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Eidesstattliche Erklärung

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(Karin Kirschner)

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List of used abbreviations

8-PN	(±)-8-prenylnaringenin
6-MITC	6-methylthiohexyl isothiocyanate
20-HE	20-hydroxyecdysone
α-MEM	minimum essential medium in α-modification
ADO	autosomal dominant osteopetrosis
AP-1	activator protein 1
ARO	autosomal recessive osteopetrosis
ATP	adenosine triphosphate
BA	bitter acids
BMP	bone morphogenetic protein
BMU	basic multicellular unit
cAMP	cyclic adenosine monophosphate
c-Fms	a receptor-type tyrosine kinase
c-Fos	a protein encoded by the FOS gene
CTR	calcitonin receptor
CXCR4	cysteine X cysteine chemokine receptor 4
DAPI	4′,6-Diamidino-2-phenylindole
DNA	desoxyribonucleic acid
FCS	fetal calf serum
FGF	fibroblast growth factor
FSD	functional secretory domain
HCl	hydrochloric acid
HIV-1	human immunodeficiency virus type 1
HSC	hematopoietic stem cell
HSV-1, HSV-2	Herpes simplex virus type 1, type 2
IGF	insulin-like growth factor
IgG/M	immunglobulin G/M
IL	interleukin
LDL	low density lipoprotein
LSD	least significant difference
MAP	mitogen-activated protein

MMP	matrix metalloproteinase
M-CSF	macrophage colony stimulating factor
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NFATc1	nuclear factor of activated T-cells, cytoplasmic 1
NF-κB	nuclear factor kappa B
N-f-5HT	N-feruloyl-serotonin
OB	osteoblast
OC	osteoclast
OCIF	osteoclastogenesis inhibitory factor
ODF	osteoclast differentiation factor
OPG	osteoprotegerin
OPGL	osteoprotegerin ligand
PBS	phosphate-buffered saline solution
PE	plant extract
PGE_2	prostagladin E2
PKA/C	protein kinase A/C
PMA	phorbol-myristate-acetate
PS	penicillin and streptomycin
PTH	parathyroid hormone
PTHR1	receptor for PTH
PTHrP	parathyroid hormone-related peptide
RANK	receptor activator of nuclear factor kappa B ligand
RANKL	RANK ligand
Runx2	runt-related transcription factor 2
SEM	standard error of mean
TGF-β	transforming growth factor beta
TNF	tumor necrosis factor
TRANCE	tumor necrosis factor-related activation-induced cytokine
TRAP	tartrate resistant acid phophatase
UV	ultraviolet
XN	xanthohumol (2′,4′,4-trihydroxy-6′-methoxy-3′-prenylchalcone)

1. Introduction

1.1 General principles of bone organization

The skeletal system is a metabolically highly active organ being remodelled constantly throughout life (Hadjidakis and Androulakis, 2006). It is composed of bone, a complex and highly specialised connective tissue (Marks and Odgren, 2002), and cartilage. The skeleton serves two main functions, a structural and an essential metabolic one (Hadjidakis and Androulakis, 2006).

The structural function provides rigidity, strength and support while still sustaining a certain kind of elasticity (Marks and Odgren, 2002). Furthermore, internal organs as well as bone marrow are protected and muscle attachment for locomotion is possible (Hadjidakis and Androulakis, 2006).

The metabolic function is based on the fact that bone is a main resource of ions, especially of calcium and phosphate, which contribute to the maintenance of mineral homeostasis in serum (Hadjidakis and Androulakis, 2006). The skeleton also plays an important role regarding the acid-base balance (Arnett, 2003). Furthermore, bones serve as rich source of cytokines and growth factors (Taichmann, 2005).

1.1.1 Bone matrix

Cells, vessels and crystalline salts compose a porous mineralized structure, the bone (Hadjidakis and Androulakis, 2006). Primarily, calcium and phosphate compounds, in the form of hydroxyapatite, are deposited in the organic matrix and therefore strengthen the bone. Around 95 % is formed by type I collagen, the rest is constituted of proteoglycans and various noncollagenous proteins (Marks and Odgren, 2002). The role of the latter has not been fully clarified yet. The main noncollagenous protein is osteocalcin; another occurring component is the proteoglycan biglycan (Hadjidakis and Androulakis, 2006).

1.1.2 Bone types

Two types of bone are morphologically existing; the cortical (compact) and the cancellous or trabecular (spongy) one (Marks and Odgren, 2002). The cortical bone is solid and dense, whereas the cancellous one appears like a honeycomb-like network of connected trabecular plates and bars enclosing the bone marrow (Dempster, 2006). Though the two forms look macro- and microscopically different, they are chemically identical (Hadjidakis and Androulakis, 2006). Both types of bone are composed of basic structural units called osteons (Dempster, 2006). Differences between the two types are both functional and structural, which are related to their basic functions (Marks and Odgren, 2002) and their location (Dempster, 2006).

The skeleton is comprised to 80 % of cortical bone with a slow turnover rate. It constitutes the outer part of the skeletal structures and has a high resistance to bending and torsion. Its function is mainly to provide protection and mechanical strength (Hadjidakis and Androulakis, 2006).

Though trabecular bone only represents 20 % of the skeletal mass it makes up 80 % of the bone surface. It has a higher turnover rate and is more elastic. Its major metabolic function is to provide the primary supply of minerals in acute deficiency states (Hadjidakis and Androulakis, 2006).

1.1.3 Bone remodelling and basic multicellular units (BMU)

213 bones comprise the adult human skeleton; each one is formed by a procedure termed modelling and continuously renewed by a procedure called remodelling. The bone remodelling is done by the sequential action of osteoblasts and osteoclasts and regulated by osteocytes (Dempster, 2006). Osteoclasts are responsible for removing the mineralized bone, whereas osteoblasts are in charge of forming the bone matrix while they subsequently become mineralized. Through this progress bone architecture is adapted and regulated, microdamages are repaired, the accumulation of old bone is prevented (Hadjidakis and Androulakis, 2006) and the bone strength is preserved (Dempster, 2006).

Bone modelling is the process where bones are shaped or reshaped, for example, in response to mechanical loads which lead to a change of the shape of the bones (mechanical adaption) (Dempster, 2006).

Normally, resorption and formation are well-balanced and old bone is constantly replaced (Hadjidakis and Androulakis, 2006). The balance is tightly regulated and maintained to make sure that after every remodelling cycle no major changes in bone mass or mechanical strength occur. Nevertheless, an imbalance between resorption and formation might be caused by certain pathological conditions, leading to abnormal bone remodelling and development of bone disorders (Feng and McDonald, 2011). Especially the role of osteoclasts has become evident in various skeletal diseases. As they degrade bone mineral and organic bone matrix, the specific inhibition of their function has become a primary strategy to deal with osteoporosis and other metabolic bone diseases (Väänänen and Laitala-Leinonen, 2008).

1.1.3.1 Basic multicellular unit (BMU)

The actions of osteoblast and osteoclasts are organized and coordinated by the basic multicellular units (BMU) (Hadjidakis and Androulakis, 2006). A BMU is about 1 – 2 mm long and 0.2 – 0.4 mm wide. In front of the BMU a couple of osteoclasts, in the rear part a couple of osteoblasts can be found. Furthermore, a BMU has a nerve supply, a central vascular capillary and associated connective tissue (Parfitt, 1994). 3 – 4 million BMUs are launched in healthy human adults each year and around 1 million are operating permanently (Manolagas, 2000).

The organisation of the BMUs is morphologically different in cortical and trabecular bone. The BMU forms a cylindrical tunnel in cortical bone (Hadjidakis and Androulakis, 2006). During a run, 10 osteoclasts are burrowing this canal in the dominant loading direction (Petrtyl et al., 1996) and are followed by thousands of osteoblasts filling the tunnel (Parfitt, 1994). Thereby, around 2 – 5 % of the cortical bone is remodelled per year. In trabecular bone, osteoclasts move across the bone surface, digging a trench (Hadjidakis and Androulakis, 2006).

In both types of bone, the cells of the BMU keep a well-coordinated spatial and temporal connection with each other. The osteoclasts attach to the bone and afterwards resorb it by acidification and proteolytic digestion. After the osteoclasts have left the resorption site, there is room for the osteoblasts which cover the excavated area and begin the procedure of new bone formation by secreting osteoid (Manolagas, 2000).

The lifespan of BMUs is 6 – 9 months which is much longer than of its executive cells. Consequently, constant supply of new osteoblasts and osteoclasts is needed and the balance between lifespan and new cells is crucial for maintaining the bone homeostasis (Manolagas, 2000).

1.1.3.2 Phases of bone remodelling

Bone remodelling means that the resorption lacunae are filled by osteoblasts to the original level (Matsuo and Irie, 2008). The process of bone remodelling occurs over several weeks. The five sequential phases of bone remodelling are: activation, resorption, reversal, formation and termination (Raggatt and Partridge, 2010).

Activation Phase – To start bone remodelling, an initiating remodelling signal has to be detected. It can either be a mechanical strain on the bone resulting in structural damage or a hormone action (e.g. estrogen or PTH) on bone cells (Raggatt and Partridge, 2010). In everyday life mechanical strain is placed on the skeleton continuously. Osteocytes obviously sense changes in these physical forces and furthermore translate them into biological signals which initiate the bone remodelling (Bonewald, 2007).

Resorption Phase – Osteoblasts respond to the signals that are generated by the osteocytes or the direct endocrine activation and thus osteoclast precursors are recruited to the remodelling site (Raggatt and Partridge, 2010). The osteoclasts create an isolated area beneath the cell which is named “sealed zone”. Hydrogen ions are pumped into this area, creating an acidic space. Hence mineralized matrix dissolutes and Howship’s resorption lacunae are formed (Teitelbaum, 2000). Different collagenolytic enzymes with a low pH

optimum (e.g. cathephsin K) then degrade the remaining organic bone matrix (Saftig et al., 1998).

Reversal Phase – After the resorption by the osteoclasts, the Howship lacunae stay covered with demineralized but undigested collagen matrix (Everts et al., 2002). Those collagen remnants are removed by a mononuclear cell of undetermined lineage which prepares the bone surface for the following osteoblast-mediated bone formation. Primarily, this "reversal" cell was thought to be a monocytic phagocyte (Tran et al., 1982), nowadays it is proposed to be from the osteoblast lineage (Everts et al., 2002). The importance of the reversal cells may lay in the receiving or producing of coupling signals within the BMU, allowing transition from bone resorption to bone formation (Raggatt and Partridge, 2010).

Formation Phase – The origin of the coupling signal that coordinates the transition and guides bone formation accurately to the sites of bone resorption is not clearly figured out by now. The anatomical constitution of the BMU avoids permanent cell contact between osteoblasts and osteoclasts. Consequently, different mechanisms, including both soluble signals as well as direct contact, might be necessary to achieve coupling. When early osteoblast progenitors or mesenchymal stem cells return to the resorption area, they differentiate and furthermore secrete molecules that finally form replacement bone (Raggatt and Partridge, 2010).

Termination Phase – After a sufficient quantity of bone has been replaced, the remodelling cycle ceases. The signal or signals which conclude the work of the remodelling machinery are mostly unknown, but osteocytes my play an important role. After mineralization, the mature osteoblasts undergo apoptosis, go back to a bone lining phenotype or differentiate into osteocytes and become embedded in the mineralized matrix. The bone surface maintains in a resting state until the next cycle of remodelling is started (Raggatt and Partridge, 2010). The termination phase is a long lasting phase since osteoblastic bone formation is much slower than osteoclastic bone resorption. Osteoclastic differentiation is evidently inhibited during the termination phase (Matsuo and Irie, 2008).

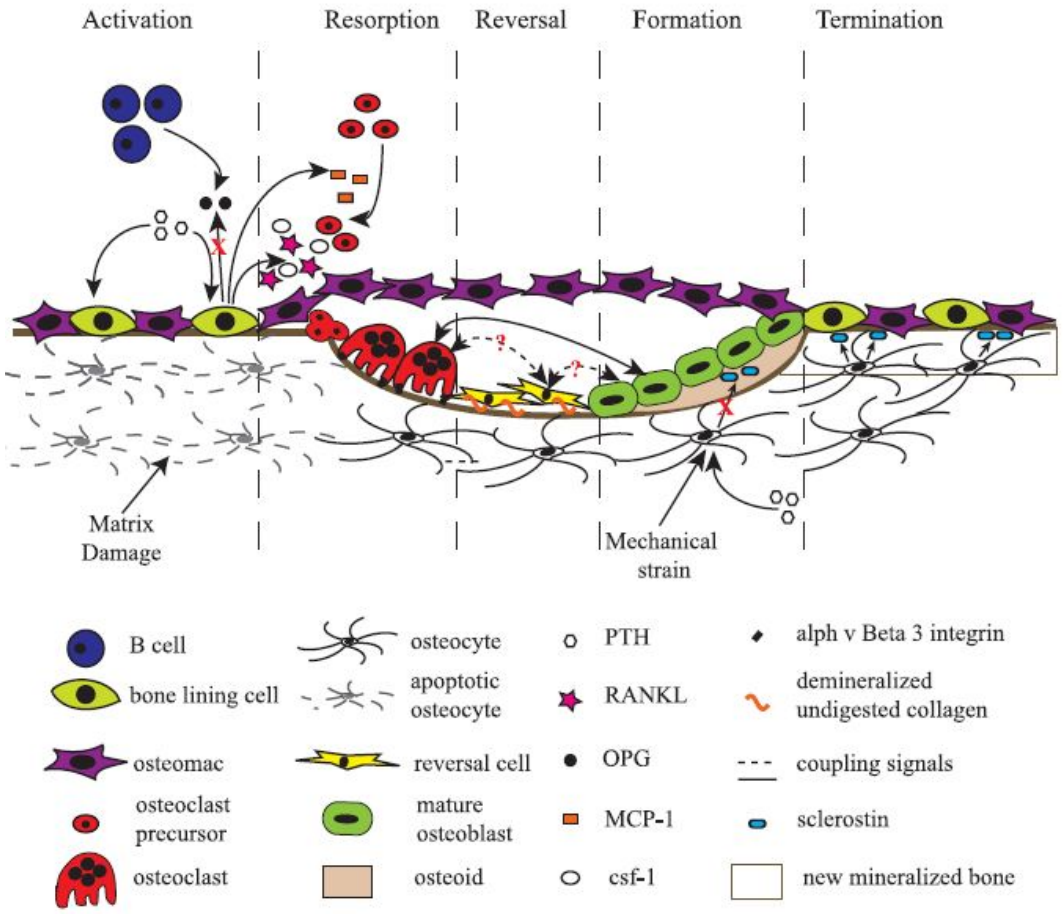


Figure 1.1: Schematic representation of a BMU and the associated bone remodelling process

(Raggatt and Partridge, 2010)

1.1.3.3 Regulation of bone remodelling

Hormones and other proteins secreted by bone cells and hemopoietic bone marrow cells seem to control the overall integrity of bone. Systemic and local regulations of bone cell function are existing (Hadjidakis and Androulakis, 2006). In addition to systemic and local factors, the osteoclast itself can produce regulatory cytokines stimulating and inhibiting osteoclast formation. Osteoclasts obviously are secretory cells stimulating their own activity (Roodman, 2006).

1.1.3.3.1 Systemic hormones

Parathyroid hormone (PTH) – Calcium homeostasis is primarily regulated by parathyroid hormone. Through stimulating bone resorption, increasing the renal tubular calcium reabsorption and the renal calcitriol production, the serum calcium concentrations are maintained. Interestingly, PTH stimulates bone formation when it is given intermittently, but stimulates bone resorption when it is secreted continuously (Kim et al., 2003).
The parathyroid glands produce the peptide PTH (Feyen et al., 1989). The osteoblast seems to be the primary target cell for PTH (Rodan and Martin, 1981). Isolated osteoclasts do not resorb bone in response to PTH. They only resorb when osteoblasts or osteoblastic cell lines are added to the cultures (McSheehy and Chambers, 1986). In addition, RANK ligand expression by marrow stromal cells is induced by PTH (Yasuda et al, 1998).

Calcitriol – Calcitriol is the most active metabolite of vitamin D3 (cholecalciferol), correctly named as 1,25-$(OH)_2$ cholecalciferol (Roodman, 1999). It is promoting bone mineralization through enhancing intestinal calcium and phosphorus absorption (Chapuy et al., 1992). The metabolites of vitamin D3 are potent stimulators of osteoclast formation and osteoclastic bone resorption (Roodman, 1999); meaning vitamin D3 has an important anabolic effect on bone turnover (Chapuy et al., 1992).
Osteotropic factors, including 1,25-$(OH)_2$ cholecalciferol, up-regulate the expression of the RANKL gene. RANKL activates nuclear factor kappa B (NF-κB) and enhances dendritic cell function as well as T cell growth. NF-κB activation seems to be important for

osteoclastogenesis (Yasuda et al., 1998). RANKL-mediated immune responses might be influenced by vitamin D and other osteotropic factors. Vitamin D has a potential immunological role (Lemire, 1997).

Calcitonin – The parafollicular cells of the thyroid gland secrete the peptide hormone calcitonin which is a potent inhibitor of osteoclastic bone resorption (Roodman, 1999). Calcitonin receptors are expressed on mature osteoclasts as well as on their precursors (Lee et al., 1995).
The effect of calcitonin on osteoclasts is a stimulation of adenylcyclase activity and cAMP accumulation, resulting in contraction of the osteoclast away from the bone surface and immobilization of them (Gorn et al., 1995). Calcitonin also mediates the loss of the ruffled border and an inhibition of the secretion of proteolytic enzymes through its receptor on osteoclasts. These effects, however, appear in pharmacologic doses and its physiologic role in the adult skeleton is minimal (Hadjidakis and Androulakis, 2006).

Glucocorticoids – Glucocorticoids exert both inhibitory and stimulatory effects on bone cells. They are necessary for osteoblast maturation by promoting their differentiation from mesenchymal progenitors, but they also decrease osteoblast activity. In addition, they sensitize bone cells to regulators of bone remodelling and raise osteoclast recruitment (Weinstein et al., 1998).

Thyroid hormones – Thyroid hormones stimulate both bone formation and resorption (Britto et al., 1994).

Estrogens – Estrogens are considered to be important steroid hormones regulating bone metabolism in females as well as in males (Smith et al., 1994). Deficiency of these sex steroids caused, for example, by menopause or removal of the ovaries, results in increasing osteoclastic bone resorption followed by bone loss (Jilka et al., 1992).
The responsiveness of the osteoclast progenitor cells to RANKL is decreased by estrogens resulting in prevention of osteoclast formation (Srivastava et al., 2001). Moreover, they reduce the life span of osteoclasts (Kameda et al., 1997), stimulate osteoblast proliferation and decrease their apoptosis (Manolagas, 2000).

