The role of phytomolecules in the prevention of bone cell apoptosis–potential role in bone health

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DECLARATION

The work in this thesis is the result of my own investigations and the views expressed are those of myself, except the modification of the two phytoestrogens Genistein and Daidzein which was carried out by Dr. Sinan Battah, visiting fellow at Essex University, School of Biological Science.

ABSTRACT

Estrogen withdrawal after the menopause leads to an increased rate of bone remodeling, excessive osteoclast activity and a greater fracture risk. Hormone replacement therapy (HRT) was prescribed as a treatment for postmenopausal women to prevent bone loss; however, HRT is associated with an elevated incidence of cardiovascular disease, stroke and cancer. These side effects led to an interest in naturally occurring compounds with estrogenic action such as phytoestrogens (PEs), which are non-steroidal-plant derived compounds. The aim of this study describes investigation of the protective effect of phytoestrogen molecules (Genistein, Daidzein, Resveratrol, 6-Gingerol and Fucosterol) in the prevention of reactive oxygen species induced by H_2O_2 cell death in MLOY4 osteocyte like cell line. All compounds tested significantly ($p<0.05$) reduced osteocyte apoptosis and decreased reactive oxygen species activity. PEs have multiple effects on different bone cells, reducing osteoblast apoptosis and inhibiting RANKL inducing osteoclast formation. The protective effect of Genistein, Daidzein and Resveratrol was not prevented by estrogen receptor inhibitor ICI 182780, suggesting a potential direct antioxidant effect. Additionally, this study offers evidence for bone protection conferred by PEs through demonstrating their potent inhibitory effect on cytokine release and activating antioxidant enzymes such as Catalase (CAT). The antioxidant effect of PEs was thought to be due to their similar phenolic structure to estrogen; therefore Genistein and Daidzein were modified by masking their hydroxyl groups and tested for their protective effects on osteocyte apoptosis. Not all modified compounds protected osteocytes from oxidative stress induced by hydrogen peroxide (H_2O_2). This study

provides novel evidence that PEs have multiple effects on bone cell activity. Thus, PEs may not be the only treatment but possible supplementation of pharmacological or alternative to pharmacological treatment of post-menopausal osteoporosis.

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ABBREVIATIONS GLOSSARY

1,25(OH)3	VitaminD3
ALP	Alkaline phosphatase
ATP	Adenosine triphosphate
BMU	Basic multicellular unit
BSUs	Basic structure units
BH3	BCL-2 homology 3
Bcl-2	B cell leukemia/lymphoma 2-like proteins
BMSCs	Bone marrow stromal cells
BLCs	Bone lining cells
BMD	Bone mineral density
BRU	Bone remodelling unit
BMP	Bone morphogenic protein
Caspase	Cysteine Aspartate Protease
CFU-O	Colony- forming unit-osteoprogenitor
CX43	Connexin43
DC-STAMP	Dendritic specific transmembrane protein
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor Beta
ERK1/2	Extracellular signal-regulated kinase 1/2
FADD	FAS-associated death domain
FGF	Fibroblast growth factor
FOXO	Forkhead box-O
FPPs	Farnesyl diphosphate synthase
GLa	Carboxyglutamic acid
Gpx1	Glutathione peroxidase 1
GM-CSF	Granulocyte macrophage colony stimulating factor
HRT	Hormone replacement therapy
H_2O_2	Hydrogen peroxide
IFN-γ	Interferone-gamma
IGF-1	Insulin-like growth factor-1
IL-1α	Interlukin 1 alpha

IL-1β	Interlukin 1 Beta
JNK	c-Jun NH2-terminal kinase
LPS	Lipopolysaccharides
LPR5	Low-density lipoprotein receptor-related protein 5
MAPKs	Mitogen-activated protein kinases
M-CSF	Macrophage colony stimulating factor
MITF	Microphthalmia-associated transcription factor
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor kappa B
NFATc1	Nuclear factor of activated T-cells, cytoplasmic 1
NO	Nitic oxide
OABs	Osteocyte apoptotic bodies
O2-	Superoxide
OH^-	Radical hydroxyl group
OPG	Osteoprotegrin
OPG-L	Osteoprotergerin ligand
OPs	Osteoclast precursors
OVX	Ovariectomy
PEs	Phytoestrogens
PE2	Prostaglandin E2
PKC	Protein kinase C
PKB	Protein Kinase B
RANK	Receptor activator of nuclear activator kB
RANKL	Receptor activator of nuclear activator kB ligand
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TNF-β	Tumor necrosis factor beta
TRANCE	TNF-related activation induced cytokine
TRAIL	TNF-related apoptosis-inducing ligand
TRAFs	TNFR-associated factors
TRAP	Tartrate-resistant acid phosphatase
PHEX	Phosphate regulating endopeptidase homolog X-linked
PTH	Parathyroid hormone

PTHR1	Parathyroid hormone receptor 1
Pyk2	Proline-rich tyrosine kinase 2
ROS	Reactive oxygen species
Runx2	Runt-related transcription factor 2
SERMs	Selective estrogen receptor modulators
SOD	Superoxide dismutase
WHI	Women’s Health Initiative
WNT	Wingless/integrated

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CHAPTER ONE: INTRODUCTION

1. 1 Bone biology

Bone is a rigid connective tissue whose function is to bear mechanical loads upon the body during movement. Other functions of bone include maintaining plasma calcium homeostasis, and supporting hematopoiesis within the bone marrow (Clarke 2008). Recent studies suggest that in addition to its classic roles, bone can also be considered as an endocrine organ. Evidence has been accumulating that FGF23 that is derived from bone cells and osteocalcin, which is derived from osteoblast cells has important roles in regulating kidney function and glucose metabolism. Osteocalcin has an endocrine action on pancreatic β-cells, stimulating proliferation and insulin production (Fukumoto and Martin, 2009). Bone can be woven or lamellar. Woven bone is immature bone, which is formed very rapidly during growth or fracture healing. It is so called because its collagen fibers are loosely arranged in irregular arrays and therefore also called nonlamellar bone. Woven bone contains more cells per unit area than lamellar bone (Hernandez *et al*., 2004). In contrast, lamellar bone is mature bone, in which the collagen fibers are arranged in a lamellar structure (Hernandez *et al*., 2004, Ross and Pawlina 2006). Lamellar bone is mainly composed of cylindrical units called osteons or Haversian systems, which consist of a central canal surrounded by concentric lamellae. The central canal is called osteonal or Haversian canal and contains blood vessels and nerves (Pawlina Ross and 2006). Woven bone is often replaced by lamellar bone as growth continues (Bandyopadhyay-Ghosh 2008). Bone is categorized as being long, short, flat, or irregular according to its shape and function. For instance, long bones such as the femur and ulna are tubular in structure and longer than they are wide, and are the major

weight-bearing bones of the body. Short bones are small bones that act as shock absorbers and force distributors. Flat bones protect internal organs and consequently contain broad surfaces for muscle attachment to bone. Irregular bones such as the vertebra and the skull are termed irregular because their shape determines their specific functions within the skeleton (Clarke 2008). Skeletal bone is also categorized according to the proportion of trabecular and cortical bone, which are the two major types of bone tissue. Trabecular bone is a spongy like porous material and is composed of plates or rods of bone that are distributed within bone marrow. The high porosity of the plates or rods, known as trabeculae, allows the bone to absorb and distribute mechanical energy when loaded. Cortical bone is a dense material with approximately 15% porosity (Hamill 2009). Due to its increased density, cortical bone is stiffer and is able to tolerate large tensile and compressive loads. Approximately 80% of the skeleton is made of cortical bone which forms the outer protective shell of all bones, whereas the other 20% is trabecular bone. Each type of bone has various ratios of trabecular and cortical bone, which in turn is correlated to each bone's specific mechanical requirements. For instance, vertebral bone is made of 25% cortical bone and 75% trabecular bone, and the femoral neck is 50% cortical and 50% trabecular bone (Clarke 2008). Long bones are the primary weight-bearing bones of the skeleton and they are structured to transfer loads from the ends of the bone to the middle. Thus, sections of long bone have different trabecular to cortical bone ratios with age to fulfil their respective mechanical requirements. The three regions within a long bone are the epiphysis, metaphysis, and diaphysis (Fig.1.1).

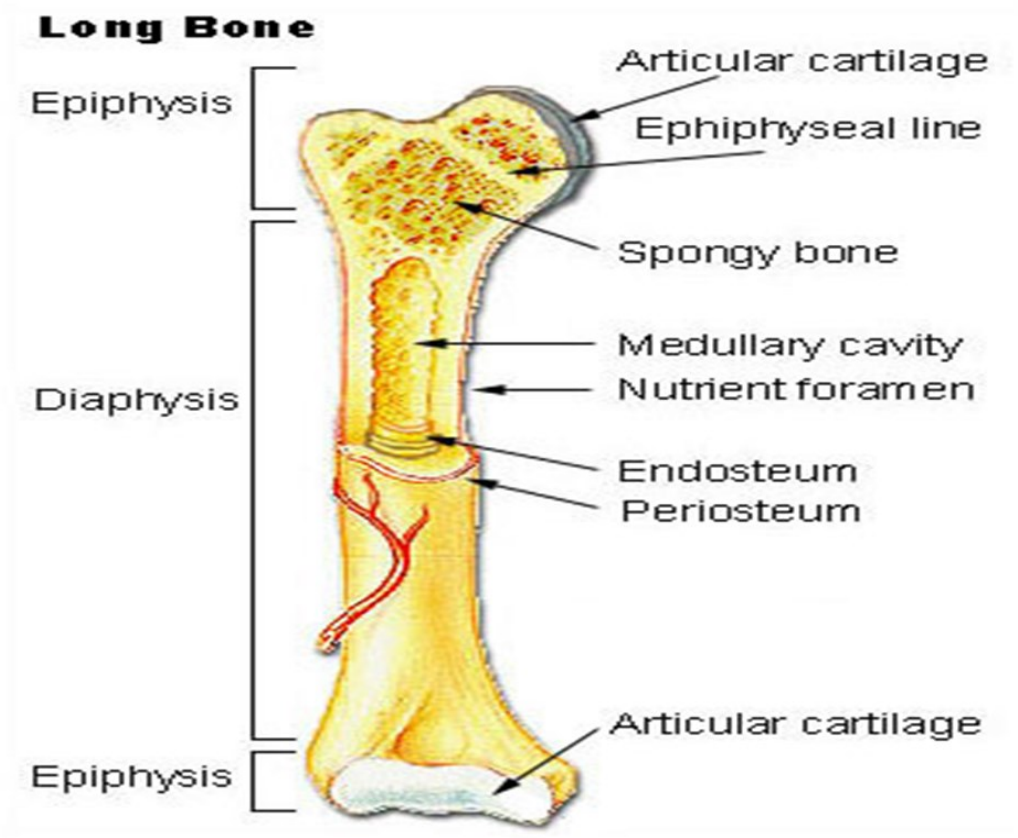

Figure1.1: The basic anatomy of bone. showing the diaphysis (shaft of bones) and epiphysis (ends of bones) with the periosteum membrane and medullary cavity highlighted (Umadevi and Geethalakshmi 2011).

The epiphysis is located on either end of a long bone and is covered with articular cartilage to protect the bone from friction within an articulating joint. The epiphysis is composed mainly of trabecular bone with a thin cortical bone outer layer. Beneath the epiphysis is the metaphysis. The cone-shaped metaphysis of long bone is composed of a cartilaginous core that allows the bone to continuously grow via endochondral ossification during childhood. Upon adulthood (18-25 years old) the cartilaginous region ossifies and fuses with the epiphysis and diaphysis, thereby stopping bone growth.

Like the epiphysis, the metaphysis is mostly composed of trabecular bone that is bordered by a thin outer layer of cortical bone. The diaphysis is a long tubular shaft comprised of dense cortical bone, and is filled with bone marrow, when loaded, the wide trabecular ends of long bone allows for compressive forces exerted from locomotion to be transferred to the stronger and stiffer cortical bone within the diaphysis (Turner 2006).

The outer layer of bone is a well-vascularized connective tissue known as the periosteum. The periosteum is a fibrous connective tissue sheath that surrounds the outer cortical surface of bone, except at joints where bone is lined by articular cartilage, which contains blood vessels, nerve fibers, osteoblasts and osteoclasts. The periosteum is tightly attached to the outer cortical surface of bone by thick collagenous fibers, called Sharpeys' fibers, which extend into underlying bone tissue (Clarke 2008). The periosteum provides osteoprogenitor cells to the bone that are essential for appositional bone growth and fracture repair. As a result, the periosteum experiences more bone formation than bone resorption (Turner 2006). The endosteum is the layer of connective tissue lined with bone lining cells that separates the bone from the bone marrow compartment within the bone shaft. The endosteum typically experiences more bone resorption than bone formation, as the endosteum has a large surface area exposed to the bone marrow space that is the home of formation of osteoclast precursor, the haematopoietic origin of osteoclasts, the bone resorbing cells. Bone has a stiff structure to bear loading, resist deformation, it is flexible in turn to absorb energy from loading and light enough to help in mobility (Bilezikian 2008). The external layer of bone is a double-layered membrane (periosteum), which is the external surface of bone

(Fig.1.1). The outer fibrous layer serves as an insertion point for ligaments and tendons where as its inner osteogenic layer is more cellular and vascular. Cortical and porous trabecular bone lies beneath the periosteum and compose the main bone matrix. The main structural unit of the cortical bone is the osteon, concentric rings of lamella or matrix tubules form an osteon. They have cylinder shape arranged parallel to the bone long axis to enable loading and bending stress (Fig.1.2).

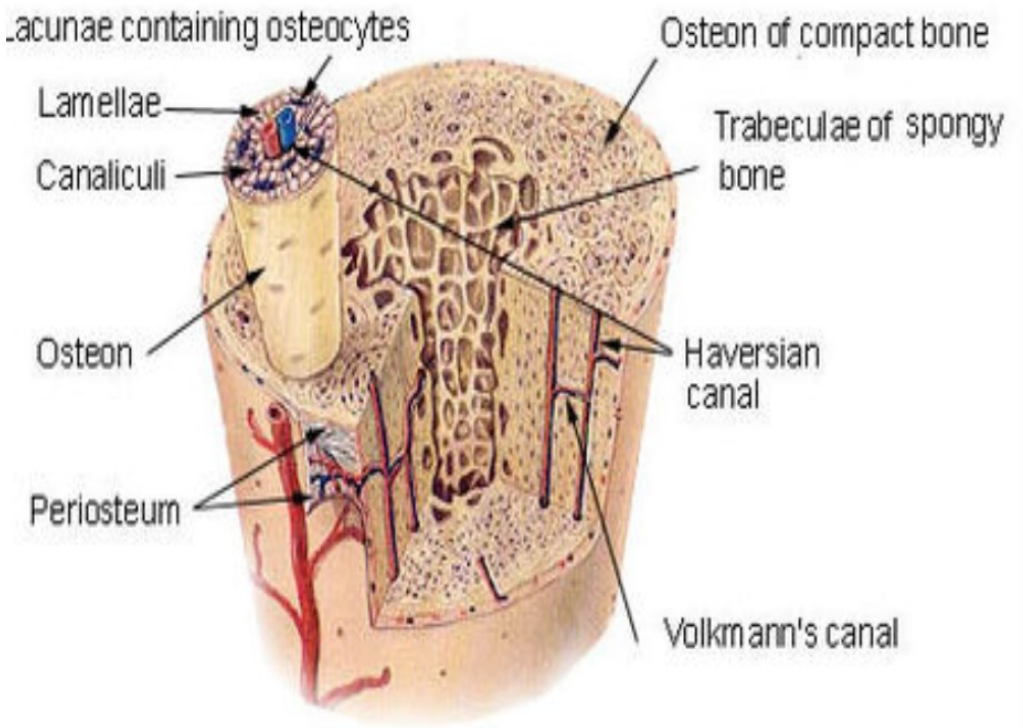

Figure1.2: Cortical and trabecular bone structure. Each osteon consists of concentric layers or lamellae of compact tissue that boarders a central canal (the Harversian canal which contains bone nervous supply and blood supply). Osteons are connected to each other by Volkmann's channels ((Umadevi and Geethalakshmi 2011)

Bone tissue is composed of an organic matrix of type I collagen. It is mineralized with an inorganic phase comprising calcium hydroxyapatite crysctals. The organic matrix provides flexibility, whereas increasing amounts of mineral confer increasing degrees of material stiffness. The mineralized tissue is organized into basic structure units (BSUs) that are the footprint of a remodeling event (Follet *et al.,* 2004). In addition to collagen, there are also non-collagenous proteins which are synthesized by bone cells and make up 15% of bone protein. Osteoblasts produce several proteins that play an important role in bone matrix mineralization including enzymes such as alkaline phosphatase, ALP (Xiao et al., 2007). ALP is an important molecule in mineralization of bone formation and an osteoblastic differentiation marker that is expressed in the initial phases of the process, in addition, this enzyme is necessary for mineralization and formation of bone and its immobilization on a collagen scaffold leads to an increase in the concentration of inorganic phosphate in cell culture and increases the rate of osteoblast differentiation (Jafary *et al*., 2017).

1.2 Bone Cells

Bone tissue is continuously remodelled through the action of bone cells, which include bone resorption by osteoclasts, bone formation by osteoblasts, whereas osteocytes act as mechanosensors, and regulate the bone remodelling process (Florencio-Silvaet al., 2015). Osteoblasts produce new extracellular matrix and control mineralization. The lining cells, which are covering the entire bone surface, are thought to control the access to the pure bone

matrix at the bone surface, which is important for Ca^{+2}-homeostasis and attachment of osteoblasts and osteoclasts (Bonewald and Johnson 2008). The osteocytes are embedded throughout the mineralized matrix and are interconnected via cell membrane extensions known as canaliculi and are thought to play a pivotal role in mechanosensing and Ca^{+2}, PO_4^{-2}, homeostasis. In the adult skeleton, the osteocytes make up 90-95% of all bone cells compared with 4-6% osteoblasts and 1-2% osteoclasts (Bonewald and Johnson 2008).

1.3 The osteoblast

Osteoblasts are derived from multipotent marrow stromal stem cells called mesenchymal stem cells (MSC) (Aubin and Triffitt, 2002). They are responsible for matrix secretion and mineralization of bone. Osteoblasts arise from osteoprogenitors found in the bone marrow (Dennis and Charbord 2002), periosteum (Ogita*et al.,* 2008), and pericytes that surround blood vessels (Shi and Grothos 2003). Recent work has found that the osteoblast linage comprises the supporting unit for haematopoietic stem cells (HSC), which reside in endosteal niches (often called osteoblast niches) in bone marrow to exert their function and a small proportion of HSC and mostly haematopoietic progenitor cells traffic countiously from BM into circulation and concurrently re-home to the BM, leading to the release of small proportion of haematopoietic stem cells and their progenitor from their niches into the blood (Lévesque *et al*., 2010). Osteoprogenitors with appropriate stimulation undergo proliferation, differentiation into pre-osteoblasts, and commitment into terminally dif-

ferentiated osteoblasts. In addition, osteoblasts contribute to the regulation of bone resorption through the expression of receptor activator of nuclear activator kB (RANK) ligand (RANKL), which binds to its receptor RANK, on the surface of osteoclast progenitor cells, inducing their differentiation and bone resorption (Wada *et al*., 2006). Osteoblasts also produce the soluble receptor decoy osteoprotegerin (OPG), which can block the interaction of RANK/ RANKL through binding to RANKL, therefore, preventing osteoclast differentiation and activation, which in turn can reduce bone resorption (Gori *et al.,* 2000). It has been proposed that RANKL / OPG ratio can be regulated by several factors, which in turn regulate osteoclastogenesis. Among these factors are Vitamin D3 (Simonet *et al.,* 1997), IL-1α, IL-1β, TNF-α, TNF-β, bone morphogenic protein (BMP) 2, transforming growth factor β(TGFβ) and 17β-estradiol all of which increase OPG levels, whereas prostaglandin E2 (PGE2), parathyroid hormone (PTH) (Khosla 2001), glucocorticoids and insulin-like growth factor-1 (IGF-1) decrease OPG level (Walsh and Choi 2003). Recent studies have demonstrated that Protein Kinase B (PKB), extracellular signal-regulated kinase 1/2 (ERK1/2) and canonical wingless/integrated (WNT) signalling pathways regulate osteoblast proliferation and differentiation. In response to fibroblast growth factor (FGF) that inhibits osteoblast differentiation, the PKB signalling pathway promotes osteoblast survival while ERK1/2 strongly regulates osteoblast proliferation (Rauccie*t al.,* 2008). Osteoblasts are specialized fibroblasts that secrete osteoid matrix proteins to form the foundation of newly formed bone. Osteoblasts secrete both collagenous and non-collagenous proteins such as type 1 collagen, osteocalcin, osteopontin, osteonectin, bone sialoprotein, and alkaline phosphatase (Flor-

encio-Silva*et al*., 2015). Some osteoblasts undergo apoptosis (programmed cell death) when the deposition of bone matrix terminates, while others differentiate into flattened 'bone lining cells' on the bone surface, whereas others become trapped in bone matrix known as osteocytes (Marks and Odgren, 2002; Parfitt, 2002). Estrogen loss in menopause is known to have a great impact on skeletal mass and calcium metabolism, and both osteoblasts and osteoclasts express estrogen receptors (Kholsa 2010). Estrogen promotes the differentiation of osteoblasts from mesenchymal stem cells and suppresses expression of lipoprotein lipase, a marker of adipocyte differentiation (Okazaki *et al.,* 2002, Dang *et al.,* 2002), prolongs the lifespan of the osteoblast (Kousteni *et al.,* 2001), increases osteoblast expression of proteins that play a role in bone formation (e.g., type I collagen, IGF-1, bone morphogenic protein-6) (Kholsa 2010) and inhibits apoptosis in osteoblasts and osteocytes (Kousteni *et al.,* 2001). Estrogen suppresses osteoclast activity via stimulation of osteoclast apoptosis (Nakamura *et al*., 2007 and reducing osteoblasts expression of RANK-L (Eghbali-Fatourechi *et al.,* 2003). In addition, to the direct effects of estrogen on osteoblasts and osteoclasts, estrogen also inhibits inflammatory cytokine production from bone marrow stromal and mononuclear cells (Kitazawa *et al.,* 1994; 1992, Jilka *et al.,* 1992). This is an important bone protective-mechanism because inflammatory cytokines such as interleukin (IL)-1, IL-6, and TNF-α are known to be potent stimulators of osteoclastogenesis (Strand and Kavanaugh 2004).

1.4 The osteoclast

Osteoclasts are multinucleated cells that are derived from hematopoietic stemcells and are responsible for bone resorption. Differentiation and activation of osteoclasts are regulated by different factors such as RANKL, OPG, TNF-α, IL-1, IL-6, M-CSF, parathyroid hormone and 1,25(OH) D3, which are produced by different cell types including osteoblasts and lymphocytes (Teitelbaum and Ross 2003). Activated multinucleated osteoclasts secrete hydrogen ions via H^+-ATPase proton pumps and chloride channels within their ruffled border cell membranes into the resorption pits beneath them to lower the pH within the bone-resorbing compartment to a PH of below 4, which helps solubilize bone mineral (Ross and Teitelbaum, 2005). Resorbing osteoclasts secrete tartrate-resistant acid phosphatase (TRAP), cathepsin K, matrix metalloproteinase-9 (MMP-9), and gelatinase from cytoplasmic lysosomes to digest organic matrix, resulting in the formation of saucer-shaped Howship's lacunae on the surface of trabecular bone and resorption tunnels in cortical bone. The resorption phase is completed when the multinucleated osteoclasts stop functioning and undergo apoptosis (Reddy 2004).

1.4.1 Factors that affect osteoclastogenesis

1.4.1.1 Receptor activator of NF-kB (RANKL)

A member of the TNF family of proteins, RANKL also known as osteoprotergerin ligand (OPG-L), exists as a homotrimeric protein and is typically membrane-bound on osteoblastic and activated T cells or is secreted by some cells, such as activated T cells (Kearns *et al.,* 2008), being involved in pro-

moting the survival of dendritic cells and contributing to efficient priming of T cells (Akiyama*et al.,* 2012). However, the discovery of OPG, which binds RANKL and inhibits osteoclastogenesis, identified that RANKL is an essential regulator of osteoclastogenesis (Simonet et al., 1997; Yasuda et al., 1998). RANKL is a type II transmembrane protein that consists of an intracellular domain, a transmembrane domain and an extracellular TNF family homologous domain. RANKL is expressed as an integral membrane protein, but a soluble form of RANKL is produced by cleavage of TNF-related activation induced cytokine (TRANCE) or several metalloproteases (Bezerra *et al.,* 2005). In addition, RANKL exists as a homotrimer that binds a cysteine-rich site on the extracellular domain of RANK, the specific receptor of RANKL (Lacy *et al.,* 2012). Similar to other members of the TNF receptor superfamily, the binding of RANKL to RANK causes receptor clustering to form functional trimeric receptors resulting in the activation of signaling molecules in the cells (Lacy *et al.,* 2012). The dimerization of OPG occurs through its carboxyl terminal domain, which binds and inhibits RANKL. OPG has a unique C-terminus domain that binds heparin and TRAIL, a member of the TNF family (Lamoureux *et al.,* 2010). Despite the role of the RANKL/RANK pathway in osteoclastogenesis, it has been reported that RANKL plays an important role in cell proliferation and differentiation in different cells, particularly in epithelial cells in mammary gland lobules during pregnancy and is required for hyperplasia of these cells during lactation and thus milk production in mice (Fata *et al.,* 2000)). RANKL is also known to be an important factor of bone damage associated with cancer metastasis.

RANKL, which was previously reported as a dendritic cell survival factor, was identified as a ligand for OPG. Other studies conducted have showed that the addition of RANKL to bone marrow culture induces osteoclast differentiation in the presence of M-CSF (Jimi *et al.,* 1999). RANKL and RANK knockout mice displayed osteopetrosis; this reveals that RANKL signals are essential for *in vivo* osteoclastogenesis (Kong *et al.,* 1999, Dougall *et al.,* 1999). RANKL is also involved in the stimulation of multinucleation from osteoclast precursor cells and bone resorption activity of mature osteoclasts (Jimi *et al.,* 1999). The survival of mature osteoclasts is regulated by several factors including RANKL. Therefore, RANKL is involved in the differentiation, activation and survival of osteoclasts. In normal physiology, RANKL regulates bone homeostasis by working with OPG. RANKL expression is induced by stimulating osteoblasts with several osteotropic factors such as parathyroid hormone (PTH), Vitamin D3 and IL-6, which enhance osteoclastogenesis (Takahashi *et al.,* 1999). The process of osteoclastogenesis is thought to be affected by the membrane-bound RANKL, which is expressed on the surface of osteoblasts. However, recent studies conducted, have shown that high expression of RANKL in osteocytes plays an important role in osteoclastogenesis, especially in bone remodeling (Nakashima *et al.,* 2011, Xiong *et al.,* 2011). Soluble and/or membrane forms of RANKL derived from osteocytes are thought to be involved in osteoclastogenesis. RANKL is also expressed in lymphocytes, its expression by T or B-lymphocytes does not play an important role in bone remodeling under normal physiological conditions (Nakashima *et al.,* 2011, Onal *et al.,* 2012); however, RANKL expressed in B-cells was partially involved in a mouse model of bone loss induced by estro-

gen deficiency (Onal *et al.,*2012). RANKL expression in osteoclast precursor cells is stimulated by several factors including M-CSF, TNF-alpha, LPS and WNT5A (Arai *et al.,* 1991, Maeda *et al.,* 2012). Binding of RANKL to RANK receptors which are expressed on osteoclast precursor cells induces intracellular signals essential for osteoclast differentiation and activation (Takayanagi 2007). Signaling through RANK is mediated by recruitment of TRAFs. Among the TRAF family of proteins, TRAF6 is essential for RANKL-induced osteoclastogenesis (Naito *et al.,* 1999). TRAF6 activates signaling pathway of NF-kB and MAPK. RANKL promotes osteoclast differentiation via activation of the NF-kB pathway. RANK signaling also activates the transcription factor AP-1 by inducing its component c-Fos. Activation of NF-kB and c-Fos leads to the induction of NFATc1, which stimulates the expression of osteoclast-specific genes such as cathepsin K. RANKL also induces the phosphorylation of Microphthalmia-associated transcription factor (MITF), which regulates the development and function of several cell lineages, including osteoclasts and involved in osteoclastogenesis which activates downstream of MAPK (Hu *et al.,* 2007). The transcription factor complex, including transcription factors such as NFATc1 and MITF, stimulates the expression of osteoclast-specific genes such as cathepsin K. In osteoclastogenesis, the expression of NFATc1 is strongly induced via auto amplification in the later stages of differentiation. Transcriptional activity of NFATc1 is dependent on the localization of protein in the nuclei, which is regulated by calcium signaling. Co-stimulatory molecules possessing tyrosine-based activation motif-accomodating adaptors induce calcium signalling in osteoclastogenesis (Negishi-Koga andTakayanagi 2009). In addition, RANK has a unique cyto-

plasmic domain that recruits adaptor proteins Gab2 and PLCg2, which also induce calcium signalling (Taguchi *et al.,* 2009). Phosphorylation of PLCg2 in conjunction with co- stimulatory signals lead to the activation of NFATc1 (Mao *et al.,*2006). NFATc1 also stimulates transcription of the D2 isoform of the vacuolar ATPase Vo domain and of the dendritic cell-specific transmembrane protein DCSTAMP, which are involved in multinucleation of osteoclasts (Lee *et al.,* 2006, Yagi *et al*., 2005). Thus, it is generally accepted that NFATc1 is involved in the differentiation and multinucleation of osteoclasts.

1.4.1.2 Tumor Necrosis Factor - α (TNF-α)

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine with multifunctional properties, it belongs to the tumor necrosis factor ligand superfamily, with multi activities such as regulation, differentiation, proliferation and apoptosis. TNF can be found in two different forms (α andβ) with similar biological effects. TNF-α plays an important role in modulating the acute inflammatory response to injury or infection, however high levels of TNF-α production contributes to the development of chronic diseases including bone resorption (Azuma *et al.,* 2000). TNF-α is expressed on T- lymphocytes (Gillespie 2007), and promotes bone loss in different conditions such as postmenopausal osteoporosis and could be the key regulator of bone loss in rheumatoid arthritis (Teitelbaum 2007). The first mechanism of TNF-α, is that it promotes osteoclast formation and activation in the inflamed joint by stimulating RANKL, produces RANKL, M-CSF, and IL-1 production by marrow stromal cells and by directly stimulating differentiation of osteoclast pre-

cursors (Lam *et al.,* 2000). TNF-α and RANKL are synergistic, and minimal levels of one markedly enhance the osteoclastogenic capacity of the other (Lam *et al.,* 2000). TNF-α also has effective anti-apoptotic effects on osteoclasts, extending their lifetime (Weitzmann and Pacifici, 2005). The second mechanism occurs after exacerbation of inflammatory process, hence TNF-α may support osteoclast formation through the activation of transforming growth factor (TGF)-β (Teitelbaum 2007). Studies showed that mice whose CD4 cells express a dominant-negative TGF-βII (such that these cells do not respond to TGF-β) have a lower bone mineral density (BMD) than wild-type animals (Gillespie 2007). TNF-α attracts monocytes and lymphocytes to the site of infection and stimulates apoptosis in different cell types via stimulation of cytokine production. The effect of TNF-α is mediated by transmembrane receptor TNFR1 (p55r) and TNFR2 (p75r). TNFR1 (P55r) contains a death domain that can prompt apoptosis, while p75 lacks a death domain (Kobayashi *et al.,* 2000). TNFR1 (p55r) is an important receptor involved in osteoclast differentiation and bone resorption, and treatment with anti-p55r antibody completely inhibits TNF-α-induced osteoclastogenesis (Kobayashi *et al.,* 2000, Azuma *et al.,*2000). Activation of a TNF receptor associated death domain which is caused by binding of TNF-α to its receptor, stimulates two pathways, JNK, protein kinase C and IkB leading to activation of NFκB which translocates to the nucleus and induces the transcription of TNFα-responsive genes (Kruppa *et al.,* 1992). TNF-α also has the ability to trigger the Fas activated death domain which provokes the apoptotic signaling cascades (Nanes 2003). TNF-α affects bone remodeling by direct and indirect action through collaboration with other signal pathways via TNF receptor-associated

factor (TRAF) and NF-κB (Kim *et al.,* 2005). Treatment with TNF-α can induce TRAP positive osteoclast formation on the surface of bone in the absence of RANKL, suggesting that TNF-α may act independently of the RANKL/RANK axis (Kim *et al.,* 2005). Similarly, TNF-α stimulates osteoclast formation in cultures of M-CSF dependent precursors by a TNFR1 dependent but RANKL-independent mechanism (Kitaura*et al.,* 2005). In addition, it has been shown that TNF-α directly induced human osteoclast formation by a RANKL-independent mechanism (Kudo 2002), alsoTNF-α can stimulate osteoclast formation in the presence of M-CSF with less effect than RANK, while RANKL increased the level of TNF-α m-RNA and induced TNF-α release from osteoclast progenitors (Zou 2001).

1.4.1.3 Interleukin-6 (IL-6)

IL-6 is a pro-inflammatory cytokine, secreted by T-cells and macrophages, it possesses pro anti-inflammatory functions and mediates immune response during an infection, and it elevates differentiation of B cells into plasma cells, activates cytotoxic T cells, and regulates bone hemostasis. IL-6 signals through soluble IL-6 receptors binding to gp130, which is ubiquitous in all cells (Barkhausen*et al.,* 2011). IL-6 is known as a potent stimulator of osteoclast induced bone resorption and subsequent bone loss in the context of chronic inflammation (Edwards and Williams 2010). Animal studies suggest that over expression of IL-6 in transgenic mice exhibit severe impairment in cortical and trabecular bone with an increase in osteoclastogenesis and decreased osteoblastogenesis (De Benedetti *et al.,* 2006). Estrogen has the

ability to inhibit IL-6 production in osteoblast lineage cells (Stein and Yang 1995). Moreover, studies conducted on wild type animals showed that estrogen deficiency resulted in bone turnover in these animals, but ovariecctomy failed to cause any change in bone mass and bone remodeling in IL-6 deficient mice (Poli *et al.,* 1994). IL-6 is produced by osteoblasts, however the biological effect is recognized on osteoclast cells, it regulates RANKL production and binding to its receptor on osteoblasts which indirectly promotes osteoclastogenesis and bone resorption (Udagawa *et al.,*1995). Although the direct effects of IL-6 on osteoclasts are inhibitory (Yoshitake *et al.,* 2008, Axmann*et al.,* 2009), IL-6 is thought to contribute to bone loss in osteoporosis, which is caused as a result of estrogen deficiency leading to a mild increase in pro-inflammatory cytokines (Terauchi 2011; Jilka*et al.,* 1992). This finding has led to the development of an anti-IL-6 receptor agents (tocilizumab) which has been used in patients with moderate to severe rheumatoid arthritis (Agarwal 2011). Studies on indirect mechanism of IL-6 on osteoclast formation have demonstrated that IL-6 stimulates osteoclastogenesis when co-cultured with osteoblasts with no effects on purified osteoclasts culture (Hattersley *et al.,* 1988). Further studies suggested that IL-6 receptors are required on osteoblasts but not on osteoclasts for osteoclastogenesis regulation, though higher levels of IL-6 receptors are found on osteoclastic cells (Udagawa *et al.,* 1995). IL-6 mostly binds to IL-6R and forms a complex with gp130 to stimulate the associated intracellular signaling machinery (JAK/STAT or MAPK pathway) and subsequent gene expression (Ohsugi and Kishimoto 2008). Osteoclast formation and activity is mainly affected by IL-6, which stimulates signaling molecules especially RANKL, produced by

osteoblasts (Bellido *et al.,* 1995). Additionally, IL-6 activity on osteoclasts commonly interacts with IL-1, TNF, and IL-6 increases their effect on bone resorption by increasing osteoclastic progenitor pool (Devlin *et al.,* 1998). As IL-6 is essential for the differentiation of immature $CD4^+$ T cells to Th17 cells, it is an effective osteoclastogenic inducer in chronic inflammation and auto-immune diseases.

1.5 Osteocytes

Osteocytes are generated from osteoblasts, which become encased in the bone matrix they produce and are left within the bone matrix after bone re-modelling (Manolagas*et al.,* 2006, Seeman*et al.,* 2006). Osteocytes are situated in lacunae and are connected to each other by canals called canaliculi within the bone matrix (Fig. 1.3). They also retain nutrient and dispose waste through this lacunar-canalculi network (Palumbo *et al.,* 1990b; Zhang *et al.,* 2006b). Osteocyte processes are connected via gap junctions which enable them to communicate with each other and with bone lining cells (BLCs) at the surface of bone (Batra *et al.,* 2011; Ishihara *et al*., 2008). Gap junctions are facilitated by a group of proteins called connexion (Goodenough *et al.,* 1996). Connexin 43 (CX43) is expressed on the cell membrane of osteocytes as well as on the cell processes (John *et al.,* 1993). Osteocytes can sense changes in the level of circulatory hormones (Manolagas 2000), induce signals for bone resorption by activating death signalling pathway through apoptosis (Kogianni*et al.,* 2008) and communicate with bone lining cells through gap junctions to stabilize bone mineral by preserving an adequate local ionic

environment (Cowin 2001). It has been suggested that osteocytes act as the sensor for mechanical loading of bone and transmit signals to the bone cells at the surface of the bone to control bone remodelling (Bonewald 2011).

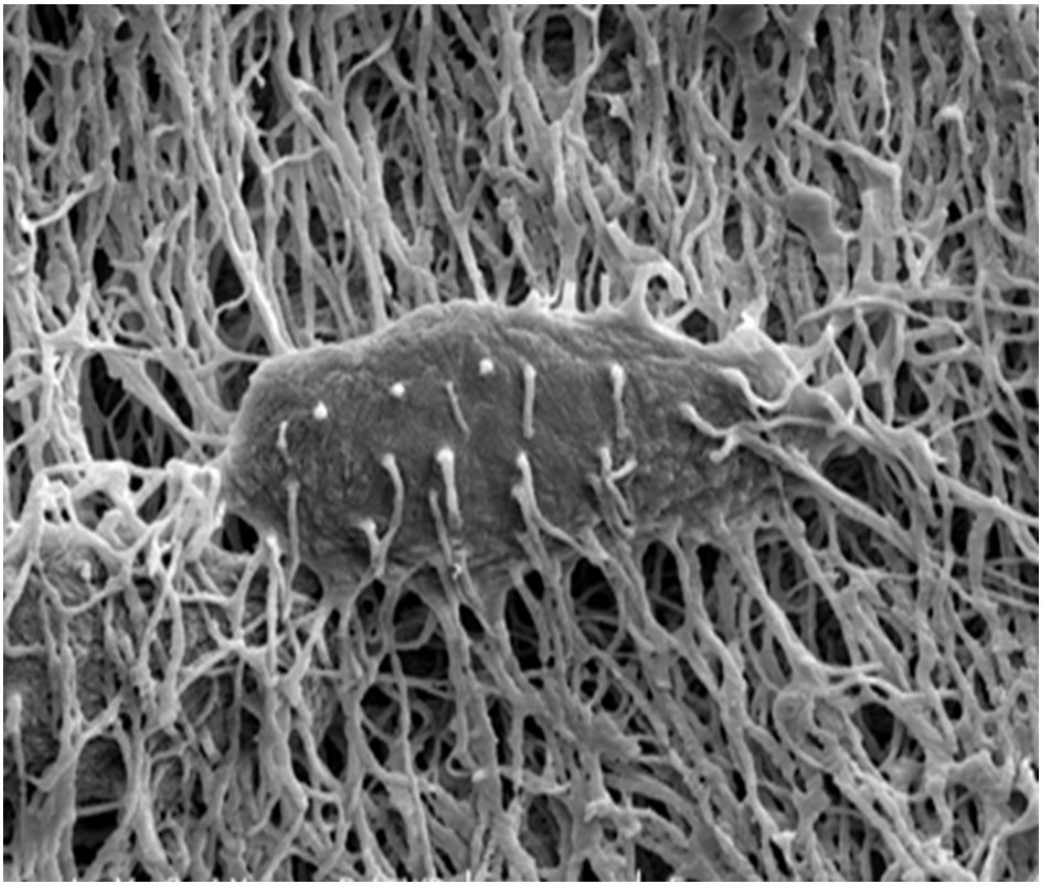

Figure 1.3: Scanning Electron Microscopy (SEM) image of osteocyte-canalicular system. From 4 month old mouse long bone that shows an osteocyte lacuna connecting with bone lining cells through a network of canaliculi toward the bone surface (Pajevic 2009).

1.6 Osteocyte function

1.6.1 Blood-calcium/phosphate homeostasis

Osteocytes play an important role in the metabolism and maintenance of bone. The bone tissue may utilize two advantages that this unique network offers:

1) Intracellular communication system between osteocytes and bone lining cells by a gap junction based network and an extracellular communication system between located osteocytes within the bone and the bone surface via hemichannels.

2) The communication area of cell-bone surface between osteocytes and bone lining cells, that is about twice as large as the communication area between the osteoblasts and bone lining cells at the bone surface (Johnson, 1966).

Osteocytes might be responsible for blood-calcium homeostasis; later observations supported this theory (Palumbo *et al.,* 1990a, Sissons*et al.,* 1990). Osteoblasts and osteoclasts transport calcium into and out of bone (Marotti *et al.,* 1992), however the osteocytes may aid the circulation of calcium into and out of the bone tissue (Bonucci *et al.,* 1990). Thus, osteocytes may play a key functional role in the regulation of blood-calcium homeostasis. Osteocytes also regulate phosphate and bio-mineralization through molecules such as PHEX, DMP-1 and FGF-23 (Bonewald 2007), which are expressed by osteocytes (Thompson *et al.,* 2002). Both DMP1 and PHEX appear to down regulate FGF-23 expression, which, in turn, allows reabsorption of phosphate by the kidney, thereby maintaining sufficient circulating phosphate to preserve normal bone mineral content. In the absence of either DMP1*o*r Phex, FGF-23 is elevated in the osteocyte and in the circulation, leading to phosphate excretion by the kidney, thereby reducing circulating phosphate. Therefore, it has been proposed that the osteocyte lacunocanalicular network can function as an endocrine system, targeting distant organs such as kidney (Feng *et al.,* 2006).

1.6.2 The osteocyte as a mechanosensor

Osteocytes reside in a fluid-filled widely spaced lacuna and are connected via cellular processes contained within channels called canaliculi. Osteocytes have been proposed to act as the mechanosensor in bone through their ability to detect the mechanical strain and load generated factors such as fluid flow, streaming and pressure through a connected network (Bonewald 2011). They may also sense load, such as through the cell body, the dendritic processes, or bending of Cilia (Bonwald 2006). It has been proposed that the osteocyte senses load only through its processes (Han *et al.,* 2004) or through both the cell body and the processes (Nicolella *et al.,* 2008). However, a recent study has shown that osteocyte dendritic processes sence mechanical loading, which is likely to be transmitted through the glycocalyx, leading to the opening of hemichannels on the cell body (Burra *et al.,* 2010). This suggests the role of dendrites and the glycocalyx in transducing mechanical signals and in the regulation of the opening of hemichannels (Burra *et al.,* 2010). In addition, it has been proposed that the osteocyte senses load through cilia. Primary cilia are sensory, microtubule-based organelles that grow from the centrosome and project from the cell surface in many vertebrate tissues including bone cells and they might regulate homeostasis in diverse tissues by allowing mechanical signals to alter cellular activity via tissue-specific pathways (Xiao *et al.,* 2006; Malone *et al.,* 2007). Mechanical signals sensed by osteocytes are converted to chemical signals, and the lacuno-canalicular network plays a critical role in conveying these signals to osteoblasts, osteoclasts, and bone-lining cells (Klein-Nulend *et al.,* 2005). Rapid anabolic signals that are released within seconds after loading in oste-

ocytes include nitric oxide (NO), prostaglandins, and other small molecules such as ATP. NO, a short-lived free radical that inhibits resorption and promotes bone formation is generated within seconds in both osteoblasts and osteocytes in response to mechanical strain (Bakker*et al.,* 2001). Primary osteocytes and primary calvarial bone cells have also been shown to release prostaglandins in response to fluid flow treatment, and a number of studies have suggested that osteocytes are the primary source of these load-induced prostaglandins (Ajubi*et al*., 1999). *In vivo* studies have shown that new bone formation induced by loading can be blocked by the prostaglandin inhibitor, indomethacin (Forwood 1996), and agonists of the prostaglandin receptors have been shown to increase new bone formation (Hagino *et al.,* 2005). NO is a mechanical mediator that appears to be released around the same time as PGE2 from osteocytes (Klein-Nulend *et al*., 1995) and endothelial NO synthase is found in osteocytes (Zaman *et al.,* 1999). In bone, NO inhibits resorption and promotes bone formation. Both osteoblasts and osteocytes release NO in response to mechanical strain or fluid-flow shear stress (Bakker *et al.,* 2001). ATP and intracellular calcium can also be released from osteocytes in response to extracellular calcium or mechanical stimulation (Genetos *et al.,* 2005; Kamioka *et al.,* 1995); additionally, osteocytes are considered to be the most sensitive bone cells to mechanical loading because of their distribution throughout the bone matrix and their ability to respond to strain with biochemicals such as nitic oxide and prostaglandins (Skerry *et al.,* 1989). Many studies have also confirmed the rapid changes in metabolic activity of osteocytes after mechanical loading; this confirms the mechanosensory role of osteocytes in bone (Dallas and Bonewald, 2010, Lee *et al.,* 2002).

