Development of cucurbitacin derivative through *in-silico* and *in-vitro* methods to control prostate cancer cell proliferation

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MASTER OF PHARMACY
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Submitted By
A. Balasachidanandam

(Reg. No. 261525902)

Under the Guidance of

Dr. M. Ramanathan, M. Pharm, Ph.D.,

Department of Pharmacology
PSG COLLEGE OF PHARMACY
PEELAMEDU,
COIMBATORE-641 004
MAY-2017

Dr. M. Ramanathan, M. Pharm., Ph.D.,
Principal,
PSG College of Pharmacy,
Peelamedu,
Coimbatore - 641 004.

CERTIFICATE

This is to certify that the dissertation work entitled "**Development of cucurbitacin derivative through *in-silico* and *in-vitro* methods to control prostate cancer cell proliferation**" submitted by University Reg. No**.** 261525902 is a bonafide work carried out by the candidate under the guidance of **Dr. M. Ramanathan, M. Pharm., Ph.D.,** and submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmacology** at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2016-2017.

Dr. M. Ramanathan, M. Pharm., Ph.D.,
Principal

DECLARATION

I do hereby declare that the dissertation work entitled "**Development of cucurbitacin derivative through *in-silico* and *in-vitro* methods to control prostate cancer cell proliferation**" submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of Master of Pharmacy in Pharmacology, was done by me under the guidance of **Dr. M. Ramanathan, M. Pharm., Ph.D.,** at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore, during the academic year 2016-2017.

Reg No. 261525902

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled "**Development of cucurbitacin derivative through *in-silico* and *in-vitro* methods to control prostate cancer cell proliferation**" submitted by University Reg. No 261525902 to the Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmacology** is a bonafide work carried out by the candidate at the Department of Pharmacology, PSG College of Pharmacy, Coimbatore and was evaluated by us during the May 2017.

Examination Center: PSG College of Pharmacy, Coimbatore.

Date:

Internal Examiner **External Examiner**

Dedicated

To

Beloved Parents,

Respectful Guide,

&

The Almighty

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ABBREVIATIONS

DHT	: Dihydrotestosterone
AR	: Androgen receptor
ADT	: Androgen deprivation therapy
CAB	: Combined androgen blockade therapy
GnRH	: Gonadotropin releasing hormone
LHRH	: Lutenizing hormone releasing hormone
PSA	: Prostate specific antigen
LNCaP	: Lymph node carcinoma of the prostate
PI3K	: Phosphoinositide 3-kinase
Bcl2	: B-cell lymphoma 2
Bax	: Bcl2 associated X protein
JAK/STAT	: Janus kinase/signal transducer and activators of transcription
LBD	: Ligand binding domain
TAU	: Transactivation units
AF	: Activation function
LBP	: Ligand binding pocket
HSP	: Heat shock proteins
PDB	: Protein Data Bank
ER β	: Estrogen receptor β
TNF	: Tumour necrosis factor
TST	: Testosterone
APAF	: Apoptotic protease activating factor
ADMET	Absorption, distribution, metabolism, elimination and toxicity

DMEM	: Dulbecco's modified eagle medium
RPMI	: Roswell park memorial institute
Asn	: Asparagine
Gln	: Glutamine
Arg	: Arginine
Leu	: Leucine
Phe	: Phenylalanine
Met	: Methionine
Trp	: Typtophan
Val	: Valine
PC-3	: Prostate cancer cell line-3

1. INTRODUCTION

1.1. Prostate cancer

Prostate cancer is the second most common malignancy among men in the world. Prostate cancer occurs in prostate gland, which is a small walnut-shaped gland that produces the seminal fluid that nourishes and transports sperm. Prostate cancer usually grows slowly and initially remains confined to the prostate gland, where it may not cause serious harm. While some types of prostate cancer grow slowly and may need minimal or no treatment, other types are aggressive and can spread quickly. Prostate cancer begins when cells in the prostate gland start to grow uncontrollably.

The prostate is below the bladder and in front of the rectum. The size of the prostate changes with age. In younger men, it is about the size of a walnut, but it can be much larger in older men. Just behind the prostate are glands called *seminal vesicles* that make most of the fluid for semen. The *urethra,* which is the tube that carries urine and semen out of the body through the penis, goes through the center of the prostate. Prostate cancer occurrence is high in developed countries and recorded the lowest in central Asian countries. (Ahmedin *et al.,* 2011)

Causative factors for prostate cancer are multiple which include increasing age, race, ethnicity, family history, environmental pollution, diet and obesity, smoking, frequent sex and sexually transmitted diseases. (Osamu *et al.,* 2002)

1.2. Types and treatment of prostate cancer

- Localized prostate cancer
- Metastatic prostate cancer

Localized prostate cancer treatment is stage specific. Radical prostatectomy (surgical removal of the prostate gland), image guided radiotherapy which includes 3D conformal radio therapy and intensity modulated radio therapy are widely used to treat

localized prostate cancer. For metastatic prostate cancer the therapy is aimed at reducing the circulating prostate specific antigen and the androgen level. This can be established by androgen deprivation therapy (ADT) and combined androgen blockade therapy (CAB).

ADT includes:

- Surgical castration - bilateral orchiectomy (removal of both the testes) and the standard castrate level of testosterone (≤ 20ng/dl) is achieved within 12hr.
- Pharmacological castration using Gonadotropin releasing hormone (GnRH) receptor agonist or GnRH receptor antagonist. There will be an initial rise in the testosterone concentration by using GnRH receptor agonist but after 2 to 4 weeks castration level of testosterone is maintained.

CAB includes:

- Pharmacological or surgical Castration.
- Androgen receptor (AR) antagonists are used to prevent AR activation by dihydrotestosterone. There are two types of AR antagonist, steroidal and non-steroidal. They competitively inhibit the binding of androgens to AR ligand binding pocket.
- DHT synthesis inhibitors (Table 1). (Heidenreich *et al.,* 2011).

Table 1: Drugs used in prostate cancer treatment

Hormonal therapy	
GnRH agonist	Leuprolide, Goserilin, Triprorelin, Histrelin, Busrelin
GnRH antagonist	Abarelix, Degarelix, Cetrorelix, Ganirelix
Non-steroidal anti-androgens	Flutamide, Bicalutamide, Nilutamide
Steroidal anti-androgens	Cyproterone acetate, Megesterol, Medroxyprogesterone
DHT synthesis inhibitors	Ketoconazole, Abiraterone acetate
Non hormonal therapy	
Cytotoxic agents	Docetaxel

1.3. Prostate cancer targets

1.3.1. AR mediated pathway

The normal development and maintenance of the prostate is dependent on androgen acting through the AR. AR remains important in the development and progression of prostate cancer. AR expression is maintained throughout prostate cancer progression, and the majority of androgen-independent or hormone refractory prostate cancers express mutated AR. Mutation of AR, especially mutations that result in a relaxation of AR ligand specificity, may contribute to the progression of prostate cancer and the failure of endocrine therapy. Similarly, alterations in the relative expression of AR co-regulators have been found to occur with prostate cancer progression and may contribute to differences in AR ligand specificity or transcriptional activity. Prostate cancer progression is also associated with increased growth factor production and an altered response to growth factors of prostate cancer cells. (Heinlein *et al.,* 2004).

Androgen action can be considered to function through an axis involving the testicular synthesis of testosterone, its transport to target tissues, and the conversion by

5- α reductase to the most active metabolite 5-dihydrotestosterone (DHT). Testosterone (TST) and DHT exert their biological effects through binding to AR and inducing AR transcriptional activity. The androgen-induced transcriptional activation of AR is modulated by the interaction of AR with co-regulators and by phosphorylation of AR and AR co-regulators in response to growth factors. (Heinlein *et al.,* 2002) (Buchanan *et al.,* 2001). Approximately 80 -90% of prostate cancers are dependent on androgen at initial diagnosis, and endocrine therapy of prostate cancer is directed toward the reduction of serum androgens and inhibition of AR. (Denis *et al.,* 2000).

The prostate specific antigen is the biological marker for androgen mediated prostate cancer. The prostate specific antigen (PSA) test clearly provides the opportunity for clinically relevant prostate cancer to be detected. However, in some patients the PSA test may lead to investigations which can identify clinically insignificant cancers, which would not have become evident in a man's lifetime. In addition, a raised PSA may often indicate benign prostatic enlargement, and this may provide an opportunity for treatment of this condition before complications develop. (Kirby *et al.,* 2016).

1.3.2. Estrogen receptor mediated pathway

Although AR signaling is the main molecular tool regulating growth and function of the prostate gland, estrogen receptor β (ER-β) is involved in the differentiation of prostatic epithelial cells and numerous anti-proliferative actions on prostate cancer cells. ER-β agonist is promising as an anticancer therapy and in the prevention of prostate cancer. (Paraskevi *et al.,* 2014)

1.3.3. Phosphoinositide 3-kinase (PI3K) pathway

The phosphoinositide 3-kinase (PI3K) pathway, a critical signal transduction system linking oncogenes and multiple receptor classes to many essential cellular

functions, is perhaps the most commonly activated signaling pathway in human cancer. Emerging evidence demonstrates a key role for the PI3K-AKT-mTOR signaling axis in the development and maintenance of Castration resistant prostate cancer. This pathway, which is deregulated in the majority of advanced Prostate cancer, serves as a critical nexus for the integration of growth signals to downstream cellular processes such as protein synthesis, proliferation, survival, metabolism and differentiation, thus providing mechanisms for cancer cells to overcome the stress associated with androgen deprivation.(Pixu *et al.,* 2009)(Edlind *et al.,*2014). Studies suggest that PI3K/Akt/mTOR signaling is upregulated in 30-50% of prostate cancers, often through loss of PTEN. Molecular changes in the PI3K/Akt/mTOR signaling pathway have been demonstrated to differentiate benign from malignant prostatic epithelium and are associated with increasing tumor stage, grade, and risk of biochemical recurrence. (Todd *et al.,* 2016).

1.3.4. Bcl-2 over expression

The Bcl-2 family constitutes both agonists and antagonists of apoptosis that function at least in part through protein-protein interactions between various members of the family. The final outcome depends on the relative ratio of death agonists and antagonists. The Bcl-2 expression has been closely associated with the androgen independent phenotype of prostate cancer. Cytotoxic chemotherapy may be used as palliative therapy in androgen independent prostate cancer but has not been found effective. Most chemotherapeutic cytotoxic agents induce apoptosis in cancer cells by direct and indirect action on the cell cycle. In vitro and in vivo studies have established that the Bcl-2 expression confers an anti-apoptotic activity against androgen withdrawal and cytotoxic chemotherapy. It thus offers a tempting potential target for therapeutic manipulations of prostate cancer. The up-regulation of BCL-2 after androgen ablation

in prostate carcinoma cell lines and in a castrated-male rat model further established a connection between BCL-2 expression and prostate cancer progression. (Catz *et al.,* 2003)

1.4. Cucurbitacin

1.4.1. Phytochemistry

Cucurbitacins which are structurally diverse triterpenes with cucurbitane skeleton, found in the members of Cucurbitaceae and several other plant families possess immense pharmacological potential. They differ from most other tetracyclic triterpenes by being highly unsaturated and containing numerous keto-, hydroxyl- and acetoxy groups. Chemically, cucurbitacins are ranked according to the presence of various functional groups on rings A and C, diversity in the side chain and stereo-chemical considerations. They are widely distributed in the plant kingdom, where they act as heterologous chemical pheromones that protect plants from external biological insults. Chemically, Cucurbitacins are ranked according to the presence of various functional groups on rings A and C, diversity in the side chain and stereochemical considerations. (Zheng *et al.,* 2007)

1.4.2. Mechanism of anti-cancer activity

In general, cucurbitacins are considered to induce apoptosis by selectively inhibiting the JAK/STAT pathways. However, other mechanisms are also implicated in their apoptotic effects. These include the MAPK pathway, PARP cleavage, expression of active caspase-3, decreased pSTAT3 and JAK3 levels, as well as decreases in various downstream STAT3 targets such as Mcl-1, Bcl-2, Bcl-XL, and cyclin D3. All the above mentioned markers were implicated in apoptosis and the cell cycle control (Ríos *et al.,* 2012). Being a triterpenoid, cucurbitacin might be a precursor of steroid. The structure of cucurbitacin resembles the structure of steroids. Approximately 80-

90% of prostate cancers are dependent on androgen at initial diagnosis, and endocrine therapy of prostate cancer is directed toward the reduction of serum androgens and inhibition of AR (Heinlein *et al.,* 2004).

The initial evidence of docking studies had revealed that the cucurbitacins have high binding for the AR. However, there has been no biological evidence to prove the same. Hence, in this study, we evaluated the prostate cancer activity of cucurbitacins by AR antagonism. Cucurbitacin is a novel compound for steroid dependent prostate cancer study.