Prostaglandins – Osteoclastic bone resorption in bone organ culture systems and osteoclast formation in murine marrow cultures is stimulated by prostaglandins (Takahashi et al., 1988). Though, osteoclastic bone resorption and formation in human marrow systems is inhibited by prostaglandin (PGE_2) (Chenu et al., 1990).
Prostaglandins show a strong influence on the differentiation of osteoclast precursors. This effect is not only depending on the dose and type of prostaglandin administered, but also on the nature of bone-derived stromal cells supporting osteoclast formation (Roodman, 1999).

Androgens – With their effect on the androgen receptor, which is present in all types of bone cells, androgens are essential for skeletal growth and maintenance (Sato et al., 2002).

1.1.3.3.2 Local regulation

Osteoblast-osteoclast communication

At least three types of osteoclast-osteoblast communication exist. The two cell types can be in direct contact allowing membrane-bound ligands and receptors to interact and initiate intracellular signalling, whereas they can also form gap junctions which allow the passage of small water-soluble molecules between osteoblasts and osteoclasts. They can as well communicate through diffusible paracrine factors, for instance cytokines, growth factors, chemokines and other small molecules secreted by one of the cells, which then act on the other via diffusion (Matsuo and Irie, 2008).

Bone remodelling is regulated by all three types of communication. The osteoblast-osteoclast communication is occurring in the BMU. Initiation of osteoclastogenesis is largely depending on the interaction between RANKL-expressing cells of the osteoblast lineage and RANK-expressing osteoclast precursors (Matsuo and Irie, 2008).

A variety of skeletal disorders can originate from abnormalities of bone remodelling. Recent advances concerning systemic and local regulation of bone remodelling are leading to new approaches in diagnosis and treatment of skeletal disorders. Especially newer

methods in cellular and molecular biology help defining the abnormalities in cells of the osteoblastic and osteoclastic lineages, which are leading to bone diseases. The better understanding of the pathogenetic mechanisms aids to develop new therapeutic approaches, involving development of inhibitory peptides, specific inhibition of key signalling pathways and production of recombinant molecules of cytokines and their soluble receptors (Hadjidakis and Androulakis, 2006).

OPG/RANKL/RANK system

Several laboratories identified around 1997 the essential osteoclastic ligand RANKL, which is a transmembrane glycoprotein produced as a trimer by stromal cells and belongs to the tumor necrosis factor (TNF)-α superfamily (Matsuo and Irie, 2008). RANKL, receptor activator of NF-κB ligand, is also called tumor necrosis factor-related activation-induced cytokine (TRANCE), osteoprotegerin ligand (OPGL) or osteoclast differentiation factor (ODF) (Väänänen et al., 2000). RANKL has two receptors, RANK and osteoprotegerin (OPG), also called osteoclastogenesis inhibitory factor (OCIF). The balance of expression level between RANKL and OPG is critical for the osteoclast formation (Miyamoto and Suda 2003).

RANKL/RANK

RANKL, which is found on the surface of preosteoblastic/stromal cells, binds to RANK on osteoclastic precursor cells, which results in differentiation, fusion into multinucleated cells and activation of osteoclastic cells. Furthermore, the RANKL/RANK interaction is crucial for the survival of osteoclastic cells because it suppresses apoptosis (Hsu et al., 1999). RANKL is not only expressed in osteoblasts but also in other cells like T cells and spleen cells (Miyamoto and Suda, 2003).

Through RANK signalling an osteoclastogenic cascade of transcription factors is activated. This includes NF-κB, AP-1 (c-Fos) and NFATc1 (Matsuo and Irie, 2008). Mice lacking RANKL lack as a result also osteoclasts (Li et al, 2000). Injected soluble forms of RANKL quickly induce formation of osteoclasts and furthermore bone resorption, also in wild-type mice (Tomimori et al., 2009). Osteoclastogenesis is enhanced by various soluble factors through RANKL induction in osteoblasts. Such factors are for instance PTH, parathyroid hormone-related peptide (PTHrP), IL-1, IL-10, IL-11, TNF-α, 1,25-$(OH)_2$-

Cholecalciferol, prostaglandin E_2 and thyroid hormone. Most of them are modulating the cyclic AMP/protein kinase A (PKA) pathway (Matsuo and Irie, 2008). TNF-α and IL-10 primarily stimulate M-CSF production and so directly increase RANKL expression (Hofbauer et al., 1999). The identification of the pathways which are involved in induction of RANKL in marrow stromal cells is just beginning. The MAP kinase pathway and the PKA as well as the PKC pathways have been implicated in the up-regulation of RANKL by cytokines and hormones (Xing et al., 2002; Lee and Lorenzo, 2002).

Osteoprotegerin (OPG)

OPG, a decoy RANKL receptor, is also expressed by osteoblasts and masks RANKL. This makes it for osteoclasts and their precursors impossible to bind RANKL resulting in inhibited RANK signalling (Simonet et al., 1997). OPG is interfering with osteoblast-osteoclast interactions and the RANKL/OPG ratio is therefore an indicator of the osteoclastogenic activity in several bone remodelling diseases (Matsuo and Irie, 2008). The levels of OPG are normally much higher than those of RANKL, so few osteoclasts are present in healthy bone. During inflammatory conditions, for example rheumatoid arthritis, myeloma, bone metastasis and Paget's disease, the ratio of OPG to RANKL is decreased, leading to increased osteoclastogenesis (Hofbauer et al., 2004; Terpos et al., 2003; Vanderborght et al., 2004). Estrogens decrease RANKL but increase OPG expression in osteoblasts, therefore favoring bone formation. Postmenopausal bone loss is linked to lower estrogen levels leading to increased bone resorption (Khosla, 2001).

Different laboratories using the techniques of homologous recombination to delete the RANKL gene or its receptor RANK in mice, proofed that RANKL is a crucial factor in osteoclastogenesis (Odgren et al., 2003; Dougall et al., 1999). Those mice showed severe osteopetrosis, whereas mice with overexpression of RANKL or deletion of OPG developed severe osteoporosis with sustaining spontaneous fractures as a result of their notably decreased bone mass (Bucay et al., 1998). Also mice which fail to express functional M-CSF or its receptor (c-Fms), are severely osteopetrotic (Dai et al., 2001)

Macrophage colony stimulating factor (M-CSF)

The cytokine M-CSF is, beside RANKL, also modulating osteoclast proliferation and differentiation (Khosla, 2001; Suda and Takahashi, 2008). M-CSF is produced by osteoblasts in bone tissue (Suda and Takahashi, 2008) and induces the expression of RANK in osteoclast precursors, therefore priming these cells to differentiate in response to RANKL (Arai et al., 1999).

M-CSF is furthermore required for the survival of cells in the macrophage-osteoclast lineage (Lagasse and Weissman, 1997) and is also controlling cell migration and cytoskeletal reorganization in macrophages and osteoclasts. Both express c-Fms, the tyrosine kinase receptor for M-CSF (Pixley and Stanley, 2004). M-CSF is also essential for the formation of the bone marrow cavity (Suda and Takahashi, 2008).

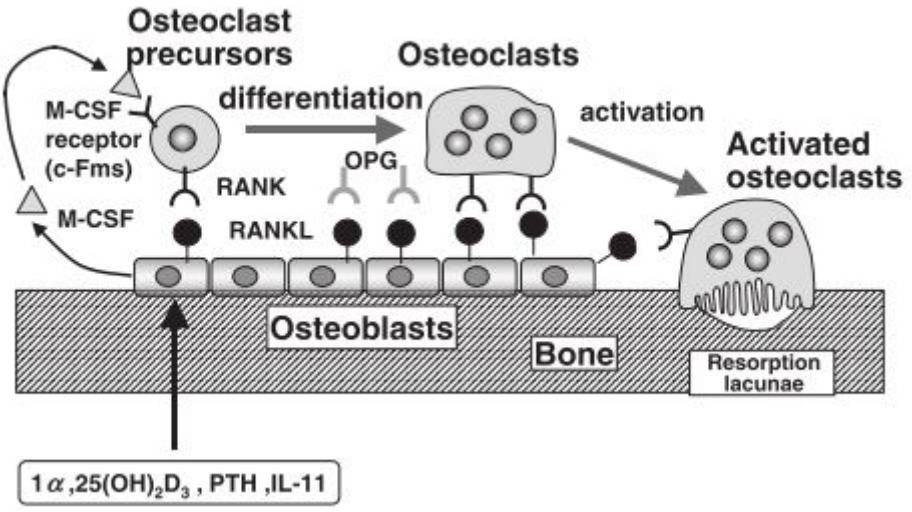


Figure 1.2: Molecular mechanism of osteoclast differentiation and activation
(Suda and Takahashi, 2008)

1.1.4 Bone cells

Four different bone cells compose bone. Osteocytes are present in the mineralized interior, whereas osteoblasts, osteoclasts and bone lining cells can be found on the bone surface. Osteocytes, together with osteoblasts and bone lining cells, originate from local osteoprogenitor cells, whereas osteoclasts arise from the fusion of mononuclear precursors originating in the various hemopoietic tissues (Marks and Odgren, 2002).

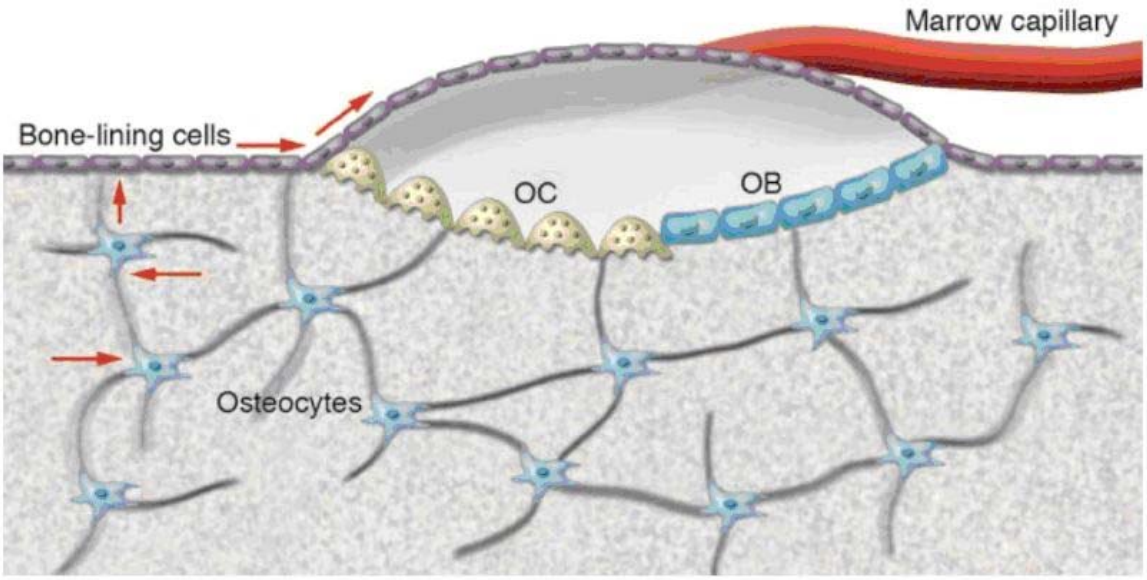


Figure 1.3: Scheme of the connections between the osteocyte network, surface bone-lining cells, osteoclasts (OCs) and osteoblasts (OBs).
All cells in this network are connected with gap junctions. The direct physical contact between OCs and OBs allows signalling between these cells.
(Eriksen et al., 2007)

1.1.4.1 Bone lining cells

Bone lining cells are inactive cells, flat and elongated. They cover the bone surface and are neither undergoing bone formation nor resorption. Because of their inactivity, few cytoplasmic organelles are needed. In general little is known concerning the function of bone lining cells (Marks and Odgren, 2002).

1.1.4.2 Osteoblasts

Osteoblasts are mesenchymal cells deriving from mesodermal and neural crest progenitor cells (Eriksen, 2010). As cells of the osteoblast lineage differentiate, they produce molecules necessary for supporting hematopoiesis and angiogenesis, and for the construction of mineralized bone matrix (Khosla et al., 2008). The formation of the osteoblasts comes along with differentiation from progenitors into proliferating preosteoblasts, then into bone matrix-producing osteoblasts, and eventually into bone-lining cells or mechanosensory osteocytes (Eriksen, 2010; Khosla et al., 2008).

Runt-related transcription factor 2 (Runx2) is crucial for progenitor cell differentiation along the osteoblast lineage (Komori et al., 1997). Runx2 and also osterix are transcription factors controlling the differentiation of osteoblasts (Nakashima et al., 2002).

A large number of endocrine, autocrine and paracrine factors affect osteoblast maturation and development. For instance bone morphogenetic proteins (BMPs), growth factors like IGF and FGF, angiogenic factors like endothelin-1, hormones like prostaglandin agonists and PTH modulate osteoblast differentiation (Qin et al., 2003).

Activation of Wnt signalling pathways is closely associated with the action of PTH and BMPs (Westendorf et al., 2004). Wnts are secreted glycoproteins essential for the renewal and development of many tissues, including bone. Osteoblast differentiation pathways are dominated by Wnt signalling (Eriksen, 2010) which is modulated by Runx2 and osterix (Hill et al., 2005). The suppression of canonical Wnt signalling causes bone loss while activation leads to improved bone strength (Bodine and Komm, 2006).

The coexpression of type I collagen and alkaline phosphatase characterizes the fully differentiated osteoblast. Both are important for the synthesis of bone matrix and its subsequent mineralization (Murshed et al., 2005). Primarily, osteoblasts produce osteoid by rapidly depositing collagen. They are found in clusters along the bone surface, lying on the layer of bone matrix which they are producing. Depositing collagen is followed by an increase in the mineralization rate to equal that of collagen synthesis. In the final stage the mineralization continues until the osteoid becomes fully mineralized while the rate of collagen synthesis decreases (Hadjidakis and Androulakis, 2006).

Regulators of matrix mineralization are also produced by mature osteoblasts, for example: osteocalcin, osteopontin and ostenectin, the receptor for PTH (PTHR1) as well as RANKL which is necessary for osteoclast differentiation. At the end of their lifespan osteoblasts develop into either bone lining cells or osteocytes which become embedded in the mineralized matrix (Eriksen, 2010).

1.1.4.3 Osteocytes

Osteocytes derive from osteoblasts (Franz-Odendaal et al., 2006). While bone formation, some osteoblasts undergo terminal differentiation and become engulfed by unmineralized osteoid. Those cells are then named osteoid-osteocytes (Palumbo, 1986). It is not known yet why some osteoblast progenitors are designated to differentiate into polygonal matrix-producing cells and further become osteocytes (Franz-Odendaal et al., 2006). As soon as the matrix around the osteoid-osteocytes is mineralized they are referred to as mature osteocytes. Within the mineralized bone matrix the osteocytes are embedded in fluid-filled cavities (lacunae). Osteocytes have been described as inactive, passive cells acting as a placeholder in bone (Bonewald, 2007).

But osteocytes have been found to have several functions, being far from the “passive placeholder in bone”. They function as an orchestrator of bone remodelling through regulation of osteoblast and osteoclast activity and can also act as an endocrine cell. Both healthy and dying osteocytes, for example, can recruit osteoclasts to sites of remodelling as well as osteocyte apoptosis can occur at sites of microcracks (Bonewald, 2011). Also a

protein called sclerostin, highly expressed in osteocytes, can target osteoblasts to inhibit bone formation (Van Bezooijen et al., 2005). Other arguments against osteocytogenesis being a passive process are the changes taking place in the entombed cells. During the transformation of the polygonal cells, the becoming osteocytes extend dendrites towards the vascular space or the bone surface (Bonewald, 2011).

Those dendrites travel through narrow tunnels, called osteocytic canaliculi, and build up a network within the bone matrix (Bonewald, 2007). Hence osteocytes are connected to other osteocytes in different lacunae and also with osteoblasts on the bone surface. These processes are even extended into the bone marrow (Kamioka et al., 2001). The osteocytic canaliculi are filled with extracellular fluid which provides a large surface for the exchange and transport of ions and other bioactive substances. Compared to osteoblasts, osteocytes possess less intracellular organelles and much fewer protein biosynthetic/secretory capacities (Ikeda, 2008).

Osteocytes are highly abundant and compose more than 90 – 95 % of all bone cells in the human adult skeleton. Osteoblasts make up less than 5 % and osteoclasts less than 1 % (Bonewald, 2007). Osteocytes are characterized as the longest lived, most numerous but also least studied cell type in bone (Seemann and Delmas, 2006). Osteocytes can exist inside bone up to 10 – 20 years, whereas osteoblasts live for a period of several weeks to months. The lifespan of osteoclasts is extremely short; they are viable for a period of some days but not even 2 weeks (Ikeda, 2008). In the end osteocytes are phagocytosed and digested through osteoclastic bone resorption (Elmardi et al., 1990).

1.1.4.4 Osteoclasts

The osteoclast is defined as “bone resorbing cell” (Miyamoto and Suda, 2003). Osteoclasts exhibit typical ultra-structural features (Väänänen et al., 2000), for instance multinucleation through cell fusion of mononuclear osteoclasts with the result to cover larger areas (Miyamoto and Suda, 2003). Before fusing with each other and forming multinuclear osteoclasts, the mononuclear osteoclasts adhere tightly to the bone. The fused cells

are further reorganized, polarized and construct their osteoclast-specific structures (Nesbitt and Horton, 1997).

Other examples for the characteristic differentiation of osteoclasts, beside the multinucleation, are the synthesis of a vacuolar proton pump and acid for dissolving bone mineral, formation of a ruffled border with the function of secreting protons and acid, as well as the formation of a sealing zone to prevent the leakage of proton and acid (Miyamoto and Suda, 2003). In addition to the mentioned morphological and phenotypic characteristics which are routinely used to identify osteoclasts, expression of calcitonin receptor (CTR) and the tartrate-resistant acid phosphatase (TRAP) activity are convenient markers to detect osteoclasts (Teitelbaum and Ross, 2003), beside their bone resorbing activity (Miyamoto and Suda, 2003).

Osteoclasts are giant multinucleated cells up to 100 mm in diameter and deriving from pluripotent hematopoietic stem cells through the monocyte/macrophage lineage (Roodman, 2006; Teitelbaum, 2000). They are formed by cellular fusions from their mononuclear progenitors. A number of cells and their products regulate their differentiation, especially RANKL and M-CSF (Väänänen and Laitala-Leinonen, 2008).

