQO1)	5'-AAG GAT GGA AGA AAC GCC TGG AGA-3'	5'-GGC CCA CAG AAA GGC CAA ATT TCT-3'
Glutathione S-transferase mu 2 (GSTm2)	5'-ACT AAA GCC AGC CTG ACC TTC CTT-3'	5'-AAT GCT GCT CCT TCA TGC AAC ACG-3'
Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)	5'-TGC TTT ATA GCG TGC AAA CCT CGC-3'	5'-ATC CAT GTC CCT TGA CAG CAC AGA-3'
Hemeoxygenase-1 (HO-1)	5'- ACG CGT TGT AAT TAA GCC TCG CAC-3'	5'-TTC CGC TGG TCA TTA AGG CTG AGT-3'
UDP-Glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1)	5'-ATG ACC CGT GCC TTT ATC ACC CAT-3'	5'-AGT CTC CAT GCG CTT TGC ATT GTC -3'
Superoxide dismutase 1 (SOD1)	5'-GCA GGG CAT CAT CAA TTT CGA GCA-3'	5'- TGC AGG CCT TCA GTC AGT CCT TTA-3'

Table .3

Human oligonucleotide primers of histone deacetylases (HDACs) used for qPCR

Gene	Forward (5') primer	Reverse (3') primer
HDAC1	5′- GCT GGC AAA GGC AAG TAT TAT G -3′	5′- CTA GGC TGG AAC ATC TCC ATT AC -′3
HDAC2	5'- GCT ACT ACT ACG ACG GTG ATA TTG -3'	5'- GTT ATG GGT CAT GCG GAT TCT A -3'
HDAC3	5'- GAC GGT GTC CTT CCA CAA ATA -3'	5'- GGC ACG TTC AGA CAG TAG TAG -3'
HDAC6	5'- GGA GGG TCC TTA TCG TAG ATT G -3'	5'- GTA GCG GTG GAT GGA GAA ATA G -3'

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