A natural product Decursin enhances the radio-sensitization of IR against DMBA-induced tumor

By

HIRA ZAHID

NUST201463172MSMME62414F

Supervised by: **Dr. Adeeb Shehzad**

School of Mechanical and Manufacturing Engineering
National University of Sciences and Technology
H-12 Islamabad, Pakistan

FORM TH-4

MASTERS THESIS WORK

We hereby recommend that the dissertation prepared under our supervision by **Hira Zahid** under registration no. NUST201463170MSMME62424F, titled: "A natural product Decursin enhances the radio- sensitization of IR against DMBA-induced tumor" be accepted in partial fulfillment of the requirements for the award of Masters Degree with **A** grade.

Examination Committee Members

1. Name: Dr Nabeel Anwar Signature:_____

2. Name: Dr Syed Omer Gilani Signature:_____

3. Name: Dr. Umer Ansari Signature:_____

Supervisor's name: Dr. Adeeb Shehzad Signature:_____

_____ _____

(Head of Department) (Date)

COUNTERSIGNED

Dated: _____ _____

(Dean / Principal)

DECLARATION

It is hereby declared that this research study has been done for partial fulfillment of requirements for the degree of Masters of Sciences in Biomedical Sciences. This work has not been taken from any publication. I hereby also declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification in this university or other institute of learning.

Hira Zahid

Every breath I take in my life and every little drop of blood running in my body, are dedicated to my family to whom I owe everything.

ACKNOWLEDGEMNT

"In the name of Allah the most Merciful and Beneficent"

First and foremost, praises and thanks to Allah, the Almighty, who is my Lord and who is my all who never let efforts go wasted. His blessings and guidance throughout made me complete this project successfully.

I don't have words to thank to my family to whom I owe everything, the ones who can never ever be thanked enough, for the overwhelming love and care they bestow upon me. Without their proper guidance it would have been impossible for me to complete my study. Thanks to my parents for their encouragement affection, support, care, prayers and giving me real happiness whenever I am depressed, to my brothers, sister, grandparents and friends for their consistent prayers and care.

I would like to express my profound and sincere gratitude to my research supervisor, Dr. Adeeb Shehzad, for giving me the opportunity to work under his supervision. His timely contribution, encouragement, kindness and valuable pieces of advice helped me shape my work into its final form.

I am also deeply indebted to my committee members especially to Dr. Nabeel Anwar for kindness in examining the research work and providing suggestions for improvement and for generously supporting the thesis with his expertise throughout. Thanks to Dr Umar Ansari for his encouragement, pieces of advice and moral support and Dr. Omer Gilani for his helpful comments and administrative support.

I would like the whole hearted thank my friend Maryam Shahid who helped me passionately and sincerely, Mehak Fatima and Aisha Tahir who have listened to my moaning and complaining and to all my lab fellows for providing me friendly and peaceful environment in whole tenure of my research work.

The special thanks goes to students of Prosthetics Lab, Amber Zahoor and Javeria Yousaf, who have always been there whenever needed for any kind of help. Last but not

the least, I would like to thank each and every person for supporting me throughout this research phase in any way.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic Acid
RT	Radiation Therapy
CDKs	Cyclin Dependent Kinases
SSBs	Single Stranded Breaks
DSBs	Double Stranded Breaks
Gy	Gray (Unit of Radiation)
PKC	Protein Kinase C
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PI3k	Phosphatidylinositide 3-kinases
Akt	Protein Kinase B
STAT3	Signal transducer and activator of transcription 3
Caspase	Cysteine-aspartic proteases
DMSO	Dimethyl sulfoxide
DMBA	7,12-Dimethylbenz[a]anthracene
BSA	Bovine Serum Albumin
NC	Nitrocellulose
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
ECL	Enhanced Chemiluminescence
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

ABSTRACT

In healthy cells signaling networks control key functions and the overall fate of the cell and are activated through a myriad of signals from the extracellular and the intracellular environment. In cancerous cells however, these signaling networks are disrupted as a result of mutations or genetic alterations. These mutations interfere with the normal signaling and cause Radio-resistance. Recent advances in cancer therapeutics are aimed at targeting these pathways through inhibition of various molecules e.g. tyrosine kinase inhibitors are used to target kinases. Decursin is the active coumarin compound, isolated from the roots of Angelica gigas Nakai (Umbelliferae) and exhibits anti-inflammatory activities. We hypothesized that the anti-inflammatory activity of Decursin will consecutively contribute to its radio-sensitization effect, when administered together with radiation therapy. The western blot results showed that Decursin together with ionizing radiation, inhibited the p52 subunit of NF-κB and also inhibited the degradation of IκB. Decursin also lead a decrease in the expression of P-Akt. Tumor volume was also monitored and showed a decrease over the period of treatment.

INTRODUCTION

1.1What Is Cancer?

Normal functionality of living organisms is maintained by a finely tuned balance between cell proliferation and cell death. If this balance is lost or even one of the cells becomes rebellious and starts uncontrolled proliferation, a tumor will be formed and, if metastasized, can lead to any fatal kind of cancer. This can result from any of the several process like (a) Activation of proto-onco genes, which is turning of normal gene into onco gene via mutation of or over expression, (b) inactivation of tumor suppressor genes, which are there to stop the metastasis, (c) changes in gene repair mechanisms, which are there to minimize the unwanted alterations in gene expression.

Cancers are classified into various types on the basis of their origin and tissue types. (a) Carcinoma is the cancer of epithelial origin. It occurs in all the internal as well as external lining of body parts including skin and gastrointestinal track. Carcinomas has further two subtypes: one of them is Adenocarcinoma which appears in organs or glands and the other one is Squamous cell Carcinoma which appears in squamous epithelium. (b) Sarcoma is the type of cancer which appears in connective of supportive tissues like muscles, bones, cartilage etc. (c) Myeloma originates from plasma cells and bone marrows. (d) Leukemia tends to originate from the blood and flows in blood stream. (e) Lymphoma occurs in the lymphatic system, lymph nodes etc

.

1.2 Melanoma

Skin, the largest organ of the body, has three layers. (a) Epidermis is the outer most layer of skin which protects the body from external environment. It is 0.01 inches thick. The main types of cells present in epidermis include: *Squamous cells* which are flat and outer most cells that shed all the time as the new ones are made; *Basal cells* are present in the lower part of epidermis and they keep on dividing and eventually replacing the squamous cells; *Melanocytes* are the cells that are involved in the brown pigmentation of the skin called melanin and they protect the skin from the harmful effects of sun rays. These cells have the potential to become 'melanoma' cancer. (b) Dermis is the middle layer of the skin which contains the hair follicles, sweat glands, blood vessels etc. (c) Subcutis is the deepest layer of skin which is involved in making a network of collagen and fat cells, conserves energy and protects the body organs from external shocks.

Melanoma skin cancer begins in melanocytes that have become cancerous. They appear brown or black if the melanin is being made and they appear pink or even white is the production of melanin stops because of cancer. Melanoma is one of the most dangerous forms of cancer. It can appear on any part of skin and can spread many bounds if not treated properly. Melanoma has the potential to spread to other parts of the body via dermis and if it goes deeper into the dermis, it can also reach the blood vessels causing way more adversity.

1.3 Radiation Therapy

One of the treatments of melanoma cancer is radiation therapy (RT). Radiations are used to destroy the cancer cells by altering their genes which cause their growth and division.

The radiation used is called ionizing radiation because it forms ions and deposits energy in the cells of the tissues it passes through. This deposited energy can kill cancer cells or cause genetic changes resulting in cancer cell death. This is how radiations can be used to kill cancer cells (Baskar et al., 2012). RT is known to be the most effective non-surgical method of locoregional therapy of melanoma cancer (Strojan, 2010).

Ionizing radiations are categorized into two main types. One of them is photon radiation which includes X-rays and gamma rays while the other one is particle radiation which includes electrons, protons, neutrons, alpha and beta particles, etc.

The normal life cycle of the cells has 5 phases. The phases of cell cycle are controlled by the proteins known as cyclin dependent kinases (CDKs). (a) The first phase of the cell cycle is *Go phase*. In this phase, the cells are in resting phase. They do not tend to divide and they keep on doing their normal work in the body. It can last from hours to years. (b) The second phase of the cell cycle, *G1 phase*, is the time where the cell gathers the information about when to go the next phase. In this phase, the cells get ready to divide. It lasts for 18-30 hours. (c) The *S phase* of the cell cycle is involved in the synthesis of DNA so that both the cells get enough and same amount of DNA. It takes about 18-20 hours. (d) The *G2 phase* builds up the apparatus for the process of mitosis and gathers the information about the starting point of mitosis. It takes place for 2 to 10 hours (e) *M phase* is the phase where mitosis occurs. One cell divides into two exactly same cells within 30 to 60 minutes in this phase.