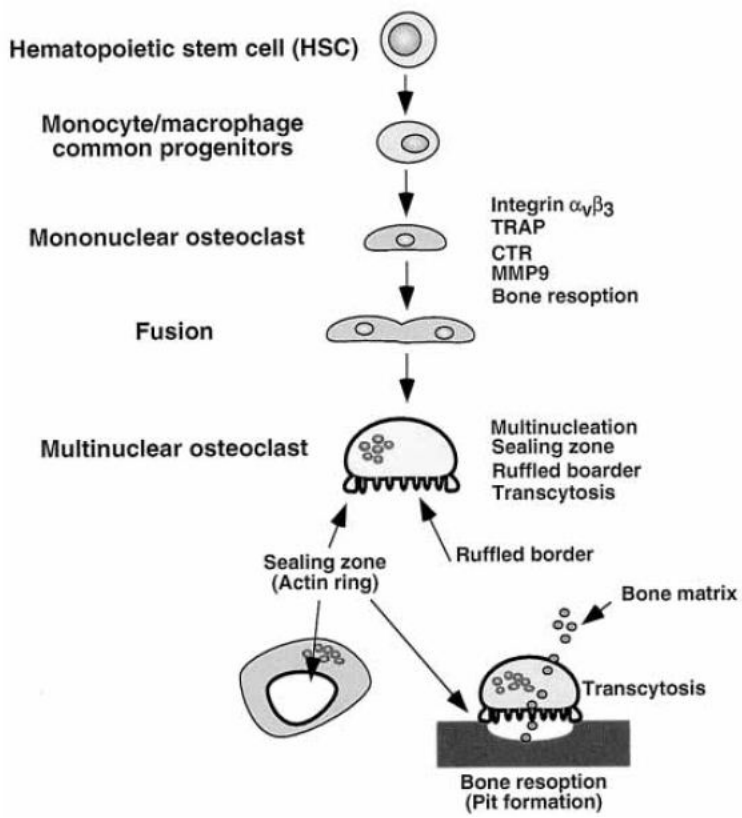


Figure 1.4: Development of osteoclasts from hematopoietic stem cells (HSCs)
Tatrate resistant acid phosphatase (TRAP); Calcitonin receptor (CTR); matrix metalloproteinase 9 (MMP-9)
(Miyamoto and Suda, 2003)

Common human osteoclasts found at resorption sites normally contain five to eight nuclei (Väänänen and Laitala-Leinonen, 2008). As a result of their own resorptive activity, they lay in a lacuna called Howship's lacunae. They have numerous Golgi complexes, transport vesicles filled with lysosomal enzymes and mitochondria (Hadjidakis and Androulakis, 2006). Though in vitro it has been demonstrated that even mononuclear osteoclasts are able to resorb, formation of large multinucleated cells often happens when effective and rapid bone resorption is needed (Väänänen and Laitala-Leinonen, 2008).

Osteoclast formation is only happening on the bone surface; therefore bone is obviously providing a suitable environment for osteoclastogenesis (Miyamoto and Suda, 2003).

Most of the osteoclasts are attached to mineralized matrix which suggests that they spend most of their lifetime resorbing bone. Both, the preliminary stages and also the post-resorption time must be relatively short compared to the actual resorption time (Lakkakorpi et al., 1989). Bone resorbing osteoclasts survive about 2 weeks in the marrow (Marks and Seifert, 1985) and then die by apoptosis. Factors enhancing osteoclast apoptosis include TGF-β and bisphosphonates (Weitzmann et al., 2000; Mundy et al., 2001).

1.1.4.4.1 Sealing zone and ruffled border

The osteoclasts show deep finger-like foldings of the plasma membrane in the area facing the bone matrix, called ruffled border, and the surrounding zone of attachment, called sealing zone (Väänänen et al., 2000). They form under the osteoclast after migration to a resorption site through attaching the plasma membrane tightly to the bone matrix (Väänänen and Horton, 1995). The sealing zone is totally devoid of cellular organelles, with the exception of actin filaments (Suda and Takahashi, 2008). Integrins play an important role in the molecular interactions between the plasma membrane and the bone matrix (Väänänen et al., 2000).

The presence of ruffled borders and sealing zones are the most characteristic features of osteoclasts (Suda and Takahashi, 2008). Building these specific plasma membrane domains, polarization of the osteoclasts and bone resorption need continuous modulation of the cytoskeleton and membrane trafficking (Väänänen and Laitala-Leinonen, 2008).

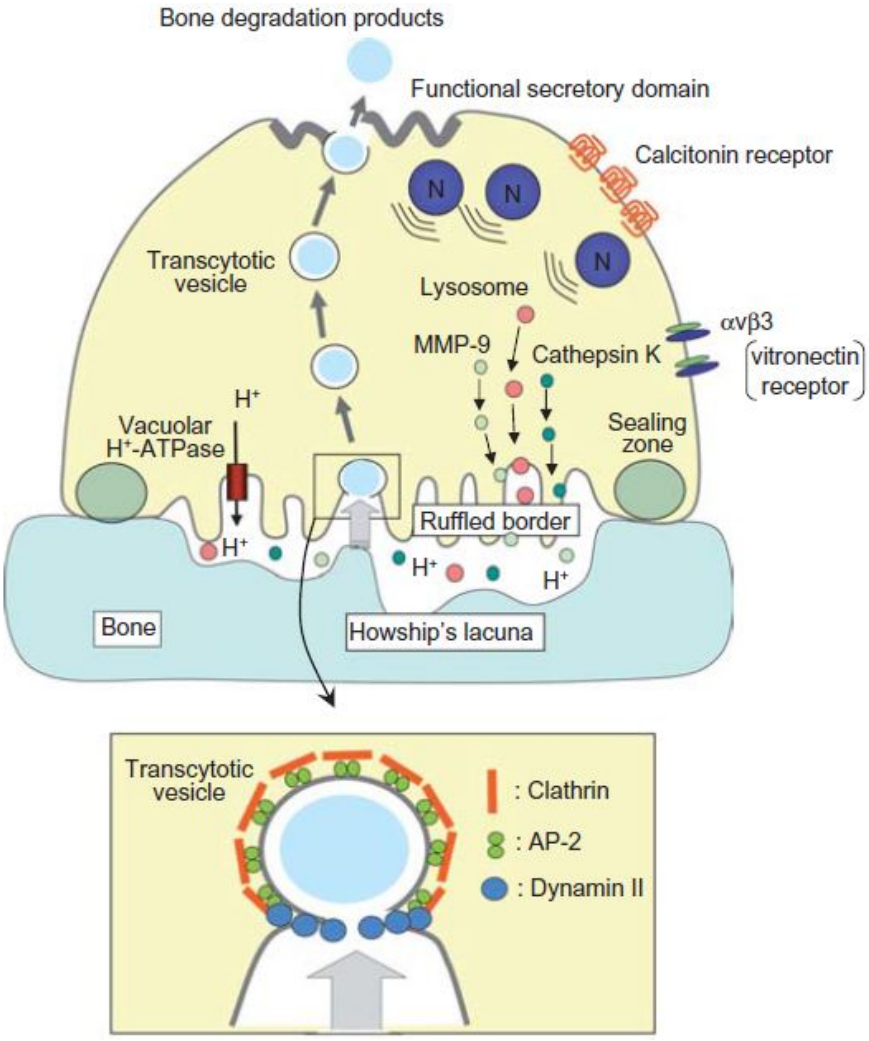


Figure 1.5: Ultrastructures of a functioning osteoclast
(Takahashi et al., 2007)

Sealing zone

The function of the sealing zone, defined as a unique thick band of actin, is, beside attaching the osteoclasts to the bone surface, the separation of the bone resorbing area, Howship's lacuna, underneath the ruffled border from the unresorbed area. This guarantees an isolation of the Howship's lacuna from its surroundings and the extracellular fluid (Takahashi et al., 2007). Dynamic structures, called podosomes, usually arrange the attachment (Hadjidakis and Androulakis, 2006). Podosomes are columnar arrays of actin and observed as ringed structures of F-actin dots, the actin ring (Takahashi et al., 2007).

Through their continuous assembling and disassembling, the osteoclasts can move across the bone surface proceeding bone resorption (Hadjidakis and Androulakis, 2006).

Only resorbing osteoclasts show a typical organization of F-actin into a belt or ring-like structure (actin ring). This can be used as marker for resorbing cells (Lakkakorpi and Väänänen, 1996).

Ruffled border

When the osteoclast prepares to resorb bone, the attachment to the bone matrix through the sealing zone is followed by forming another specific membrane domain, the ruffled border. It is the resorbing organelle and is formed by fusion of intracellular acidic vesicles with the region of plasma membrane facing the bone (Blair et al., 1989; Väänänen et al., 1990). Through this fusion process, much internal membrane is transferred and formed to long, finger-like projections penetrating the bone matrix (Väänänen et al., 2000).

Degradation of bone matrix

The main physiological function of the osteoclast is the degradation of mineralized bone matrix, involving dissolution of crystalline hydroxyapatite and proteolytic cleavage of the organic matrix rich in collagen. The mineral is dissolved by targeted secretion of HCl into the resorption lacuna through the ruffled border (Blair et al., 1989; Väänänen et al., 1990).

To reach the low pH in the resorption lacuna, an ATP-consuming vacuolar proton pump is needed. It can be found both in intracellular vacuoles as well as at the ruffled border. Obviously the resorption lacuna is further acidified by direct secretion of protons through the ruffled border (Väänänen et al., 2000). After dissoluting the mineral phase, numerous proteolytic enzymes degrade the organic bone matrix. Lysosomal cysteine proteinases and matrix metalloproteinases (MMPs) are the two major classes of proteolytic enzymes. High levels of MMP-9 and cathepsin K expression suggest that these enzymes play a central role in the resorption process (Drake et al., 1996). Cathepsin K is selectively expressed in osteoclasts, while other cathepsins are virtually absent (Saftig et al., 1998).

Matrix degradation is followed by removal of the degradation products from the resorption lacuna through a transcytotic vesicular pathway from the ruffled border to the functional secretory domain, where they are furthermore liberated into the extracellular space (Nesbitt and Horton, 1997; Salo et al., 1997).

1.1.4.4.2 Basolateral membrane and functional secretory domain

Resorbing osteoclasts contain not only the sealing zone and the ruffled border, but also at least two other specialized membrane domains: a basolateral membrane and a functional secretory domain (Väänänen et al., 2000).

The basolateral membrane is the plasma membrane which is not facing the bone and divided into two distinct domains. The functional secretory domain (FSD) is formed in the centre of the basolateral membrane (Salo et al., 1997).

1.2 Skeletal disorders

1.2.1 Osteopetrosis

Osteopetrosis is called the “marble bone disease” and refers to a heterogeneous group of rare, heritable bone remodelling disorders, which are characterized by increased bone density (Stark and Savarirayan, 2009; Tolar et al., 2004).

Two types of osteopetrosis are existing, autosomal recessive osteopetrosis (ARO) and autosomal dominant osteopetrosis (ADO). ARO has an incidence of 1 in 250.000 births, whereas the incidence of ADO is 1 in 20.000 births (Stark and Savarirayan, 2009).

Osteopetrosis is caused by a defect in bone resorption by osteoclasts (Tolar et al., 2004), namely by a failure of osteoclast function or development (Stark and Savarirayan, 2009).

Osteopetrosis is subclassified upon the mode of inheritance, severity, age of onset and clinical symptoms. Several gene mutations have been identified by molecular genetic studies. All of them encode proteins that involve osteoclast mediated bone resorption (Tolar et al., 2004). The classifications are continually updated as new clinical syndromes and mutations are being identified (Feng and McDonald, 2011).

Phenotypic features, such as macrocephaly and altered craniofacial morphology, can be caused by the increased bone mass. Even more important is the impact on other tissues and organs, for example the nervous systems and the bone marrow (Stark and Savarirayan, 2009).

1.2.2 Osteoporosis

"Osteoporosis is one of the major public health problems associated with aging" (Sipos et al., 2009).

It is a common disorder of bone remodelling characterized by structural degeneration of bone and as a consequence, low bone mass. Osteoporosis leads to bone fragility and an increased risk of fracture (Natl. Inst. Health Consens. Dev. Panel., 2001). There is a clear correlation between the risk of fracture and any standard deviation decrease in bone mineral density (Kanis et al., 1994).

Estrogen deficiency is primarily leading to involutional osteoporosis, but there are also many other additional factors which contribute to the pathogenesis of osteoporosis. Lifestyle and genetic factors play an important role as well as nutrition and the intake of drugs (Sipos et al., 2009).

Osteoporosis is classified into a primary and a secondary type (Natl. Inst. Health Consens. Dev. Panel., 2001; Marcus and Bouxsein, 2008). The primary type of osteoporosis is divided into two subtypes: type I and type II osteoporosis (Riggs et al., 1982; Riggs et al., 2001). Type I osteoporosis is also known as postmenopausal osteoporosis. It is a common skeletal disorder and primarily caused by estrogen deficiency in postmenopausal women. Type II osteoporosis is also called senile or age-related osteoporosis. It is primarily associated with aging in both men and women (Natl. Inst. Health Consens. Dev. Panel., 2001; Marcus and Bouxsein, 2008).

The secondary type of osteoporosis is referring to bone disorders which are secondary complications of different other medical conditions, adverse results of therapeutic interventions or a consequence of changes in physical activity (Natl. Inst. Health Consens. Dev. Panel., 2001; Marcus and Bouxsein, 2008). Two examples for the secondary type of osteoporosis would be glucocorticoid-induced osteoporosis and immobilization-induced osteoporosis (Feng and McDonald, 2011).

1.3 Test substances

1.3.1 Plant extracts

A lot of different effects of compounds of the investigated plant extracts are described in numerous studies. With the exception of Wasabi japonica, no studies investigating an effect of a plant extract on bone, bone metabolism or bone resorption could be found.

1.3.1.1 Extr. Leuzeae e rad. aquos. sicc. (PE 2)

Leuzea carthamoides DC. (Asteraceae) (syn. Rhaponticum carthamoides [Willd.] Iljin) is a Siberian perennial herb, commonly known as a maral root or Russian leuzea (Kokoska and Janovska, 2009). Due to its adaptability to rough climates, the plant is suited for cultivation as fodder crop and medicinal plant in Eastern and Northern Europe (Gaube et al., 2008). Its rhizomes and roots have been traditionally used in folk Siberian medicine mainly as roborant, tonic and stimulant (Havlik et al., 2009). Products derived from roots of Leuzea carthamoides are nowadays being promoted as dietary supplements with anti-aging, anabolic and adaptogenic activity (Gaube et al., 2008).

Various classes of compounds are already isolated from different parts of Leuzea carthamoides. The main groups are steroids, particularly ecdysteroids and phenolics (flavonoids and phenolic acids) accompanied with sesquiterpene lactones, polyacetylenes, terpenes (essential oil) and triterpenoid glycosides (Kokoska and Janovska, 2009).

Several different types of preparations, individual compounds and extracts derived from Leuzea carthamoides have been found to possess a broad spectrum of pharmacological effects on several organs such as the brain, blood, nervous and cardiovascular systems as well as on different physiological functions and biochemical processes including proteosynthesis, work capacity, sexual function and reproduction. Furthermore, the preparations and extracts from the herb exhibit various additional biological effects e.g. antioxidant, anticancerogenic, immunomodulatory, antiparasitic and insect antifeedant or repellent

activities (Kokoska and Janovska, 2009). Ethanol extracts of Leuzea species also show antimicrobial activity against Gram-positive and Gram-negative bacteria (Chobot et al., 2006). The results of data analysis on pharmacological, chemical and toxicological characteristics of Leuzea carthamoides indicate that this species has beneficial therapeutic properties and a high potential for being used as an effective adaptogenic herbal remedy (Kokoska and Janovska, 2009).

Logvinov et al. (2001) showed that Leuzea extract has a cerebroprotective activity. Peroral administration of 150 mg/kg Leuzea extract to rats with cerebral ischemia for 5 days prevented decrease in the density of synapses and destructive changes in the cerebral cortex (Logvinov et al., 2001).

Chobot et al. (2003) investigated the antifungal activity of constituents of Leuzea carthamoides. They isolated the thiophene polyine (E)-2-w5-(hept-5-en-1,3-diynyl)-thien-2-ylx-ethan-1,2-diol from an ethanolic extract of underground parts of Leuzea carthamoides, which demonstrated significant antifungal activity against all tested fungi (Chobot et al., 2003).

The influences of a plant preparation, a combination of dried ethanol/water extracts from roots of Leuzea carthamoides, Rhodiola rosea, Eleutherococcus senticosus and fruits of Schizandra chinensis, on immunity in ovarian cancer patients were studied by Kormosh et al. (2006). Patients who took the plant preparation had increased numbers of the four T cell subclasses as well as the mean amounts of IgG and IgM were increased. This suggests that combination of extracts from adaptogenic plants may boost the suppressed immunity in ovarian cancer patients who are subject to chemotherapy (Kormosh et al., 2006).

The effects of N-feruloylserotonins, substances isolated from the seeds of Leuzea carthamoides, on nociception and anxiety were studied by Yamamotová et al. (2007) in rats. N-feruloylserotonins reduced anxiety in high-pain threshold rats but did not influence anxiety in low-pain threshold rats. N-feruloylserotonins obviously have selective stress-reducing effects in stress-sensitive animals (Yamamotová et al., 2007).

The in vitro antiplatelet activity of four flavonoids from Leuzea carthamoides and ethanol summary extract was determined in human platelet-rich plasma. Two of the tested flavonoids inhibited platelet aggregation, but further evaluation of Leuzea carthamoides, in order to discover other antiplatelet active compounds and possible adverse health effects, is needed (Koleckar et al., 2008).

The effects of a lipophilic Leuzea root extract and the major phytoecdysteroid, 20-hydroxyecdysone (20-HE), were investigated in human breast adenocarcinoma MCF-7 cells. The roots contain up to 0.6 % phytoecdysteroids (Varga et al., 1986), mainly 20-HE, which were believed to be the active principles. While the extract significantly modulates cellular activities, the results showed that the phytoecdysteroids are most likely not the active components of Leuzea carthamoides (Gaube et al., 2008).

