Study the effect of phytochemicals phenethyl isothiocyanate (PEITC) and Quercetin on mitochondrial biogenesis in cancer and normal cell lines

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For the award of

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In

Department for Human Genetics and Molecular Medicine

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May, 2018

CERTIFICATE

I declare that the dissertation/thesis entitled "**Study the effect of phytochemicals phenethyl isothiocyanate (PEITC) and Quercetin on mitochondrial biogenesis in cancer and normal cell lines**" has been prepared by me under the guidance of Dr. Harish Chander, Assistant Professor, Department for Human Genetics and Molecular Medicine, School of Health Sciences, Central University of Punjab. No part of this dissertation/thesis has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that **ANCHAL THAKUR** has prepared his/her dissertation/thesis entitled " **Study the effect of phytochemicals phenethyl isothiocyanate (PEITC) and Quercetin on mitochondrial biogenesis in cancer and normal cell lines**", for the award of M.Sc. degree of the Central University of Punjab, under my guidance. She has carried out this work at the Department for Human Genetics and Molecular Medicine, School of Health Sciences, Central University of Punjab.

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ABSTRACT

Study the effect of phytochemicals phenethyl isothiocyanate (PEITC) and Quercetin on mitochondrial biogenesis in cancer and normal cell lines.

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Keywords: Phytochemicals, PEITC, Quercetin, Cancer, NRF-2 (Nuclear respiratory factor-2), mitochondrial biogenesis

Phytochemicals are plant-derived chemicals generally are biologically active compounds and mediate positive health benefits by targeting genes or metabolic pathways of a cell. The phytochemicals examined can be classified into main categories, such as carotenoids and polyphenols, which include phenolic acids, flavonoids and stilbenes / lignans. Quercetin is isolated from the *Tridax procumbens* (Linn.). Its anti-cancer activity has been well documented in vitro and in vivo. It could be pro-apoptotic as well as anti-apoptotic depending upon its concentration of it and time of exposure. Isothiocyanates are cruciferous derived phytochemicals. PEITC majorly isolated from *Nasturtium officinale* (watercress) has shown to mediate its anti-cancer activity through ROS-mediated pathway. It is a basic leucine zipper protein involved in protection against oxidative damage triggered by stress like injury or inflammation through regulation of the expression of anti-oxidant proteins. Under oxidative stress, inactivation of Kelch-like ECH-associated protein 1 (Keap1) occurs which is a cytosolic repressor protein that binds to Nrf2. This results in Nrf2-Keap1 complex dissociation, and hence, promoting the translocation of Nrf2 to the

nucleus where it binds to ARE (anti-oxidant response element), and induce the transcription of anti-oxidative proteins. Quercetin and PEITC treatment to the cancer cells led to decreased mitochondrial biogenesis as the NRF-2 levels diminishes as the concentration of the drug increases. The anti-oxidant levels are getting down in the cancer cells leading to ROS accumulation in the cancer cells leading ultimately to the death. Quercetin and PEITC treatment to the normal HBL-100 cells induced the mitochondrial biogenesis by increasing NRF-2 levels as the concentration of the drug increases. Confocal microscopy results also proved that treatment of quercetin or PEITC or the combination of both drugs was found to be effective in cancer cells as the mitochondria size and shape got decreased interpreted through the intensity of green dye. To conclude our study, it has been shown that quercetin and PEITC lead to increased mitochondrial biogenesis in normal cells whereas decreased mitochondrial biogenesis in cancer cells.

Anchal Thakur **Dr. Harish Chander**

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Anchal Thakur

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LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviations
1	Deoxyribonucleic acid	DNA
2	Tri-carboxylic acid	TCA
3	Oxidative Phosphorylation	OXPHOS
4	Peroxisome proliferator-activated receptor co- activator-1	PGC-1
5	Nuclear respiratory factor	NRF
6	Mitochondrial transcription factor A	mtTFA
7	Transcription factor B	TFBs
8	Phenethyl Isothiocyanate	PEITC or P
9	Reactive oxygen species	ROS
10	Glutathione	GSH
11	Glutathione peroxidase	GPX
12	Quercetin	Que or Q
13	Adenosine Triphosphate	ATP
14	Superoxide dismutase	SOD
15	Cytochrome C oxidase	COX
16	Sirtuin	SIRT
17	Poly ADP ribose polymerase	PARP
18	American type culture collection	ATCC
19	Roswell park memorial institute	RPMI
20	Dulbecco’s modified eagle medium	DMEM
21	Dimethyl sulfoxide	DMSO
22	Phosphate buffered saline	PBS
23	Phenylmethylsulfonyl fluoride	PMSF
24	Bovine serum albumin	BSA
25	Sodium Dodecyl sulfate	SDS
26	Ammonium Persulfate	APS

27	Phosphate buffered saline with Tween 20	PBST
28	4’,6-diamidino-2-phenylindole	DAPI
29	Mito-tracker green dye	MTG
30	Glyceraldehydes 3-phosphate dehydrogenase	GAPDH
31	Anti-oxidant response element	ARE
32	Kelch-like ECH-associated protein 1	KEAP-1

Chapter 1

Introduction

1.1 Background

Cancer is a multifactorial disease that occurs due to changes in cells at genetic level leading to uncontrolled and abnormal cell growth either they might be malignant (which can metastasize to other parts of the body from its original site of fromation) or benign tumors. Cancer arises from a loss of the normal regulatory events that control cellular growth and proliferation. Cellular proliferation greatly depends upon the amount of mitochondria which is governed by the process of mitochondrial biogenesis. Various studies have linked the tumorigenesis to the increased mitochondrial biogenesis *(Martinez-Outschoorn et al., 2011)*. In many types of human cancer reduced anti-oxidants levels with increased oxidative stress has been observed. As many of the anti-cancer drugs act through free radical mechanism to carry out their effects so the reduced anti-oxidant defense can actually make the cells susceptible to the chemotherapeutics.

Mitochondrial biogenesis is a process resulting in higher mitochondrial mass and DNA and increased expression of metabolic enzymes related to -oxidation, TCA cycle and OXPHOS (Oxidative phosphorylation) in response to environmental stimuli, cellular stress or to fuel actual energy demands *(lee and Wei,2005)*.. Various factors govern the mitochondrial biogenesis and regulate the expression of proteins which are involved in biogenesis pathway. Mainly the factors involved are PGC-1 (Peroxisome proliferator-activated receptor co-activator-1), NRF (Nuclear respiratory factor) and mtTFA (mitochondrial transcription factor A). PGC-1 , master regulator of the mitochondrial biogenesis and co-transcriptional regulation factor, instigates the activation of Nrf-1 and Nrf-2 which regulates the mitochondrial transcription factor A (Tfam) and transcription factor B (TFBs) proteins gene expression which are the major regulators of mitochondrial DNA replication and transcription. PGC-1 is also involved in mitochondrial biogenesis regulation but its expression doesn't gets increased on metabolic changes like stress, cold, exercise etc as PGC-1 does *(Ventura-Clapier et al., 2008)*.

1.2 phytochemicals

Phytochemicals are plant-derived chemicals generally are biologically active compounds and mediate positive health benefits by targeting genes or metabolic pathways of a cell. The phytochemicals investigated can be classified into main categories, such as carotenoids and polyphenols. Polyphenols is group which includes phenolic acids, flavonoids and stilbenes / lignans. Based on their similar chemical structure flavonoids are branched into groups, such as anthocyanins, flavones, flavones, and isoflavones and flavonoids. Quercetin is a plant flavonol comes under flavonoid group of polyphenols. The flavonols are also classified as catechins, epicatechins and proanthocyanidins. Glucosinolates are natural components of many acidic plants such as mustard, cabbage and horseradish. They protect plants against pests and diseases but are also believed to contribute for the human health. PEITC, a glucosinolate, is a natural component of vegetables such as broccoli, cabbage, etc. It acts as an antitumor agent with low toxicity to normal cells. There is a greater production of ROS in tumor cells that leads to the dependence of tumor cells on the antioxidant system to eliminate ROS and their survival *(Chen et al., 2010)*. PEITC acts by deactivating the antioxidant system, either directly by conjugating with the GSH set or by exporting it outside the cell, which results in the exhaustion of cellular GSH or in the inhibition of the enzyme modulating redox glutathione peroxidase (GPX) *(Xu and Thornalley, 2001)*.

The flavonols are also classified as catechins, epicatechins and proanthocyanidins.Quercetin (Que), 3, 3 ’, 4’, 5, 7-pentahydroxyflavone, is a member of the polyphenolic flavonoid family found more abundantly in fruits and vegetables *(Rayamaji et al., 2013)*. It has wide-ranging bioactive effects and through modulation of various enzymes and transcription factors in inflammatory signaling cascade or by reducing ROS production in various cell types it can regulate the mitochondrial biogenesis *(Lee et al., 2015, Hock and Kralli, 2009).)*. It has already been reported that higher doses of quercetin (40-100 μM) cause a decrease in cell survival rates and an increase in antitumor effects by decreasing the levels and activities of antioxidants. While low doses (5-30 μM) increase the antioxidant capacity of tumor cells and antagonize the cytotoxic effects of antineoplastic drugs on tumor cells. Similarly, short-term treatment with quercetin has been found to be effective in inducing antioxidant and anti-

apoptotic effects, while long-term treatment causes pro-oxidative and pro-apoptotic effects *(Gibellini et al., 2015)*.

1.3 Nuclear respiratory factor-2

Due to various intracellular oxygen metabolism or in response to various exogenous stimuli including ultraviolet, ionizing radiation, chemotherapeutics, hypoxia etc the generation of ROS (Reactive oxygen species) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) occurs *(Liou & Storz, 2010)*. They are required for normal physiological pathways but overproduction of ROS can lead to toxic effects on cells either by causing damage to cellular proteins, polysaccharides, DNA and lipids or by inducing apoptosis. It is a basic leucine zipper protein involved in protection against oxidative damage triggered by stress like injury or inflammation through regulation of the expression of anti-oxidant proteins. Under oxidative stress, inactivation of Kelch-like ECH-associated protein 1 (Keap1) occurs which is a cytosolic repressor protein that binds to Nrf2. This results in Nrf2-Keap1 complex dissociation, and hence, promoting the translocation of Nrf2 to the nucleus where it binds to ARE (anti-oxidant response element), thereby inducing the transcription of phase-II detoxifying enzymes (e.g. glutathione S-transferase), antioxidant enzymes (e.g. NADH quinone oxidoreductase 1(NQO1), heme oxygenase-1 (HO-1) and drug transporting proteins *(de Oliveira et al., 2016)*. It provides both benefits as well as risks to the cells. Its activation in normal tissues has been found to be beneficial for cancer chemoprevention. However, in some cancers it enhanced the survival of damaged cells, tumorigenesis, and drug resistance during chemotherapy and also decreased the apoptosis in them. Its stable over-expression led to enhanced resistance of cancer cells to chemotherapeutic agents, whereas its depletion or silencing or over-expression of keap-1 enhances the sensitivity *(Lee, Y. J.et al., 2015)*.

This study aimed to investigate the effects of quercetin and PEITC (Phenethyl isothiocyanate) on NRF-2 and subsequently mitochondrial biogenesis in cancer cells as well as normal cells.

Aims and objectives

Aim: To study the effects of natural compounds on mitochondrial biogenesis

Objective: To study the effect of natural compounds on mitochondrial biogenesis in normal and cancer cell lines.