2. LITERATURE REVIEW

2.1. Role of AR in prostate cancer

The normal development and maintenance of the prostate is dependent on androgen acting through the AR. AR remains important in the development and progression of prostate cancer. AR expression is maintained throughout prostate cancer progression, and the majority of androgen-independent or hormone refractory prostate cancers express AR. Mutation of AR, especially mutations that result in a relaxation of AR ligand specificity, may contribute to the progression of prostate cancer and the failure of endocrine therapy by allowing AR transcriptional activation in response to anti-androgens or other endogenous hormones. Similarly, alterations in the relative expression of AR co-regulators have been found to occur with prostate cancer progression and may contribute to differences in AR ligand specificity or transcriptional activity. Prostate cancer progression is also associated with increased growth factor production and an altered response to growth factors by prostate cancer cells. The kinase signal transduction cascades initiated by mitogenic growth factors modulate the transcriptional activity of AR and the interaction between AR and AR co-activators. The inhibition of AR activity through mechanisms in addition to androgen ablation, such as modulation of signal transduction pathways, may delay prostate cancer progression. (Heinlein *et al*., 2004)

2.2. AR structure and functions

AR is a nuclear receptor encoded by a single gene located on human X-chromosome at Xq11-12 region that spans more than 90kb and contains 8 exons. Human AR is a 900-920 amino acid containing protein and the variations are due to the polymorphism in the length of polyglutamine (CAG repeats) and polyglycine (GGN repeats) tracts of the first exon.

Like other nuclear receptors AR is also divided into 4 regions

- Variable amino terminal domain or Activation function 1 (AF1),
- DNA binding domain,
- Hinge Region,
- Ligand binding domain (LBD) / Activation function-2 (AF-2).

Two isoforms of AR (A&B) are expressed in humans. AR-B isoform is predominantly expressed over AR-A isoform and the ratio of AR-B to AR-A isoform is 10:1 (Nigel *et al.,* 2010).

2.2.1. Structure and function of AR amino terminal domain

The amino terminal domain approximately contains half the molecule of AR and is required for full transcriptional activity. The activity of AR depends on various transactivation units (TAU) within the amino terminal domain. The amino terminal domain of AR contains two transactivation units namely TAU-1 and TAU-5. TAU-1 appears to be important in ligand dependant activation and TAU-5 is responsible for constitutive activation of AR. The ^{23}FQNLF^{27} motif is involved in the ligand dependant intramolecular amino/carboxyl terminal interaction which is essential for full activation of AR because this interaction is essential for receptor stabilization, prevents ligand dissociation from AR, increases the DNA binding affinity, creates surface area for recruitment of co-activators etc. This ligand dependant amino/carboxyl terminal interaction occurs between the first motif of amino terminal domain with helices 3, 4 and 12 of AF-2 domain overlapping the binding site of p160/SRC family of co-activators (Scott *et al.,* 2007). The second motif WXXLF is responsible for constitutive activation of AR in the absence of ligand or amino terminal domain. The ^{433}WXXLF^{437} motif interacts with LBD outside of AF-2 domain (Bin *et al.,* 2000)

2.2.2. Structure and Function of AR DNA binding domain

DNA binding domain of AR is highly conserved and plays a major role in AR nuclear localization, receptor dimerization, specific and non-specific DNA binding. DNA binding domain is made of 3 α-helices having 2 zinc finger motifs and a short C-terminal extension. Each zinc finger consists of 4 cysteine residues and a Zn^{-1} ion. The first zinc finger (P-box) is involved in the sequence recognition and directly binds to major groove of hormone response element (HRE) in the DNA. The second zinc finger (D-box) is involved in the stabilization of DNA bound AR interactions and mediate DNA dependant intermolecular protein-protein interactions (homo dimerization) (Scott *et al.,* 2007).

In AR the carboxy terminal extension amino acids provide an additional dimer interface along with second zinc finger that is required for selective and high affinity binding of AR to specific androgen response elements (Edward *et al.,* 2002).

2.2.3. Structure and functions of hinge region

Hinge region is flexible and separates the DNA binding domain from Ligand binding domain. The hinge region of AR has a transcriptional inhibiting motif made of highly basic amino acids and has an attenuating effect on AF-2 by interfering with the recruitment of co-activators. Deletion of the inhibiting motif enhances the intramolecular N/C terminal interaction which is necessary for AR transcriptional function as discussed in Variable amino terminal domain but impairs the hormone dependant nuclear translocation because of the deletion of nuclear localization signal and also affects DNA binding and receptor stability (Annemie *et al.,* 2007).

2.2.4. Structure and function of LBD

The LBD of AR consists of 12 α-helices and 1 β-sheet that fold to form triple layered α-helical sandwich which creates a hydrophobic ligand binding pocket in which ligand binds (Fig.1).

Agonist binding to LBD causes helix-12 to lay over the ligand binding pocket and exposes the groove necessary for intramolecular N/C interaction. Antagonist binding displaces the helix-12 away from ligand binding pocket and unveils the binding surface for co-repressor NcoR/SMRT interaction and inhibition of AR transcriptional activity. (Osguthorpe *et al.,* 2011).

Fig. 1: Crystal structure of wild AR showing α-helices and β-strand folding

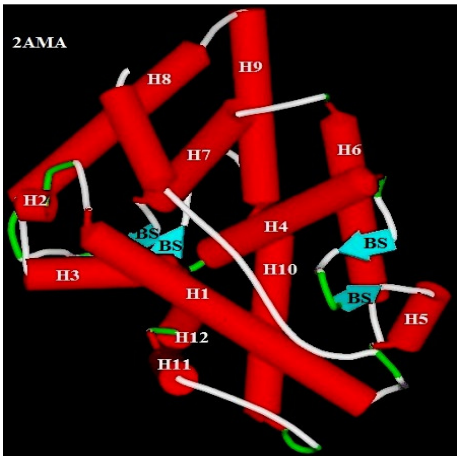
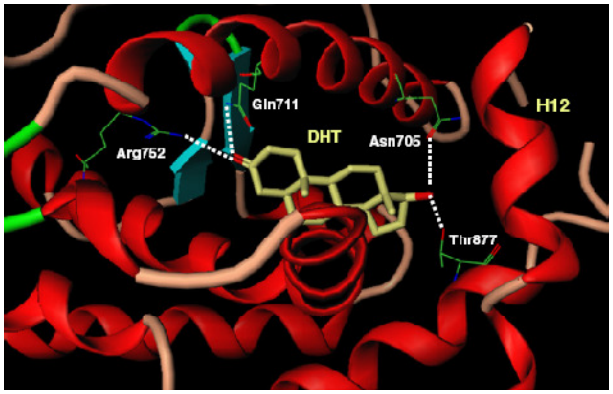


Fig 2: Essential Hydrogen bond interactions of DHT



2.2.5. Ligand dependant AR activation

The ligand dependant activation is required for maximum activation of full length AR. Upon androgen binding to AR LBP, AR dissociates from heat shock proteins (HSP), undergoes ligand dependant intramolecular amino-carboxy terminal interaction, phosphorylation, nuclear translocation, DNA dependant homodimerization, co-activator recruitment, binding to specific androgen response elemnts and initiation of transcription. AR resides predominantly in cytoplasm is associated with HSP and chaperons. HSP also have active role in transcriptional activation of the receptor because HSP 90 binds to AR LBD and keeps it in an active conformation so that AR is able to bind androgens. Androgen binding to LBP results in the dissociation of HSP from AR, helix 12 to fold over the LBP to enclose the ligand unmasking the necessary grooves to form intramolecular amino-carboxy terminal interaction, dimerization and exposes nuclear localization signal. Phosphorylation of AR also plays an important role in transcriptional activity of AR. The newly translated AR is phosphorylated at serine residues 506, 641, 653 as a post-translational modification which increase the ligand acquiring properties. Antagonist binding does not enhance receptor phosphorylation. After translocation into nucleus AR undergoes DNA dependant intermolecular homodimerization (Jin *et al.,* 2009).

Table 2: Drugs available for prostate cancer

S. No	Drug	Classification
1.	Bicalutamide	Anti-androgen
2.	Enzalutamide	Anti-androgen
3.	Flutamide	Anti-androgen
4.	Nilutamide	Anti-androgen
5.	Cyproterone acetate	Anti-androgen

6.	Abiraterone acetate	Anti-androgen and CYP17A1 inhibitor
7.	Cabazitaxel	Taxane derivative and anti-microtubular agent
8.	Docetaxel	Taxane derivative and anti-microtubular agent
9.	Degarelix	Testosterone inhibitor
10.	Goserelin acetate	LHRH agonist
11.	Leuprolide acetate	LHRH agonist
12.	Triprorelin acetate	LHRH agonist
13.	Mitoxantrone hydrochloride	Anti-tumor antibiotic
14.	Sipulencel – T	Cellular immunological agent
15.	Histrelin	GnRH inhibitor
16.	Buserelin acetate	GnRH inhibitor
17.	Ketoconozole	Inhibitor of CYP17 and TST synthesis
18.	Radium 223 dichloride	Radiopharmaceutical
19.	Prednisone	Corticosteroid
20.	Hydrocortisone	Corticosteroid
21.	Dexamethasone	Corticosteroid
22.	Denosumab	Monoclonal antibody
23.	Zoledonic acid	Bisphosphonate

Ref: (National cancer institute) (Gerald *et al*., 2017)

Table 3: Drugs in clinical trial for prostate cancer

S. No	Drug	Phase
1.	Vaccine-Based Immunotherapy Regimen (VBIR)	I
2.	AZD8186	I
3.	ZEN003694	I

4.	Abiraterone Acetate With or Without Cabazitaxel	II
5.	ARN-509+Abiraterone acetate+Leuprolide with Stereotactic, Ultra-Hypofractionated Radiation	II
6.	JNJ-56021927 in Combination with Abiraterone Acetate and Prednisone Versus Abiraterone Acetate and Prednisone in Subjects with Chemotherapy-naive Metastatic Castration-resistant Prostate Cancer	III

Ref: (Memorial Sloan Kettering cancer centre, 2017)

2.3. The role of ER-β in prostate cancer

ER-β is encoded by chromosome locus 14q22–24 and it is expressed in both stromal and luminal epithelial cells of the human prostate. ERα is expressed mainly in prostate stroma. (Fixemer *et al.,* 2003). As a member of the nuclear receptor family, ERβ acts individually, forming homodimers (ERβ/β) or heterodimers (ERβ/α). Ligand-induced dimerization leads to translocation of dimer to the nucleus, binding with co-regulatory proteins and interaction with responsive elements (binding sites) in the promoter regions including nuclear factor-κB (NF-κB) and activator protein 1 (AP-1). ERβ binds indirectly to these alternative binding sites through the recruitment of cofactors to the receptor. (Heldring *et al.,* 2007). However, less is known about the interactions between ERβ and transcriptional cofactors. ERβ/β and ERα/β homo- and hetero dimers, respectively, exhibit anti-proliferative effects as they activate different target genes. Interestingly, ERα/β heterodimer is more stable than the ERβ/β homodimer. Overall, ER dimerization is a crucial step in defining ER signaling.

Interestingly, prostate morphogenesis occurs under the control of androgens and is modulated by estrogens (Marker *et al., 2003*). However, ERβ is not required in early stages of prostate development, as it appears to be expressed in the prostate after 2 wks

in the life of newborn mice following ERα expression (Omoto *et al., 2005*). Moreover, in the developing rodent prostate gland, ERα-induced excessive estrogenic exposure leads to permanent alternation of the gland including squamous metaplasia, inflammation and epithelial dysplasia as reported by an *in utero* study (Prins *et al., 2006*). Notably, the developmental pattern for ERβ in the human prostate is different from the rodent. Apparently, ERβ is the only detectable ER in the developing human fetal prostate. However, by year 11 post-natally, expression of ERβ is restricted to the basal epithelial cells and prostate stromal compartments, similar to adult human prostate. (Shapiro *et al.,2005*). Thus, in the developing human prostate, ERβ is the predominant ER in both stromal and epithelial cells (Adams *et al., 2002*).

In the adult human prostate, ERβ is characterized as an important mediator of epithelial differentiation (Imamov *et al., 2004*). The mechanisms through which ERβ maintain differentiation involve the degradation of hypoxia-inducible factor 1α (HIF-1α) (Mak *et al.,* 2010). ERβ enhances transcription of prolyl hydroxylase domain-containing protein 2 (*PHD2*) that hydroxylates HIF-1α and marks HIF for destruction by the von Hippel-Lindau tumor suppressor (VHL) (Mak *et al., 2013*). Additionally, ERβ appears to have antiproliferative actions which are independent from the alternations of systemic androgen concentration and the activation of ERα, as documented in aromatase-knockout mice treated with ERβ-specific agonists (McPherson *et al., 2007*). There, ERβ seemed to have a suppressive role in the proliferation process, stimulating the differentiation of adult prostate epithelial cells.