20-HE and 20-HE-containing extracts from Leuzea carthamoides are sold with claims of anabolic and immunomodulatory effects, but their effect on the activation of NF-κB, a key player in immune response and cell fate, and their influence on the NF-κB-inhibiting activity of steroidal anti-inflammatory drugs is still obscure. 20-HE inhibits NF-κB activation but less active than other plant metabolites (xanthohumol, withaferin). Leuzea carthamoides extracts with low 20-HE content significantly inhibited NF-κB activation but some extracts with high content in 20-HE only had a fair activating effect. 20-HE does not explain the NF-κB modulation achieved by Leuzea carthamoides extracts, in fact the extracts but not 20-HE itself showed a significant modulation of the NF-κB inhibitory effect. Therefore 20-HE is unlikely a major player in the NF-κB inhibitory effects displayed by some Leuzea carthamoides extracts in vitro. The results also indicate interaction potential of Leuzea carthamoides with steroidal anti-inflammatory drugs (Peschel et al., 2011).

The effect of four N-feruloyl-serotonin (N-f-5HT) isomers isolated from the seeds of Leuzea carthamoides was analysed by Nosal et al. (2011). N-f-5HT inhibits dose-dependent oxidative burst in human whole blood and isolated neutrophils in vitro stimulated with phorbol-myristate-acetate (PMA). In isolated neutrophils stimulated with PMA, N-f-5HT isomers were effective against intracellular as well as extracellular reactive oxygen species. Western blot analysis documented that N-f-5HT isomers significantly decrease PMA-induced phosphorylation of PKC α/β II. The results suggest that

N-f-5HT isomers represent naturally occurring substances potently affecting the oxidative burst in human neutrophils and should be further investigated for their pharmacological activity against oxidative stress in inflammation, ischaemia-reperfusion and other pathological conditions (Nosal et al., 2011).

1.3.1.2 Extr. Cichorie aquos. sicc. (PE 6) / Extr. Cichorie e rad. spir. sicc. (PE 7)

The genus Cichorium belongs to the Asteraceae family and is well known because of two widely cultivated species, Cichorium intybus, whose root is used as a source of inulin or for the production of a coffee substitute, while the leaves of selected varieties of Cichorium endivia (endive) are grown as a salad green (Koudela and Petrikova, 2007).

Cichorium intybus is a bushy perennial herb, growing as a wild plant on roadsides in Europe and in North America (Wang and Cui, 2009). Seeds of Cichorium intybus are commonly known as chicory (Gadgoli and Mishra, 1997). Chicory has a long history of herbal use. It is especially of great value for its tonic effects upon the digestive tract and the liver. The root and the leaves are appetiser, depurative, cholagogue, diuretic, hypoglycemic, digestive, laxative and tonic. A decoction of the root has been used in the treatment of gout, rheumatism, jaundice and liver enlargement (Pushparaj et al., 2007). Fructans extracted from the root of Cichorium intybus have been authorized as food ingredients in the U.S., Canada, Japan and in all European countries. Inulin and oligfructose derived from chicory have the ability to specifically stimulate the growth of bifidobacteria in the human colonic microbiota (Roberfroid et al., 1998).

Cichorium intybus is a potent antihepatotoxic plant and a major component of indigenous drugs (Zafar and Mujahid Ali, 1998). The alcoholic extract of Cichorium intybus is also used against gingival inflammation or pyorrhea (Patel and Bhatt, 1985). Alcoholic and aqueous extracts showed anti-inflammatory activity against formalin induced oedema (Jindal et al., 1975). The main constituents of chicory reported to be present in the root are reducing sugars, sucrose and inulin (Wight and Niekerk, 1983) as well as sesquiterpene lactones have been reported (Seto et al., 1988).

Zafar and Mujahid Ali (1998) compared the natural root and root callus extracts of Cichorium intybus for their anti-hepatotoxic effects in rats against carbon tetrachloride induced hepatic damage. Increased levels of bilirubin and serum enzymes (aspartate transaminase, alanine transaminase) in rats treated with carbon tetrachloride were substantially reduced through the treatment with root callus extracts and natural root. The decreased levels of albumin and proteins after carbon tetrachloride treatment increased in rats treated with root callus extracts and natural root (Zafar and Mujahid Ali, 1998).

A histopathological study of the liver showed swelling and necrosis in hepatocytes in carbon tetrachloride treated rats. Administration of different extracts of Cichorium intybus exhibited a significant recovery of hepatocytes. The methanolic fraction and the phenolic compound showed almost complete normalization of the tissues. Neither necrosis nor fatty accumulation occurred. The central vein appeared clearly indicating a potent antihepatotoxic activity (Ahmed et al., 2003).

Upur et al. (2009) investigated the protective effect of Cichorium glandulosum root extract on carbon tetrachloride-induced and galactosamine-induced hepatotoxicity in mice. The extract showed noticeable antioxidant activity, comparable with standard antioxidants. This activity is explainable through its efficiency against lipid peroxidation and its ability to scavenge several free radicals. Therefore, the results of the study suggest that Cichorium glandulosum root extract is a potent hepatoprotective agent that could protect the liver against acute injury. This ability might be attributed to its antioxidant potential (Upur et al., 2009).

Hassan and Yousef (2010) showed that it is worth considering chicory as a natural substance for ameliorating the hepatic injury and oxidative stress induced by nitrosamine compounds. The obtained effects may be due to the immunostimulating effect of chicory which is obtained by enhancing the capacity of influencing the hemopoiesis and stimulating the production of cytokines and antibodies (Kocsis et al., 2003).

Cichorium intybus contains two 1beta-hydroxyeudesmanolides, named magnolialide and artesin. Magnolialide appears to induce differentiation of human leukemia HL-60 and U-937 cells to monocyte/macrophage-like cells and inhibits the growth of several tumor

cell lines. Artesin and other constituents were inactive. The content of magnolialide was shown to be highest in the leaves (Lee et al., 2000).

Aqueous extract of Cichorium intybus showed a remarkable antioxidative effect on LDL, inhibitory effects on the degradation of fatty acids in LDL and the production of thiobarbituric acid reactive substance. Vitamin E and unsaturated fatty acids in LDL were protected from the effects of metal catalyzed LDL oxidation by adding Cichorium intybus extract. (Kim and Yang, 2001). The pro- and anti-oxidant activity of water soluble components in Cichorium intybus var. silvestre was investigated by Papetti et al. (2002). A boiled Cichorium juice, obtained by centrifugation, only showed strong antioxidant activity, the not boiled one possessed both pro- and anti-oxidant activity, proving that the vegetable prooxidant components are thermally instable (Papetti et al., 2002).

Kim et al. (2002) indicated in their study that immunotoxicity in mice, induced by ethanol, is significantly prevented or restored by treatment with Cichorium intybus extract orally administered at a dose of 300 mg/kg. Treatment with the plant extract results in a marked recovery of the immunosuppression caused by ethanol (Kim et al., 2002).

The antibacterial activity of ethanol, water and ethyl acetate extracts of Cichorium intybus were investigated by Petrovic et al. (2004). All tested extracts showed antibacterial activity against at least four tested bacteria, the ethyl acetate extract was the most active. Generally, root extracts had more antibacterial activity than extracts from the whole plant (Petrovic et al., 2004). Moreover, at least two antimalarial compounds can be found in Cichorium intybus. Lactucin and Lactucopicrin, both have been previously reported in chicory, show antimalarial activity (Bischoff et al., 2004). Other compounds, namely the two main sesquiterpene lactones, 8-deoxylactucin and 11β,13-dihydrolactucin are discussed to be potential antifungals (Mares et al., 2005).

Pushparaj et al. (2007) investigated the hypolipidemic and hypoglycemic properties of an ethanolic extract of Cichorium intybus, which is widely used in India as a traditional treatment for diabetes mellitus. Their results support the traditional belief that Cichorium intybus can ameliorate an existing diabetic state (Pushparaj et al., 2007). Antidiabetic potential of chicoric acid (2,3-dicaffeoyltartaric acid), a phenolic compound extracted from Cichorium intybus, was reported for the first time by Tousch et al. (2008). The study

describes the ability of chicoric acid to increase glucose uptake as well as to enhance insulin secretion in a glucose-dependent manner (Tousch et al., 2008). Dietary supplementation with caffeoylquinic acid-rich extract from chicory seeds can improve glycemia, decrease serum atherogenicity and increase blood antioxidant status in rats (Jurgonski et al., 2011).

1.3.1.3 Extr. Wassabiae e herb. aquos. sicc. (PE 10)

Effects of food and plant factors on bone metabolism

Isoflavones, including daidzein and genistein, which are contained in soybeans, have an inhibitory effect on osteoclastic bone resorption and a stimulatory effect on osteoblastic bone formation. Menaquinone-7, an analogue of vitamin K2 abundant in fermented soybeans, shows the same effects as well as various carotenoids. Supplementation of these factors has preventive effects on bone loss in rats. Factors with anabolic effects on bone metabolism were also found in extracts obtained from wasabi leafstalk (Wasabi japonica Matsum). Food chemical factors play a more and more important role in bone health and may help preventing bone loss with increasing age (Yamaguchi, 2006).

Effects of wasabi leafstalk extract on bone metabolism

The effects of 20 % ethanol extracts derived from different food and plants on bone calcium content were examined. Of various food and plants (including loquat leaf, cherry leaf, raw shiitake, dried shiitake, green tea, gabaron tea, muskmelon, tomato and blueberry) investigated, wasabi leafstalk extract was found to have an anabolic effect on bone calcification in mouse calvaria tissue culture in vitro (Suzuki et al., 1997).

Wasabi leafstalk extract was obtained from a homogenate with 20 % ethanol and orally administered in different concentrations (10 – 40 mg/100 g body weight) once per day for 7 days in young and aged rats to determine its anabolic effect on bone components in vivo (Yamaguchi et al., 2003). The wasabi leafstalk extract (10 mg/ml) caused a significant increase in alkaline phosphatase activity and calcium content in bone tissues. The effect was weakened with higher concentrations (50 and 250 mg/ml) (Yamaguchi, 2006). Additionally wasabi leafstalk shows an inhibitory effect on PTH-induced osteoclast-like cell

formation in a mouse marrow culture system. This suggests that the component is inhibiting bone resorption (unpublished data).

The components of the wasabi leafstalk extracts increasing bone calcium content were stable when treated with acidity, alkalinization or heating. Therefore the active component of wasabi leafstalk extract may not be a protein. The active component in wasabi leafstalk was further purified, its chemical structure is currently being identified (Yamaguchi, 2006).

Wasabi (Wasabia japonica, Miq. Matsum, syn. Wasabia pungens, Eutrema wasabi, Alliaria wasabi, Cochlearia wasabi,) belongs to the Brassicaceae family. The plant is harvested for its rhizomes which develop a characteristic pungent taste and odour when crushed. Wasabi is mainly used as a condiment, in fresh form or as dry powder, mainly in the Japanese cuisine (Weil et al., 2004).

Ono et al. (1998) screened, isolated and identified antibacterial compounds, contained in some common food, for bacterial use. Cruciferae plants, coriander and banana showed antibacterial activity, but the highest activity among tested food was found in stems of wasabi. An ethereal extract of wasabi stems had potent antibacterial activity. The active compound was isolated from the extract and identified as 6-methyl-sulfinylhexyl isothiocyanate (Ono et al., 1998).

The effect of 6-methylthiohexyl isothiocyanate (6-MITC), isolated from wasabi, on lung tumorigenesis in mice was studied by Yano et al. (2000). Treatment with 6-MITC suppressed lung tumorigenesis suggesting that it inhibits the development of lung tumours in mice due to suppression of the initiation stage. Therefore, the anticarcinogenic effect of 6-MITC is likely (Yano et al., 2000). Also Hou et al. (2000) suggest that dietary wasabi 6-MITC might be implicated in cancer chemoprevention, respectively play a role in cellular protection against carcinogens. 6-MITC is also a potential inhibitor of human platelet aggregation (Morimitsu et al., 2000) and was found to induce apoptotic cell death in human monoblastic leukemia U-937 cells and human stomach cancer MKN45 cells (Watanabe et al., 2003). Therefore Watanabe et al. (2003) also mention 6-MITC as a potent natural anti-cancer agent. 6-MITC also directly affects human cancer cells in vitro. It inhibits their growth in culture as well as it influences the survival of the cells. The

severely suppressed cell lines include melanoma and breast cancer cell lines. Because of its sufficiently small size, 6-MITC is a new possible candidate for controlling cancer cells (Nomura et al., 2005). The effect of administration of 6-MITC to inhibit macroscopic pulmonary metastasis was studied by Fuke et al. (2006). Together with previous results wasabi appears to inhibit, beside tumor cell growth, also tumor metastasis. So far 6-MITC from wasabi appears to be a useful dietary candidate for controlling tumor progression (Fuke et al., 2006).

6-MITC is also discussed as a principal enhancer of neuritogenesis (Shibata et al., 2008). It is also reported that 6-MITC suppresses lipopolysaccharide-induced cyclooxygenase-2 transcription and inducible nitric oxide synthase and in mouse macrophages. 6-MITC therefore has a variety of biological activities and is a potent chemopreventive compound in wasabi (Yoshida et al., 2011). A new biological activity of 6-MITC was discovered as an inhibitor of glycogen synthase kinase-3β (Yoshida et al., 2011), which is involved in the molecular pathogenesis of human diseases such as Diabetes mellitus type 2 and Alzheimer's disease (Balaraman et al., 2006).

Moreover, the effect of wasabi rhizome extract on atopic dermatitis model mice was investigated. The wasabi extract was fed to the HR-1 hairless mice developing, with a special diet, atopic dermatitis like symptoms. The wasabi rhizome extract improved those symptoms. A repressing effect on the scratching behaviour and reduction effects on the histopathological index were shown (Nagai and Okunishi, 2009). Wasabi extracts also exhibit significant anti-oxidant activities and anti-hypercholesterolemic action in high cholesterol diet rats, companied with modulations of cholesterol metabolism and decrease in liver xanthine oxidase activity (Lee et al., 2010). The suppressive effects of a leaf extract of wasabi on Helicobacter pylori infection and on stress loading in Mongolian gerbils were examined. The extract was administered orally. The results suggest that the simultaneous loading of H. pylori infection and physical stress might induce oxidative DNA damage additively. On the contrary the leaf extract attenuated the DNA damage in the stomach as well as in the peripheral erythrocytes (Sekiguchi et al., 2010).

The effect of isosaponarin derived from wasabi leaf on the synthesis of type I collagen in human fibroblasts and the mechanism of its action were investigated by Nagai et al. (2010). The type I collagen production in human fibroblasts was increased with treatment

of wasabi leaf extract. Isosaponarin was isolated from wasabi leaves and belongs to the group of flavone glycoside. It was the key compound in collagen synthesis from the wasabi leaf ingredients. The type I collagen production was increased by isosaponarin at the mRNA gene level (Nagai et al., 2010).

Also allylisothiocyanate is a potent component in wasabi. It shows inhibitory effect on the growth of food poisoning fungi and bacteria (Kinae et al., 2000). Kinae et al. (2000) also investigated several functional properties of leaves and roots from wasabi in vitro. All samples showed peroxidase activity, exhibited antioxidative and superoxide scavenging potency as well as antimutagenic activity was observed. This data suggests that wasabi might be a potent functional food for keeping human beings healthy (Kinae et al., 2000).

1.3.1.4 Extr. Humuli lupuli sicc. (PE 16)

Humulus lupulus L. is a climbing perennial plant belonging to the Cannabaceae family. It is growing in the temperate regions throughout the world and is well-known because of the wide usage of female inflorescences (hop cones or “hops”) to give beer a characteristic aroma and flavour as well as to preserve it (Zanoli and Zavatti, 2008). Hop cones contain bitter acids, essential oil, catechins, flavonoid glycosides and prenylated chalcones (De Keukeleire et al., 2003). Humulus lupulus has long been used for medicinal purposes. In particular, it is reputed a central nervous system depressant traditionally used to relief anxiety, insomnia, restlessness and excitability associated with gastrointestinal spasm and tension headache (Blumenthal, 1998). Indian tribes drank hop tea to allay nervousness and heated small bags of leaves to apply in cases of toothache or earache (Moerman and Cole, 1982).

Furthermore, hops have been reported to have estrogen-like properties (Chadwick et al., 2006). Among female pickers of hops evidence of menstrual disturbances was found, whereas in Germany, hop baths were used to reduce hot flashes in menopausal women (Goetz, 1990). Estrogenic activity of a methanol hop extract was demonstrated in different in vitro tests by Liu et al. (2001). Hops contain (±)-8-prenylnaringenin (8-PN), one of the most potent in vitro estrogenic substances originating from the plant kingdom. It is

able to mimic the effects of 17β-estradiol (Kitaoka et al., 1998). The estrogenic property of 8-PN was shown in diverse in vivo experiments (Milligan et al., 2002; Diel et al., 2004), including the ovariectomized rat model (Overk et al., 2008). The extract of Humulus lupulus also has an effect on the sexual behavior of female rats (Di Viesti et al., 2011).

Xanthohumol (2´,4´,4-trihydroxy-6´-methoxy-3´-prenylchalcone) (XN) is a major prenylated chalcone isolated from hop plant. It has a wide range of biological activities and is commonly used in beer brewing due to its bitter flavours. XN shows anti-inflammatory, free radical-scavenging and anti-carcinogenic activities, but its specific mechanisms are not indentified yet (Lee et al., 2011). XN has an anti-angiogenic effect on human breast cancer MCF-7 cells and shows a chemopreventive effect on many other cancer cell lines (Monteiro et al., 2008). The chemopreventive mechanism of XN is occuring through induction of the detoxification enzyme NAD(P)H:quinone oxidoreductase-1 (Dietz et al., 2005). XN also shows immunomodulatory activity in macrophage cell lines (Cho et al., 2008; Turchini et al., 2009), but the precise mechanisms of its anti-inflammatory action are not clarified yet (Lee et al., 2011). Lee et al. (2011) suggest that XN can be an attractive candidate for the regulation of inflammatory responses in the brain. Mentionable is also the antioxidant activity of XN in inhibiting low-density lipoproteins oxidation (Miranda et al., 2000a,b).

XN could also be a promising new therapeutic agent contributed to cancer treatment because of being an inhibitor of Cysteine X Cysteine chemokine receptor 4 (CXCR4). CXCR4 inhibitors have great potential to abrogate tumor metastasis and XN suppressed CXCR4 expression in various cancer cell types. Furthermore, XN could block endogenous activation of NF-κB, a key transcription factor regulating the expression of CXCR4 in cancer cells. Therefore, XN abolished cell invasion in both colon and breast cancer cells (Wang et al., 2012).