The cell cycle is necessary for understanding the radiation mechanism because the actively dividing cells tend to be killed earlier by radiations as compared to the cells in the resting phase or slowly dividing cells. The more speedily the cell divides, the earlier it

gets the chance to be killed by radiation. Since the cancer cells are uncontrolled and follow rapid division, they are easily killed by RT. But radiation therapy can also kill the normally dividing cells which is one of the side effects of RT. The required balance between destruction of cancer cells and the protection of normal cells should be maintained properly. How much the cell is damaged by the radiation is called *radiosensitization*. RT kills the tumor cells by causing double stranded breaks in the DNA of the cells and hence interrupting the cell cycle. The more number of DSBs result by more absorbance of radiation dose(Huber et al., 2013).

1.4 Radio-resistance

Melanoma has always been believed to be more radio-resistant as compared to other types of cancers (Chang et al., 2006). The radio-resistance of a tumor is determined by innate ability of the cells to repair the DNA damages either by non-homologous end joining or by homologous recombination. Tumor mass is not restored again if the irradiated tumor cells leave the DSBs unrepaired. Radio-resistance and hence the survival of the tumor can be a result of cellular stress response caused by ion transports to DNA damage by controlling various cell functions like cell cycle, DNA repair or metabolic adaptations(Huber et al., 2013). Many protein pathways occurring within the tumor cell can be involved in the mechanism of radio-resistance which are discussed further.

1.5 Decursin

Decursin is a coumarin compound isolated from the roots of the Korean plant, Angelica Gigas Nikai. It has been used in Korean folk medicine for the treatment of a number of diseases and is known to have antibacterial and anamnestic effects (Yim et al. 2005). The

most common method of administration is the boiling of the dried roots in varying concentrations. Decursin and its derivative, decursinol angelate have been used to treat condition like anemia, pain, infection, articular rheumatism etc. Its alcoholic extracts have been used in *in vitro* experiments on various cancer models including breast cancer, lung cancer, colon cancer, prostate cancer, sarcoma, myeloma and leukemia. Although there is a lack of *in vivo* studies which have only been focused on a number of organs (Zhang et al. 2012).

1.6 Scope of Study

The study was conducted to determine whether Decursin has any synergistic effect when administered together with radiation therapy. Radiation therapy is of the main tools that are used to treat many types of cancer. Melanoma or skin cancer is known to be resistant to Ionizing radiation therefore doctors often resort to surgery as a means of treating melanoma. Research is being focused on the development of novel therapeutic agents that sensitize radio-resistant tumors such as skin tumors to radiation therapy.

Decursin has been well studied for its anti-cancer and anti-inflammatory effects. It is documented to be involved in inhibition of cell proliferation, angiogenesis, cell migration, and is also known to cause cell cycle arrest at various phases of the cell cycle. The scope of this study was to compare the individual effects of radiation and drug therapy to the combined effects of both. Reduction in tumor volume was observed over the period of treatment and the localization of various proteins in the cytoplasm was observed. The expression of the NF-κB, IκB and Akt was observed and visualized through western blot experiments. The expressions of these proteins was compared

amongst the experimental and control groups. The results were seen to be conclusive with the fact that Decursin exerts its anti-cancer effect alone and in combination with Ionizing radiation.

To elucidate the mechanism of action of Decursin by which it induces radio-sensitivity and inhibits pathways that are known to contribute to radio-resistance. A dose- dependent effect of Decursin was also studied where human colon cancer cell lines were subjected to increasing concentrations of Decursin and the difference in cell viability of the cancer cells was observed.

The effect of Decursin on the expression levels of NF-κB, IκB and Akt was investigated The expression level of NF-κB was reduced in the experimental groups compared to the control group. The expression of IκB was seen to be more in the experimental groups and this can be accounted to the inhibition of degradation of IκB by Decursin.

The expression of phospho-Akt was reduced in the drug group and remained constant in the radiation + drug group. It can however be seen that that in the group where radiation was administered alone the expression of phospho-Akt increased dramatically which can be a result of the aberrant activation of the PI3k/Akt pathway upon exposure to ionizing radiation.

1.7 Objective

The objective of this study was to highlight the concomitant effect of Decursin when administered together with radiation. Decursin has anti-inflammatory and anti-tumor activity and when administered together with Radiation it acts as a radio-sensitizer. Two main pathways were studied that are known to contribute to radio-resistance; the NF-κB

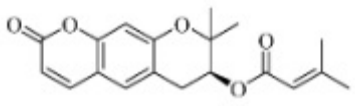
pathway and the PI3k/Akt pathway. Both these pathways are involved in cell survival and cell growth and hence when over-active they cause radio-resistance in tumors.

LITERATURE REVIEW

2.1 Decursin Pharmacology

Decursin is a naturally occurring coumarin compound that is isolated from the roots of Korean Angelica also known as Angelica Gigas Nakai. Korean Angelica is a native plant that thrives well in moist soil. It is a biennial or herbaceous perennial plant (Kim et al. 2015). Decursin was first discovered in a research carried out in 2007 to target Androgen receptor signaling that is crucial in the onset of Prostate cancer (Lu et al. 2007). Decursin and its components are known to have anticancer activities. A study carried out the biopharmaceutical characterization of Decursin and its components. There are four main components of Angelica Gigas Nakai; I) Decursin (which is the main component), II) an isomer of Decursin known as Decursinol Angelate III) the ether form of Decursin (JH714) IV) epoxide Decursin (Mahat et al. 2013) V) Oxim Decursin and VI) Diketone Decursin (Mahat et al. 2012).

Decursin has a molecular weight of 328 and its molecular formula is C19H20O5. Decursin is traditionally known to have therapeutic effects in a number of diseases like anemia. A number of studies have shown various pharmacological effects of Decursin including antibacterial, antiamnestic and anti-tumor effects (Yim et al. 2005). Decursin has also been used for the treatment of dysmenorrhea, amenorrhea, menopause, abdominal pain, injuries, migraine, and arthritis (Mahat et al. 2012).

Compound	Molecular Weight	Structure
Decursin (Main Component)	328.4	

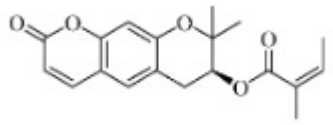
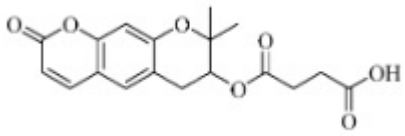
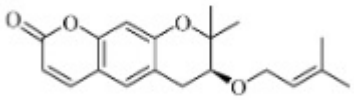
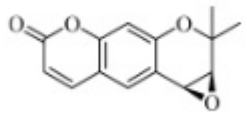
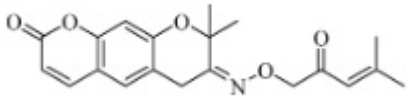
Decursinol Angelate (Isomer)	328.4	
Diketone Decursin (Derivative)	346.3	
Ether Decursin (Derivative)	314.4	
Epoxide Decursin (Derivative)	244.3	
Oxim Decursin (Derivative)	341.4	

Table 1: Decursin and its derivatives

2.2 Radio-Resistance

Radio-resistance is the one of the main hurdles during radiation therapy of many tumors. Generally, radio-resistance is considered to be the level of ionizing radiation that organisms can withstand. When a tumor becomes radio-resistant, it stops decreasing in size or volume by irradiation or when there is recurrence after regression of a tumor (Lacombe et al. 2013).

A number of characteristics determine tumor resistivity such as the number of clonogeneic cells at the start of the treatment, proliferating tumor cells that survive (Lacombe et al. 2013), the hypoxic or anoxic environment of the tumor that protects

tumor cells against damage by radiation (GRAY et al. 1953) and the most important factor which is the intrinsic radiosensitivity of a tumor. The intrinsic radiosensitivity of tumors include the cells intrinsic response to induce resistance towards radiation. This includes various molecular mechanisms including DNA damage repair, intercellular communication of a cell and its response to growth factors which includes various signaling pathways (Tucker & Thames 1989).