2.4. Role of Bcl-2 protein family in regulation of apoptosis in prostate cancer

In-vitro studies have highlighted the role of Bcl-2 proteins as important regulators of the apoptotic pathway in several cell types Bcl-2, the prototype of this

family, was discovered by studies of t(14:18) chromosomal translocations, which are frequent in non-Hodgkin lymphomas and follicular lymphomas. The name Bcl-2 (B cell lymphoma/leukemia gene2) signifies the close association of this gene to these malignancies in which enhanced expression was initially believed to arise solely as a result of these translocations, resulting in the juxta-position of the Bcl-2 gene to a potent enhancer element sequence of the Ig H gene. Several genes have been identified and designated as the Bcl-2 family based on their sequence homology to Bcl-2. These genes include both positive and negative regulator of apoptosis.(Chaudhary *et al.,1999*)

2.5. Role of PI3K/Akt/mTOR pathway in prostate cancer

One pathway with a prominent role in prostate cancer is the PI3K/Akt/mTOR pathway. Current estimates suggest that PI3K/Akt/mTOR signaling is upregulated in 30-50% of prostate cancers, often through loss of PTEN. Molecular changes in the PI3K/Akt/mTOR signaling pathway have been demonstrated to differentiate benign from malignant prostatic epithelium and are associated with increasing tumor stage, grade, and risk of biochemical recurrence. Multiple inhibitors of this pathway have been developed and are being assessed in the laboratory and in clinical trials, with much attention focusing on mTOR inhibition. Current clinical trials in prostate cancer are assessing efficacy of mTOR inhibitors in combination with multiple targeted or traditional chemotherapies, including bevacizumab, gefitinib, and docetaxel. (Todd *et al., 2009)*

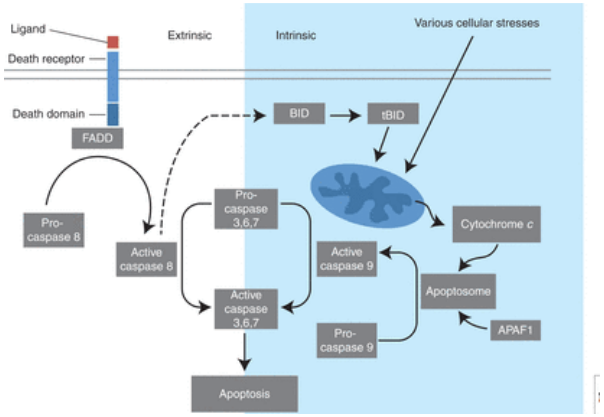
2.6. Caspase pathway

Caspases involved in apoptosis have been subclassified by their mechanism of action and are either initiator caspases (caspase-8 and -9) or executioner caspases (caspase-3, -6, and -7). (David *et al.,* 2013). Initiator caspases activate executioner

caspases that subsequently coordinate their activities to demolish key structural proteins and activate other enzymes.

The extrinsic apoptosis pathway is activated through the binding of a ligand to a death receptor, which in turn leads, with the help of the adapter proteins (FADD/TRADD), to recruitment, dimerization, and activation of caspase-8. Active caspase-8 then either initiates apoptosis directly by cleaving and thereby activating executioner caspase (-3, -6, -7), or activates the intrinsic apoptotic pathway through cleavage of BID to induce efficient cell death. The intrinsic or mitochondrial apoptosis pathway can be activated through various cellular stresses that lead to cytochrome *c* release from the mitochondria and the formation of the apoptosome, comprised of APAF1, cytochrome *c*, ATP, and caspase-9, resulting in the activation of caspase-9. Active caspase-9 then initiates apoptosis by cleaving and thereby activating executioner caspases (Fig 3).

Fig 3: Caspase pathway



2.7. Cucurbitacin

Cucurbitacins are found in many of the cucurbitaceous plants (Kaushik *et al.*, 2015). This diverse group of compounds may prove to be important lead molecules for future research. Research focused on these unattended medicinal leads from the nature

can prove to be of immense significance in generating scientifically validated data with regard to their efficacy and possible role in various diseases. Medicinal and toxic properties of these compounds have stimulated a continuing interest in them. (Kupchan *et al.,* 1978). Many genus of Cucurbits viz. *Trichosanthes*, *Cucurbita*, *Cucumis* and *Citrullus* are affluent in cucurbitacins. These compounds have also been discovered in other plant families like Scrophulariaceae, Cruciferae, Datiscaceae, Primulaceae, Rubiaceae etc. The diversity of cucurbitacins lies in variety of its side chain derivatives that contribute to their disparate pharmacological actions. (Stuppner *et al.,* 1993). The bitter taste of plant species like cucumber has been attributed to the presence of cucurbitacins.

The first cucurbitacin was isolated as a crystalline substance in 1831 and was named α-elaterin. Certain plant species rich in cucurbitacins like *Momordica* hold coveted position in different system of traditional medicines for curative effects in metabolic disease like diabetes. All cucurbitacins contain a basic 19-(10→9β)-abeo--10α-lanost-5--ene ring skeleton. A common feature among all compounds in the category of Cucurbitacins is the presence of 5, (6) --double bond. The difference of Cucurbitacins from steroidal nucleus lies in the fact that in basic structure of Cucurbitacins a methyl group is located at C-9 rather than C-10. (Dinan, *et al.,* 2001) Most of the Cucurbitacins are tetracycline, but some representatives have an extra ring due to formal cyclization between C--16 and C--24 as in cucurbitacins S and T. (Gamlath *et al.,* 1998) The Cucurbitacins differ from most of the other tetracyclic triterpenes by being highly unsaturated and contains numerous keto--, hydroxyl--, and acetoxy--groups. (Jorn *et al.,* 1998) Certain Cucurbitacins have been discovered in the form of glycosides and some of them lack C--11 carbonyl function (Stuppner *et al.,*

1991). Cucurbitacin B inhibits ATP citrate lyase which is a key enzyme that plays crucial role in cancer cell metabolism in prostate cancer (Gao *et al*., 2014).

The structural composition of following Cucurbitacins are known and have been designated by the letters: A, B, C, D, E, F, G, H, I, J, K, L, O, P, Q, R and S. Cucurbitacin I caused reduction of growth in breast and prostate carcinoma cell lines (MDA-MB-231, MDA-MB-468, Panc-1), in vitro, as well as in nude mice xenograft models (Sun *et al*., 2008). Cucurbitacin Q induces apoptosis more potently in human and murine tumors. Furthermore, in HeLa cells, cucurbitacins inhibited DNA, RNA, and protein synthesis (Witkowski *et al*., 1984). The 23, 24-dihydrocucurbitacin B and cucurbitacin R, inhibit proliferation and/or induce apoptosis in colon cancer cell lines. Cucurbitacin E inhibits the proliferation of prostate cancer cells and caused disruption of the cytoskeleton structure of actin and vimentin (Duncan *et al*., 1996). Cucurbitacin A and I act by inhibition of only JAK2 and STAT3 respectively. It has been reported that cucurbitacin E inhibited tumor angiogenesis by inhibiting JAK-STAT3 and mitogen activated protein kinases (MAPK) signaling pathways. It has been reported that cucurbitacin B exerts an anticancer effect by inhibiting telomerase via down-regulating both the human telomerase reverse transcriptase and c-Myc expression in breast cancer cells (Kaushik *et al*., 2015).

2.7.1. Cucurbitacin and Anti-inflammatory activity

Cucurcitacin analogues viz. Cucurbitacin R and DHCB have been reported to possess anti-inflammatory potential and their action is reported to be mediated by inhibition of tumor necrosis factors (TNF)-α and other mediators of inflammation such as nitric-oxide synthase-2 and cyclo-oxygenase-2 (Escandell *et al.,*2008). Cucurbitacins B, D, E and I have been reported to inhibit cyclo-oxygenase -2 enzymes with no effect on COX-1 enzymes. (Jayaprakasam *et al.,*2003). The anti-inflammatory response of 23,

24-dihydrocucurbitacin D (DHCD) have been hypothesized to get mediated through blocking of NF-κ B activation thereby obstructing the release of nitrous oxide. DHCD can be taken up as probable lead and appraised for providing a promising anti-inflammatory agent. (Park *et al.,* 2004).

2.7.2. Cucurbitacin and Antitumor activity

Very less information is available on the role of Cucurbitacins at molecular level which has lead to slow advancement in the development of Cucurbitacins as anti-cancer agents. (Kee *et al.,* 2008). In relation to cancer, targets of Cucurbitacin actions involve growth inhibition, arrest of cell cycle at G2/M phase and induction of apoptosis in cancer cell. (Liu *et al.,* 2000). The mechanisms underlying anti-tumorigenic potentials of Cucurbitacins involve inhibition of Janus kinase/Signal Transducer Activator of Transcription 3 (JAK/STAT3) signaling pathway whose activation is required for the proliferation and sustainment of cells. (Bowman *et al.,* 2000). The role of Cucurbitacin I in suppressing phosphotyrosine STAT3 in cancer cell lines and cancerous lung cells of humans has been reported. (Blaskovich *et al.,* 2003). Although Cucurbitacin B, E, and I act by inhibiting the activation of both JAK2 and STAT3, Cucurbitacin A and I acts by inhibition of only JAK2 and STAT3 respectively. (Sun *et al.,* 2005). It has been reported that Cucurbitacin E inhibited tumor angiogenesis by inhibiting JAK-STAT3 and mitogen activated protein kinases (MAPK)- signaling pathways (Dong *et al.,* 2010). The role of interference with actin cytoskeleton has been attributed to anti-proliferative effects of Cucurbitacin B and E. The anti-proliferative activities have been correlated directly with the disruption of the F-actin cytoskeleton. (Duncan *et al.,* 1996). It has been proposed that the combination of Cucurbitacin B with docetaxel may augment the chemotherapeutic effects by suppression STAT3 in patients with laryngeal cancer. (Liu *et al.,* 2000). It is expected that cucumber fruits have anti-

tumor effects since they have been reported to contain Cucurbitacin C. (Higashio *et al.,* 2002) It has been reported that cucurbitacin B exerts an anticancer effect by inhibiting telomerase via down-regulating both the human telomerase re verse transcriptase and c-Myc expression in breast cancer cells. (Duangmano *et al.,* 2010)

2.7.3. Cucurbitacin and Anti-artherosclerotic activity

There have been reports on Cucurbitacin B and E in glycosidic form to exhibit inhibitory effect on lipid oxidation products like- malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE). (Tannin-Spitz *et al.,*2007). These reports bolster the therapeutic role of Cucurbitacins in artherosclerosis, which involves modification of lipoproteins by involvement of- MDA and 4-HNE. (Saba *et al.,* 2010)

2.7.4. Cucurbitacin and Anti-diabetic activity

There have been a plethora of reports on the role of Cucurbitacins for their cytotoxic, hepatoprotective, cardiovascular, and antidiabetic effects. (Park *et al.,* 2004). Cucurbitane triterpenoids present in momordica fruits are noted for antidiabetic and anticancer activities, this may provide leads as a class of therapeutics for diabetes and obesity. (Tan *et al.,* 2008). The 5'-adenosine monophosphate-activated protein kinase (AMPK) pathway is suggested as a probable mechanism for the stimulation of GLUT4 translocation by triterpenoids from *M. charantia*. It is particularly interesting in relation to diabetes and obesity because activation of AMPK increases fatty acid oxidation, inhibits lipid synthesis, and can improve insulin action. An analogue of 23,24-dihydrocucurbitacin F from *Hintonia latiflora* has been reported to possess significant hypoglycemic and antihyperglycemic effects. The probable mechanism underlying--antihyperglycemic effect could be stimulation of insulin release and regulation of hepatic glycogen metabolism. (Jose *et al.,* 2007)

2.7.5. Miscellaneous activity of cucurbitacin

It has been reported that the concentration of Cucurbitacin C in the leaves is an important parameter in spider mite resistance in *Cucumis sativus*, perhaps by acting as an antagonist of a spider mite ecdysteroid receptor. (Balkema *et al.,* 2003). The steroid like resemblance of Cucurbitacin D may possess therapeutic effects via inhibition of Na^+/K^+-ATPase. (Chen *et al.,*2010). The role of Cucurbitacins as preventive and radical scavenging antioxidant has also been reported. Cucurbitacins have also been reported to possess adaptogenic activity. Cucurbitacins have been reported to increase the rat capillary permeability and to demonstrate antifertility effects in female mice. (Shohat *et al.,*1972). Cucurbitacin D has been reported to inhibit ovulation in mice. There has been protective role of Cucurbitacins acting as allomones in many plant species. Role of Cucurbitacins as anti-feedants for few insects, birds and as kairomones (Cucurbitacin B, E, D, I and L) for diabroticite beetles have been reported. (Subbiah. 2011). It is reported that Cucurbitacins act via Cuc receptors located on the maxillary palpi. They arrest the searching behavior of diabroticite beetles and produce a compulsive feeding behavior. Role of Cucurbitacin B and D in controlling diabrotic beetles can be an interesting approach. (Escandell *et al.,*2007).