Chapter 2

Review of literature

2.1 Mitochondrial structure and function

Mitochondria, double membrane-bound organelle, also known as powerhouse of the cell as it generates most of the cell's energy supply molecule "ATP" (Adenosine Triphosphate). Mitochondria are a dynamic network subject to continuous fission and fusion processes *(scott and youle, 2010)*. Intermembrane space is present in between the inner and outer membranes. Inner membrane is folded in a manner that it forms cristae like structures to accommodate large surface areas. The respiratory chain, made up of five complexes, is embedded in the inner mitochondrial membrane where oxidative phosphorylation takes place. ATP synthesis at complex V occurs due to the proton gradient formed across the inner membrane *(Hatefi 1985).* Mitochondrial respiration is not only the source for ATP production but also for free radical or ROS production *(Nohl, Gille and Staneik, 2005).* Mitochondrial DNA is about 16.6 Kb size in humans and 16.3 Kb in mice. Only 13 protein subunits, which are essential components of mitochondrial electron transport chain (ETC) are encoded by mitochondrial DNA *(Boengler et al., 2011).* However, transcription of these 13 mtDNA gene products is regulated by nuclear transcription factor mtTFA (mitochondrial transcription factor A). It also encodes 2 ribosomal RNAs (rRNA) and 22 transfer RNAs (tRNA). One of the strands of mtDNA is termed as heavy (H) and light (L) strand. Though there are no introns present in the DNA but it has a long non-coding region, the D loop, which plays role in controlling transcription and replication of mtDNA *(Shadel and Clayton, 1997).*

The **mitochondrial biogenesis** is a process resulting in higher mitochondrial mass and DNA and increased expression of metabolic enzymes related to -oxidation, TCA cycle and OXPHOS in response to environmental stimuli, cellular stress or to fuel actual energy demands *(lee and Wei,2005).* Inhibition of mitochondrial biogenesis disrupts energy production, metabolism, and oxidative resistance and also may lead to development of degenerative diseases in brain and muscles which are dependent on high energy *(Melser et al., 2015)*. Intact sensing or signalling in or between nucleus and mitochondria is responsible

for intense relationship between both the compartments. Most of the proteins involved in mitochondrial biogenesis are encoded by nucleus, such as cytochrome C, cytochrome C oxidase IV, NADH dehydrogenase, citrate synthase, superoxide dismutase (SOD), ATP synthetase, mtTFA are transcribed in nucleus and are then imported to mitochondria *(Brenmoehl and Hoeflich,2013).*

2.2 Mitochondrial protein transport

A mechanism for transport of proteins from nucleus to mitochondria exists. After activation of nuclear genome, various mRNAs are translated to precursor proteins. An N-terminal positively charged pre-sequence is also present as a marker in the precursor proteins and is capable of forming a basic, amphipathic -helix. Basic, amphipathic -helix Protein translocases present in mitochondrial membrane helps in spanning the mitochondrial outer and inner membranes in an unfolded conformation. The mitochondrial membrane potential and action of Hsp70 are the driving tools for translocation *(Jornayvaz and Shulman, 2010).* Matrix protease cleaves the sequence and molecular chaperones aid in folding of imported protein. Different protein pathways diverge at TOM (translocase of the outer membrane), which is a prevalent path for the import of all proteins in mitochondria *(Hill et al., 1998, Stojanovski et al., 2012).* TIM (translocase of the inner membrane) is involved in sorting matrix-targeted precursors *(Stojanovski et al., 2012, Schatz G, 1996)*. The PAM (pre-sequence translocase-associated motor) functions in driving precursors into the matrix through regulating the matrix Hsp70 action *(Frazier et al., 2004,, Hutu et al., 2008).* SAM (sorting and assembly machinery) is located in the outer membrane which inserts -barrel proteins into outer membrane *(Milenkovic et al., 2004, Wiedemann et al., 2003).*

2.3 Factors involved in mitochondrial biogenesis regulation

- **Peroxisome proliferator-activated receptor- co-activator-1 (PGC-1)**
  Mitochondrial biogenesis is instigated by a co-transcriptional regulation factor, PGC-1, through activation of distinctive transcription factors, including NRF-1 and NRF-2, which promotes the expression of Tfam *(Ventura-Clapier et al., 2008).* An experiment was conducted which lead to the discovery of PGC-1 . An elevated increase in PGC-1 mRNA expression and subsequently increased expression of mitochondrial

enzymes such as COX subunits (II and IV), ATP synthetase was seen while mice are exposed to cold *(Puigserver et al., 1998).* PGC-1 is also responsible for co-activation of different other transcription factors, for example, PPARs *(Vega et al., 2000),* thyroid hormone, glucocorticoid, estrogen and ERRs (oestrogen-related receptors) and . It had also been reported that the induction of UCP2 (uncoupling protein 2) and mitochondrial biogenesis in muscle cells by PGC-1 lead to the stimulation of respiration through NRF-1 and NRF-2 gene expression *(Wu et al., 1999).*

PGC-1 and have been found to have similar structure and function as well as binding to the nuclear receptors and transcriptional activation is also quite similar. It had also been found that PGC-1 co-activates HNF4 , GR, and PPAR and all of these functions in gluconeogenesis and fatty acid oxidation *(Lin et al., 2002).* Unlike PGC-1 , whose expression gets altered on metabolic changes like cold, stress etc, PGC-1 mRNA expression doesn't get altered *(Jornayvaz and Shulman, 2010).*

- **Nuclear respiratory factors (NRFs)**

Transcription of mitochondrial genes is prominently being regulated by nuclear respiratory factors 1 and 2. Through sequence analysis of electron transport gene cytochrome C promoter, NRF-1 had been found to be an important regulator of gene expression *(Evans and Scarpulla, 1989).* Expression of various proteins which are constituents of five respiratory complexes, mitochondrial import and heme biosynthesis are principally observed to be controlled by NRF-1 *(Scarpulla, 2008).* NRF-1 is the common factor that integrates the nuclear control of transcriptional and replicative activity of mitochondrial genome. It modulates the gene expression of mitochondrial transcription factor A (Tfam) and transcription factor B (TFBs) proteins which are the major regulators of mitochondrial DNA replication and transcription. Further it had also been found that binding of TFB1M to the carboxy-terminal transcriptional activation domain of Tfam facilitates promoter specific recognition *(Gleyzer et al., 2005).* The role of NRF-1 transcription factor in cardiac mitochondrial biogenesis has been explained by a research showing the enhanced NRF-1 expression on electrical stimulation of neonatal cardiomyocytes leading to increased mitochondrial content *(Xia Y et al., 1997).*

NRF-2 is also linked to the regulation of expression of proteins in electron transport chain. Through various studies, it had been found that it specifically binds to essential cis-acting elements in cytochrome oxidase subunit IV (COXIV) promoter *(Scarpulla, 2008).* NRF-2 promotes the expression of genes that encode for mitochondrial complex IV cytochrome C oxidase *(Gugneja et al. 1995).* NRF-2 also controls the expression of Tfam and TFBs.

- **Mitochondrial Transcription Factor A**

  The replication and transcription of mitochondrial DNA is driven by the nuclear-encoded mitochondrial transcription factor A or Tfam. Tfam translocates to mitcohondria and binds to the upstream enhancer of promoter sites of mitochondrial DNA strands. The interaction of TFB1M and TFB2M with mammalian mitochondrial RNA polymerase and Tfam is required for the promoter-specific mtDNA transcription *(Ventura-Clapier et al., 2007).* The promoters of the genes encoding oxidative phosphorylation (OXPHOS) complex subunits and mtDNA metabolic enzymes contain complex and specific binding motifs. Both are able to participate in a coordinated response. Various motifs present are OXBOX/ REBOX motif, Mt motif, Sp1 and NRF motifs.

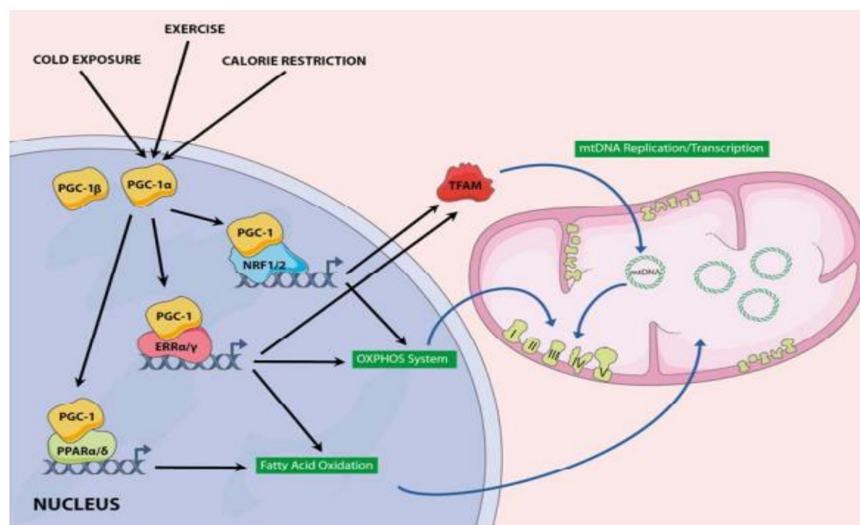


Figure 1: Transcriptional regulation of mitochondrial biogenesis

Source: - Zamora, M., & A Villena, J. (2014). Targeting mitochondrial biogenesis to treat insulin resistance. *Current pharmaceutical design*, *20*(35), 5527-5557.

- **Sirtuin proteins**

  These are NAD^+-dependent protein deacetylases. Seven sirtuin proteins have been reported in mammals SIRT1, SIRT6, and SIRT7 are nuclear proteins while SIRT3, SIRT4, and SIRT5 are imported to mitochondria, and SIRT2 is principally cytoplasmic (*Dominy and Puigserver, 2013).* Their deacetylation activity modulates biological activities by targeting various transcription factors, histones and other molecules that influence energy metabolism, stress response and cell survival**.** SIRT1, SIRT3, SIRT4, and SIRT5 have an efficient role in modulating the function of mitochondria. Evidence indicates that SIRT1 activates PGC-1 which translocates to nucleus exerting its effect on transcriptional control of mitochondrial genes *(Zhong and Mostoslavsky, 2011).* It had been suggested that SIRT1 controls the expression of PGC-1 gene in skeletal muscle, probably with the contribution of myogenic factors such as MEF2 and MyoD. The complex of SIRT1-MyoD-PGC-1 binds to PGC-1 promoter creating positive feedback and auto-regulatory loop for PGC-1 expression *(Amat et al., 2009).*

  SIRT3 gene transcriptional activation through estrogen-related receptor binding element (ERRE) is essential for PGC-1 mediated mitochondrial biogenesis. PGC-1 , UCP1 and other mitochondria-related genes expression increases by activation of CREB phosphorylation through forced expression of SIRT3. PGC-1 -dependent gene expression of subunits I and II, as well as subunit VIIa from cytochrome C oxidase, is blocked due to knockdown of SIRT3 *(Kong et al., 2010).*

2.4 Signalling events controlling mitochondrial biogenesis

- **AMPK (AMP-activated protein kinase)**

  In response to acute energy crisis (when ATP synthesis gets impaired or AMP is generated at a high rate due to consumption of ATP), AMPK regulates the intracellular energy metabolism *(Hardie 2007).* AMPK on activation phosphorylates catabolic pathway enzymes such as glucose transport and fatty acid oxidation and inhibits the anabolic pathway such as lipogenesis and glycogen synthesis *(Kahn et al., 2004).* -GPA treatment resulted in AMPK activation in skeletal muscle leading to increased NRF-1 binding activity, -ALAS mRNA expression, cytochrome C protein expression and mitochondrial content *(Bergeron et al., 2001).* AMPK activation is responsible for

increased PGC-1 expression by phosphorylation of two residues, threonine-177 and serine-538 *(Jager et al., 2007).* -GPA (-guanadinopropionic acid), creatine analogue, is a pharmacological activator of AMPK and hence promotes mitochondrial biogenesis through modulation of PGC-1 and NRFs *(Bergeron et al., 2001).*