The quantitatively dominating secondary metabolites in hops are the bitter acids (BA). They are responsible for the preserving effect of hop and for the flavour and bitterness of the beer. About 95 % of the worldwide cultivated hop is used for brewing purposes, the rest is utilized for the production of dietary supplements and phytomedicines

(van Cleemput et al., 2009a). BA consist of α-acids (humulones) and β-acids (lupulones). They exhibit numerous biological properties with promising effects in, as already mentioned, cancer therapy and prevention. Hence BA inhibit angiogenesis, cell growth of invasive cancer cells and induce apoptosis in fast-growing tumor cells (Lamy et al., 2007). Furthermore, it was shown that BA could play a role in the prevention of lifestyle-related disorders because they exhibit positive effects on glucose tolerance, lipid metabolism and body weight in vivo (Miura et al., 2006; Yajima et al., 2005). Recent studies indicate that BA may inhibit NF-κB activity which plays a key role in the activation process of hepatic stellate cells, they key event of hepatic fibrosis. Saugspier et al. (2011) suggest that BA inhibit the activation and development of the profibrogenic phenotype of hepatic stellate cells. Therefore BA appear as potential functional nutrient for the treatment and also prevention of hepatic fibrosis in chronic liver diseases (Saugspier et al., 2011).

BA also block the TNF-α induced production of the cytokine IL-6 (van Cleemput et al., 2009b). Both play a crucial role within the complications of obesity (Lupinacci et al., 2009). BA are, because of their effectiveness against metabolic and inflammatory disorders, a challenging candidate for the treatment of cardiovascular diseases, diabetes mellitus and metabolic syndrome (van Cleemput et al, 2009a).

Magalhães et al. (2007) identified ergosterol and ergocalciferol for the first time in hop. Ergosterol (provitamin D2) in plant tissue is converted to ergocalciferol (vitamin D2) by UV irradiation, having the same biological activity as vitamin D3. Therefore, the presence of ergosterol should have great potential for the quality of this raw material and assessment of hop (Magalhães et al., 2007).

A xanthohumol-enriched hop extract, containing 8.4 % XN, displayed weak to moderate antiviral activity against herpesviruses HSV-1 and HSV-2 and bovine viral diarrhea virus (Buckwold et al., 2004). XN is classified as sprenylchacone flavonoid and flavonoids have shown anti-HIV-1 activity with emphasis on inhibiting HIV-1 reverse transcriptase (Matthee et al., 1999). Wang et al. (2004) demonstrated that XN is effective against HIV-1 and might represent a novel chemotherapeutic agent for HIV-1 infection. However, the mechanism of its anti-HIV-1 effect needs further investigation.

1.3.2 Pamidronate

Pamidronate, (3-amino-1-hydroxypropylidene)-1,1-bisphosphonate (Peretz et al., 1996), is an aminobisphosphonate and was used as positive control group in the experiments to have comparison considering the inhibition.

Bisphosphonates represent the medication of choice for most patients suffering from osteoporosis. They are the best studied drugs for prevention of bone loss and decreasing occurrence of fractures (Watts, 2003). Bisphosphonates are antiresorptive drugs and also used by several congenital bone diseases with systemic or local defects as well as a step in the treatment of cancer (Syversen and Halse, 2011).

Bisphosphonates are derivatives of pyrophosphate which binds with high affinity to hydroxyapatite. Aminobisphosphonates inhibit an enzyme in the mevalonate pathway, which leads to inhibition of osteoclastic activity and osteoclast apoptosis. After treatment with aminobisphosphontes, the incidence of vertebral and hip fractures was reduced (Syversen and Halse, 2011). Bisphosphonates reduce the risk of fractures quickly through increasing the bone mineral density (Watts, 2003).

Intravenous bisphosphonates ensure compliance and are relatively simple to use. They are effective in the treatment of Paget's disease and bone marrow edema (Syversen and Halse, 2011). Pamidronate is approved by the U.S. Food and Drug Administration in adults for treatment in Paget disease, hypercalcemia of malignancy and osteolytic bone metastases of breast cancer or multiple myeloma (Steelman and Zeitler, 2003).

1.4 Aim of the study

The aim of my thesis was to get an overview of the effect of twenty different plant extracts on osteoclasts in vitro and the possible inhibition of bone resorption by those plant extracts. A screening was done by performing a resorption assay and staining the osteoclast marker enzyme tartrate resistant acid phosphatase (TRAP).

Finally, five promising plant extracts of the original twenty were chosen and the structure of the osteoclasts was morphologically investigated. For this reason, the nuclei and the cytoskeleton of the cells were stained with Alexa Fluor/Phalloidin and DAPI. Images were taken to illustrate the effect of the test substances on the morphological characteristics of the osteoclasts and to figure out their eventual potential to be used as a novel pharmaceutical product treating skeletal disorders.

2. Material and methods

2.1 Equipment

Equipment	Company
Autoclave	SX-300E, Tomy Digital Biology, Japan
Bone saw + Saw blade	Isomet Low speed saw, Buehler, USA Diamond wafering blade/series 15 HC diamond, Buehler, USA
Centrifuge Eppendorf centrifuge	Hermle Z323K, Bartelt, Austria SORVALL® RT7, Sorvall, USA Eppendorf centrifuge 5415 C, Eppendorf, Germany
Computers	Power Macintosh PC (Windows)
Digital camera	Coolpix 995, Nikon, Japan Color View III, Olympus, Japan XM 10, Olympus, Japan (fluorescence)
Incubator	HERAcell® 240, Kendro, USA
Laminar airflow workbench	EHRET Labor- und Pharmatechnik, Germany
Light source for microscope	KL 1500 electronic, Schott, North America X-Cite® 120PC Q, Lumen Dynamics Group Inc., Canada
Magnetic stirrer	Ikamag® RCT, Janke & Kunkel, Germany
Microscopes	Diaphot 300, Nikon, Japan BX51, Olympus, Japan
pH-meter	Metrohm, Switzerland
Scales	MC 210 P, Sartorius, Austria
Shaker	Swip, Edmund Bühler, Germany

Sterilizer	WTC, Binder, Germany
Ultrasound bath	Transsonic 570, Elma, Germany
Ultra pure water system	Milli-QUF PLUS, Millipore, USA
Vortex	Vortex Genie 2TM, Bender & Hobein AG, Switzerland
Water bath	GFL-1086, GFL, Germany
Water purification system	Milli-RO 5 PLUS, Millipore, USA

Table 2.1: List of all equipment used

2.2 Material

Material	Company
Centrifuge tube: 15 ml, 50 ml	Becton Dickinson, USA Greiner Bio-One, Germany
Cover slips (22x22 mm) and microscope slides (76x26 mm)	Menzel-Gläser, Germany Assistent, Glaswarenfabrik Karl Hecht KG, Germany
Cryogenic vials	Becton Dickinson, USA
Eppendorf tube	Eppendorf, Germany
Glass pipettes, 5 ml, 10 ml, 20 ml, 25 ml	Brand, Germany
Glue	Fixo Gum, Marabu, Germany
Plastic pipettes, 10 ml	Costar, Corning Incorporated, USA
Parafilm "M"	American National Can Group, USA
Pasteur pipette	Copan, Italy
Petri dish	Iwaki, Japan Sterilin, Bibby Sterilin Ltd., U.K.
Polystyrene tubes, 5 ml	Becton Dickinson, Falcon®, USA
Reaction tubes, 1.5 ml	Greiner Bio-One, Germany
Silicon	GE Bayer Silicones, USA
Tissue culture plates	Iwaki, Japan Nunc, Denmark Costar, USA Greiner Bio-One, Germany

Table 2.2: List of all material used

2.3 Substances

Substances	**Company**
α-MEM	Gibco BRL, USA
1,25-$(OH)_2$-Cholecalciferol	Hoffmann-La Roche, Switzerland
4´,6-Diamidino-2-phenylindole (DAPI)	Sigma, USA
Acetone	Riedel-de Haen, Germany
Alexa Fluor®546 phalloidin	Molecular Probes, USA
$CaCl_2$ (calcium chloride)	Sigma, USA
Diethyl ether	Riedel-de Haen, Germany
Ethanol absolute	Sigma, USA
Fast-Red-Violet-Salt	Sigma, USA
Fetal calf serum	PAA Laboratories, Austria
Formaldehyde	Sigma, USA
HCl (hydrogen chloride)	Merck, Germany
Isopropanol 70 %	Riedel-de Haen, Germany
KCl (potassium chloride)	Merck, Germany
KH_2PO_4 (monopotassium phosphate)	Merck, Austria
Methanol	Merck, Germany
$MgCl_2.6H_2O$ (magnesium chloride hexahydrate)	Sigma, USA
Mounting medium (FluorSave) Mounting medium (Vectashield®)	Calbiochem-Novabiochem, USA Vector Laboratories, USA
N,N – dimethylformamide	Sigma, USA
$Na_2C_4H_4O_6$ (sodium tartrate)	Sigma, USA
NaCH3CO2 (sodium acetate)	Sigma, USA
NaCl (sodium chloride)	Sigma, USA
$Na_2HPO_4.2H_2O$ (disodium phosphate dihydrate)	Merck, Germany
NaN_3 (sodium azide)	Sigma, USA
NaOH (sodium hydroxide)	Merck, Germany

Naphtol AS-Mx-Phosphate	Sigma, USA
Pamidronate (AREDIA)	Ciba-Geigy, Switzerland
Penicillin	Gibco BRL, USA
Plant extracts	PhytoLab, Germany
Silicon grease	Wacker-Chemie, Germany
Sodium borate	Sigma, USA
Streptomycin	Gibco BRL, USA
Toluidine blue	Sigma, USA

Table 2.3: List of all substances used

2.4 Solutions

Phosphate Buffered Saline Solution (PBS), 10x dilution:

2.00 g KCL
2.00 g KH_2PO_4
80.00 g NaCl
27.07 g $Na_2HPO_4.2H_2O$
1000 ml H_2O

To prepare a 10x diluted solution KCl (2.6 mM), KH_2PO_4 (1.4 mM), NaCl (140 mM) and $Na_2HPO_4.2H_2O$ (15.2 mM) were dissolved in 1000 ml double distilled water.
The solution used was PBS 1x. For this purpose, the PBS 10x was diluted 1:10 with double distilled water and stored at 4 °C.

Phosphate Buffered Saline Solution (PBS) with Ca^{2+} and Mg^{2+}, 10x dilution:

2.00 g KCL
2.00 g KH_2PO_4
80.00 g NaCl
14.34 g $Na_2HPO_4.2H_2O$
1.00 g $CaCl_2$
1.00 g $MgCl_2.6H_2O$
1000 ml H_2O

KCl (2.6 mM), KH_2PO_4 (1.4 mM), NaCl (140 mM), $Na_2HPO_4.2H_2O$ (8 mM) and $MgCl_2.6H_2O$ (0.5 mM) were dissolved in double distilled water. The pH was adjusted to a value of 2 – 3 with 5 M HCl because $CaCl_2$ is dissolved best at this pH. $CaCl_2$ (0.9 mM) and then double distilled water was added until a total volume of 1000 ml was reached.

The solution used was PBS with Ca^{2+}/Mg^{2+} 1x. For this purpose, the PBS with Ca^{2+}/Mg^{2+} 10x was diluted 1:10 with double distilled water. The pH was adjusted to a value of 7.2 with 5 N NaOH and the solution was stored at 4 °C.

Fixing Solution:

The fixing solution (= 3.7 % formaldehyde) was made of 37 % formaldehyde solution which was diluted 1:10 with PBS with Ca^{2+}/Mg^{2+} 1x.

NaN_3 500 solution:

0.10 g NaN_3 was dissolved in 1 ml double distilled water and stored at 4 °C. This solution was diluted 1:500 with PBS with Ca^{2+}/Mg^{2+} 1x for use.

TRAP - buffer solution:

0.271786 g	$NaCH_3CO_2$
0.115714 g	$Na_2C_4H_4O_6$
50 ml	H_2O

$NaCH_3CO_2$ (40 mM) was dissolved in 50 ml double distilled water. The pH was adjusted to a value of 5.0 using 1 M HCl and then $Na_2C_4H_4O_6$ (10 mM) was added.

TRAP - staining solution:

0.015 g	Fast-Red-Violet-Salt
0.005 g	Naphtol AS-Mx-Phosphate
500 µl	N,N – dimethylformamide
25 ml	TRAP – buffer solution

Naphtol AS-Mx-Phosphate (0.24 mM) was dissolved in N,N – dimethylformamide. 250 µl of this solution were mixed with 25 ml TRAP - buffer solution. Fast-Red-Violet-Salt (1.59 mM) was added and dissolved by 5 minutes sonication in the ultrasound bath.

1 % Toluidine blue staining solution:

1.00 g	sodium borat
1.00 g	toluidine blue O
100 ml	H_2O

Sodium borate and toluidine blue O were dissolved in 100 ml double distilled water and stored at room temperature.

4´,6-Diamidino-2-phenylindole (DAPI) - dye:

DAPI stock solution (5 µg/ml), stored at -20 °C, was diluted 1:50 with PBS with Ca^{2+}/Mg^{2+}.

Phalloidin-Alexa Fluor® 546-dye:

Phalloidin Alexa Fluor® 546 stock solution (200 units diluted in 1 ml of methanol), stored at -20 °C, was diluted 1:40 with PBS with Ca^{2+}/Mg^{2+} after methanol was evaporated. Therefore 5 µl of phalloidin Alexa Fluor® 546 stock solution were diluted in 200 µl PBS with Ca^{2+}/Mg^{2+} for one slide.

2.5 Culture medium and maintenance conditions

Minimal Essential Medium by Harry Eagle, α-modification (α-MEM), was used as nutrient substance for the cell culture.

Two kind of α-MEM were used, a liquid one and one made of powder. The liquid α-MEM was ready to use. The powdered α-MEM was dissolved in 10 liters of sterile water, then 22 g of sodium bicarbonate (2.2 g/l) were added. The pH was adjusted to a value of 7.1 using 1 M HCl or 1 M NaOH. The medium was sterilized by sterile filtration through a filter with the pore size of 0.2 µm.

The α-MEM deriving from powder was used for preincubation, isolation of osteoclasts and washing. The ready to use α-MEM was used for seeding of osteoclasts and diluting the test substances.

To provide growth factors for the cells, fetal calf serum (FCS) was added to the medium in certain concentrations. For preincubation α-MEM + 10 % heat inactivated FCS was used. For isolation of osteoclasts, washing, seeding of osteoclasts and diluting of test substances, α-MEM + 5 % heat inactivated FCS was used.

The heat inactivation was achieved by warming the serum for 30 minutes up to 56 °C in a water bath. This process destroyed disturbing substances such as lactate dehydrogenase, clotting factors and complement factors.

To prevent microbial contamination a solution of penicillin and streptomycin (PS) was added to the medium in a concentration of 100 µg/ml.

All incubations were performed at 37 °C in a humidified atmosphere with a concentration of 5 % CO_2.

2.6 Test substances

Twenty different plant extracts were investigated within the scope of this thesis. Five of those were selected and investigated to further extent. The plant extracts came from the company "PhytoLab GmbH & Co. KG" from Germany.

The five selected plant extracts had the internal numbers 2, 6, 7, 10 and 16. The initial condition was a dry and small-grained powder. The colour of the powders ranged from a yellowish brown/red to green, the smell was earthy, partly spicy and similar to the smell of herbal tea.

The powdered plant extracts were stored in the dark at room temperature.

Plant extract nr.	Name	Composition	Solvent
2	Extr. Leuzeae e rad. aquos. sicc.	80 % native 20 % maltodextrin	water
6	Extr. Cichorie aquos. sicc.	80 % native 20 % maltodextrin	water
7	Extr. Cichorie e rad. spir. sicc.	100 % native	ethanol 70 % (V/V) (heptane)
10	Extr. Wassabiae e herb. aquos. sicc.	63 % native 30 % maltodextrin 7 % SiO_2	water
16	Extr. Humuli lupuli sicc.	70 % native (Mixture of (1152310) + CO_2 – extract)	ethanol 40 % (V/V) and CO_2 – extract

Table 2.4: List of further investigated plant extracts

(aquosus (*aquos.*) – aqueous, Extracturn (*Extr.*) – extract, Herba (H*erb.*) – herb, Radix (*Rad.*) – root, siccatus (*sicc.*) – dried, sprituosus (*spir.*) – spirituous)

2.6.1 Design of the experiments

Five different concentrations were examined of every plant extract. Considering the fact that bone cells tolerate a maximal ethanol concentration of 1 ‰, the highest concentration was always set as high as it was needed to reach a final concentration of 1 ‰ ethanol in the well.

The solvents used for the dissolution of the powdered plant extracts were 70 % ethanol, 40 % ethanol and double distilled water. To make sure that the different solvents did not harm the cells, a group with culture medium alone (control group) and a group with culture medium containing the according solvents (vehicle group) were included in every experiment for comparison. These two groups were always put on the same well plate together with the groups of the different concentrations of the corresponding plant extract treatment.

To have a comparison regarding the inhibition, a positive control group was included as well in every experiment on every well plate. For this purpose, a group with culture medium containing the bisphosphonate pamidronate in a concentration of 1×10^{-4} M was used.

Initially, five different stock solutions were prepared. Each stock solution was prepared directly from the powdered plant extract and not by diluting the highest concentrated solution. The stock solutions contained plant extract concentrations of 10 %, 3 %, 1 %, 0.3 % and 0.1 %. The solutions were prepared freshly for every experiment.

- 10 % stock solution: 0.1 g powdered plant extract / ml ethanol or water
- 3 % stock solution: 0.03 g powdered plant extract / ml ethanol or water
- 1 % stock solution: 0.01 g powdered plant extract / ml ethanol or water
- 0.3 % stock solution: 0.003 g powdered plant extract / ml ethanol or water
- 0.1 % stock solution: 0.001 g powdered plant extract / ml ethanol or water

2.6.1.1 Calculation of the concentrations of the solutions

As bone cells tolerate a maximal ethanol concentration of 1 ‰, more than 1 µl of the solution could be added per ml culture medium, as the ethanol in use was not absolute ethanol.

Plant extract 7 with the solvent ethanol 70 %:

To reach a concentration of 1 ‰ ethanol per ml culture medium, 1.428 µl of the plant extract solution in 70 % ethanol / ml culture medium could be added.

Final concentration of the plant extract in the well:

10 % stock solution: 0.1 g plant extract in 1 ml 70 % ethanol
=> in 1.428 µl are: 0.000143 g = 0.143 mg

3 % stock solution: 0.03 g plant extract in 1 ml 70 % ethanol
=> in 1.428 µl are: 0.000043 g = 0.043 mg

The calculations for the other stock solutions were done like above.