2.2.1 Radio-Resistance and Pi3k/Akt Pathway

One of the pathways associated with radio-resistance is the PI3k/Akt pathway. It is known to be associated with intrinsic radio-resistance, tumor cell proliferation and hypoxia related radio-resistance (Bussink et al. 2008). The Phosphatidylinositide 3-Kinase (PI3K) consists of two main subunits, the P110 subunit and a regulatory P85 subunit. The P110 subunit phosphorylates PIP2 in the plasma membrane hence activating the pathway (Zhan & Han 2004). c-Akt is a proto-oncogene which encodes for the 57kDa serine-threonine protein kinase. This protein kinase is the homologue for the v-akt oncogene found in viral genome (Bellacosa et al. 1991). The PI3k/Akt pathway is a major mediator in radioresponse of cancer cells. It is activated as a result of radiation as demonstrated in A549 lung cancer cell line where increased expression of VEGF-C was seen as a result of the PI3k/Akt/mTOR pathway activation (Chen et al. 2014). A number of studies have been conducted where inhibitors of the PI3k/Akt/mTOR pathway have enhanced the radiosenisitivity of various cancer cell lines both *in vitro* and *in vivo*. In one study carried out on Radio-resistant Prostate carcinoma cell lines it was shown that dual PI3k/mTOR inhibitors, BEZ235 and PI103, when administered together with radiation therapy could overcome radio-resistance in these cell lines by inducing more apoptosis (Chang et al. 2013). In a similar study conducted on 5-8F xenograft model it was shown that two inhibitors, GSK2126458 and PKI-587 when administered together with ionizing radiation, induced radiosensitivity in NPC cells in a concentration and time dependent manner. The concomitant administration of these inhibitors with IR also inhibited the phosphorylation of Akt and mTOR (Liu et al. 2015). In melanoma, aberrant activity of

the PI3k/Akt pathway has been demonstrated to promote melanogenesis (Madhunapantula & Robertson 2009).

ThePI3k/Akt pathway also has the ability to activate other signaling pathwyas. One of the more well-known pathways involved in radio-resistance which is the NF-κB pathway is also activated through Akt activation. NF-κBi s one of the targets of the PI3k/Akt pathway. In a proposed pathway, PDGF activates NF-κB by inducing Akt to associate with and activate IKK (Romashkova & Makarov 1999). NF-κB activation constitutively leads to radio-resistance.

2.2.2 Radio-Resistance and NF-κB pathway:

NF-κB is activated by ionizing radiation thus reducing the cytotoxic effects of chemotherapy. Inhibition of NF-κB activation can lead to an increase in the efficacy of radiation therapy. NF-κB is found in all cells in association with IκB. IκB Kinase phosphorylates IκB which is the ubiquitinated and degraded by a proteasome assembly. NF-κB is then free to translocate to the nucleus and bind to the DNA to allow the transcription of a number of genes that lead to cell survival and growth. NF-κB is activated by both radiation and chemotherapeutic agents alike. This leads to radio-resistance and chemoresistance and adds to the survival of the tumor. Therfore, down-regulation of NF-κB is a promising target to enhance radiosensitivity (Nakanishi & Toi 2005).

A study was conducted on colorectal cancer cell lines which showed that pre-treatment with PS-341 (an inhibitor of the proteosome that degrades IκB)or IκB alpha super-repressor, inhibited the activation of NF-κB with a 7-41% increase in radiosensitivity (Russo et al. 2001). In a similar study it was shown that sorafenib when combined with Ionizing radiation reduced the binding activity of Nf-KappaB/DNA when compared to control and radiation. This sensitized human OSCC to radiation and suppressed the expression of carcinogenic proteins (Hsu et al. 2015). Prostate cancer PC3 cells transduced with IκB super repressor gene showed repressed binding of DNA to Nf-KappaB. The clonogenicity of these cells also declined to 19.6% compared to that of the

control (Pajonk et al. 1999). The role of NF-κBin radio-resistance was elucidated in the study conducted on ataxia-telangiectasia (AT) fibroblasts. AT fibroblasts have extremely dysregulated NF-κB activation and also exhibit extreme radiosensitivity (Lee et al. 1998). Very limited research has been carried out to elucidate the role of NF-κB as a modifier of radiosensitivity of cancer cells. A study was carried out to investigate whether resistant melanoma cell lines could be made susceptible to radiation by inhibiting the activation of Nf-KappaB. These cell lines were treated with MG132 or by stable expression of IκBαM and down-regulation of anti-apoptotic proteins was observed. Survival of clonogenic cells on exposure to radiation was also reduced (Munshi et al. 2004).

2.3 Anti-Cancer Role of Decursin

The anti-cancer activity of Decursin has been determined in various studies and a considerable amount of data has been collected so far that elucidates the role of Decursin as a possible therapeutic agent in cancer therapy. Decursin also activated caspase-3 thus inducing apoptotic cell death in these cells (Kim et al. 2015). Decursin exerts its anti-cancer effects by inducing apoptosis through activation of caspase 8, 9 and 3 , down-regulation of anti-apoptotic proteins such as bcl-2 and bcl-xL (Kim et al. 2011), by inhibiting VEGF induced angiogenesis (Jung et al. 2009) , by inducing cell cycle arrest through the downregulation of cyclin D1(Yim et al. 2005) and by modulating various signaling pathways including the STAT3 signaling pathway (Kim et al. 2011), the NF-κB pathway (Kim et al. 2006a), the PI3K pathway (Lee et al. 2012).

Decursin induced cell cycle arrest in human prostate carcinoma DU145, PC-3, and LNCaP cells. Depending on the time and dose of the treatment, the cell cycle was arrested in different phases i.e. G^1, S and G^2-M phase (Yim et al. 2005). In a study conducted on B16F10 melanoma cell lines, Decursin inhibited cell proliferation on a dose dependent manner by phosphorylating p38 and increasing the expression of Bax while repressing the phosphorylation of extracellular signaling-regulated kinase (ERK). Decursin also activated caspase-3 thus inducing apoptotic cell death in these cells (Kim et al. 2015). Decursin and its derivatives have been investigated for their effects in

modulating signaling pathways. In one such study a derivative of Decursin CSL-32 was used to modulate the NF-κB and the PI3k pathways by modulating the activity and migration of inflammatory mediators involved in theses pathways. Human fibrosarcoma cell line HT1080 was treated with tumor necrosis factor α with and without treatment with CSL-32. TNF-α induced the expression of MMP-9 and IL-8 which are pro-inflammatory mediators. CSL-32 inhibited the activation of NF-κB by suppressing the phosphorylation and degradation of IκB, phosphorylation of p65 subunit of NF-κB and hence inhibiting its translocation to the nucleus. CSL-32 also suppressed the proliferation of HT1080 by inhibiting cellular adhesion to the ECM protein, fibronectin. CSL-32 also inhibited PI3k activity in a dose dependent manner (Lee et al. 2012). Decursin also has a role in inhibiting multiple pathways that cross-talk and are simultaneously activated in many cancers. Two of such pathways are the STAT3 signaling pathway and the NF-κB pathways. STAT3 is known to bind with the p65 subunit of the NF-κB (YU et al. 2002). STAT3 and NF-κB have been shown to be activated in response to lipopolysaccharides in macrophages (Carl et al. 2004). Decursin is known to inhibit the inflammatory mediators induced by lipopolysaccharides by inactivation of NF-κB (Kim et al. 2006b) thus we can conclude that Decursin has multiple targets for the suppression of different pathways.

Decursin has also been investigated for its synergistic effect with bortezomib. Decursin has the ability to potentiate the apoptosis that is induced by bortezomib. In human multiple myeloma cell lines U266 decursin induced cytotoxicity in a dose dependent manner. In addition to this expression of several gene products was down regulated including surviving, bcl-2, bcl-xL and activated caspase 3. Decursin also suppressed activation of STAT3 and JAK2 (Kim et al. 2011).

METHODOLGY

3.1 MTT Assay

MTT Assay was carried out to find the effect of increasing Decursin concentrations on the viability of cells. MTT solution (5mg/ml) was first prepared using distilled water and 1x PBS. The solution was filtered and stored at 4°C. A 96 well plate was used which contained Human colon cancer cell lines (HCT-15) which were grown for 24 hours. After 24hours they were treated with different concentrations of Decursin and left for another 24 hours. After this media was removed and 50ul of MTT solution was added to each well. After 3 hours the MTT solution was removed and 200ul of DMSO was added. Pipetting was done and the cells were analyzed for percentage of cell viability.

3.2 Animals

BALB/C mice were purchased from the National Institute of Health (NIH) Islamabad. 20 female mice were purchased that were 6 weeks old and were divided into 4 groups with 5 mice per group; control, Radiation, Radiation plus drug and drug. The mice were given a week acclimatize. The mice were weighed and the weights of these mice was then averaged to calculate the dose of Decursin.