- **Calcium signalling**

  Mitochondrial gene expression is affected by the intracellular calcium dynamics which is dependent on the signaling pathways and secondary messenger, calcium. A study revealed that **Calcineurin** (calcium/calmodulin-dependent protein phosphatase) overexpressing transgenic mice found to have increased number of slow-twitch muscle fibres as well as increased PGC-1 expression *(Chin et al., 1998).* Calcineurin upregulates a number of genes involved in mitochondrial energy metabolism in cardiomyocytes *(Schaeffer et al., 2004).* Although calcineurin plays an important role in mitochondrial biogenesis but it is not fully responsible for adaptations in skeletal muscle induced by exercise-training. This has been supported by the research in which Cyclosporin, calcineurin inhibitor, treatment to the rats did not prevent the mitochondrial markers upregulation *(Garcia-Roves et al., 2006).*

- **mTOR pathway**

  PI3K/AKT/mTOR pathway is a mechanistic pathway important in regulating the cell cycle and hence monitors the cell growth and cell size. It can have an effect on mitochondrial biology through several mechanisms. One of them is the direct contact of mTOR-raptor with the mitochondria and stimulation of respiration *(Schieke et al., 2006).* The other mechanism is mTOR modulation of mitochondrial gene expression through regulation of different transcription factors. In skeletal muscle, binding of mTOR to the transcription factor YY1 (Ying Yang 1) recruits PGC-1 resulting in the increased ability of YY1 to activate mitochondrial gene expression *(Cunningham et al. 2007).*

- **p38 MAPK (mitogen-activated protein kinase)**

  p38 participates in signaling cascades controlling the cellular responses to cytokines and stress such as osmotic shock, inflammatory cytokines, lipopolysaccharides (LPS), ultraviolet light (UV) and growth factors. It's important role have been found in myogenic cell differentiation *(Zetser et al., 1999).* A study revealed that enhanced PGC-1 expression and increased mitochondrial proteins are found to be linked to overexpression of p38 MAK in skeletal muscle of transgenic mice *(Akimoto et al., 2005).* Exercise leads to the increased p38 MAPK which is sufficient to stimulate the PGC-1 transcription *(Wright et al 2007, Akimoto et al 2005).*

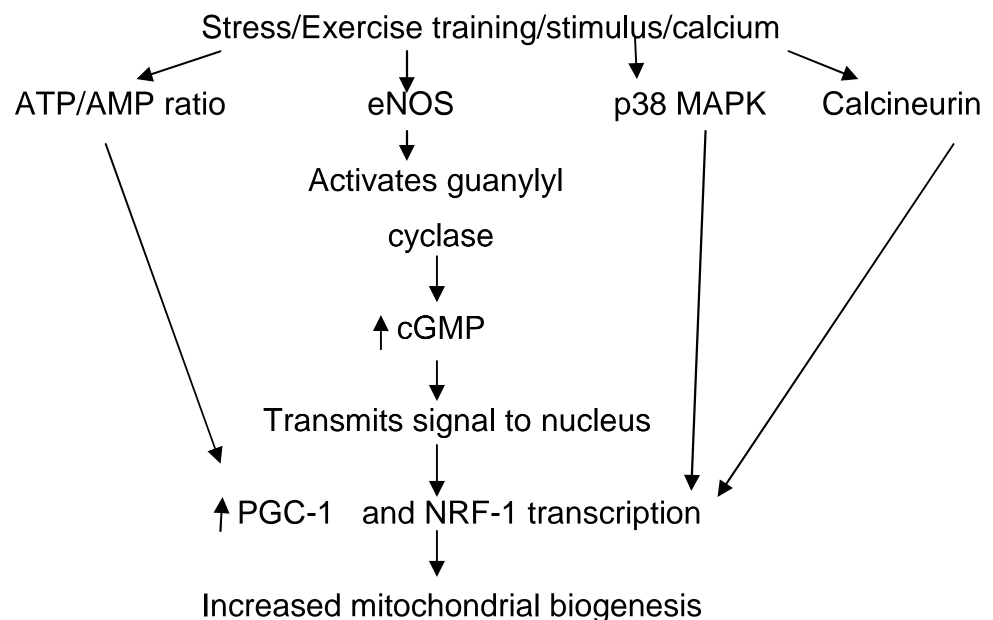


Figure 2: Various signaling events regulating mitochondrial biogenesis

2.5 Natural compounds targeting mitochondrial biogenesis

- **PEITC (-Phenylethyl isothiocyanate)**

  Increased leakage of electrons from the mitochondrial respiratory chain is the cause of elevated ROS production in cancer cells. Cancer cells defense itself from harmful effects of ROS by strengthening the cellular antioxidant system and hence becoming resistant to the exogenous compounds that lead to the ROS overproduction *(Chen et al., 2010).*

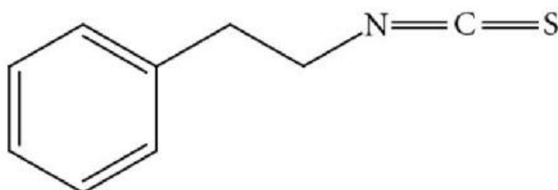


Figure 3: Chemical Structure of Phenethyl isothiocyanate (PEITC)

PEITC, a glucosinolate, is a natural constituent of vegetables like broccoli, cabbage etc. It acts as an anti-cancer agent with low toxicity to the normal cells. There is increased ROS production in cancer cells leading to the dependence of cancer cells on the anti-oxidant system for scavenging the ROS and their survival. PEITC functions by disabling the anti-oxidant system either by directly conjugating with GSH pool and its export out of the cell resulting in depletion of cellular GSH *(Xu and Thornalley, 2001)* or by inhibiting the redox-modulating enzyme glutathione peroxidase *(Trachootham et al., 2009)*.

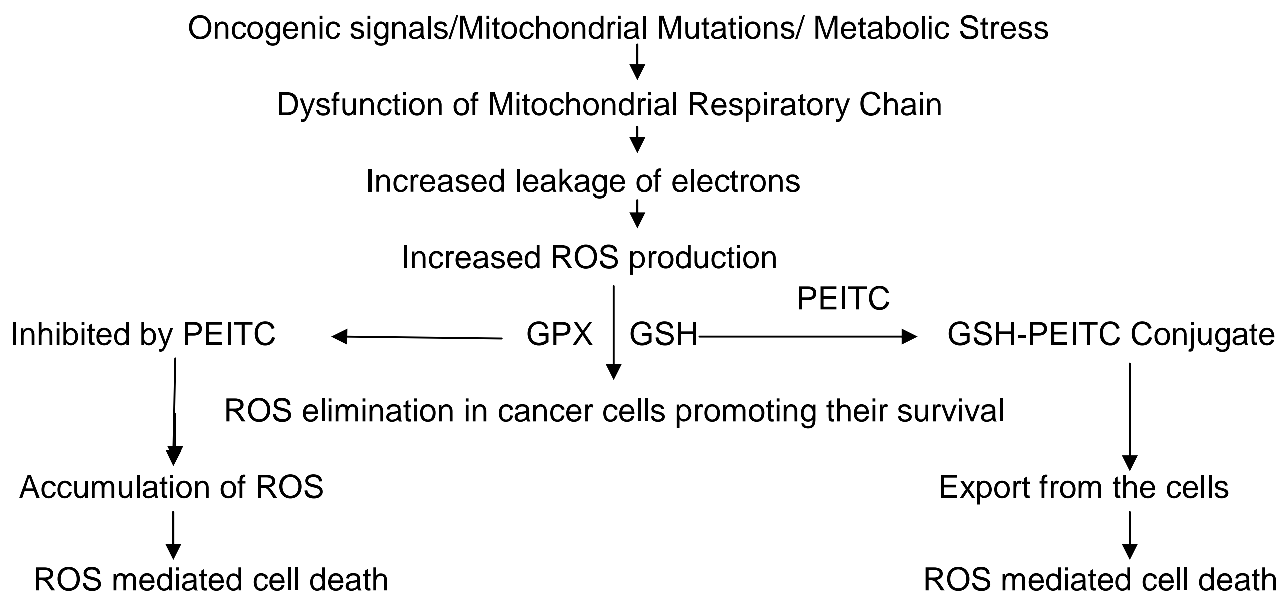


Figure 4: PEITC mechanism of action leading to ROS mediated cancer cell death

- **Quercetin**

  Quercetin (Que), 3, 3', 4', 5, 7-pentahydroxyflavone, is a naturally occurring polyphenolic flavonoid family member which is found most abundantly in fruits and vegetables. It has wide-ranging bioactive effects and through modulation of various enzymes and transcription factors in inflammatory signaling cascade or by reducing ROS production in various cell types it can regulate the mitochondrial biogenesis *(Lee, W. J. et al., 2015, Hock and Kralli, 2009).*

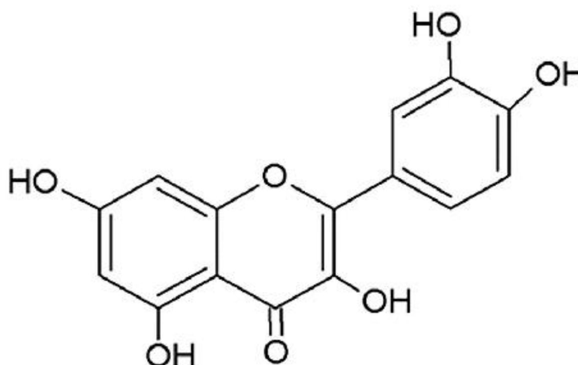


Figure 5: Chemical structure of Quercetin

Study conducted by Rayamajhi et al. revealed that the increased amount of mitochondrial DNA is induced by quercetin in cultured HepG2 cells *(Rayamajhi et al., 2013).* Expression of PPAR and PGC-1 got enhanced through Nrf-2/HO-1-dependent mechanism after quercetin treatment in primary hepatocytes *(Kim et al.,2015).* Davis et al study revealed the effect of quercetin on mitochondrial biogenesis in mice brain and muscle by enhancing the mtDNA through the mechanism including NAD-dependent protein deacetylase sirtuin-1, PGC-1 and cytochrome C upregulation *(Davis et al., 2009).* Increased mitochondrial area, length, number, and DNA content was induced by the quercetin in the hippocampus of rats exposed to hypobaric hypoxia *(Liu et al., 2015).* 50-200μM concentration of quercetin induced decrease in viability of human prostate carcinoma LNCaP cells in a dose dependent manner. PARP cleavage as well as caspase-8, caspase-9 and caspase-3 activation were also observed after quercetin treatment *(Lee et al.,2008).* Another study analyzed the effect of quercetin with cisplatin on human non-small cell lung carcinoma H-520 cells which revealed that it led to up-regulation of Bax and decreased Bcl-X_L and Bcl-2 levels. Authors also found enhanced release of cytochrome C to the cytosol *(Kuhar et al.,2006).* Interestingly, quercetin can act either as an anti-oxidant or a pro-oxidant depending on concentration and duration of exposure. Already reported that higher doses of quercetin (40-100μM) lead to decreased cell survival rates and enhanced anti-tumor effects by diminishing the levels and activities of anti-oxidants. Whereas low doses (5-30μM) augment the anti-oxidant capacity of cancer cells and antagonize the cytotoxic effects of anti-neoplastic drugs in cancer cells *(Lee, Y. J.et al.,2015).*

2.6 Cancer and its association with mitochondrial biogenesis

It is a group of diseases which involves uncontrolled and abnormal cell growth either having potential to invade or spread to other parts of the body or they might be the benign tumors. Various genetic changes leads to cancer development and many of them may be due to inherited genetic defects from parents. Cancer can be detected by certain signs and symptoms and further can be investigated by medical imaging and confirmed by biopsy. Cancers can be prevented by accommodating to healthy lifestyle. It can be treated with combination of radiation therapy, surgery, chemotherapy and targeted therapy.