Plant extract 16 with the solvent ethanol 40 %:

To reach a concentration of 1 ‰ ethanol per ml culture medium, 2.5 µl of the plant extract solution in 40 % ethanol / ml culture medium could be added.

Final concentration of the plant extract in the well:

10 % stock solution: 0.1 g plant extract in 1 ml 40 % ethanol
=> in 2.5 µl are: 0.00025 g = 0.25 mg

3 % stock solution: 0.03 g plant extract in 1 ml 40 % ethanol
=> in 2.5 µl are: 0.000075 g = 0.075 mg

The calculations for the other stock solutions were done like above.

Plant extracts 2, 6 and 10 with the solvent water:

Within the twenty investigated plant extracts, some were dissolved with the solvent ethanol 20 %. To achieve comparability between the different plant extracts with the solvent ethanol and the solvent water, the concentrations of the groups of the plant extracts with the solvent water were set like the groups of the plant extracts with the solvent ethanol 20 %. Therefore, the calculations for these three plant extracts are done for the solvent ethanol 20 %, though the solvent was water.

To reach a concentration of 1 ‰ ethanol per ml culture medium, 5 µl of the plant extract solution in 20 % ethanol / ml culture medium could be added.

Final concentration of the plant extract in the well:

10 % stock solution: 0.1 g plant extract in 1 ml 20 % ethanol
=> in 5 µl are: 0.0005 g = 0.5 mg

3 % stock solution: 0.03 g plant extract in 1 ml 20 % ethanol
=> in 5 µl are: 0.00015 g = 0.15 mg

The calculations for the other stock solutions were done like above.

Stock solution	**Solvent**		
	Ethanol 70 % (mg/ml culture medium)	**Ethanol 40 % (mg/ml culture medium)**	**Ethanol 20 % (mg/ml culture medium)**
10 % equals	0.143	0.250	0.500
3 % equals	0.043	0.075	0.150
1 % equals	0.014	0.025	0.050
0.3 % equals	0.004	0.008	0.015
0.1 % equals	0.001	0.003	0.005

Table 2.5: Overview of the plant extract concentrations in the stock solutions at a concentration of 1 ‰ ethanol

2.6.1.2 Preparation of the solutions

After weighing in the powdered plant extracts in Eppendorf tubes, the corresponding solvent was added to produce the stock solutions. To dissolve the powder the tubes were vortexed. If the powdered plant extract did not dissolve completely, the tubes were put into an ultrasound bath for 3 times for 1 minute each. Ice was added to the water in the ultrasound bath to prevent heating in case the plant extracts do not tolerate heat. If the powdered plant extracts dissolved completely after vortexing, putting them in the ultrasound bath was not necessary.

After vortexing, respectively the treatment in the ultrasound bath, the Eppendorf tubes were centrifuged for 5 minutes at 3000 rpm at room temperature to solidify insolvable ingredients, for instance the maltodextrin.

Though 2.5 µl, respectively 5 µl, of all stock solutions with the solvent ethanol 40 %, respectively water, could have been taken per ml culture medium to reach a final concentration of 1 ‰ ethanol in the well, this was only done for the group with the highest concentration (0.25, respectively 0.5, mg/ml culture medium). To maintain the comparability between the groups of the different plant extracts, 1.428 µl of the stock solutions were taken for the lower concentrated groups of the plant extracts with the solvent ethanol 40 % and water, like for the one with the solvent ethanol 70 %. This was done to get the same concentrations as in some groups of the plant extract with the solvent ethanol 70 %. Therefore, independent of the solvent, at least three groups had the same concentration comparing all plant extracts among each other.

Plant extract nr.	Solvent	Investigated concentrations (mg/ml culture medium)
2	Water	0.500 – 0.250 – 0.143 – 0.043 – 0.014
6	Water	0.500 – 0.250 – 0.143 – 0.043 – 0.014
7	Ethanol 70 %	0.143 – 0.043 – 0.014 – 0.004 – 0.001
10	Water	0.500 – 0.250 – 0.143 – 0.043 – 0.014
16	Ethanol 40 %	0.250 – 0.143 – 0.043 – 0.014 – 0.004

Table 2.6: Overview of the investigated concentrations

Pipetting and well plate schemes:

	C	Water	0.500	0.250	0.143	0.043	0.014	Pamidronate
Pit Assay								
TRAP								

Figure 2.1: Scheme of a well plate for the plant extracts 2, 6 and 10

	C	EtOH 70 %	0.143	0.043	0.014	0.004	0.001	Pamidronate
Pit Assay								
TRAP								

Figure 2.2: Scheme of a well plate for the plant extract 7

Wells	Pipetting scheme
C	0.5 ml culture medium
Vehicle (Water)	5 µl double distilled water / ml culture medium
0.500	5 µl 10 % stock solution / ml culture medium
0.250	2.5 µl 10 % stock solution / ml culture medium
0.143	1.428 µl 10 % stock solution / ml culture medium
0.043	1.428 µl 3 % stock solution / ml culture medium
0.014	1.428 µl 1 % stock solution / ml culture medium
Pamidronate	0.5 ml pamidronate solution (1×10^{-4})

Table 2.7: Pipetting scheme for the groups of the plant extracts 2, 6 and 10

Wells	Pipetting scheme
C	0.5 ml culture medium
Vehicle (Ethanol 70 %)	1.428 µl ethanol 70 % / ml culture medium
0.143	1.428 µl 10 % stock solution / ml culture medium
0.043	1.428 µl 3 % stock solution / ml culture medium
0.014	1.428 µl 1 % stock solution / ml culture medium
0.004	1.428 µl 0.3 % stock solution / ml culture medium
0.001	1.428 µl 0.1 % stock solution / ml culture medium
Pamidronate	0.5 ml pamidronate solution (1×10^{-4})

Table 2.8: Pipetting scheme for the groups of the plant extract 7

2.7 Methods

2.7.1 Resorption assay (“pit assay”)

2.7.1.1 Preparation of bone slices

The bone used for the resorption assay was derived from cattle. After removing the muscles and the bone marrow, the bones were dried for 24 hours at 50 °C and then stored at 4 °C. The bone was further cut into slices with a thickness of 300 – 350 µm using a low speed saw with diamond blade. These slices were cut with a knife into small equal squares (4 mm x 4 mm), so 4 of them could fit in a well of a 48-well plate.
Cleaning of the bone pieces was done by sonicating them 3 times for 5 minutes in double distilled water and a fourth time for 5 minutes in 70 % ethanol in an ultrasound bath.

After that, the bone pieces were marked with a pencil on one side and then each side was treated with UV-light for 15 minutes under sterile conditions. The sterilized bone pieces were then glued with sterile silicone grease into the wells of a 48-well plate (4 pieces per well). The wells were preincubated with α-MEM + 10 % heat inactivated FCS for at least 2 hours or overnight.

2.7.1.2 Preparation of rabbit osteoclasts

Isolation
Osteoclasts were isolated from femora and tibiae of 4 – 5 days old rabbits. After removing skin and muscles from the bones with a scalpel, they were placed in a petri dish on ice containing sterile PBS. Under the laminar airflow workbench, the bones were cut into small pieces which were afterwards transferred to a centrifuge tube containing 6 ml α-MEM + 5 % heat inactivated FCS for the bones of one rabbit.

The bone pieces were cut with scissors and then more α-MEM + 5 % heat inactivated FCS was added. The supernatant cell suspension was transferred to a second centrifuge tube. This process was repeated twice. More α-MEM + 5 % heat inactivated FCS was added to the rest of the bones. The cell suspension was vortexed and after sedimentation, the supernatant was also transferred to the other centrifuge tube. The collected cell suspensions were centrifuged. Afterwards the supernatant medium was removed and the remaining pellet was redispersed in α-MEM + 5 % heat inactivated FCS.

Seeding

Cell suspension was added to each well of a 48-well plate and was put on each cover slip of a 6-well plate. After incubating the plates, the wells were rinsed with medium and afterwards new medium, α-MEM + 5 % heat inactivated FCS including the respective concentrations of the different test substances or the vehicle (controls) was added to the wells. The rinsing was done to remove most of the contaminating cells. Finally, the well plates were incubated for 24 – 48 hours.

Because of the size of the osteoclasts, either pipettes with large openings had to be used for all working steps, or the tip of the pipette tip had to be cut, to avoid destroying of the cells.

2.7.1.3 Cleaning and staining of bone slices

After the end of the cultivation, the bone slices were taken out of the wells and given into polypropylene tubes with 70 % isopropanol. All bones of one group (8 bone slices) were put together into one tube. To wash off the medium, silicon and the adherent layer of cells, the bone slices were sonicated for 15 minutes in an ultrasound bath. Then the slices were dried on a towel paper and put separately in a well of a 24-well plate. 700 µl of 1 % toluidine blue were added to each well. The bones were incubated for 4 minutes on a shaker at room temperature. Staining with toluidine blue made it possible to see the resorption areas under the microscope. After the staining, the bones were washed 3 times with double distilled water to rinse off the remaining dye. The stained bone slices were dried and stored in a 96-well plate.

2.7.1.4 Pit area measurement

The areas of osteoclast resorption were visualized using an Olympus BX51 microscope with a 5x magnification. Two images were taken of each bone slice, one of the upper left corner and one of the lower right corner, the edges had to be visible. The images were taken with an Olympus Color View III digital camera which was connected to the microscope. The two images were then merged together to one image, using the imaging software program cell^F. The pit areas were marked and analysed with the same program. (Figure 2.3)

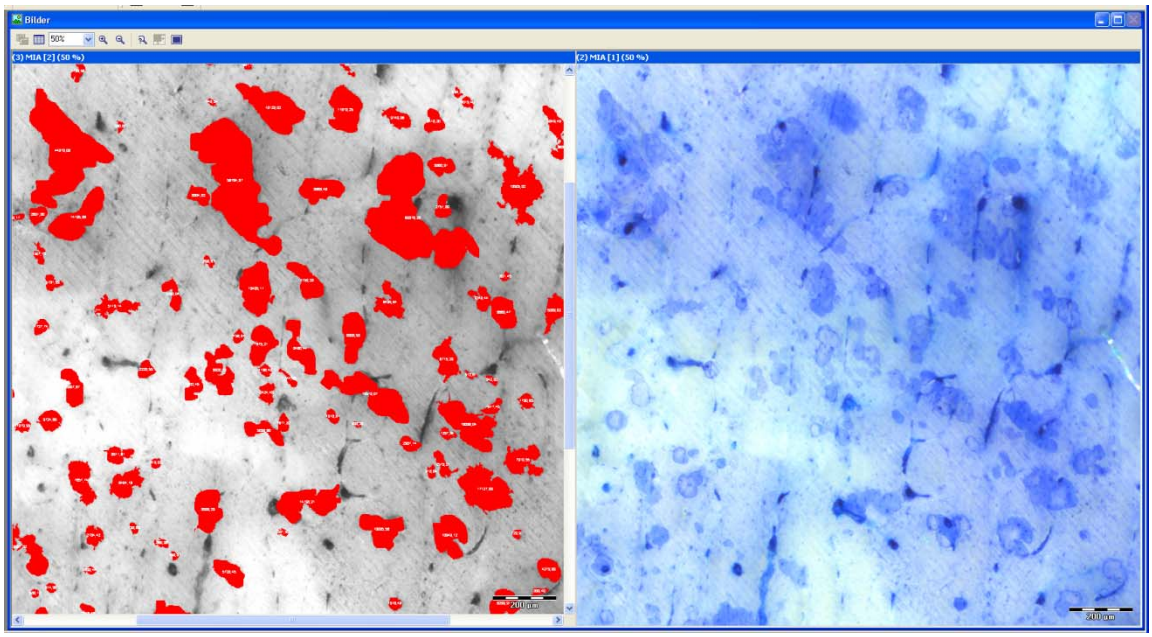


Figure 2.3: Stained and marked bone slice with resorption pits in the imaging software cell^F

Left side: Image of a stained bone slice in black-and-white with already marked (red) resorption pits.

Right side: Image of the same stained bone slice with unmarked resorption pits in colour.

The total area of resorption pits, their total number and the average pit area were calculated by the program as well as the total area of the whole bone slice visible in the image. These data was then copied to a Microsoft Excel sheet for calculation of the resorption (%) / bone slice.

- $\text{Average pit area} = \frac{\text{total area of pits}}{\text{total number of pits}}$
- $\%\ \text{Resorption} = \frac{\text{total area of pits} * 100}{\text{total area of bone slice}}$

This method was carried out with 8 samples for each group (8 bone slices in 2 wells / group). The graphical presentations were made with the program Prizm 5.0.

2.7.2 Quantification of TRAP-positive cells

Through the staining, TRAP-positive cells got a characteristic red colour and could be counted easily under a light microscope. With this method, the number of osteoclasts in each group (different test substances and concentrations of treatments or controls) could be determined.

TRAP staining was always performed with a corresponding resorption assay. The osteoclasts were seeded in empty wells directly on the plastic surface of a 48-well plate next to the wells with the bone slices (for the resorption assay) of the same group.

2.7.2.1 Preparation of rabbit osteoclasts

Done as described in chapter 2.7.1.2.

2.7.2.2 Fixation

At the end of the incubation period, the medium was removed from the wells and 37 °C warm PBS with Ca^{2+}/Mg^{2+} was added to the wells. The PBS with Ca^{2+}/Mg^{2+} was removed again (rinsing). The cells were fixed by adding 3.7 % formaldehyde solution to the wells twice, first for 3 minutes and then for 10 minutes with fresh solution. After the fixation, the cells were washed 3 times with PBS with Ca^{2+}/Mg^{2+}. For storage at 4 °C, the wells were filled with PBS with Ca^{2+}/Mg^{2+} containing NaN_3 (500x) to prevent infection.

2.7.2.3 Staining

The cells were incubated with the TRAP-buffer for 30 minutes and then treated with an acetone/ethanol 1:1 solution for 30 seconds at room temperature to make the cell membranes permeable. The cells were dried for 2 minutes and then treated with the TRAP-staining solution for 5 – 10 minutes in the incubator. The staining was stopped by washing the cells 3 times with double distilled water. The cells were stored in double distilled water at 4 °C for further investigation.

This method was carried out with 3 samples for each group (3 wells / group).

2.7.2.4 Counting of TRAP-positive cells

Cells were visualized using a Nikon Diaphot 300 light microscope with a 10x magnification. Only TRAP-positive multinucleated cells with 3 or more nuclei were counted as osteoclasts. Images were taken with a Nikon Coolpix 995 digital camera which was connected to the microscope. All tested concentrations of the five different plant extracts and their control groups were photographed. (Figure 2.4)

The graphical presentations were made with the program Prizm 5.0.

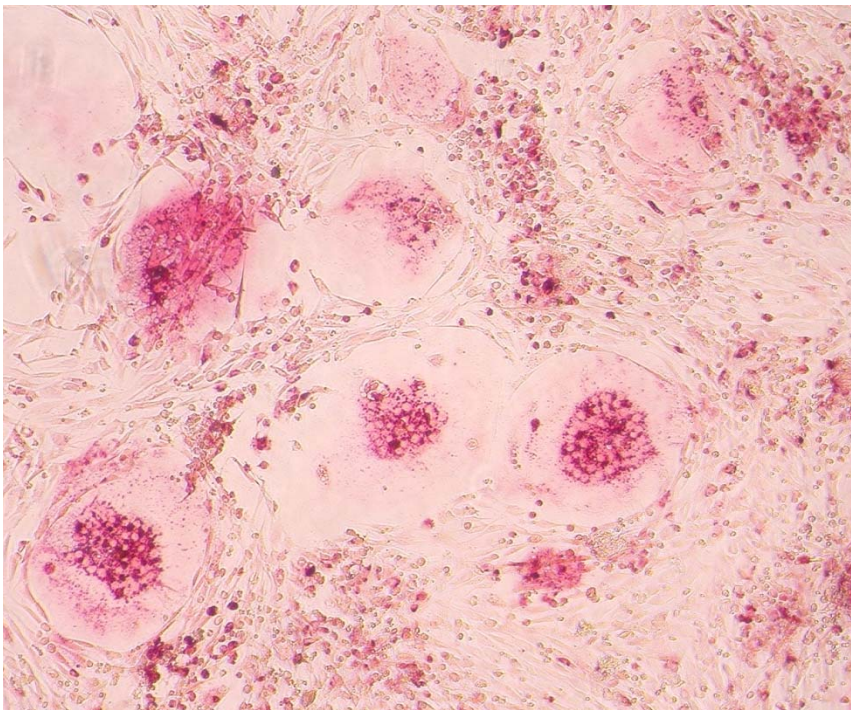


Figure 2.4: TRAP-positive cells (control group) under the light microscope

2.7.3 Morphological analysis

The morphology was examined by staining the cytoskeleton and nuclei of the osteoclasts.

The cytoskeleton was labelled with phalloidin which binds with high affinity to the F-actin present in osteoclasts. The phalloidin was conjugated with Alexa Fluor® 546 dye (excitation/emission maxima ~556/573 nm) which made the distribution of F-actin in the osteoclasts, and further, the actin rings of active osteoclasts visible under the fluorescence microscope. In the images the F-actin appears in red colour.

The nuclei were made visible with 4´,6-Diamidino-2-phenylindole (DAPI). DAPI (excitation/emission maxima ~350/470 nm) forms fluorescent complexes with AT-rich sequences of double-stranded DNA. Through this staining method, the number of nuclei could be determined easily as well as signs of apoptosis were revealed. In the images the nuclei appear in blue colour.

2.7.3.1 Preparation of cover slips

The cover slips were cleaned with 100 % ethanol overnight and then sterilized at 180 °C for about 2 hours. Afterwards the sterilized cover slips were preincubated in α-MEM + 10 % FCS for at least 2 hours or overnight. Finally the cover slips were placed in a 6-well plate, one in each well.

2.7.3.2 Preparation of rabbit osteoclasts

Done as described in chapter 2.7.1.2.

2.7.3.3 Fixation

At the end of the incubation period, the medium was removed from the wells and 37 °C warm PBS with Ca^{2+}/Mg^{2+} was added to the wells. The PBS with Ca^{2+}/Mg^{2+} was removed again (rinsing). The cells were fixed on the cover slips by adding 1 ml 3.7 % formaldehyde solution to each well twice, first for 3 minutes and then for 10 minutes with fresh solution. After the fixation, the cover slips were washed with PBS with Ca^{2+}/Mg^{2+}. For storage at 4 °C, the wells were filled with PBS with Ca^{2+}/Mg^{2+} containing NaN_3 (500x) to prevent infection.