2.7.6. Cucurbitacin I (JSI-124) induces apoptosis via p53 pathway in HepG2 cells

JSI-124 inhibited the proliferation and induced Hoechst 33258-stained chromatin condensation in HepG2 cells in a concentration- and time-dependent manner. Flow cytometry revealed that 1.00 μmol/L JSI-124 treatment increased the apoptotic rate significantly in HepG2 cells compared with the control cells. Furthermore, JSI-124 significantly enhanced the mRNA expressions of p53 and its downstream apoptotic factors, including Bax and Fas, but did not change the gene expression of the p53 tumor suppressor, MDM2. The 48-hour treatment of JSI-124 in HepG2 cells significantly

increased the levels of p53 and cleaved caspase-3 proteins. Conclusion JSI-124 induces the apoptosis of HepG2 cells through the activation of p53 and its downstream pro-apoptotic factors. (Wu *et al.,* 2017)

Cucurbitacin I dose- and time-dependently inhibited the proliferation of five OS cell lines. Following cucurbitacin I treatment, STAT3 was inactivated and analysis of Mcl-1, cleaved PARP and caspase-3 indicated apoptosis induction. Expression of cell cycle regulator proteins, such as phospho-cyclin D1, c-Myc and survivin, were suppressed. Finally, cucurbitacin I potently inhibited the tumor growth of human OS 143B cells in nude mice. *In-vitro* and *in-vivo* results suggest that STAT3 inhibition by cucurbitacin I will be an effective and new approach for the treatment of Osteosarcoma. (Oi *et al., 2016*)

2.7.7. Cucurbitacin I and cardio-toxicity

The mechanisms of cucurbitacin-I-induced cardiotoxicity are examined by investigating the role of MAPK-autophagy-dependent pathways. After being treated with 0.1-0.3μM cucurbitacin-I for 48h, H9c2 cells showed a gradual decrease in the cell viabilities, a gradual increase in cell size, and mRNA expression of ANP and BNP (cardiac hypertrophic markers). Cucurbitacin-I concentration-dependent apoptosis of H9c2 cells was also observed. The increased apoptosis of H9c2 cells was paralleling with the gradually strong autophagy levels. Furthermore, an autophagy inhibitor, 3-MA, was used to block the cucurbitacin-I-stirred autophagy, and then the hypertrophy and apoptosis induced by 0.3μM cucurbitacin-I were significantly attenuated. In addition, cucurbitacin-I exposure also activated the MAPK signaling pathways, including ERK1/2, JNK, and p38 kinases. Interestingly, only the ERK inhibitor U0126, but not the JNK inhibitor SP600125 and p38 MAPK inhibitor SB203580, weakened the induction of 0.3μM cucurbitacin-I in hypertrophy, autophagy and apoptosis. The

findings suggest that cucurbitacin-I can increase the autophagy levels of H9c2 cells, most likely, through the activation of an ERK-autophagy dependent pathway, which results in the hypertrophy and apoptosis of cardiomyocytes. (Wu *et al.,* 2016)

2.7.8. Mechanism of cucurbitacin I in gastric cancer cells

Deng *et al.,* for the first time systematically studied the underlying molecular mechanisms of Cu-I-induced gastric cancer cell death both in vitro and in vivo. In this study, they show that Cu-I markedly inhibits gastric cancer cell growth by inducing G2/M phase cell cycle arrest and apoptosis at low nanomolar concentrations via a STAT3-independent mechanism. Notably, Cu-I significantly decreases intracellular GSH/GSSG ratio by inhibiting NRF2 pathway to break cellular redox homeostasis, and subsequently induces the expression of GADD45α in a p53-independent manner, and activates JNK/p38 MAPK signaling. Interestingly, Cu-I-induced GADD45α and JNK/p38 MAPK signaling form a positive feedback loop and can be reciprocally regulated by each other. Therefore, the present study provides new insights into the mechanisms of antitumor effects of Cu-I, supporting Cu-I as an attractive therapeutic drug in gastric cancer by modulating the redox balance. (Deng *et al.,* 2016)

2.7.9. Cucurbitacin I and Breast cancer

Qi *et al* used JSI-124 (Cucurbitacin I), a selective JAK/STAT3 signaling pathway inhibitor, to investigate the role of STAT3 in tumor angiogenesis of a human BC cell line in vitro. JSI-124 inhibited cell viability, proliferation, adhesion, migration and tube formation of a human BC cell line MDA-MB-468. After transfection with pMXs-Stat3C, a dominant active mutant, the inhibitory effects of JSI-124 on MDA-MB-468 were abolished. Furthermore, JSI-124 reduced the phosphorylation of STAT3. These results suggested that JSI-124 inhibited tumor angiogenesis of the human BC cell

line in vitro through the reduction of STAT3 phosphorylation. In addition, JSI-124 could reduce VEGF transcription and secretion, suggesting that JSI-124 is also involved in the inhibition of the VEGF autocrine loop in the tumor microenvironment. (Qi *et al.,* 2015)

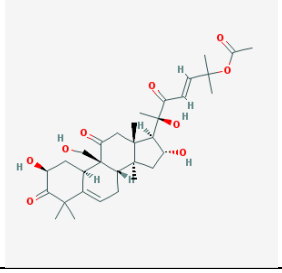
The small GTPase Rac1 has been widely implicated in mammary tumorigenesis and metastasis. Previous studies established that stimulation of ErbB receptors in breast cancer cells activates Rac1 and enhances motility via the Rac-guanine nucleotide exchange factor P-Rex1. As the Janus tyrosine kinase 2 (Jak2)/signal transducer and activator of transcription 3 (Stat3) pathway has been shown to be functionally associated with ErbB receptors, Lopez *et al* asked if this pathway could mediate P-Rex1/Rac1 activation in response to ErbB ligands. They found that the anticancer agent cucurbitacin I, a Jak2 inhibitor, reduced the activation of Rac1 and motility in response to the ErbB3 ligand heregulin in breast cancer cells. However, Rac1 activation was not affected by Jak2 or Stat3 RNA interference, suggesting that the effect of cucurbitacin I occurs through a Jak2-independent mechanism. Cucurbitacin I also failed to affect the activation of P-Rex1 by heregulin. Subsequent analysis revealed that cucurbitacin I strongly activates RhoA and the Rho effector Rho kinase (ROCK) in breast cancer cells and induces the formation of stress fibers. Interestingly, disruption of the RhoA-ROCK pathway prevented the inhibitory effect of cucurbitacin I on Rac1 activation by heregulin. Lastly, they found that RhoA activation by cucurbitacin I is mediated by reactive oxygen species (ROS). The ROS scavenger N-acetyl L-cysteine and the mitochondrial antioxidant Mito-TEMPO rescued the inhibitory effect of cucurbitacin I on Rac1 activation. In conclusion, these results indicate that ErbB-driven Rac1 activation in breast cancer cells proceeds independently of the Jak2 pathway. Moreover,

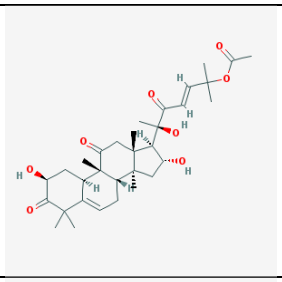
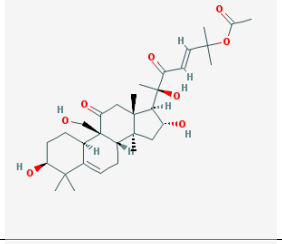
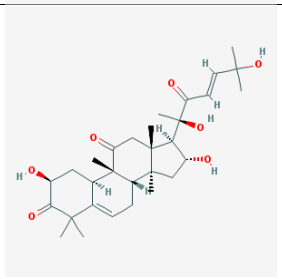
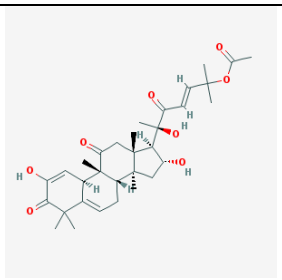
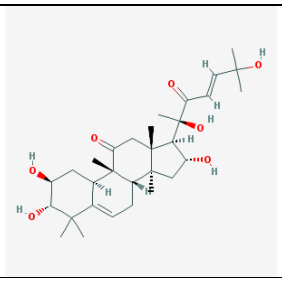
they established that the inhibitory effect of cucurbitacin I on Rac1 activity involves the alteration of the balance between Rho and Rac. (Lopez *et al.,* 2013)

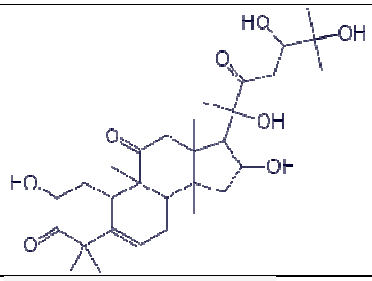
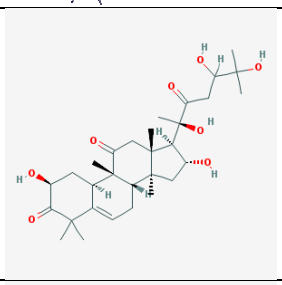
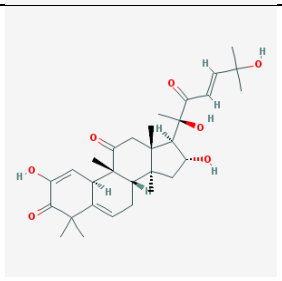
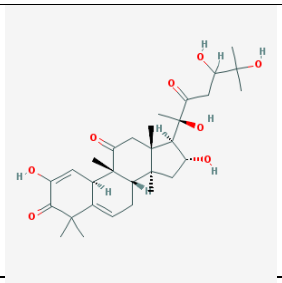
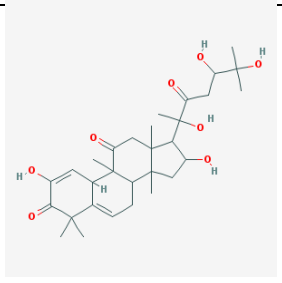
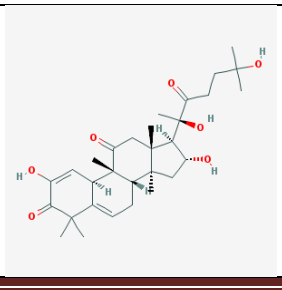
2.7.10. Cucurbitacin I and colon cancer

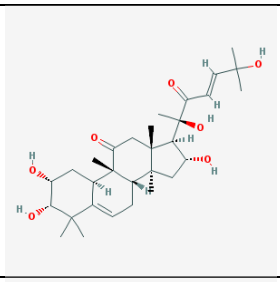
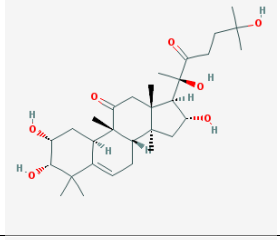
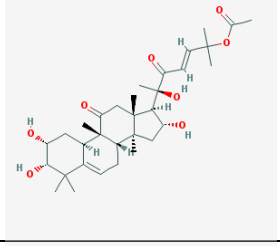
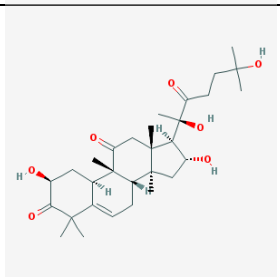
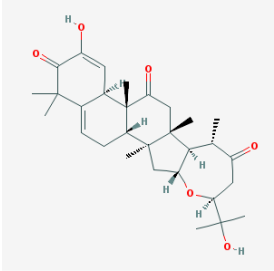
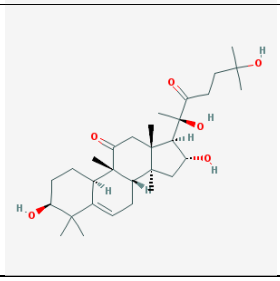
Song *et al.,* examined the chemopreventive potential of cucurbitacin I, a natural component extracted from plants of the Cucurbitaceae family, in the colon cancer cell line COLO205. They hypothesized that cucurbitacin I would prevent colon cancer cell migration and invasion, and sensitize colon cancer cells to chemotherapy. The data demonstrated that exposure of the COLO205 cells to cucurbitacin I significantly decreased cell viability. Furthermore the data demonstrated for the first time that in the COLO205 cells, cucurbitacin I could suppress the cell migration and invasion, and harbor chemosensitization activity against colon cancer. The anticancer activity of cucurbitacin I was accomplished by downregulating p-STAT3 and MMP-9 expression. Collectively, the results suggest that cucurbitacin I may be a potent adjuvant chemotherapeutic agent for colon cancer with anti-migration, anti-invasion and chemosensitizing activities. (Song *et al.,* 2015). Cucurbitacin-I reduced colon cancer cell proliferation by enhancing apoptosis and causing cell cycle arrest at the G2/M phase. (Kim *et al.,* 2014).