Cellular proliferation greatly depends upon the amount of mitochondria which is governed by the process of mitochondrial biogenesis. Various studies have linked the tumorigenesis to the increased mitochondrial biogenesis. *In vivo* experiments revealed the decreased tumorigenesis with impaired oxidative phosphorylation due to the loss of mtDNA *(Martinez-Outschoorn et al., 2011)*. p32 expression is found to be upregulated in specifically breast cancers. It is involved in promoting oxidative phosphorylation and has been found to be pro-tumorigenic *in vivo*. Reducing the p32 expression in tumor cells suppresses tumor growth. Knocking down p32 expression shifted cancer cells metabolism from OXPHOS to glycolysis, accompanied by increased lactate production and elevated glucose consumption *(Fogal et al., 2010)*. Decreased expression of mitochondrial COX1 (cytochrome C oxidase 1, a component of complex IV), ND2 and ND6 (NADH dehydrogenases 2 and 6) have been shown in p53-/- chronic lymphocytic leukemia cells. But a compensatory increase in mitochondrial biogenesis was found in p53-/- CLL cells. Tfam, NRF-1 expression, and NF- B pathway was found to be elevated in p53 knocked down CLL cells (Ogasawara et al., 2016).

Otto Warburg hypothesis revealed irreversible respiration as the primary cause for the origin of cancer. He showed the altered mitochondrial metabolism along with high glycolysis rates, high lactate production, and impaired mitochondrial oxidative phosphorylation. Contrary, Sidney Weinhouse revealed that cancer cells could exhibit normal oxidative phosphorylation if NAD^+ is supplemented. Within tumors, there is heterogeneity of cellular metabolism with metabolic coupling between hypoxic regions with high levels of glycolysis and lactate production and well-oxygenated regions that have high rates of oxidative phosphorylation *(Martinez-Outschoorn et al., 2011).* Frequent mutations in mtDNA have been detected in ovarian cancer. Various studies revealed that mitochondrial biogenesis is enhanced in oncogene-transformed (HRAS) epithelial cancer cells. Nuclear respiratory factor-1 (NRF-1), mitochondrial transcription termination factor (MTERF), peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1), estrogen-related receptor alpha (ERR) and calcium/calmodulin-dependent protein kinase type II delta (CAMK2D), all these genes are found to be strongly associated with epithelial ovarian cancer risk *(Uddin et al., 2014)*.

Chapter 3

Materials and Methods

3.1 Materials

1. **Cell culture**

   All cell lines were obtained from ATCC. HBL-100, T47D were cultured in Roswell Park Memorial institute (RPMI) and MDA-MB 231 was cultured in Dulbecco's Modified Eagle Medium (DMEM) with addition of 10% fetal bovine serum and antibiotics. They were maintained in a cell culture incubator at 37°c. Trypsin was used for detachment of cells and subsequently passaging in T25 and T75 flasks. Culturing media, serum and all other culturing reagents were accessed from Invitrogen.

2. **Sub-culturing of cell lines**

   Cells are cultured in the complete media having FBS and antibiotic. When the cells are confluent enough around 70-80%, they are sub-cultured by trypsinizing them for 2 to 3 minutes. Detachment of the cells is followed by addition of media to stop the action of trypsin. Media containing cells is transferred to the microfuge tubes and centrifuged at 1500 rpm for 6-8 minutes at 37°c. Supernatant discarded and resuspended cells in complete media. Then added 300 µl of this media to dishes and 7ml respective media were added to the cells.

3. **Chemicals**

   Quercetin, PEITC, DMSO, 1X PBS, Lysis buffer, Protease inhibitor, PMSF, Bradford's reagent, BSA, acrylamide bis-acrylamide, SDS, APS, TEMED, Running buffer, Transfer buffer, stacking gel buffer, resolving gel buffer, 1X PBST, ECL substrate, 4% paraformaldehyde, DAPI, Mito-tracker green dye.

3.2 Methods and protocols

Confocal Microscopy

Reagents: - Quercetin, PEITC, 1X PBS, paraformaldehyde, DAPI (4,6-diamidino-2-phenylindole), MTG (Mito-tracker green dye), mounting media.

Equipment: - 6-well microplate, confocal microscope.

Methodology

- Cultured cells were seeded on coverslips in 6-well microplate in each well with a final volume of 2ml culture medium per well. Plates were incubated for 24 hrs in an incubator (5% CO_2, 37°c).
- After 24 hrs, treatment with PEITC and Quercetin was given in the same order as control, Q20, Q40, P20, P40 and Q+P (20).
- After 36 hrs, media was discarded and washed with 1X PBS twice.
- Cells were fixed with 4% paraformaldehyde for 5 minutes.
- After wash DAPI (1µl:2000µl) was added to the cells and waited for 30 minutes.
- Then wash was given with PBS twice and added Mito tracker green dye (1µl:2400µl) to wells and left for 30 minutes.
- After wash with PBS, mounting of coverslip on slides was done using mounting media.
- Cells were imaged using confocal microscopy.

Western blot protocol

Reagents: - Quercetin, PEITC, 1X PBS, lysis buffer, protease inhibitor, Phenylmethyl sulfonyl flouride, Bradford's reagent, SDS, TEMED, APS, Acrylamide bis-acrylamide, loading dye, nitrocellulose membrane, GAPDH and NRF2 primary antibody, anti-mouse and anti-rabbit secondary antibody, ECL substrate, running buffer, transfer buffer

Equipment: - microfuge tubes, falcon tubes, electrophoresis assembly, nitrocellulose assembly, centrifuge, ThermoBlotter, BioRad-Chemidoc.

Solutions: -

1. **10% APS: -**

   Ammonium Persulphate – 10g

   It is dissolved in 100ml of distilled water.

2. **30% Acrylamide solution: -**

   Acrylamide – 30g

   Bis-acrylamide – 0.8g

   Both were dissolved in 100 ml water. It is then filtered through 0.2μM filter.

3. **SDS running buffer: -**

   Tris base – 6.04g

   Glycine – 28.8g

   SDS – 2g

   All were dissolved in 1.8L water resulting in 1X running buffer.

4. **6X loading Dye: -**

   1M Tris 6.8 – 3.75ml

   SDS – 1.2g

   Glycerol – 6ml

   Bromophenol Blue – 0.006g

   5%   -mercaptoethanol – 0.5ml

5. **Resolving and stacking gel components**

Component	10% Resolving Gel	4% Stacking Gel
Double Distilled Water	4mL	2.975mL
30% Acrylamide	3mL	670μL
Tris-Hcl	2.5mL (pH=8.8 of 1.5M)	1.25mL (pH=6.8 of 0.5M)
10% SDS	100μL	50μL
10% APS	100μL	50μL
TEMED	10μL	5μL

Table 1: Components of resolving and stacking gel

Cell lysate preparation

- Firstly, the cells are plated on to the 100mm culture dishes (Total 6 plates).
- After 24 hours, treatment to the cells with quercetin and PEITC (phenethyl isothiocyanate) was given.
  I. Label the dishes as control, Q20, Q40, P20, P40 and Q20+P20.
  II. Control was treated with 28µl DMSO in 7ml media.
  III. 56 µl of 10mM PEITC was added to 13944 µl (~ 14ml) for making 40µM PEITC. Add 8ml of this solution to P40 dish. 6ml media was added to the remaining 6ml solution making it 12 µl of 20µM PEITC. Now added 8ml of this solution to P20 dish and 4ml was added to the Q20+P20 dish.
  IV. 56 µl of 10mM Quercetin was added to 13944 µl (~14ml) for making 40 µM Quercetin. Add 8ml of this solution to Q40 dish. 6ml media was added to the remaining 6ml solution making it 12 µl of 20µM Quercetin. Now added 8ml of this solution to Q20 dish and 4ml was added to the Q20+P20 dish.
- After 36 hours, media was taken and put into the 6 falcons (15ml) but around 2ml media was kept in those dishes. Scraping was done in those dishes and all media taken into the falcons.
- Centrifuge them at 2000 rpm for 5mins at 37°c.
- After centrifuge, discard the media from falcons and add 2ml phosphate buffered saline (1X) to the pellet. Mixing of the pellet with PBS was done.
- Again centrifuge is done at 2000 rpm for 5mins at 37°c and 1ml PBS (1X) was added to the pellet and mixed well.
- Distribute the mixture in ratio of 600:400 µl in 12 eppendorfs. Centrifuge them all again at 1500-2000 rpm.
- Store the RNA isolation ones at -20°c.

Protein isolation

- 200µl PI-lysis buffer solution (1000µl lysis buffer + 10µl Protease inhibitor + 10µl phenylmethyl sulfonyl fluoride) was added to the pellets kept on ice.
- ½ hour incubation at ice only and gave short spins at every 10min interval.
- Centrifuge all the eppendorfs at 12000 rpm for 10mins at 4°c. After centrifuge supernatant was transferred to another eppendorfs and discarded the pellets.

Protein estimation was done through Bradford's assay using 2µl of each sample and BSA (1mg/ml) for making standards. Blank was just 2µl Lysis buffer. At last added 200 µl Bradford's reagent and took the readings at 595 nm. The protein concentration in various samples was found through this assay.

SDS-PAGE

- Glass plates were cleaned with spirit and fit into the vertical gel cast holder.
- 1.5Mm 10% resolving gel was prepared by using materials mentioned in the table.
- Poured the liquid in between the two vertical glass plates and added isopropanol on the top to remove the bubbles on the top.
- Left the gel undisturbed until it polymerizes. In the mean time, stacking gel was prepared till SDS.
- When resolving gel polymerizes, add the remaining components to stacking gel solution.
- Then the solution is loaded on to the resolving gel and immediately followed by comb insertion.
- As soon as the gel gets polymerized, it is assembled in the running unit.
- Running buffer was added to the tank and can also ice pack to keep the run in a cool temperature.
- Protein samples were prepared by calculating the volume of the samples needed to load 50µg of the samples in the gel. The samples were prepared by equalizing volume using lysis buffer and lastly adding 6X loading dye to all the samples.
- These prepared samples were heat activated in boiling water at 95°c for 5 minutes.

- The combs were removed from the gel and loaded 2μl of protein marker and sample on their respective wells.
- Run the gel for 20 minutes at 70v until protein passes from stacking to resolving gel and then changed it to 200v for around 45 minutes.

Transfer of Gel

- After the run, the glass plates were removed and gel was transferred to transfer unit.
- In the transfer cassette, firstly at the bottom, filter paper placed and on the top nitrocellulose membrane was kept. Gel was placed on that membrane and lastly another filter paper was put on the gel. All of these should be wet in the 1X transfer buffer before placing in the thermoblotter cassette.
- The cassette was put in the TurboBlotter and voltage kept at 25v and current at 1.3 Amperes for 13 minutes.
- After transfer the cassette was removed and the gel was put in the staining solution for 1 hour.
- Gel was destained by incubating it for 1 hour in destaining solution. It was done to just check the loading or transfer efficiency.