1.6.3 Bone remodeling

Bone remodelling is a metabolic process, which regulates the structure or remodelling function of bone during adult life (Boyd*et al.,* 2003). Bone modeling is a periodic replacement of old bone by new which is responsible for the complete regeneration of the adult skeleton approximately every 10 years (Manolagas and Weinstein 1999). Bone remodeling confers repair of microarchitectural damage and maintenance of calcium homoeostasis and acid–base balance. It results from the synchronized action of bone cells in the bone remodelling unit (BRU). Bone remodeling occurs in both cortical and cancellous bone but is much greater in surface area within cancellous bone, the process predominates within this compartment (Peel 2009). Bone remodeling occurs via the activity of a functional group of cells called the basic multicellular unit (BMU), which consists of the osteoclasts resorbing bone, the osteoblasts forming bone, the osteocytes within the bone matrix, the bone lining cells covering the bone surface and the capillary blood supply (Kular*et al.,* 2012) and under the control of various circulating factors including calcium, phosphate and parathyroid hormone (PTH) (Henriksen *et al.,* 2009). Bone remodeling starts with an initiation phase which includes the recruitment of osteoclast precursors, then their differentiation into mature osteoclasts as well as activation of bone resorption. This phase is followed by a resorption phase, which starts with the reaction of osteoclasts in response to mechanical or endocrine signals via the engagement of preosteoclastic cells to the location of remodeling. Cytokines secreted by osteoblasts, promote the propagation of these osteoclastic cells along with coordinating the differentiation to multinucleated mature osteoclasts, which further stimulate

bone resorption. This phase is followed by the reversal phase whereby osteoclast cells remove matrix debries before producing signals to initiate transition from bone resorption to bone growth in BMUs (Delaisse 2014). The final stage is bone formation by the osteoblsats known as the termination stage, after comparable quantity of resorbed bone is restored with new stronger bone, the remodelling cycle is concluded (Raggatt *et al.,* (2010) (Fig. 1.4). Mature osteoclasts adhere to bone and remove it by acidification and proteolytic digestion and release minerals, this process is known as resorption, then osteoclasts die by apoptosis after leaving the resorption site. Osteoblast precursors then enter the excavated area and differentiate into osteoblasts, which begin secreting osteoid and are subsequently mineralized to new bone. The lifespan of an osteoclast is two weeks, after which they are replaced by new pre-osteoclasts that originate in the bone marrow and travel via the circulation to the site of resorption. Osteoblasts have a longer lifespan (approximately 3 months). They form most rapidly initially, slowing progressively with some becoming embedded as osteocytes, some dying and the remainder becoming lining cells (Manolagas 2000). Bone is able to avoid damage by minimizing stress (load/area) through modeling and remodeling bone structure, mainly during growth. Signals from damaged areas within mineralized matrix as a result of impact or fatigue loading and damaged osteocytic processes within their canaliculae are transported to the flattened osteoblasts lining the bone surface adjacent to marrow in the case of cortical and trabecular bone, and adjacent to a haversian canal on the intracortical surface.

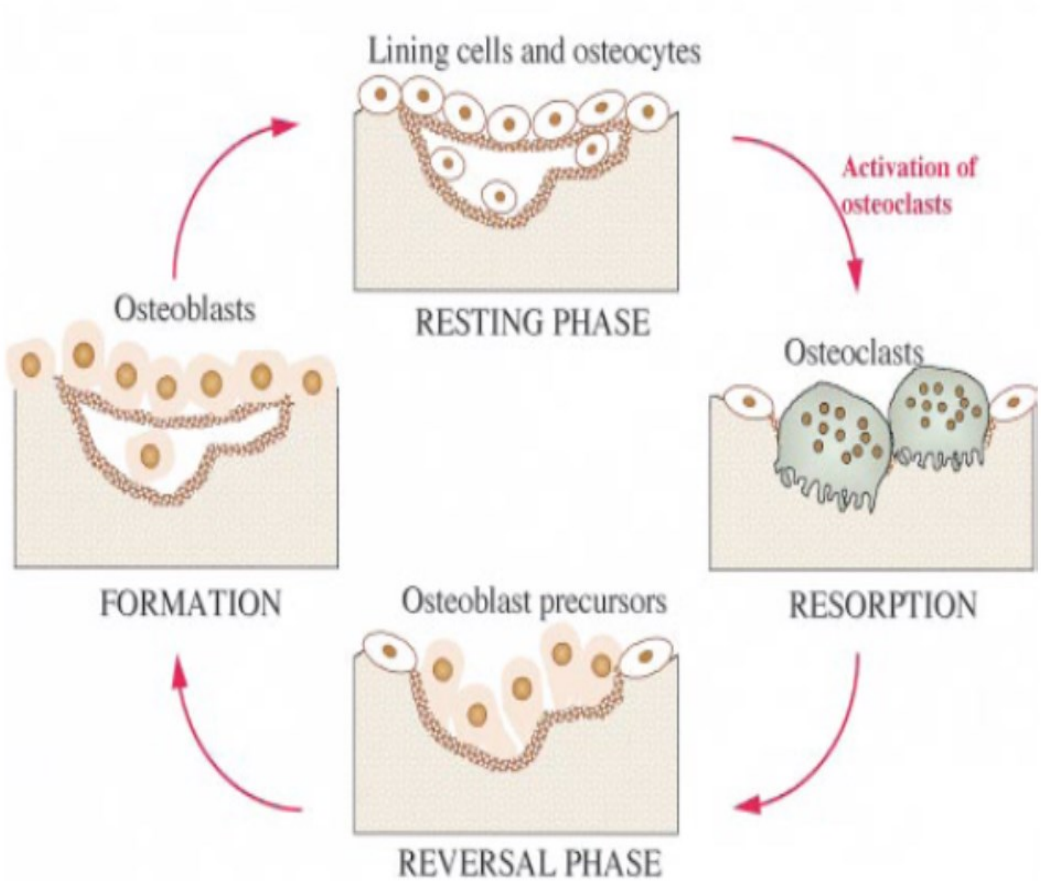

Figure 1.4: The bone remodellingcycle. Bone remodeling cycle begins with attraction of osteoblast precursor which is derived from mesenchymal stem cells to the site of the resorption. Followed by reversal phase whereby osteoclast bone formation is inhibited and osteoclasts undergo apoptosis. The final stage is bone formation by the osteoblats and called termination stage Osteoblast can be buried as osteocyte or remain in the bone surfaceas bone lining cells or undergo apoptosis. (Hill 1998).

Bone develops fatigue damage, the effecter cells are able to detect the damage location to removes and replace it with new bone to restore material composition and micro-/macro-architecture (Parafitt 2002). The resorptive phase of the remodelling cycle removes damaged bone, while extended remodeling suppression using anti-resorptive therapy may result in micro-

damage accumulation, fractures, and reduced bone healing (Odvina 2005). An equal amount of bone will be removed and replaced during the remodeling process in the healthy situation. The newly deposited osteoid undergoes rapid primary mineralization as it is laid down, and then secondary mineralization (enlargement of deposited calcium hydroxyapatite-like crystals) during the next 12 months (Martin *et al.,* 2008).

1.7 Hormone receptors on osteocytes

Parathyroid hormone (PTH) has an intense effect on the skeleton, and its increased level in the circulation can cause both catabolic and anabolic effects on bone depending on the temporal profile of its increase (Bellido*et al.,* 2005). Chronic excess of PTH, as in primary hyperparathyroidism or secondary to calcium deficiency, increases the rate of bone remodeling, and can result in bone loss. In contrast, intermittent PTH elevation, as achieved by daily injections, cause bone gain, and it is the only current bone anabolic therapy. High bone remodeling rates and bone loss with chronic PTH elevation are associated with excessive production of osteoclasts coupled to increased osteoblasts, with imbalance between formation and resorption within each bone multicellular unit (BMU). On the other hand, the main effect of intermittent PTH elevation is a rapid increase in osteoblasts and bone formation, attributed to the ability of PTH to increase proliferation of osteoblast precursors, to reduce osteoblast apoptosis, to re-stimulate lining cells to become matrix synthesizing osteoblasts, or a combination of these effects (Jilka 2007, Kim *et al.,* 2012). PTH has an important role in the viability of osteo-

cytes and the efficiency of cell-cell communication in the osteocytic network (Bivi*etal.,* 2011, Kimmel et al., 2011, Kimmel *et al.,* 2010, Miyauchi *et al.,* 2000). It has been determined that PTH inhibits the expression of the osteocyte specific gene Sost, which is identified as a novel potent negative regulator of bone formation. Consistent with negative regulation of bone formation, overexpression of SOST in transgenic mice produced osteopenia due to reduced bone formation (Winkler *et al.,* 2003). Most importantly, SOST loss-of-function mutations in humans are the cause of the autosomal recessive bone dysplasias Sclerosteosis and Van Buchem disease, which are characterized by massive bone overgrowth throughout life and increased bone strength and serum markers of bone formation (Demiralp *et al.,* 2002). It has been thought that PTH possibly stimulates bone formation via direct actions on osteocytes (Bellido *et al.,* 2005; Keller and Kneissel 2005). Consistent with this idea, expression of parathyroid hormone receptor 1 (PTHR1) has been demonstrated in osteocytes (Fermor and Skerry 1995). It has been reported that activation of PTHR1 signalling exclusively in osteocytes in transgenic mice is sufficient to decrease sclerostin (the protein product of Sost gene) expression, increase Wnt signaling, and increase bone mass (Glass and Karsenty 2007). PTHR1 activation in osteocytes also accelerates the rate of bone remodeling. Deletion of the Wnt co-receptor LRP5 attenuates the high bone mass phenotype induced by the transgene, but does not affect the increased remodeling. Thus, PTH signaling in osteocytes stimulates the increase of bone mass and increases the rate of bone remodeling by LRP5-dependent and independent mechanisms, respectively.Therefore, the skele-

tal effects of PTH and PTHrP may be due in part to the actions of PTHR1 activation in osteocytes (O'Brien*et al.,* 2008).

1.7.1 Estrogen

Estrogen, a well-known steroid hormone is essential to maintain bone health. In addition, mechanical loading, in which estrogen signaling may interact with the Wnt/β-catenin pathway, is essential for bone maintenance (Kondohv *et al.,* 2014). Estrogen's osteo-protective effects are also demonstrated by regulating the life span of osteoclast through osteoclastic and osteoblastic ERα and also inhibiting osteoblast and osteocyte apoptosis (Martin-Millan *et al.,* 2010, Almeida *et al.,* 2010). The effects of sex hormones on bone tissue can be considered as direct effects on bone cells, and indirect effects on other tissues (Imai *et al.,* 2010). The indirect effects of estrogen on bone through other tissues have been well described, such as modulation of cytokine production by immune cells and the increased induction of pituitary gland hormones (Sun *et al.,* 2006, Pacifici 2008). However, the direct effect of estrogens on bone tissue is not fully understood. ERα in osteocytes was found to play an important role in maintaining bone mass by regulating osteoblastic bone formation only in females and supports maintenance of trabecular bone mass not only under normal loading conditions but also under tail suspension-induced unloading, which can be considered as experimental recapitulation of immobilization or space flight (Lee *et al.,* 2003). However, these results show that, the absence of this receptor protected against cortical bone loss and bone mass adaptation induced by mechanical loading was impaired

in ERα total KO mice (Lee *et al.,* 2003). Together, these results indicate that osteocyte mechanosensation may, at least in part, occur via osteocytic ERα. Osteocytic ERα might play a role in estrogen's osteoprotective action by controlling the expression of Wnt antagonists, which regulate osteoblastic bone formation in trabecular bone (Kondoh *et al.,* 2014).

Almost all osteocytes in the rat tibia express ERα (Braidman *et al.,* 2000). Osteocytes in the cortical bone of the midshaft express significantly higher levels per cell than those in the trabeculae of the metaphysis and epiphysis (Bord*et al.,* 2001). Ovariectomy (OVX) is associated with a decrease of ERα per osteocyte in the cortex, where levels are initially high, and has no effect in the secondary spongiosa where initial levels are low. Strain has only a small but positive correlation with the level of ERα per osteocyte (Ehrlich *et al.,* 2002). Strain as well as estrogen stimulates transient translocation of ERα to the nucleus. Strain but not estrogen also induces isolate membrane localization of ERα. Bone cells response to strain and estrogen involve ERα, but only estrogen regulates its cellular concentration. Bone loss associated with estrogen deficiency, is a consequences of decreased number and activity of ERα which is associated with lower estrogen concentration reducing the anabolic response of bone to strain (Zaman *et al.,* 2006). Generally, ERα orchestrates most actions of estrogen on bone cells (Barkhem *et al*., 1998, Hall and McDonnell1 999). In vitro studies conducted have suggested that estrogen's bone-sparing effects are mediated by both estrogen and androgen receptors (Kousteni *et al*., 2001). The mechanism in which information is transduced from ligand-bound receptors had been thought to be via the binding of ligands to the ERs causing a conformational change leading to recep-

tor dimerization and binding to specific DNA sequences known as estrogen response elements (EREs) (Smith and O'Malley 2004). The formation of a complex along with co-activator proteins results in the activation of general transcriptional machinery and leads to increased expression of target genes via chromatin remodelling. In addition, ERs also have the ability to recruit co-repressors, which play a role in negatively regulating ER-dependent gene expression. Furthermore, other mechanisms of activation besides the traditional mode of gene activation have been stipulated that are responsible for the ability of estrogen in the stimulation and repression of gene expression encoding critical osteoclastogenic factors such as IL-6, TNF-α, and M-CSF. For instance, the activated ERs have the ability to bind to transcription factors such as NF-κB and therefore, blocking binding to DNA, which demonstrates in which how estrogen represses IL-6 production (Steinand Yang 1995). Similarly, estrogen exerts its effects on many families of kinases. For example, casein kinase 2 (CK2) activity has been known to be decreased by estrogen resulting in reduced phosphorylation of the nuclear protein Egr-1. Dephosphorylated Egr-1 has increased affinity for the transcriptional activator Sp-1, a factor critical for expression of the MCSF gene (Srivastava *et al*., 1998). During estrogen deficiency the formation of aEgr-1/Sp-1 complex occurs, causing a decrease in the nuclear level of free Sp-1 and thus reducing MCSF transcription. Moreover, Estrogen also has the ability of reducing JNK activity. The decreased production of activator protein 1 (AP1) factors explains the repressive effects of estrogen on TNF gene expression (Srivastava*et al*., 1999) as well as why estrogen decreases the sensitivity of maturing OCs to the osteoclastogenic factor receptor activator of NF-κB (RANK) ligand

(RANKL) (Srivastava *et al*., 2001). Although many estrogenic effects are mediated by nuclear ERs, some responses initiate in the plasma membrane. In fact, estrogen produces rapid effects in various cell types, including in bone cells. These nongenomic actions are due to signaling by a membrane receptor. The ability of estrogen to induce OC apoptosis and inhibit OB apoptosis is linked to its ability to increase ERK1 and ERK2 phosphorylation and inhibit JNK activity (Kousteni*et al*., 2001, Kousteni*et al*., 2003). The phosphorylation of these cytoplasmic kinases and their transport to the nucleus modulate the activity of transcription factors required for antiapoptotic actions of estrogen (Kousteni*et al*., 2003).

1.8 Apoptosis

Apoptosis is programmed cell death characterized by morphological changes, which include cytoplasmic shrinkage, nuclear chromatin condensation, and DNA fragmentation into apoptotic bodies (Fig.1.5). The content of dying cells is eliminated without inflammatory response within the body (Kerr 2002). In contrast, necrosis is a cell death characterized by a chromatin flocculation, loss of membrane integrity, and a general local inflammatory response (Kerr *et al.,* 1972).

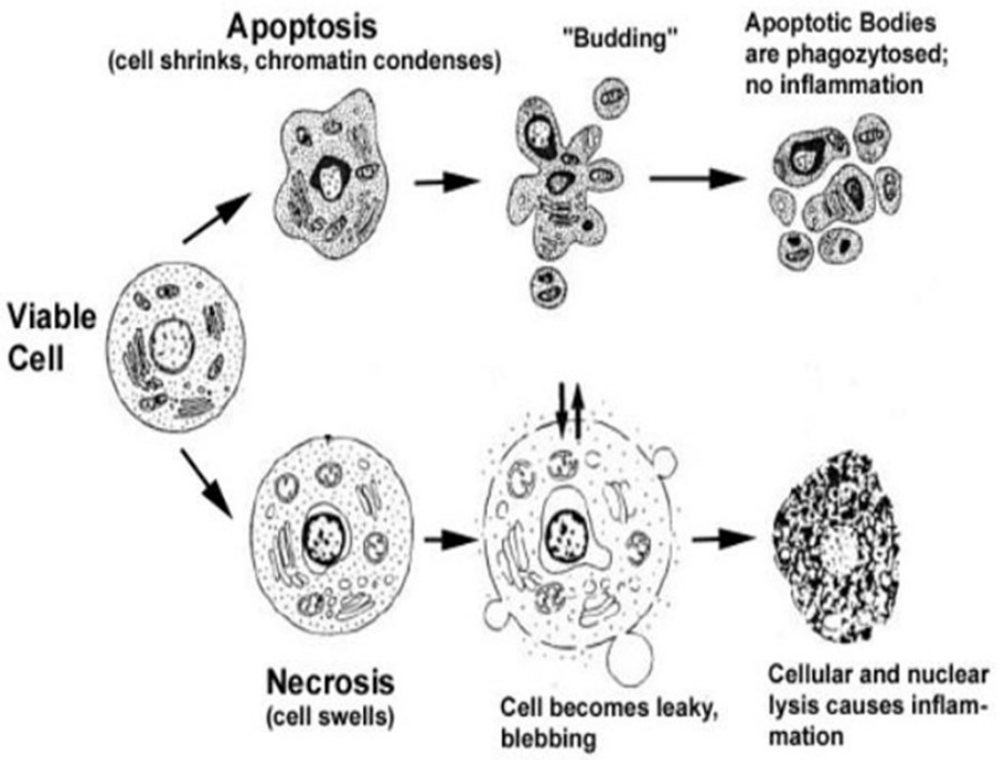

Figure 1.5: Schematic diagram illustrating the basic differences between apoptotic and necrotic cells. The onset of apoptosis is characterized by cellular shrinking, chromatin condensation and margination at the nuclear periphery with the formation of membrane-bound apoptotic bodies that contain organelles, cytosol and nuclear fragments are phagocytosed without triggering inflammatory processes. The Signs of early necrosis in an irreversibly injured cell include clumping of chromatin and gross swelling of organelles. At a later stage, membranes rupture and the cell disintegrates (Gewies 2003).

1.9 Bcl-2 family and mitochondrial role in apoptosis

The Bcl-2 family (B cell leukemia/lymphoma 2-like proteins) is a main regulator of apoptotic events regulated by mitochondria and is a member of a growing gene family consisting of two subfamilies that can inhibit (pro-survival) or promote (pro-apoptotic) apoptosis (Marsden *et al.,* 2003). The interaction between pro-apoptotic and pro-survival proteins plays an important role in

determining the final ending (death or survival). Bax is a pro-apoptotic member of the Bcl-2 family which acts as a death receptor and exists in both cytoplasm and mitochondria and during apoptosis a substantial fraction of the cytoplasmic Bax moves to the mitochondria to form dimmers (Tsujimoto 1998). Previous studies have shown that the Bcl-2 (pro-survival)-to-Bax (pro-apoptotic) ratio in the cell determines initiation of cell apoptosis or alternatively, re-enters the cell cycle (Delpoeta*et al.,* 2003). This was represented by the finding that over expression of Bcl-2 (therefore, a higher Bcl-2: Bax ratio) permitted for continued cellular proliferation through antagonism of apoptotic signals transported by the pro-apoptotic Bax, as seen in the continuous growth and division of malignant cells (Zhang and Insel 2001).

1.10 Caspases (Cysteine Aspartate Protease)

Caspases are a family of genes important for maintaining homeostasis through regulating cell death and inflammation (McIlwain.*et al.,* 2013). Apoptotic caspases are divided into two classes: initiator and executioner caspases. Initiator caspases (caspase 2, caspase 8 and caspase 9) are the important caspases in signaling cascades and their activation is normally required for executioner caspase (caspase 3, caspase 6 and caspase 7) activation. The content of initiator caspase substrates is limited and includes self-cleavage, Bcl-2 homology 3 (BH3)-interacting domain death agonist (BID) and executioner caspases. Executioner caspases cleave hundreds of different substrates and are mostly responsible for the observable characteristic changes seen during apoptosis. Initiator caspase activation first involves

dimerization of inactive caspase monomers. In the case of caspase 8, its monomers are recruited following death receptor ligation, and dimers formation through their pro-domains to the adaptor molecule FAS-associated death domain protein (FADD). Dimerization and interdomain cleavage are required for the activation and stabilization of mature caspase 8 (Stephen *et al.,* 2010).

1.11 Death receptors

Death receptors are part of a family of tumour necrosis factor receptor superfamily (Bhardwaj and Aggarwal 2003). Eight members of the death receptor-family have been recognized, Fas, TNFR1, or the TRAIL receptors DR4 and DR5 are implicated in diverse diseases. Death receptor of the Fas/tumor necrosis factor receptor (TNF) family is transmembrane protein characterized by cytoplasmic death domain (DD) of 65-80 amino acids. The death receptor family comprises TNFR1, death receptor (DR3), (APO-3/TR-MP/WSI/LARD), DR4 (TRAIL), DR5 (TRAIL-R2/TRICK2/killer) DR6 and CD95 (APO-1Fas) (Ashkenazi 1999). All death receptors have the ability to induce apoptosis when ligated by their similar ligands. Therefore, ligand binding of the Fas receptor triggers receptor trimerization and aggregation of the intracellular death domains. These trigger interaction with C-terminal DD of the adaptor molecule FADD which recruits caspase-8 and creates Fas-FADD-Caspase-8 complex (Fig. 1.6) (Boldin *et al.,* 1996). Although Caspase-8 is characterized as a major up-stream caspase effecter of death receptor signaling, some evidence indicates that it is also recruited by other apoptotic signals and impli-

cated in detachment- induced apoptosis. However, the exact mechanism of its activation is unclear as it is inhibited not only by a dominant negative FADD, suggesting death receptor involvement, but also by Bcl-2, suggesting mitochondrial involvement and does not require downstream caspase -3 or -9(Rytomma*et al*., 1999).

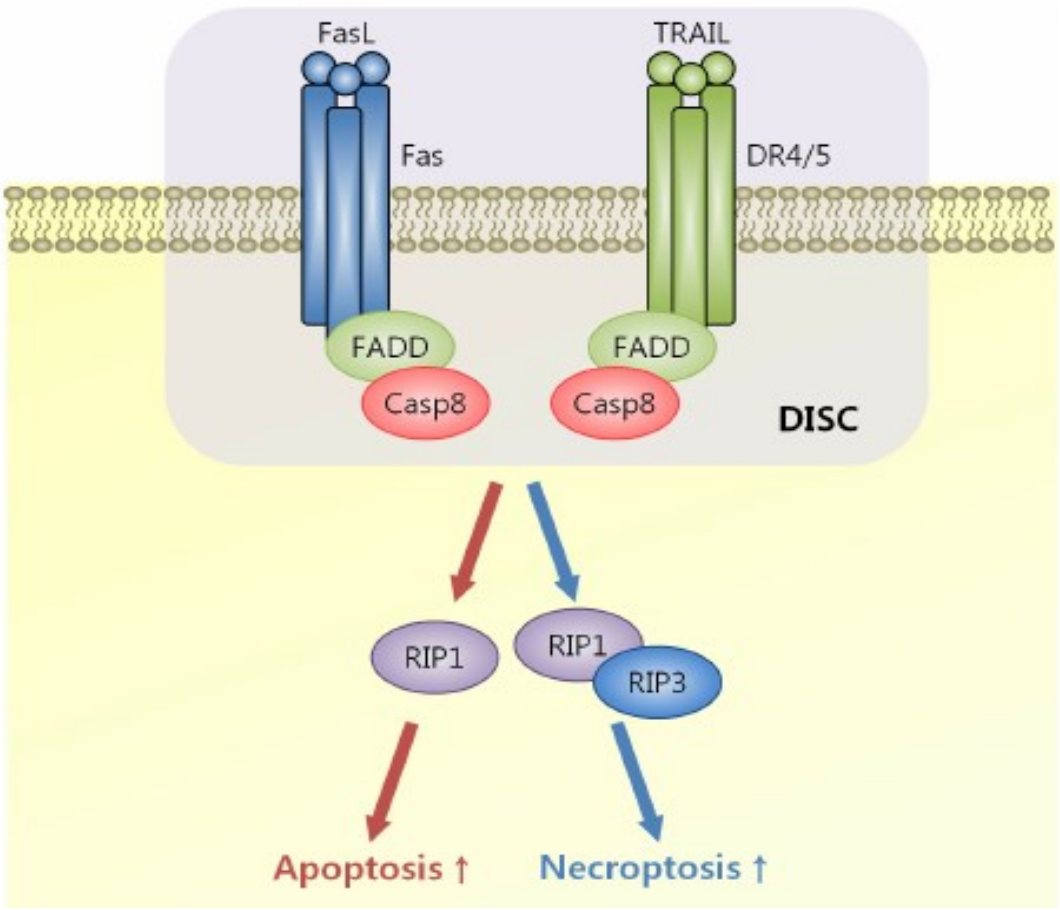

Figure 1.6: The role of FADD in Fas and TRAIL signaling. Fas and TRAIL-R are activated by FADD, an essential adaptor protein that links death receptor for FasL or TRAIL to caspase-8, thereby inducing DISC formation. These complexes have a capability to induce both apoptosis and necroptosis (Lee *et al.,* 2012).

1.12 Osteocyte Apoptosis

Osteocyte death occurs as a result of ageing, after the menopause, reduced bone loading and due to pathological conditions such as microcracks and the death of osteocytes is strongly associated with bone resorption (Noble and Reeve 2000; Cardoso et al., 2009; Emerton *et al.,* 2010). Osteocytes die through one of three ways, apoptosis, autophagic cell death and necrosis (Bursch2001), (Fig. 1.7). It has been suggested that apoptotic bodies derived from osteocytes, independent of pro-osteoclastogenic factors, are associated with efficient osteoclastogenic activity capable of bone local resorptive activity (Kogiani *et al*., 2008). Bone fragility is thought to be as a result of failure of apoptotic osteocytes to sense microdamage and subsequently they fail to signal to the bone surface for repair (Noble 2008; Noble 2000; Noble *et al.,* 2003, Manolagas 2010; Manolagas 2000). Osteocyte apoptosis also occurs in association with the area surrounding a microcrack leading to remodelling and repair of the damaged area (Noble *et al.,* 2003). In contrast to overloading, which induces microdamage and microcracking, a physiological level of load on bone *in vivo* may inhibit apoptosis in the osteocyte network (Noble. *et al.,* 2003). On the other hand, lack of mechanical loading or disuse can lead to apoptosis of osteocytes by oxygen deprivation, especially if associated with immobilization (Aguirre *et al.,* 2006; Basso and Heersche 2006). It appears that mechanical stimulation provides essential oxygen levels to keep osteocytes viable (Gross *et al.,* 2005). Thus, it appears that apoptotic osteocytes disrupt the integrity of the osteocyte network, this disruption functions as a signaling mechanism for the bone to initiate repair and adaptation to mechanical strain resulting from mechanical stimulation. Age-

ing results in accelerating osteocyte death through a mechanism associated with oxidative stress, as evidenced by a reduction in antioxidant enzymes, and phosphorylation of oxidative stress markers, p53 and p66Shc in bone from ageing mice (Almeida *et al.,* 2007). One of the characteristics of osteocyte apoptosis is glucocorticoids- induced bone disease in human and mice (Noble 2000). The pro-apoptotic effect of the steroids, mediated via the glucocorticoid receptor, is independent of gene transcription and results from the activation of the focal adhesion related kinase Pyk2/JNK signaling pathway (Poltkin *et al*., 2007). This leads to cell detachment-induced apoptosis. In addition, the action of glucocorticoid on osteoblasts might induce osteocyte apoptosis. This includes the local suppression of the synthesis of survival factors such as insulin-like growth factor 1 (IGF-1), interleukin 6 (IL-6), matrix metaloproteinases (MMPs) (Canalis and Delany 2002) and the induction of the Wnt antagonist SFRP- 1 (Wang *et al*., 2002). The effect of glucocorticoids has also been associated with increased oxidative stress (Almeida 2011; Jia *et al.,* 2011) and contributes to the increase in reactive oxygen species and osteocyte apoptosis during ageing. Both estrogens and androgens prevent osteocyte apoptosis (Kousteni *et al.,* 2002). Similar effect has been demonstrated with the selective estrogen receptor modulator (SERM) (Van *et al.,* 2007). The effect of the sex steroids can be exerted via activation of estrogen receptor is independent of gene transcription (Kousteni *et al.,* 2001).

Apoptotic osteocytes show development of osteoclastogenesis *in vitro* (Kogianni *et al.,* 2008; Al-Dujaili *et al.,* 2011; Cheung *et al.,* 2012). Studies conducted have shown that osteoclastogenesis can be induced by addition of

osteocyte apoptotic bodies (OABs) to the surface of bone-forming areas in murine calvariae *in vivo* and also the potential for OABs to promote osteoclastogenesis *in vitro* after their introduction to bone marrow–derived osteoclast precursors (OPs) in the absence of pro-osteoclastogenic factors RANKL and macrophage-colony stimulating factor (M-CSF) (Kogianni *et al.,* 2008).

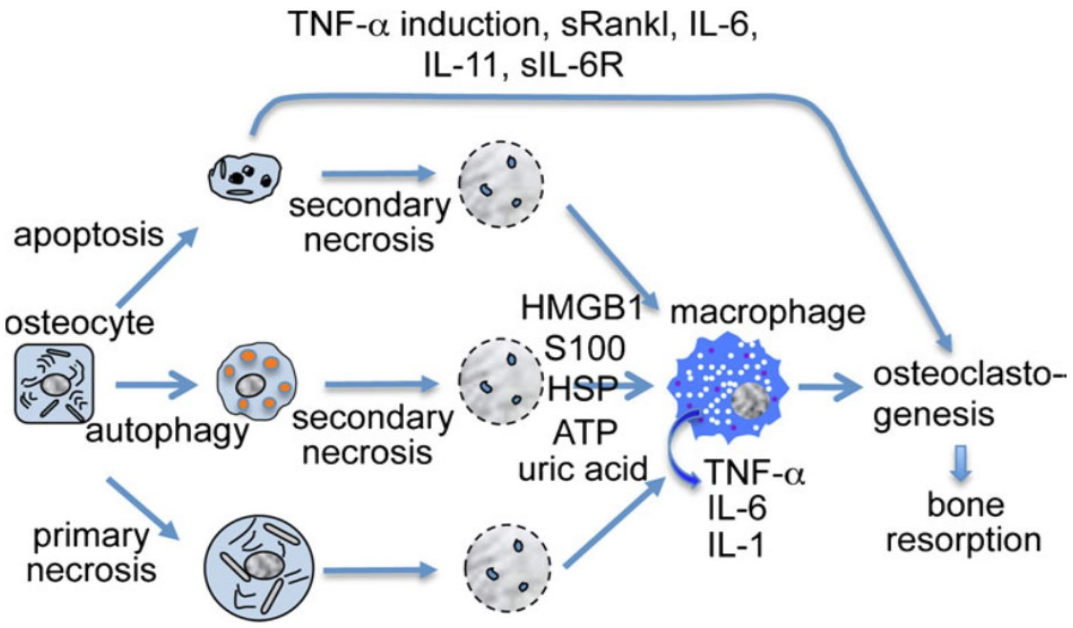

Figure 1.7: **Osteocytes die mainly through one of three pathways:** apoptosis, autophagic cell death and necrosis During the terminal phase of apoptosis autophagic cell death, osteocytes which are not engulfed by phagocytes undergo secondary necrosis. Immunostimulatory molecules including high-mobility group box 1 (HMGB1), S100, heat shock protein (HSP), adenosine triphosphate (ATP) and uric acid are released from lacunae through canaliculi to the bone surface and vascular channels,where they assist macrophages activation, thus promoting the production of pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α), interleukin 6 (IL-6) and interleukin1 (IL-1), which induce osteoclastogenesis and bone resorption. Apoptotic osteocytes release osteoclastogenesis-promoting factors, including soluble receptor activator of nuclear factor κ-B ligand (sRankl), IL-6 and soluble IL-6 receptor (sIL-6R) and induce TNF-α expression in osteoclast precursors *in vitro* (Komori 2013).

1.12.1 The role of osteocytes in bone disease

Osteocytes communication network can detect mechanical load and micro-damage. Drugs and bone active hormones control the bone strength and integrity of this network through the regulation of osteocyte cell death (Plotkin et al., 2005). Osteocyte apoptosis has a critical role in determination of bone strength, as osteocyte apoptosis is the main feature of conditions of increased bone fragility (Manolagas 2006). Osteocyte cell death can occur in association with some pathologic conditions such as osteoporosis and osteoarthritis, leading to increased skeletal fragility (Weinstein *et al.,* 2000). Such fragility is considered to be due to loss of the ability to sense microdamage and/or signal repair. Oxygen deprivation that occurs as a result of immobilization has been shown to promote osteocyte apoptosis (Dodd *et al.,* 1999). In addition, glucocorticoid treatment (Weinstein *et al.,* 2000) and low levels of estrogen that result in bone loss is thought to be the product of the imbalance between bone formation and resorption, this is possibly caused by increased osteoclast survival, as estrogen promotes osteoclast apoptosis (Tomkinson *et al.,* 1997 and Emerton *et al.,* 2010). Tumour necrosis factor-α (TNF-α) and interleukin 1 (IL-1) have been reported to increase with estrogen deficiency and also induce osteocyte apoptosis (Bonewald *et al.,* 2007). Studies on fluid shear stress in osteocytes, in which the mechanical bone loading was mimicked by applying pulsating fluid flow, the TNF-α induced osteocyte apoptosis was inhibited by mechanical loading. However, this effect was just observed in osteocytes but not osteoblasts. Since apoptotic osteocytes attract osteoclasts, these results indicate that osteocyte apoptosis

plays a key role in osteoclastic bone resorption, which is in part modulated by TNF-α (Tan *et al.,* 2006).

1.13 Osteoclast apoptosis

The number and activation of osteoclasts play a critical role in the determination of the balance between bone formation and resorption (Manolagas 2000). Osteoclast number is dependent upon relative rates of cell differentiation and death. Osteoclast differentiation *in vitro* is dependent on at least two extracellular factors, macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL). Other factors, such as IL-1, IL-6, TNF-α, and TGF-β, also affect osteoclast differentiation (Teitelbaum and Ross 2003). Osteoclast apoptosis has been reported to be induced by some agents such as estrogen, tamoxifen and some bisphpsphonate molecules, however the mechanism is not fully clear (Boyce *et al.,* 2002). Bisphosphonates induce apoptosis in osteoclast-like cells expressing the Fas gene, thus implicating Fas in osteoclast apoptosis (Wu *et al.,* 2003). Estrogen has been reported to directly targets RANKL-induced osteoclast differentiation by repressing c-Jun activation and that this response was ER dependent (Shevde, *et al*., 2000) or through the production of transforming growth factor beta (TGF-β) produced by osteoblasts (Hughes *et al.,* 1996). Estrogen regulates the life span of osteoclast through upregulation of Fas ligand (FasL) expression in osteoclasts of the trabecular bone of mice. The expression of ERα was also required for the induction of apoptosis by tamoxifen and estrogen in cultured osteoclasts. These results are a possible

explanation for the osteoprotective function of estrogen as well as SERMs (Nakamura *et al.,* 2007).

1.14 Osteoblast apoptosis

Osteoblasts undergo an orderly developmental progression that finally ends in apoptosis. It is still unclear what signal transduction pathway is used in programmed cell death in osteoblasts. Glucocorticoid treatment and estrogen withdrawal promotes apoptosis in osteoblasts and osteocytes (Tomkinson *et al.,* 1997and 1998, Weinstein *et al*., 1998). Osteoblast apoptosis is in part responsible for osteoporosis in sex steroid deficiency, glucocorticoid treatment, and ageing, however overexpression of Bcl-2 in osteoblasts inhibits osteoblast differentiation, reduce osteocyte processes, and causes osteocyte apoptosis (Moriishi *et al.,* 2011). The Fas/FasL death receptor pathway has been implicated in human and murine osteoblast apoptosis through interaction with T cells (Kawakami *et al*. 1997) or exposure to pro-inflammatory cytokines such as TNF-α, IFN-γ (Ozeki *et al.* 2002). Inorganic phosphate acts as local apoptogenes for osteoblasts, which is enhanced by increasing Ca^{2+} concentration (Adams *et al.,* 2001). Apoptosis of mature osteoblasts is influenced by cytokines such as IL-6 and TGF-β (Jilka *et al.,* 1998), but survival and differentiation of osteoblast into osteocyte was dependent on matrix metalloproteinase activity, which is activated by p44/p42 MAPK pathway (Karsadal *et al.,* 2002).

1.15 Oxidative stress and its role in promotion of osteoporosis

The activity of reactive oxygen species, a by-product of aerobic respiration plays an important role in bone turnover particularly in bone resorption (Östman*et al.,* 2009). The most common theory of ageing states that oxidative stress is resulting from an increase in intracellular ROS which is the major determinant of ageing (Giorgio *et al.,* 2007, Lu and Finkel 2008), as well as the cause of several degenerative disorders associated with ageing (Balaban *et al.,* 2005). ROS formation occurs primarily in the mitochondria from the escape of electrons passing through the electron transport chain during aerobic metabolism, the process that is supported by nutrients such as glucose and is responsible for the formation of ATP (Giorgio *et al.,* 2007, Newmeyer and Ferguson-Miller 2003). ROS are also generated during fatty acid oxidation or in response to external stimuli, such as inflammatory cytokines, growth factors, environmental toxins, chemotherapeutics, UV light, or ionizing radiation. Free electrons are added to molecular oxygen to generate superoxide (O2-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH-). Among these species, H_2O_2 has the highest oxidative activity and the highest stability. As a result of its properties, H_2O_2 performs a critical role in signaling for the replicative capacity of regenerative cells, apoptosis, and changes in gene expression leading to ageing and ageing-related diseases (Giorgio *et al.,* 2007). The production of H_2O_2 is amplified considerably by the adapter protein p66shc, which is released from an inhibitor complex in the inner mitochondrial membrane in response to a variety of pro-apoptotic stimuli and acts as a redox enzyme catalyzing the reduction of O_2 to H_2O_2 through electron transfer from cytochrome C (Giorgio et al., 2005; Camiciet *al.,* 2007). H_2O_2 in

turn causes opening of the permeability transition pore, swelling, and apoptosis. Deletion of the p66shc gene enhances cellular resistance to apoptosis induced by H_2O_2 or UV light, and mice deficient in p66shc not only exhibit increased resistance to oxidative stress but also have an increase in lifespan (Migliaccio*et al.,* 1999). Oxidative stress is characterized by increased level of reactive oxygen species (ROS) which is involved in bone homeostasis and affects bone remodelling by promoting bone resorption. Many studies have suggested the role of oxidative stress in bone diseases such as osteoporosis, diabetes-induced bone diseases and vascular diseases.

Oxidative stress is a physiological or pathophysiological condition which increases with age and results in deregulation of redox balance in tissues, characterized by an excess formation of free radicals. These free radicals are implicated in the apoptosis of osteoblasts, osteocytes and also in osteoblastogenesis, therefore in bone resorption as shown *in vivo* and *in vitro* studies (Ostman*et al.,* 2009), through the activation of a protein kinase C (PKC) β/P66shc/NF-kB signaling cascade. P66shc is an essential mediator of the stimulating effect of H_2O_2 on the apoptosis of osteoblastic cells as well as being able to activate NF-kB (Almaida *et al.,* 2010). ROS can either have beneficial or harmful effects on tissues depending on concentration and have been known as a major contributor to the immune response (Bogdan 2001) and are involved in defence mechanism during inflammatory responses as well as cell signaling. It has been shown that H_2O_2 in bone, oxidizes those proteins which are involved in cell differentiation and modulates their activity, either by inhibition or stimulation which includes signaling pathways involved

in bone cell differentiation, mitogen-activated protein kinases (MAPKs), Wnt/b-catenin and NF-kB (Lee *et al.,* 2005).

ROS have been reported to play an important role in ageing associated complications (Krause 2007) and development of different types of degenerative diseases including cancer, cardiovascular disease, ischemia–reperfusion injury, arthritis, diabetes and neurological disorders (Valko *et al*., 2007).

ROS are also involved in defence mechanisms during inflammatory responses and generating cell signals. It has been suggested that lower concentration of ROS induce mitogenic response, whereas higher concentrations would induce cell death (Bergamini*et al*., 2004). ROS are produced abundantly in mitochondria and its production is regulated by many complex enzymes such as cyclo-oxygenase, NADPH oxidase and cytochrome P450 (Kadenbach*et al*., 2009). It has been reported that ROS are involved in osteoclastogenesis regulation induced by RANKL (Lee *et al*., 2005) and in the apoptosis of osteoblasts/osteocytes, therefore influencing bone resorption (Almeida *et al*., 2007). This has been shown *in vitro* and in *in vivo* animal studies (Garrett *et al*., 1990). Wnt/β-catenin are widely used molecules in osteoblast function and bone formation. The accumulation of β-catenin in the cytoplasm is promoted through Wnt signalling by preventing its degradation. β-catenin is translocated into the nucleus and is involved in the expression of osteoblastic markers hence, osteoblast differentiation (Manolagas and Almedia, 2007). However, in conditions of oxidative stress, as induced by the lack of estrogen and ageing, Wnt-induced osteoblastic gene expression is inhibited through a mechanism which involves Forkhead box-O (FOXO)

transcription factor, and results in osteoblast and osteocyte apoptosis (Manolagas and Almedia 2007).

In vivo experiments support the role of ROS in age-related osteoporosis. In ageing mice, male and female mice showed decreased bone formation rates, whereas ROS levels were up regulated, this might depend on antioxidant defence failure and that the same changes in oxidative stress were reproduced by gonadectomy (Almeida *et al*., 2007). In addition to ageing, ovariectomy induces oxidative stress in rat femurs with a decreased activity of antioxidant systems (Almeida *et al*., 2007; Muthusamiet *al*., 2005). It has been demonstrated that RANKL-induced osteoclastogenesis is regulated by ROS production (Lee *et al.,* 2005). RANKL binds to its receptor, RANK at the surface of pre-osteoclasts. This binding has been shown to induce differentiation of pre-osteoclast into mature osteoclast and induce bone resorption (Fig. 1.8). On the other hand OPG, the decoy receptor for RANKL produced by osteoblasts, prevents RANKL from binding to RANK on the surface of osteoclast, thus inhibiting osteoclast differentiation and suppressing apoptosis induction (Theoleyre *et al.,* 2004).

Similarly over-expression of glutathione peroxidase 1 (Gpx1), one of the main antioxidant enzymes produced by osteoclasts, is responsible for the degradation of hydrogen peroxide as well as the inhibition of RANKL-induced osteoclastogenesis (Lee *et al*., 2005). This suggests that H_2O_2 plays an important role in the formation of osteoclasts. These changes in glutathione, influenced by increased ROS, contribute to decreased rate of remodelling and increased osteoblast/osteocyte apoptosis.

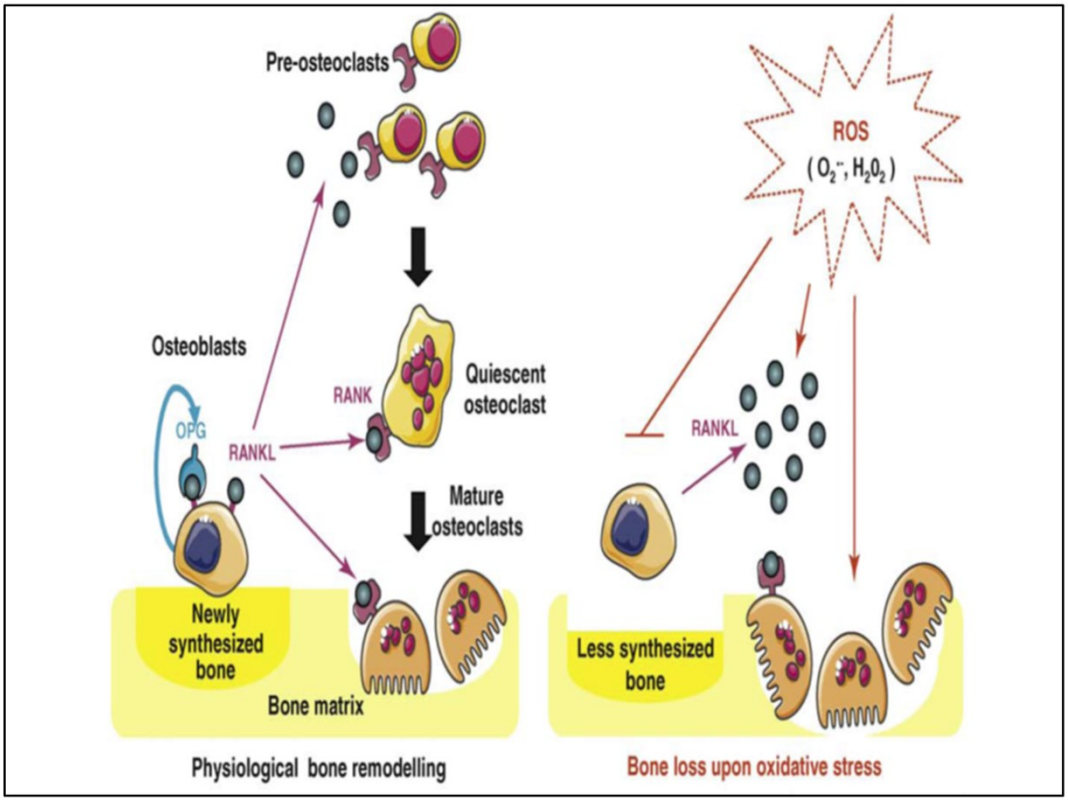

Figure 1.8: Bone remodelling and oxidative stress. In the presence of the receptor activator of NF-kB ligand (RANKL), expressed by osteoblasts RANKL binding to the receptor RANK at the surface of pre-osteoclasts stimulates cell fusion, activates resorption capabilities and enhances cell survival. OPG, a decoy receptor for RANKL, prevents osteoclast differentiation As a result of oxidative stress this bone coupling is unbalanced, and bone formation by osteoblasts is reduced, whereas osteoclast differentiation and activities and subsequent bone resorption are enhanced directly or indirectly through an increased RANKL production (Wauquier *et al.*, 2009).