Table 4: Cucurbitacins and their activity

Cucurbitacin	Structure	Activity	Reference
A		No reported biological activity found.	-----

B		Anti-proliferative activity in prostate and breast cancer.	Gao *et al.*,2014. Duangmano *et al.*,2010.
C		No reported biological activity found.	-----
D		Inhibits proliferation and induce apoptosis of T-cell leukemia cells correlating NF-κB inhibition and down-regulation of the expression of antiapoptotic proteins Bcl-xL and Bcl-2	Ding *et al.*,2011
E		Cucurbitacin E-induced disruption of the actin and vimentin cytoskeleton in prostate carcinoma cells and inhibits tumor angiogenesis through VEGFR2 mediated JAK2/ STAT3 signaling pathway.	Duncan *et al.*, 1996. Dong *et al.*, 2010.
F		Cucurbitacin F induces cell cycle G2/M arrest and apoptosis in human soft tissue sarcoma cells.	Lohberger *et al.*, 2015.

G		No reported biological activity found.	-----
H		No reported biological activity found.	-----
I		Anti-tumor activity against colon cancer, gastric cancer, osteosarcoma breast cancer.	Deng *et al.,*2016, Qi *et al.,*2015, Ren *et al.,*2014, Song *et al.,*2015. Kim *et al* 2014. Oi *et al.,2016.*
J		No reported biological activity found.	-----
K		No reported biological activity found.	-----
L		No reported biological activity found.	-----

O		No reported biological activity found.	-----
P		No reported biological activity found.	-----
Q		Selective STAT3 activation inhibitor with potent antitumor activity	Sun *et al.*, 2008.
R		Reduces the inflammation and bone damage associated with adjuvant arthritis in Lewis rats by suppression of tumor necrosis factor-alpha in T lymphocytes and macrophages.	Escandell *et al.*, 2007.
S		No reported biological activity found.	-----
U		No reported biological activity found.	-----

3. OBJECTIVES AND PLAN OF STUDY

3.1 Objectives

- To identify Cucurbitacin derivative, which is having high affinity towards prostate cancer targets by *in-silico* method.
- To evaluate the cytotoxic activity of the cucurbitacin derivative in prostate cancer cell lines by MTT assay.
- To elucidate the apoptotic mechanism of cucurbitacin in prostate cancer cell line by caspase assay, acridine orange\ethidium bromide staining methods and gene expression studies.

3.2 PLAN OF STUDY

3.2.1 Phase I

3.2.1.1 In-Silico study

- *In-silico* study comprises of molecular docking of cucurbitacin derivatives (A – U) with prostate cancer target proteins like AR, ER-β, PI3K-α, Bcl2 and AKT.
- Cucurbitacin which is having Glide score more than the standard in concerned protein will be studied further.
- Selected cucurbitacins will be examined in QikProp for its ADME properties and toxicity.
- One lead molecule will be taken for *in-vitro* studies in cancer cell lines.

3.2.2 Phase II

3.2.2.1 In-Vitro study

- Culturing LNCaP and PC3 cell lines using RPMI + 10% FBS and DMEM + 10% FBS respectively in 5% CO_2 at 37˚ C.

- MTT assay of standard bicalutamide and cucurbitacin will be done in LNCaP and PC3 cell lines to evaluate the IC_{50} value.
- Determination of apoptosis mechanism by ethidium bromide/acridine orange staining method and caspase 3, 8, 9 assay.
- PCR studies – to detect the expression of genes such as Bax, Bcl2 and PSA (Prostate Specific Antigen).

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Chemicals used in this study

Table 5: Chemicals used for this study

S. No	Chemical	Manufacturer
1.	Cucurbitacin I	Phytolab
2.	DMSO	Thermo fisher scientific
3.	DMEM	Gibco
4.	RPMI	Gibco
5.	Trypsin	Sigma
6.	Phosphate buffer saline	Gibco
7.	FBS	Sigma
8.	MTT	Himedia
9.	Dihydrotestosterone	Himedia
10.	Bicalutamide	Santa cruz
11.	Acridine orange	Himedia
12.	Ethidium bromide	Himedia
13.	Caspase 3 assay kit	Biovision
14.	Caspase 8 assay kit	Biovision
15.	Caspase 9 assay kit	Biovision
16.	TRI reagent	Sigma
17.	Chloroform	Nice chemicals
18.	High capacity cDNA conversion kit	Applied biosystems

Table 6: Instruments used in this study

S. No	Instruments	Manufacturer
1.	Glide	Schrodinger
2.	Biosafety working hood	Esco
3.	CO_2 incubator	Thermo scientific
4.	ELISA reader	Multiskan Go – Thermo scientific
5.	Autoclave sterilizer	Everflow autoclave
6.	Refrigerated centrifuge	Thermo scientific
7.	Deep freezer	Thermo scientific
8.	Inverted fluorescence microscope	Nikon
9.	Arthik thermal cycler	Thermo scientific
10.	Micropipettes	Eppendorf
11.	Gel doc image analyser	Syngene
12.	Microcentrifuge	Eppendorf

4.2. Methods

4.2.1. In-silico screening

4.2.1.1. Molecular Modelling

The cucurbitacin derivatives are downloaded from the PubChem compound database and are prepared for docking using LigPrep (Ligprep, Schrödinger). LigPrep helps to convert 2D structure to 3D representation. LigPrep can also produce a number of structures from each input structure with various ionization states, tautomers, stereochemistries, and ring conformations, and eliminate molecules using various criteria including molecular weight or specified numbers and types of functional groups

present. Subsequently the structures were optimized by means of OPLS-2005 using a default setting in LigPrep.

4.2.1.2. Protein preparation

Proteins used in the study are downloaded from the RCSB protein data bank (PDB). Protein structures are prepared by using Maestro software (Maestro, Schrödinger) and aligned using the protein structure alignment module in Prime (Prime, Schrödinger). Bond order and formal charges were added for heterogroups and hydrogens were added to all atoms in the system. Protein was inspected visually for accuracy in the χ^2 dihedral angle of Asn and His residues and the χ^3 angle of Gln, and rotated by 180˚ when needed to maximize hydrogen bonding. The proper His tautomer was also manually selected to maximize hydrogen bonding. All Asp, Gln, Arg and Lys residue were left in their charged state. Water molecules for crystallization were removed from the complex except in the active site. A brief relaxation was performed on structure using the protein preparation module in Maestro with the "Refinement only" option. This is a two – part procedure that consists of optimizing hydroxyl and thiol torsion in the first stage followed by an all-atom constrained minimization carried out with the impact refinement module (Impref) using the OPLS-2005 force field to alleviate steric clashes that may exist in the original PDB structures. The minimization was terminated when the Root Mean Square Deviation (RMSD) reached a maximum cutoff of 0.30 Å.

4.2.1.3. Grid generation and ligand docking

Grids were defined by centering them on the ligand in the crystal structure using the default box size setting in Glide. Scaling of van der Waals radii of protein atom's partial atomic charge of less than 0.25 in 1.0. Hydrogen bond constraints were not applied. The prepared ligands were docked against the target proteins. All docking

calculations were performed using the "Extra precision" (XP) mode of Glide program. Glide uses a hierarchical series of filter to search for possible locations of the ligand in the active site region of the receptor. The initial filters test the spatial fit of the ligand to the defined active site and examined the ligand-receptor interactions using a grid-based method. Poses that pass these initial screens enter the final stage of the algorithm, which involves evaluation and minimization of a grid approximation to the OPLS-AA non-bonded ligand-receptor interaction energies. Final scoring is then carried out on the energy-minimized poses. The minimized poses are restored using Schrödinger's proprietary Glide score (G score) scoring function. G score is a modified version of ChemScore, but includes a steric-clash term and adds buried polar terms devised by Schrödinger to penalize electrostatic mismatches.

G Score = (a x vdW) + (b x Coul) + Lipo + Hbond + Metal + BuryP + RotB + Site

Where, vdW – van der Waal energy, Coul – Coulomb energy, Lipo – Lipophilic contact term, Hbond – Hydrogen bond, Metal – Metal binding term, BuryP – penalty for buried polar groups, RotB – penalty for freezing rotatable bonds, Site – polar interaction of the active site.

4.2.1.4. ADMET property Prediction

Many drugs often fail to enter the market as a result of poor pharmacokinetic profiles. Thus, it has become imperative nowadays to design lead compounds which can be easily orally absorbed, easily transported to their desired site of action, not easily metabolized into toxic metabolic products before reaching the targeted site of action and easily eliminated from the body before accumulating in sufficient amounts that may produce adverse side effects. The sum of the above mentioned properties is often referred to as ADME (absorption, distribution, metabolism and elimination) properties, or better still ADMET, ADME/T or ADMETox (when considerations are given to

toxicity issues). The inclusion of pharmacokinetic considerations at earlier stages of drug discovery programs using computer-based methods is becoming increasingly popular. The rationale behind *in silico* approaches are the relatively lower cost and the time factor involved, when compared to standard experimental approaches for ADMET profiling. As an example, it only takes a minute in an *in silico* model to screen 20,000 molecules, but takes 20 weeks in the "wet" laboratory to do the same exercise. A set of ADMET-related properties (a total of 46 molecular descriptors) were calculated by using the QikProp program (Schrödinger) running in normal mode. QikProp generates physically relevant descriptors, and uses them to perform ADMET predictions. An overall ADME-compliance score – drug-likeness parameter (indicated by #stars), was used to assess the pharmacokinetic profiles of the test compounds. The #stars parameter indicates the number of property descriptors computed by QikProp that fall outside the optimum range of values for 95% of known drugs.(QikProp, Schrodinger). The methods implemented were developed by Jorgensen and Duffy and among the calculated descriptors are: the logarithm of predicted binding constant to human serum albumin, log K_{HSA} (range for 95% of drugs: -1.5 to 1.2) the logarithm of predicted blood/brain barrier partition coefficient, log B/B (range for 95% of drugs: -3.0 to 1.0) the predicted apparent Caco-2 cell membrane permeability (BIP_{caco-2}) in Boehringer–Ingelheim scale, in nm s^{-1} (range for 95% of drugs: < 5 low, > 100 high) the predicted apparent Madin-Darby canine kidney (MDCK) cell permeability in nm s^{-1} (< 25 poor, > 500 great) calculated from the number of hydrogen bond acceptors (HBA), donors (HBD) and the predicted IC_{50} value for blockage of HERG K^+ channels, log *HERG* (concern < −5) the predicted skin permeability, log K_p (−8.0 to −1.0 for 95% of drugs) and the number of likely metabolic reactions, #metab (range for 95% of drugs: 1–8) (Fidele Ntie-Kang, 2013).

4.2.2 In-*vitro* screening

4.2.2.1. Mammalian cell culture

The LNCaP and PC3 cell lines are obtained from NCCS, Pune. LNCaP cells are maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS and penicillin–streptomycin, whereas PC-3 cells were maintained in the Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, penicillin–streptomycin, L-glutamine, pyruvate sodium. Cells were grown at 37°C with 5% CO2. PC3 cells are grown in culture flasks with polystyrene surface. The LNCaP cells are grown in culture flasks with corning Cell BIND surface. 0.25 % trypsin is used for detachment of the cells. (Claudia *et al*., 2007).

4.2.2.2. Maintaining and storage of cell lines

LNCaP cells show a steady growth rate with a doubling time of 60 hrs whereas PC3's doubling time is 48 hrs. The cells reached confluence in 6 to 7 days and were passed to get cells for experiment and also to store liquid nitrogen. Passaging was done as follow as.

Culture medium was removed from T25 flask by decanting into a clean container inside the laminar airflow hood, cells were rinsed with Phosphate Buffer Solution (PBS) to remove the traces of serum. 2ml of trypsin was added to the flask containing cells and incubate at 37˚C for 2 to 2.5 minutes. As soon as, cells started dislodging from the surface, the flask was rinsed with culture medium to arrest trypsination. The suspension of cells was collected in a sterile 15ml centrifuge tube using serological pipette and the cells were pelleted at 1500rpm for 5 mins. The cell pellet was resuspended in PBS and again centrifuged. The resulted pellet is resuspended in Culture medium and a part of it was seeded back into a sterile flask. The remaining cells were pelleted and resuspend in cryopreservative medium (10% DMSO in serum)

in a cryovial and frozen at -80˚C for short term storage and at liquid nitrogen for long time storage.

4.2.2.3. Drug Preparation

Cucurbitacin I was subjected to solubility test with different organic solvents and found to be dissolved in DMSO, 50mM concentration was prepared. The desired doses such as 0.1, 0.5, 0.10 and 50 mm were prepared from the stock using DMSO.

4.2.2.4. Anti-proliferative assay

Approximately 1000 to 3000 cells/well were added in 96 well plate from well grown culture, the viability is tested using tryptan blue dye with the help of haemocytometer, 95% viability should be confirmed. The anti-proliferative activity is measured by adding standard and test compounds (0.1 nM to 500 nM). The proliferative activity of the standard and test compound was evaluated without 1nM DHT. After 24 hours, fresh medium containing the drug replaces the old medium and incubated for another 24 hour. At the end of 48th hr 10µl of 3-[4, 5-dimethyl thiazol-2-yl] 2, 5- diphenyl tetra-zolium bromide (MTT) is added and the plates were incubated for an additional 4 hour. The formazon crystals were dissolved in 100µl of DMSO/well. The optical density was measured at 570nm. (Sanchez *et al.,* 2006). By plotting dose response curve the IC50 value will be calculated. The cell viability can be calculated using the formula,

Cell viability = (O.D of test cells/O.D of control) X 100

4.2.2.5. Dose finalization

Based on the IC50 value of test compound of different doses in LNCaP and PC3, final dose concentration is fixed for further studies on mechanism of apoptosis.