Blocking and antibody incubation

- After the bands are transferred from gel to the nitrocellulose membrane, the membrane was kept in 5% milk (20ml) for blocking for 1 hour for blocking the non-specific binding sites.
- Membrane was then washed twice with 1X PBST.
- Then membranes was cut from the respective markers and transferred to primary antibody solutions (GAPDH and NRF2). The membranes were kept for overnight in the rotor at 4°c.
- After overnight incubation washing was done with 1X PBST for 3 times (changed PBS at 10mins intervals).
- Then the membranes were transferred to respective secondary antibody (anti-mouse and anti-rabbit) and kept for 2 hrs on a dancing shaker.
- After 2hrs again washing done as mentioned above.
- Membrane was developed using ECL substrate and BioRad's ChemiDoc for visualizing the protein bands observed on BioRad software Image Lab 3.0.

Chapter-4

Results

Summary of all the research work done is explained in the following sections

1. Analysis of mitochondrial size and shape change in T47D cells after quercetin treatment using confocal microscopy.
2. Analysis of mitochondrial size and shape change in T47D after PEITC treatment using confocal microscopy.
3. Western blot analysis of Nrf-2 in HBL-100 cells treated with quercetin and PEITC.
4. Western blot analysis of Nrf-2 in MDA-MB 231 cells treated with quercetin and PEITC.

4.1 Analysis of mitochondrial size and shape change in T47D cells after quercetin treatment using confocal microscopy

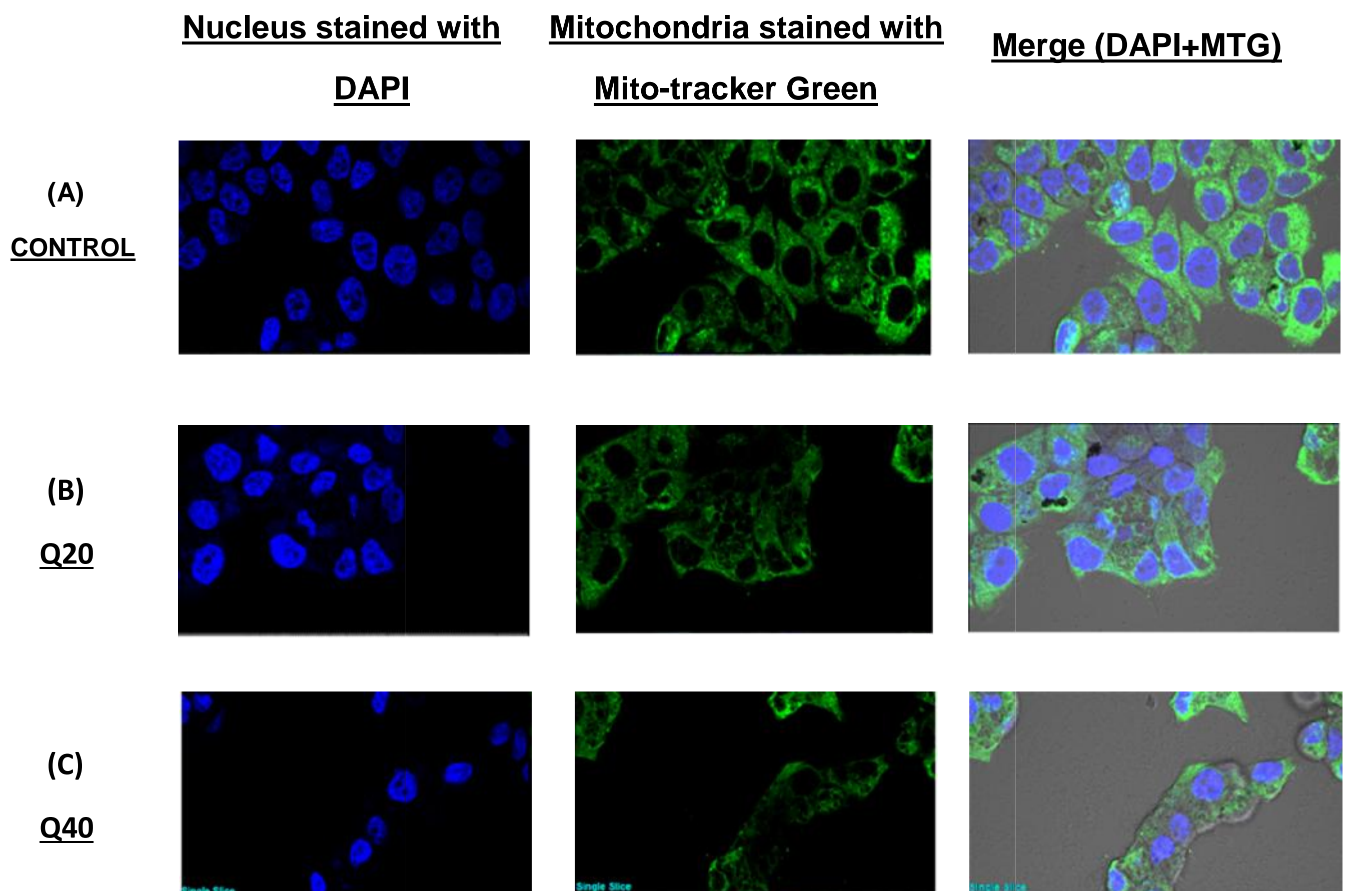


Figure 6: Morphology of mitochondria in T47D cells under confocal microscope at 60X (A) control (without treatment) (B) treated with 20μM quercetin (C) treated with 40μM quercetin

T47D cells are breast carcinoma cells cultured in RPMI-1640 media. As they get 70-80% confluent, they were split and plated in 6-well microplates. After 24 hrs incubation, quercetin treatment (20μM and 40μM) was given. After 36 hrs the cells were stained with DAPI and MTG dye and mounted on slides for confocal microscopy. The results obtained showed clearly the decreased intensity of the MTG dye after treatment as shown in Fig.6 (B) and (C) revealing the decreased mitochondria size and shape which is linked to decreased mitochondrial biogenesis in T47D cells. The cells were observed under confocal microscope at 60X. As the drug concentration is increasing the mitochondria size and number are getting decreased.

4.2 Analysis of mitochondrial size and shape change in T47D cells after PEITC treatment using confocal microscopy

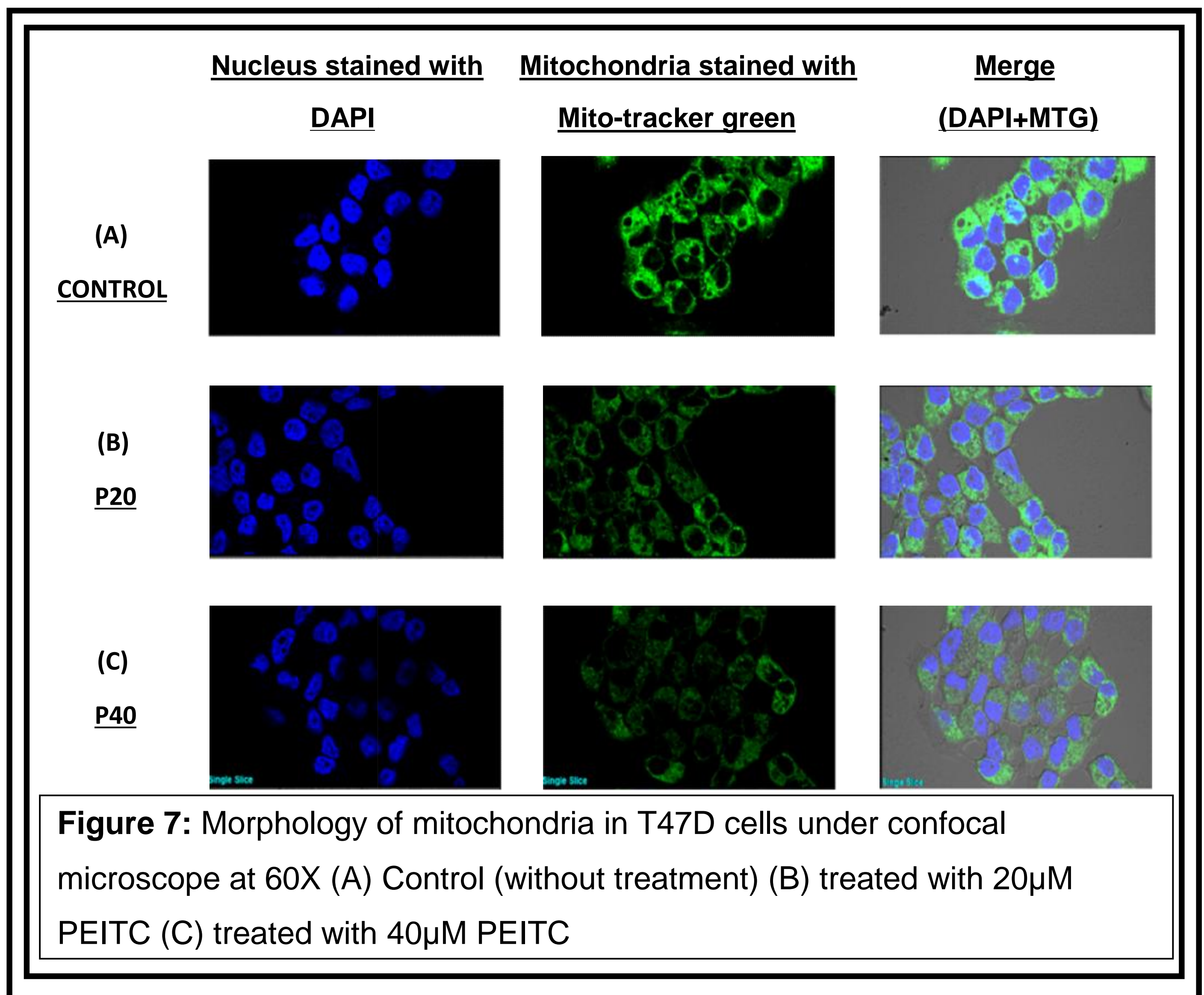


Figure 7: Morphology of mitochondria in T47D cells under confocal microscope at 60X (A) Control (without treatment) (B) treated with 20μM PEITC (C) treated with 40μM PEITC

T47D cells are breast carcinoma cells cultured in RPMI-1640 media. As they get 70-80% confluent, they were split and plated in 6-well microplates. After 24 hrs incubation, PEITC treatment (20µM and 40µM) was given. After 36 hrs the cells were stained with DAPI and MTG dye and mounted on slides for confocal microscopy. The results obtained showed clearly the decreased intensity of the MTG dye after treatment as shown in Fig.7 (B) and (C) revealing the decreased mitochondria size and shape which is linked to decreased mitochondrial biogenesis in T47D cells. The cells were observed under confocal microscope at 60X. As the drug concentration is increasing, the mitochondria size and number are getting decreased.

4.3 Western blot analysis of Nrf-2 in HBL-100 cells treated with quercetin and PEITC

Nrf-2 is a basic leucine zipper protein involved in protection against oxidative damage triggered by stress like injury or inflammation through regulation of the expression of anti-oxidant proteins. Under normal conditions, it is sequestered in cytoplasm by KEAP1 and Cullin3 which are responsible for degradation of Nrf-2 by ubiquitination. On exposure to oxidative stress the comples of KEAP1-Nrf2 disrupts and Nrf-2 translocates into nucleus and binds to anti-oxidant response element (ARE) in upstream promoter of many anti-oxidant genes and initiate transcription. Its activation has been found to be beneficial for cancer chemoprevention in normal tissues. To confirm that phytochemicals PEITC and Quercetin induced NRF-2 expression western blot analysis was done in HBL-100 cell line. Levels of activation of Nrf-2 and GAPDH (loading control) were measured using anti-Nrf-2 and anti-GAPDH antibodies respectively.