Studies in rodent bone marrow-derived osteoclasts have shown that 17β-estradiol stimulates the expression of Gpx1; whilst estrogen deficiency results in the ROS-mediated expression of TNF-α and induce bone loss by lowering thiol antioxidants in osteoclasts and ultimately bone resorption (Lean *et al.*, 2003). Also CAT (another antioxidant enzyme) administration prevents the bone loss caused by ovariectomy and the effect of CAT on bone loss also has further significance. CAT, unlike hydrogen peroxide, is unable

to diffuse across cell membranes. Therefore, suppression of bone loss by CAT is due to degradation of H_2O_2 in the extracellular space. This suggests that H_2O_2 causes bone loss through a paracrine or autocrine action (Lean *et al*., 2005).

H_2O_2 is also known to induce inhibition of osteoblastic differentiation in primary mouse BMSCs (Liu *et al.,* 2004), which is characterized by the reduction of ALP. Similar results have been shown in term of suppression of osteoblastic differentiation marker when primary bone marrow stromal cells from rabbit BMSCs were treated with H_2O_2 (Bai *et al.,* 2005). This included, the nuclear phosphorylation of Runx2 (a transcription factor), ALP, colony- forming unit-osteoprogenitor (CFU-O), and type I collagen. Many studies have reported that ROS has a direct effect on the life span of osteocytes, and induce apoptosis in the same way as mentioned above and leading to bone loss (Bonewald 2011).

1.16 Osteoporosis

Osteoporosis is a systemic skeletal disease characterized by decreased bone strength and an increased risk of fractures (NIH Consensus 2001). It is a chronic disease of bone, characterized by reduced bone mass and micro-architectural deterioration of bone tissue, leading to increased bone fragility and resulting in an increase fracture risk (Ray *et al.,* 1997). Osteoporosis is also associated with the normal ageing process and is a result of imbalance between the activity of osteoblasts and osteoclasts, ultimately leading to

bone loss (Dempster1995). Primary osteoporosis mostly occurs in postmenopausal women due to estrogen deficiency that affects circulating levels of cytokines such as IL-1, TNF-α, granulocyte macrophage colony stimulating factor (GM-CSF) and IL-6. The level of theses cytokines increases because of decreased estrogen levels, thus enhancing bone resorption by increasing differentiation and activation of osteoclasts (Horowitz 1993, Manolagas 1995). This process is mediated by RANKL, produced from osteoblasts and other cell types, leading to increased bone resorption (Eghbali *et al.,* 2003). Secondary osteroporosis occurs in men and women older than 75 years, but could also be induced in younger individuals by various diseases or by continual use of certain medications, such as corticosteroids. Secondary osteoporosis is the result of other medical complications, changes in physical activity or as a consequence of therapy for certain diseases (NIH Consensus 2001). Osteoporosis is defined as a bone mineral density (BMD) 2.5 standard deviations or more below the mean peak BMD for healthy adults, as measured by dual energy X-ray absorption (DXA) (World Health Organization 1994). Besides hip fractures, other debilitating fractures such as compression fractures of a vertebra are severe consequences of osteoporosis. In addition, several other types of bone fractures such as Colles'fractures are seen at highly elevated rates in osteoporotic patients. Osteoporosis is a mutifactorial disease with potential contributions from genetic, endocrine functional, exercise related and nutritional factors (Aaseth *et al.,* 2012).

1.16.1 Osteoporosis and estrogen

Bone fracture incidence increases clearly in women during the first decade of post menopause; this has been attributed to the rapid loss of bone mass and trabecular architecture that occurs with a decline in estrogen production associated with menopause (Raisz 2001).

Estrogens exert a protective action in maintaining bone health by increasing osteoclast apoptosis (Kameda *et al.,* 1997) and decreasing cytokines, which promote osteoclast activity (Pacifici*et al.,* 1996). It has been proposed that osteocytes may regulate bone remodeling activation via connection with bone lining cells (Bonewald 2007); therefore it is likely that osteocytes mediate the anti-remodeling effects of estrogen. Estrogen deficiency is associated with increased osteocyte apoptosis in humans (Tomkinson *et al.,* 1997). Also estrogen is thought to have a great impact on skeletal mass and calcium metabolism, suppresses both directly, and indirectly bone resorption (Khosla *etal.,* 2011) and its acute effect is blocking the new osteoclast formation. In addition, estrogen modulates RANK signaling in ostoclastic cells through the suppression of receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF)-induced differentiation of myelomonocytic precursors into multinucleated osteoclasts (Srivastava *et al.,*1999; Shevde*et al.,* 2000) and induces osteoclast apoptosis (Nakamura *et al.,* 2007, Martin-Millan*et al.,* 2010). In term of bone formation, estrogen promotes osteoblast differentiation from mesenchymal stem cells (Okazaki *et al.,* 2002; Dang *et al.,* 2002), prolongs osteoblast life span (Kousteni*et al.,* 2001) and increases the expression of osteoblast proteins such as (type 1 collagen, IGF and bone morphogenic protein-6) (Khosla *et al.,* 2010) and inhibits osteoblast and os-

teocyte apoptosis (Kousteni*et al.,* 2001).

Estrogen suppresses osteoclast activity by stimulating osteoclast cell apoptosis (Hughes *et al.,* 1996) and by reducing osteoblast, T cell and B cell expression of RANK-L (Eghbali-Fatourechi*et al.,* 2003). In addition to having direct effects on the osteoblasts and osteoclasts, estrogen inhibits inflammatory cytokine production from bone marrow stromal and mononuclear cells (Kitazawa *et al.,* 1994; Jilka*et al.,* 1992). This is an important bone protective-mechanism because inflammatory cytokines such as interleukin (IL-1, IL-6) and TNF-α are known to be potent stimulators of osteoclastogenesis (Strand and Kavanaugh 2004). Taken together, estrogen plays an anabolic role in the skeleton, and the decline in estrogen levels during menopause is correlated with loss of bone mass and increased risk of fracture (Marcus *et al.,* 1994). However, hormone replacement therapy (HRT) is able to reduce bone loss and fracture risk in postmenopausal women (Blank and Bockman, 1999).

1.17 Current treatments for osteoporosis

1.17.1 Calcium & Vitamin D

One of the first steps in the treatment of osteoporosis is considered to be Calcium and Vitamin D as they are essential for normal skeletal homeostasis. Reduced calcium absorption can result from an insufficient intake of Ca^{2+} and Vitamin D which lead to a rise in parathyroid hormone, an increased rate of bone remodelling and increased bone loss (Holick 2007). It has been docu-

mented that calcium and vitamin D levels have positive association with bone mineral density (BMD) (Bischoff-Ferrari 2004 and Dawson-Hughes 1997). Some trial results have shown that vitamin D supplementation had positive results and suggests that individuals would benefit by having 25 hydroxy vitamin D no lower than 70 nmol/l protecting against fracture by decreasing PTH and increasing bone mass (Dawson-Hughes and Bischoff-Ferrari 2007). Calcium supplements, alone or in combination with vitamin D are commonly used for the prevention of osteoporosis. The recommended dose of calcium, usually in the form of calcium carbonate, is 1g daily with common side effects that include dyspepsia and constipation; however a meta-analysis report showed that calcium supplements without co-prescribed vitamin D have increased risk of myocardial infarction (Bolland *et al.,* 2010). Vitamin D plays an important role in calcium absorption and bone health, this led to a consensus that calcium should not be prescribed to patients with osteoporosis without Vitamin D.

1.17.2 Bisphosphonates

Bisphosphonates inhibit bone resorption and are now used in the treatment of osteoporosis (Russell 2011). The molecular mechanism action of bisphosphonate is classified into two different classes, the nitrogen-containing and non nitrogen-containing bisphosphonates, which have a structure similar to pyrophosphate and thus inhibit the activation of enzymes that use pyrophosphate (Dunford 2010). The simple bisphosphonates such as (clodronate , etidronate and tildronate) can be metabolically incorporated into

non-hydrolysable analogues of adenosine triphosphate (ATP) that accumulate intracellularly in osteoclasts, resulting in induction of osteoclast apoptosis. In contrast, the more potent, nitrogen-containing bisphosphonates (such as pamidronate, alendronate, risedronate, ibadronate and zoledronate) act as analogues of isoprenoid diphosphate lipids, thereby inhibiting farnesyl diphosphate synthase (FPPs) synthase, an enzyme in the mevalonate pathway necessary for prenylation of small GTPase that are critical for osteoclast cytoskeletal association and function, leading to osteoclast apoptosis inducing bone resorption (Russell 2011, van beek *et al.,* 1999). Despite their positive effect, bisphosphonates have relatively common adverse side effects that limit their use such as upset stomach, oesophageal inflammation, flu-like symptoms, as well as a risk of renal toxicity and in some cases have also been associated with osteonecrosis of the jaw and mid-shaft and subtrochanteric femur fractures (Russell 2011, Rizzoli *et al.,*2008).Therefore, alternative treatments should be investigated.

1.17.3 Hormone replacement therapy (HRT)

HRT is a possible treatment option for women during menopause. Estrogen is approved for the prevention, but not for treatment of postmenopausal osteoporosis (Vokes and Favus 2010). It was demonstrated by studies conducted by Women's Health Initiative (WHI), that increased risk of breast cancer, coronary heart disease, stroke and venous thromboembolism are all associated with estrogen treatment in combination with progesterone and this is despite the fact that estrogen treatment was found to reduce risk of osteopo-

rotic fractures (Nelson et al. 2002). Unopposed estrogen therapy has been shown to increase stroke and thromboembolism only. HRT is not considered as first line therapy in postmenopausal osteoporosis management and this is due to the overall health risks of HRT exceeding the potential benefits.

1.17.4 Selective estrogen receptor modulators (SERMs)

Selective estrogen receptor modulators (SERMs) are a group of nonsteroidal compounds that are chemically different from estradiol and act as estrogen agonists in some tissues, such as bone, and as estrogen antagonists in other tissues, such as breast, through specific, high-affinity binding to the ER (Goldstein *et al.,* 2000). Raloxifene and tamoxifene are currently SERMs used for osteoporosis treatment; they have been proven to show beneficial effects on vertebral and nonvertebral fractures. Raloxifene increases bone mineral density in postmenopausal women(Delma *et al.,* 1997), reduces the risk of vertebral fracture in postmenopausal women with osteoporosis (Ettinger *et al.,* 1999). Tamoxifen which has been approved for prevention of breast cancer also may reduce fractures (Cooke *et al.,* 2008) but appears to be less potent with a greater variability in the number of responders. Therefore, raloxifene is a preferred treatment for women who have more severe cases of osteoporosis than tamoxifene and, thus, may not be suitable for use in early postmenopausal women with severe vasomotor symptoms. Raloxifene has been shown to increase thromboembolic risk, but not the risk of coronary heart disease (Barrett-Connor *et al.,* 2006).

1.17.5 Parathyroid Hormone

Recombinant Parathyroid Hormone (PTH) 1-34 (teriparatide) is the only anabolic therapy currently used for osteoporosis (Hodsman *et al*., 2005). Teriparatide binds to the PTH receptor type I on osteoblasts and stimulates osteoblastic bone formation more than bone resorption leading to an increase in bone mass (Boron and Boulpaep 2005). It has been found that once daily injection of teriparatide reduced vertebral and non-vertebral fractures in women with osteoporosis (Neer et al. 2001). Intermittent treatment with PTH over a limited period supports bone formation; in contrast, prolonged treatment with PTH supports bone resorption by stimulating proteosomal degradation of the critical transcription factor Runx2 in the osteoblast, shortening its anti-apoptotic effect, and by stimulating induction of RANKL that in turn stimulates osteoclastogenesis (Ishizuya *et al.,* 1997; Bellido *et al.,* 2003; Walker *et al.,* 2011). Teriparatide is not recommended for patients at risk of sarcoma due to theoretical concerns that teriparatide risks of osteosarcoma, which was based on rodent toxicity studies in which prolonged high-dose therapy was associated with osteosarcoma in rats. There have been no reported cases of osteosarcoma in humans receiving teriparatide (Favus and Vokes 2010). Teriparatide is a potentially exciting treatment for osteoporosis; it endorses the regeneration of trabecular connectivity and cortical thickness, which translates into improved mechanical strength of bone (Bouxsein and Radloff 1997). The bone mass which is gained during Teripatatide treatment may be lost in the months after completion of treatment, therefore follow on anti-resorptive treatments may be needed to maintain the new bone mass (Favus and Vokes 2010).

1.17.6 Calcitonin

Calcitonin is a 32-amino acid linear polypeptide hormone that is secreted by thyroid C cells (Pagonis *et al.,* 2014). It acts to reduce blood calcium (Ca^{2+}), opposing the effects of PTH (Boronand Boulpaep 2004). Calcitonin interacts with G-protein-coupled receptors on osteoclast surfaces during the final stages of differentiation and causes osteoclasts to withdraw from active sites of bone resorption by flattening their ruffled border in the resorption pit (Adami 2008). Calcitonin is an anti-resorptive of modest potency. In clinical trials, calcitonin reduced vertebral but not hip fractures (MacLean et al. 2008), and is generally not a first choice anti-resorptive.

1.18 Phytoestrogens

Phytoestrogens (PEs) are naturally occurring plant compounds that are structurally and/or functionally similar to mammalian estrogens and their active metabolites (Whitten et al 1997). They are non-steroidal plant derived compounds which can exhibit estrogen effects in estrogen target tissues, including bone (Viereck et al., 2002; Weaver & Cheong, 2005). Most PEs are phenolic compounds, among these compounds, isoflavones and coumestans are the most widely researched groups (Adlercreutz 2002). Isoflavones are present in berries, wine, grains and nuts, but are most abundant in soybeans and other legumes (Kurzer and Xu 1997). Epidemiological studies indicated that populations consuming a large amount of soy bean have lower risk of chronic diseases such as osteoporosis (Coxam 2008). In contrast to these beneficial health claims, the anti estrogenic properties of PEs have also

raised concerns since they might act as endocrine disruptors, indicating a potential to cause adverse health effects (Wuttke *et al.,* 2007; Andres *et al.,* 2011; Rietjens *et al.,* 2013). Altogether, the health benefits or risks of isoflavones and other PEs are still controversial, and the question of whether PEs are beneficial or harmful to human health remains unresolved. 17β-estradiol (E2) is the dominant form of estrogen in the body, although any compounds which can bind estrogen receptors (ER) inducing receptor dimerization and consequent binding to estrogen response elements, can be considered estrogens (Cornwell *et al.,* 2004). This includes the PEs, which are able to induce similar responses in various cells including bone tissues and cell lines as estradiol and mimic estrogen regardless of their mechanism via estrogen receptor (Adlercreutz 1999). They can act as estrogen agonists and antagonists by blocking or altering ERs, thus they are more similar to natural selective estrogen receptor modulators (SERMS) (Brzezinski & Debi 1999). PEs have been found in almost all plants (Ososki and Kennelly 2003); they are sub-divided into many classes and the most used in human are isoflavones, lignans and coumestans (Adlercreutz 2002). Soybeans contain a large amount of isoflavones, including genistein (4', 5',7-trihydroxyisoflavone), daidzein (4', 7-dihydroxy isoflavone), glycitein (6-methoxydaidzein) and their glycosides (Murphy *et al.,* 1982). Experimental evidence suggests that soy isoflavones have many properties including estrogenic (Song *et al.,* 1999), antioxidant (Carroll 1991), hypocholesterolemic (Anthony *et al*., 1998) and inhibition of cell proliferation and DNA synthesis (Pan *et al.,* 2001, Santell*et al.,* 2000). PEs exert estrogen agonist and antagonist characteristics (Zhao

et al 2002); they have differential binding affinities for the estrogen receptor (ER) isoforms; with higher affinity for ERβ than for ERα (Kuiper *et al.,* 1998). PEs have a phenolic group (Fig.1.9), which is essential for binding to ER, and have similar molecular weight to E2.

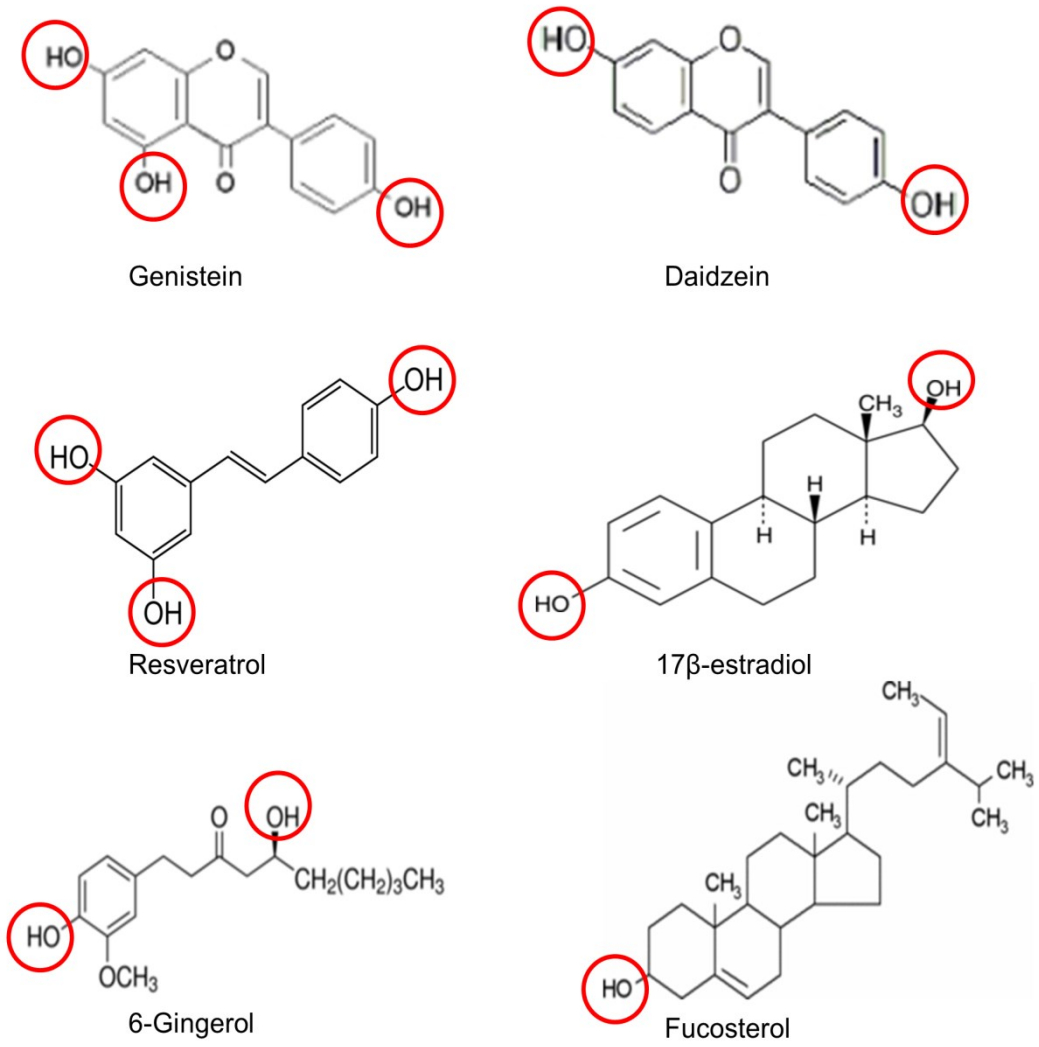

Figure 1.9: Chemical structures of 17β-estradiol and PEs. All have the presence of phenolic ring containing a free-OH group thought to be responsible for the reaction with oxyradicals by H atom transfer due to the weakness of O-H bond of the hydrogen group.

The cellular effects of PEs are affected by different factors, including conctrtion, ER status, presence or absence of endogenous estrogens, and the target tissue (Setchell 1998). PEs exert agonistic and antagonistic estrogenic effects and also induce differentiation as well as inhibit angiogenesis, cell proliferation, tyrosine kinase, and topoisomerase II (Oseni *et al.,* 2008). The presence of a phenolic ring and the distance between the two opposing phenolic oxygen atoms in isoflavone structure is similar to E2. This resemblance confers the binding of isoflavones to the ER, effectively displacing E2 (An *et al.,* 2001), which may help to explain how PEs protect against breast cancer, because ERβ signalling inhibits mammary cell growth (Lazennec *et al.,* 2001). The antioxidant activity of PEs is related to the presence of free hydroxyl groups in the aromatic ring and ability to bind between aromatic rings (Burda and Oleszek, 2001). The presence of C3-OH grouping in PEs is thought to assist the reduction of oxidative stress or inhibit the oxidative stress-induced apoptosis of osteoblasts/osteocytes (Behl *et al.,* 1997). This protection was not restricted to bone cells, but also neurons were reported to be protected from oxidative stress by estrogen through the A ring of the steroid which has the C3-OH group (Behl *et al.,* 1997).

Although PEs generally, have positive effects, consumption of high amount of PEs may lead to undesirable health effects. Many studies have shown prenatal exposure to Genistein resulted in effects in the progeny e.g. fetal erythropoiesis and altered gene expression as well as altering DNA methylation of hematopoietic cells which result in long-lasting effects on the endocrine and immune system and altered stress response and post weaning growth (Vanhees *et al.,* 2011). Soy consumption in pregnant mice, in doses below

the range of human consumption standardized per weight, showed Genistein accumulation in the fetus. The effect of genistein includes changes in blood composition observed in mice that were prenatally exposed to Genistein, which was associated with increased granulopoiesis and erythropoiesis together with moderate macrocytosis; these changes may be attributed to estrogenic properties of genistein, due to its structural similarity to 17β-estradiol (Miodini *et al*., 1999). This was supported by other studies which showed that genistein inhibited testosterone secretion in fetal Leydig cells (interstitial cells of Leydig, in the testicle) during early fetal development, suggesting that genistein concentrations relevant for human consumption, may affect the development and function of the male reproductive system (Lehraki *et al.,* 2011, Lee *et al.,* 2004). Therefore, future studies on PEs could help to identify the safest dose for these dietary levels and explain the mechanism of health risks and/or therapeutic action involved.

1.19 Genistein (GEN)

Phytoestrogens are classified in four main distinct classes: isoflavones, lignans, coumestans and stilbenes Isoflavones, originated from soy and soy derivatives are the most common PEs with Genistein and Daidzein being the most abundant and frequently studied (Dixon 2004). Isoflavones, including GEN and DZ, are polyphenolic compounds commonly found in legumes. They have a broad variety of physiological and pharmacological functions and are known to act as antioxidants *in vivo* and *in vitro*; their activity includes anti-estrogenic (Muller *et al.,* 2004; Ratna *et al.,* 2002), anticancer

(Chen 2005; Miltyk 2003; Tsuchiya 2002), anti-inflammatory (Marotta *et al*., 2006), cardio protective (Park *et al.,* 2005) and enzyme-inhibitory effects (Woclawek-Potocka *et al.,* 2005). Isofavones have similar structure to estrogen molecules (Fig.1.9), which is thought to play an important role in the prevention of osteoporosis (Ward *et al.,* 2007), and have also been reported to inhibit resorption activity and survival of osteoclasts *in vitro* and prevent trabecular bone loss *in vivo* following ovariectomy (OVX) in rodents (Reinwald and Weaver, 2006). The hydroxyl group in the B-ring is important to enable the scavenging of reactive nitrogen or oxygen species (Sekher*et al.,* 2001). Isoflavones such as GEN have also been known to donate hydrogen atoms from the phenolic group (Heim *et al*., 2002).

GEN aglycone is an isoflavone found in soybeans (Messina *et al.,* 2004), structurally resembles E2 and may positively regulate bone cell metabolism without harmful estrogenic activity in the breast and uterus (Cassidy 2003). This safe profile results from the greater affinity of GEN for estrogen receptor-β, which is more abundant in bone, than for estrogen receptor-α, which is abundant in reproductive tissue. Observational studies suggest that postmenopausal Asian women who consume diets high in isoflavones have a lower rate of fracture than other groups (Setchell *et al*., 2002). However, the mechanism of action of genistein on bone is not yet fully understood. In postmenopausal women daily treatment with genistein at concentration of 54mg increased BMD in lumbar spine and femoral neck with no undesirable effects on breast and uterus (Morabito *et al.* 2002, Crisafulli *et al.,* 2004, Marini *et al.,* 2007). Also GEN decreases the ratio of soluble receptor activator of nuclear factor-B ligand to osteoprotegerin, which may partly account for its

positive effects on BMD (Crisafulli*etal.,* 2004). Furthermore, studies conducted show that GEN at doses adjusted for rat body weight was more effective than alendronate or raloxifene or estradiol in treating primary osteoporosis induced by ovariectomy (Bitto *et al.,* 2008) and secondary osteoporosis (Bitto *et al.,* 2009). According to these studies, it has been suggested that GEN aglycone may be an option for osteoporosis prevention.

1.20 Daidzein (DZ)

DZ, a soybean isolavone, is metabolized to equol in the gastrointestinal tract by gut microflora (Fujioka *et al.,* 2004). Equol possesses a stronger affinity for estrogen receptors than DZ (Fujioka *et al.,* 2004), and is derived from certain plants such as soybeans, legumes and peas. DZ in soy products can be found in two chemical forms, aglycones (unconjugated form) and glucosides (which are bound to a sugar molecule). The main dietary source of daidzein is the biologically active glucoside daidzin. As with GEN, fermentation or digestion of soy products results in the release of the sugar molecule from the glucoside DZ, which results in the formation of the other form, the aglycone DZ (Rowland *et al.,* 2003). This process normally occurs in the stomach through acid hydrolysis and in the intestine through the action of the bacterial enzymes (Rowland *et al.,* 2003). Daidzein has a direct stimulatory effect on bone formation in cultured osteoblastic cells *in vitro*, which may be mediated by increased production of bone morphogenic protein (BMP-2), a potent inducer of osteogenic differentiation and a target for isoflavones (Jia *et al.,* 2003). DZ has been shown to increase bone formation in an animal model of

bone defect healing (Wong and Rabie 2009). This was demonstrated by measuring bone forming ability *in vivo* for the repair of bone defects by using a carrier to enable its use in the clinical setting and the amount of new bone formed in collagen matrix carrier with DZ grafted into bony defects and compared with that of the collagen carrier alone. Using DZ in collagen matrix significantly enhanced new bone formation locally when grafted into skull defects. It may be the long-sought-after agent for bone induction and bone defect repair (Wong and Rabie 2009).

1.21 Resveratrol (Resv)

Many studies have shown that resveratrol (3,4-,5-trihydroxystilbene), a polyphenolic constituent in grapes and red wine, has various benefits, such as protecting against neurodegeneration, cardiovascular disease, cancer, diabetes, as well as obesity-related disorders (Baur and Sinclair, 2006). This wide range of positive biological effects might be explained by resveratrol's antioxidant properties which include increases in catalase and superoxide dismutase (SOD) activity (Rubiolo *et al*., 2008). In addition, the antioxidant capability of resveratrol might be mediated by activation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) which controls the orchestrated expression of phase II enzymes and genes involved in oxidative defence such as glutathione (GSH) S transferase (GST), heme oxygenase-1 (HO-1), NAD(P)H quinone oxido-reductase1 (Rubiolo *et al.,* 2008). The antioxidant effect of resveratrol has been suggested to be due to the presence of hydroxyl phenolic groups in its chemical structure (Leonard *et al.,* 2003).

Resveratrol has been known as an excellent scavenger of superoxide, hydroxyls and other radicals (Leonard *et al.,* 2003). Furthermore, it protects DNA from oxidative stress-induced damage and therefore cell apoptosis (Wang *et al.,* 2007; Gehm*et al.,* 1997). Resveratrol has been reported tohave the capacity to bind to estrogen receptors and can turn on estrogen responsive genes. It is, therefore, postulated that resveratrol can act as a phytoestrogen in the absence of estrogen in postmenopausal women as well as elderly men. The protective effect of resveratrol has also been investigated in bone loss induced by disuse, which in turn has been linked with increased intracellular ROS (Boissy *et al.,* 2005). *In vitro* studies have shown that resveratrol inhibits osteoclast formation and promotes osteoblast differentiation (Boissy *et al.,* 2005). On the other hand, several studies have reported that it has a low bioavailability and weak ability to scavenge ROS (Leonard *et al.,* 2003). *In vitro* and *in vivo* study studies show that resveratrol is absorbed and metabolized. Around 75% of this polyphenol are secreted via faeces and urine (Walle *et al.,* 2004). The oral bioavailability of reseveratrol has been thought to be almost zero due to rapid and extensive metabolism and the consequent formation of various metabolites as resveratrol glucuronides and resveratrol sulfates.(Wenzel and Somoza 2005). However other studies suggested that, dietary Resveratrol can have beneficial effects in mice (Baur *et al*., 2006; Lagouge *et al*., 2006), it is thought that when Resveratrol enters circulation, retention of some pharmacological activity of these metabolites occurs, thereby releasing the pharmacologically active parent compound. The potential biological activity of resveratrol should be considered in future

to assess the significance of resveratrol in humans, and understanding of its role in estrogenic actions.

1.22 Fucosterol

Fucosterol (24-ethylidene cholesterol) is a sterol that can be isolated from algae, seaweed and diatoms. Fucosterol exhibits a range of biological activity with therapeutic potential that include antioxidant and anti-osteoporotic effects (Abdul *et al.,* 2016). Fucosterol is isolated from brown algae and has a similar structure to human estrogen (Fig. 1.9) (Gaulin *et al.,* 2010) and can be found abundantly in some species of brown algae such as Hizikiafusiforme. Recent studies have shown that Fucosterol promotes cell proliferation in human osteoblast like cells MG63 and elevated ALP and inhibits osteoclast differentiation, RANK expression in TRAP assay, thus it has been suggested that Fucosterol has the dual potential to activate osteoblastic bone formation and suppress osteoclastic differentiation. This dual effects represents a benefit for treatment of postmenopausal osteoporosis, as an alternative to using a purely anti-resorptive approach, in order to inhibit bone remodeling (Lee *et al.,* 2014). Fucosterol, through its biological activities, may play an important role in preventing osteoporosis and might be useful as a supplement for postmenopausal women. Further clinical studies will be vital to identify the specific role of Fucosterol in the mechanism of bone metabolism and assess its estrogen-like actions in postmenopausal women.

1.23 6-Gingerol

Ginger has been used historically as a traditional medicine in India. Several studies have shown evidence that compounds found in ginger contribute to relief of symptoms from chronic inflammatory diseases (Srivas, 1984; Kiuchi *et al.,* 1992; Tjendraputra *et al.,* 2001). Ginger consists of different phyto-chemicals such as 6-Gingererol, 8-Gingerol and 6-Shogaol. Among these compounds, 6-Gingerol (Fig.1.9) exhibits a wide range of biochemical and pharmacological properties and has been reported that it possesses antioxi-dant and anti-inflammatory properties as well as anti-mutagenic potential (Shukla *et al.,* 2007). 6-Gingerol has been shown to induce apoptosis in cancer cells through depolarization of mitochondrial membrane potential, cleavage of caspase 3 and results in DNA fragmentation (Chakraborty *et al.,* 2012). Earlier studies showed the anti-cancer potential of 6-Gingerol in the treatment of colorectal cancer cells through mitochondrial damage and cell survival pathway (Lee *et al.,* 2008). 6-Gingerol also stimulates osteoblast dif-ferentiation in normal physiological andinflammatory settings and improves TNF-α-suppressed osteoblast differentiation. Thus, it is suggested that 6-Gingerol may have beneficial effects on bone as a therapeutic agent for treat-ing bone disorders such as osteoporosis (Fan *et al*., 2015).

Hypothesis

PEs have antioxidant activity that prevent oxidative stress induce bone c ell death.

Aim of study

The primary goal of this project is to study the potential protective effect of phytoestrogen against osteocyte apoptosis induced by H_2O_2. Specifically, the objectives and their associated aims of this project are as follow:

- Firstly, to study the effect of PEs (Genistein, Daidzein and Resveratrol, Fucosterol and 6-gingerol) in the protection of osteocyte like cells MLO-Y4 from apoptosis induced by H_2O_2.
- Secondly to study the protective effect of PES on different bone cells such as osteoblast like cells MC3T3-E1 cells and whether these PEs inhibit osteoclastogenesis by testing their effect on differentiation of RAW264.7 cells into osteoclast cells.
- Thirdly, we sought to determine whether the effct of these PEs is direct or indirect.
- To evaluate the effect of the chemical structure of PEs (Genistein and Daidzein) in protecting MLO-Y4 cells against oxidative stress through modification of OH group.
- To evaluate the effect of PEs on cytokines release in MLO-Y4 cells in response to oxidative stress induced by H_2O_2.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Materials:

Al the chemicals were purchased from Sigma UK and all tissue culture reagents from Gibco/ Invitrogen UK unless otherwise stated. Tissue culture flasks and well plates were purchased from Coring USA. Annexin V and Propidium iodide from Invitrogen life technology USA. 17β-estradiol, Genistein, Daidazein, Resveratrol, Fucosterol and 6-Gingerol from (Sigma Aldrich). Estrogen receptor inhibitor (ICI 182780) from Tocris Bioscience (R&D system). IL-6 and TNF-α ELISA kit from (Biolegend). RANKL ELISA kit from (Boster Biological Technology). Catalase activity assay kit from (Cell Biolabs, INC.).

2.2 Methods:

2.2.1 Cell culture:

The cell lines used in the present study were murine long- bone derived osteocyte like MLO-Y4 cell lines, a gift from Prof. Lind Bonewald, MC3T3-E1 osteoblast cells and RAW264.7 cells. MLO-Y4 cells were grown in 75 cm^2 tissue culture flasks, which were pre-coated with 0.15mg/ml type 1 collagen solution prepared in 0.1M acetic acid. The cells were cultured under standard sterile conditions in pre-coated T-75 flask comprising growth medium: Minimal Essential Medium alpha (αMEM) supplemented with 10% foetal bovine serum 1% penicillin/streptomycin (P/S) (Gibco)and 1% (1ml) Glutamax (Gibco). Cells were grown in continuous monolayer and maintained in an incubator at 37^0C in a humidified atmosphere of 5% CO_2. Sub culturing was performed twice a week upon reaching 90% confluence. The monolayer of cells

were detached by adding 1 ml of 2.5 % trypsin for 3 minutes followed by adding 5ml of fresh growth medium to stop trypsin reaction, cell suspension at dilution of 1:10 was added to a new flask which was pre coated with type 1 collagen to help maintain cells.

2.2.2 MC3T3-E1 cell culture

MC3T3-E1 cells were grown in Minimal Essential Medium alpha (αMEM) supplemented with 10% foetal bovine serum (FBS, Sigma) 1% penicillin/ streptomycin (P/S) (Gibco) and 1% L-Glutamine (Gibco) in 75 cm^2 tissue culture flask. Cells were maintained in 75 cm^2 tissue culture flasks as described in 2.2.1.

2.2.3 Cell culture for RAW264.7

Murine macrophage RAW264.7 cells are adherent cells and were cultured in DMEM medium supplemented with 10% foetal calf serum, 1%l glutamine, 1% penicillin/streptomycin. Cells were maintained as described by replacing medium with fresh medium every 2-3 days.

2.2.4 Osteoclast differentiation

RAW 264.7 cells were seeded in 12-well plates (3×10^4 cells/well) in appropriate medium. In test samples, the growth medium DMEM was replaced by differentiation medium, comprising (100 ng/mL RANKL and 50 ng/ml M-

CSF). The differentiation medium was changed every 2 days. After 5 days, the medium was removed, and the cell monolayer was gently washed twice using PBS, fixed and stored at 4^0C for microscopic imageing.

2.2.5 Assesment of Tartrate-Resistant Acid Phosphatase activity

Osteoclast formation was evaluated by staining for the specific osteoclastic marker TRAP. Cells were fixed prior to immuno-histochemistry in 4% para-formaldehyde for 10 min and washed with distilled water. The cells were incubated at 37^0C in a humid and light-protected incubator for 1 h in the reaction mixture of a leukocyte acid phosphatase assay kit (Sigma-Aldrich), as directed by the manufacturer. The cells were washed three times with distilled water, and TRAP-positive multinucleated cells containing three or more nuclei were counted under a light microscope. The number of TRAP-positive cells was counted and the results expressed as the number of cells per 5 fields of view. All experiments were performed in triplicate.

2.2.6 Fixation

Following experimental procedure, cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature. The cells were then washed with 1x PBS and stained with DAPI.

2.2.7 DAPI staining

Cells were stained with 4', 6- diamino-phenylindole (DAPI) 4mg/ml for 15 minutes in the dark at room temperature. Cells were then washed twice with 1X PBS and visualized under microscope.

2.2.8 Induction of apoptotic bodies using H_2O_2

MLO-Y4 cells were plated on 6 well plates coated with collagen at a density of $5x10^4$ cells/ml. H_2O_2 was used at different concentrations (0.3, 0.4, 0.5mM) for 2 hours, (n=2 wells per treatment). These concentrations were used based on studies conducted in previous literatures (Mann *et al.,* 2007). Cells without H_2O_2 treatment were used as a control. Cells were incubated at 37^0c for 2 hours after treatment with H_2O_2. Media was removed, centrifuged and cells washed with PBS, then stained with 10 µl of (1mg/ml) of Annexin V, followed by 5 µl of (1mg/ml) propidium iodide (PI) for 15 minutes and then visualized under fluorescence microscope (Carl Zeiss inverted microscope) at 494nm/518nm for Annexin V and 535nm/617nm for PI.

2.2.9 Characterization of apoptotic bodies by Annexin V FITC binding and PI staining.

Osteocyte apoptotic bodies (OAB) were collected from cells, medium was removed and cells washed in PBS, centrifuged for 5 minutes at 6000rpm and supernatant was removed. Cell density was determined and diluted in Annexin binding buffer to prepare sufficient volume to have 10000 OAB/ 100µl

per assay. Annexin binding buffer was prepared as follows (10mM HEPES, 140mM NaCl, 2.5mM $CaCl_2$). A quantity of 10 µl of (1mg/ml) FITC conjugated Annexin V was added to each 100µl of OAB suspension followed by 5 µl of (1mg/ml) propidium iodide (PI) and incubated at room temperature for 15 minutes, 50µl was then spotted on a slide and visualized under fluorescence microscope to distinguish between viable (FITC negative, PI negative), early apoptosis (FITC positive, PI negative) and late apoptotic (FITC positive, PI positive) or necrotic cells (FITC negative, PI positive). Apoptotic cells were expressed as the percentage of ratio of Annexin V positive and PI negative from 3 fields over the total number of cells estimated from those fields. The total cell numbers were counted from using bright field microscopy.

$$\text{Percentage of apoptotic cells} = \frac{\text{Average number of apoptotic cells from 3 fields}}{\text{Average number of cells from 3 fields}} \times 100$$

2.2.10 Treatment of MLO-Y4 cells with 17β-Estradiol or phytoestrogens (Genistein, Diadzein and Resveratrol):

Cells were seeded at a density of $3x10^4$ cells/well 24 hours prior to the experiment (n= 3 wells per treatment). Untreated cells were used as a negative control and cells treated with H_2O_2 only were used as a positive control. Cells were pre-treated with a dose range of (10nm, 100nM and1µM) for each of E2, Geistein, Diadzein and Resveratrol for 1 hour prior to incubation with 0.4mM H_2O_2 for two hours to induce apoptosis. Cells were fixed and stained

with DAPI as described previously and visualized under fluorescent microscope.

2.2.11 Treatment of MLO-Y4 cells with Estrogen Receptor Inhibitor prior to treatment with 17β-Estradiol or phytoestrogen (Genistein, Diadzein and Resveratrol):

Cells were treated with 10nM ICI 182780(RI) for 1 hour prior to treatment with 10nM 17β-Estradiol or phytoestrogens. Untreated cells were used as a negative control and cells treated with H_2O_2 only were used as a positive control. Apoptosis was induced by treatment with 0.4mM H_2O_2 for 2 hours. After incubation, cells were fixed and stained with DAPI. Images were taken with an Inverted Carl Zeiss microscope, 3 images from 3 different fields of view per well (n=9 in total per treatment).

2.2.12 Detection of Caspase 3 and 7 activities

Caspase activity is a key event in apoptosis. Untreated cells were used as a negative control. Apoptotic cells were detected by staining the cells with Image live red caspase detection kit (FLICA)from (Sigma) for 1 hour. Cells were washed with culture media, stained with Hoechst stain for 2-10 minutes then washed with wash buffer and visualized under fluorescence microscope at 595nm for FLICA.

2.2.13 Detection of intracellular reactive oxygen species

Detection of reactive oxygen species was determined with 2, 7'-dichlorodihydrofluorescein-diacetate (H_2DCF-DA) which is a non-fluorescent substrate used as an indicator for reactive oxygen species (ROS). Upon cleavage of the acetate groups by intracellular esterases and oxidation, the non-fluorescent H_2DCF-DA is converted to the highly fluorescent 2', 7'-dichlorofluorescein.

H2DCF-DA was added to the cell culture media at a concentration of 10 μM for 45 minutes followed by incubation with either E2 or other PEs (Genistein, Daidzein and Resveratrol) at a concentration of 10nM. Untreated cells were used as control. The cells were incubated with these reagents for 1 hour prior to incubation with 0.4mM H_2O_2 for 2 hours. All the reagents remained in the culture media during treatment with H_2O_2, which were then removed and washed twice with PBS. The rate of oxidation was monitored by a fluorescence microscopy. ROS positive cells were detected at 490nm FITC excitation and images were captured at 20 x with the inverted microscope.

2.2.14 Enzyme-linked immunosorbent assay (ELISA) for cytokines (RANKL, IL-6 and TNF-α)

ELISA kit for RANKL was obtained from Booster Biological Technology, and ELISA kit for IL-6 and TNF-α were obtained from Biolegend. Cytokine production was measured in supernatants obtained from experimental culture following pre-treatment of MLO-Y4 cells in 24 well plates at a density of $3x10^4$ cells/ml. Cells were treated with E2 and PEs (Genistein, Didzein and

Resveratrol) at a concentration of 10nM each for 1 hour prior to treatment with H_2O_2 for 2 hours. Supernatants from samples were collected and stored at -80^{o}C for later analysis. ELISA experiments were carried out according to the manufacturer's instructions, 96 wells palates were coated with 100µl/well of capture antibody in coating buffer and incubated over night at 4^{o}C. The wells that had been coated previously were aspirated and washed 5 times with wash buffer and followed by blocking them with 200 µl/well of 1xAssay diluent. The plate was incubated at room temperature for 1 hour followed by washing as previously. Standard curve was obtained by adding 100 µl/well of diluted standard to specific wells. Performing 2 fold serial dilution of the top standard to generate a standard curve to calculate the concentration of RANKL, IL-6 and TNF-α released in the cells supernatant, then 100 µl/well of samples supernatants were added to the appropriate wells. The Plate was covered and incubated at room temperature for 2 hours followed by a total of 5 washes. Then the detection antibody (100 µl/well) diluted in 1x assay diluent was added and incubated in room temperature for 1 hour. Following washing, 100 µl/well of Avidin–HRP was added to the all wells and the plate was sealed and incubated at room temperature for 30 minutes. Aspirating and washing was carried out for 5 times. Substrate solution was then added to each well at 100 µl/well, and incubated at room temperature for another 15 minutes. After that 50 µl/well of stop solution (1M H_3PO_4) was added to each well. Finally, the plate was read by micro plate reader at 450nm and 570nm.

2.2.15 Modification of Genistein and Daidzein

Due to high cost and limited funding, experimentation with Resveratrol, Fucosterol and 6-Gingerol was not possible. However, Genistein and Daidzein were readily available to be modified. DZ approximately (0.54 g, 2mmol) was dissolved in acetone (50 ml) in two necked round bottom flask. Potassium carbonate (2g) was added to this solution and the mixture was stirred under argon via magnetic stirrer then refluxed for 10 minutes. Iodomethane (0.124 ml, 2mmol) was added to 20ml of acetone, then added dropwise to the mixture over 30 minutes and the mixture was refluxed for 2h. The solvent was evaporated under reduced pressure and the residue was dissolved in dichloromethane and washed twice with pure water. The solvent was dried over magnesium sulphate then evaporated under reduced pressure. The crude Genistein and Daidzein were purified with flash chromatography by PuriFlash instrument (Interchem) after determining the eluent solvent system by TLC (Thin Liquid Chromatography), a technique used to separate the components of a mixture using silica gel cartridge (High Performance Spherical Silica 15 μ). The crude product was fractionated with dichloromethane and methanol (9:1) and the fractions were collected and the solvents evaporated under vacuum. Each fraction were purified again with semi-preparative HPLC over isomer's separation column (COSMOSIL PYE, Pyrenylethyl group bonded, 4.6x15 mm), eluted with (0.5% formic acid, methanol and acetonitrile, and water, 80:15:4.5). The fractions were collected and solvents were evaporated in vacuum, ready for structural analysis. Genistein (0.5g, 1.9mmol) was dissolved in acetone (50ml) in two necks round bottom flask and the procedure for purification was repeated as above.