4.2.3. Elucidation of apoptotic mechanism

4.2.3.1. Gene expression study

RNA isolation

Homogenize cell samples in TRIZOL reagent. Lyse the cells directly in the culture dish. Use 1 ml of the TRIZOL reagent per 10 cm^2 of glass culture plate surface area. After addition of the reagent, the cell lysate was passed several times through a pipette to form a homogenous lysate. Isolate cells by centrifugation and then lyse in TRI reagent by repeated pipeting. To ensure complete dissociation of nucleoprotein complexes, allow samples to stand for 5 minutes at room temperature. Add 0.2 ml of chloroform per ml of TRI reagent used. The sample was shaken vigorously for 15 seconds, and allowed to stand for 2–15 minutes at room temperature. Centrifuged the resulting mixture at 12,000 × g for 15 minutes at 2–8 °C. Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an inter phase (containing DNA), and a colorless upper aqueous phase (containing RNA). The aqueous phase was transferred to a fresh tube and add 0.5 ml of 2-propanol per ml of TRI reagent used in sample preparation. Allow the sample to stand for 5– 10 minutes at room temperature. Centrifuged the sample at 12,000 × g for 10 minutes at 2–8 °C. The RNA precipitate will form a pellet on the side and bottom of the tube. Remove the supernatant and wash the RNA pellet by adding a minimum of 1 ml of 75% ethanol per 1 ml of TRI reagent used in sample preparation. The sample was vortexed and then centrifuged at 7,500 × g for 5 minutes at 2–8 °C. Briefly dry the RNA pellet for 5–10 minutes by air-drying. The RNA pellet should not be dried completely, as this will greatly decrease its solubility. Appropriate volume of DEPC water was added to the RNA pellet. To facilitate dissolution, mix by repeated pipeting with a micropipette at 55–60 °C for 10–15 minutes.

Preparation of cDNA from RNA

The cDNA was converted from 1µg of the total RNA using high capacity cDNA conversion kit. The synthesis of DNA from an RNA template, via reverse transcription, produces complementary DNA (cDNA). Reverse transcriptases (RTs) use an RNA template and a short primer complementary to the 3' end of the RNA to direct the synthesis of the first strand cDNA, which can be used directly as a template for the Polymerase Chain Reaction (PCR). This combination of reverse transcription and PCR allows the detection of low abundance RNAs in a sample, and production of the corresponding cDNA, thereby facilitating the cloning of low copy genes. After initial denaturation for 10 min at 95 °C, further denaturation done for 30 sec at 95 °C followed by annealing at 60 °C for 45sec and extension at 72 °C for 15sec thirty five amplification cycles were performed for PSA, Bax and Bcl2.

Table 7: Primers for gene expression

Gene	Primer	NCBI
BAX	F: 5'-GGGGACGAACTGGACAGTAA-3' R: 5'-CAGTTGAAGTTGCCGTCAGA-3'	NM_001291428
Bcl2	F: 5'- CAGGATAACGGAGGCTGGGATG-3' R: 5-GACTTCACTTGTGGCCCAGAT-3'	NM_000633.2
PSA	F: 5'-CCGGAAGTGGATCAAGGACA-3', R: 5'- GGCCTGGTCATTTCCAAGGT-3',	NM_001648.2

4.2.3.2. Caspase assay

Caspase 3, 8, 9 enzyme activity will be assayed by using a calorimetric caspase assay kit. LNCaP cells were treated with 25nM of Cu I and concurrently incubated a control culture without induction. Count cells and pellet 1-5 × 10^6 cells. Resuspend

cells in 50 µl of chilled cell lysis buffer and incubate cells on ice for 10 minutes. Centrifuge for 1 min in a microcentrifuge (10,000 × g). Transfer supernatant to a fresh tube and put on ice for immediate assay or aliquot and stored at -80°C. Assay protein concentration. Dilute 50-200 µg protein to 50 µl cell lysis buffer for each assay. Add 50 µl of 2X reaction buffer that contains 10 mM DTT to each sample. Add 5 µl of 4 mM DEVD-pNA (caspase 3), IETD-pNA (caspase 8), LEHD-pNA (caspase 9) substrate and incubate at 37°C for 1-2 hour. Read samples at 400 or 405 nm in a microtiter plate reader. Fold increase in caspase activity can be determined by comparing these results with the level of the un-induced control.

4.2.3.3. Acridine orange and ethidium bromide staining

Incubate 25µl of cell suspension (0.5×10^6 to 2.0×10^6 cells/ml) with 1 µl of AO/EB solution. Mix gently. Each sample should be mixed just prior to microscopy quantification. The sample must be evaluated immediately. Place the plate onto a microscopic stage of Fluorescence microscope with fluorescence filter and 10X objective. Acridine orange is a vital dye and will stain both live and dead cells. Ethidium bromide will stain only cells that have lost membrane integrity. Live cells will appear uniformly green. Early apoptotic cells will stain green and contain bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells will also incorporate ethidium bromide and therefore stain orange, but, in contrast to necrotic cells, the late apoptotic cells will show condensed and often fragmented nuclei. Necrotic cells stain orange, but have a nuclear morphology resembling that of viable cells, with no condensed chromatin.

5. RESULTS

5.1. *In-silico* screening results

Cucurbitacin derivatives were evaluated for their binding affinity with AR, ERβ, Bcl2, PI3Kα and AKT proteins. Cucurbitacins had binding affinity with AR, Bcl2 and AKT and not with PI3Kα and ERβ protein. Among the proteins studied, cucurbitacin exhibited better binding affinity with androgen receptors (Table 8). In androgen receptor, cucurbitacins had similar and more binding affinity as of bicaluatmide (G score -9.177) (Table 8). The standard bicalutamide forms H-Bond interactions with amino acids Gln711, Arg752, HOH108 and Asn705 (Table - 9, Fig 4). Among the cucurbitacins, cucurbitacin I had number of H-Bond interactions with androgen receptors. Cucurbitacin I forms H-Bond interaction with Gln711, Arg752, Asn705, HOH108, Met749, HOH109, Val746, Met745 and Trp741 (Table 9, Fig 5).

Cucurbitacin G, H, K, L, D and I which had a better binding affinity than bicalutamide were further evaluated for their ADME and toxicity properties using QikProp 4.0, Schrodinger software. Except for molecular weight, the test compounds were within the prescribed range of selected properties (Table 10 & 11). Among the cucurbitacins, cucurbitacin I have low molecular weight (514 Dalton). Based on the H-bond interactions and predicted ADME properties, we have selected cucurbitacin I as a lead compound and used that for *in-vitro* studies.

5.2. In-Vitro results

5.2.1. Growth optimization

LNCaP cells were cultured in RPMI medium supplemented with 10% fetal bovine serum at 37˚C with 5% CO_2. PC3 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum at 37˚C with 5% CO_2. The doubling time of

LNCaP cells and PC3 cells was observed at 60 hour and 34 hours respectively. The fully grown LNCaP cell was in spindle shape and PC3 was in spheroid shape.

5.2.2. MTT assay result

The test and standard compounds were evaluated in LNCaP (AR +) and PC3 (AR-) cell lines. The test and standard compounds were initially evaluated for their cytotoxicity from 1µM to 100µM. Cucurbitacin I exhibits 100% cytotoxic effect in the lowest tested dose (0.1µM) on LNCaP and PC3 cell lines. Hence the cucurbitacin I was evaluated between 1nM to 100nM in LNCaP and PC3 cell lines. Cucurbitacin I inhibited the proliferation of LNCaP cells in a dose dependent manner, whereas in PC3 the inhibition is less. The IC_{50} value of Cucurbitacin I in androgen receptor positive cell line (LNCaP) was found to be 25 nM whereas in androgen receptor negative cell line (PC3) was found to be 791 nM (Table 12) (Fig 6). The Cucurbitacin I was 31.4 times selective towards the AR positive cell line than the AR negative cell line (Table 14). The IC_{50} of bicalutamide in LNCaP cell line was found to be 45.5 µM whereas in PC3 cell line it was 66.6µM (Table 13) (Fig 7). Inhibition in PC3 cell lines by bicalutamide was dose independent, whereas in LNCaP it was dose dependent. The bicalutamide was 1.48 times selective towards the AR positive cell line than the AR negative cell line. The results indicate that the cucurbitacin I was more selective towards the LNCaP cell line than the bicalutamide. Also, the cucurbitacin I was more cytotoxic in LNCaP cell line than bicalutamide. This is because the cucurbitacin I was 1796 times more potent than bicalutamide in LNCaP cell line.

5.2.3. Acridine orange/Ethidium bromide staining result

Acridine orange/Ethidium bromide staining was done in LNCaP cell line treated with 25nM cucurbitacin I and solvent DMSO for 48hr. Cells were counted manually for total cells, live cells and apoptotic cells. Cells with greenish color indicate live cells and

cells with dark red denote necrotic cells. Late apoptotic cells are in reddish orange color with condensed chromatin and early apoptotic cells have green condensed chromatin. (Fig: 8a, 8b). LNCaP cells treated with 25 nM of Cucurbitacin I had 49.8 % apoptotic cells. (Table: 15). Significant increase in apoptosis of Cucurbitacin I treated cell was observed in comparison with solvent control. The report of acridine orange and ethidium bromide assay confirms the result of MTT assay.

5.2.4.Caspase assay

The Caspase 3, 8, 9 assay was done in LNCaP cell line treated with 25 nMCucurbitacin I and solvent DMSO for 48hr. The protein concentration assay was done by Bradford's method. Fold increase in caspase 3, 8, 9 was determined by comparing the absorbance of Cucurbitacin I treated cells with the control. Fold increase of caspase 3, 8, 9 activities in LNCaP cells was found to be 2.3, 2.8 and 2.6 respectively. The caspase activity was found to be increased ($P<0.001$) in Cucurbitacin I treated cells in comparison to DMSO treated cells. (Table: 16)

5.3. Gene expression study

LNCaP cells were treated with standard bicalutamide, Cucurbitacin I and DMSO was taken as solvent control.Bax, Bcl2 and GAPDH gene expression pattern of control, bicalutamide and cucurbitacin I treated cells are shown in Fig 9a, 9b, 9c. PSA and GAPDH gene expression pattern control, DHT, bicalutamide and cucurbitacin I treated cells are shown in Fig. 9d and9e. Cells were treated with 10 NM of DHT, which serve as a negative control, for the Prostate Specific Antigen gene expression study.

5.3.1. PSA Gene expression

A significant increase in PSA gene expression was observed in DHT treated cells (~2.1 fold) in comparison with control ($P<0.001$). Cucurbitacin I significantly

decreased the expression of PSA gene (~0.56 fold) followed by bicalutamide (~0.73 fold) ($P<0.01$) (fig 10).

5.3.2. Bax-Bcl2 gene expression

Expression of Bcl2 was low ($P<0.01$) in Cucurbitacin I (~0.56 fold) treated cells in comparison to solvent control and standard drug bicalutamide (~0.87 fold) (fig 11). Cells treated with Cucurbitacin I (~1.20 fold) showed upstream Bax expression as like standard (~1.29 fold). (Fig.12). Cucurbitacin I inhibited the expression of anti-apoptotic protein Bcl2 (~0.56 fold) and stimulated the expression of pro-apoptotic protein Bax (~1.20 fold). The ratio between Bax and Bcl2 of bicalutamide and Cucurbitacin I was found to be 1.48 and 2.14 respectively. Significant increase in Bax/Bcl2 ratio was observed for Cucurbitacin I in comparison with bicalutamide and solvent control. (Table: 17)

Table 8: Docking of cucurbitacin derivatives with prostate cancer targets

S. No	Cucurbitacin derivatives	AR (PDB Id : 2OZ7)	ERβ (PDB Id : 4ZI1)	PI3Kα (PDB Id : 4YKN)	Bcl2 (PDB Id : 2W3L)	AKT (PDB Id: 2KEL)
1	Cu A	×	×	×	×	-3.302
2	Cu B	×	×	×	×	-3.274
3	Cu C	-5.733	×	×	-4.919	-1.507
4	Cu D	-9.201	×	×	-4.026	-3.473
5	Cu E	-7.857	×	×	×	-5.168
6	Cu F	-5.977	×	×	-.4.256	-5.043
7	Cu G	-10.886	×	×	×	-8.047
8	Cu H	-11.075	×	×	×	-4.364
9	Cu I	-9.043	×	×	-4.011	-3.378
10	Cu J	-8.621	×	×	-4.912	-5.027
11	Cu K	-9.634	×	×	-4.268	-6.232
12	Cu L	-9.338	×	×	×	-3.646
13	Cu O	-4.906	×	×	-4.555	-6.164
14	Cu P	×	×	×	-5.390	-4.859
15	Cu Q	×	×	×	-4.576	-4.260
16	Cu R	×	×	×	×	-3.664
17	Cu S	×	×	×	×	-2.357
18	Standard	-9.177	-8.628	-9.733	-9.895	-11.107