3.3 DMBA and Croton oil

7,12-Dimethylbenz[a]anthracene (DMBA) was used to initiate the development of skin tumor in mice. DMBA was prepared by dissolving in acetone. 100mg DMBA was dissolved in 100 ml of acetone to get a final solution of 0.1% w/v. This was stored at -20°C. 100ul DMBA was used in each application which gave a 400nmol dose of DMBA per application.

Croton oil is used a promoter for the development of tumors. Croton oil was purchased from Sigma Aldrich. 100ul of croton oil was applied to the skin using a brush to ensure maximal application.

3.4 Induction of Tumor

The mice were prepared for DMBA application by first shaving off hair from their back. After shaving they were given a week before the first application of DMBA. After a week mice from each group were given the first application of the prepared DMBA solution. DMBA was applied six times until skin lesions were observed over a time period of 4 weeks. Similarly, Croton oil was applied 2 times per week on alternate days to DMBA application.

3.5 Decursin

Decursin was available in the form of two stock solutions; 20mmol and 1mmol. The drug calculations were carried out using the average weight of the mice from each group and the dosage was prepared using the 20mmol solution. The calculations were made on the basis of 20mg/kg bodyweight of mice.

3.6 Treatment

50ul of Decursin was administered to each mouse via IP injections. The treatment was continued for 4 weeks. After 2 weeks of drug administration, a one-time dose (5Gy) of radiation was administered.

3.7 Tumor Volume:

Tumor volume was measured over the period of 8 weeks. Volume was measured by using the formula:

$V = (W(2) \times L)/2$

The lengths were measured using a Vernier caliper. W is the width or the widest length whereas L is the longest length.

3.8 Sample Preparation:

The mice were sacrificed and the tumor was isolated and kept in liquid nitrogen. It was then transferred and stored in the cryo-freezer at -80°C for further use. The samples needed preparation before being used for protein quantification and western blot analysis.

Samples from each group were taken and 1-2 ug sample was immersed in 1ml of lysis solution and was homogenized to extract protein. 1ml of lysis solution was taken in a falcon tube along with 1mg sample and rapid pipetting was used to lyse the cells to extract proteins. The resulting solution was then centrifuged at 10,000 rpm for 20 mins. The supernatant was extracted using a pipette while the pellet was discarded. The extracted protein was then stored at -80°C to be used in protein quantification and western blot analysis.

3.9 Bradford Assay:

Protein quantification is carried out using the Bradford protein assay. A standard curve is first drawn using BSA (Bovine serum albumin) to make different protein concentrations. 6 samples are made, with the following concentrations; 0,2,4,6,8,10ug/ml. 0.1mg/ml stock solution of BSA was used to make these concentrations.

ug/ml	H2O (ul)	BSA (ul)	Bradford Reagent (ul)
0	800	0	200
2	780	20	200
4	760	40	200
6	740	60	200
8	720	80	200
10	700	100	200

Table 2: sample preparation using BSA for standard curve

The samples were vortexed after preparation and were incubated at 37°C for 5mins. Absorbance was then checked at 595nm using UV spectrophotometer. A standard curve was prepared which was then used to check the concentration of proteins in our samples.

A standard curve was prepared using the absorbance values and the regression line plotted with an R^2 value of 0.99.

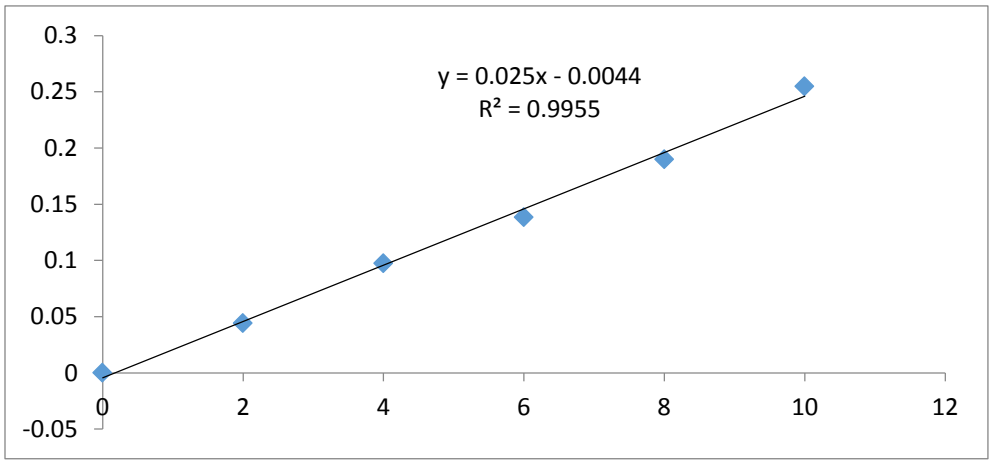


Figure 1: Standard curve for BSA using Bradford Assay

3.10 Protein Quantification:

Protein quantification was then carried out using the tumor samples. 3 samples from each group were taken for quantification. The samples were prepared at a concentration of 2ug/ml. For each sample 200ul of Bradford reagent, 798ul of H2O and 2ul of sample was added in separate Eppendorf tubes. The tubes were then vortexed and incubated at 37°C for 5 minutes. Absorbance for each sample was checked at 595nm.

Using the equation from the regression line obtained above, values for sample loading were calculated.

3.11 Western Blotting:

Western blot analysis was carried to identify proteins of interest. Mini Trans-Blot cell from BIO-RAD was used. The regression line equation was used to calculate the loading values for the samples.

	absorbance	2ug	1ug	20ug load	H2O	4x	Total
control	0.11	4.45	2.23	8.98	12.02	7	28
drug	0.10	4.26	2.13	9.40	11.60	7	28
rad	0.05	2.16	1.08	18.48	2.52	7	28
rad + drug	0.21	8.63	4.32	4.63	16.37	7	28

Table 3: Preparation of samples for loading in western blot analysis

3.11.1 SDS PAGE electrophoresis:

The concentration of the running gel was optimized and it was found that the best results were obtained at 12%. The samples were prepared by mixing with distilled water and 4x loading buffer. They were then kept at $95°C$ for 5 – 10 minutes and were then centrifuged. They were then loaded onto 12% SDS polyacrylamide gel. The gel was run for 2 hours first at 90v and then at 120v using 1x running buffer. A broad range protein marker (Santa Cruz) was run alongside the samples.

3.11.2 Transfer to NC membrane

After running the transfer of proteins onto Nitrocellulose membrane was carried out. The gel was sandwiched between two sponges, blotting paper and NC membrane. Transfer was carried out for 25 minutes for a single gel and 50 minutes for two gels at 90v. When two gels were being transferred simultaneously their sides were switched at 25 minutes. 1x transfer buffer was used for this process.

3.11.3 Ponceau staining

Ponceau stain (Merck chemicals) was prepared using acetic acid and was used to stain the NC membranes. The stain made the bands and the protein marker visible for marking before moving onto the next step i.e. blocking. The membranes were marked at specific places using the protein marker to identify the proteins of interest.

3.11.4 Blocking with non-fat milk

5% non-fat milk prepared in 1xTBS (prepared by dissolving Tris and Nacl in distilled water). The membranes were marked and cut into strips. These strips were then kept in 5% non-fat milk for 2 hours at 4c on a shaker. Blocking is done to prevent non-specific binding of the primary and secondary antibodies with the membrane.

3.11.5 Preparation of Antibodies

Antibodies were prepared using 1x TBS. Primary antibodies were prepared in the ratio of 1;1000 and secondary antibodies were prepared in the ratio of 1:2000. Primary antibodies included NF-κB, Akt,IκB and actin while the secondary antibodies included those of rabbit, mice and goat.

3.11.6 Primary Antibody Incubation:

The strips were removed from the non-fat milk and were incubated in primary antibody overnight at 4°C. The next day they were removed from the primary antibody solution and washed with TBST for 5,5,10,10 and 10 minutes before being incubated in secondary antibody.

3.11.7 Secondary Antibody Incubation

After washing the strips were then incubated in secondary antibody for two hours with shaking. After the two-hour incubation period was over they were washed again with TBST for 5,5,10,10 and 10 minutes and were then used for signal detection.

3.11.8 Signal Detection using X-ray development:

Signal detection was done using the ECL kit (Santa Cruz). The strips were laid onto a clean paper and ECL reagent was dropped onto each strip. The strips were then transferred onto the plastic sheet laid inside the X-ray cassette. X-ray sheets were fixed in the cassette which was then left for 30 – 40 minutes in a dark room. After fixing the sheets were then used to detect the proteins by using the developer and fixer buffers. One x-ray sheet was left over-night and was developed after 24 hours.