2.2.16 Catalase Activity Assay

Catalase activity assay is a direct measurement of catalase activity from cell lysate. MLO-Y4 cells were seeded at a density of $1x10^6$ cells / well for 24 hours then treated with E2 or PEs for 1 hour prior to treatment with H_2O_2 for 2 hours. Media was removed and cells washed with cold PBS. Cells were harvested with a rubber scraper, and homogenized on ice in 1 ml cold PBS, 1mM EDTA, centrifuged at 10,000 x g for 15 minutes at 4°C. Supernatant was removed and stored on ice then in -80 ^{0}C freezer until further use. The procedure was carried out by adding 20 µL of diluted catalase standard or test samples to a 96-well microtiter plate, then 50 µL of the Hydrogen Peroxide working solution (12mM) was added to each well and incubated for 1 minute. The reaction was stopped by adding 50 µl of the Catalase Quencher into each well and mixed thoroughly by using pipette, and then 5 µl of each reaction was transferred to a fresh well, 250 µl of chromogenic working solution to each well and plate was incubated 40-60 minutes with vigorous mixing. Optical densityat 520nm was measured on an Omega Microplate Reader (Thermo scientific).

Statistical analysis

Statistical differences between groups were evaluated by using ANOVA test followed by Tukey post-hoc test. The Statistical significance was indicated on graphs by $*p \leq 0.05$ and $**p \leq 0.001$ when compared to H_2O_2 treatment and # for significance when compared to control. Analyses were performed with the software programm “Graphpad Prism 5”.

CHAPTER THREE: INVESTIGATION OF THE EFFECT OF PHYTOESTROGENS IN THE PREVENSION OF OXIDANT INDUCED APOPTOSIS IN MLO-Y4 CELLS

3.1 Introduction

Osteoporosis is a chronic bone disease, characterized by a decrease in bone density, ultimately osteoporosis leads to loss in bone strength and increased-susceptibility to fracture (Kular *et al*., 2012). Estrogen exerts a protective ac tion in maintaining bone health by increasing osteoclast apoptosis (Manolagas *et al*., 2013) and decreasing cytokines which promote osteoclast activity (Pacifici 1998). Estrogen deficiency promotes bone loss at every age (Seeman *et al*., 2002) and causes an imbalance between the lifespan of osteoclasts and osteoblasts, this leads to a decrease in bone formation (Hughes *et al*., 1996). On the contrary, hormone replacement therapy (HRT) is able to reduce bone loss and related fractures in postmenopausal women (Prelevic *et al*., 2005). In 2002, the Women's Health Initiatives (WHI) was the first large clinical trial to show a significant overall reduction in osteoporotic fractures in women receiving HRT compared with placebo, but this study reported an increased risk of breast and endometrium cancer and of heart disease in the HRT group (Manson *et al*., 2003). Therefore, potential supplementary treatments that do not have these negative effects should be considered. Women are at a greater risk of bone loss and eventually osteoporosis due to estrogen withdrawal after the menopause. It has been reported that osteocytes respond to estrogen, therefore, estrogen deficiency has been linked to osteocyte death in both human and rat bone (Tomkinson *et al*., 1997 and 1998). The main aim for an effective osteoporosis treatment is to reduce fracture risk and increase bone mineral density. The reason for age related osteocyte loss is unclear but osteocyte apoptosis is caused by different conditions including glucocorticoid treatment (Kogiani *et al., 2004*), micro

damage (Noble *et al*., 2003), estrogen loss (Tomkinsin *et al.,* 1997) and oxidant stress (Kikuyama *et al*., 2002). The free radical theory of ageing is based on the evidence suggesting that ROS production and the response to oxidative stress contribute to a number of age related phenomena (Finkel and Holbrook 2000).

Phytoestrogens are non-steroidal plant derived compounds that exhibit the effect of estrogen in estrogen target tissues, including bone (Viereck *et al*. 2002; Weaver & Cheong, 2005). Isoflavones are the main flavones in soybeans (Wang *et al.,* 1994) and have gained great attention in protection against several diseases such as cardiovascular disease, cancer, osteoporosis, and postmenopausal syndrome (Adlercreutz *et al.,* 1999), and have been reported to reduce risk of endometerial cancer in women when compared with women on low levels of isoflavone consumption (Horn-Ross*et al.,* 2003). Isoflavones have structural similarity to estrogen-like molecules, and it has been proposed that they play an important role in the prevention of osteoporosis (Ward *et al*., 2007) through the presence of hydroxyl group in the B-ring to enable the scavenging of reactive nitrogen or oxygen species (Sekher et al., 2001), which might act be directly through lowering oxidative stress (Hassan and Abdel-Wahhab, 2012). Therefore, the potential anti-oxidant effects of phytoestrogens in the prevention of oxidative stress induced apoptosis in the osteocyte like cell line MLO-Y4 was investigated.

Phytoestrogens such as isoflavones are plant-derived substances that struc turally resemble estrogen and display hormonal activity (Dan *et al.,* 2005). A

range of phytomolecules such as Fucosterol and 6-Gingerol were used to investigate their protective effect on MLO-Y4 cells from apoptosis induced by H_2O_2. Fucosterol was first isolated in 1934 from brown algae, where it is abundant and its structural backbone is similar to human estrogen (Gaulin *et al.,* 2010). Previous studies have shown that Fucosterol has anti-inflammatory and anti-cancer effects, and protects skin from ultraviolet-induced damage (Khanavi *et al.,* 2012). However, this study investigated the ability of Fucosterol to counter osteocyte apoptosis induced by H_2O_2. 6-Gingerol is another phytochemical compound which contains a phenolic group and has a number of diverse effects such as anticancer, antioxidant, and anti-inflammatory. It is of interest to investigate the effect of this compound on bone cells. Previous studies have determined the effect of 6-gingerol on proliferation and differentiation of osteoblasts by measuring alkaline phosphatase (ALP) activity, collagen type I synthesis, and bone mineralization in 6-gingerol-treated human osteoblast-like MG-63 cells (Fan *et al*., 2015). Previous studies showed that 6-gingerol had no cytotoxic effects on MG-63 cells. Furthermore, 6-gingerol was shown to possess anti-inflammatory activity in MG-63 cells stimulated with TNF-α (Fan *et al*., 2015). 6-Gingerol and estrogen share a similarity in structure, as both compounds possess hydroxyl groups. Therefore, it would be beneficial to explore the effect of this compound on MLO-Y4 cells and whether it can protect these cells from oxidative stress induced by H_2O_2.

3.2 Characterisation of osteocyte apoptotic bodies

In order to establish the cell assay and to determine dose and time of exposure of MLO-Y4 to H_2O_2 that induced apoptosis (but did not reduce cell number). MLO-Y4 cells were exposed to H_2O_2 in a dose dependent manner over a 2 hour incubation period. The percentage of apoptotic cells stained with Annexin V (early stage of apoptosis) was increased at a concentration of 0.4mM of H_2O_2 at 2 hours (% apoptosis 0.4mM H_2O_2 40% ± 1.7 vs. Control 5.4% ± 1.6; p=0.0001) and the number of apoptotic cells stained positive for Annexin V and PI) was 10% ± 2.2 (Fig.3.1 and Fig.3.2), meanwhile the percentage of cells at early stage apoptosis at concentration of 0.5mM of H_2O_2 were stained positive for Annexin V decreased (37%± 2.1), and the number of apoptotic cells that were stained positive for Annexin V and (PI) (late stage of apoptosis were increased 22.6% ± 2.08). The optimial concentration of H_2O_2 used to induce apoptosis was 0.4mM, which is in line with previous studies (Mann *et al*., 2007). Therefore, this concentration was used to induce apoptosis in subsequent experiments. DAPI staining was also used to determine MLO-Y4 apoptosis as evidenced by morphological changes such as chromatin condensation, shrinkage of nuclei and the fragmentation of nuclear material into small blebs (Elmore 2007) Fig.3.3.

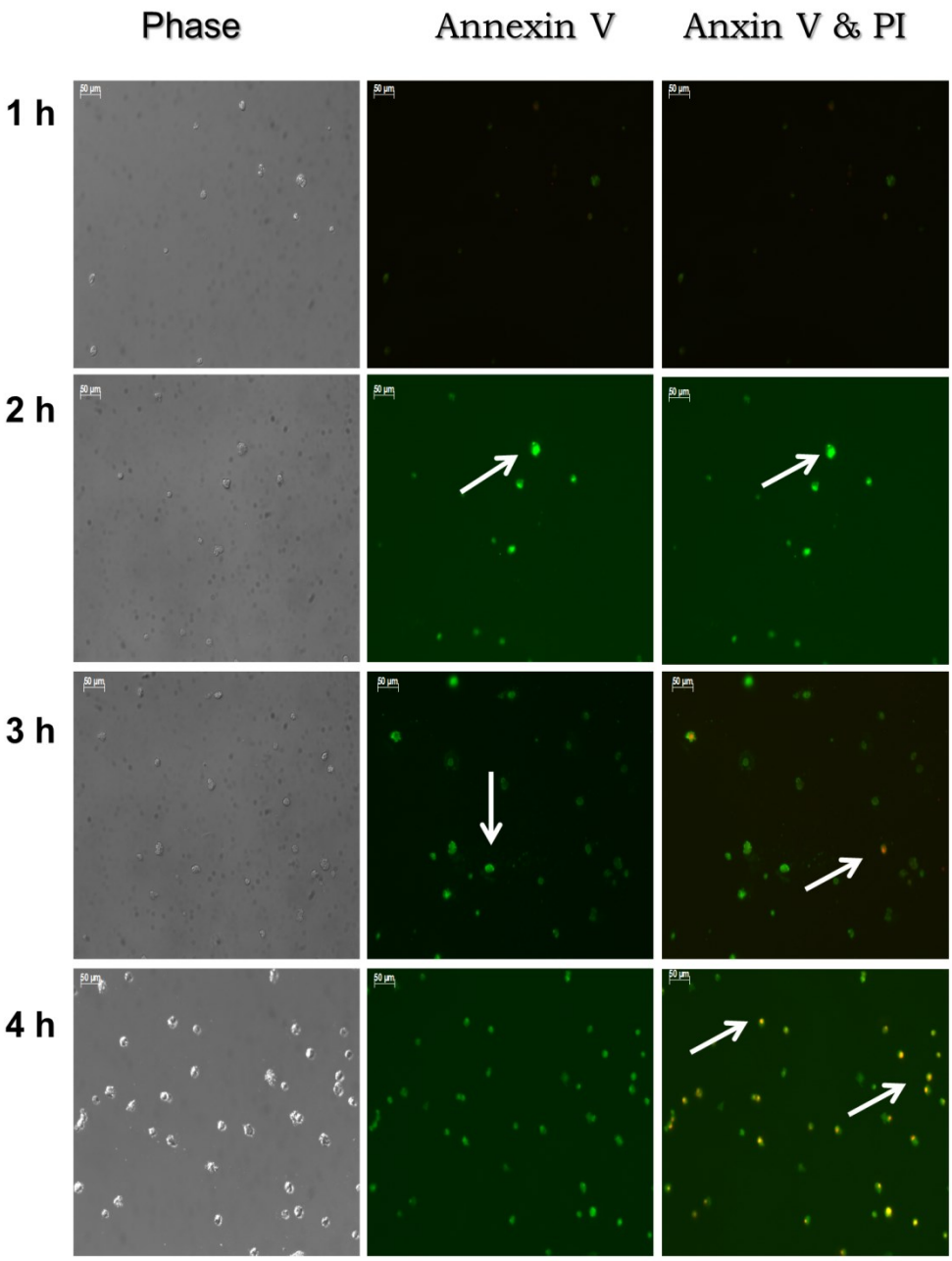

Figure 3.1: Induction of apoptotis in MLO-Y4 cells on treatment with0.4mM H_2O_2. Representative Phase contrast, Annexin V and Annexin V PI images for MLOY4 treated with 0.4mM H_2O_2 for 1 hour; 2 hours; 3 hours and 4 hours. FITC staining (green) represents Annexin V positive cells (early stage of apoptosis), PI staining (red) represents necrotic cells and orange colour represents Annexin V and PI positive cells (late stage of apoptosis) are indicated with white arrows. Scale bar = 50µm.

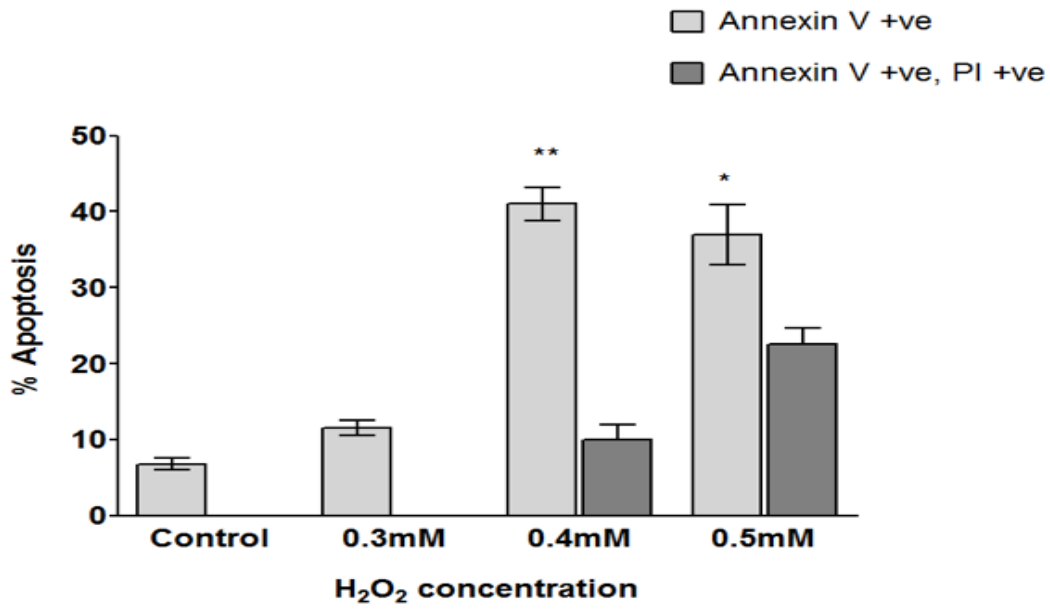

Figure 3.2: Induction of apoptosis in MLO-Y4 cells on treatment with different concentrations of H_2O_2 for 2 hours. A 2 h incubation of MLO-Y4 cells with varying concentration of H_2O_2 between 0.3 and 0.5mM was used to induce oxidative stress cell death. The percentage of apoptotic cell death increased significantly compared to control at a concentration of 0.4mM. Data were expressed as mean ± SD of triplicate wells (three fields per well counted) $^{**}p<0.001$ versus control and $^{*}p<0.05$.

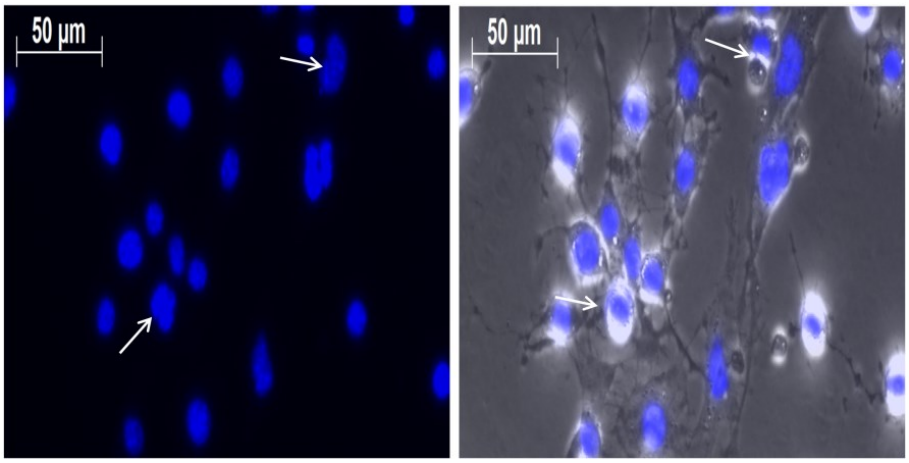

Figure 3.3: MLO-Y4 cells apoptosis. Cells were treated with 0.4 mM H_2O_2 for 2 hrs and stained with DAPI. Apoptotic cells exhibiting membrane blebbing indicated with white arrow.

3.3 The anti-apoptotic effect of E2 on H_2O_2 induced apoptosis in MLOY4

This assay was conducted in oreder to provide a relative level of protection in the comparison between E2 and PEs. MLO-Y4 was cultured and treated as described in section 2.2.8. The percentage of apoptosis induced by 0.4mM H_2O_2 (32% ± 4.06) was significantly reduced on pre-treatment with E2 for 1 hour at a concentration of 10nM (16% ± 0.86, p=0.002), 100nM (20% ± 3.9, p=0.011) and 1μM (18% ±1.61, p=0.02). Treatment with E2 alone at concentration of 1μM induced MLOY4 apoptosis when compared to control (Control 15.5% ± 3.5) vs 1μM E2 (18.94% ±2.9, p=0.0001) (Fig 3.4).

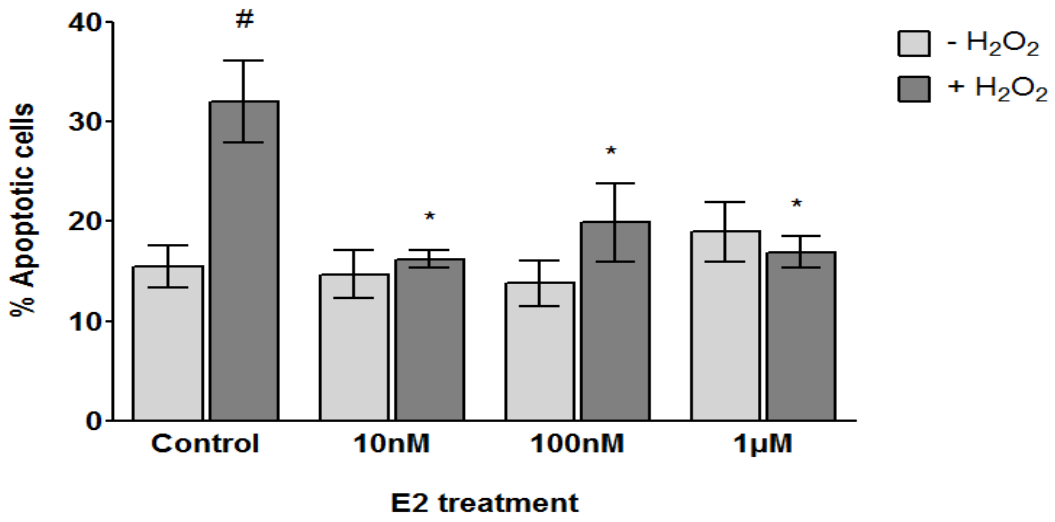

Figure 3.4: Effect of 17β-estradiol (E2) on H_2O_2 induced apoptosis in MLOY4. Pre-treatment of MLO-Y4 with (E2) at a concentration of 10nM, 100nM and 1μM all significantly (p<0.001) reduced the percentage of apoptosis induced by treatment with [0.4mM] H_2O_2 for 2 hours. Data were expressed as mean ± SD of triplicate wells (nine fields of view per well were counted).*p<0.05 versus H_2O_2, # versus control.

3.4 The anti-apoptotic effect of Genistein, Daidazein and Resveratrol on osteocyte apoptosis induced by H_2O_2.

MLO-Y4 at a density of 3 × 10^4 cells/well were plated in 24 well plates for 24 hours, and then treated with Genistein for 1 hour at concentrations of 10nM, 100nM and 1μM prior to treatment with 0.4mM H_2O_2 for 2 hours. The percentage of apoptotic cells induced by H_2O_2 (48% ± 3.8) was significantly reduced after pre-treatment with Genstein at a concentration of 10nM (25% ±1.7, p=0.0013), 100nM (27% ± 2.5, p=0.0017) and 1μM (31% ± 2.3, p=0.012) Fig. 3.5.

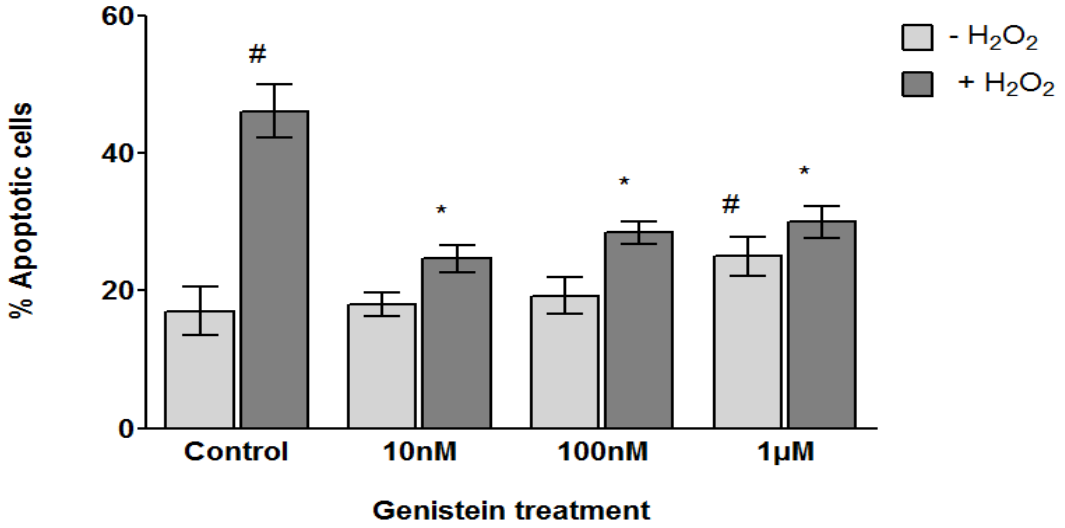

Figure 3.5: Effect of Genstein on on H_2O_2 induced apoptosis in MLOY4 cell line. Pre-treatment of MLO-Y4 cells with Genistein at concentrations of 1μM, 100nM and 10nM reduced the percentage of MLOY4 apoptosis induced by treatment with 0.4mM H_2O_2. Data were expressed as mean ± SD of three wells (three fields per well were counted), n=9, *p<0.05 versus H_2O_2, # versus control

Similarly, Daidazein was able to reduce H_2O_2 induced apoptosis. The percentage of apoptotic cells induced by H_2O_2 (26.9% ± 2.1) was significantly reduced after pre-treatment with Daidzein at a concentration of 10nM (15.6% ± 1.5, *p*=0.001), (17% ± 2.06, p=0.0165) at a concentration of 100nM, (15% ± 2.2, *p*=0.021) at a concentration of 1μM (Fig. 3.6), (*p*<0.05).

Pre-reatment of MLO-Y4 cells with Resveratrol at a concentration of 10nM significantly reduced the percentage of apoptosis induced by H_2O_2 (48% ± 3.8) at a concentration of 10nM (16.5% ± 2.4, *p*=0.001), (20% ± 2.1, *p*=0.016) at 100nM and (22% ± 0.5, *p*=0.017) at 1μM (Fig. 3.7).

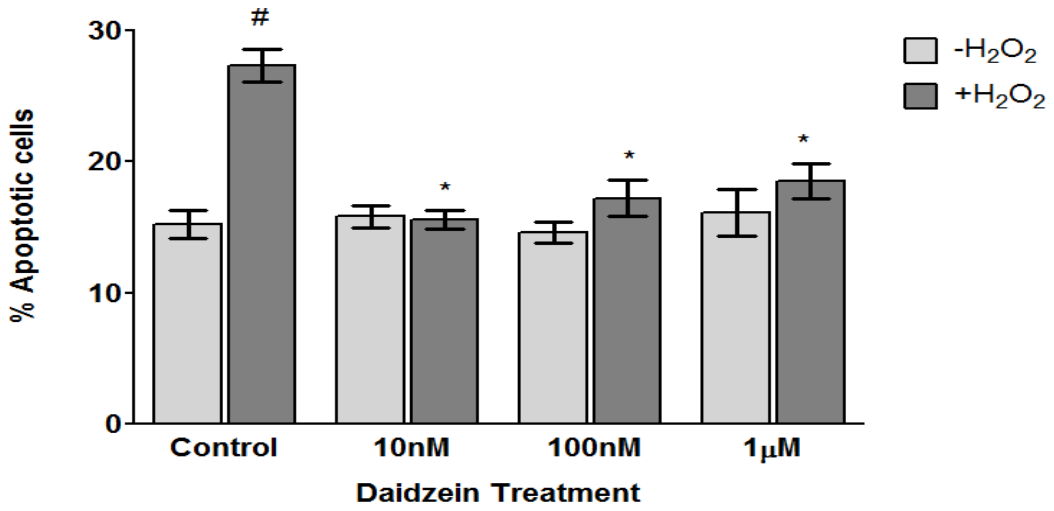

Figure 3.6: Effect of Daidzein on H_2O_2 induced apoptosis in MLOY4 cell line. Pre-treatment of MLO-Y4 cells with Daidazein at concentrations of 10nM, 100nM and 1μM reduced the percentage of apoptotic bodies induced by treatment with 0.4mM H_2O_2. Data represent mean ± SD of three wells (three fields per well were counted), n=9, *p<0.05 versus H_2O_2, # versus control

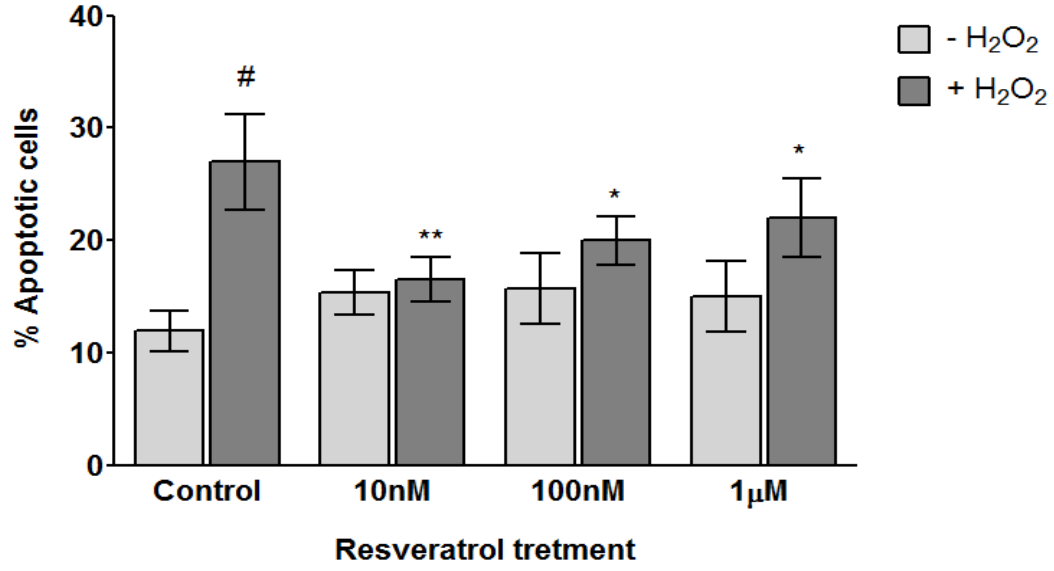

Figure 3.7: Effect of Resveratrol on H_2O_2 induced apoptosis in MLOY4 cell line. Pre-treatment of MLO-Y4 cells with Resveratrol at concentrations of 10nM, 100nM and 1μM reduced the percentage of apoptotic osteocytes induced by treatment with H_2O_2 at concentration of 0.4mM. Pre-incubation of Resveratrol at a concentration of 10nM reduced the percentage of apoptosis in MLO-y4 cells significantly, but the reduction was not significant at other concentrations. Data is expressed as mean ± SD of three wells (three fields per well were counted), n=9, *p<0.05, **p<0.001 versus H_2O_2, # versus control.

3.5. Effect of Fucosterol on oxidative stress induced by Hydrogen Peroxide in MLO-Y4 cells

The effect of Fucosterol on protecting MLO-Y4 cells from apoptosis induced by $H_2O_2$was investigated. MLO-Y4 were plated in 24 well plates for 24 hours, and then treated with Fucosterol for 1 hour at a range of concentrations (10nM-1μM) before treatment with 0.4mM H_2O_2 for 2 hours. The percentage of apoptosis induced by 0.4mM H_2O_2 (11.5% ± 2.2) was significantly reduced on pre-treatment with Fucosterol for 1 hour at concentrations of 10nM (6.2% ± 1.39, p=0.02), 100nM (6.3% ± 2.92; p=0.011) and 1μM (8.97% ± 2.63; p=0.02) Fig.3.8.

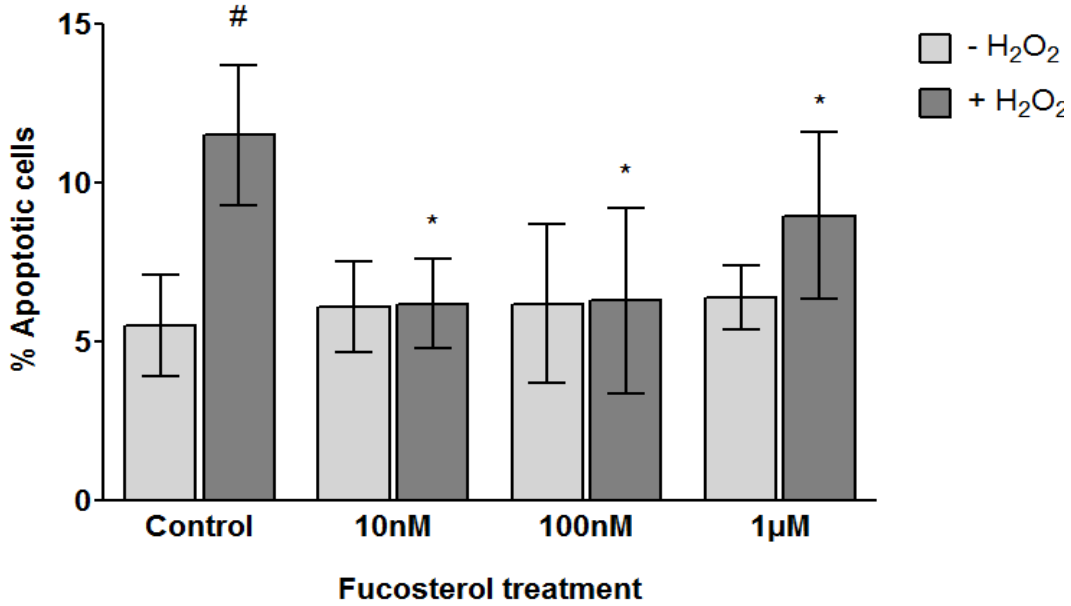

Figure 3.8: Effect of Fucosterol treatment on H_2O_2 induced apoptosis in MLOY4 cell line. Pre-treatment of MLO-Y4 with Fucosterol at concentrations of 10nM, 100nM and 1μM all significantly reduced the percentage of apoptosis induced with H_2O_2 at concentration of 0.4mM for 2 hours. Data were expressed as ± SD of three wells (three fields per well were counted), *p<0.05 versus H_2O_2, # versus control

3.6 Effect of 6-Gingerol on protecting MLO-Y4 cells from apoptosis

The protecting effect of 6-Gingerol on osteocytes from H_2O_2 induced apoptosis was investigated. MLO-Y4 were plated in 24 well plates for 24 hours, prior to treatment with 6-Gingerol for 1 hour at a concentration of 10μM, prior to treatment with 0.4mM H_2O_2 for 2 hours. The percentage of apoptosis induced by 0.4mM H_2O_2 (12.10% ± 1.58) was significantly reduced on pre-treatment with 6-Gingerol for 1 hour (6.2± 2.7, p=0.015) Figure 3.9.

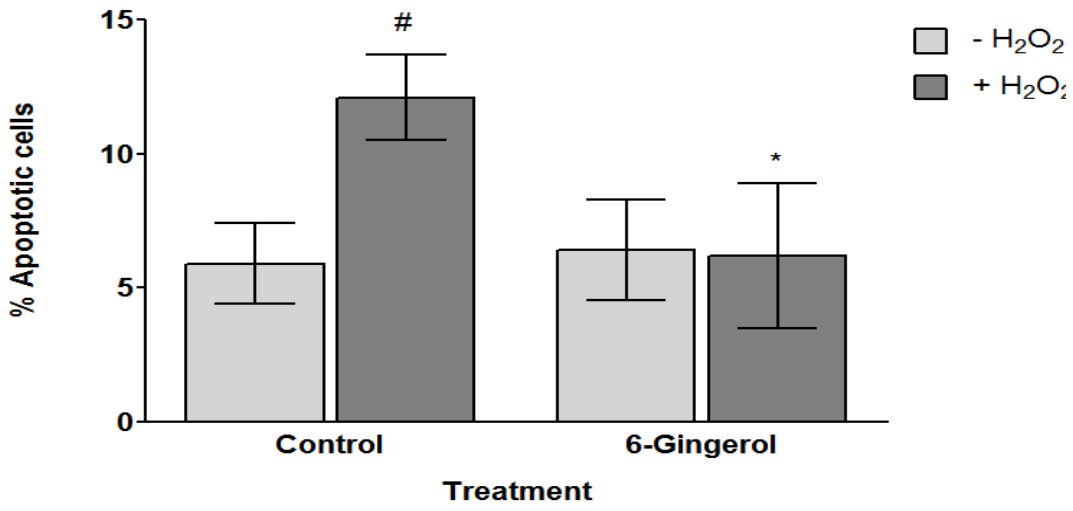

Figure 3.9: Effect of 6-Gingerol treatment on H_2O_2 induced apoptosis in MLOY4 cells. Pre-treatment of MLO-Y4 cells with 10μM of 6-gingerol reduced the percentage of apoptosis induced with H_2O_2 at a concentration of 0.4mM for 2 hours. Data were expressed as ± SD of three wells (three fields per well were counted), n=9, *p<0.05 versus H_2O_2, #versus control.

3.7 Detection of intracellular ROS

ROS is the key factor with regards to ageing and is involved in several degenerative disorders and could be the cause of osteocyte death on E2 withdrawal (Bonwald 2011). The majority of cellular ROS are generated by the mitochondrial electron transport chain during normal metabolism. In order to determine whether phytoestrogen could reduce ROS generation induced by H_2O_2 in MLO-Y4 cells, cells were exposed to E2 or Genistein, Daidazein and Resveratrol at a concentration of 10nM for 1 hour prior to incubation with H_2O_2 at a concentration of 0.4mM for 2 hours. These concentrations were used based on the previous experiments. The ability of H_2O_2 to generate ROS was investigated using 2', 7'-dichlorofluorescein (H2DCF-DA) Fig.3.10. The proportion of ROS positive cells was increased after 2 hours of treatment with H_2O_2 when compared to control (ROS Positive cells 2.8% ±1.1 vs. H_2O_2 56.5% ± 1.79, p=0.001). Treatment with E2, which was used as a positive control to compare with PEs reduced the percentage of ROS positive cells (10.37% ± 2.12, p=0.0001) Fig.3.11. Also treatment with Genistein, Daidzein, and Resveratrol reduced the level of positive ROS cells at concentration of 10nM each which was similar to that seen in treatment with E2 at a concentration of 10nM. The percentage of ROS positive cells in pre-treatment with Genistein was (10.7% ± 1.5, p=0.0001) Fig. 3.12, Daidzein (11.87% ± 1.526%, p=0.0001) Fig.3.13 and Resveratrol (10.27% ± 1.535, p=0.0001) Fig.3.14.

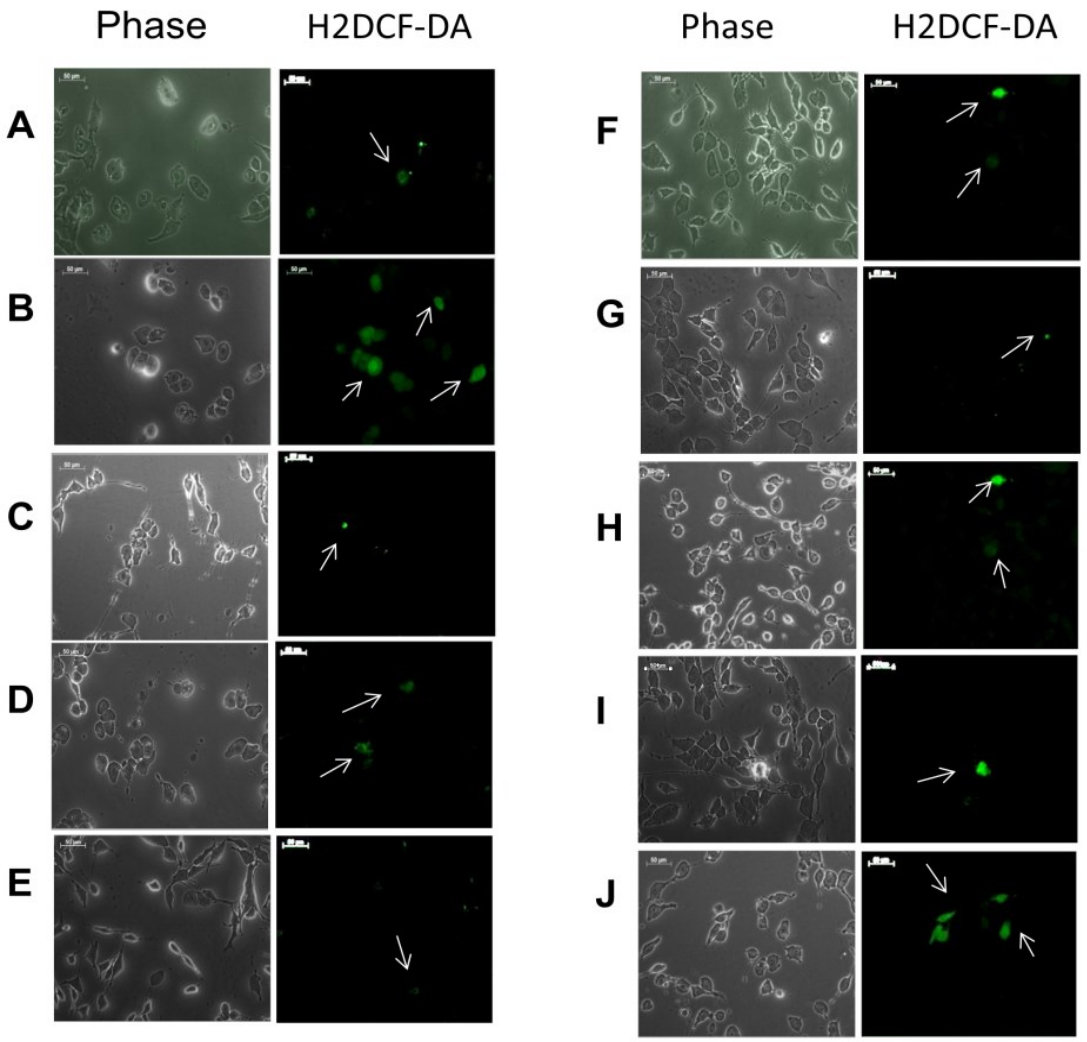

Figure 3.10 Representative images of ROS activity in MLO-Y4 cells. Phase contrast and H2DCF-DA. **(A)** Represents control. **(B)** Treatment with H_2O_2 at a concentration of 0.4mM. **(C)** Treatment with E2 at a concentration of 10nM (**D)** Treatment with E2 at a concentration 10nM prior to treatment with H_2O_2. **(E)** Treatment with Genistein at concentration of 10nM. **(F)** Treatment with Genistein at a concentration of 10nM prior to treatment with H_2O_2. **(G)** Treatment with Daidazein at a concentration of10nM. **(H)** Treatment with Daidzein at a concentration of 10nM prior to treatment with H_2O_2**. (I)** Treatment with Resveratrol at concentration of10nM. **(J)** Treatment with Resveratrol at a concentration of 10nM prior to H_2O_2 treatment. White arrows indicate ROS positive cells. Scale bar 50µm.

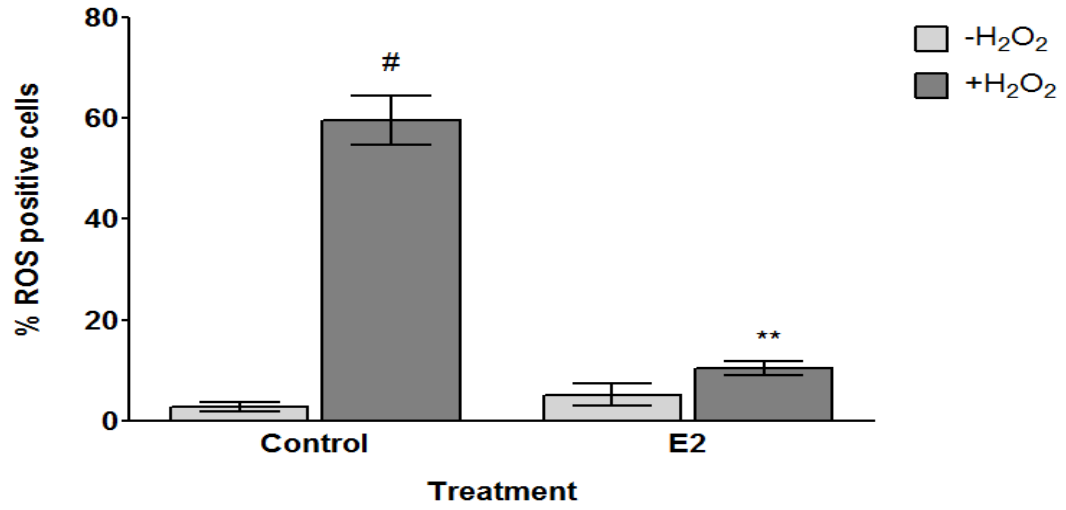

Figure 3.11: Percentage of ROS positive cells in MLO-Y4 cells pre-treated with E2 at a concentration of 10nM (E2) prior to treatment with H_2O_2 at a concentration of 0.4mM. E2 Pre-treatment significantly reduced the percentage of ROS positive cells on H_2O_2 treatment. Data were expressed as means ± SD of triple wells (3 fields per well were counted)* p<0.0001 versus H2O2, # versus control.

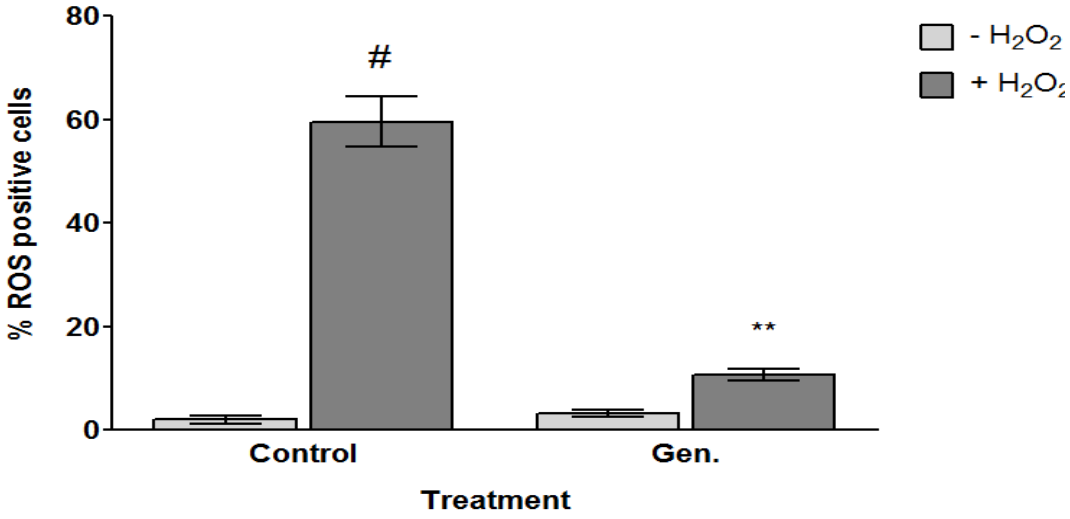

Figure 3.12: Percentage of ROS positive cells in MLO-Y4 cells pre-treated with Genistein at a concentration of 10nM prior to treatment with 0.4mM H_2O_2. Pre-treatment of MLO-Y4 cells with 10nM of Genistein significantly reduced the percentage of ROS positive cells on H_2O_2 treatment. Data were expressed as means ± SD of triple wells (3 fields per well were counted) ** *p*< 0.0001versus H_2O_2, # versus control.

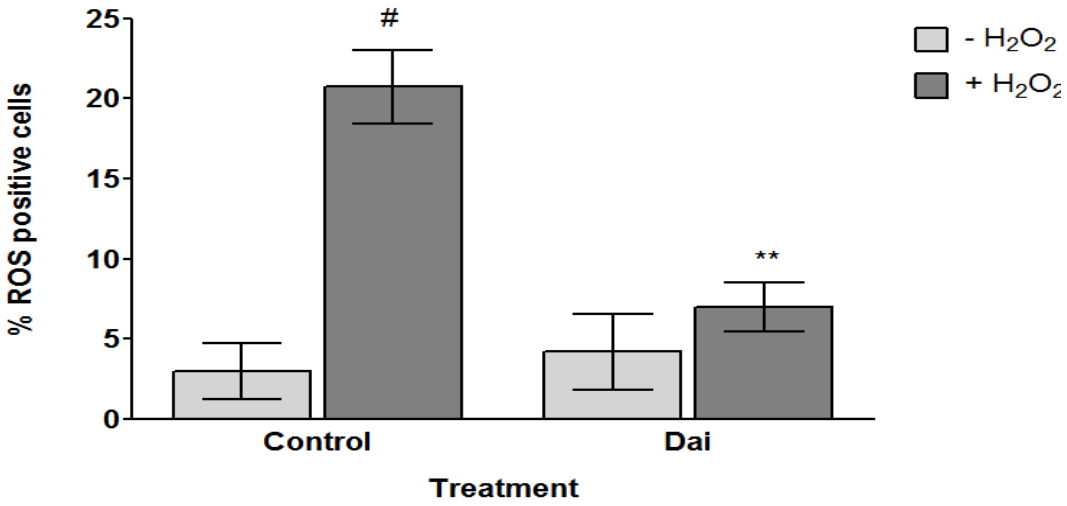

Figure 3.13: Percentage of ROS positive cells in MLO-Y4 cells treated with Daidzein at a concentration of 10nM prior to treatment with 0.4mM H_2O_2. Pre-treatment with 10nM of Daidzein significantly reduced the percentage of ROS positive cells on H_2O_2 treatment. Data were expressed as means ± SD of triple wells (3 fields per well were counted) * p< 0.0001 versus H_2O_2, # versus control.