2.7.3.4 Alexa Fluor/Phalloidin and DAPI staining

The Alexa Fluor/Phalloidin dye was stored dissolved in methanol at -20 °C. 5 µl Alexa Fluor/Phalloidin dye per cover slip were mixed with 200 µl PBS with Ca^{2+}/Mg^{2+} and shortly vortexed.

The DAPI stock solution was stored as well at -20 °C. 200 µl of the stock solution (5 µg/ml – 50x) were mixed with 10 ml PBS with Ca^{2+}/Mg^{2+} to stain 5 cover slips.

The cover slips were washed with PBS with Ca^{2+}/Mg^{2+} and then treated with ice cold acetone for 3 minutes. Afterwards 200 µl of the Alexa Fluor/Phalloidin staining solution was dropped on the cover slips. After 30 minutes of light free incubation at room temperature, the cover slips were put back in a 6-well staining plate and washed with PBS with Ca^{2+}/Mg^{2+} to stop the staining. 2 ml of the DAPI staining solution were added to each well for 10 minutes and incubated at 37 °C. To stop the DAPI staining the cover slips were washed with PBS with Ca^{2+}/Mg^{2+} and afterwards with double distilled water.

The cover slips were then put on an object slide on a drop of mounting fluid. The edges were sealed and stored at 4°C.

During the entire time the dyes and staining solutions had to be protected against light.

2.7.3.5 Analysis

The cells were visualized using an Olympus BX51 microscope with a 40x or 60x magnification, using the filters FITC and DAPI. 10 – 15 images of each cover slip were taken with an Olympus Color View III digital camera which was connected to the microscope. The images, displaying the nuclei blue and the cytoskeleton red, were saved in the database of the cell^F program. The difference between the osteoclasts in the control groups and the groups with treatment were examined and documented regarding the health of the nuclei, the actin ring structure and the cytoskeleton organization. (Figure 2.5)

Each treatment was carried out on 2 cover slips with cells.

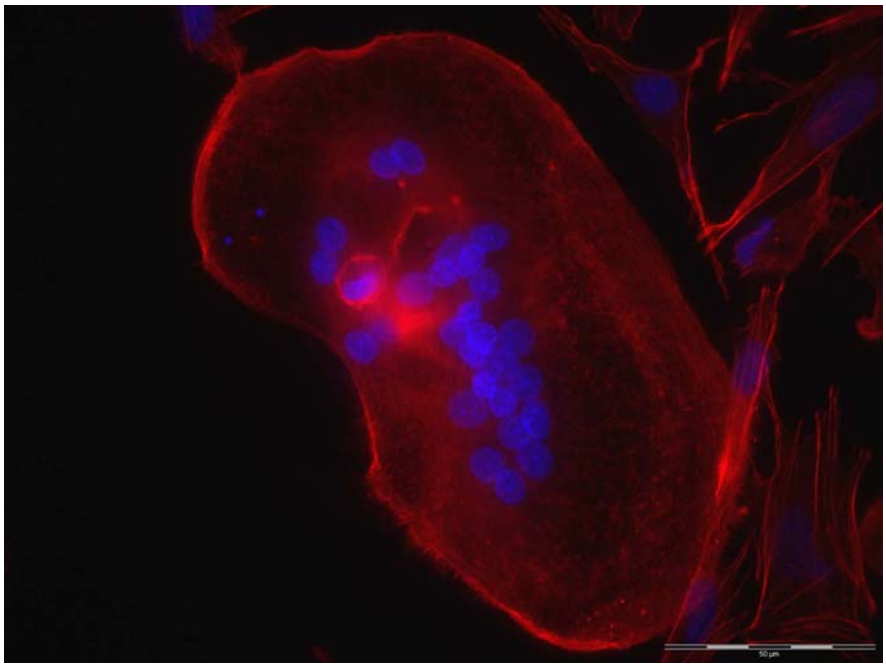


Figure 2.5: Alexa fluor® 546/Phalloidin and DAPI stained OC (control group) (60x magnification, scale: 50 µm)

2.7.4 Statistical analysis

Data was analysed with the statistical program StatView. Statistical significance was defined by using one factor ANOVA (analysis of variance). Statistical differences between the groups were determined by Fisher's least significant difference (LSD) test. Data is presented as the mean ± standard error of mean (SEM). Significant differences are presented as $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***) compared to the respective control group.

3. Results

3.1 Resorption assay

3.1.1 Extr. Leuzeae e rad. aquos. sicc. (PE 2)

PE 2 was used in the concentrations 0.5, 0.25, 0.143, 0.043 and 0.014 mg/ml. One control group was culture medium alone; the other control group was culture medium containing the same amount of solvent of PE 2, which was water. The control group regarding the inhibition was culture medium containing pamidronate in a concentration of 1×10^{-4} M.

PE 2 inhibited the formation of pits in the resorption assay in a dose-dependent manner; the number of pits was significantly decreased. Bone resorption, respectively osteoclast function, was clearly reduced. (Figure 3.1)

PE 2 in the concentration of 0.5 and 0.25 mg/ml inhibited bone resorption with a significance of $p<0.001$ compared to the control group with the solvent water. These two treatments are comparable with the inhibition of bone resorption caused by pamidronate.

PE 2 in the concentration of 0.143 mg/ml still showed an inhibition of bone resorption with a significance of $p<0.01$ compared to the control group with the solvent water. The concentrations of 0.043 and 0.014 mg/ml did not show a significant inhibitory effect compared to the control group with the solvent water.

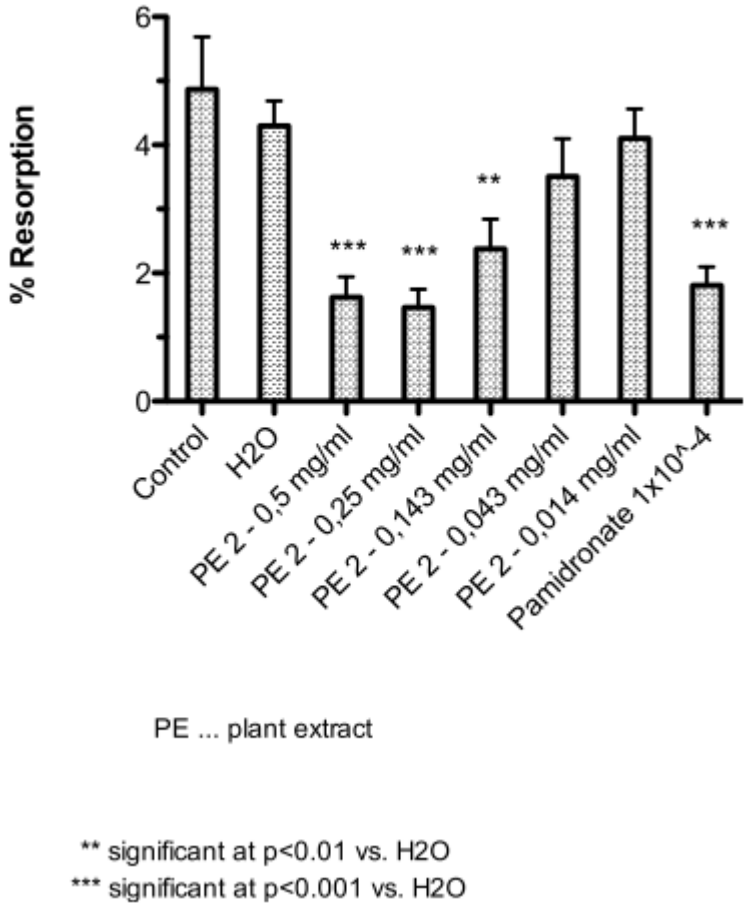


Figure 3.1: Effect of PE 2 on the resorption of rabbit OCs on bovine bone slices. Rabbit osteoclasts on bovine bone slices; time of treatment was 48 hours.

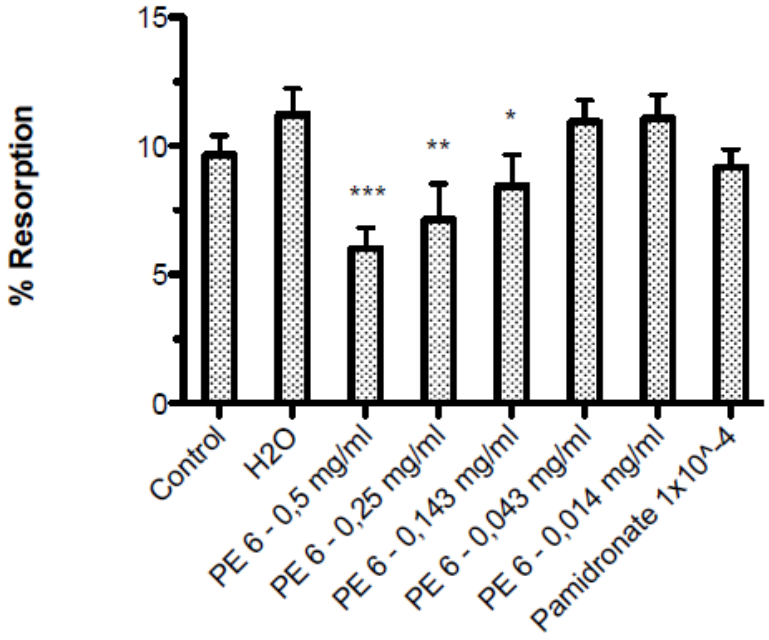
3.1.2 Extr. Cichorie aquos. sicc. (PE 6)

PE 6 was used in the concentrations 0.5, 0.25, 0.143, 0.043 and 0.014 mg/ml. One control group was culture medium alone; the other control group was culture medium containing the same amount of solvent of PE 6, which was water. The control group regarding the inhibition was culture medium containing pamidronate in a concentration of 1 x 10^{-4} M.

PE 6 inhibited the formation of pits in the resorption assay in a dose-dependent manner; the number of pits was significantly decreased. Bone resorption, respectively osteoclast function, was clearly reduced. (Figure 3.2)

PE 6 in the concentration of 0.5 mg/ml inhibited bone resorption with a significance of $p<0.001$ compared to the control group with the solvent water. In the concentration of 0.25 mg/ml, bone resorption was inhibited with a significance of $p<0.01$, whereas PE 6 in the concentration of 0.143 mg/ml inhibited bone resorption with a significance of $p<0.05$ compared to the control group with the solvent water. The concentrations of 0.043 and 0.014 mg/ml did not show an inhibitory effect compared to the control group with the solvent water.

Inhibition of bone resorption by pamidronate did not work properly in this experiment. Through comparing the inhibitory effect of the pamidronate groups of the other experiments, it can be assumed that the resorption of the pamidronate group in this experiment would have also been much lower, namely around 2 – 3 %.



PE ... plant extract

* significant at p<0.05 vs. H2O
** significant at p<0.01 vs. H2O
*** significant at p<0.001 vs. H2O

Figure 3.2: Effect of PE 6 on the resorption of rabbit OCs on bovine bone slices.
Rabbit osteoclasts on bovine bone slices; time of treatment was 48 hours.

3.1.3 Extr. Cichorie e rad. spir. sicc. (PE 7)

PE 7 was used in the concentrations 0.143, 0.043, 0.014, 0.004 and 0.001 mg/ml. One control group was culture medium alone; the other control group was culture medium containing the same amount of solvent of PE 7, which was 70 % ethanol. The control group regarding the inhibition was culture medium containing pamidronate in a concentration of 1×10^{-4} M.

PE 7 inhibited the formation of pits in the resorption assay. The number of pits was significantly decreased, especially in the treatment with the highest concentration of the plant extract (0.143 mg/ml). At this concentration, bone resorption, respectively osteoclast function, was clearly reduced. (Figure 3.3)

PE 7 in the concentration of 0.143 mg/ml inhibited bone resorption with a significance of $p<0.001$ compared to the control group with the solvent 70 % ethanol. Bone resorption was almost totally inhibited and therefore even stronger than the inhibition caused by pamidronate.

In the concentration of 0.043 mg/ml bone resorption was inhibited with a significance of $p<0.05$ compared to the control group with the solvent 70 % ethanol. PE 7 in the concentrations of 0.014, 0.004 and 0.001 mg/ml did not show an inhibitory effect compared to the control group with the solvent (70 % ethanol).

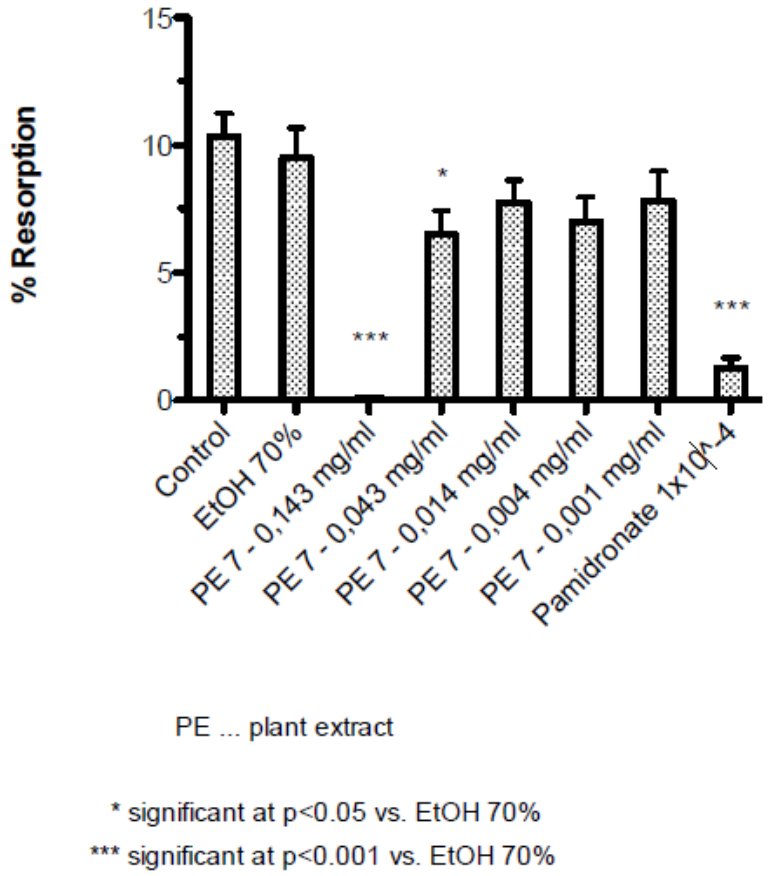


Figure 3.3: Effect of PE 7 on the resorption of rabbit OCs on bovine bone slices.
Rabbit osteoclasts on bovine bone slices; time of treatment was 48 hours.

3.1.4 Extr. Wassabiae e herb. aquos. sicc. (PE 10)

PE 10 was used in the concentrations 0.5, 0.25, 0.143, 0.043 and 0.014 mg/ml. One control group was culture medium alone; the other control group was culture medium containing the same amount of solvent of PE 10, which was water. The control group regarding the inhibition was culture medium containing pamidronate in a concentration of 1×10^{-4} M.

PE 10 did not inhibit the formation of pits in the resorption assay; the number of pits was not decreased. Bone resorption and osteoclast function were not reduced. (Figure 3.4)

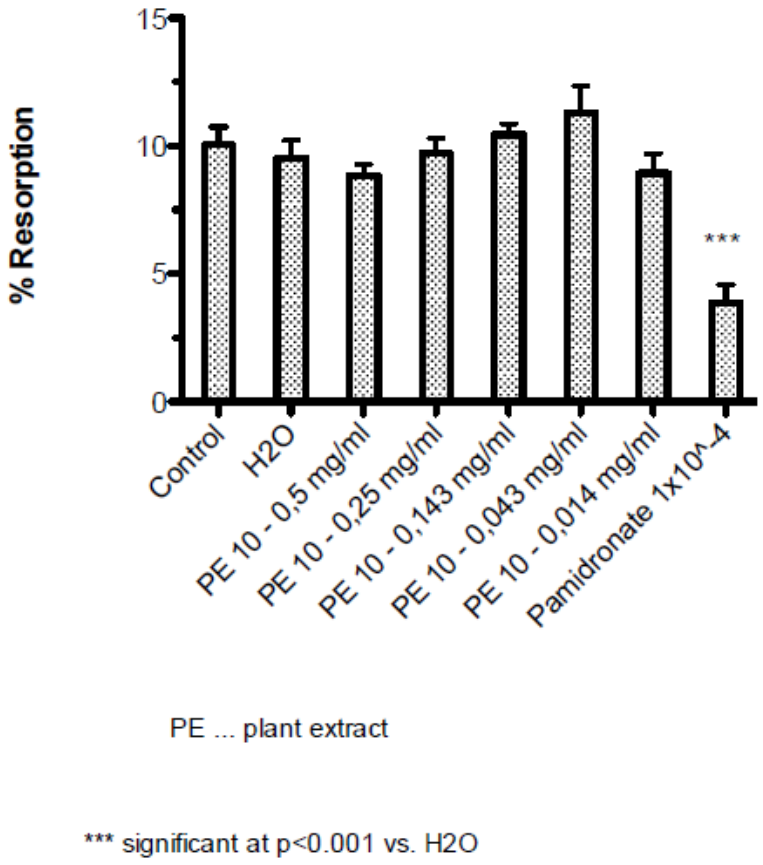


Figure 3.4: Effect of PE 10 on the resorption of rabbit OCs on bovine bone slices. Rabbit osteoclasts on bovine bone slices; time of treatment was 48 hours.

3.1.5 Extr. Humuli lupuli sicc. (PE 16)

PE 16 was used in the concentrations 0.25, 0.143, 0.043, 0.014 and 0.004 mg/ml. One control group was culture medium alone; the other control group was culture medium containing the same amount of solvent of PE 16, which was 40 % ethanol. The control group regarding the inhibition was culture medium containing pamidronate in a concentration of 1×10^{-4} M.

PE 16 inhibited the formation of pits in the resorption assay in a dose-dependent manner. The number of pits was significantly decreased, especially in the treatment with the highest concentration of the plant extract (0.25 mg/ml). In the three treatments with the highest plant extract concentrations (0.25, 0.143 and 0.043 mg/ml), osteoclast function was clearly reduced. (Figure 3.5)

PE 16 in the concentration of 0.25 mg/ml inhibited bone resorption with a significance of $p<0.001$ compared to the control group with the solvent 40 % ethanol. Bone resorption was almost totally inhibited and therefore even stronger than the inhibition caused by pamidronate.