RESULTS

4.1 **Cell viability assay**

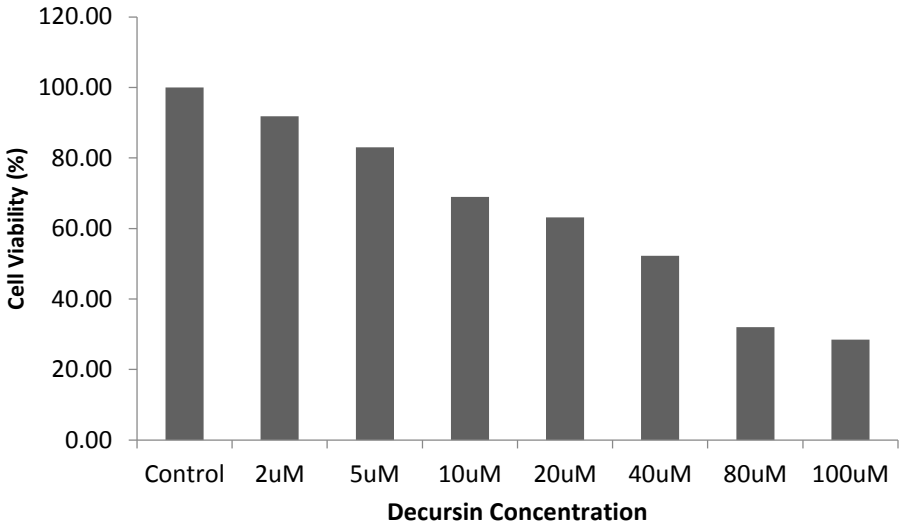


Figure 2: Dose Dependent effect of Decursin of HCT-15 cell line

Cell viability assay determines how capable cells are in keeping themselves viable in this case cancer cells. HCT-15 cell lines were subjected to the cell viability assay with increasing Decursin concentrations. It was seen that with increase in Decursin concentration the percentage of 'live' cells decreased or less percentage of cells were able to maintain or recover cell viability. The control is considered to be at 100% i.e. all the cells in this state are viable, 50% reduction in cell viability was observed between 20uM and 40uM concentrations of Decursin. This shows the dose dependent effect of Decursin on colon cancer cell lines.

4.2 **Tumor Volume**

Tumor Volume was induced using the two-stage chemical carcinogenesis method. The process of induction took 4 weeks. After the induction of tumor treatment was started. A

decrease in the tumor volume was observed in the three groups compared to the control group. One way Anova was used for multiple group comparison. Multiple comparison was carried out using the Holm-Sidak method. The results of the test showed an overall p value of p=0.003 which is statistically significant.

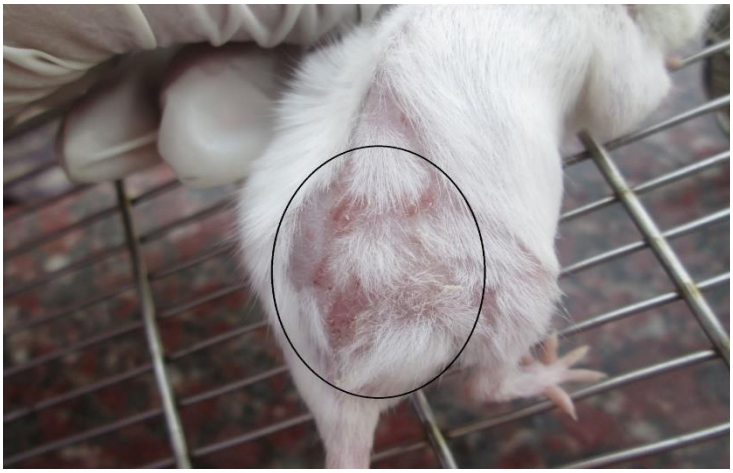


Figure 3:Tumor induction by DMBA.

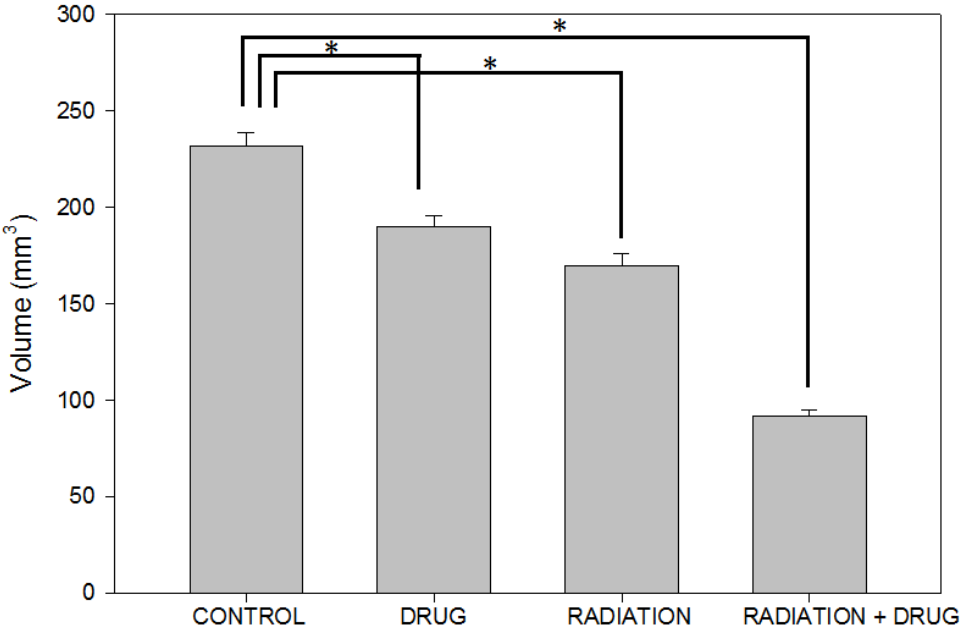


Figure 4:The tumor volume of experimental groups in comparison to the control group. One way Anova was used.

The multiple comparison with the control group also gave a p value of $p = 0.001$; $p<0.05$ when compared to the drug group, $p=0.007$; $p<0.05$ when compared to the radiation group and $p=0.007$; $p<0.05$ when compared with the radiation + drug group.