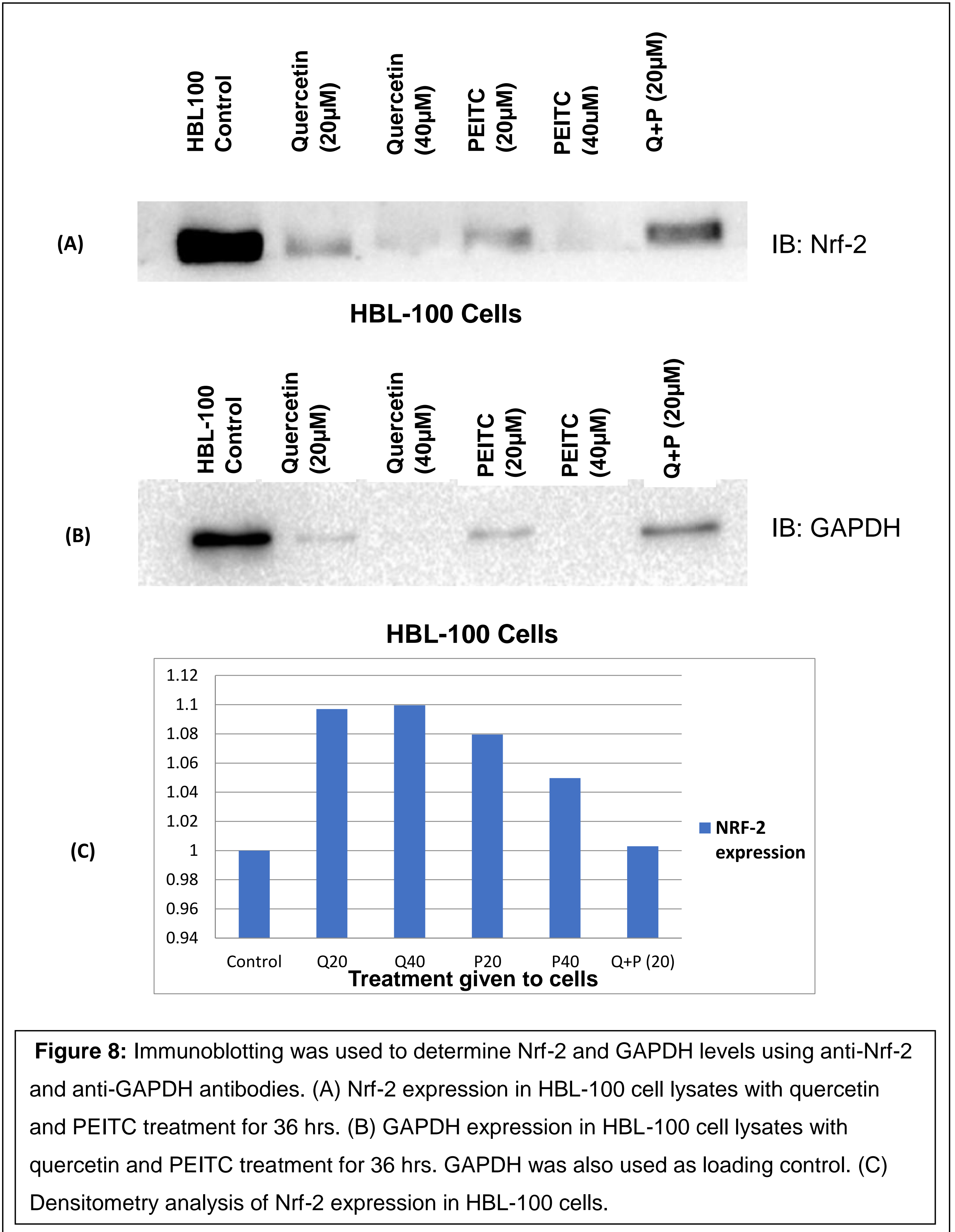
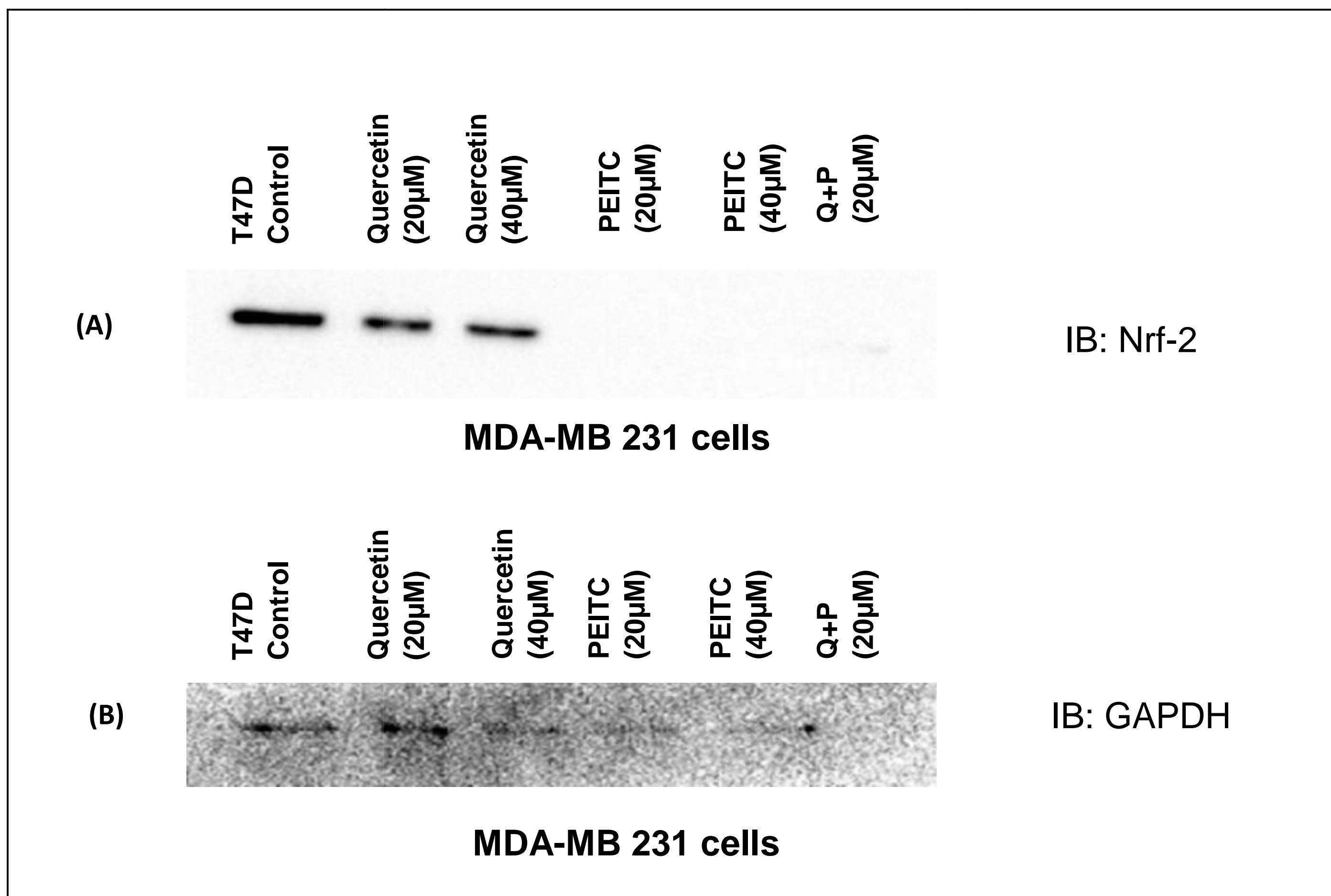


Figure 8: Immunoblotting was used to determine Nrf-2 and GAPDH levels using anti-Nrf-2 and anti-GAPDH antibodies. (A) Nrf-2 expression in HBL-100 cell lysates with quercetin and PEITC treatment for 36 hrs. (B) GAPDH expression in HBL-100 cell lysates with quercetin and PEITC treatment for 36 hrs. GAPDH was also used as loading control. (C) Densitometry analysis of Nrf-2 expression in HBL-100 cells.

Western blotting analysis for Nrf-2 expression in Figure 8 (A) demonstrates a dose-dependent increase in Nrf-2 levels in drug-treated cells as compared to control (Fig. 8 (B)) in HBL-100 cell line. This is indicative of increased mitochondrial biogenesis in normal cells after treated with quercetin and PEITC. Due to improper loading the bands obtained were not sharp. But densitometry results confirmed the increased Nrf-2 expression in normal cells.

4.4 Western blot analysis of Nrf-2 in MDA-MB 231 cells treated with quercetin and PEITC

MDA-MB 231 is an epithelial triple negative breast cancer cell line. Cellular proliferation greatly depends upon the amount of mitochondria which is governed by the process of mitochondrial biogenesis. As many of the anti-cancer drugs act through free radical mechanism to carry out their effects so the reduced anti-oxidant defense can actually make the cells susceptible to the chemotherapeutics. To confirm that phytochemicals PEITC and Quercetin decreased NRF-2 expression western blot analysis was done in MDA-MB 231 cell line. Levels of inhibition of Nrf-2 and GAPDH (loading control) were measured using anti-Nrf-2 and anti-GAPDH antibodies respectively.



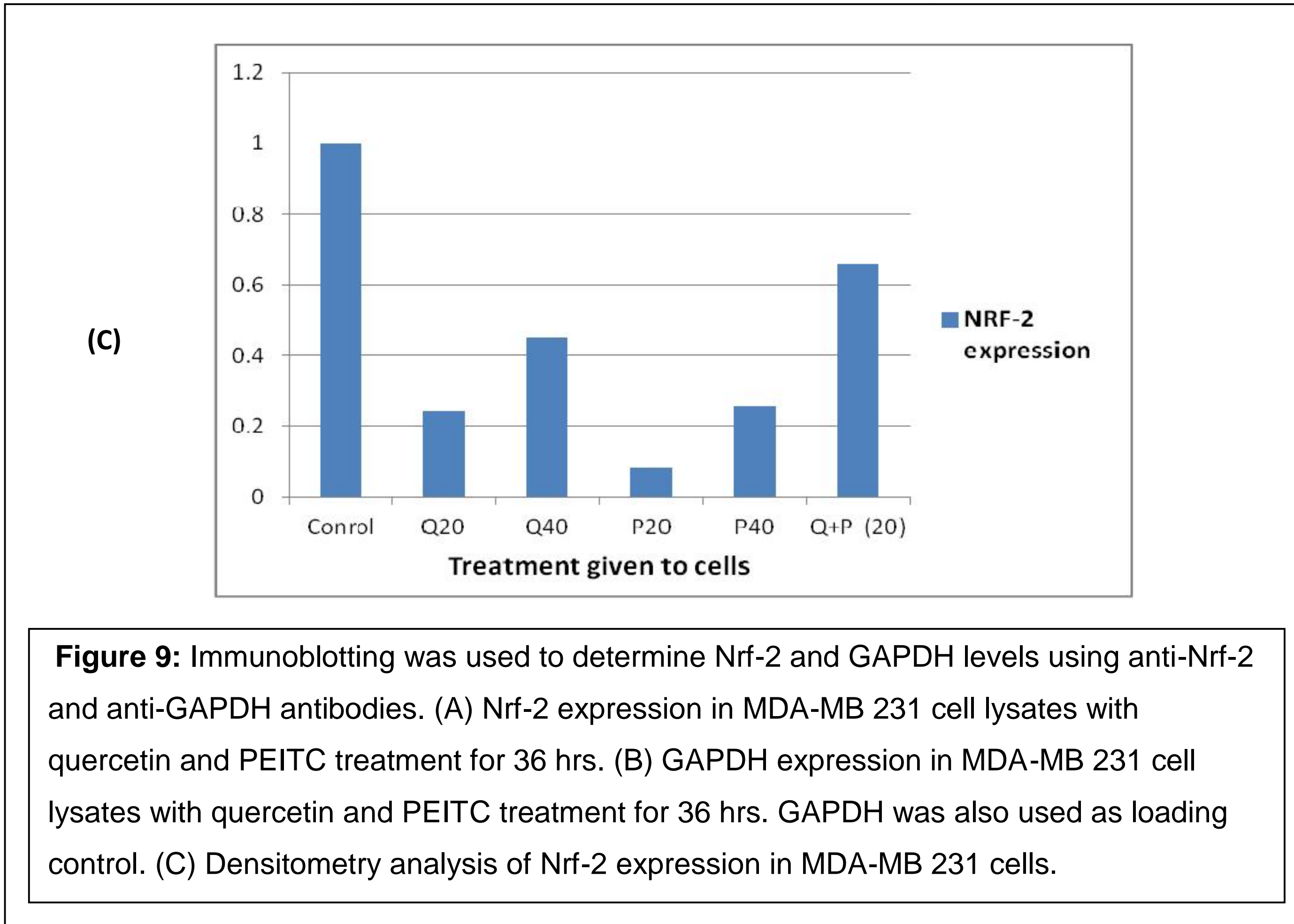


Figure 9: Immunoblotting was used to determine Nrf-2 and GAPDH levels using anti-Nrf-2 and anti-GAPDH antibodies. (A) Nrf-2 expression in MDA-MB 231 cell lysates with quercetin and PEITC treatment for 36 hrs. (B) GAPDH expression in MDA-MB 231 cell lysates with quercetin and PEITC treatment for 36 hrs. GAPDH was also used as loading control. (C) Densitometry analysis of Nrf-2 expression in MDA-MB 231 cells.