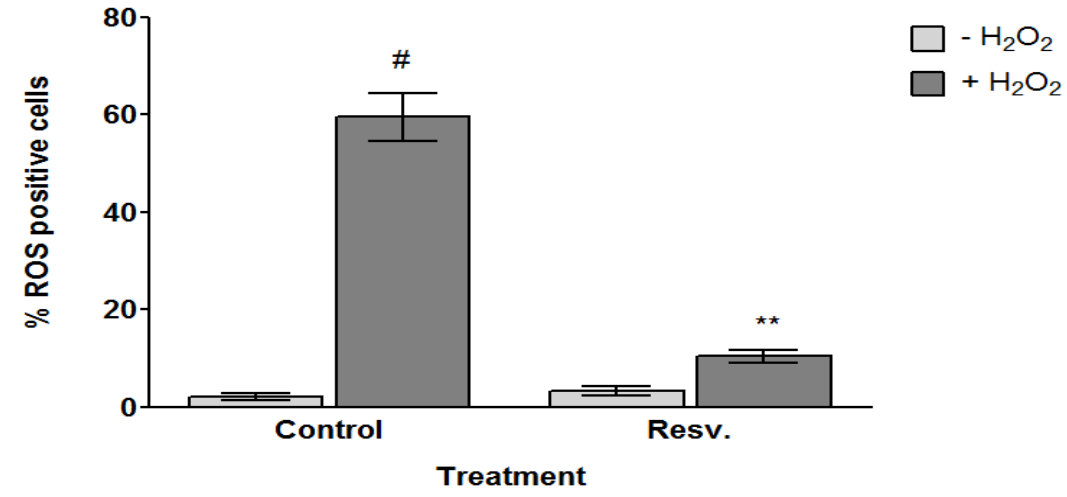

Figure 3.14: Percentage of ROS positive cells in MLO-Y4 cells pre-treated with Resveratrol at a concentration of 10nM prior to treatment withH_2O_2. Pre-treatment with 10nM of Resveratrol significantly reduced the percentage of ROS positive cells on treatment with H_2O_2. Data were expressed as means ± SD of triple wells (3 fields per well were counted) **p< 0.0001versus H_2O_2, # versus control.

3.7.1 Effect of Fucosterol on intracellular ROS generation

To determine the effect of Fucosterol on ROS generation induced by H_2O_2 in MLO-Y4 cells, cells were treated with fucosterol at a concentration of 10nM for 1 hour prior to incubation with H_2O_2 for 2 hours. The ability of ROS generation by H_2O_2 was investigated using 2', 7'-dichlorofluorescein (H2DCF-DA). The level of ROS positive cells was detected by counting ROS positive cells which had increased after 2 hours of treatment with H_2O_2 compared to control (H_2O_2 25.85% ± 4.5 vs. Control 5.52% ± 1.5, p=0.000). Treatment with Fucosterol at a concentration of 10nM significantly reduced the level of ROS positive cells to (12.16% ± 2.89, p=0.0001) Fig. 3.15.

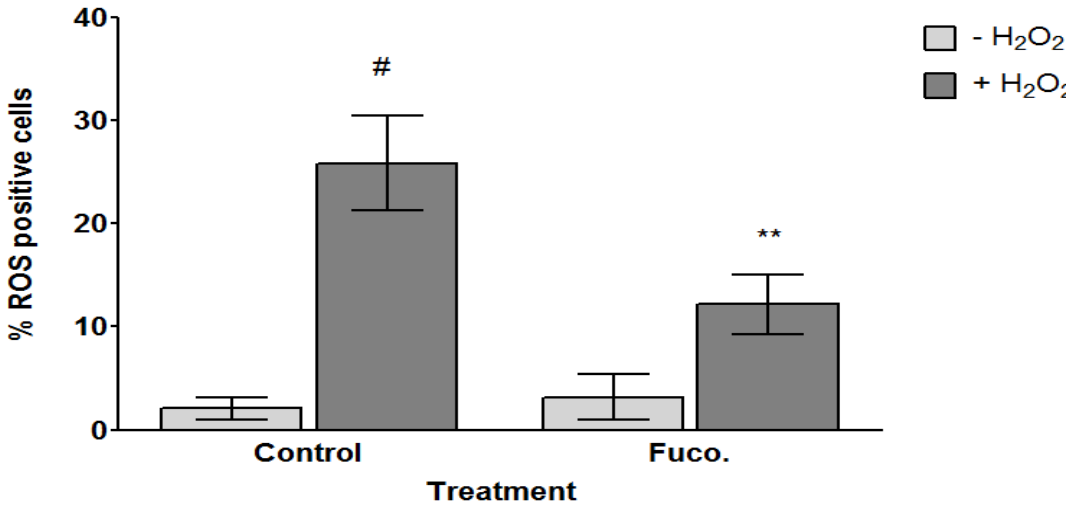

Figure 3.15: Percentage of ROS positive cells in MLO-Y4 cells treated with Fucosterol at a concentration of 10nM prior to treatment with H_2O_2. Pre-treatment with 10nM Fucosterol significantly reduced the percentage of ROS positive cells on treatment with H_2O_2. Data were expressed as means ± SD of triple wells (3 fields per well were counted) **p< 0.0001 versus H_2O_2, # versus control.

3.7.2 Effect of 6-Gingerol on intracellular ROS generation

The ability of ROS generation in MLO-Y4 cells was investigated by H_2O_2 using 2', 7'-dichlorofluorescein (H2DCF-DA). The level of ROS positive cells was detected by counting ROS positive cells which had increased after 2 hours of treatment with H_2O_2 (56.93% ± 2.7). Treatment with 10μM of 6-gingerol significantly reduced the percentage of ROS generation (8.10 ± 4, *P*=0.0001) Fig. 3.16.

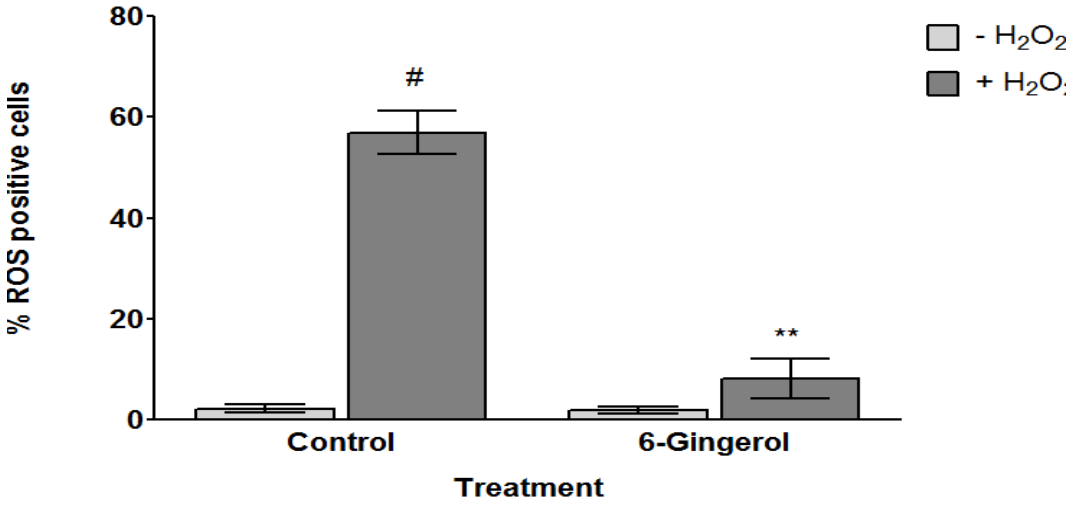

Figure 3.16: Percentage of ROS positive cells in MLO-Y4 cells treated with 6-Gingerol at a concentration of 10 μM prior to treatment with H_2O_2. Pre-treatment with 6-Gingerol at a concentration of 10μM significantly reduced the percentage of ROS positive cells on treatment with H_2O_2. Data were expressed as means ± SD of triple wells (3 fields per well were counted**)** ***p*< 0.0001 versus H_2O_2, # versus control.

3.8 Investigation of role of E2 and Genistein, Daidzein and Resveratrol on caspase3, 7 activation

A distinctive feature of the early stages of apoptosis is the activation of caspase enzymes, these enzymes participate in a series of reactions that are triggered in response to pro-apoptotic signals and result in the cleavage of protein substrates and in the subsequent disassembly of the cell. The effect of E2 and PEs on caspase activation induced by H_2O_2 in MLO-Y4 cells was investigated. Cells were pre-treated with either E2, Genistein, Daidzein or Resveratrol followed by treatment with H_2O_2 as described in material and method section 2.2.12. Caspase activation was detected using Image-iT ™ live red Caspase-3 and -7-detection kit, (Figure 3.17 and 3.18). Incubation of MLOY4 with H_2O_2 at a concentration of 0.4mM increased caspase activity. The percentage of cells staining positive for Caspase -3 and -7 were decreased in MLOY4 following pre-treatment with E2 (6.81% ± 0.7 vs. H_2O_2 treatment 12.81% ± 3.7; p=0.002) Fig.3.19.

In a similar way treatment with Genistein, Daidzein and Resveratrol significantly inhibited caspase activity. The percentage of cells stained positive for caspase 3, 7 in cells pre-treated with Genistein was (6.97% ± 1.41, p=0.0363) Fig.3.20, cells pre-treated with Daidzein (8.8% ± 1.1, p=0.038) Fig.3.21 and in cells treated with resveratrol is (6.82% ± 1.11, p=0.02) Fig.3.22.

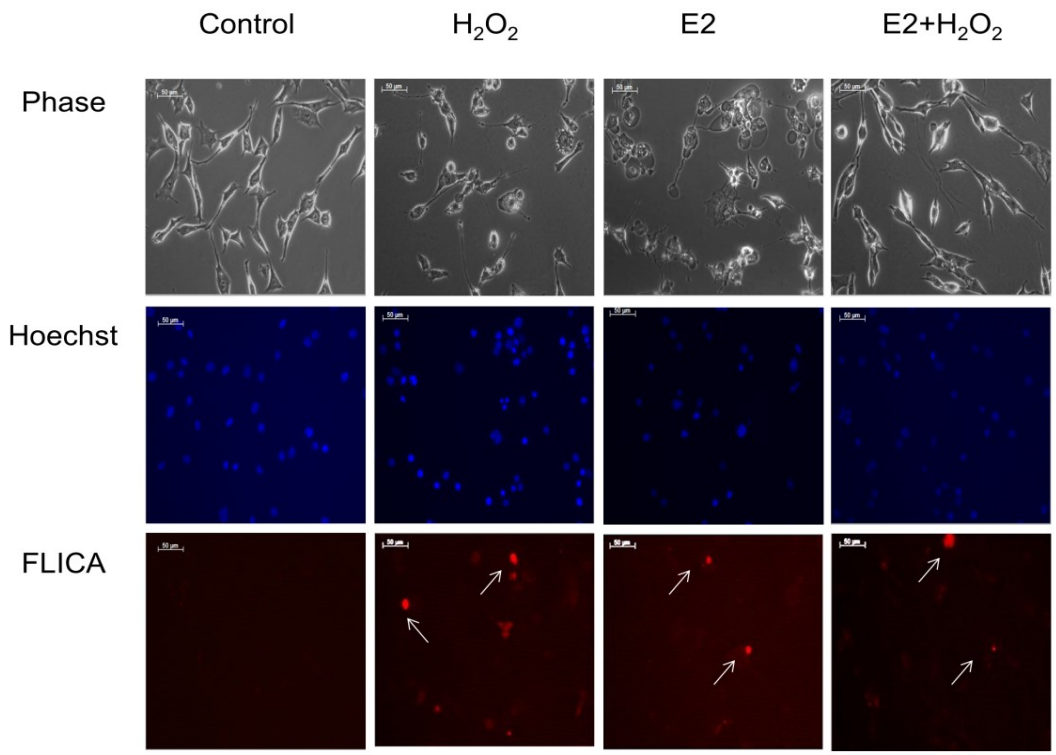

Figure 3.17: Detection of caspase activity in MLO-Y4 cells induced by H_2O_2. Cells were pre-treated with 10nM E2 for 1 hour followed by treatment with H_2O_2 at a concentration of 0.4mM for 2 hours**.** Cells were labelled with FLICA reagent and viewed under fluorescence. White arrows indicate cells staining positive for Caspase 3 and 7.

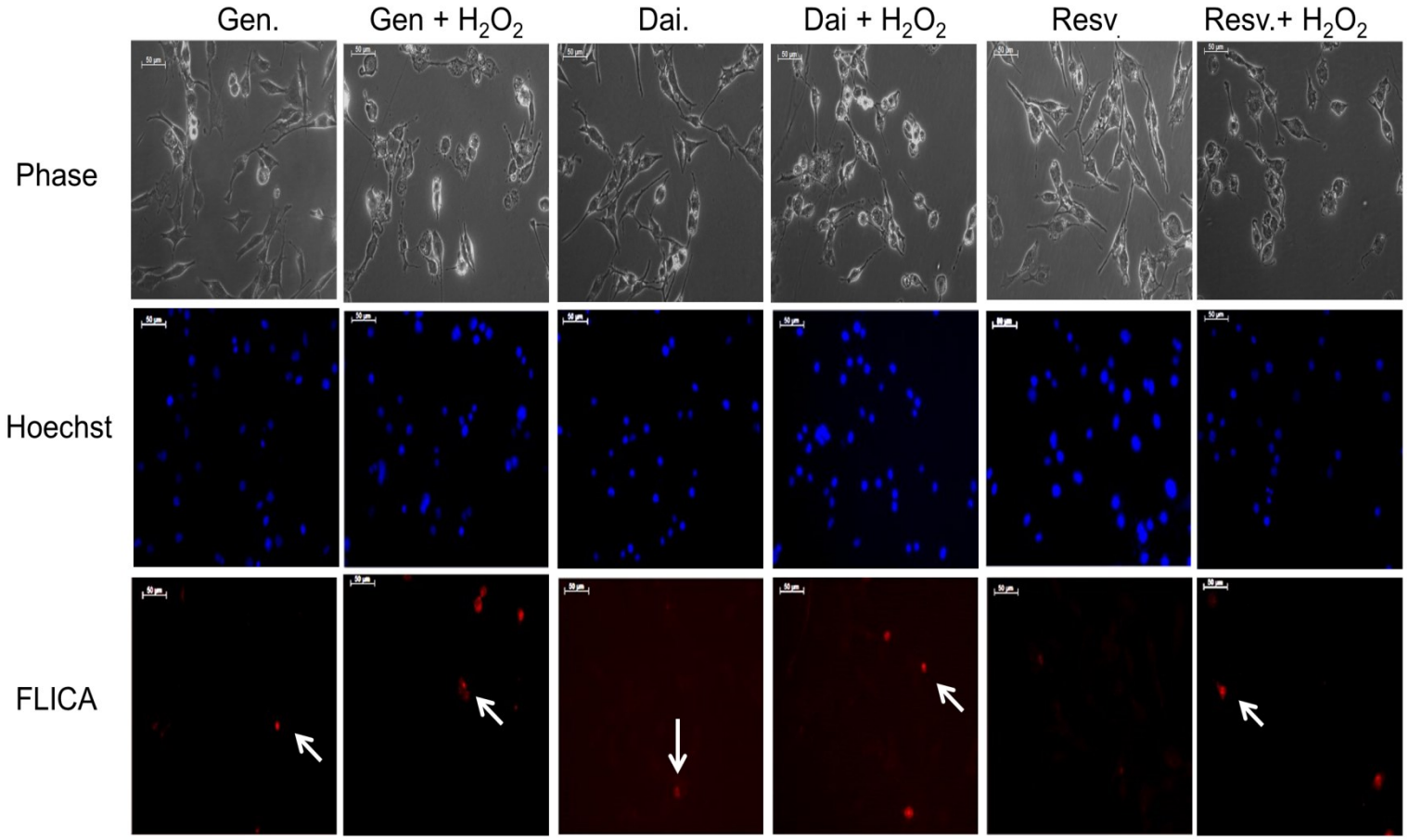

Figure 3.18: Detection of caspase activity in MLO-Y4 induced by H_2O_2. Cells were pre-treated with 10nM Genistein, Daidzein and Resveratrol for 1 hour followed by treat-ment with 0.4mM of H2O2 for 2 hours. Cells were labelled with FLICA reagent and viewed under fluorescence. White arrows indicate cells staining positive for Caspase 3 and 7.

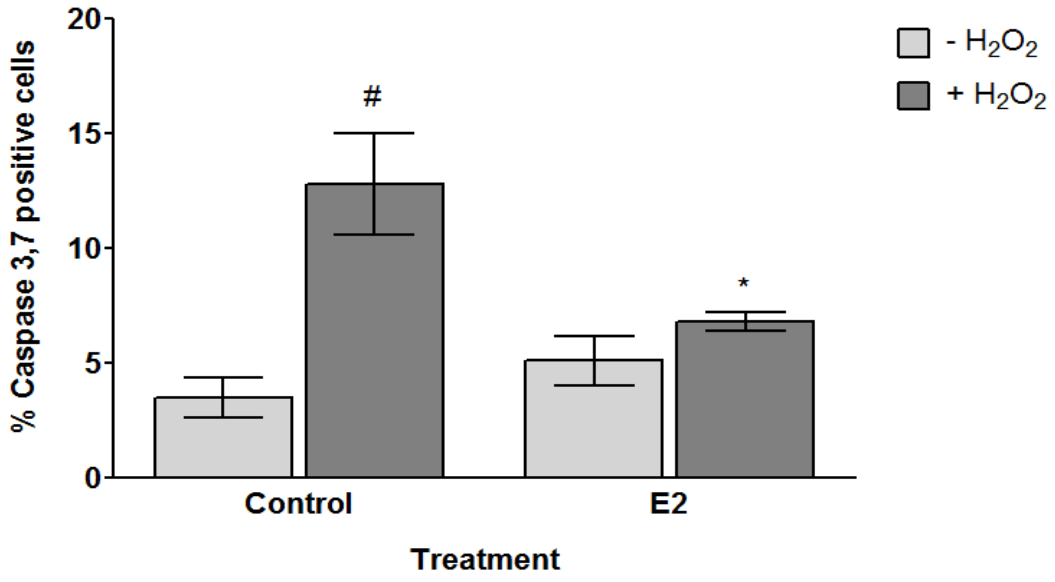

Figure 3.19: Detection of caspase 3 and 7 activity in MLO-Y4 cells pre- treated with E2 prior to H_2O_2 treatment at a concentration of 0.4mM. Pre-treatment of cells with E2 decreased Caspase activity on challenge with H_2O_2. Data were expressed as means ± SD of triple wells (three fields per well counted) *p<0.05 versus H_2O_2, # versus control.

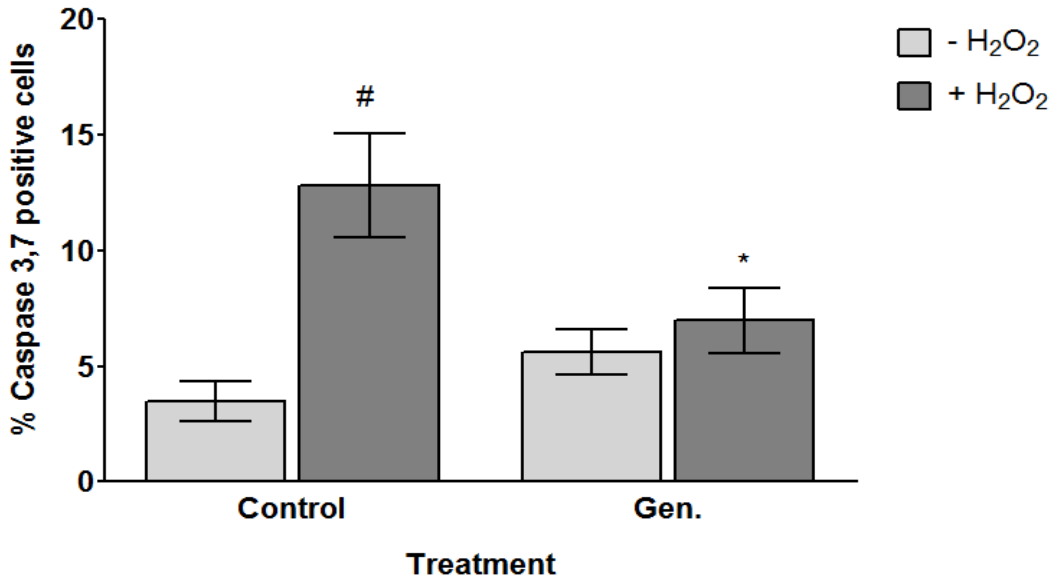

Figure 3.20: Detection of caspase 3 and 7 activity in MLO-Y4 cells pre-treated with Genistein prior to H_2O_2 treatment at a concentration of 0.4mM. Pre-treatment of MLO-Y4 cells with 10nM of Genistein significantly reduced caspase activity on challenge with H_2O_2. Data were expressed as means ± SD of triple wells (three fields per well counted) *p<0.05 versus H_2O_2, # versus control.

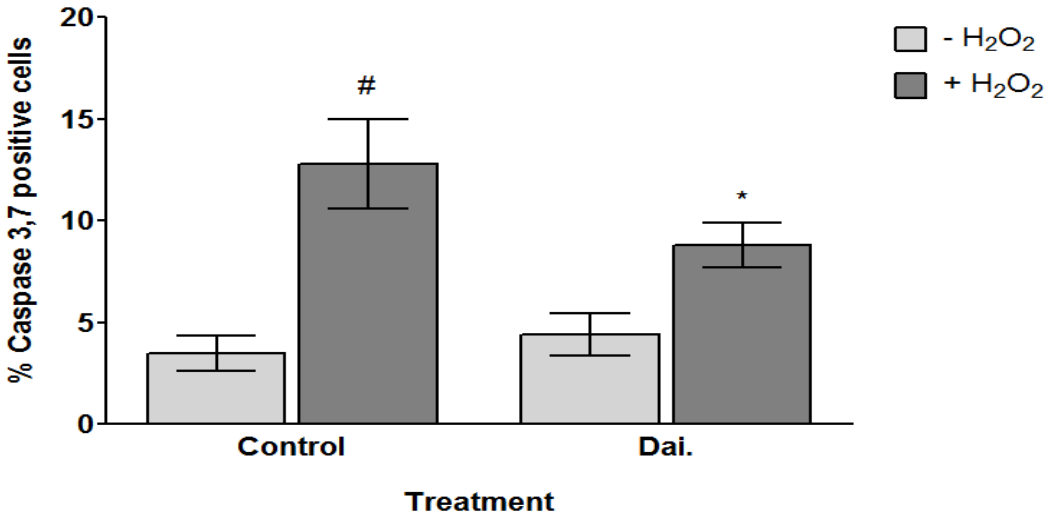

Figure 3.21: Detection of caspase 3 and 7 activity in MLO-Y4 cells pre- treated with Daidzein prior to H_2O_2 treatment at a concentration of 0.4mM. Pre-treatment of MLO-Y4 cells with Daidzein at a concentration of 10nM significantly reduced caspase activity on challenge with H_2O_2. Data were expressed as means ± SD of triple wells (three fields per well counted) *p<0.05 versus H_2O_2, # versus control.

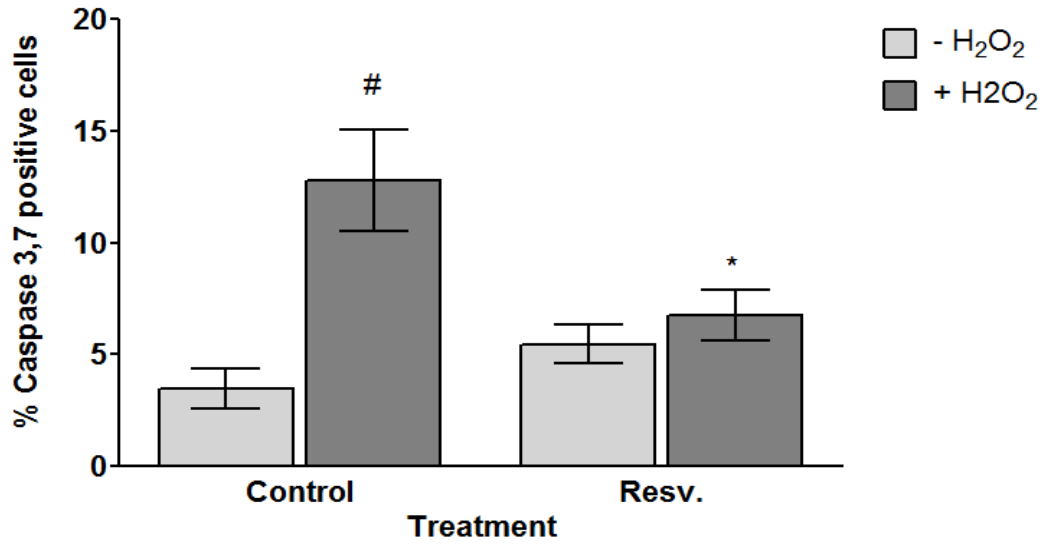

Figure 3.22: Detection of caspase 3 and 7 activity in MLO-Y4 cells pre-treated with Resvertrol prior to H_2O_2 treatment at a concentration of 0.4mM. Pre-treatment of MLO-Y4 cells with Resveratrol at a concentration of 10nM significantly reduced caspase activity on challenge with H_2O_2. Data were expressed as means ± SD of triple wells (three fields per well counted) *p<0.05 versus H_2O_2, # versus control.

3.9 Effect of 6-Gingerol on caspase activity

The effect of 6-Gingerol on caspase activity was detected using Image-iT ™ live red Caspase-3 and 7 detection kit after treatment with 10μM of 6-Gingerol before treatment with 0.4mM H_2O_2 for 2 hours. H_2O_2 tratment increased the caspase activity in MLO-Y4 cells. The percentage of cells stained positive for caspase 3, 7 were decreased in MLOY4 cells following pre-treatment with 6-Gingerol (6.61% ± 0.90, vs H_2O_2 treatment 16.69% ± 1.58, p =0.002) Fig 3.23.

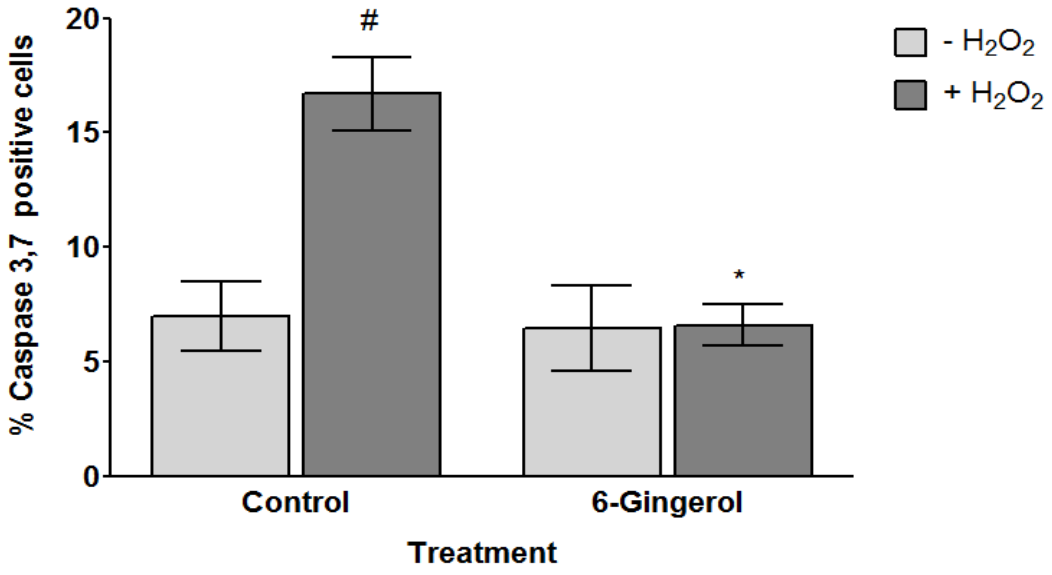

Figure 3.23 : **Detection of caspase 3 and 7 activity in MLO-Y4 cells pre-treated with 6-Gingerol prior to H_2O_2 treatment at a concentration of 0.4mM.** Pre-treatment of MLO-Y4 cells with 6-Gingerol at a concentration of 10μM significantly reduced caspase activity. Data were expressed as means ± SD of triple wells (three fields per well counted) *p<0.05 versus H_2O_2, # versus control.

3.10 Discussion

In this study, H_2O_2 has been shown to induce MLO-Y4 apoptosis as confirmed by Annexin V staining. H_2O_2 induced osteocyte apoptosis in a dose dependent manner, which is consistent with other studies (Mann *et al.,* 2007). Many conditions such as ischemia, reperfusion and myocardial infarction can indirectly lead to oxidative stress, which could influence osteocytes resulting in an increase in intracellular ROS that is the major determinant of ageing (Manolagas 2010). Increased levels of ROS generation has been involved in tissue homeostasis in bone tissue and affects bone remodelling by promoting bone resorption (Wauquier *et al*., 2009). Studies have suggested the role of oxidative stress in bone diseases such as osteoporosis, diabetes-induced bone diseases and vascular diseases (Hamada *et al.,* 2014). Oxidative stress results in the disregulation of redox balance in the tissue. In this study, H_2O_2 was used as a model of ROS to induce apoptosis in MLO-Y4 osteocyte like cell line. The concentration of H_2O_2 used was based on other literature (Mann *et al.,* 2007).

Estrogen deficiency in postmenopausal women increases the incidence of osteogenesis-associated fracture and estrogen HRT has been suggested as the first-line treatment for osteoporosis, but prolonged usage of HRT is one of the risk factors for incidence of breast, endometrial and high level of ovarian cancer as well as cardiovascular diseases (Beral 2003).

In this study, the effect of a range of phytomolecules, Genistein, Daidzein, Resveratrol, Fucosterol and 6-Gingerol on H_2O_2 induced apoptosis in MLO-Y4 cells were examined. Apoptosis was induced in MLOY4 by exposure to H_2O_2, as evidenced by a range of methods such as Annexin V staining, mor-

phology characterisation such as membrane blebbing evidenced by DAPI staining (Fig.3.3) and caspase activation (Fig.3.16). It has been observed that MLO-Y4 cells pre-incubated with 17β-estradiol prior to H_2O_2 treatment reduced the number of apoptotic nuclei. This protective action was dose dependent namely 1μM, 100nM and 10nM. A maximal effect was detected at a concentration of 17β-estradiol 1μM however, at this concentration, apoptosis was also increased when compared to control (Fig.3.4); this result was consistent with other work published by (Mann *et al.,* 2007) and provides a comparator for the potential protecting effects of phytoestrogen molecules. Cell treatment with 17β-estradiol at a concentration of 10nM has shown to be non-toxic to MLO-Y4 cells, on the other hand, blocked the apoptotic effect induced by H_2O_2, as previously described (Mann *et al*., 2007). Geistein, Daidazein and Resveratrol were also shown to block MLOY4 apoptosis induced by H_2O_2. This protection is thought to be due to the structural similarity of these phytomolecules to estrogen which is related to the presence of hydroxyl group at the C3 position of the A ring of the hormone (Behl *et al.,* 1997). Genistein and Daidzein have been found to have a stimulatory effect on protein synthesis and on alkaline phosphatase release by osteoblastic MC3T3-E1 cells *in vitro* (Sugimoto *et al.,* 2000, Yamaguchi *et al*., 2000). More recently, Genistein has been found to stimulate the production of osteoprotegerin by human osteoblasts, providing a further mechanism for the bone-sparing effects of soy isoflavones (Viereck *et al*., 2002). Genistein and Daidzein both inhibit osteoclast activity by a number of possible mechanisms, including induction of apoptosis, activation of protein tyrosine phosphatase, inhibition of cytokines, changes in intracellular Ca^{2+}, and membrane depolari-

zation (Ming *et al*., 2013, Williams *et al.,* 1998). The presence of estrogen receptors in osteoblasts, osteoclasts and osteocytes (Arts *et al.,* 1997, Onoe *et al.,* 1997) and the wide-ranging biological properties of these non-steroidal dietary estrogens (Akiyama *et al.,* 1987, Kim *et al.,* 1998) provide good foundation for thinking that dietary phytoestrogens could play a role in bone remodelling.

Resveratrol has been thought to be involved in promotion of osteopontin and osteocalcin expression, the main osteoblastic markers in human bone marrow mesenchymal stem cells and regulates the expression of 1, 25-dihydroxy vitamin D3 and calcium uptake to help in bone formation. Additionally, *in vitro* studies suggested that Resveratrol prevents osteoclast formation through inhibiting receptor activator of nuclear factor-κB (NF-κB) ligand–induced formation of tartrate-resistant acid phosphatase (TRAP)–positive multinucleated cells, TRAP activity in the medium, up-regulation of cathepsin K gene expression, and bone resorption (Boissy *et al.,* 2005). These inhibitions are associated with a down-regulation of RANK expression at both mRNA and cell surface protein levels, and a decrease of NFATc1 stimulation and NF-κB nuclear translocation, whereas the gene expression of c-fms, CD14, and CD11a is up-regulated. Finally, Resveratrol promotes dose-dependently the expression of osteoblast markers like osteocalcin and osteopontin in human bone marrow mesenchymal stem cells (Boissy *et al.,* 2005). Moreover, it has been proven that Resveratrol prevents bone resorption and enhances osteoblast differentiation and has been proven to act as phytoestrogen (Gehm *et al.,* 1997). The anti-oxidative activity of Resveratrol

is claimed to be due to the presence of hydroxyl phenolic group in its chemical structure (Leonard *et al.,* 2003).

In this study, PEs have also been shown to influence caspase activation in apoptotic MLO-Y4 cells induced by H_2O_2, as assessed by selective caspase-3, 7. Activation of caspase enzyme is the distinctive feature for early stage apoptosis and involves an intrinsic proteolytic cascade within which caspase family of enzymes is critical (Nicholson and Thornberry 1997). The detection of caspase is based on the fluorescent inhibitor of caspase (FLICA), which is a cell inhibitor, cell permeant and non-cytotoxic.

One of the fascinating roles of ROS at a cellular level is controlling normal cell signalling, regulation of growth and apoptosis and at system level controlling blood pressure and immune function (Brieger *et al*., 2012). Conversely, ROS participate in normal ageing and age related diseases such as cancer, atherosclerosis and neurodegeneration (Finkel 2000). Many studies suggest that osteoporosis is one of the consequences of oxidative stress which is associated with reduced bone mineral density (BMD) (Basu et al 2001). ROS generation causes bone resorption by active osteoclasts (Garrett et al 1990), and their activity is dependent upon several intracellular signals that are sensitive to ROS (Lean et al 2003). Regarding antioxidant potency of phytoestrogens, this study shows that these plant derivatives are capable of reducing the level of ROS.

In this study, a range of phytomolecules were also used such as Fucosterol and 6-Gingerol, which have been known to have antioxidant effects on different cell types, therefore, these effects on MLO-Y4 were assessed.

Fucosterol has been known to possess anti-inflammatory, anti-cancer properties as well as being effective in the protection of skin from ultraviolet induced damage through inhibition of ROS generation (Yoo *et al.,* 2012, kim*et al.,* 2013). However, to date, the effects of Fucosterol on bone degeneration have not been thoroughly investigated. In this study, the ability of Fucosterol in counter act oxidative stress induced by H_2O_2 treatment of MLO-Y4 was investigated. The results obtained showed that treatment with Fucosterol reduced the percentage of apoptosis in MLO-Y4 cells when compared to H_2O_2 treatment and intracellular ROS generation was also reduced. Previous studies have suggested the role of Fucosterol in bone formation by increasing osteogenesis through cell proliferation and ALP expression in comparison to those of estradiol in an osteoblastic cell line (MG63) which has a potential to activate osteoblast (Lee *et al.,* 2014).

Ginger (*Zingiber officinale roscoe*, Zingiberaceae) is a medicinal plant that has been widely used in Chinese medicine, and is a commonly used spice in many parts of the world (Ali *et al.,* 2008), which contains 6-Gingerol, 8-gingerol and 6-shogaol all of which are phytochemical compounds. Among all these fractions, 6-Gingerol displays a wide array of biochemical and pharmacologic activities. 6-Gingerol, the major pharmacologically active component of Ginger, was reported to exhibit antioxidant and anti-inflammatory properties (Shukla *et al.,* 2007). Previous studies have not actively tested the anti-apoptotic effect of 6-Gingerol and mostly focused on the apoptosis-promoting properties in certain cancer cells. However, an active component of dried Ginger, Shogaol, attenuated apoptotic cell death in a spinal cord injury model by inhibiting poly(ADP-ribose) polymerase activity and

reducing astrogliosis (change in astrocyte phenotype in response to all CNS injuries and diseases) (Kyung *et al.,* 2006).

An earlier study reported that 6-Gingerol had genotoxic effects on liver carcinoma and it could destabilize the lysosomal membrane with higher ROS induction (Elmore 2007), leading to apoptosis of HepG2 cells (Yang *et al.,* 2010). Other studies have reported that 6-Gingerol had no toxic effect on normal cells (Singh *et al.,* 2009). However, the mechanism of action of 6-Gingerol remains unclear, and therefore needs further study. In this study, the main objective was to explore its role in prevention of oxidant-induced apoptosis in MLO-Y4 cells. It was also interesting to see whether 6-Gingerol pre-treatment was able to protect MLO-Y4 cells against oxidative stress induced by H_2O_2 and decrease the percentage of apoptosis compared to treated cells with H_2O_2.

The anti-oxidative effect of 6-Gingerol was detected by H2DCFDA and as predicted, 6-Gingerol as an antioxidant was shown to protect MLO-Y4 cells. Moreover, it induced inhibition of caspase 3 activation which is the most important type of caspase which actively takes part in proteolytic cleavage of PARP (poly-ADP ribose polymerase) protein. Increased expression of PARP is indication of a greater extent of DNA degradation. Caspase 3 and PARP are therefore considered as significant markers of cells undergoing apoptosis (Biswas *et al.,* 2011). To further determine the involvement of caspase 3 in 6-Gingerol treated cells, caspase 3 activation was assessed and activation subjected with red (FLICA) reagent was also assessed. The results suggested that the inhibition of caspase 3 activation in MLO-Y4 cells might account for the mechanism of 6-Gingerol protection from apoptosis. This sug-

gest that 6-Gingerol protect cells from apoptosis by mediating reactive oxygen species and inhibition of caspase activation, as would be the case for other PEs, as these all had the same effect on caspase activation.

Fucosterol and 6-Gingerol which share structural similarities to E2 also have similar effects to that of Genistein, Daidzein and Resveratrol on protecting cells from oxidative stress and inhibition of ROS generation. These results provide further evidence that PEs have a potential in the prevention of oxidants induced in MLO-Y4 cells. Therefore, it is worth exploring the potential protecting effects of Genistein, Daidzein and Resveratrol in osteoblasts induced by H_2O_2 and the potential to influence osteoclast differentiation.

CHAPTER FOUR: THE EFFECT OF PHYTOESTROGEN ON HYDROGEN PEROXIDE INDUCED APOPTOSIS IN MC3T3-E1 AND RAW 264.7 CELLS DIFFERENTIATION

4.1 Introduction

Bone remodelling is a constant process in the skeleton to ensure correct mineral homeostasis and to maintain the structural integrity and thereby bone strength (Martin and Seeman, 2008, Henriksen, *et al*. 2009). This process is maintained by osteoblasts, bone forming cells and osteoclasts, bone resorbing cells.

Ageing and loss of sex steroids have adverse effects on skeletal homeostasis. Changes in bone strength which is associated with a decrease in bone remodelling as evidenced by decreased osteoblast and osteoclast numbers and decreased bone formation rate; as well as increased osteoblast and osteocyte apoptosis (Almeida*, et al*. 2007). Low E2 levels in post-menopausal women results in an increase in bone remodeling and increased bone resorption (Manolagas *et al*. 2002). Thus, bone loss increases fracture risk in trabecular areas such as the femoral neck and distal radius and ulna.

Soy is a component of several foods which are thought to maintain bone health (Katsuyama *et al*. 2009). Several studies noted decreases in resorption markers following PEs supplementation (Weaver *et al*., 2009) and *in-vitro* studies show a direct suppressive effect of PEs on cytokine-induced osteoclast differentiation (Karieb and Fox, 2011). In addition to suppressing resorption, PEs have also been shown to increase bone formation markers such as serum ALP and osteocalcin levels in post-menopausal women (Morabito *et al*. 2002, Roudsari *et al*., 2005). Genistein has also been shown to increase mineral apposition and bone formation rates in ovariectomised rats (Dai *et al*. 2008) and PEs stimulate osteoblast differentiation and mineralisation *in-vitro* (Kanno *et al*. 2004; Wu *et al*. 2009). Estrogen loss has been

shown to increase the levels of reactive oxygen species (ROS) and consequently, increased bone porosity as a result of an imbalance in the bone remodeling process. Therefore, it is important to look at the effect of PEs on other bone cells such as osteoblasts and osteoclasts. Also if PE's have the potential to augment therapeutic interventions it will be important to understand the effects on other bone cells. Murine cells MC3T3-E1 and RAW 264.7 cell lines were used as models for osteoblasts and osteoclasts respectively.

4.2 Saving effect of PEs on MC3T3-E1 cells

The effect of E2 and PEs (Genistein, Daidzein and Resveratrol) on H_2O_2 induced apoptosis were evaluated in MC3T3-E1 cells. Cells were cultured as described in section 2.2.1 and treated with E2 or PEs (Genistein, Daidzein and Resveratrol) at a concentration of 10nM. Apoptotic cells were detected via morphological changes as mentioned in section 2.2.7. The percentage of apoptosis induced by 0.4mM H_2O_2 (18.05% ± 1.33, p=0.0002 vs control (6.04% ± 1.4, p=0.0002) was significantly reduced on pre-treatment with E2 for 1 hour at a concentration of 10nM (9.49% ± 1.23 p=0.009). Furthermore, pre-treatment with PEs at a concentration of 10nM reduced the percentage of MC3T3-E1 apoptosis. Genistein (9.58% ± 0.97 p=0.006), Daidzein (10.7% ± 2.16, p=0.0056) and Resveratrol (10.9% ± 1 p=0.0022) Figure 4.1.

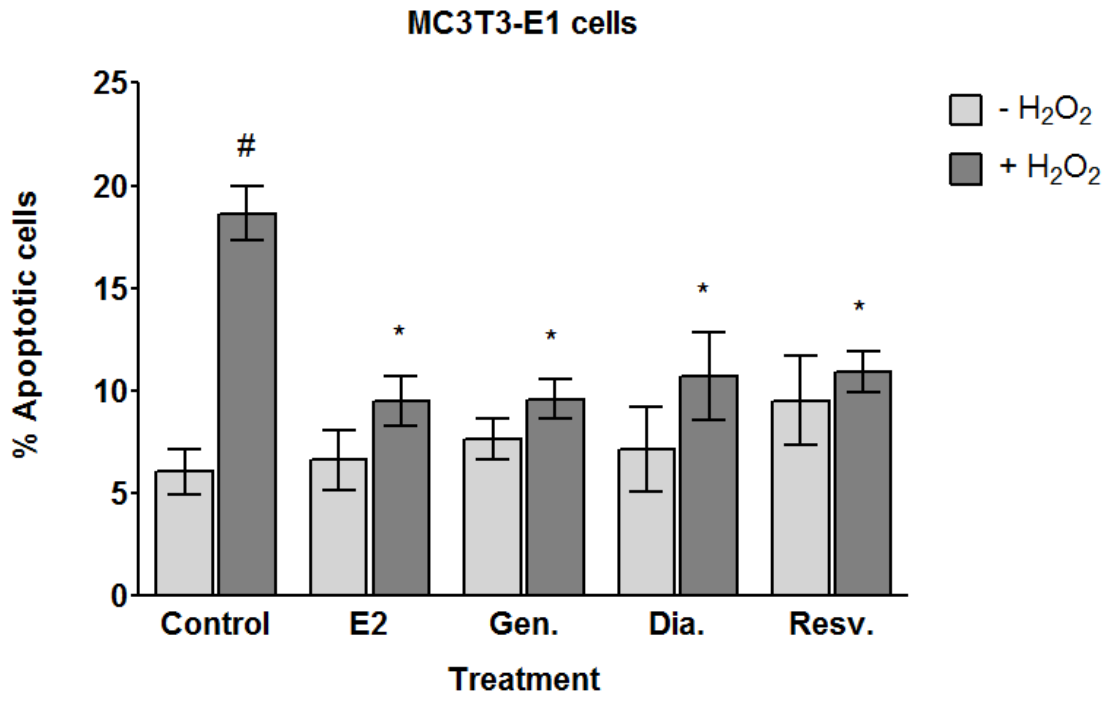

Figure 4.1: Effect of E2 and Ps on MC3T3-E1 cell apoptosis induced by H_2O_2. Pre-treatment of MC3T3-E1 cells with E2 and different PEs (Genestein, Daidzein. and Resveratrol) at a concentration of 10nM decreased the percentage of MC3T3-E1 cells apoptosis induced by H_2O_2. Data were expressed as ± SD of three wells (three fields per well were counted), n=9, *p<0.05 versus H_2O_2, # versus control.