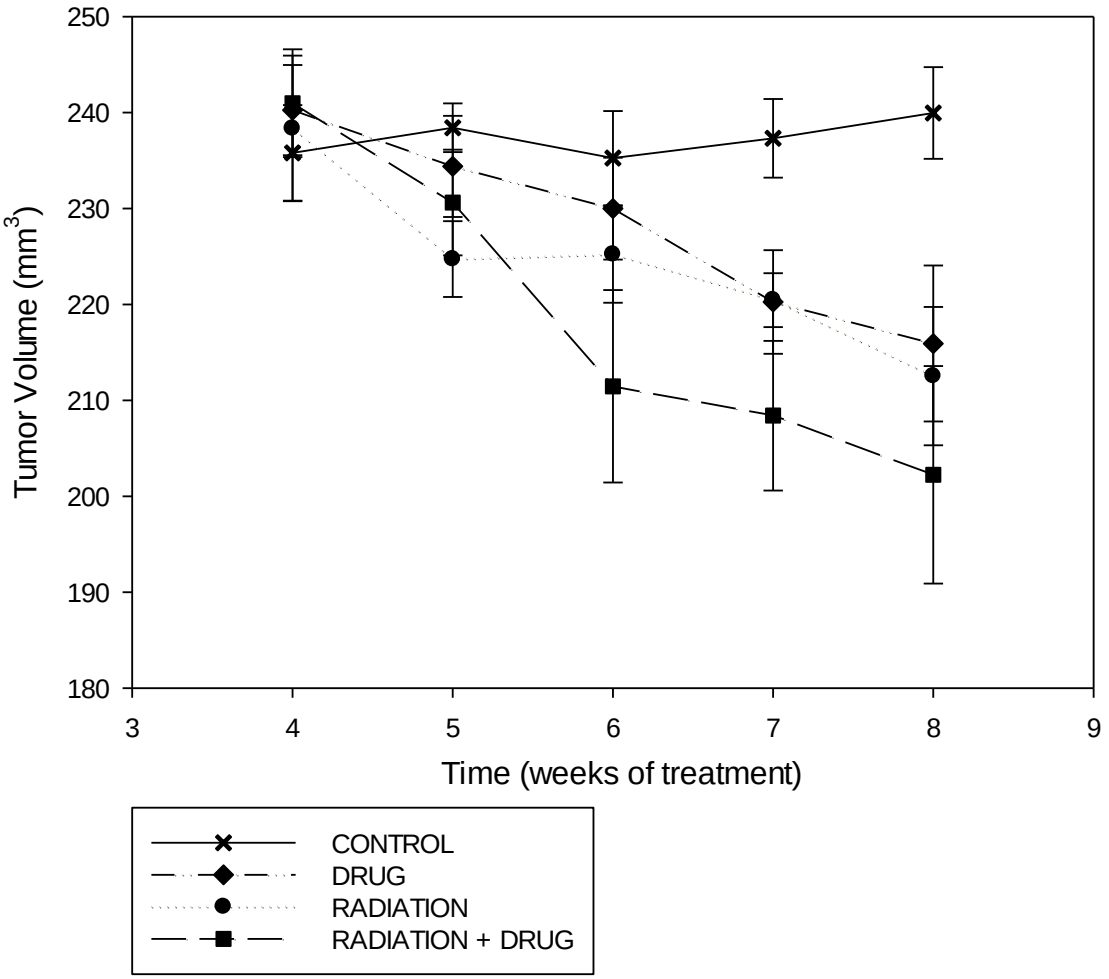


Figure 5:Reduction in tumor volume over the period of treatment.

Treatment was begun after 4 weeks of tumor induction. The Concomitant treatment of Decursin with radiation gave greater reduction in tumor volume compared to the other two groups. The mice were sacrificed for the isolation of tumor after the 8^{th} week.

4.3 **Western Blot analysis**

Western blot analysis was carried for the qualitative analysis of proteins in tumor samples. The lysates were loaded onto the gel, transferred to a NC membrane and then incubated with primary and secondary antibody. The signal was then detected via X-ray development using ECL.

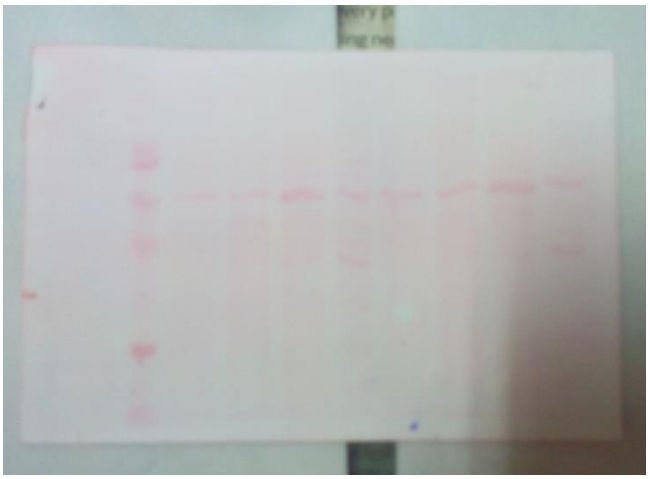


Figure 6: NC membrane after staining with ponceau solution to visualize bands and protein marker.

4.3.1 Effect of The Concomitant Treatment of Decursin and Radiation On Skin Tumor

To evaluate the radio-sensitizing effect of Decursin when administered together with radiation, mice with tumors were divided into four groups. The control group was left untreated, one group was treated with Decursin, one was subjected to a single dose of 5Gy radiation and the third group was treated with both Decursin and Gamma radiation. Protein expression in of NF-κB, IκB and Akt in cytoplasmic extracts was analyzed.

The results show that the expression of NF-κB decreased after treatment with Decursin. There was a decrease in its expression upon treatment with Decursin and radiation but a greater decrease in expression was observed with Decursin and Radiation combined. IκB which is an inhibitor of NF-κB, also increased in expression upon treatment with

Decursin. Its expression increased in the samples which were treated with Decursin. The expression of IκB was also sustained in the radiation and the radiation + drug groups. When comparing the drug group with the radiation + drug group, the expression of IκB was more in the drug group i.e. the Radiation + drug group.

Similarly, for the samples tested for the expression of P-Akt it was seen that P-Akt decreased in all the drug group when compared to the control group. When comparing the radiation group with the Radiation + drug group it was seen that the expression of P-Akt was significantly higher in the Radiation group compared to the Radiation + drug group.

Actin was used as the loading control whose control remained constant in all the groups.

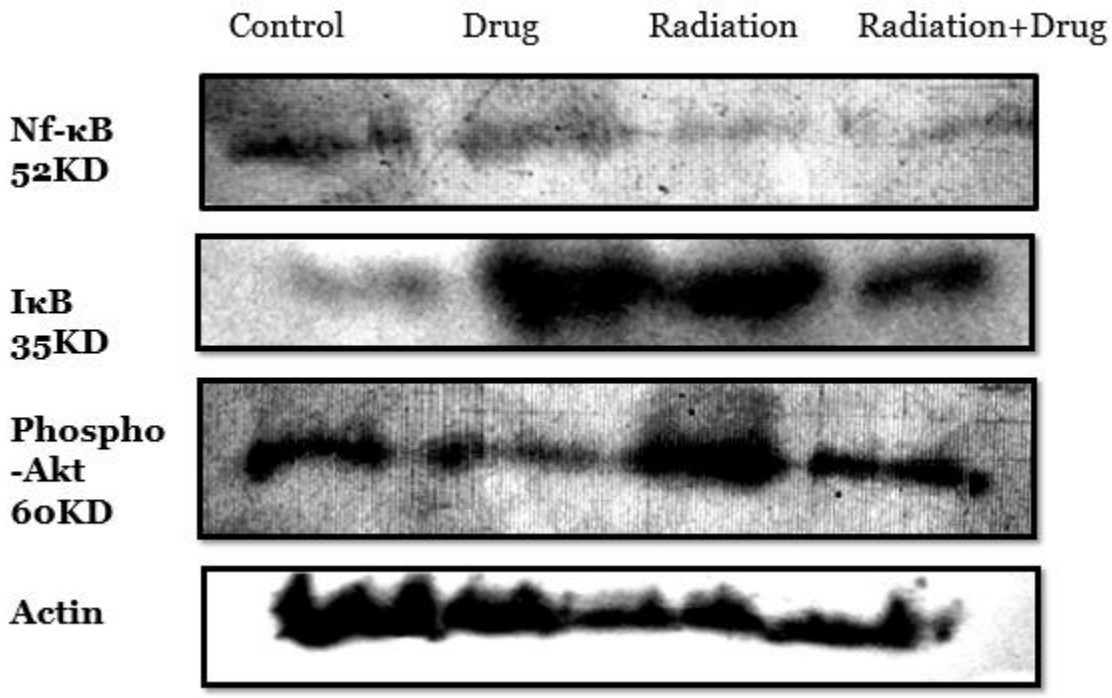


Figure 7: Results for Western blot analysis

Tumor lysates were treated with Decursin and a single dose of 5 Gy Radiation. They were then subjected to western blot analysis and analyzed for the expression of NF-κB,

IκB and Akt. Actin was used as the loading control. Change in expression levels shows the concomitant effect of Decursin and Radiation.

DISCUSSION

Melanoma or skin cancer is one of the leading cancers world-wide and its incidence is increasing day by day. Melanomas are well known to be resistant to radiation and therefore radiation therapy is not commonly used to treat them. Surgery is the common mode of treatment for this disease. Combined with a poor prognosis of its metastatic state, melanoma can easily prove to be fatal (Munshi et al. 2004). Several studies have focused on the

The above study shows that Decursin is a potential radio-sensitizer. It helps in the downregulation of the key proteins in two of the most documented pathways in radio-resistance. There is accumulating evidence that a number of signaling pathways are involved in radio-resistance. Ionizing radiation is capable of activating the NF-κB pathway by degradation of the IκB and translocation of the NF-κB to the nucleus for the transcription of various anti-apoptotic proteins and pro-inflammatory cytokines for the proliferation of cancer, modulation of the inflammatory response, cell growth and differentiation (Baeuerle et al. 1996). The results showed that Decursin inhibited the p52 subunit of NF-κB when compared to the control group. An overall reduction in the size of the tumor volume was also observed. This can be correlated with the results of IκB which show the inhibition of degradation of IκB in turn inhibiting the activation of NF-κB. The expression of IκB was also increased in the group treated with decursin. Hence it is safe to conclude that decursin inhibits the degradation of IκB thereby inhibiting activation of NF-κB. The effects of decursin on NF-κB/IκB have been well documented in literature. Decursin blocked phosphorylation of IκB and nuclear translocation of NF-κB in THP-1 cells activated with LPS (Kim et al. 2006a). KINK-1 is a small molecule inhibitor of the Iκκ which phosphorylates IκB. KINK-1 activated a number of cytotoxic agents *in vitro* and inhibited the doxorubicin activated NF-κB. When C57BL6 mice were injected with melanoma cells along with KINK-1 and doxorubicin it was seen that the pulmonary tumor mass reduced compared to the other individual groups. This shows that down-regulation or inhibition of the NF-κB pathway can sensitize the tumor to chemotherapy and the same can be implied for radiotherapy as well (Schön et al. 2008).