Western blotting analysis for Nrf-2 expression in Figure 9 (A) demonstrates a dose-dependent decrease in Nrf-2 levels in drug-treated cells as compared to control (Fig. 9 (B)) in MDA-MB 231 cell line. This is indicative of decreased mitochondrial biogenesis in cancer cells after treated with quercetin and PEITC. Due to improper loading the bands obtained were not sharp. But densitometry results confirmed the decreased Nrf-2 expression in cancer cells. This indicates that anti-oxidative status in cancer cells is lowered by the drug might be leading to ROS-mediated cell death.

Collectively from the results it can be deduced that Quercetin and PEITC lead to decreased mitochondrial biogenesis in cancer cells by lowering the expression of NRF-2 leading to decreased anti-oxidative status of the cancer cells, hence, ultimately might be resulting in ROS-mediated cancer cell death. Quercetin and PEITC treatment also lead to increased NRF-2 expression in normal cells resulting in increased anti-oxidative status of the cells beneficial for cancer chemoprevention in normal tissues. The combination of both phytochemicals didn't proved to be more effective in cell lines experimented on.

Chapter-5

Discussion

Phytochemicals have a long history of health benefits. In recent years, phytochemicals have emerged as a new path in cancer therapies. They have anti-cancer properties that are exploited to prevent the growth and progression of the tumor. Phenethyl isothiocyanate (PEITC) and Quercetin have been found to have anti-cancer properties by targeting mitochondrial biogenesis *(Chen et al., 2010, Gibellini et al., 2015).* The tumorigenesis has been linked to the increased mitochondrial biogenesis in many studies. In many types of human cancer reduced anti-oxidants levels with increased oxidative stress has been observed. As many of the anti-cancer drugs act through free radical mechanism to carry out their effects so the reduced anti-oxidant defense can actually make the cells susceptible to the chemotherapeutics. Targeting Nrf-2 (regulates anti-oxidative genes) using phytochemicals is a promising approach for cancer therapeutics.

In our study we revealed that PEITC and quercetin treatment led to decreased mitochondria shape, size and number in T47D (mammalian breast cancer cell line) cell line. This suggests that the two drugs are effective in targeting the mitochondria in cancer cells resulting in cancer cells killing. As the mitochondria decreases in cancer cells, it leads to deprivation in energy levels and hence becoming sensitive to the chemotherapies which mainly functions through ROS mediated pathway for killing cancer cells.

In our study we have also found that on exposing HBL-100 (normal mammalian cell line) cell line to quercetin, PEITC and combination of both drugs led to increased mitochondrial biogenesis through enhanced Nrf-2 expression in them resulting in increased anti-oxidative genes expression, hence, beneficial for cancer prevention in normal cells. On exposure of MDA-MB 231 (mammalian breast cancer cell line) cell line to quercetin, PEITC and combination of drugs led to decreased mitochondrial biogenesis through diminished Nrf-2 expression in them resulting in decreased anti-oxidative genes expression, hence, might be leading to ROS-mediated cell death. Quercetin and PEITC, both had negative effect on Nrf-2 expression in cancer cells.

FUTURE PERSPECTIVE

Treatments available for the cancer involve chemotherapy, radiotherapy, surgical therapy and immunotherapy which even fail due to side effects or drug resistance development. So, it is necessary to develop more efficient therapies that can overcome the drug resistance and show least side effects. Combination of phytochemicals with other anti-cancer compounds will increase the efficacy of treatment and ensure improved quality of life for the cancer patient. More phytochemicals are required to be tested and studied in vitro and in vivo and which are found to be effective should be proposed for clinical trials. Quercetin and PEITC can be used to carry on study and design the drugs for cancer treatment. Decreasing anti-oxidative status in cancer cells can lead to their exposure to oxidative stress leading to increased ROS levels in them ultimately leading to cancer cell death. So, targeting Nrf-2 and mitochondrial biogenesis can be a promising path for cancer treatment.

Summary

Background: Phytochemicals are bioactive compounds that have the chemopreventive and chemotherapeutic property. Quercetin is isolated from the *Tridax procumbens* (Linn.). Its anti-cancer activity has been well documented in vitro and in vivo. It could be pro-apoptotic as well as anti-apoptotic depending upon its concentration of it and time of exposure. PEITC majorly isolated from *Nasturtium officinale* (watercress) has shown to mediate its anti-cancer activity through ROS-mediated pathway. The key objectives of our study were to test the anti-cancer activity of both the drugs by targeting mitochondrial biogenesis in cancer cell lines.

Methods and Materials: Confocal microscopy was used to visualize the mitochondria size, shape interpreted through the intensity of MTG dye in cancer cells after treated with the two drugs. Expression levels of NRF-2 were also examined using western blotting.

Results and Discussion: In this study, we found that on exposure of HBL-100 and MDA-MB 231 to PEITC, Quercetin and combination of both drugs led to increased NRF-2 expression in HBL-100 normal mammalian cell line whereas decreased NRF-2 expression in MDA-MB 231 mammalian breast cancer cell line. It could be interpreted that the decreased NRF-2 expression is linked to decreased mitochondrial biogenesis. As the anti-oxidative genes expression goes down, the ROS accumulation in cancer cells can lead to their death. Even the confocal microscopy results also showed the decreased mitochondria size, shape and number in cancer cells after treatment with Quercetin and PEITC. To conclude our study it has been shown that quercetin and PEITC lead to decreased mitochondrial biogenesis in cancer cells and increased mitochondrial biogenesis in normal cells. Both the phytochemicals target the cancer cells by lowering the anti-oxidative status of the cancer cells and hence might be leading to ROS accumulation and hence ultimately causing cancer cell death.

References

Akimoto, T., Pohnert, S. C., Li, P., Zhang, M., Gumbs, C., Rosenberg, P. B., ... & Yan, Z. (2005). Exercise stimulates Pgc-1 transcription in skeletal muscle through activation of the p38 MAPK pathway. *Journal of Biological Chemistry*, *280*(20), 19587-19593.

Amat, R., Planavila, A., Chen, S. L., Iglesias, R., Giralt, M., & Villarroya, F. (2009). SIRT1 controls the transcription of the peroxisome proliferator-activated receptor- co-activator-1 (PGC-1) gene in skeletal muscle through the PGC-1 autoregulatory loop and interaction with MyoD. *Journal of biological chemistry*, *284*(33), 21872-21880.

Bergeron, R., Ren, J. M., Cadman, K. S., Moore, I. K., Perret, P., Pypaert, M., ... & Shulman, G. I. (2001). Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. *American Journal of Physiology-Endocrinology And Metabolism*, *281*(6), E1340-E1346.

Boengler, K., Heusch, G., & Schulz, R. (2011). Nuclear-encoded mitochondrial proteins and their role in cardioprotection. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1813*(7), 1286-1294.

Brenmoehl, J., & Hoeflich, A. (2013). Dual control of mitochondrial biogenesis by sirtuin 1 and sirtuin 3. *Mitochondrion*, *13*(6), 755-761.

Chen, G., Wang, F., Trachootham, D., & Huang, P. (2010). Preferential killing of cancer cells with mitochondrial dysfunction by natural compounds. *Mitochondrion*, *10*(6), 614-625.

Chin, E. R., Olson, E. N., Richardson, J. A., Yang, Q., Humphries, C., Shelton, J. M., ... & Williams, R. S. (1998). A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes & development*, *12*(16), 2499-2509.

Cunningham, J. T., Rodgers, J. T., Arlow, D. H., Vazquez, F., Mootha, V. K., & Puigserver, P. (2007). mTOR controls mitochondrial oxidative function through a YY1–PGC-1 transcriptional complex. *nature*, *450*(7170), 736.

Damirchi, A., Babaei, P., Gholamali, M., & Ranjbar, K. (2012). Mitochondrial Biogenesis in Skeletal Muscle: Exercise and Aging. In *Skeletal Muscle-From Myogenesis to Clinical Relations*. InTech.

Davis, J. M., Murphy, E. A., Carmichael, M. D., & Davis, B. (2009). Quercetin increases brain and muscle mitochondrial biogenesis and exercise tolerance. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *296*(4), R1071-R1077.

de Oliveira, M. R., Nabavi, S. M., Braidy, N., Setzer, W. N., Ahmed, T., & Nabavi, S. F. (2016). Quercetin and the mitochondria: a mechanistic view. *Biotechnology advances*, *34*(5), 532-549.

Dominy, J. E., & Puigserver, P. (2013). Mitochondrial biogenesis through activation of nuclear signaling proteins. *Cold Spring Harbor perspectives in biology*, *5*(7), a015008.

Evans, M. J., & Scarpulla, R. C. (1989). Interaction of nuclear factors with multiple sites in the somatic cytochrome c promoter. Characterization of upstream NRF-1, ATF, and intron Sp1 recognition sequences. *Journal of Biological Chemistry*, *264*(24), 14361-14368.

Fogal, V., Richardson, A. D., Karmali, P. P., Scheffler, I. E., Smith, J. W., & Ruoslahti, E. (2010). Mitochondrial p32 protein is a critical regulator of tumor metabolism via maintenance of oxidative phosphorylation. *Molecular and cellular biology*, *30*(6), 1303-1318.

Frazier, A. E., Dudek, J., Guiard, B., Voos, W., Li, Y., Lind, M., ... & Bilanchone, V. (2004). Pam16 has an essential role in the mitochondrial protein import motor. *Nature Structural and Molecular Biology*, *11*(3), 226.

Garcia-Roves, P. M., Huss, J., & Holloszy, J. O. (2006). Role of calcineurin in exercise-induced mitochondrial biogenesis. *American Journal of Physiology-Endocrinology and Metabolism*, *290*(6), E1172-E1179.

Gleyzer, N., Vercauteren, K., & Scarpulla, R. C. (2005). Control of mitochondrial transcription specificity factors (TFB1M and TFB2M) by nuclear respiratory factors (NRF-1 and NRF-2) and PGC-1 family coactivators. *Molecular and cellular biology*, *25*(4), 1354-1366.