4.3 Measurement of intracellular ROS

The oxidative stress induced by H_2O_2 treatment in MC3T3-E1 cells was evaluated by measuring ROS generation. Oxidative stress may initiate a mitochondrial permeability transition event, which is an early mediator of cell apoptosis. When cells were treated with 0.4mM H_2O_2, the level of cells positive for ROS was significantly increased when compared with control (Control 3% ±1.7 vs H_2O_2 20.73% ± 2.28, p=0.0004), while treatment with E2 reduced the level of ROS generation (4.7% ± 0.89, p=0.0003) Fig.4.2, also PEs (Genistein, Daidzein and Resveratrol) at 10nM in the presence of H_2O_2 reduced ROS positive cells, Genistein (6.3% ±1.6, p=0.0009), Daidzein (7% ±1.5, p=0.001) and Resveratrol (6.6% ±1.4, p=0.0008).(Fig.4.3, 4.4 and 4.5) respectively.

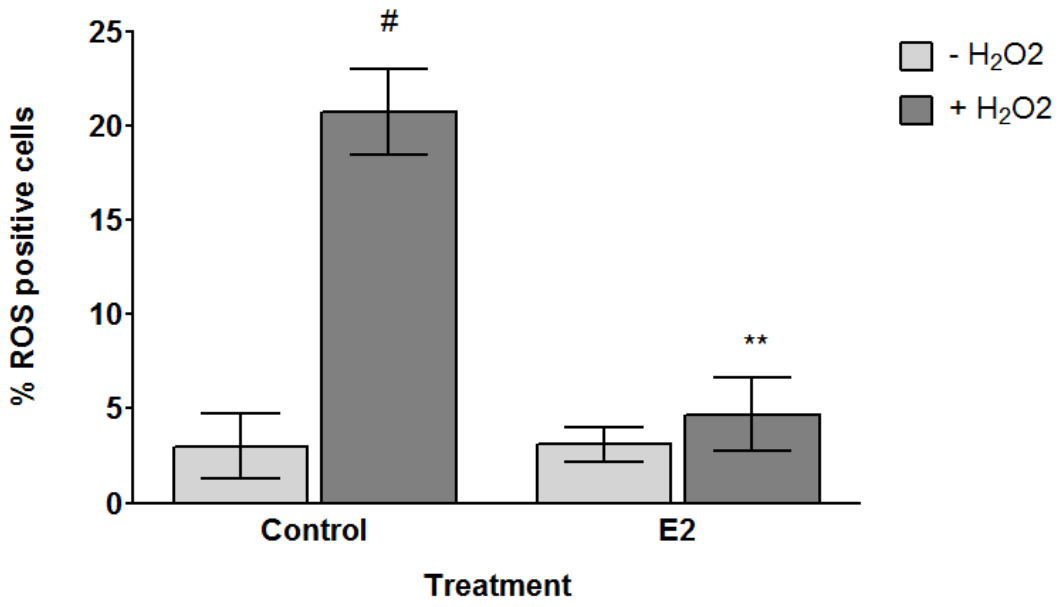

Figure 4.2: ROS activity in MC3T3 cells treated with 17-β estradiol (E2) prior to treatment with H_2O_2. Pre-treatment with E2 significantly reduced the percentage of ROS positive cells on treatment with H_2O_2. Data were expressed as means ± SD of triple wells (3 fields per well were counted) **p<0.0001 versus H_2O_2, # versus control.

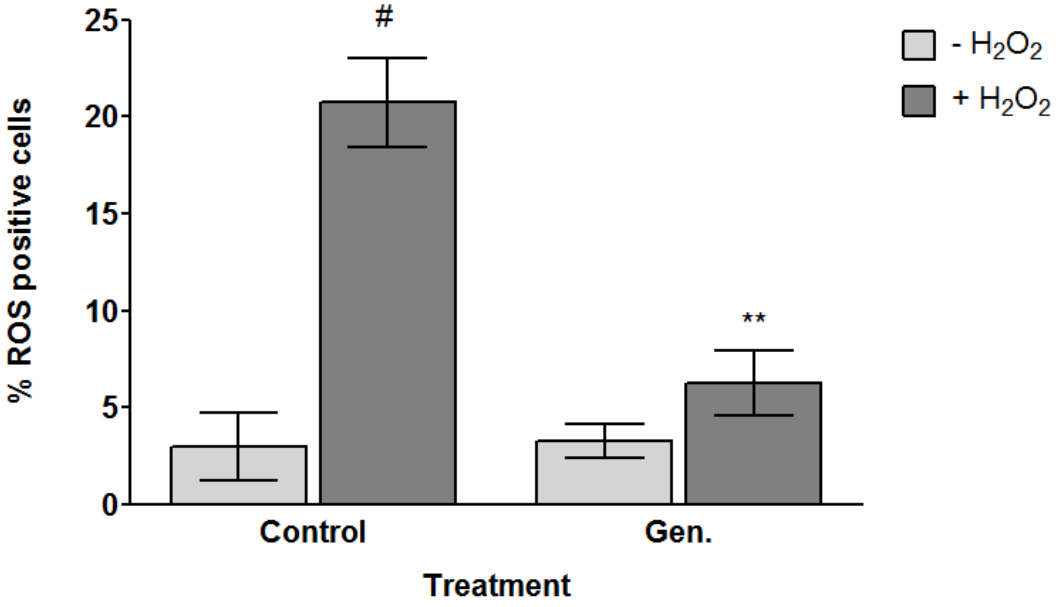

Figure 4.3: ROS activity in MC3T3 cells treated with Genistein prior to treatment with H_2O_2. Pre-treatment with Genistein significantly reduced the percentage of ROS positive cells on treatment with H_2O_2.Data were expressed as means ± SD of triple wells (3 fields per well were counted) **p<0.0001 versus H_2O_2, # versus control.

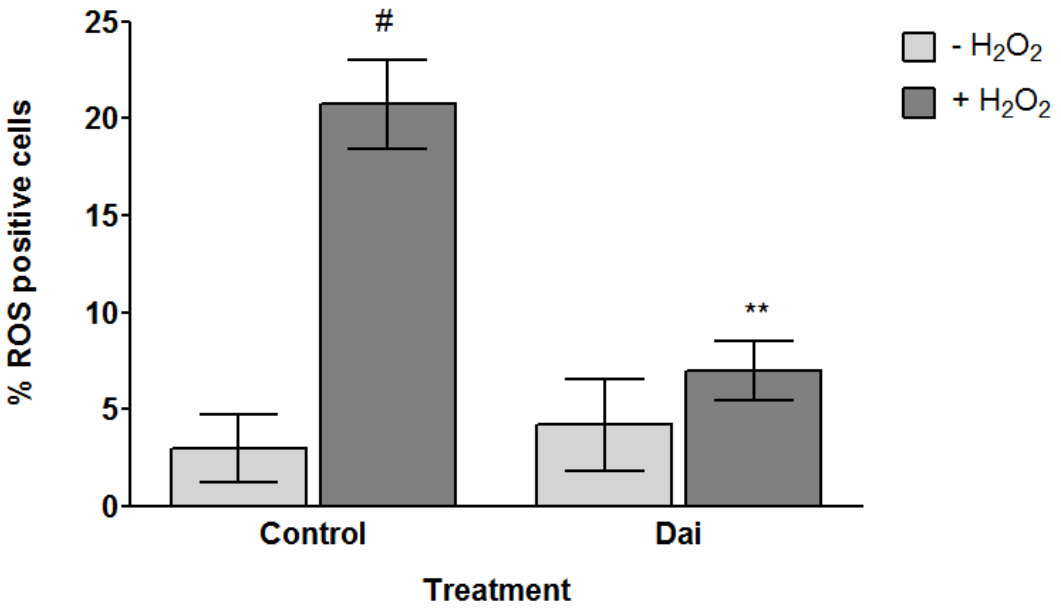

Figure 4.4: Ros activity in MC3T3 cells treated with Daidzein prior to treatment with H_2O_2. Pre-treatment with Daidzein significantly reduced the percentage of ROS positive cells on treatment with H_2O_2. Data were expressed as means ± SD of triple wells (3 fields per well were counted) ***p*<0.0001 versus H_2O_2, # versus control.

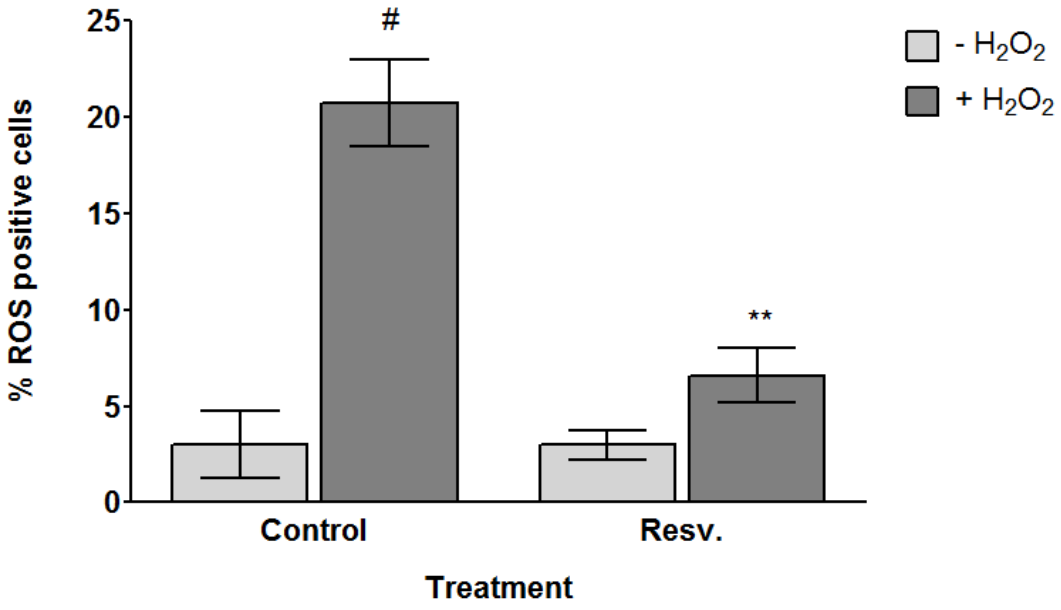

Figure 4.5: ROS activity in MC3T3 cells treated with Resveratrol prior to treatment with H_2O_2. Pre-treatment with Resveratrol significantly reduced the percentage of ROS positive cells on treatment with H_2O_2. Data were expressed as means ± SD of triple wells (3 fields per well were counted) ***p*<0.0001 versus H_2O_2, # versus control.

4.4 Effects of PEs on differentiation of RAW 264.7 cells and Tartrate-resistant Acid Phosphatase

Bone homeostasis is preserved by the balance between bone formation and resorption (Boyle *et al.,* 2003). Osteoclasts are bone-resorbing multinucleated cells, formed by the fusion of their mononuclear precursor's monocytes and macrophages. Osteoclast differentiation of precursors can be stimulated by (RANKL) produced by osteoblasts. Binding of RANKL to its receptor induces small non-toxic amounts of ROS, various growth factors, as well as cytokines including TNF-α. A low-level increase of ROS may be required as a secondary messenger in RANKL-induced signalling pathways for osteoclast differentiation. In this study, the effect of PEs on osteoclast differentiation from murine macrophage RAW264.7 cells induced by RANKL and MCSF was investigated. The murine macrophage/osteoclast precursor cell line RAW264.7 is largely used pre-osteoclast model that differentiates into osteoclasts when stimulated with M-CSF and RANKL (Wei *et al.,* 2001).

The effect of PEs on osteoclast differentiation was investigated and RAW 264.7 cells were exposed to the receptor activator of nuclear factor Kappa-B ligand (RANKL) and MCSF, as mentioned in section 2.2.4. Treatment with RANKL and MCSF induced osteoclast formation in these cells, identified via light microscopy as large TRAP + cells. The number of TRAP + cells were (480 ± 20, p=0.0001), while treatment with E2 reduced the number of TRAP + cells to (244 ± 8.7, p=0.0001), Genistein (215 ± 27.8, p=0.0002), Daidzein (248 ± 9.6, p=0.0001) and Resveratrol (252 ± 16.25, p=0.0001) compared to control (Figure 4.6 B).

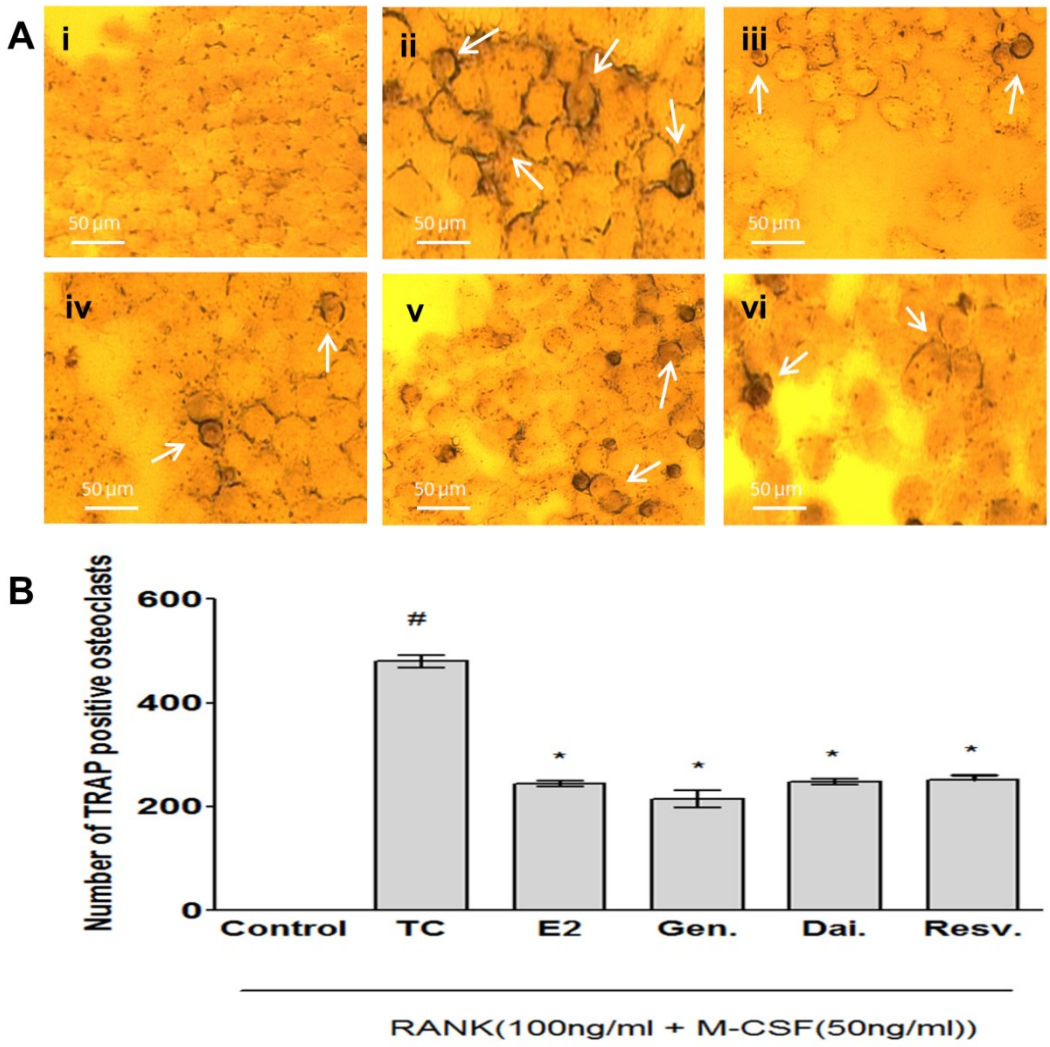

Figure 4.6: Effect of E2 and PEs on RANKL-induced osteoclast differentiation. Treatment with E2 or PEs (Genistein, Daidzein and Resveratrol) significantly reduced RANKL induced osteoclast differentiation.**[A] (**i–vi) Representative images (20×) of osteoclasts differentiated in the presence of E2, Genistein, Daidzein and Resveratrol after treatment of RAW 264.7 with macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL). Cells were subsequently stained with TRAP to identify osteoclasts; **(i)** represents control without any treatment, **(ii)** treatment with RANKl and M-CSF, **(iii)** treatment with E2, **(iv)** treatment with Genistein **(v)** treatment with Daidzein and **(vi)** treatment with Resveratrol. Osteoclasts were identified as large, TRAP+, (white arrows in panels i-vi) and quantified as osteoclasts/field of view of culture dish, n = 5. **[B]** Data are expressed as the numbers of osteoclasts which were counted after taking randomly fields in different areas of each well. At least 5 fields were used for each tested reagent and the values are expressed as mean ± SD. $p < 0.05$ compared with RANKL treatment (TC: treated control).

4.5 Discussion

Osteoblast and osteoclast activity is tightly coupled during each remodelling cycle, such that bone resorption is linked to a subsequent formative phase. This coupling is lost in post-menopausal women which therefore contributes to the bone loss during both the initial and later stages of the disorder. This study examined the effect of PEs on osteoblast apoptosis induced by H_2O_2. Genistein, Daidzein and Resverartrol have the ability to inhibit apoptosis induced by oxidative stress in osteoblast cells MC3T3-E1 evidenced with DAPI staining and assessment of ROS generation. H_2O_2 at concentration of 0.4mM was used to induce oxidative cell injury in osteoblastic MC3T3-E1 as previously described (Zhang *et al*. 2013). Yet, pre-treatment with E2 and PEs (Genistein, Daidzein and Resveratrol) revealed that H_2O_2 toxicity was decreased. Oxidative stress affects cell viability and differentiation, this was observed in previous studies which have shown that H_2O_2 results in cell injury and inhibits osteogenic differentiation in osteoblastic MC3T3-E1 cells (Liu *et al*., 2004) and also resulted in decreased calcium mineralization, ALP activity and lowered the production of osteogenic genes. Studies have indicated that H_2O_2 was correlated with a decrease in calcium mineralization and ALP activity, and a lowered production of osteogenic genes, including BGLAP, COL1A1 and SPP (Huang *et al*., 2015). Isofavones including Genistein and Daidzein are contained in soybean with high concentration; they are hydrolysed by β-glucosidase in the gastrointestinal system. Genistein has been found to inhibit tyrosine kinase enzyme activity and cause apoptosis in leukemic cells (Liu *et al*., 1994). Isoflavones are thought to be an important tool for preventing bone loss that is due to ageing. The

dietary intake of Genistein was shown to prevent bone loss in ovariectomized rats, an animal model of postmenopausal osteoporosis (Blair *et al*., 1996). Additionally, Genistein stimulates osteoblastic bone formation and mineralization *in vitro* (Pan *et al.,* 2005), and can stimulate protein synthesis in osteoblastic cells (Sugimoto and Yamaguchi 2000, Yamaguchi and Sugimoto 2000). In this study, investigation of MC3T3-E1, treated with PEs showed that Genistein, Daidzein and Resveratrol, like E2, significantly reduced the percentage of H_2O_2 induced apoptosis in these cells. It has been found through the molecular examination of Genistein that it has an anabolic effect on MC3T3-E1 (Sugimoto and Yamaguchi 2000).

Furthermore, previous studies have shown that Genistein inhibited osteoclastogenesis, in part through inhibition of protein kinase activation and suppression of protein tyrosine phosphatase activity as well as by inducing apoptosis of mature osteoclasts through a Ca^{2+} signalling mechanism (Gao and Yamaguchi 2000). Osteoblasts and osteocytes are derived from mesenchymal stem cells, which also differentiate into fibroblasts, chondrocytes, myoblasts and adipocytes. Osteoclasts interact with osteoblasts at various stages of differentiation (Matsuo and Irie, 2008). Several studies recognized the essential osteoclastogenic ligand RANKL (also called TRANCE), a transmembrane glycoprotein belonging to the tumor necrosis factor (TNF-α1) superfamily and plays a key role in osteoclast development and in resorbing activity (Theill *et al.,* 2002). Increased osteoclast activity has been reported in many osteopenic disorders, including postmenopausal osteoporosis (Jones and Penninger 2002; Wang *et al.,* 2002). TRANCE interacts with two receptors: a secreted decoy receptor osteoprotegerin, OPG (Simon *et al.,* 1997),

and a transmembrane receptor, RANK (Anderson *et al.,* 1997). TRANCE and RANK interaction is essential for osteoclastogenesis because RANK is activated by TRANCE, and then associates with TNF receptor-associated family members to trigger downstream signaling events (Hsu *et al*., 1999).

In the present study, the inhibitory effects of E2 and PEs (Genistein, Daidzein and Resveratrol) on osteoclast differentiation were investigated to establish their anti-osteoporotic activity through suppressing excessive bone resorption. Osteoclast differentiation from murine macrophage RAW 264.7 cells was induced by RANKL and M-CSF, which is essential for the terminal differentiation of monocytes/macrophages into osteoclasts (Ming and Xian 2013). TRAP-positive multinucleated osteoclasts were visualized by light microphotography (Figure 3.24 A). RANKL and MCSF treatment induced osteoclast formation from RAW 264.7 cells. Treatment with E2 and PEs reduced the number of multinucleated TRAP positive cells (Figure 3.24 B) which is similar to previous studies using Genistein (Lee *et al.,* 2014). The RANKL-induced differentiation of RAW 264.7 could be through controlling ROS levels (Lee *et al.,* 2014). The inhibitory effects of natural flavonoids, such as Resveratrol (He *et al.,* 2010), Curcumin (Kim *et al.,* 2012) and Fisetin (Sakai *et al.,* 2013) as potent antioxidants on osteoclast differentiation from osteoclastic precursors through controlling ROS generation have also been reported. The suppressive effect on osteoclastogenesis via attenuating ROS production in RANKL-treated RAW 264.7 cells was also observed in other flavonoids such as luteolin, which showed a potent cellular antioxidant activity in rat C6 astroglioma cells (Lee *et al.,* 2009). It has been found that Genistein, Daidzein

and Resveratrol, like E2, inhibit the differentiation of osteoclasts from cultures of RAW 264.7 cells.

Genistein has been shown previously to increase osteoprotegerin/ receptor activator of nuclear factor kB ligand (RANKL) ratio and reduce osteoclast differentiation from hematopoietic stem cells which results in less bone resorption *in vivo* (Li *et al.,*2005). Therefore, soy-based products containing Genistein may be used for the development of potential therapeutic agents preventing bone diseases, such as osteoporosis.

In vitro studies have indicated that Resveratrol prevents bone resorption, enhances osteoblast differentiation, and has been shown to act as a phytoestrogen (Gehm *et al.,* 1997). The anti-oxidative activity of Resveratrol is claimed to be due to the presence of hydroxyl phenolic group in its chemical structure (Leonard *et al.,* 2003).

Studies conducted have shown that Resveratrol, in addition to its stimulatory effect on osteoblasts, can inhibit RANKL-induced osteoclastogenesis and thus induce apoptosis of differentiated osteoclasts (He *et al*., 2010). A study conducted on an ovariectomized rat model of postmenopausal osteoporosis reported that Resveratrol supplementation attenuated estrogen deficiency induced bone loss and trabecular structural deterioration (Tou 2015). Furthermore, the dual effects, anti-resorptive and anabolic, on bone remodeling together with its tolerance in rat and human studies (Williams *et al.,* 2009; Almeida *et al.,* 2009) indicate that resveratrol is a potential candidate for anti-osteoporosis therapy. Despite the health benefits of Resveratrol, it's molecular targets have not been fully determined. A broad range of intracellular proteins, including sirtuins, kinases, lipo- and cyclooxygenases, as well as ster-

oid receptors, have been shown to be involved in Resveratrol signaling (Pirola and Fröjdö 2008). Among these targets, Sirt1, a NAD+ dependent protein deacetylase, is thought to be the major mediator for stress resistance and considered as anti-ageing effects (Howitz *et al.,* 2003). However, it has been found that Sirt1 is not involved in inhibition of osteoclastogenesis by Resveratrol and its effect could not be attenuated by ICI-182780, a high affinity estrogen receptor antagonist. This shows that the effect of Resveratrol is ER independent and also its inhibitory effect on osteoclastogenesis is mediated via ROS inhibition (He *et al.,* 2010).

CHAPTER FIVE: INVESTIGATION OF POTENTIAL SAVING MECHANISM OF PHYTOESTROGEN

5.1 Introduction

PEs have multiple mechanisms of action including estrogen agonist and antagonist activity. They are markedly similar in chemical structure to the mammalian estrogen, estradiol, and bind to ERα and ERβ with a high affinity to ERβ (Younes and Honma 2011; Rietjens *et al.,* 2013; Paterni *et al.,* 2014). These receptors after binding with their ligand are able to move from cytoplasm to the nucleus, bind and affect the transcription-control regions of DNA and therefore, the expression of specific genes. Furthermore, steroids are able to bind to receptors of cell surfaces, promote formation of cytoplasmic cyclic nucleotides and related protein kinases, which in turn via transcription factors control the expression of target genes (Sirotkin, 2014; Yanagihara *et al.,* 2014). Therefore, PEs can potentially affect all the processes regulated by estrogens including induction sex hormone binding globulin and inhibition aromatase (Wang 2002). ERs are present in different tissues such as central nervous system, reproductive tract, placenta, mammary gland, bones, gastrointestinal tract and lung. This suggests that PEs may exert tissue specific hormonal effects (Cassidy 2003; Younes and Honma 2011; Böttner *et al.,* 2013). Additionally, ERα is considered as a promoter of cell proliferation, whilst ERβ is responsible for promoting cellular apoptosis (Rietjens *et al.,* 2013). PEs besides their ability to bind to ERs, have other biological effects which are not mediated through these receptors such as activation of serotoninergic receptors (Hajirahimkhan *et al.,* 2013), IGF-1 receptors (Bourque *et al.,* 2012), binding of free radicals (Wang 2002; Cassidy 2003; Vina *et al.,* 2011) and other intracellular regulators of cell cycle and apoptosis. These abilities are probably responsible for anti-oxidant, anti-proliferative, anti-

mutagenic and anti-angiogenic effects of PEs and their ability to promote human health (Kurzer and Xu 1997; Wang 2002; Cassidy 2003; Vina *et al.,* 2011; Hajirahimkhan *et al.,* 2013; Ming *et al.,* 2013).

The estrogenic effect of Genistein and Daidzein is dependent on two main properties. The first being the presence of a phenolic ring A that mimics E2's receptor binding A ring; and the second, a similar distance between the 7- and 4'-hydroxyl groups in isoflavones and the C3 and C17 hydroxyl groups of estradiol (Dixon 2004). The presence of a phenolic ring enables PEs to bind to ERs and provides the major basis of their estrogenic action (Wuttke *et al.,* 2003). In addition to their estrogenic effect, PEs also function as antioxidants and many such as Genistein can modify tyrosine kinase activity (Anderson and Garner 1998).

5.2 Investigation of Estrogen Receptor Inhibitor effect on anti-apoptotic effect of estrogen and phytoestrogen

Estrogen receptor inhibitor, ICI 182 780, is a specific ER inhibitor which is an estradiol derivative containing alkyamide extension in the 7α position. The non-receptor mechanism of protection was investigated for E2 and PEs whether they exert anti-apoptotic effect. MLO-Y4 cells were pre-treated with (10nM) of the receptor antagonist ICI 182 780 prior to treatment with E2 and PEs at a concentrations of 10nM did not block the anti- apoptotic effect of E2 (15.77% ± 1.98 vs. H_2O_2 treatment 27.78% ± 4, p=0.0096) (Fig. 5.1). In a similar way the anti-apoptotic effects of Genistein, Daidazein and Resveratrol were not blocked by pre-treatment with ICI 182 780 (Gen.17% ± 1, *p*=0.0106,

Fig.5.2), (Dai.13.79% ± 1.94, *p*=0.005, Fig. 5.3) and (Resv. 20.2% ± 3, *p*= 0.05, Fig. 5.4). Treatment with ICI 182 780 alone also significantly reduced H_2O_2 induced apoptosis, indicating that ICI 182780 has anti-apoptotic activity. In order to further elucidate the potential contribution of estrogen receptor mediated in this response, HEK293 human embryonic kidney epithelial cell line which is ER-α and ER-β negative were used (Zhao *et al.,* 2007). Treatment of H_2O_2 induced apoptosis in HEK293 cells at a concentration of 0.4mM for 2 hours indicated the percentage of apoptosis was (18.49% ± 2.4 vs. control 10.7% ±1.3, *p*=0.002). Pre-incubation of HEK293 cells with PEs significantly reduced the percentage of apoptosis Genistein (12.11% ± 1.7, *p*=0.0021), Daidzein (12.74% ± 1.5, *p*=0.0011) and Resveratrol (10.63% ± 1.4, *p*=0.001). The saving effect of these PEs on HEK293 cells were similar to that of E2 (11.22% ± 1.2, *p*=0.001) Fig. 5.5, indicating an estrogen receptor-independent effect of these compounds. The effect of E2 on HEK cells was reported previously by (Mann *et al.,* 2007).

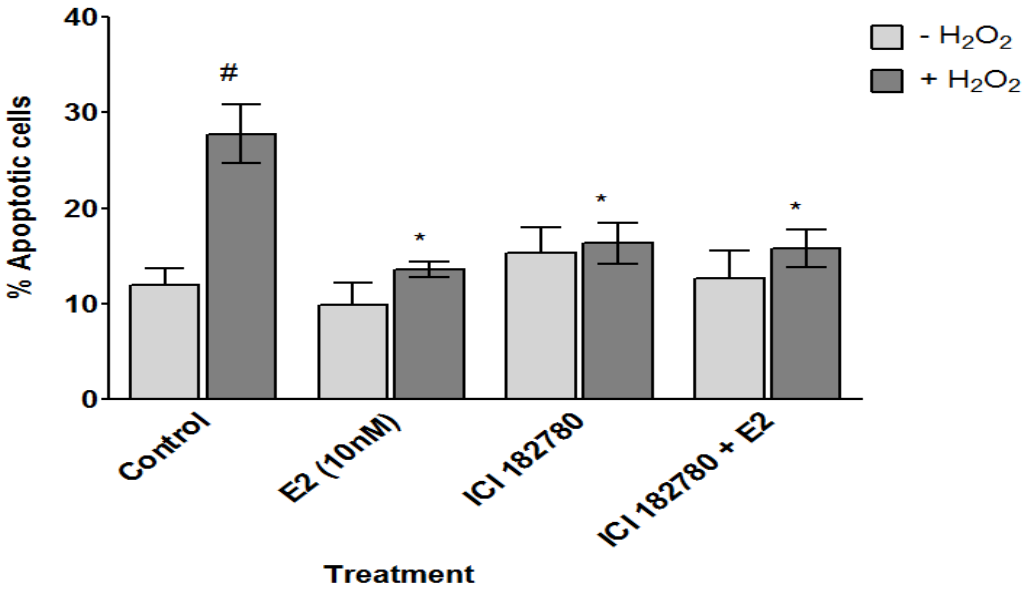

Figure 5.1: Effect of estrogen receptor inhibitor (ICI 182780) on protecting effect of E2. Pre-treatment of MLO-Y4 cells with 10nM of estrogen receptor inhibitor ICI 182 780 prior to treatment with 10nM of E2 and 0.4mM of H_2O_2 did not block the protecting effect of E2. Data are expressed as mean ± SD of three wells (three fields per well were counted), *p<0.05 versus H_2O_2, # versus control.

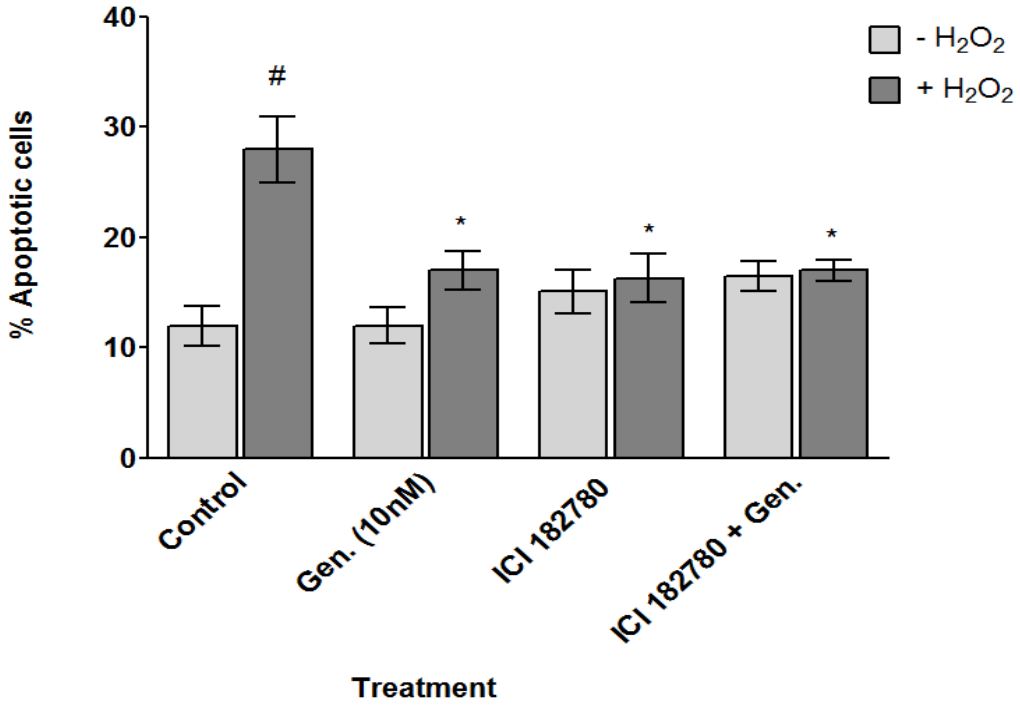

Figure 5.2: Effect of estrogen receptor inhibitor (ICI 182780) on saving effect of Genstein. Pre-treatment of MLO-Y4 cells with 10nM of estrogen receptor inhibitor ICI 182 780 prior to treatment with 10nM of Genistein and 0.4mM of H_2O_2 did not block the protecting effect of Genistein. Data are expressed as mean ± SD of three wells (three fields per well were counted), *p<0.05 versus H_2O_2, # versus control.

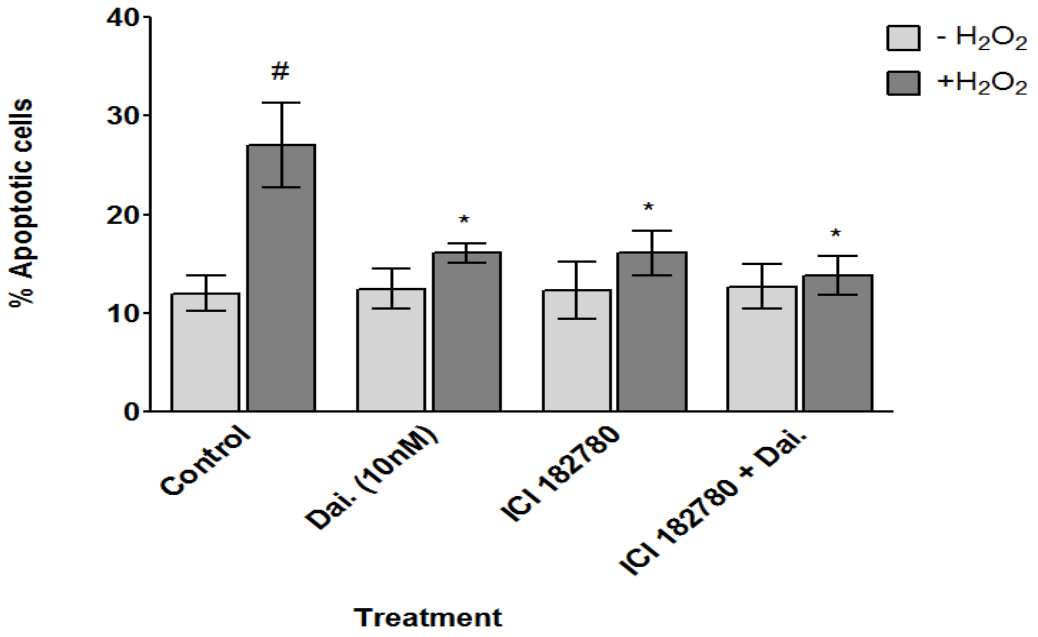

Figure 5.3: Effect of estrogen receptor inhibitor (ICI 182780) on saving effect of Daidzein. Pre-treatment of MLO-Y4 cells with 10nM of estrogen receptor inhibitor ICI 182 780 prior to treatment with 10nM of Daidazein and 0.4mM of H_2O_2 did not block the protecting effect of Daidzein. Data are expressed as mean ± SD of three wells (three fields per well were counted), * p<0.05 versus H_2O_2, # versus control.

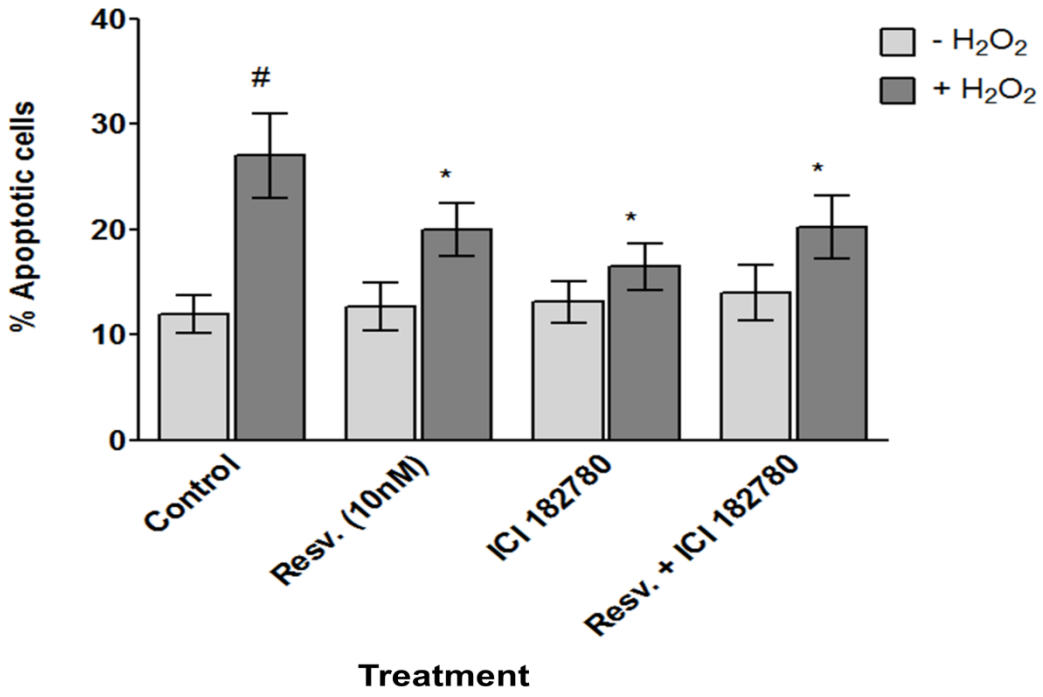

Figure 5.4: Effect of estrogen receptor inhibitor (ICI 182780) on saving effect of Resveratrol. Pre-treatment of MLO-Y4 cells with 10nM of estrogen receptor inhibitor ICI 182 780 prior to treatment with 10nM of Resveratrol and 0.4mM of H_2O_2 did not block the protecting effect of Resveratrol. Data are expressed as mean ± SD of three wells (three fields per well were counted), * p<0.05 versus $H_2O_2$and # p< 0.05 versus Control

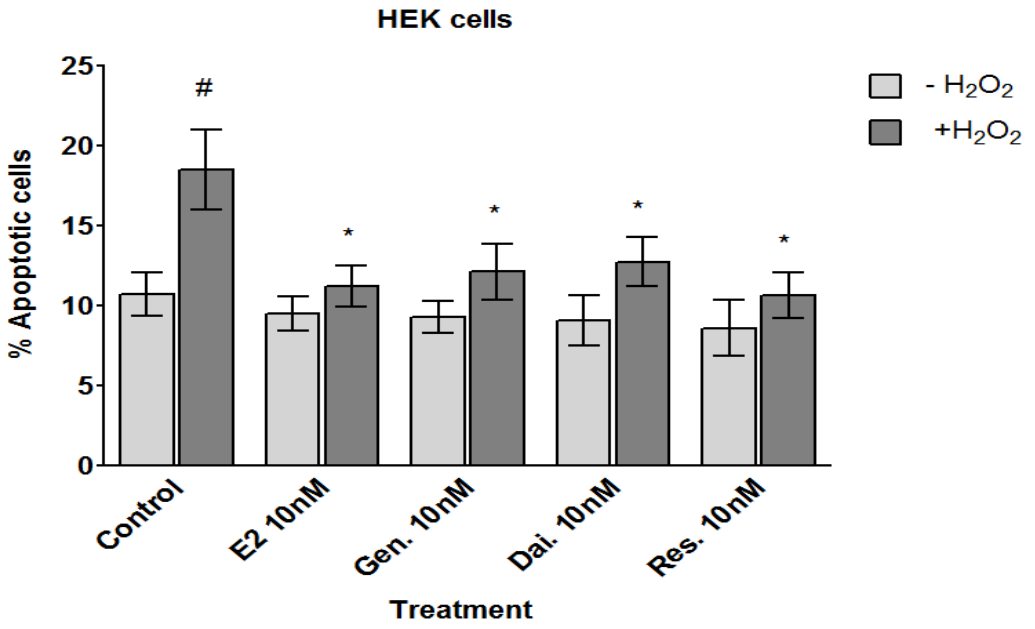

Figure 5.5: Effect of 17β-estradiol (E2) and PEs on HEK 293 cells apoptosis. Pre-treatment of ER negative HEK293 cells with E2 0r PEs (Genstein., Daidzein, and Resveratrol) all significantly reduced H_2O_2 induced apoptosis. Data are expressed as mean ± SD of three wells (three fields per well were counted), n=9, *p<0.05 vs H_2O_2, # vs control.

5.3 Effect of Genistein, Daidzein and Resveratrol on catalase (CAT) enzyme activity

Among the antioxidant enzymes, Catalase is a ubiquitous antioxidant enzyme that is present in most aerobic cells. Catalase (CAT) is involved in the detoxification of H_2O_2, which is a toxic product of both normal metabolism and pathogenic ROS production. CAT catalyses the conversion of two molecules of H_2O_2 to molecular oxygen and two molecules of water. The experiment was carried out as previously described in section 2.2.1 by culturing MLO-Y4 cells and treating them with PEs prior to treatment with H_2O_2 (Fig.5.6). Catalase assay was carried out to determine the effects of E2, Genistein, Daidzein and Resveratrol on enzyme activity, described in section

2.2.16. Catalase activity was examined in the supernatants and the Catalase assay was performed using a kit which exploits the peroxidatic function of CAT for the determination of enzyme activity. Treatment of MLO-Y4 cells with E2 or PEs alone did not increase catalase activity significantly, while treatment with H_2O_2 increased the activity of CAT enzyme in the media compared with control (44.4Unit/ml ± 6.2 vs H_2O_2 treatment 212.1Unit/ml ± 3, *p*=0.001). Treatment of cells with 10nM of E2 or PEs prior to treatment with H_2O_2 decreased the activity of CAT in the supernatant when compared to treatment with H_2O_2 alone, E2 (124.4Unit/ml ± 1.9, *p*=0.004), Genistein (95.9Unit/ml ± 1.4, p=0.0025), Daidzein (87.8Unit/ml ± 2.5, *p*= 0.0022), Resveratrol (138Unit/ml ± 1.7, *p*=0.012).

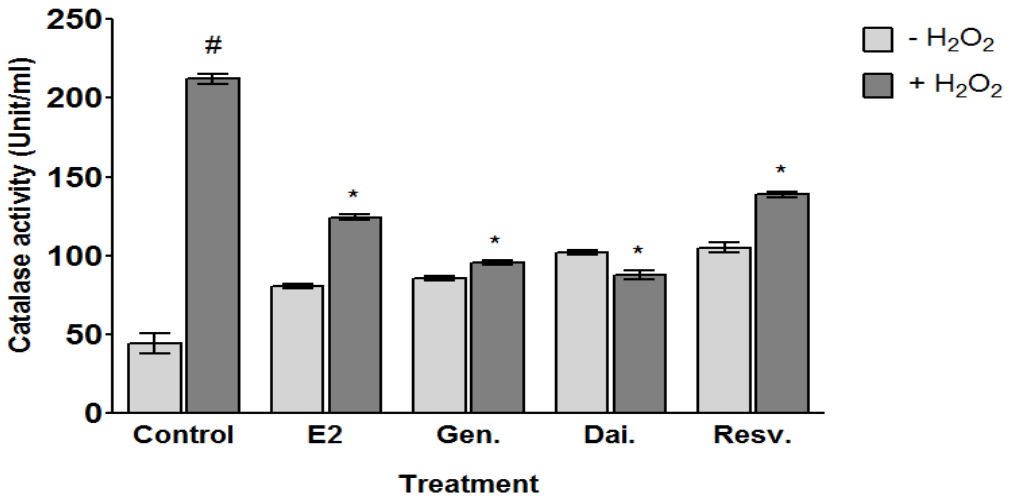

Figure 5.6: Catalase activity in MLO-Y4 cells after PES exposure. Cells were exposed to 10nM of E2 and PEs (gen., dai. And resv.) for 1 hour prior to H_2O_2 for 2 hours. The assay was carried out using catalase activity assay kit. The assay utilizes visible light (520 nm), which reduces sample interference. The kit is designed for use in microplate readers. The reaction was carried out for one minute, at which time the catalase was quenched with sodium azide. The result is expressed as the amount of catalase enzyme produced in unit /ml. Data is expressed as means of ±SD, n=3 different experiments; *P<0.05 vs. H_2O_2, # vs. control.