Therefore, constitutive inhibition of the NF-κB pathway is a possible and promising strategy to increase the efficacy of chemotherapy as well as radiation therapy. Moreover, decursin is a natural compound that has no adverse effects on healthy cells. Our results show that decursin inhibits the p52 subunit of NF-κB in both the drug and radiation groups. However, in the group where decursin was administered together with ionizing radiation, a very low expression of the p52 subunit was observed compared to that of the control and the drug group. Our results combined with previous studies validate the therapeutic potential of decursin in radio sensitization of tumors, particularly melanoma.

The PI3k/Akt pathway is another well documented pathway involved in radio resistance. Our results demonstrated that the expression of Akt reduced in the group treated with decursin. This is because Decursin suppresses the phosphorylation of Akt thus inhibiting the PI3k/Akt pathway. One such pathway that is associated with the activation of Akt3 in melanomas is due to the loss PTEN. Loss of PTEN causes an increase in the cellular concentration of PIP3 which in turn binds to the PH domains of Akt3 and facilitates its translocation to the membrane and its phosphorylation (Stahl et al. 2004; Sale & Sale 2008). Akt3 inhibits apoptosis by decreasing the activity of caspase 3/7 and by increasing the activity of cleaved caspase 3 and PARP. Increase in chemo resistance in melanoma can be accounted for by the decrease in apoptosis (Stahl et al. 2004). A number of inhibitors of Akt have been investigated in melanoma studies that inhibit metastasis and tumor development. It is therefore safe to conclude that inhibition of P-Akt is promising therapeutic strategy to induce radio-sensitivity in melanoma.

CONCLUSION

We can draw several inferences from the results obtained. One being that Decursin inhibits the p-52 subunit of the NF-κB thereby inhibiting this pathway. Inhibition of NF-κB inhibits its translocation to the nucleus thereby inhibiting the transcription of a number of pro-inflammatory and anti-apoptotic proteins.

Decursin also inhibits the degradation of IκB. IκB is phosphorylated and degraded by a proteasome assembly. Once degraded, NF-κB and IκB assembly is dissociated the pathway is activated. This pathway is activated by a number of signaling molecules.

Decursin leads to the suppression of phosphorylation of Akt as can be seen from its expression in the drug group. However, the expression of Akt is very high in the radiation group this can be the result of over-expression of the P110 subunit of PI3k which phosphorylates PIP2 (Zhan & Han 2004).

The overall mechanism of action of decursin can be depicted in the figure below:

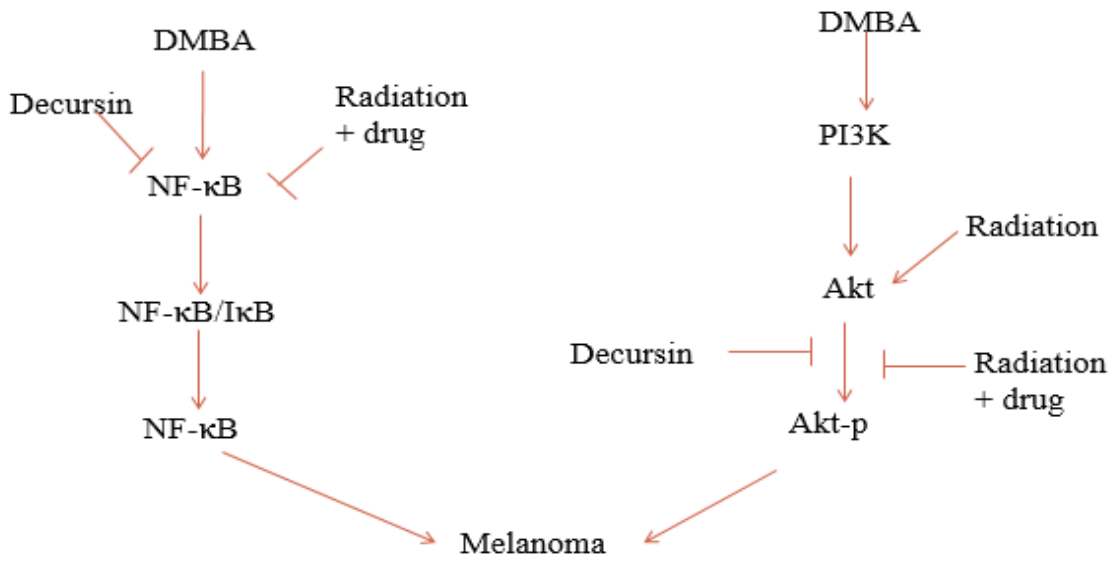


Figure 8: Proposed mechanism of action of Decursin on the NF-κB pathway and the Akt pathway

REFERENCES

Aggarwal, B.B. & Sung, B., 2011. NF-κB in Cancer: A Matter of Life and Death. *Cancer Discovery*, 1(6).

Anon, 2000. Cancer multidrug resistance. *Nature biotechnology*, 18 Suppl, p.IT18-20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11221708 [Accessed October 17, 2016].

Baeuerle, P.A. et al., 1996. NF-kappa B: ten years after. *Cell*, 87(1), pp.13–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8858144 [Accessed October 17, 2016].

Baskar, R. et al., 2012. Cancer and radiation therapy: current advances and future directions. *International journal of medical sciences*, 9(3), pp.193–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22408567 [Accessed October 17, 2016].

Bellacosa, A. et al., 1991. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science (New York, N.Y.)*, 254(5029), pp.274–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1833819 [Accessed October 16, 2016].

Bussink, J. et al., 2008. Activation of the PI3-K/AKT pathway and implications for radio-resistance mechanisms in head and neck cancer. *The Lancet. Oncology*, 9(3), pp.288–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18308254 [Accessed October 13, 2016].

Carl, V.S. et al., 2004. Role of endogenous IL-10 in LPS-induced STAT3 activation and IL-1 receptor antagonist gene expression. *Journal of leukocyte biology*, 76(3), pp.735–42. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15218058 [Accessed October 17, 2016].

Chang, D.T. et al., 2006. Adjuvant radiotherapy for cutaneous melanoma: comparing hypofractionation to conventional fractionation. *International journal of radiation oncology, biology, physics*, 66(4), pp.1051–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16973303 [Accessed October 17, 2016].

Chang, L. et al., 2013. Acquisition of epithelial-mesenchymal transition and cancer stem cell phenotypes is associated with activation of the PI3K/Akt/mTOR pathway in prostate cancer radio-resistance. *Cell death & disease*, 4, p.e875. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24157869 [Accessed October 16, 2016].

Chen, Y.-H. et al., 2014. Radiation-induced VEGF-C expression and endothelial cell proliferation in lung cancer. *Strahlentherapie und Onkologie : Organ der Deutschen Röntgengesellschaft ... [et al]*, 190(12), pp.1154–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24989178 [Accessed October 16, 2016].

Didenko, V. V et al., 2002. Caspase-3-dependent and -independent apoptosis in focal brain ischemia. *Molecular medicine (Cambridge, Mass.)*, 8(7), pp.347–52. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12393932 [Accessed October 17, 2016].

Faustino-Rocha, A. et al., 2013. Estimation of rat mammary tumor volume using caliper and ultrasonography measurements. *Lab animal*, 42(6), pp.217–24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23689461 [Accessed October 17, 2016].

GRAY, L.H. et al., 1953. The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. *The British journal of radiology*, 26(312), pp.638–48. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13106296 [Accessed October 13, 2016].

Hsu, F.-T. et al., 2015. Synergistic Effect of Sorafenib and Radiation on Human Oral Carcinoma in vivo. *Scientific Reports*, 5, p.15391. Available at: http://www.nature.com/articles/srep15391 [Accessed October 16, 2016].

Huber, S.M. et al., 2013. Ionizing radiation, ion transports, and radio-resistance of cancer cells. *Frontiers in physiology*, 4, p.212. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23966948 [Accessed October 17, 2016].

Jung, M.H. et al., 2009. Decursin and decursinol angelate inhibit VEGF-induced angiogenesis via suppression of the VEGFR-2-signaling pathway. *Carcinogenesis*, 30(4), pp.655–661.