Table 9: Interaction of cucurbitacin derivatives with AR

S.No	Cucurbitacinderivative	H bond	π – π bond	Ionic bond	Hydrophobic
1	Cu C	Asn 705, Ala 877	×	×	×
2	Cu D	Asn 705	×	×	×
3	Cu E	Gln 711, Met 745, Asn 705	×	×	×
4	Cu F	Leu 704, Asn 705	×	×	×
5	Cu G	Asn 705, Arg 752, Gln 711, Leu 873, Gln 709	×	×	×
6	Cu H	Gln 711, HOH, Leu873, Asn 705, Ala 877	×	×	×
7	Cu I	Gln 711, Arg 752, Met 749, Asn 705, Trp 741, Met 745, Val 746	×	×	×
8	Cu J	Glu 709	×	×	×
9	Cu K	Asn 705, Gln 711	×	×	×
10	Cu L	Asn 705	×	×	×
11	Cu O	Asn 705	×	×	×
12	Standard	Gln 711, Arg 752, HOH, Asn 705	Phe 764	×	×

Table 10: ADME property prediction

Drug	CNS	QPLog HERG	QPPCaCO	BB	QPMD CK	QPLogkhs a	Metabolism
Cu G	-2	-3.671	98.6	-1.8	40.4	0.23	9
Cu H	-2	-3.671	55.0	-2.5	21.5	0.16	9
Cu L	-2	-3.684	76.6	-1.8	48.2	0.31	9
Cu D	-2	-3.696	85.00	-1.9	30.7	0.39	8
Cu K	-2	-3.855	116.1	-1.8	34.4	0.28	7
Cu I	-2	-3.647	80.4	-1.8	32.4	0.42	7
Bic	-2	-3.306	163.6	-1.3	562.3	-0.04	2
TST	-2	-5.806	1125.9	-0.2	487.6	0.50	3

- **CNS** - is predicted central nervous system activity on a -2 (inactive) to +2 (active) scale.
- **QPlogKhsa** - is predicted binding to human serum albumin, which should in the range of 1.5 to 1.5

- **QPPCaco** - is predicted apparent Caco-2 cell permeability in nm/sec. Caco 2 cells are a model for the gut-blood barrier, recommended value is <25 poor, >500 great.
- **QPlogBB** - is predicted blood/brain partition coefficient, which should be in the range of -3.0 to 1.2.
- **QPMDCK** - predicted apparent MDCK cell permeability in nm/Sec. It's a good mimic for blood brain barrier, recommended value is <25 poor, >500 great.
- **QPlog HERG** - is predicted IC_{50} for blockage of HERG K^+ channels, which should be under -5.
- **Metabolism** is number of likely metabolic reactions, the range is 1 to 8.

Table 11: Lipinski Rule of five

Drug	Mol. Wt<500 daltons	Donor HB(<5)	Acceptor HB (>10)	Log P (< 5)
Cu G	534.6	4	11.6	2.5
Cu H	534.6	4	11.6	2.2
Cu L	532.6	4	10.6	2.8
Cu D	516.6	4	9.9	2.7
Cu K	516.6	4	10.9	2.4
Cucurbitacin I	514.6	4	9.9	2.7

Bicalutamide	430.3	1	7.7	2.9
Testosterone	288.4	1	3.7	3.3

Table 12: % Inhibition of Cucurbitacin I in LNCaP and PC3 cells

S.No	Cucurbitacin I (nM)	% Inhibition	
		LNCaP	PC3
1	1	0.15±0.02	0.125±0.00
2	5	2.15±0.09	0.425±0.17
3	10	7.04±6.3	0.83±7.4
4	15	21.44±5.2	4.80±3.9
5	20	49.37±6.6	11.09±3.4
6	25	58.84±8.2	16.98±5.1
7	30	85.21±7.4	22.24±4.6
8	50	92.58±5.7	39.24±5.7
9	100	100.10±3.1	45.24±3
10	500	103±6.6	47.24±3.1

Table 13: % inhibition of Bicalutamide in LNCaP and PC3 cells

S.No	Bicalutamide (μM)	% Inhibition	
		LNCaP	PC3
1	0.1	0.47±0.1	0.16±0.1
2	0.5	1.82±1.5	0.22±0.2
3	1	3.03±2.6	0.30±0.3
4	5	15.14±2.5	0.66±0.1
5	10	19.57±2.6	0.68±0.1
6	50	40.91±3.4	1.30±0.7
7	100	60.04±6.6	12.24±3.4
8	500	100.58±4.8	92.65±2.32

Table 14: IC_{50} value and selectivity of cucurbitacin I and bicalutamide

S.No	**Drug**	**IC_{50} (nmol/L)**		**Selectivity (PC3/LNCaP)**
		PC3	LNCaP	
1	Cucurbitacin I	790.99 ± 107.30	25.18±7.09	31.41

2	Bicalutamide	66963.33±2453.38	45239±2586.24	1.48

Table 15: % Apoptosis incucurbitacin I treatedLNCaP cells

Treatment	**Total no. of cells**	**No. of Live cells**	**No. of Apoptotic cells**	**% apoptosis**
CONTROL	121	117	4	3.30
Cucurbitacin I (25nM)	129.5	65	64.5	49.80***

All the values are expressed in mean ± S.D. One way ANOV followed by Post hoc analysis Turkey's multiple comparison test. *** denotes statistical significance of treatment group compared to control group at P<0.001.

Table 16: Caspaseactivity of Cucurbitacin I in LNCaP cells

S. No	Cucurbitacin I in LNCaP	Control (abs 405nm)	Cucurbitacin I treated (25nM) (abs 405nm)	Fold Increase in Caspase activity
1	Caspase 3	0.06±0.00	0.14±0.02	2.33***
2	Caspase 8	0.06±0.00	0.17±0.011	2.83***
3	Caspase 9	0.06±0.00	0.16±0.010	2.66***

All the values are expressed in mean ± S.D. One way ANOVA followed by Post hoc analysis Turkey's multiple comparison test. *** denotes statistical significance of treatment group compared to Control group at P<0.001.

Table 17: Bax- Bcl2 ratio

S.No	Group	Bax mRNA expression	Bcl2 mRNA expression	Bax/Bcl2 ratio
1	**Control**	1	1	1
2	**Bicalutamide**	1.29	0.87	1.48***
3	**Cucurbitacin I**	1.20	0.56	2.14***

All the values are expressed in mean ± S.D. One way ANOVA followed by Post hoc analysis Turkey's multiple comparison test. *** denotes statistical significance of treatment group compared to control group at P<0.001.

Fig 4: Interaction of bicalutamide with AR

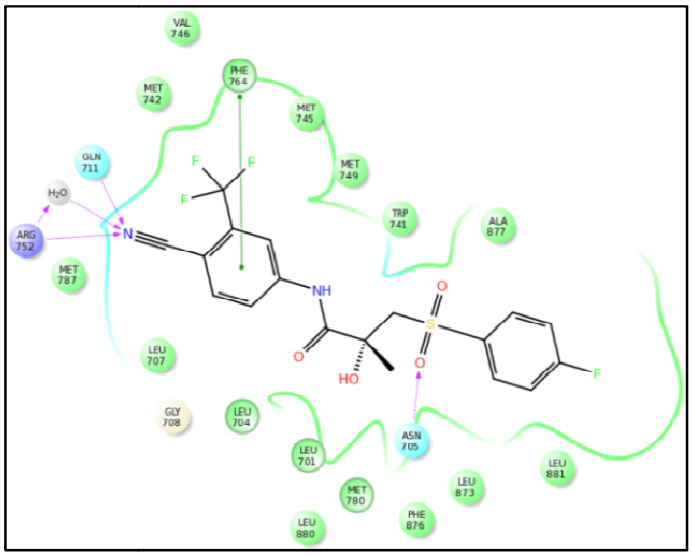


Fig 5: Interaction of Cucurbitacin I with AR

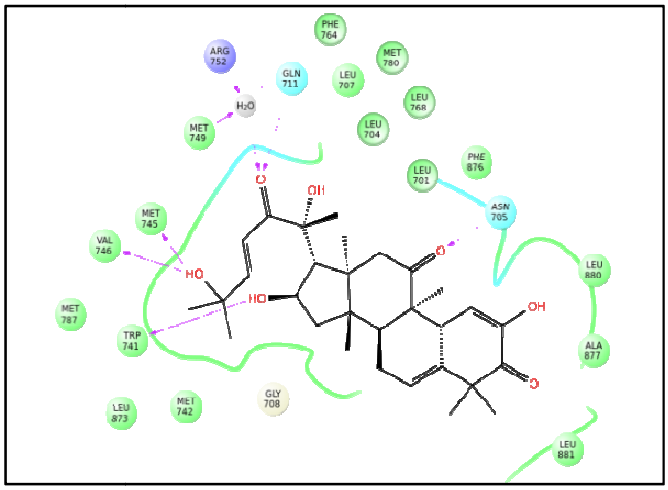
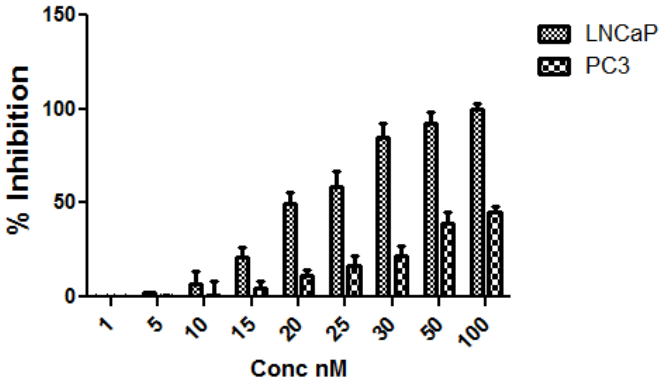
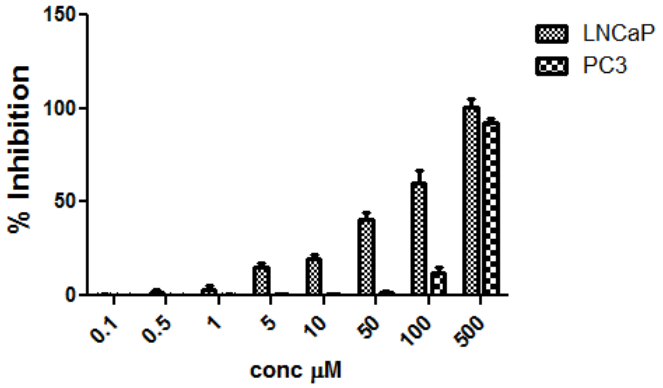


Fig 6: Inhibition of cucurbitacin I



All the values are expressed in mean ± S.D. IC_{50} value was calculated by non-linear regression curve.

Fig 7: % Inhibition of bicalutamide



All the values are expressed in mean ± S.D. IC_{50} value was calculated by non-linear regression curve.

Fig 8a:LNCaP cells treated with solvent DMSO

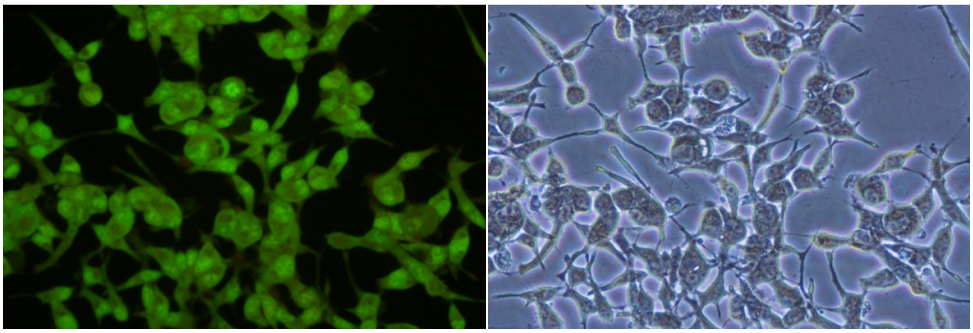


Fig 8b:LNCaP cells treated with Cucurbitacin I

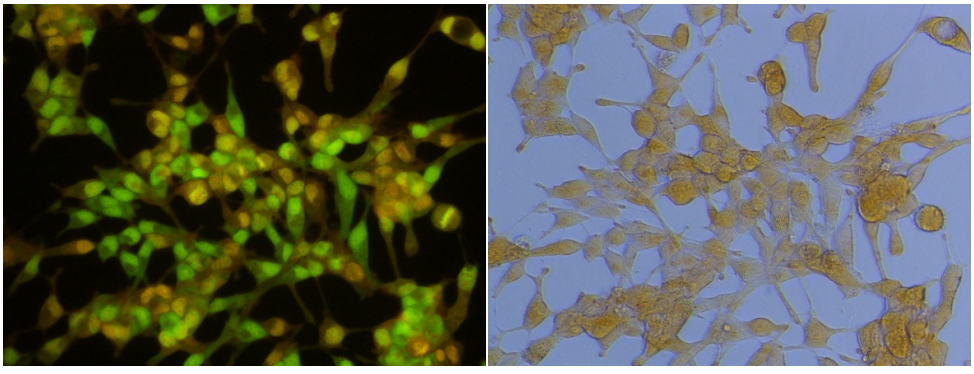
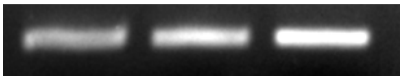