5.4 Modification and the structural rational strategy

In order to provide an insight into the role of the hydroxyl groups (OH) in the activity of Genistein and Daidezein, modification of the OH groups was carried out. The molecular weight of Genistein is 270.24g/mole and that of Daidezein is 254.24gm/mole. Both compounds could be subjected to modifications by masking one, two and/or three (in case of Genistein) of their OH groups with an alkyl group, which is non-cleavable within the cellular compartment such e.g. CH_3 to give methoxy groups. Several possibilities of mono-methoxy, di-methoxy groups or tri-methoxy could occur for Genistein, on either positions (5 & 7), (5 & 4'), or (7 & 4'), while postions (5, 7, and 4') for all the three OH groups. Daidzein could be modified on either hydroxyl position 7, 4' or both. The molecular weight of Genistein and Daidzein would change if modified, this was shown in (Table 5.1) and confirmed by Mass spectrometry analysis which indicated that the synthetic modification occurred on both OH groups of Daidzein and a mixture of two and three OH groups of Genistein, which was based on the changes in the molecular weight of these compounds. Upon isolation and purification pure di-methoxy Daidzein was obtained. While the mixture of di-methoxyand tri-methoxy were purified and separated by chromatography on normal phase silica gel. The location of the two modified OH groups in di-methoxy Genistein was identified as being on positions 5, and 7, as confirmed by mass spectroscopy fragmentation analysis MS/MS. The total ion mass of the di-methoxy Genistein m/z was 298 D (Figure 5.7). The ion masses (166 and 137 Dalton) of two fragments for Genistein gave evidence for the modified hydroxyl groups being on the same aromatic ring at position 5 and 7 (Figure 5.8). Di-

methoxy Daidzein ionic mass, m/z was 282 Dalton, confirmed by addition of sodium ion 305 and cluster of two ions of the compound with sodium to give mass of 587 Dalton (Figure 5.10). Table 5.1 shows the molecular weight of modified and unmodified compounds of Daidzein and Genistein. The modified Daidzein and Genistein alongside the unmodified counterparts were investigated *in vitro* to assess their protective effects on oxidative stress induced by H_2O_2 in MLO-Y4 cells.

Table 5.1: Molecular weight of Modified and unmodified Genistein and Daidzein. The molecular weight increased by 15 Dalton for each hydroxyl group removed and replaced with methyl group and H^+ from methanol which was used as a solvent which protonate the compound**.**

Compounds	**Molecular weight g/mole**
Daidzein	254.24
Modified Daidzein A	282.09= (CH3x2)=30+H^+- 2H from removing 2H of two OH groups
Genistein	270.24
Modified Genistein A	298.08= (CH3 x 2)=30+H^+- 2H from removing 2H of OH groups
Modified Genistein B	312.10= (CH3 x 3)=45+H^+- 3H from removing 3H of OH groups

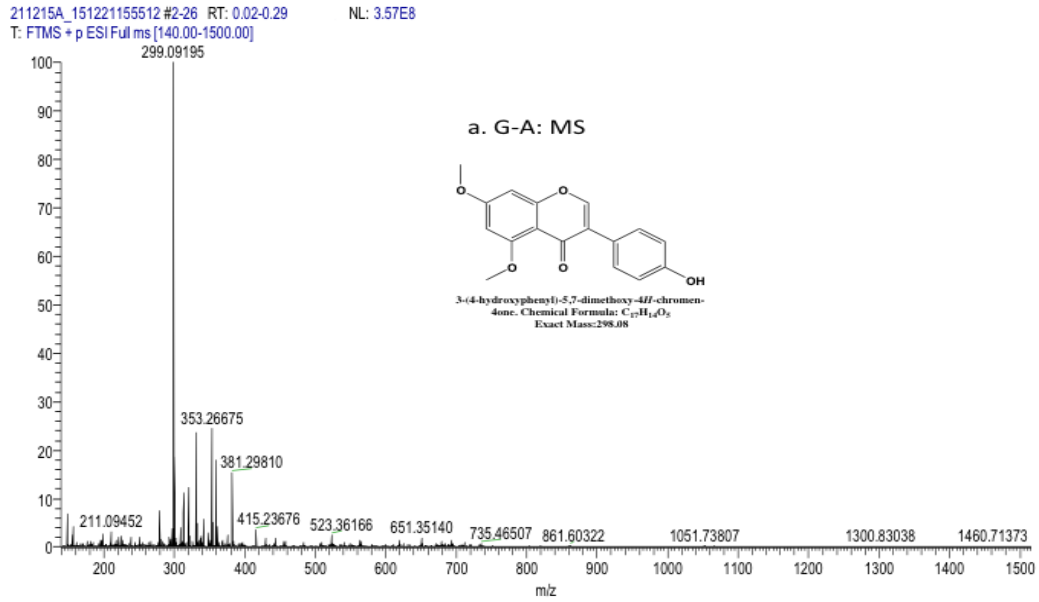

Figure 5.7: Mass spectra of di-methoxy Genistein: ESI-MS, m/z: $[M+H]^+$, 299D.The spectra above shows the fragmentation of the molecules which represented by numerous peak observed. The structure of di-methoxy substituted: 3(4-hydroxyphenyl)-5,7-dimethoxy-4H-chromen4 –one.

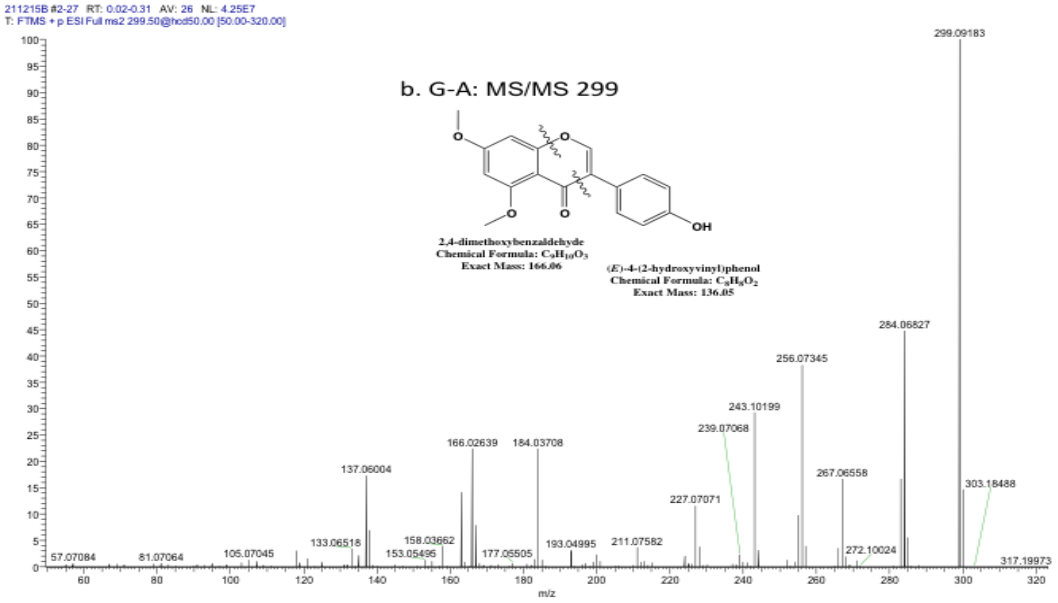

Figure 5.8: Mass spectra of MS/MS fragmentation of di-methoxy Genistein, MS/MS: 284 $[M^+-CH_3]$, 267 $[M^+-CH_5O]$, 256 [isotope $M^+-CH_3O_2$], 243 [isotope $M^+-C_2H_6O_2$, 184 $[M^+-C_9H_{12}O_4]$, 166 $[M^+-C_9H_{10}O_3]$, 137 $[M^+-C_8H_9O_2]$. The spectra above shows the fragmentation of the molecules which represented by numerous peak observed. The structure of di-methoxy substituted: 7-hydroxy-5methoxy-3-(4-methoxyphenyl)-4H-chromen-4-one.

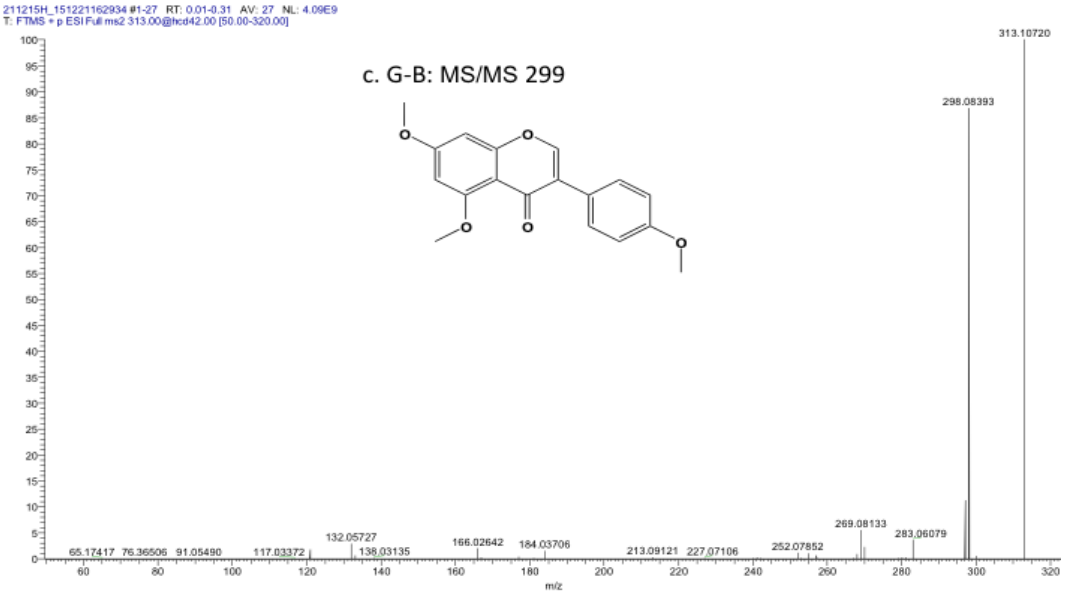

Figure 5.9: Mass spectra of tri-methoxy Genistein ESI-MS, m/z: $[M]^+$312, 351 $[M^+$+K], 663 $[2M^+$+K] The spectra above shows the fragmentation of the molecules which represented by numerous peak observed. The structure of tri-methoxy substituted: 5,7-dimethoxy-3-(4-methoxyphenyl)-4H-chromen-one.

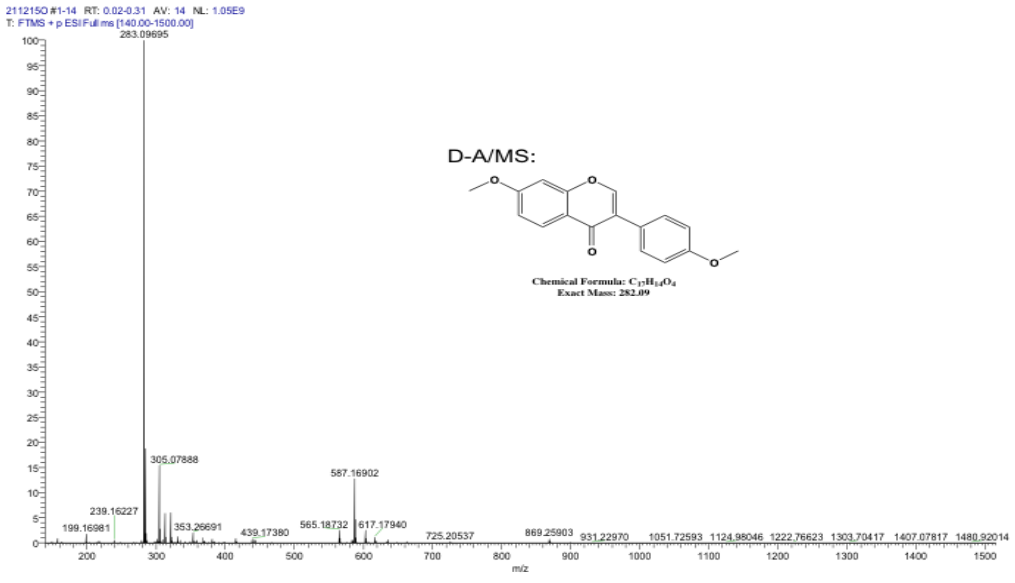

Figure 5.10: Massspectrometry analysis of di-methoxy Daizein: ESI-MS, m/z: 282$[M^+$+H] 305 $[M^+$+Na], 587 $[2M^+$+Na. The spectra above shows the fragmentation of the molecules which represented by numerous peak observed. The structure of di-methoxy substituted: 7-mehoxy-3-(4-methoxyphenyl)-4-chromen-4-one.

5.5 Fluorometric assay of MLO-Y4 cells treated with modified Genistein and Daidzein via H2DCF-DA

This method is based on a fluorometric assay using H2DCFHDA dye which is used as an intracellular probe for direct measurement of the cell redox state (Tammariello *et al.,* 2000; Ottonello *et al.,* 2001). DCFH-DA is a compound that is diffused into cells where it can be hydrolysed by intracellular esterases to cell membrane impermeable and non-fluorescent 2, 7-dichlorofluorescein (H2DCF) which does not permeate membranes. H_2DCF is then oxidized by the intracellular H_2O_2 to a highly fluorescent compound DCF, which is membrane permeable and can leak out of cells over time. Accumulation of DCF in cells or extracellular supernatant may be measured by a production of the fluorescence at 530 nm when the sample is excited at 485 nm. MLO-Y4 cells were treated with 10nM of unmodified Genistein, Genistein A, Genistein B and also, unmodified Daidzein and Daidzein A. The result in (Figure. 6.3) shows that treatment with modified Genistein did not protect the cells from oxidative stress induced by H_2O_2 and the fluorescent intensity was not significant when compared with H_2O_2 treatment (26300.66 ± 31.68, p=0.302) for Genistein A and (28328.34 ± 82.03, p=0.325) for Genistein B vs. H_2O_2 (34791.33 ± 81.32). While treatment with unmodified Genistein reduced the fluorescent intensity compared to cells treated with H_2O_2 (12265.32 ± 68.32, p=0.03) (Fig.5.11). Similar results were observed with cells treated with unmodified and modified Daidzein. The fluorescence intensity was not significant in comparison to H_2O_2 treatment (21206.67 ± 25.01 vs. H_2O_2 34791.33 ± 81.32, p=0.106), while treatment with unmodified Daidzin reduced the fluo-

rescence intensity in comparison to cells treated with H_2O_2 (12360.67 ± 23, p=0.01) (Fig.5.12).

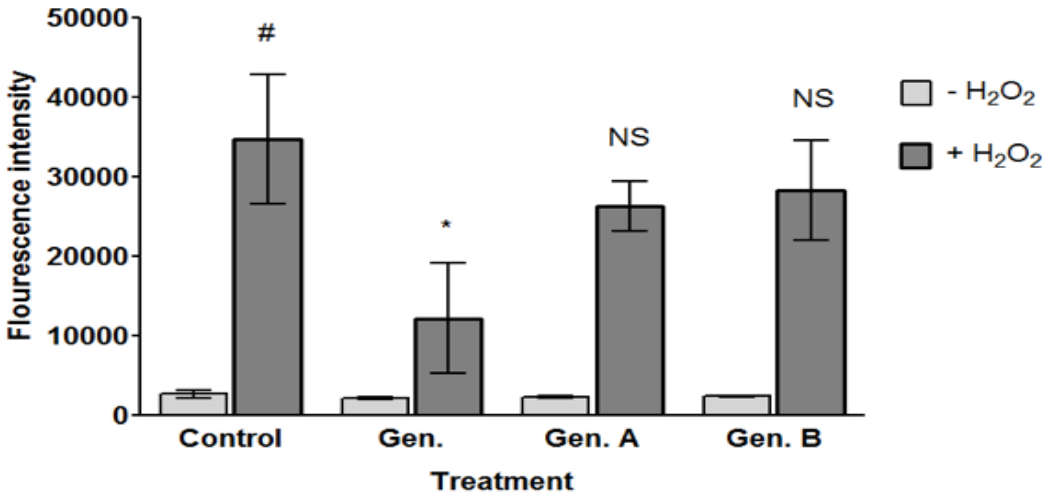

Figure 5.11: Fluorescence intensity in MLO-Y4 cells using H2DCF-DA.. Cells were pre-treated with H2DCF-DA at concentration of 10μM for 45 minutes prior to treatment with modified and unmodified Genistein at concentration of 10nM for 1 hour, then treated with 0.4mM H_2O_2 for 2 hours and then the fluorescence intensity was measured. Data are expressed as mean ± SD of 3 wells, $p<0.05$ versus H_2O_2, # versus control.

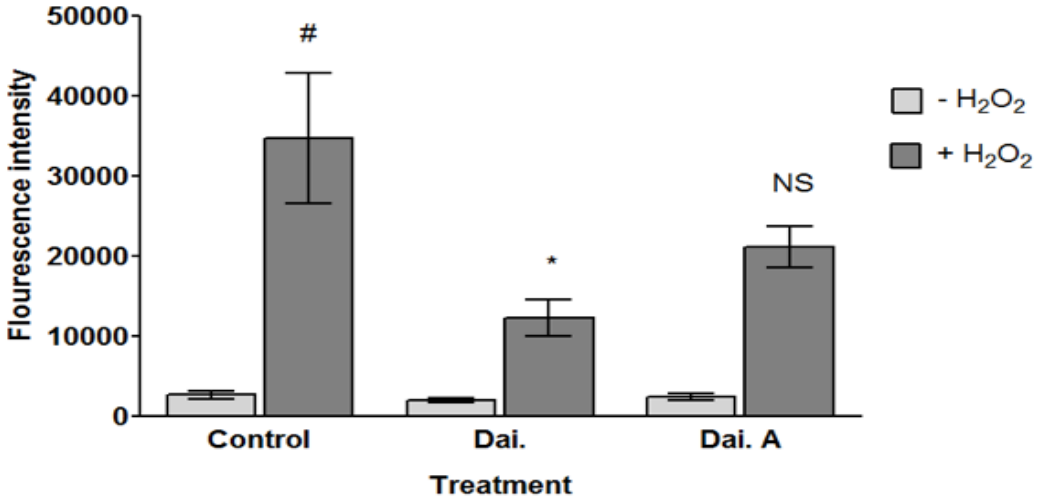

Figure 5.12: Fluorescence intensity in MLO-Y4 cells using H2DCF-DA. Cells were pre-treated with H2DCF-DA at concentration of 10μM for 45 minutes prior to treatment with modified unmodified Daidzein at concentration of 10nM for 1 hour, then treated with 0.4mM H_2O_2 for 2 hours and then the fluorescence intensity was measured. Data are expressed as mean ± SD of 3 wells, $p<0.05$ versus H_2O_2, # versus control.

5.6 The anti-apoptotic effect of modified Genistein and Daidazein on osteocyte apoptosis induced by H_2O_2.

Based on the previous data in (Figure 5.11 and 5.12), Genistein A , B and Daidzein A were used further to test their protective effect on MLO-Y4 cells from apoptosis induced by H_2O_2 subjected with DAPI staining by taking the percentage of apoptotic cells. It was found that none of the tested modified Genistein and Daidzein exhibited rescuing effect against apoptosis induced by H_2O_2. MLO-Y4 cells were plated in 24 well plates for 24 hours, and then treated with Genistein and two different groups of modified Genistein (A and B) for 1 hour at concentration 10nM prior to treatment with 0.4mM H_2O_2 for 2 hours. The percentage of osteocyte apoptosis induced by H_2O_2 ($H_2O_2$18.5% ± 1.33 vs. control 7.40 ± 0.9, p=0.01) was significantly reduced after pre-treatment with unmodified Genistein at concentration of 10nM (11.8% ± 1.09, p=0.002), but it was not significant after treatment with modified Genistein A (17.24 ± 1.7, p=0.36) and Genistein B (17.11% ± 2.1 p=0.38) Fig. 5.13. The percentage of osteocyte apoptosis was reduced after treatment with unmodified Daidzein (8.1% ±1 vs. H_2O_2 19.7% ± 1.26, p=0.0002) with no significant changes in the percentage of apoptosis in cells pre-treated with modified Daidzein (16.5% ± 1.05, p=0.106) (Fig 5.14).

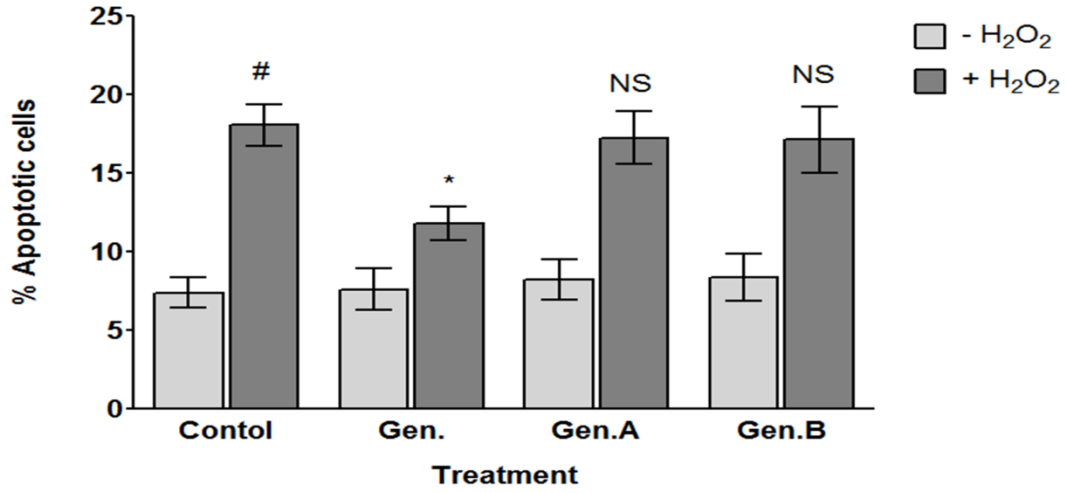

Figure 5.13: Effect of modified Genistein on osteocyte apoptosis induced by H_2O_2. MLO-Y4 cells were pre-treated with Genistein and modified Genistein at concentration of 10nM prior to treatment with 0.4mM of H_2O_2. Pre-incubation of modified Genistein A and B at a concentration 10nM did not reduce the percentage of apoptosis in MLO-Y4 cells significantly, but the reduction was significant after treatment with unmodified Genistein $p<0.05$. Data are expressed as mean ± SD of three wells (three fields per well were counted), n=9.

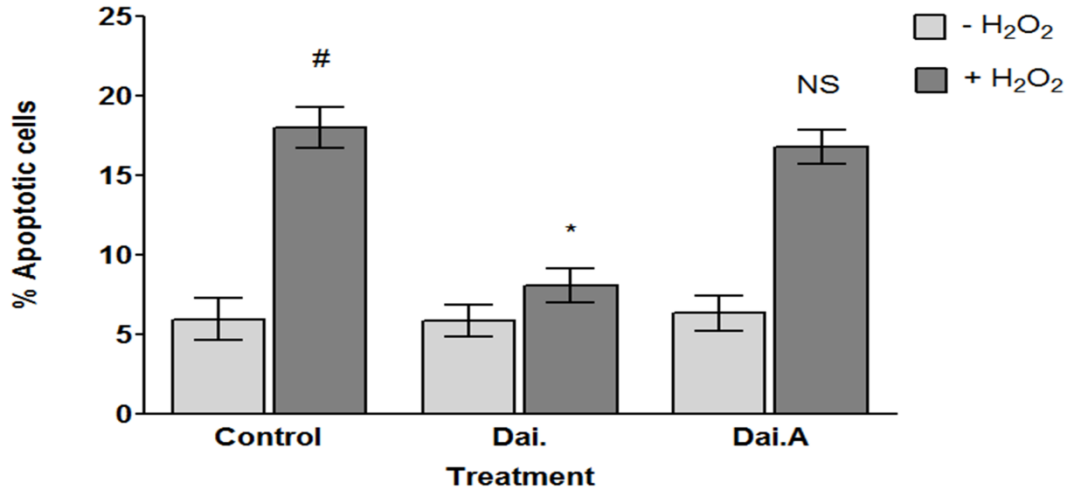

Figure 5.14: Effect of modified Daidzein on osteocyte apoptosis induced by H_2O_2. MLO-Y4 cells were treated with 10nM of unmodified and modified Daidzein A prior to treatment with 0.4mM H_2O_2. Cells were fixed and stained with DAPI and visualized under microscope. Pre-incubation of modified Daidzien A at a concentration 10nM did not reduce the percentage of apoptosis in MLO-Y4 cells significantly, but the reduction was significant after treatment with unmodified Daidzein $p<0.05$. Data are expressed as mean ± SD of three wells (three fields per well were counted), n=9.

5.7 Discussion

The mechanism of action of PEs on MLO-Y4 cells was investigated as to whether it was direct or indirect through ER. E2 was used as a control for its saving effect as it has been demonstrated previously that E2 had a direct effect on saving MLO-Y4 cells from apoptosis induced by H_2O_2 (Mann *et al*., 2007). The protective effect of PEs was investigated, whether it was mediated in a similar way to E2 directly or via ER.

Genistein has been identified to have a similar structure to E2, binds to ERs (Kurzer 2002) and it has been found to stimulate ER responsive gene through its interaction with ERs (Birt *etal.*, 2001; Kostelac *et al.*, 2003; Zierau *et al.*, 2006), with higher affinity to ERβ (Pike *et al.*, 1999). Some studies suggested that Genistein should be classified as a selective estrogen receptor modulator (SERMs), due to its similar interaction manner with raloxifene (Pike *et al.*, 1999) and similar effect on the skeletal system in ovariectimized mice (Śliwiński *et al.*, 2009).

Estrogen receptor inhibitor ICI 182780 is the most commonly known as pure anti-estrogen. It is derived from estradiol but contains alkylamide extension in the 7 alpha position, the effect of this anti-estrogen was to reduce the level of ER and also affect the receptor shuttling between nucleus and cytoplasm (Dauvoise *et al*.,1993). Pre-treatment of MLO-Y4 cells with ICI 182 780 did not block the protective effect of PEs and E2. However the incubation of cells with estrogen antagonist ICI 182 780 alone also prevented the cells from undergoing apoptosis induced by H_2O_2. Thus it is possible that ICI 180 780 is capable of antioxidant activity which can explain its ability to save osteocytes against oxidant attack in the absence of estradiol and this also indi-

cates that ER might not be involved in the pathway response. This study is consistent with previous studies which show, PEs in addition to their estrogenic activity have been reported to exert various non ER-related actions including inhibition of tyrosine kinase activity, inhibition of protein kinase C, inhibition of DNA topoisomerases I and II, inhibition of angiogenesis, antioxidant effects and modification of prostaglandin synthase activity (Kurzer and Xu, 1997, Arora *et al*., 1998, Markovits *et al*., 1995).

The inhibition of tyrosine kinase by Genistein may explain the mechanism by which it disturbs cancer cell growth (Ibarreta *et al*., 2001). PEs can also affect steroid bioavailability by interacting with key enzymes involved in the steroid synthesis such as 5α reductase, 17β-OH-steroid-dehydrogenase and aromatase (Makela *et al*., 1995, Kao *et al*., 1998, Rice *et al*., 2006, Lacey *et al*., 2005). In addition, PEs stimulate the production of sex hormone binding globulin (Pino et al., 2000), suggesting their role in altering steroid hormone bioavailability.

Also in this study, the activity of antioxidant enzyme catalase in MLO-Y4 cells exposed to H_2O_2 with or without PEs was determined. In the present study, treatment with H_2O_2 alone at concentration of 0.4mM induced cell apoptosis. PEs partially protected these cells from apoptosis and accumulation of ROS. Regulation of antioxidant enzyme in cells is an important process that balances the generation and the abolition of reactive oxygen species. Among the antioxidant enzymes, catalase is an important antioxidant enzyme, which eliminates hydrogen peroxide directly. According to the results in this study, catalase activity was higher in the hydrogen peroxide group compared to the control group. This seems to be consistent with previous studies which have

tested the neuroprotective and antioxidant effect of Resveratrol against H_2O_2 in embryonic neural stem cells, showing that H_2O_2 treatment increased CAT activity when compared with control (Konyalioglu *et al.,* 2013). However, treatment with PEs decreased the catalase activity, indicating a reduction in reactive oxygen species production. Resveratrol was known as a potent direct radical scavenger and has also been demonstrated to interact with redox systems *in vivo* by increasing the expression of antioxidant enzymes such as CAT, SOD or GSH-Px directly (Pignitter *et al.,* 2016). The prevention of oxidative stress induced apoptosis is thought to be through its direct activity and is related to the presence of C3-OH group (Heim *et al*., 2002). The protective effects of these OH groups on PEs was investigated by modifying their structure, and removing these groups to test their effect on oxidative stress induced by H_2O_2.

The presence of free hydroxyl groups on the aromatic ring of Daidzein and Genistein was assumed to play significant roles in the antioxidant activity of PEs, owed to their ability in scavenging ROS. Modification of the hydroxyl groups at positions 5 and 7 for Genisten, or all the hydroxyl groups, endorsed the significant structural relationship activity of those functional groups within the biological system. The fluorescence intensity of H2DCFDA probe correlated negatively with the strength of the ROS quenching by the hydroxyl groups. The cellular fluorescence production of H2DCFDA gave clear evidence for the reduced capacity of the modified hydroxyl groups in ROS scavenging in MLO-Y4 cell line, as compared to unmodified counterparts. Studies conducted by (Wang 2005) tested the anti-osteoporotic activity of Genistein whether they can be enhanced by their modification and substitut-

ed of their C-7 or C-4' hydroxyl with a number of chemical groups. In this study, Genistein and Daidzein were modified by masking their hydroxyl group and testing them for their effect on saving osteocytes from apoptosis induced by H_2O_2. Two groups of modified Genistein A, B and modified Daidzein A were tested for their effect on saving MLO-Y4 cell from ROS generation after treating MLO-Y4 with H2DCFDA and then treated with 10nM Genistein and Daidzein prior to treatment with H_2O_2. The assay is rapid and sensitive technique for detecting ROS generation due to oxidative stress induced by H_2O_2. Results show that treatment with modified Genistein A, B and Daidzein A did not increase oxidative stress in cells when compared with control. On the other hand they did not protect cells from oxidative stress induced by H_2O_2.

The antioxidant activity of PEs is thought to be related to the presence of free hydroxyl groups in the aromatic ring and ability to bind between aromatic rings (Burda and Oleszek, 2001). The hydroxyl group in the B-ring is important to enable the scavenging of reactive nitrogen or oxygen species (Pannala *et al.,* 2001). The mechanism by which PEs are able to scavenge free radicals is dependent on the ability of these compounds to donate a hydrogen atom or transfer an electron from C-3-OH in the B-ring to hydroxyl to peroxy and peroxynitrite radicals. This stabilizes the reactive species and gives rise to a relatively stable PE radical (Heim *et al*., 2002). Studies suggest that the antioxidant capacity of flavonoids strongly correlates with the number of hydroxyl groups in the B-ring, whereas hydroxyl groups in the A-ring may have little correlation with antioxidant activity (Heim *et al*., 2002). Interestingly, isoflavones such as Genistein have also been known to donate hydrogen atoms from the phenolic group (Heim *et al*., 2002). Therefore, PEs

scavenging ability of free radicals is attributed to the subtitutional reactivity of hydroxyl which participate in the following reaction F-OH + R= F-O· + RH (Heim *et al*., 2002). (F= phytoestrogens). Additionally, Genistein may also prevent oxidative stress by increasing the production of antioxidant enzymes such as glutathione peroxidase which maintains the reduction-oxidation reaction (redox) state by reducing oxygen peroxide, thus protecting against oxidative DNA damage (Suzuki *et al*., 2002; Raschke *et al*., 2006).

The chemical structure of isoflavones when compared with estradiol shows that the structure of isoflavones consists of two benzene rings, which are linked by a heterocyclic pyrane ring. Also, one hydroxyl group (-OH) is found attached to each benzene ring. Genistein and Daidzein differ by one hydroxyl group on the A ring of the isoflavone structure. The structures of isoflavones have simliar characteristics to the structure of 17,-estradiol. Both 17-estradiol and isoflavones have an aromatic ring with a hydroxyl group and a similar distance between two hydroxyl groups (Setchell and Cassidy 1999)). It is not surprising then, that isoflavones can bind to the estrogen receptor because of their structural similarity to estradiol.

Isoflavones and estradiol as well as other environmental estrogen like molecules bind to estrogen receptors (ERs). The isoflavones have higher binding affinities for the ERs in mammalian cells than most other estrogen like molecules but lower than that of estradiol. Isoflavones cross the cell membrane when they reach the target tissue by passive diffusion; then bind to ERs in the cytosol and form an isoflavone-ER complex; this complex then translocates into the nucleus for activation of the estrogen response element (ERE),

which is involved in the regulation of DNA-directed mRNA synthesis and the production of new proteins (Kuiper *et al.,* 1998).

Several PEs, which have structural similarities with mammalian E2 are capable of binding to ERs. They have been shown to have estrogen-like activity as they relieve postmenopausal complaints, increase bone formation and repress adipose tissue similar to E2 and they seem to have less or no side effects and have a lower cancer risk - although such risks cannot be completely excluded. Therefore, the food intake of plant hormone-containing fruits and legumes (like red grapes and soybeans) or the administrations of the active pharmaceutical ingredients have been considered as substitutes for the missing endogenous E2 production (Schlling *et al.,*2014).

CHAPTER SIX: THE EFFECT OF PHYTOESTROGENS ON CYTOKINE PRODUCTION IN MLO-Y4 IN RESPONSE TO HYDROGEN PEROXIDE

6.1 INTRODUCTION

Pro-inflammatory cytokines are known as the most powerful stimulants of bone resorption. They mediate the differentiation and proliferation of early osteoclast precursors to mature osteoclasts, which determine bone resorption capacity (Jilka et al., 1992). RANKL is a type II transmembrane TNF family protein which is expressed in different types of cell including bone marrow stromal cells, osteocytes, chondrocytes, and lymphocytes (Ikeda *et al.,* 2001). RANKL binds to the receptor activator of nuclear factor $_k$B (RANK) or OPG. RANK expressed on osteoclast progenitor cells binds to RANKL to induce osteocalastogenesis (Lacy *et al.,* 2012). Previous studies have shown that osteoclastogenesis requires cell-to-cell contact, while new studies suggest that condition media from apoptotic osteocytes which contains large amount of RANKL is sufficient for this process (Kogiani *et al.,* 2008). This suggests the important role of RANKL in osteoclastogenesis (O'Brien, 2013). While basal levels of RANKL is essential for physiological osteoclast renewal, additional cytokines either produced or regulated by T cells are responsible for the up regulation of osteoclast formation observed during estrogen deficiency (Weitzmann and Pacifici 2005). One such factor is TNF, a cytokine that enhances osteoclasts formation directly (Kim *et al.,* 2005) and by up regulating the bone marrow stromal cells production of RANKL and the responsiveness of osteoclasts precursors to this factor (Cenci *et al.,* 2000). It has been suggested that ROS may play a role in postmenopausal bone loss by generating a more oxidized bone micro-environment (Basu *et al.,* 2001).

Interleukin-6(IL-6) is a pleiotropic cytokine produced by immune cells and other organs (Papanicolaou 2000). It has been proposed that overexpres-

sion of IL-6 enhances bone remodeling and bone loss due to increasing bone resorption (De Benedetti *et al.*, 2006; Rufo *et al.*, 2011). However, *in vivo* studies showed that mice lacking IL-6 experience low bone mass, decreased osteoblast number and slow fracture healing (Yang et al., 2007).

TNF-α is a pro-inflammatory cytokine which induces apoptosis in osteoblast and fibroblast cells (Alikhani et al., 2004). Recent studies show that E2 disrupts the balance between bone formation and resorption after menopause, this imbalance is thought to be at least in part due to elevated level of TNF-α (Weitzmann and Pacifici 2007). E2 deficiency post menopause can cause elevated levels of TNF-α in human peripheral blood mononuclear cells (PBMC) (D'Amelio et al., 2005), while HRT treatment suppressed *in vivo* production of TNF-α (Bernard-poenaru et al., 2001). In addition TNF-α is involved in chronic inflammatory diseases such as rheumatoid arthritis (Boyce *et al*., 2005) and also promotes osteoclast differentiation (Fox *et al.,* 2008). The early intracellular signalling effect of TNF-α and RANKL are similar, each bind to a distinct receptors linked to different groups of tumor necrosis factor receptor (TRAF) signalling factors. RANKL activates TRAF2, 3 and 6 while TNF-α predominantly activates TRAF 2 and 3. Additionally RANKL activates more signal transduction factors than TNF-α, which are required to assist osteoclast formation (Fox *et al.,* 2008). As ROS activate the transcription factor (NF-κB) and regulate the gene expression of inflammatory cytokines (Seo *et al.,* 2002). Thus, the effect of PEs was investigated, whether they can inhibit TNF-α production in osteocytes in response to oxidative stress induced by H_2O_2. MLO-Y4 were treated with E2, Genistein, Daidzein and Resveratrol prior to treatment with H_2O_2 to measure the amount of TNF-α release in cells.

6.2 Measurement of RANKL release

The release of RANKL outside the cells was measured in MLO-Y4 cells seeded in 24-well plates at density of $3x10^4$ cells per well for 24 hours and treated with E2 and different PEs as previously described in section 2.2.14. RANKL assay was performed with ELISA kit (Booster) according to the manufacturer's instructions. The values were expressed as a total expression of RANKL concentration in pg/ml expressed relative to control. These experiments were performed to evaluate the relationship between antioxidants, apoptosis, and production of factors involved in bone remodelling such as RANKL. RANKL level in supernatant from cells cultured with H_2O_2 was increased after treatment to 70pg/ml ± 5 vs. control 9.6pg/ml ± 1.5, p=0.0001 at 2 hours, 80.3pg/ml ± 2.51 vs. control 10.5pg/ml ± 0.40, p=0.0001 after 4 hours and 102.5pg/ml ± 1.52l vs. control 8.3pg/ml ± 0.5, p=0.0001 after 24 hours. On the contrary, RANKL production in cells treated with E2 prior to H_2O_2 treatment was decreased to 30pg/ml ± 4.5, p=0.0005 at 2 hours, 44.33pg/ml ± 2.08, p=0.0001 at 4 hours and 55.6pg/ml ± 0.86, p= 0.0001 at 24 hours (Fig.6.1). Similarly, the level of RANKL production also decreased in MLO-Y4 cells treated with Genistein 41.50pg/ml ± 3.12 at 2 hours, 52.60pg/ml ± 5.03 at 4 hours and 62.32pg/ml ± 1.42 at 24 hours (Fig.6.2). Daidzein treatment reduced RANKL production to 42.76pg/ml ± 0.87 at 2 hours, 48.33pg/ml ± 3.51 at 4 hours and 65.41pg/ml ± 1.42 at 24 hours (Fig.6.3) and in treatment with Resveratrol was 41.46pg/ml ± 2.21 at 2 hours, 45.61pg/ml ± 3.51 at 4 hours and 67.04pg/ml ± 1.52 at 24 hours (Fig. 6.4).

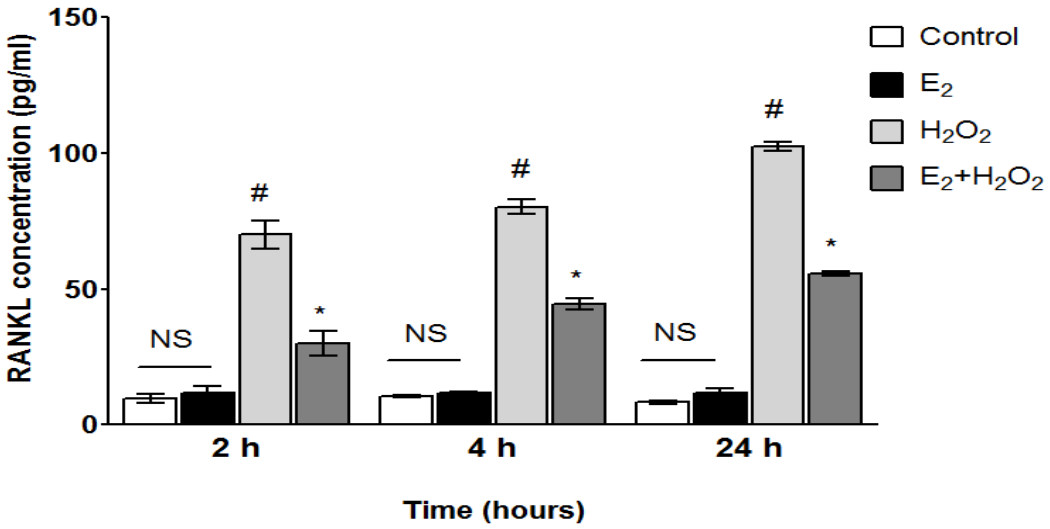

Figure 6.1: Effect of E2 on the levels of RANKL production in MLO-Y4 cells for different time. Cells were treated with E2 for one hour prior to treatment with H_2O_2 for (2, 4 and 24 hours). Media were collected and the concentration of RANKL was measured by ELISA. The levels of RANKL in cells treated with H_2O_2 was high at all hours, but lower in cells treated with E2 and * p< 0.05 versus H_2O_2, # versus control, n=3.

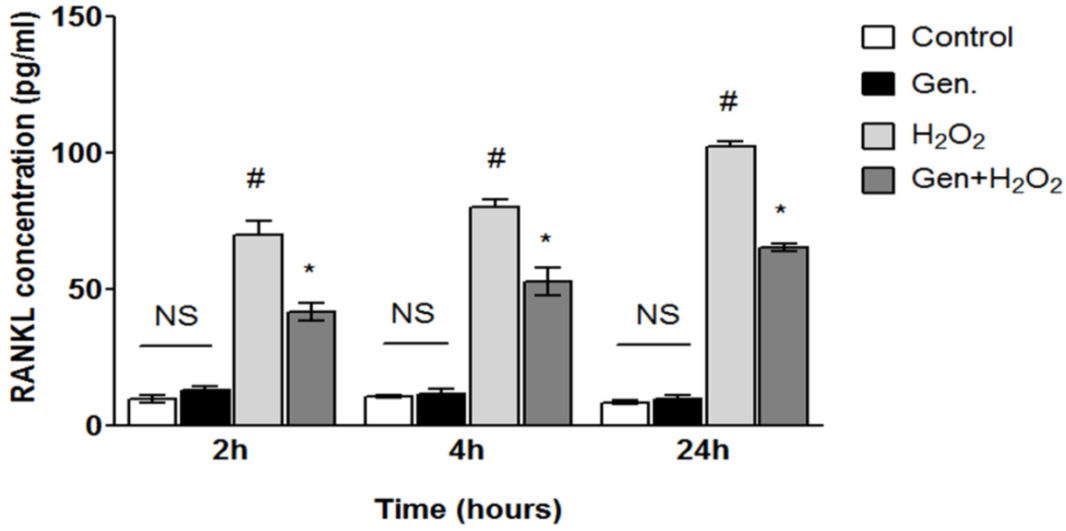

Figure 6.2: Effect of Genstein on the levels of RANKL production in MLO-Y4 cells for different time. Cells were treated with Genstein for one hour prior to treatment with H_2O_2 for (2, 4 and 24 hours). Media were collected and the concentration of RANKL was measured by ELISA. The levels of RANKL in cells treated with H_2O_2 was high at all hours, but lower in cells treated with Gen and * p < 0.05 versus H_2O_2, # versus control, n=3.

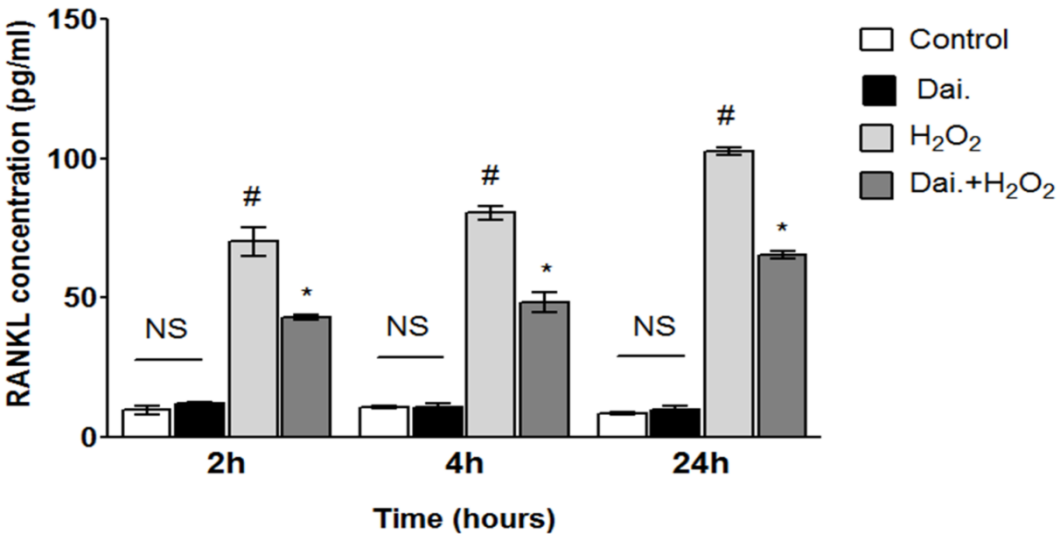

Figure 6.3: Effect of Daidzein on the levels of RANKL production in MLO-Y4 cells for different time. Cells were treated with Daidzein for one hour prior to treatment with H_2O_2 for (2, 4 and 24 hours). Media were collected and the concentration of RANKL was measured by ELISA. The levels of RANKL in cells treated with H_2O_2 was high at all hours, but lower in cells treated with Daidzein and * $p < 0.05$ versus H_2O_2, # versus control, n=3.