Kang, J.-H. et al., 2014. Protein Kinase C (PKC) Isozymes and Cancer. *New Journal of*

Science, 2014, pp.1–36. Available at: http://www.hindawi.com/journals/njos/2014/231418/ [Accessed October 17, 2016].

Kim, B.S. et al., 2015. Decursin from *Angelica gigas* Nakai Inhibits B16F10 Melanoma Growth Through Induction of Apoptosis. *Journal of Medicinal Food*, 18(10), pp.1121–1127. Available at: http://online.liebertpub.com/doi/10.1089/jmf.2014.3397.

Kim, H.J. et al., 2011. Decursin chemosensitizes human multiple myeloma cells through inhibition of STAT3 signaling pathway. *Cancer letters*, 301(1), pp.29–37. Available at: http://dx.doi.org/10.1016/j.canlet.2010.11.002.

Kim, J.-H. et al., 2006a. Decursin inhibits induction of inflammatory mediators by blocking nuclear factor-kappaB activation in macrophages. *Molecular pharmacology*, 69(6), pp.1783–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16510559 [Accessed October 17, 2016].

Kim, J.-H. et al., 2006b. Decursin inhibits induction of inflammatory mediators by blocking nuclear factor-kappaB activation in macrophages. *Molecular pharmacology*, 69(6), pp.1783–1790.

Kim, J.-M. et al., 2014. Decursin prevents TPA-induced invasion through suppression of PKCalpha/p38/NF-kappaB-dependent MMP-9 expression in MCF-7 human breast carcinoma cells. *International journal of oncology*, 44(5), pp.1607–1613.

Lacombe, J. et al., 2013. Proteomic approaches to identify biomarkers predictive of radiotherapy outcomes. *Expert Review of Proteomics*, 10(1), pp.33–42. Available at: http://www.tandfonline.com/doi/full/10.1586/epr.12.68 [Accessed October 13, 2016].

Lee, K. et al., 2010. Synthesis of (S)-(+)-decursin and its analogues as potent inhibitors of melanin formation in B16 murine melanoma cells. *European Journal of Medicinal Chemistry*, 45(12), pp.5567–5575. Available at: http://dx.doi.org/10.1016/j.ejmech.2010.09.006.

Lee, S.-H. et al., 2012. A novel derivative of decursin, CSL-32, blocks migration and

production of inflammatory mediators and modulates PI3K and NF-kappaB activities in HT1080 cells. *Cell biology international*, 36(7), pp.683–688.

Lee, S.J. et al., 1998. A novel ionizing radiation-induced signaling pathway that activates the transcription factor NF-kappaB. *Oncogene*, 17(14), pp.1821–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9778048 [Accessed October 17, 2016].

Liu, T. et al., 2015. Dual PI3K/mTOR inhibitors, GSK2126458 and PKI-587, suppress tumor progression and increase radiosensitivity in nasopharyngeal carcinoma. *Molecular cancer therapeutics*, 14(2), pp.429–39. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25504751 [Accessed October 16, 2016].

Lu, J. et al., 2007. Oriental herbs as a source of novel anti-androgen and prostate cancer chemopreventive agents. *Acta pharmacologica Sinica*, 28(9), pp.1365–72. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17723170 [Accessed October 11, 2016].

Madhunapantula, S. V & Robertson, G.P., 2009. The PTEN-AKT3 signaling cascade as a therapeutic target in melanoma. *Pigment cell & melanoma research*, 22(4), pp.400–19. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19493313 [Accessed October 16, 2016].

Mahat, B. et al., 2013. Biopharmaceutical characterization of decursin and their derivatives for drug discovery. *Drug development and industrial pharmacy*, 39(10), pp.1523–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23003006 [Accessed October 12, 2016].

Mahat, B. et al., 2012. Physicochemical characterization and toxicity of decursin and their derivatives from Angelica gigas. *Biological & pharmaceutical bulletin*, 35(7), pp.1084–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22791156 [Accessed October 12, 2016].

Munshi, A. et al., 2004. Inhibition of constitutively activated nuclear factor-κB radiosensitizes human melanoma cells. *Molecular Cancer Therapeutics*, 3(8).

Nakanishi, C. & Toi, M., 2005. Nuclear factor-κB inhibitors as sensitizers to anticancer drugs. *Nature Reviews Cancer*, 5(4), pp.297–309. Available at:

http://www.nature.com/doifinder/10.1038/nrc1588 [Accessed October 17, 2016].

Pajonk, F., Pajonk, K. & McBride, W.H., 1999. Inhibition of NF-kappaB, clonogenicity, and radiosensitivity of human cancer cells. *Journal of the National Cancer Institute*, 91(22), pp.1956–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10564680 [Accessed October 17, 2016].

Romashkova, J.A. & Makarov, S.S., 1999. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature*, 401(6748), pp.86–90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10485711 [Accessed October 16, 2016].

Russo, S.M. et al., 2001. Enhancement of radiosensitivity by proteasome inhibition: implications for a role of NF-kappaB. *International journal of radiation oncology, biology, physics*, 50(1), pp.183–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11316563 [Accessed October 16, 2016].

Sale, E.M. & Sale, G.J., 2008. Protein kinase B: signalling roles and therapeutic targeting. *Cellular and molecular life sciences : CMLS*, 65(1), pp.113–27. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17952368 [Accessed October 18, 2016].

Schön, M. et al., 2008. KINK-1, a novel small-molecule inhibitor of IKKbeta, and the susceptibility of melanoma cells to antitumoral treatment. *Journal of the National Cancer Institute*, 100(12), pp.862–75. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18544741 [Accessed October 18, 2016].

Stahl, J.M. et al., 2004. Deregulated Akt3 activity promotes development of malignant melanoma. *Cancer research*, 64(19), pp.7002–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15466193 [Accessed October 18, 2016].

Strojan, P., 2010. Role of radiotherapy in melanoma management. *Radiology and oncology*, 44(1), pp.1–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22933884 [Accessed October 17, 2016].

Tucker, S.L. & Thames, H.D., 1989. The effect of patient-to-patient variability on the accuracy of predictive assays of tumor response to radiotherapy: a theoretical evaluation. *International journal of radiation oncology, biology, physics*, 17(1),

pp.145–57. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2745189 [Accessed October 13, 2016].

Yap, T.A. et al., Poly(ADP-ribose) polymerase (PARP) inhibitors: Exploiting a synthetic lethal strategy in the clinic. *CA: a cancer journal for clinicians*, 61(1), pp.31–49. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21205831 [Accessed October 17, 2016].

Yim, D. et al., 2005. A Novel Anticancer Agent , Decursin , Induces G 1 Arrest and Apoptosis in Human Prostate Carcinoma Cells. , (3), pp.1035–1044.

YU, Z., ZHANG, W. & KONE, B.C., 2002. Signal transducers and activators of transcription 3 (STAT3) inhibits transcription of the inducible nitric oxide synthase gene by interacting with nuclear factor κB. *Biochemical Journal*, 367(1).

Zhan, M. & Han, Z.C., 2004. Phosphatidylinositide 3-kinase/AKT in radiation responses. *Histology and histopathology*, 19(3), pp.915–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15168354 [Accessed October 13, 2016].

Zhang, J. et al., 2012. Anti-cancer and other bioactivities of Korean Angelica gigas Nakai (AGN) and its major pyranocoumarin compounds. *Anti-cancer agents in medicinal chemistry*, 12(10), pp.1239–54. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22583405 [Accessed October 17, 2016].

Proposed Certificate for Plagiarism

It is certified that Masters Thesis Titled **A natural product Decursin enhances the radio- sensitization of IR against DMBA-induced tumor** by **Hira Zahid** has been examined by us. We undertake the follows:

a. Thesis has significant new work/knowledge as compared already published or are under consideration to be published elsewhere. No sentence, equation, diagram, table, paragraph or section has been copied verbatim from previous work unless it is placed under quotation marks and duly referenced.
b. The work presented is original and own work of the author (i.e. there is no plagiarism). No ideas, processes, results or words of others have been presented as Author own work.
c. There is no fabrication of data or results which have been compiled /analyzed.
d. There is no falsification by manipulating research materials, equipment or processes, or changing or omitting data or results such that the research is not accurately represented in the research record.
e. The thesis has been checked using TURNITIN (copy of originality report attached) and found with in limits as per HEC plagiarism Policy and instructions issued from time to time.

Name & Signature of Supervisor

Dr. Adeeb Shehzad

Signature**:** _____