Fig 9: Gene expression

9a. BAX gene expression in LNCaP cells

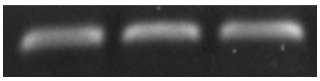


Con Bic Cu I

9b. Bcl2 gene expression in LNCaP cells

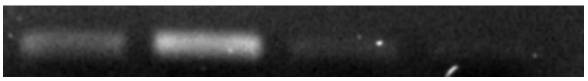
Con BicCu I

9c GAPDH gene expression in LNCaP cells



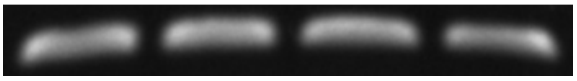
Con BicCu I

9d. PSA gene expression in LNCaP cells



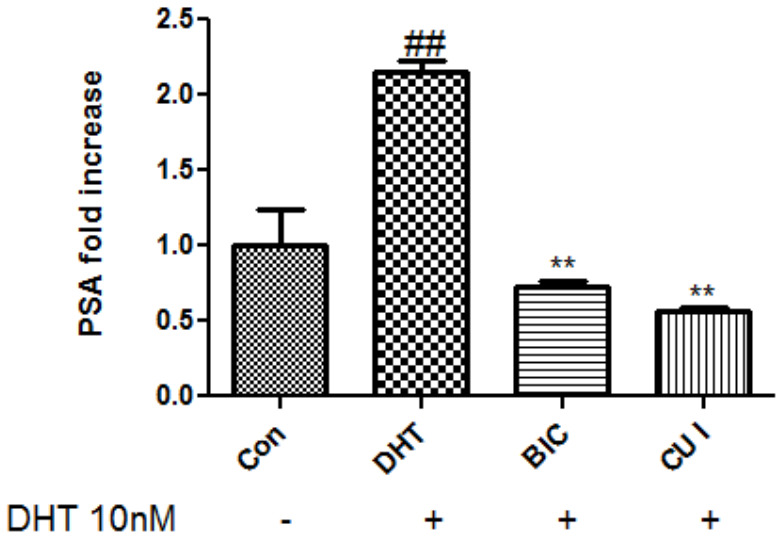
Con DHT Bic Cu I

9e. GAPDH gene expression in LNCaP cells



Con DHT Bic Cu I

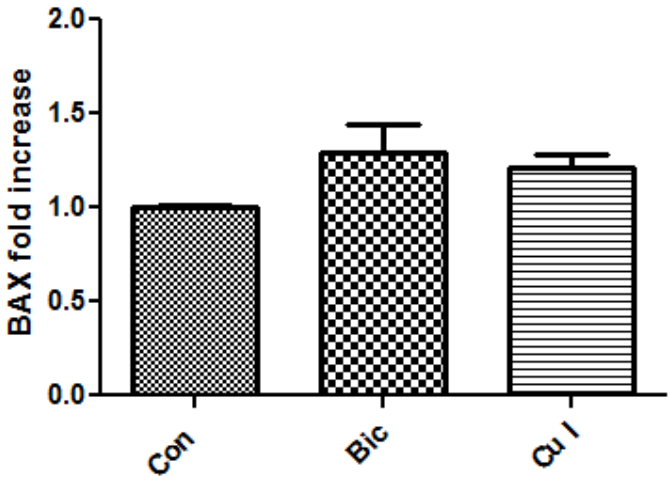
Fig 10: PSA gene expression



All the values are expressed in mean ± S.D. one way ANOVA followed by Post hoc analysis Turkey's multiple comparison test. ## denotes statistical significance of PSA

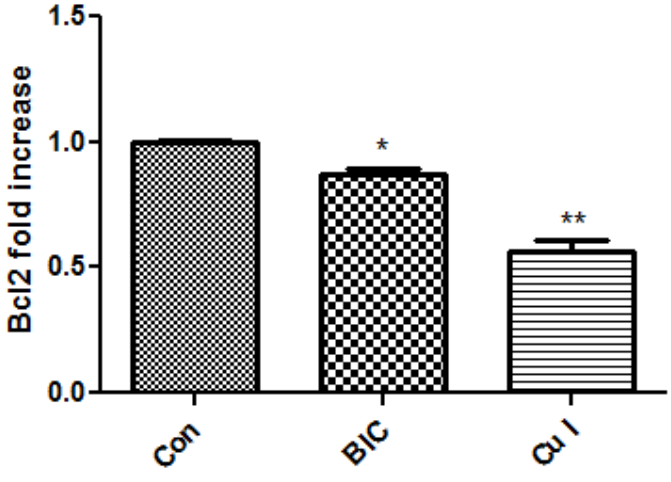
compared to control at P<0.01. ** denotes statistical significance of treatment group compared to DHT group at P<0.01.

Fig 11: Bax gene expression



All the values are expressed in mean ± S.D. One way ANOVA followed by Post hoc analysis Turkey's multiple comparison test.

Fig 12: Bcl2 gene expression



All the values are expressed in mean ± S.D. One way ANOVA followed by Post hoc analysis Turkey's multiple comparison test. *, ** denotes statistical significance of treatment group compared to control group at P<0.05 and P<0.01 respectively.

6. DISCUSSION

The present study shows that cucurbitacins are having specific binding affinity for the androgen receptor. This was proved by docking studies where cucurbitacins had a better G score for androgen receptor rather than other target of prostate cancer. Further examination of cucurbitacins for ADME and toxicity properties, we had identified cucurbitacin I was the lead molecule among the cucurbitacins A-U. The androgen receptors are nuclear receptor which is having a great binding affinity for steroidal hormones particularly Dihydrotestosterone. Cucurbitacins are triterpenoids which are the precursor of steroids (David, 1998). This may be the reason for the androgen selectivity. Additionally the presence of numerous keto-, hydroxyl and acetoxy groups are functioning as H-Bond donor/acceptor. These functional groups had formed the essential H-Bond interactions with the androgen receptor.

The ligand binding pocket of androgen receptor was made of 12α helix and 2β sheets which folds together to form a hydrophobic ligand binding pocket. The ligand-protein interaction was majorly hydrophobic , however hydrogen bond interaction also plays a major role in specificity. Docking studies and crystallographic studies revealed that ligands which are able to form H-bond interactions with amino acids Asn705, Gln711 and Arg752 andwill have strong affinity towards AR (Karine *et al*., 2006). Cucurbitacin I forms H-bond interaction at Gln711, Arg 752, Met 749, Asn 705, HOH 108, Trp 741, Met 745, HOH Val 746. Bicalutamide forms H-bond interaction with Gln 711, Arg 752, HOH 108, Asn 705. Recent studies revealed that a full antagonist to AR (ligands which function as an antagonist to wild type and mutated ARs) forms H-bond interactions with Gln711 and Arg752 (Chuangxing *et al*., 2012). The H-bond interaction with Arg 752, Gln 711 was essential for antagonist activity. The bicalutamide and cucurbitacin I had formed H-bond interaction with those amino acids.

The cucurbitacin I and bicalutamide were screened for its cytotoxicity effect in prostate cancer cell lines LNCaP (AR +ve) and PC3 (AR □ve). This method of screening in LNCaP and PC3 cells could be used to identify the AR selectivity. Expression of dihyrotestosterone stimulated prostate specific antigen was significantly reduced by cucurbitacin I, this reduction of PSA level (~0.56 fold) was due to anti-androgenic effect of Cucurbitacin I. The PSA is the Prostate cancer marker and AR response element. The cucurbitacin I was found to be 31.4 times selective towards AR positive LNCaP cell line and the standard bicalutamide was 1.48 times selective towards AR positive LNCaP cells. The potency of Cucurbitacin I in LNCaP was found to be 1796 times than bicalutamide. The tremendous potency of cucurbitacin I in LNCaP cells could be due to the synergetic effect of AR antagonism and JAK/STAT inhibition (Alghasham., 2013). Cucurbitacin I is a novel compound that could be further developed to evaluate its *in-vivo* efficacy.

Apoptosis of the Cucurbitacin I treated cells were confirmed by measuring the caspase 3, 8, 9 cleavage activity, Bax/Bcl2 gene expression ratio and Ethidium bromide/Acridine orange staining. Growth factor withdrawal and intracellular stress can induce apoptosis through the intrinsic cell death pathway, while extrinsic apoptosis is initiated through transmembrane death receptors. Initiation and execution of these processes are regulated by the BCL-2 and caspase families of proteins. Activation of the BCL-2 family members Bax and Bak results in mitochondrial outer membrane permeabilization (MOMP) and the release of pro-apoptotic proteins, including cytochrome *c*, from the inter-membrane space into the cytosol (Matthew *et al.,*2013). Cytochrome *c* can then bind apoptotic protease activating factor 1 (Apaf-1) forming the apoptosome and activating caspase-9. Once active, caspase-9 can directly cleave and activate caspase-3.

The death receptor-mediated pathway is activated upon ligand binding of cell surface death receptors such as tumor necrosis factor-α (TNF-α), initiating ligand-induced receptor trimerization and the formation of death-inducing signaling complex (DISC). Once caspase-8, the initiator caspase, is recruited in zymogen form to the DISC, it is autocatalytically processed and released from the complex to the cytosol as active tetramer to transactivate a number of downstream executioner caspases including the dominant executioner caspase, caspase-3 (Lee *et al.* 2003). The significant increase in Caspase 3, 8 and 9 activity of Cucurbitacin I treated cells indicates that apoptosis triggers by both extrinsic and intrinsic pathways. Bcl2 is an anti-apoptotic protein that was found to be increased in Prostate cancer. The anti-apoptotic protein Bcl2, has been associated with the development of androgen-independent prostate cancer due to its high levels of expression in androgen-independent tumors in advanced stages of the pathology (Catz *et al.,* 2003). Reduction of Bcl2 level is essential for apoptosis in prostate cancer cells. The level of Bcl2 mRNA level was significantly reduced in Cucurbitacin I (~0.56 folds) treated LNCaP cells, in comparison with bicalutamide (~0.87 fold) and solvent control (~ 1 fold).

The balance between pro- and anti-apoptotic members of this family can determine the cellular fate. Bax and Bcl-2 are the major members of the Bcl-2 family which has potential roles in tumor progression and prognosis of different human malignancies. Bax promotes cell death through permeabilization of mitochondrial outer membrane in response to different cellular stresses. In contrast, Bcl-2 prevents apoptosis by inhibiting the activity of Bax. (Mohan *et al.,* 2012) (Hector *et al.,* 2009). The Bax/Bcl-2 ratio can act as a rheostat which determines cell susceptibility to apoptosis (Raisova *et al.,* 2001). Lower levels of this ratio may lead to resistance of human cancer cells to apoptosis. Thus, the Bax/Bcl-2 ratio can affect tumor progression

and aggressiveness. (Hemminki *et al.,* 2010). The Bax/Bcl2 ratio of bicalutamide and Cucurbitacin I was found to be 1.48 and 2.14 respectively, significant increase in Bax/Bcl2 ratio of Cucurbitacin I treated cells in comparison with bicalutamide and solvent control was observed.

7. SUMMARY AND CONCLUSION

The present study identified that the cucurbitacin I had a better binding affinity towards androgen receptor and its H-bond interaction with AR protein was similar as of standard bicalutamide. Cucurbitacin I satisfies the ADME and toxicity parameter studies. Further evaluation of cytotoxity of cucurbitacin I in prostate cancer cell lines LNCaP (AR +ve) and PC3 (AR -ve) reveals that cucurbitacin I is more selective toward AR positive cell line LNCaP than PC3 cells. Moreover the cytotoxic potency of cucurbitacin I in LNCaP cells is 1796 times greater than standard bicalutamide. Reduction of prostate specific antigen in cucurbitacin I treated LNCaP than dihydrotestosterone treated LNCaP cells shows the anti-androgenic property of cucurbitacin I. Apoptosis was confirmed by ethidium bromide/acridine orange staining. Upregulation of caspase 3, 8, 9 in cucurbitacin I treated cells was observed. The mechanism behind the cytotoxicity could be the activation of both external and internal apoptosis pathways. Increased level of Bax/Bcl2 ratio was noted in Cucurbitacin I treated LNCaP cells. The higher levels of this ratio may lead the cancer cells to apoptosis. We conclude that Cucurbitacin I is an effective and potent anti-androgen agent, which cause apoptosis by the activation of external and internal apoptosis pathways and by inhibition of anti-apoptotic protein Bcl2 gene expression. Cucurbitacin I is a novel agent for androgen dependent prostate cancer.

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Program Coordinator
Novartis Healthcare Pvt Ltd

Dr. M. Ramanathan
Principal
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