Gugneja, S., Virbasius, J. V., & Scarpulla, R. C. (1995). Four structurally distinct, non-DNA-binding subunits of human nuclear respiratory factor 2 share a conserved transcriptional activation domain. *Molecular and Cellular Biology*, *15*(1), 102-111.

Gibellini, L., Bianchini, E., De Biasi, S., Nasi, M., Cossarizza, A., & Pinti, M. (2015). Natural compounds modulating mitochondrial functions. *Evidence-Based Complementary and Alternative Medicine*, *2015*.

Hardie, D. G. (2007). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nature reviews Molecular cell biology*, *8*(10), 774.

Hatefi, Y. (1985). The mitochondrial electron transport and oxidative phosphorylation system. *Annual review of biochemistry*, *54*(1), 1015-1069.

Hill, K., Model, K., Ryan, M. T., Dietmeier, K., Martin, F., Wagner, R., & Pfanner, N. (1998). Tom40 forms the hydrophilic channel of the mitochondrial import pore for preproteins. *Nature*, *395*(6701), 516.

Hock, M. B., & Kralli, A. (2009). Transcriptional control of mitochondrial biogenesis and function. *Annual review of physiology*, *71*, 177-203.

Hutu, D. P., Guiard, B., Chacinska, A., Becker, D., Pfanner, N., Rehling, P., & van der Laan, M. (2008). Mitochondrial protein import motor: differential role of Tim44 in the recruitment of Pam17 and J-complex to the presequence translocase. *Molecular biology of the cell*, *19*(6), 2642-2649.

Jäger, S., Handschin, C., Pierre, J. S., & Spiegelman, B. M. (2007). AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1 . *Proceedings of the National Academy of Sciences*, *104*(29), 12017-12022.

Jornayvaz, F. R., & Shulman, G. I. (2010). Regulation of mitochondrial biogenesis. *Essays in biochemistry*, *47*, 69-84.

Kahn, B. B., Alquier, T., Carling, D., & Hardie, D. G. (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell metabolism*, *1*(1), 15-25.

Kim, C. S., Kwon, Y., Choe, S. Y., Hong, S. M., Yoo, H., Goto, T., ... & Yu, R. (2015). Quercetin reduces obesity-induced hepatosteatosis by enhancing mitochondrial oxidative metabolism via heme oxygenase-1. *Nutrition & metabolism*, *12*(1), 33.

Kong, X., Wang, R., Xue, Y., Liu, X., Zhang, H., Chen, Y., ... & Chang, Y. (2010). Sirtuin 3, a new target of PGC-1 , plays an important role in the suppression of ROS and mitochondrial biogenesis. *PloS one*, *5*(7), e11707.

KUHAR, M., SEN, S., & SINGH, N. (2006). > Role of Mitochondria in Quercetin-enhanced Chemotherapeutic Response in Human Non-small Cell Lung Carcinoma H-520 Cells. *Anticancer research*, *26*(2A), 1297-1303.

Lee, D. H., Szczepanski, M., & Lee, Y. J. (2008). Role of Bax in quercetin-induced apoptosis in human prostate cancer cells. *Biochemical pharmacology*, *75*(12), 2345-2355.

Lee, H. C., & Wei, Y. H. (2005). Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. *The international journal of biochemistry & cell biology*, *37*(4), 822-834.

Lee, W. J., Hsiao, M., Chang, J. L., Yang, S. F., Tseng, T. H., Cheng, C. W., ... & Lee, L. M. (2015). Quercetin induces mitochondrial-derived apoptosis via reactive oxygen species-mediated ERK activation in HL-60 leukemia cells and xenograft. *Archives of toxicology*, *89*(7), 1103-1117.

Lee, Y. J., Lee, D. M., & Lee, S. H. (2015). Nrf2 expression and apoptosis in quercetin-treated malignant mesothelioma cells. *Molecules and cells*, *38*(5), 416.

Lin, J., Puigserver, P., Donovan, J., Tarr, P., & Spiegelman, B. M. (2002). Peroxisome proliferator-activated receptor coactivator 1 (PGC-1), a novel PGC-1-related transcription coactivator associated with host cell factor. *Journal of Biological Chemistry*, *277*(3), 1645-1648.

Liou, G. Y., & Storz, P. (2010). Reactive oxygen species in cancer. *Free radical research*, *44*(5), 479-496.

Liu, P., Zou, D., Yi, L., Chen, M., Gao, Y., Zhou, R., ... & Mi, M. (2015). Quercetin ameliorates hypobaric hypoxia-induced memory impairment through mitochondrial and neuron function

adaptation via the PGC-1 pathway. *Restorative neurology and neuroscience*, *33*(2), 143-157.

Martinez-Outschoorn, U. E., Pavlides, S., Sotgia, F., & Lisanti, M. P. (2011). Mitochondrial biogenesis drives tumor cell proliferation. *The American journal of pathology*, *178*(5), 1949-1952.

Melser, S., Lavie, J., & Bénard, G. (2015). Mitochondrial degradation and energy metabolism. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1853*(10), 2812-2821.

Milenkovic, D., Kozjak, V., Wiedemann, N., Lohaus, C., Meyer, H. E., Guiard, B., ... & Meisinger, C. (2004). Sam35 of the mitochondrial protein sorting and assembly machinery is a peripheral outer membrane protein essential for cell viability. *Journal of Biological Chemistry*, *279*(21), 22781-22785.

Nohl, H., Gille, L., & Staniek, K. (2005). Intracellular generation of reactive oxygen species by mitochondria. *Biochemical pharmacology*, *69*(5), 719-723.

Ogasawara, M. A., Liu, J., Pelicano, H., Hammoudi, N., Croce, C. M., Keating, M. J., & Huang, P. (2016). Alterations of mitochondrial biogenesis in chronic lymphocytic leukemia cells with loss of p53. *Mitochondrion*, *31*, 33-39.

Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M., & Spiegelman, B. M. (1998). A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell*, *92*(6), 829-839.

Rayamajhi, N., Kim, S. K., Go, H., Joe, Y., Callaway, Z., Kang, J. G., ... & Chung, H. T. (2013). Quercetin induces mitochondrial biogenesis through activation of HO-1 in HepG2 cells. *Oxidative medicine and cellular longevity*, *2013*.

Scarpulla, R. C. (2008). Transcriptional paradigms in mammalian mitochondrial biogenesis and function. *Physiological reviews*, *88*(2), 611-638.

Schaeffer, P. J., Wende, A. R., Magee, C. J., Neilson, J. R., Leone, T. C., Chen, F., & Kelly, D. P. (2004). Calcineurin and calcium/calmodulin-dependent protein kinase activate distinct

metabolic gene regulatory programs in cardiac muscle. *Journal of Biological Chemistry*, *279*(38), 39593-39603.

Schatz, G. (1996). The protein import system of mitochondria. *Journal of Biological Chemistry*, *271*(50), 31763-31766.

Schieke, S. M., Phillips, D., McCoy, J. P., Aponte, A. M., Shen, R. F., Balaban, R. S., & Finkel, T. (2006). The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. *Journal of Biological Chemistry*, *281*(37), 27643-27652.

Scott, I., & Youle, R. J. (2010). Mitochondrial fission and fusion. *Essays in biochemistry*, *47*, 85-98.

Shadel, G. S., & Clayton, D. A. (1997). Mitochondrial DNA maintenance in vertebrates. *Annual review of biochemistry*, *66*(1), 409-435.

Stojanovski, D., Bohnert, M., Pfanner, N., & van der Laan, M. (2012). Mechanisms of protein sorting in mitochondria. *Cold Spring Harbor perspectives in biology*, *4*(10), a011320.

Trachootham, D., Alexandre, J., & Huang, P. (2009). Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?. *Nature reviews Drug discovery*, *8*(7), 579.

Uddin, M. H., Kim, B., Suh, D. H., & Song, Y. S. (2014). Anticancer Strategy Targeting Mitochondrial Biogenesis in Ovarian Cancer. J Cancer Sci Ther 6: 422-428. doi: 10.4172/1948-5956.1000 303 Volume 6 (10) 422-428 (2014)-423 J Cancer Sci Ther ISSN: 1948-5956 JCST, an open access journal Novel Targeted Therapies for Patients with Ovarian Cancer PAK1, GSK3B etc.[36]. Other alterations are observed in RB, RAS and NOTCH pathways.

Vega, R. B., Huss, J. M., & Kelly, D. P. (2000). The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Molecular and cellular biology*, *20*(5), 1868-1876.

Ventura-Clapier, R., Garnier, A., & Veksler, V. (2008). Transcriptional control of mitochondrial biogenesis: the central role of PGC-1 . *Cardiovascular research*, *79*(2), 208-217.

Wiedemann, N., Kozjak, V., Chacinska, A., Schönfisch, B., Rospert, S., Ryan, M. T., ... & Meisinger, C. (2003). Machinery for protein sorting and assembly in the mitochondrial outer membrane. *Nature*, *424*(6948), 565.

Wright, D. C., Han, D. H., Garcia-Roves, P. M., Geiger, P. C., Jones, T. E., & Holloszy, J. O. (2007). Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1 expression. *Journal of Biological Chemistry*, *282*(1), 194-199.

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., ... & Spiegelman, B. M. (1999). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell*, *98*(1), 115-124.

Xia, Y., Buja, L. M., Scarpulla, R. C., & McMillin, J. B. (1997). Electrical stimulation of neonatal cardiomyocytes results in the sequential activation of nuclear genes governing mitochondrial proliferation and differentiation. *Proceedings of the National Academy of Sciences*, *94*(21), 11399-11404.

Xu, K., & Thornalley, P. J. (2001). Involvement of glutathione metabolism in the cytotoxicity of the phenethyl isothiocyanate and its cysteine conjugate to human leukaemia cells in vitro1. *Biochemical pharmacology*, *61*(2), 165-177.

Zamora, M., & A Villena, J. (2014). Targeting mitochondrial biogenesis to treat insulin resistance. *Current pharmaceutical design*, *20*(35), 5527-5557.

Zetser, A., Gredinger, E., & Bengal, E. (1999). p38 mitogen-activated protein kinase pathway promotes skeletal muscle differentiation participation of the MEF2C transcription factor. *Journal of Biological Chemistry*, *274*(8), 5193-5200.

Zhong, L., & Mostoslavsky, R. (2011). Fine tuning our cellular factories: sirtuins in mitochondrial biology. *Cell metabolism*, *13*(6), 621-626.

Student Approval Form

Name of the author	Anchal Thakur
Department	Human genetics and Molecular medicine
Degree	M.Sc Life Science with specialization in Molecular Medicine
University	Central University of Punjab
Guide	Dr. Harish Chander
Project title	Study the effect of phytochemicals phenethyl isothiocyanate (PEITC) and Quercetin on mitochondrial biogenesis in cancer and normal cell lines
Year of award	2018

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Signature of the candidate **Signature and seal of the supervisor**

Place: Bathinda

Date: May 2018

Central University of Punjab, Bathinda

Declaration

I declare that all the changes suggested by the external examiner in the research project entitled **"Study the effect of phytochemicals phenethyl isothiocyanate (PEITC) and Quercetin on mitochondrial biogenesis in cancer and normal cell lines"** submitted by me for the award of degree of **M.Sc. in Life sciences with specialization in Molecular Medicine** in the **Department for Human Genetics and Molecular Medicine** has been incorporated in the research project.

Anchal Thakur

Department for Human Genetics and Molecular Medicine

School of Health Sciences

Date: May 2018

Dr. Harish Chander

Department for Human Genetics and Molecular Medicine

School of Health Sciences

Date: May 2018

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