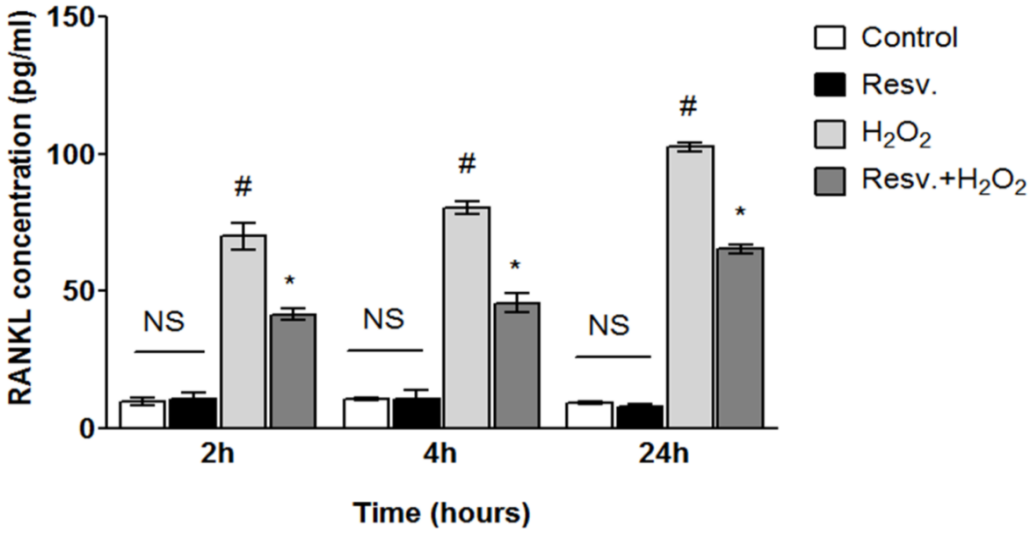

Figure 6.4: Effect of Resveratrol on the levels of RANKL production in MLO-Y4 cells for different time. Cells were treated with Resveratrol for one hour prior to treatment with H_2O_2 for (2, 4 and 24 hours). Media were collected and the concentration of RANKL was measured by ELISA. The levels of RANKL in cells treated with H_2O_2 was high at all hours, but lower in cells treated with Resv. and * $p < 0.05$ versus H_2O_2, # versus control, n=3.

Table 6.1: Concentration of RANKL released in MLO-Y4 cells in response to H_2O_2 in presence and absence of E2 and PEs (Genistein, daidzein and Resveratrol).

	2 hours	4 hours	24 hours
Control	9.6pg/ml ± 1.5	10.5pg/ml ± 0.40	8.3pg/ml ± 0.5
H_2O_2	70pg/ml ± 5	80.3pg/ml ± 2.51	102.5pg/ml ± 1.52
E2	30pg/ml ± 4.5	44.33pg/ml ± 2.08	55.6pg/ml ± 0.86
Genistein	41.5pg/ml ± 3.12	52.60pg/ml ± 5.03	62.32pg/ml ± 1.42
Daidzein	42.76pg/ml ± 0.87	48.33pg/ml ± 3.51	67.04pg/ml ± 1.42
Resveratrol	41.46pg/ml ± 2.21	45.61pg/ml ± 3.51	65.33pg/ml ± 1.52

6.3 Measurement of TNF-α release

MLO-Y4 cells were seeded in 24-well plates at density of $3x10^4$ cells per well for 24 hours and treated with E2 , Genistein, Daidzein and Resveratrol as previously described in section 2.2.14. TNF-α assay was performed with ELISA kit from (Biolegend) according to the manufacturer's instructions. The values were expressed as a total expression of TNF-α concentration in pg/ml expressed relative to control. The amount of TNF-α was measured in MLO-Y4 cells after treatment with E2 and PEs prior to treatment with H_2O_2 for 2, 4 and 24 hours. H_2O_2 increased the level of TNF-αprotein in the culture medium of MLO-Y4 cells. TNF-α level in the supernatant from cells cultured with H_2O_2 was 136.60 pg/ml ± 2.06 vs. control (50.69pg/ml ± 2.1, p=0.0001) at 2 hours, (140.60pg/ml ± 3.21 vs. control 54.17pg/ml ± 1.5, p=0.0001) at 4

hours and (187.55 pg/ml ± 2.43 vs. control 60.36pg/ml ± 1.7, *p*=0.001) at 24 hours. While treatment with E2 decreased the level of TNF-αrelease in MLO-Y4 cells to (94.62pg/ml ± 1.05, *p*=0.0001) at 2 hours, (74.16pg/ml ± 3.12, *p*=0.0001) at 4 hours and 108.61pg/ml ± 2.10, *p*=0.0005) at 24 hours (Fig.6.5). Also treatment with Genistein prior to H_2O_2 treatment reduced TNF-α level in MLO-Y4 cells (58.67pg/ml ± 0.91, *p*=0.0001) at 2 hours, (89.62pg/ml ± 2.33) at 4 hours and (98.61pg/ml ± 1.99, *p*=0.0001) at 24 hours (Fig.6.6), while TNF-α levels after Daidzein treatment was 79.65 pg/ml ± 0.91, *p*=0.0001 at 2 hours, 79.65pg/ml ± 3.2, *p*=0.0001) at 4 hours and (105.62pg/ml ± 2, *p*=0.0001) at 24 hours (Fig.6.7). Resveratrol treatment also reduced TNF-α levels to (81.15pg/ml ± 2.07, *p*=0.0001) at 2 hours, (93.79pg/ml ± 3.07, *p*=0.0001) at 4 hours and (127.61pg/ml ± 4.81, *p*=0.0001) at 24 hours (Fig.6.8).

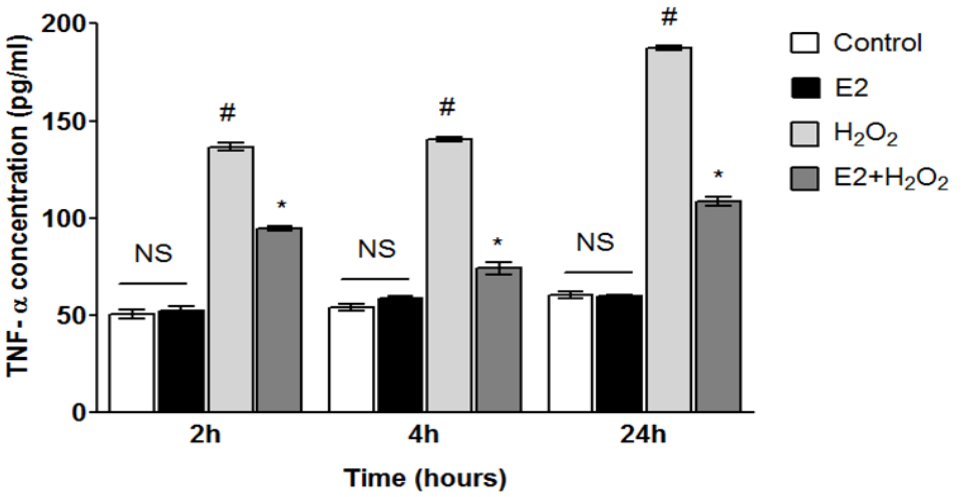

Figure 6.5: Effect of E2 on the levels of TNF-α production in MLO-Y4 cells for different time. Cells were treated with E2 for one hour prior to treatment with H_2O_2 for (2, 4 and 24 hours). Media were collected and the concentration of TNF-α was measured by ELISA. The levels of TNF-α in cells treated with H_2O_2 was high at all hours, but lower in cells treated with E2 and * $p < 0.05$ versus H_2O_2, # versus control, n=3

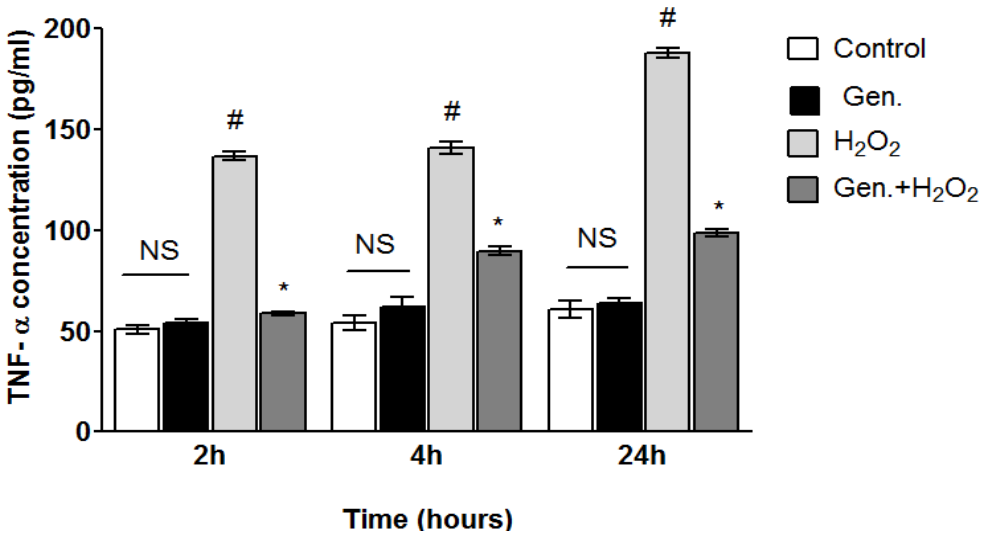

Figure 6.6: Effect of Genistein on the levels of TNF-α production in MLO-Y4 cells for different time. Cells were treated with Genistein for one hour prior to treatment with H_2O_2 for (2, 4 and 24 hours). Media were collected and the concentration of TNF-α was measured by ELISA. The levels of TNF-α in cells treated with H_2O_2 was high at all hours, but lower in cells treated with E2 and * $p < 0.05$ versus H_2O_2, # versus control, n=3.

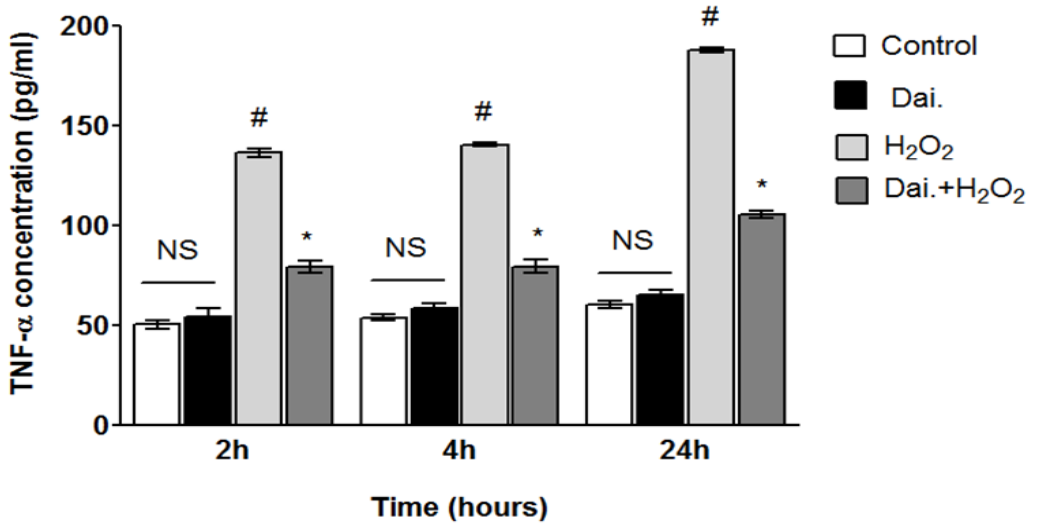

Figure 6.7: Effect of Daidzein on the levels of TNF-α production in MLO-Y4 cells for different time. Cells were treated with Daidzein for one hour prior to treatment with $H_2O_2$for (2, 4 and 24 hours). Media were collected and the concentration of TNF-α was measured byELISA. The levels of TNF-α in cells treated with H_2O_2 was high at all hours, but lower in cells treated with E2 and * $p < 0.05$ versus H_2O_2, # versus control, n=3.

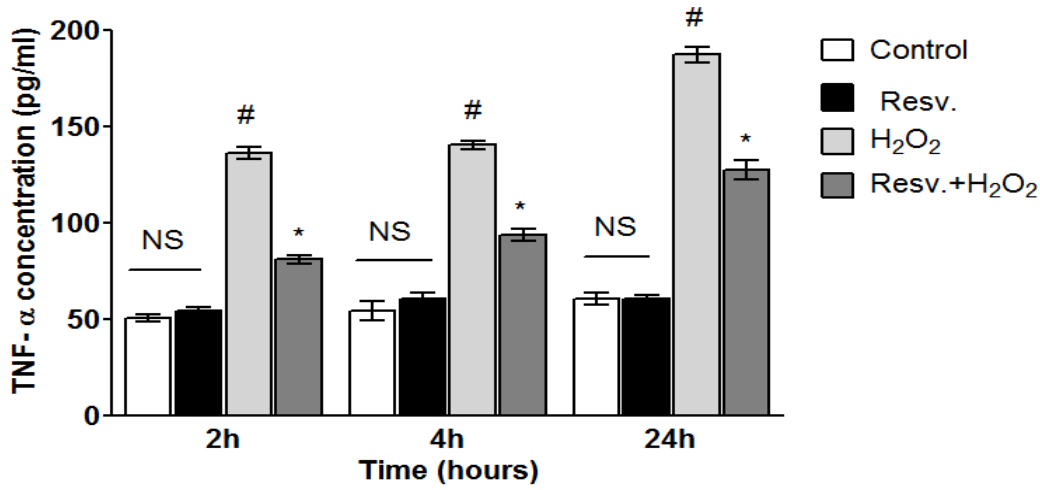

Figure 6.8: Effect of Resveratrol on the levels of TNF-α production in MLO-Y4 cells for ddifferent time. Cells were treated with Resveratrol for one hour prior to treatment with H_2O_2 for (2, 4 aand 24 hours). Media were collected and the concentration of TNF-α was measured by ELISA. The lelevels of TNF-α in cells treated with H_2O_2 was high at all hours, but lower in cells treated with E2 and * $p < 0.05$ versus H_2O_2, # versus control, n=3.

Table 6.2: Concentration of TNF-α released in MLO-Y4 cells in response to H_2O_2 in presence and absence of E2 and PEs (Genistein, daidzein and Resveratrol).

	2 hours	4 hours	24 hours
Control	50.69pg/ml ± 2.06	54.17pg/ml ± 1.5	60.36pg /ml ± 1.7
H_2O_2	136.60pg/ml ± 2.06	140.60pg/ml ± 3.21	187.55pg/ml ± 2.43
E2	94.62pg/ml ± 1.05	74.16pg/ml ± 3.12	108.61pg/ml ± 2.10
Genistein	58.67pg/ml ± 0.91	89.62pg/ml ± 2.33	98.61pg/ml ± 1.99
Daidzein	79.65pg/ml ± 0.91	79.65pg/ml ± 3.2	105.62pg/ml ± 2
Resveratrol	81.15pg/ml ± 2.07	93.79pg/ml ± 3.07	127.61pg/ml ± 4.81

6.4 Effect of PEs on IL-6 released in MLO-Y4 cells

IL-6 release was measured in MLO-Y4 cells seeded in 24-well plates at density of $3x10^4$ cells per well for 24 hours and treated with E2 and different PEs as previously mentioned in method chapter. IL-6 assay was performed with ELISA kit from (Bio-legend) according to the manufacturer's instructions. The values were expressed as the amount of IL-6 in pg/ml. H_2O_2 increased the level of IL-6 protein in the culture medium of MLO-Y4 cells. IL-6 level in cells cultured with H_2O_2 was (225.48pg/ml ± 4.7 vs. Control 110.38pg/ml ± 13.7, p=0.0001) at 2 hours, (243.4pg/ml ± 2.5 vs. control 107.5pg/ml ± 6.3, p=0.0001) at 4 hours and (315.5pg/ml ± 5.3 vs. control 65.33pg/ml ± 1.5, p= 0.0.0001) at 24 hours. While treatment with E2 decreased the level of IL-6 release in MLO-Y4 (145.4pg/ml ± 6.7, p=0.0001) at 2 hours, (147.5pg/ml ± 3.5, p=0.0001) at 4 hours and (184.50pg/ml ± 5.1, p=0.0001) at 24 hours (Fig.6.9). Treatment with Genistein prior to H_2O_2 reduced IL-6 level in cells to (152.50pg/ml ± 3.88, p=0.0001) at 2 hours, (158.16pg/ml ± 3.36, p=0.0001)

at 4 hours and (187pg/ml ± 8.08, *p*=0.0001) at 24 hours (Fig.6.10), also Daidzein treatment reduced IL-6 concentration to (155.150pg/ml ± 3.88, *p*=0.0001) at 2 hours, (166.62pg/ml ± 3.36, *p*=0.0001) at 4 hours and (195.50pg/ml ± 5.19, *p*=0.0001) at 24 hours (Fig6.11) and Resveratrol decreased the level of IL-6 to (150.16pg/ml ± 5.88, *p*=0.0001) at 2 hours, (149.65pg/ml ± 2.05, *p*=0.0001) at 4 hours and (182.95pg/ml ± 3.05, p=0.0001) at 24 hours (Fig.6.12).

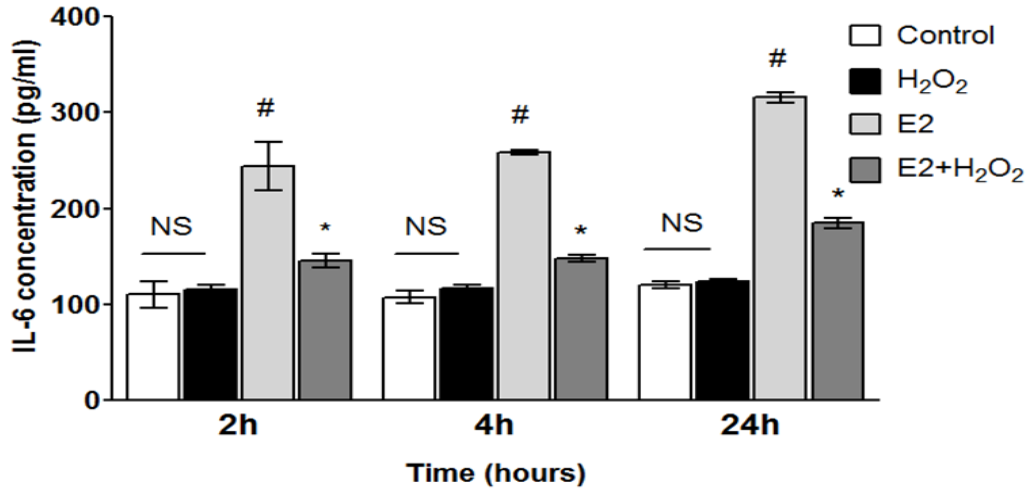

Figure 6.9: Effect of E2 on the levels of IL-6 production in MLO-Y4 cells for different time. Cells were treated with E2 for one hour prior to treatment with H_2O_2 for (2, 4 and 24 hours). Media were collected and the concentration of IL-6 was measured by ELISA. The levels of IL-6 in cells treated with H_2O_2 was high at all hours, but lower in cells treated with E2 and * p< 0.05 versus H_2O_2, # versus control, n=3.

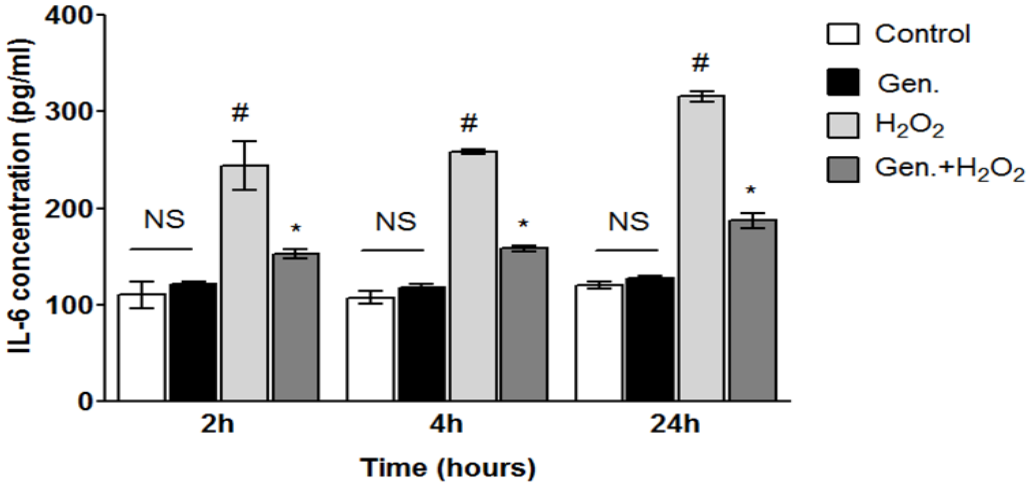

Figure 6.10: Effect of Genestein on the levels of IL-6 production in MLO-Y4 cells for different time. Cells were treated with Genistein for one hour prior to treatment with H_2O_2 for (2, 4 and 24 hours). Media were collected and the concentration of IL-6 was measured by ELISA. The levels of IL-6 in cells treated with H_2O_2 was high at all hours, but lower in cells treated with E2 and * p < 0.05 versus H_2O_2, # versus control, n=3.

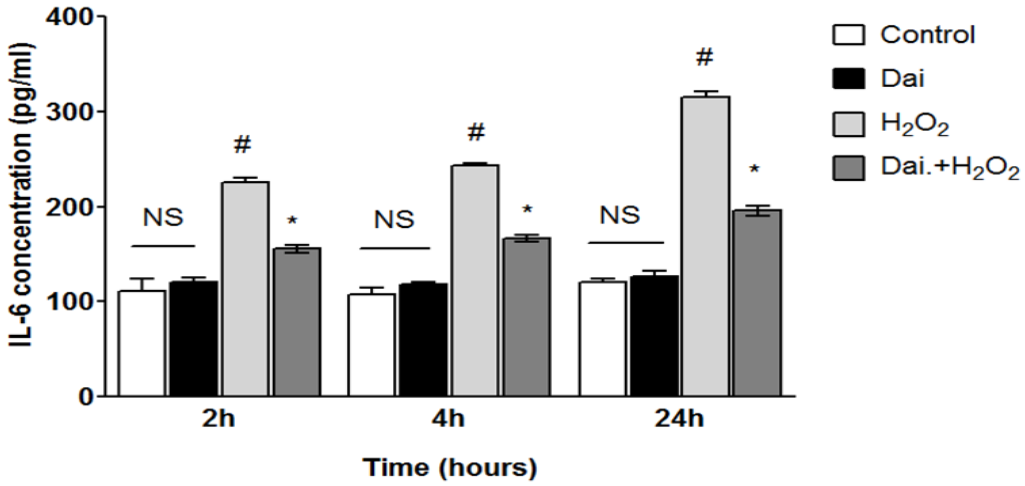

Figure 6.11: Effect of Daidzein on the levels of IL-6 production in MLO-Y4 cells for different time. Cells were treated with Daidzein for one hour prior to treatment with H_2O_2 for (2, 4 and 24 hours). Media were collected and the concentration of IL-6 was measured by ELISA. The levels of IL-6 in cells treated with H_2O_2 was high at all hours, but lower in cells treated with E2 and * $p < 0.05$ versus H_2O_2, # versus control, n=3.

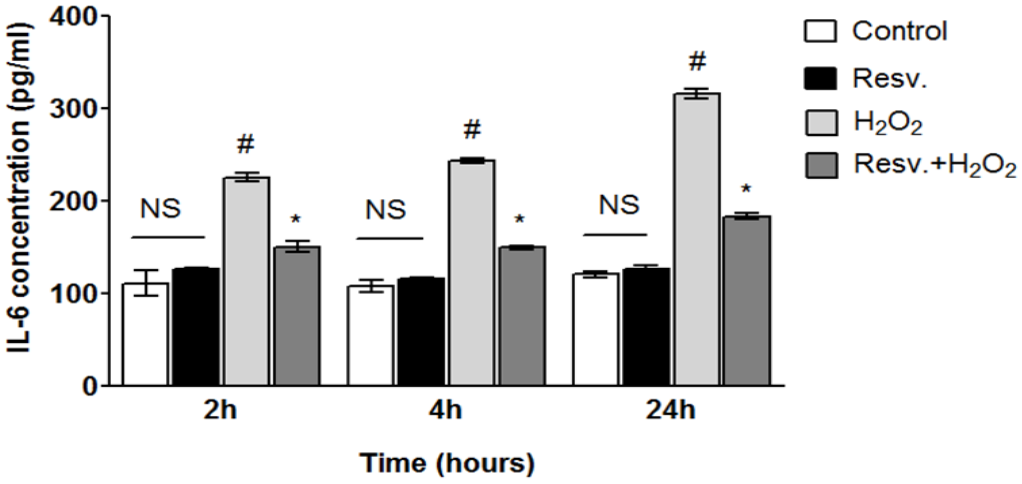

Figure 6.12: Effect of Resveratrol on the levels of IL-6 production in MLO-Y4 cells for different time. Cells were treated with Resveratrolfor one hour prior to treatment with H_2O_2 for (2, 4 and 24 hours). Media were collected and the concentration of IL-6 was measured by ELISA. The levels of IL-6 in cells treated with H_2O_2 was high at all hours, but lower in cells treated with E2 and * $p < 0.05$ versus H_2O_2, # versus control, n=3.

Table 6.3: Concentration of IL-6 released in MLO-Y4 cells in response to H_2O_2 in presence and absence of E2 and PEs (Genistein, daidzein and Resveratrol).

	2 hours	4 hours	24 hours
Control	110.38pg/ml ± 13.7	107.5pg/ml ± 6.3	107.5pg/ml ± 6.3
H_2O_2	225.48pg/ml ± 4.7	243.4pg/ml ± 2.5	315.5pg/ml ± 5.3
E2	145.4pg/ml ± 6.7	147.5pg/ml ± 3.5	184.50pg/ml ± 5.1
Genistein	152.50pg/ml ± 3.88	158.16/ml ± 3.07	187pg/ml ± 8.08
Daidzein	155.150pg/ml ± 3.88	166.62pg/ml ± 3.36	195.50pg/ml ± 5.19
Resveratrol	150.16pg/ml ± 5.88	149.65pg/ml ± 2.05	182.95pg/ml ± 3.05

6.5 Discussion

The role of oxidative stress on the release of cytokines in osteocytes was explored. Previous studies have documented increased oxidative stress participates in osteoporosis, induced by estrogen deficiency and ageing (Baek *et al.,* 2010; Maggio *et al.,* 2003). The process of bone remodeling requires close communication and balance between osteoblasts and osteoclasts. Osteoblasts play an important role not only in bone formation but also work in conjunction with osteoclasts in the release of various bone resorbing cytokines such as IL-6 and RANKL. Receptor activator of nuclear factor (NFkB) ligand more commonly known as RANKL is a cytokine of tumor necrosis factor (TNF) family of proteins, which has been known to be vital in the transduction of osteoclastogenesis (Lacey *et al.,* 1998). RANKL is highly expressed on osteoblasts, marrow stromal cells and T cells, and binds to the

RANK receptor on osteoclast progenitor cells (Boyle *et al.,* 2003). Recent studies suggest that osteocytes including MLO-Y4 cells produce RANKL at a level 40 times higher in vitro than osteoblasts (Xiong *et al.,* 2011, Nakashima *et al.,* 2011, Zhao *et al.,* 2002). Osteoclastic differentiation and activation is known to be associated with the binding of RANKL and RANK resulting in the activation of nuclear factor-kB and c-jun N terminal protein kinase. While, increased levels of ROS was found to stimulate RANKL mRNA and subsequent production of RANKL in human osteoblast-like MG63 cell line, primary bone marrow stromal cells (BMSCs) of mouse and calvarial osteoblasts. This suggests that accumulation of ROS in bone related diseases plays an important role through the stimulation of osteoclast differentiation and the initiating bone resorption (Bai *et al.,* 2005). Therefore, the development of therapies for the treatment of osteoporosis seems to be likely through the inhibition of RANKL (Gallagher and Sai, 2010).

Soybean, which contains the active component of Genistein, a part of the Isoflavonoides, has been known to possess anti-inflammatory properties. Studies have documented the anti-inflammatory properties of Genistein, in which a reduction of inflammation in collagen induced RA in (CIA) rat model was observed. For instance, it has been suggested that subcutaneous injection of Genistein exerts evident anti-inflammatory properties on CIA rats (Verdrengh *et al.,* 2003). Another study also reported the possibility for the use of Genistein in the treatment of RA-induced inflammation (Wang *et al.,* 2008). PEs have been found to enhance or suppress immune response depending on their concentrations. The mechanism in which phytoestrogens elicit such a response is not well known, therefore further studies are re-

quired to understand the underlying mechanisms involved. Studies have demonstrated the ability of Genistein in reducing the production of IL-6, an inflammatory mediator in MH7A cells activated with TNF- α *in vitro* (Li *et al.,* 2014). The anti-inflammatory properties of Genistein are known to be mediated by the interruption of the ROS/Akt/NF-κB signaling pathway in MH7A cells which results in decreased production of TNF-α (Li *et al.,* 2014).

In this study, MLO-Y4 cells were pre-treated with PEs and then exposed to H_2O_2, then the effect of PEs on the production of IL-6, RANKL and TNF- α for 2, 4 and 24 hours were investigated in which a significant decrease in level of cytokines were observed. Studies have shown that ROS plays an important role in inducing inflammatory signals through Akt and NF-κB activation (Morgan and Liu 2011, Asehnoune *et al.,* 2004). Furthermore, Genistein has been shown to reduce ROS by reducing the expression of ROS producing enzymes (Park *et al.,* 2010). A recent study has showed that Resveratrol treatment decreased in a dose- and time-dependent manner pro-inflammatory cytokines, such as TNF- a and IL-6, induced in human periodontal ligament cells by gram negative bacteria (Rizzo *et al.,* 2012).

It has been assumed that the ROS-mediated activation of the PI3K/Akt pathway is vital in the activation of NF-κB and the subsequent production of pro-inflammatory cytokines. On the other hand, *in vitro* studies have shown that phytoestrogens modulate osteoprotegerin (OPG)-receptor activator of nuclear factor κB ligand (RANKL) system which indicates the vital role it plays in the process of bone remodeling (Yamagishi *et al.,* 2001, Viereck *et al.,* 2002). Isoflavones treatment have been shown to decrease the concentration of serum cytokines such as IL-6 and TNF-α, known to be the main protein factors

responsive to the body's immune system to regulate bone metabolism and play a key role in the maturation and differentiation of osteoblasts as well as the activation of osteoclasts. These effects are worth same attention as treating bone disorders in post-menopausal women (Chi and Zhang 2013).

In summary, during the manifestation of postmenopausal osteoporosis, the interaction of estrogen with bone cells has the ability to adjust the cytokine network, which regulates the formation of bone. The sufficiency of estrogen limits the production of cytokines and maintains normal bone formation however, decreased levels of estrogen result in the loss of balance which not only leads to increased secretion of TNF-α and IL-6 in the bone marrow but also disrupt the synergy between cell factors and therefore, result in the disturbance of balance between osteoblasts and osteoclasts (Delmas *et al.,* 2000). Furthermore, studies conducted suggest that the administration of 90 mg/day isoflavone for a period of 6 months was correlated with decreased IL-6 and TNF-α serum levels which suggests that soy isoflavone can prevent osteoporosis by decreasing IL-6 and TNF-α levels in the serum. The cytokine IL-6 is thought to be a prominent factor that contributes to bone loss in osteoporosis and structural damage in rheumatoid arthritis (Terauchi 2011., Jilka *et al,* 1992). As a result, this has led to the development of anti-IL-6 receptor agents such as tocilizumab, which has been effectively used in patients suffering with moderate to severe rheumatoid arthritis (Agarwal 2011). In addition, it has been well established that the osteocytes play a critical role in bone remodeling. Similarly, to osteoblasts, osteocytes are known to produce IL-6, however, higher levels of IL-6 are observed with apoptotic osteocytes. The production of IL-6 from apoptotic osteocytes is key step in the ini-

tiation of bone remodeling by facilitating osteoclast precursors adhesion to endothelial cells (Cheung *et al.,* 2012). Moreover, previous studies showed, co-culture of MLO-Y4 cells with spleen or bone marrow derived precursor cells also promoted osteoclastogenesis *in-vitro.* However, the effects seen were secondary to RANKL and M-CSF production (Zhao *et al.,* 2002). Therefore, it is likely that the process of osteoclastogenesis is regulated by osteocytes via multiple pathways of which IL-6 plays a prominent role (Peruzzi *et al.*, 2012). Osteoclast activity is thought to be suppressed by both Genistein and Diadzein through a number of possible mechanisms such as induction of apoptosis, activation of protein tyrosine phosphatase, inhibition of cytokines, changes in intracellular Ca^{++}, and membrane depolarization (Okamoto *et al.,* 2001, Gao and Yamaguchi *et al.,* 2000). The mechanism through which PEs reduce cytokines expression is not known and could potentially be meditated through several effects. It could be caused by the antioxidant action of PEs, which could modify the redox status and inhibit TNF-α secretion. The effect of Genistein could also be mediated through its ability to suppress tyrosine kinase signalling which plays an important role in inflammation (Duan *et al.,* 2003).

CHAPTER SEVEN: GENERAL DISCUSSION

7.1 Discussion

Bone is a dynamic tissue that constantly maintains its integrity through bone remodelling cycle in response to changes in mechanical loading, serum calcium and micro-damage. Bone remodelling is dependent on the balanced activity of osteoclasts, osteoblasts and osteocytes. Local and systemic factors orchestrate a balance between bone formation and resorption during normal remodelling (Weitzmann and Pacifici, 2005). This balance is disrupted in many skeletal diseases including post-menopausal osteoporosis due to a decrease in the level of circulating E2, which is associated with decreased bone mass, and increased fracture risk. E2 has been shown to regulate bone remodelling through several mechanisms, it can directly modify osteoclast life span via the modification of cytokine production by osteoblasts and immune cells can stimulate osteoclast formation and activity (Weitzmann and Pacifici 2006a).

Several therapeutic agents have been developed to limit bone resorption in women after menopause such as HRT, which lowers the risk of vertebral and hip fracture in women (Barrett-Connor 1998) and decreases incidence of cardiovascular diseases (Collins 2002). However, other studies suggested that HRT treatment has been shown to be associated with cardiovascular disease and cancer risk (Chlebowski *et al*., 2010). PEs have been used as an alternative for HRT with fewer side effects and have beneficial effect on bone mass. This was observed in Asian women with high consumption of PEs in comparison to Western populations, and have lower incidence of fractures, breast cancer and cardiovascular disease (Peeters *et al*., 2003). Epidemiological studies and associated meta-analyses strongly suggest that

long-term consumption of diets rich in plant polyphenols offer protection against development of numerous diseases including osteoporosis (Graf *et al.,* 2005). The positive action of PEs has been thought to be via the activity of different bone cells such as inhibition of osteocyte and osteoblast apoptosis and inhibition of osteoclast differentiation. However, the molecular mechanism through which PEs act is not clear. One potential mechanism is protecting osteocyte cells from oxidative stress through inhibition of ROS generation.

Chapter three results provide evidence that PEs directly suppress oxidative stress in MLO-Y4 cells induced by H_2O_2. The data demonstrate that treatment of MLO-Y4 cells with H_2O_2 induced oxidative stress resulted in cell death with morphological characteristics of apoptosis at a concentration of 0.4mM. Treatment of MLO-Y4 cells with E2 blocked H_2O_2 effects of apoptosis and reduced the level of apoptosis to that of control. Previous studies have shown that, treatment of MLO-Y4 with H_2O_2 induced apoptotic cell death which was inhibited after pre-treatment with 17-estradiol at near physiological concentrations 10nM (Mann *et al.,* 2007). Estradiol is important in preventing the increase in osteocyte apoptosis engendered by OVX (Collishaw *et al.,* 2004). Similarly treatment of MLO-Y4 cells with Genistein, Daidzein and Resveratrol at a concentration of 10nM were found to block oxidant induced apoptosis with H_2O_2, which was evidenced by a range of methods such as morphology characterisation evidenced by DAPI staining and caspase activation. Caspase activation is the common feature of apoptosis and this study, has shown that PEs influence caspase-3,-7 activation and the percentage of caspase positive cells was decreased significantly af-

ter treatment with PEs in presence of H_2O_2. It has been demonstrated that using pan-caspase inhibitor *in vivo* prevented both, the osteocyte apoptosis and the activation of new bone remodelling. Therefore, osteocyte apoptosis plays a direct, and controlling role in the activation and targeting of a micro-damage remodelling response (Cardoso *et al.,* 2009). The identification of ROS in MLO-Y4 cells which was evidenced by the free radical indicator H_2DCF-DA, has demonstrated that PEs were capable of blocking ROS generated in cells by H_2O_2. Therefore, the effects of PEs were tested on ROS generation in MLO-Y4 cells and were found to inhibit ROS generation in these cells. A range of PEs (Genistein, Daidzein and Resveratrol) were used which were able to reduce free radical production in MLO-Y4 as in the last decade, there has been much interest in the potential health benefits of these dietary PEs as antioxidant therapy. In this study knowledge about the effects of other polyphenols are presented in the context of relevance to human health e.g. Fucosterol and 6-gingerol. There is less knowledge about these two phytomolecules in regard to their protective effects as antioxidants in MLO-Y4 cells.

Osteoblast and osteoclast activity is tightly coupled during remodelling, such that bone resorption is linked to a subsequent formative phase. This coupling is lost in post-menopausal women, which contributes to bone loss. This study examined the effect of PEs on osteoblast apoptosis induced by H_2O_2. Genistein, Daidzein and Resverartrol have the ability to inhibit apoptosis induced by oxidative stress in osteoblast cells MC3T3-E1 evidenced with DAPI staining and assessment of ROS generation. The effects of PES on osteoclast differentiation were investigated to establish their anti-osteoporotic ac-

tivity. Osteoclast differentiation from murine macrophage RAW 264.7 cells was induced by RANKL and M-CSF, which are essential for the differentiation of monocytes/macrophages into osteoclasts. TRAP-

positive multinucleated osteoclasts were visualized by light microscopy. RANKL treatment increased the induced osteoclast formation from RAW 264.7 and increased TRAP positive cells, while treatment with PEs reduced the number of TRAP positive cells. This result is consistent with previous studies which suggest that Soy phytoestrogen Genistein has been shown to enhance osteoblast differentiation and maturation and also inhibit osteoclast formation and bone resorption through inducing osteoclastogenic inhibitor OPG and blocking NF-kappaB signaling, whilst these effects are probably not mediated via estrogen receptors (Ming *et al.,* 2013). Resveratrol is also reported as a potent inhibitor of osteoclast differentiation through controlling ROS generation (He *et al.,* 2010).

These results strengthen the data for the use of PEs as agents in the treatment of post-menopausal bone loss and inflammatory osteolytic diseases where they may suppress RANKL-induced osteoclast formation.

The mechanism of action of PEs on MLO-Y4 cells was investigated as to whether it had direct action or was directed via ER. ERs inhibitor ICI 182780 was used prior to treatment with PEs (Genistein, Daidzein and Resveratrol) and H_2O_2 to induce apoptosis. Pre-treatment of MLO-Y4 cells with ICI 182780 did not block the protective effect of PEs, which indicates that the effect of PEs is ER independent. However, the incubation of cells with estrogen antagonist ICI 182780 alone also prevented apoptosis induced by H_2O_2. This study provided evidence that the ability of PEs to inhibit oxidant induced

apoptosis in HEK293 cell line which is negative for ER-α and ER-β indicated an estrogen receptor- independent mechanism of apoptosis blockade in cells exposed to oxidant attack.

Regulation of antioxidant enzyme in cells is an important process that balances the generation and the abolition of reactive oxygen species. Among the antioxidant enzymes, catalase is more important and eliminates hydrogen peroxide directly. According to the results, catalase activity was higher in the H_2O_2 group, compared to the control group. Treatment with PEs decreased the catalase activity, indicating a reduction in reactive oxygen species. The prevention of oxidative stress induced apoptosis is thought to be through its direct activity and is related to the presence of C3-OH group. Hence, the role of OH group on saving effect of PEs was investigated by modifying their structure, namely removing these groups, to test their effect on oxidative stress induced by H_2O_2.

The presence of free hydroxyl groups on the aromatic ring of Daidzein and Genistein was assumed to play significant roles in the antioxidant activity of PEs, owed to their ability in scavenging ROS. Modification of the hydroxyl groups at positions 5 and 7 for Genisten or all the hydroxyl groups, endorsed the significant structural relationship and the activities of those functional groups within the biological system. The fluorescence intensity of H2DCFDA probe correlated negatively with the intensity of the ROS quenching by the hydroxyl groups. The cellular fluorescence production of H2DCFDA gave clear evidence for the reduced capacity of the modified hydroxyl groups in ROS scavenging in MLO-Y4 cell line, as compared to original un-modified counterparts. Modified Daidzein and Genistein did not protect MLO-Y4 cells

against apoptosis caused by H_2O_2 when they were compared with cells pre-treated with H_2O_2, suggesting the important role of the hydroxyl groups in the protection of the cells from oxidative stress. This result is consistent with previous study which has reported that in most polyphenolic antioxidants, the number of OH groups substantially influence several mechanism of antioxidant activity (Burda *et al.,* 2001).

ROS is assumed to mediate the activation of NF-κB and the subsequent production of pro-inflammatory cytokines (RANKL, IL-6 and TNF- α). Therefore, the level of these cytokines which have been thought to contribute to bone resorption were measured. The results show that treatment with H_2O_2 increased the level of RANKL, IL-6 and TNF- α in MLO-Y4 cells. Treatment with E2 decreased the production of these cytokines, which was correlated with previous study that estrogen has been shown to modulate pro-inflammatory cytokine activity (Pfeilschifter *et al*., 2002). Withdrawal of estrogen is associated with increases in cytokine activity which support a link between the increased state of pro-inflammatory cytokine activity and post-menopausal bone loss (Mundy 2007). Earlier studies indicated that the pro-inflammatory cytokines IL-6 and TNF-α are important regulators of bone resorption and may play an important role in age- and estrogen deficiency-related bone loss (McLean 2009). Estrogen, as well as modulating the production of pro-inflammatory cytokines known to stimulate osteoclastogenesis, also down-regulate RANKL-induced JNK activation (Srivastava *et al.,* 2001). Therefore, the effect of PEs on cytokines release in MLO-Y4 cells was tested. E2, Genistein, Daidzein and Resveratrol significantly decreased the production RANKL at 2,4 and 24 hours when compared with treatment with

H_2O_2, also decreasing the level of IL-6 production at 2, 4 and 24 hours. TNF-α production was also decreased after treatment with PEs at 2, 4 and 24 hours.

In summary, this study has demonstrated that H_2O_2 induces production of cytokines. These responses together with recent knowledge on the connection between the saving effect of PEs on osteocyte apoptosis and initiation of bone resorption make it reasonable to suggest better treatment to control osteocytes apoptosis in diseases such as osteoporosis. Genistein and Daidzein have been shown to protect bone in ovariectomized rodent models, and most likely have a similar effects in other mammalian species (Anderson and Garner 1998). It has been reported that Genistein prevent loss of BMD in the rat model of ovaridectomy (Hooshmand *et al*.2010). Isoflavone-containing soy intake was found to protect againt osteoporosis in addition to their protective effect against breast cancer and cardiovascular disease (Zittermann *et al.,* 2004)

In conclusion, this study provides important results regarding the mechanisms by which PEs regulate bone cell activity and suggest that PEs-rich diets may assist in the treatment of hormone-dependent disease. They directly inhibit osteocyte and osteoblast apoptosis and also have an inhibitory effect on osteoclast formation. Furthermore, PEs suppressed cytokine release in osteocyte cells in response to oxidative stress. These results suggest that PEs may have a beneficial effect on skeletal health in post-menopausal osteoporosis and osteolytic inflammatory disorders such as rheumatoid arthritis.

7.2 Future work

Several areas still need further investigation to elucidate the accurate effect of PEs on bone cell. PEs have multiple activities, therefore their beneficial effect is not completely understood. The most discussed side effect of HRT is the risk of developing breast cancer, therefore it would be beneficial to investigate the effect of PEs on cancer cells to evaluate the effect of PEs on cancer cells proliferation. The antioxidant activity of PEs could be identified by investigating the protein content of mitochondrial antioxidant enzymes, SODs and GPX. Due to estrogen withdrawal, women are more prone to to develop neurodegenerative disorders such as Alzheimer's disease, hence the possible beneficial effect of PEs on brain cells apoptosis and Caspase 3 activation could be investigated.

In-vivo mouse models of bone loss would increase the strength of the data and provide further enhancement of doses necessary to alter osteocytes and osteoblasts activity during normal and diseases-induced remodelling.

The mechanism of action for isoflavones is still being investigated; it is clear from the many lines of evidence that there are probably multiple pathways, direct and indirect, that preserve the integrity and activity of bone cells to maintain stable bone mass in adults. As we found that the effect of PEs is ER independent, therefore it would be interesting to utilise ERα and ERβ-knockout mice to confirm the mechanism of action of PEs. The presence of estrogen receptors in bone and the wide-ranging biological properties of these non-steroidal dietary estrogens provide a good foundation for thinking that dietary phytoestrogens could play a role in bone remodelling.

The immune system has an important role in post-menopausal and inflammatory bone loss and in this study, PEs have been found to reduce cytokines such as RANKL, IL-6 and TNF-α. Therefore, it would be interesting to investigate the effect of PEs on circulating levels of inflammatory cytokine production in animal models of osteoporosis to identify the role of PEs on bone cells.

In conclusion, further experiments to test for the potential beneficial effect of PEs on bone and immune cells are needed to understand the precise effect of these compounds on bone health,establish effective concentration ranges *in-vivo* and determine any risks associated with long-term use.

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