In the concentration of 0.143 mg/ml, bone resorption was higher than in the treatment with the highest concentrated plant extract (0.25 mg/ml), but was still inhibited with a significance of $p<0.001$ compared to the control group with the solvent 40 % ethanol and was still less than bone resorption in the pamidronate group.

PE 16 in the concentration of 0.043 mg/ml showed an inhibition with a significance of $p<0.01$ compared to the control group with the solvent 40 % ethanol, whereas PE 16 in the concentration of 0.014 and 0.004 mg/ml did not show an inhibitory effect compared to the control group with the solvent 40 % ethanol.

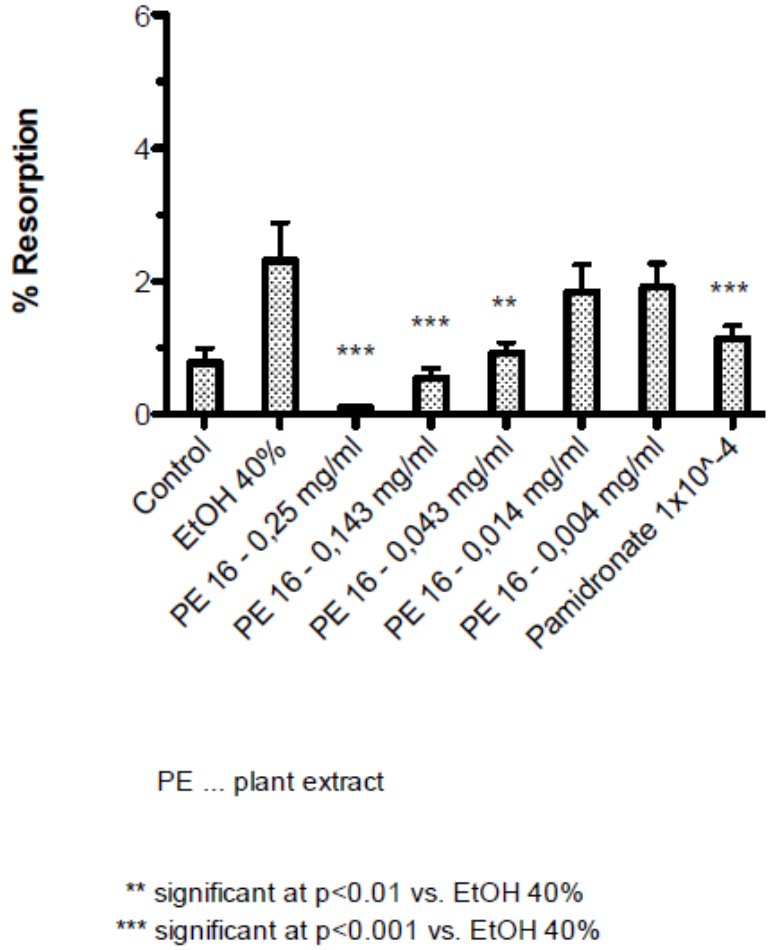


Figure 3.5: Effect of PE 16 on the resorption of rabbit OCs on bovine bone slices.
Rabbit osteoclasts on bovine bone slices; time of treatment was 48 hours.

3.2 Quantification of TRAP-positive cells

TRAP staining was always performed on the same well plate together with the corresponding resorption assay for each plant extract. The used concentrations and control groups are therefore the same as described in the corresponding resorption assay chapters above.

Images obtained of the TRAP-positive cells were taken of each control group and of each treatment of all extracts. See below the images of the control group (culture medium alone), the vehicle control groups (culture medium containing the specific solvent of each plant extract) and the control group regarding the inhibition (culture medium containing pamidronate in a concentration of 1×10^{-4} M). Two images of the pamidronate group are shown because the appearance of the cells damaged by pamidronate was not the same in all areas. (Figure 3.6)

Images of osteoclasts under plant extract treatment can be found in the corresponding chapters. Additionally, images of osteoclasts from the corresponding vehicle control groups were added for better comparability.

All images were taken from an area representative for the whole well.

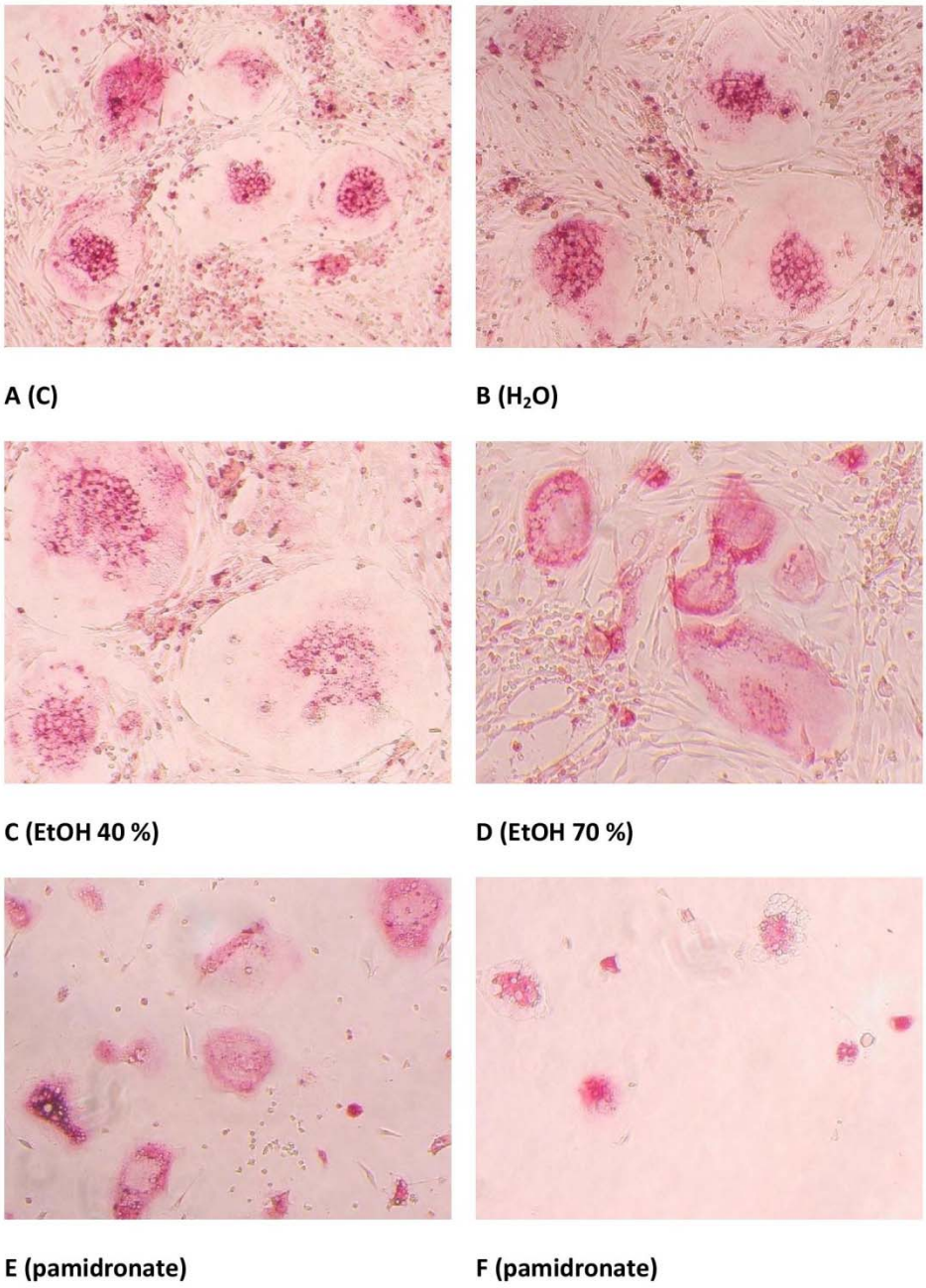


Figure 3.6: Stained TRAP-positive cells (controls) under the light microscope.

A (C) – Culture medium

B (H_2O) – Culture medium containing the solvent H_2O

C (EtOH 40 %) – Culture medium containing the solvent EtOH 40 %

D (EtOH 70 %) – Culture medium containing the solvent EtOH 70 %

E (pamidronate) and **F (pamidronate)** – Culture medium containing pamidronate in a concentration of 1×10^{-4} M

A, B, C and D show large, round OCs. E and F show the effect of pamidronate on OCs – very small TRAP-positive cells.

3.2.1 Extr. Leuzeae e rad. aquos. sicc. (PE 2)

PE 2 decreased the number of TRAP-positive cells in a dose-dependent manner. (Figure 3.7)

In the concentration of 0.5, 0.25 and 0.143 mg/ml the number of TRAP-positive cells was decreased with a significance of $p<0.001$ compared to the control group with the solvent water. PE 2 in the concentration of 0.043 mg/ml still showed a decrease of TRAP-positive cells with a significance of $p<0.01$ compared to the control group with the solvent water.

The least concentrated treatment with a concentration of 0.014 mg/ml did not show a significant difference compared to the control group with the solvent water. The pamidronate group should have shown a decrease in the number of TRAP-positive cells which was not the case in figure 3.7.

The results of the decrease in the number of TRAP-positive cells caused by PE 2, correlate with the dose-dependent result of the resorption assay (see figure 3.1).

The changes of the appearance of the osteoclasts by PE 2 can be seen in figure 3.8.

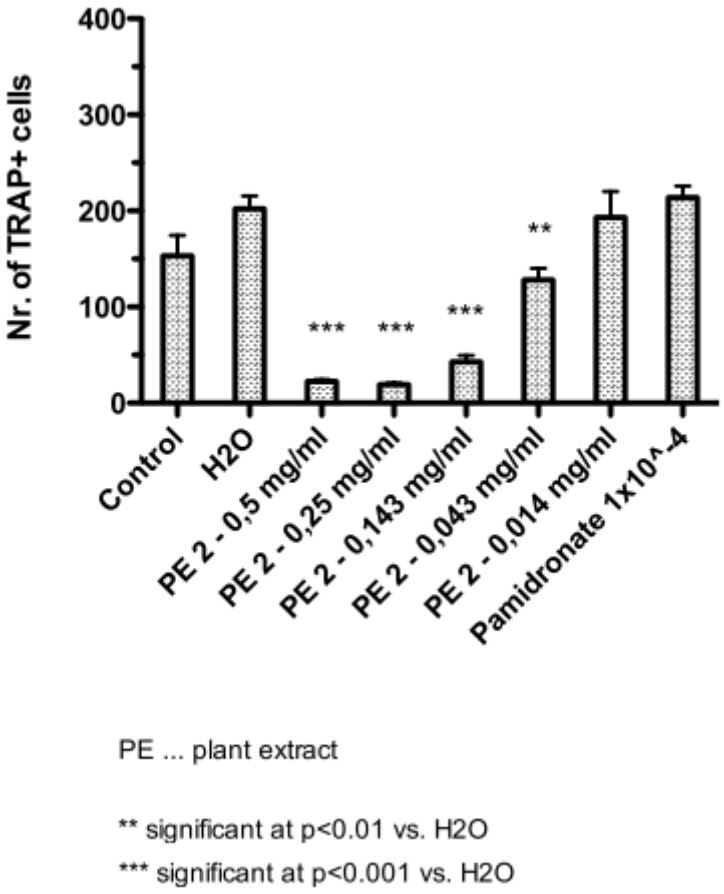


Figure 3.7: Effect of PE 2 on the number of TRAP-positive cells with three or more nuclei (osteoclasts).

Rabbit osteoclasts on well surface; time of treatment was 48 hours.

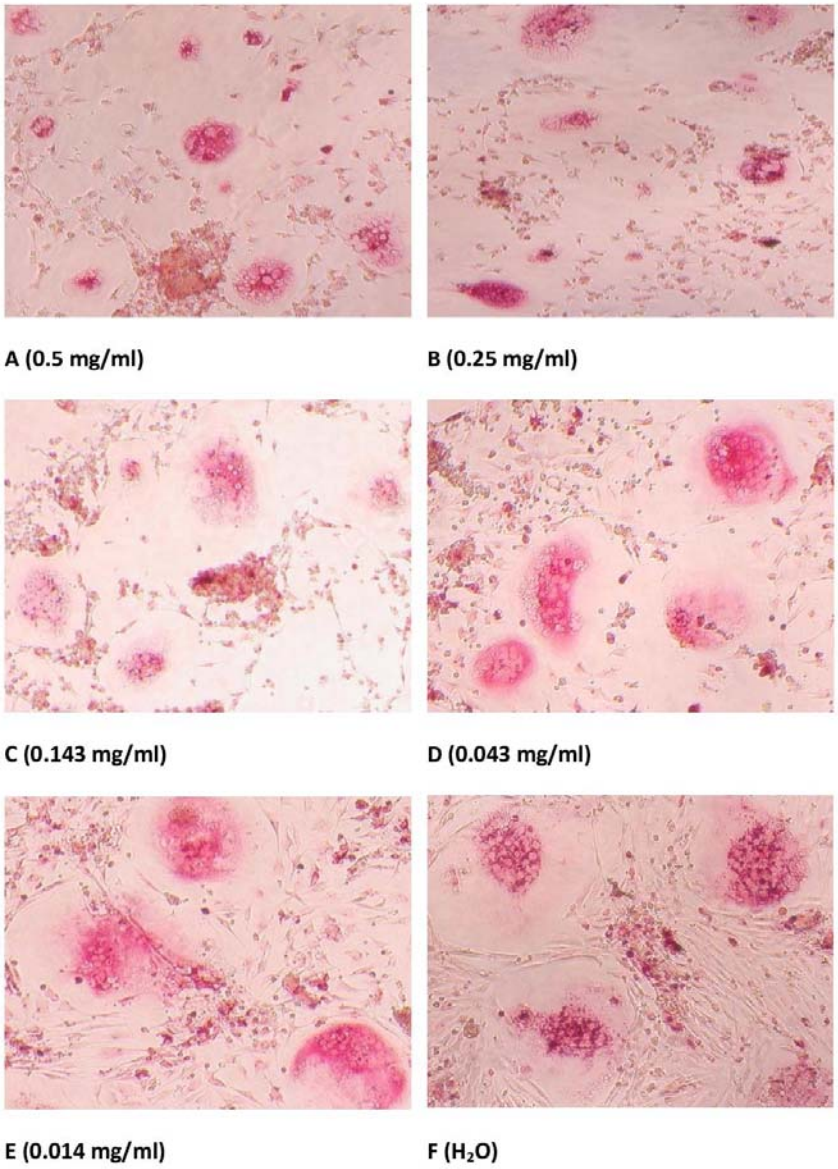


Figure 3.8: Stained TRAP-positive cells (PE 2) under the light microscope.

A – PE 2 (0.5 mg/ml)

B – PE 2 (0.25 mg/ml)

C – PE 2 (0.143 mg/ml)

D – PE 2 (0.043 mg/ml)

E – PE 2 (0.014 mg/ml)

F – Culture medium containing the solvent H_2O

A and B show small TRAP-positive cells, the nuclei are hard to detect, the cells are full of vacuoles. C and D show larger TRAP-positive cells, the nuclei are easier to detect but there are still a lot of vacuoles. E can already be compared with the control group F, the OCs are large and round.

3.2.2 Extr. Cichorie aquos. sicc. (PE 6)

PE 6 decreased the number of TRAP-positive cells in a dose-dependent manner. (Figure 3.9)

In the concentration of 0.5, 0.25, 0.143 and 0.014 mg/ml, the number of TRAP-positive cells was decreased with a significance of $p<0.001$ compared to the control group with the solvent water. PE 6 in the concentration of 0.043 mg/ml showed a decrease of TRAP-positive cells with a significance of $p<0.01$ compared to the control group with the solvent water.

All treatments showed significantly reduced numbers of TRAP-positive cells. PE 6 in the concentrations of 0.143, 0.043 and 0.014 showed a similar effect on the number of TRAP-positive cells as the pamidronate group. The two highest concentrated treatments (0.5 and 0.25 mg/ml) decreased the number of TRAP-positive cells more clearly than the pamidronate group.

The results of the decrease in the number of TRAP-positive cells caused by PE 6 correlate with the dose-dependent result of the resorption assay (see figure 3.2), though PE 6 inhibited the number of osteoclasts more than bone resorption.

The changes of the appearance of the osteoclasts by PE 6 can be seen in figure 3.10.

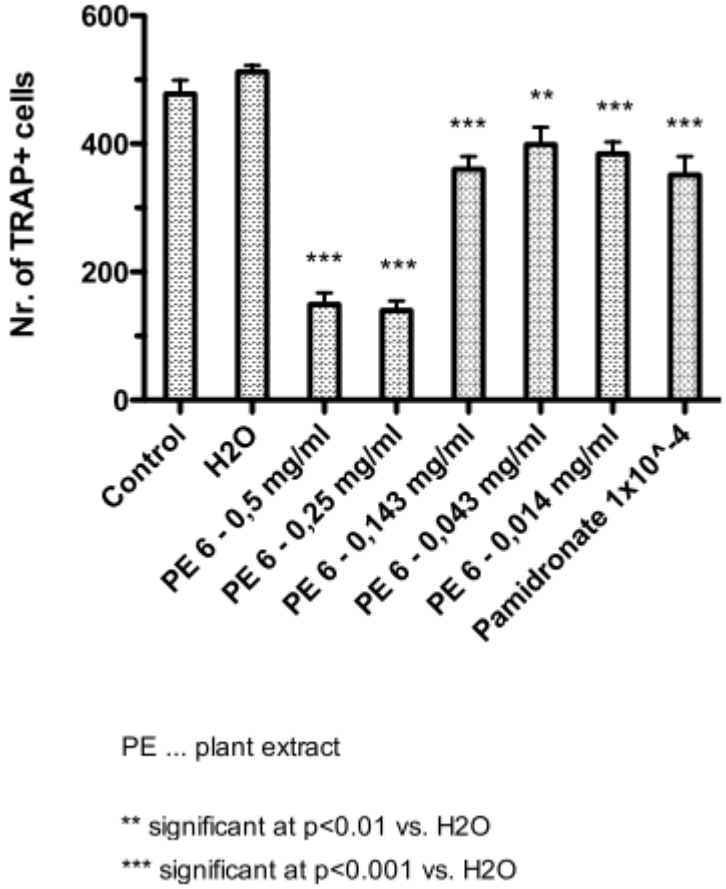


Figure 3.9: Effect of PE 6 on the number of TRAP-positive cells with three or more nuclei (osteoclasts).

Rabbit osteoclasts on well surface; time of treatment was 48 hours.

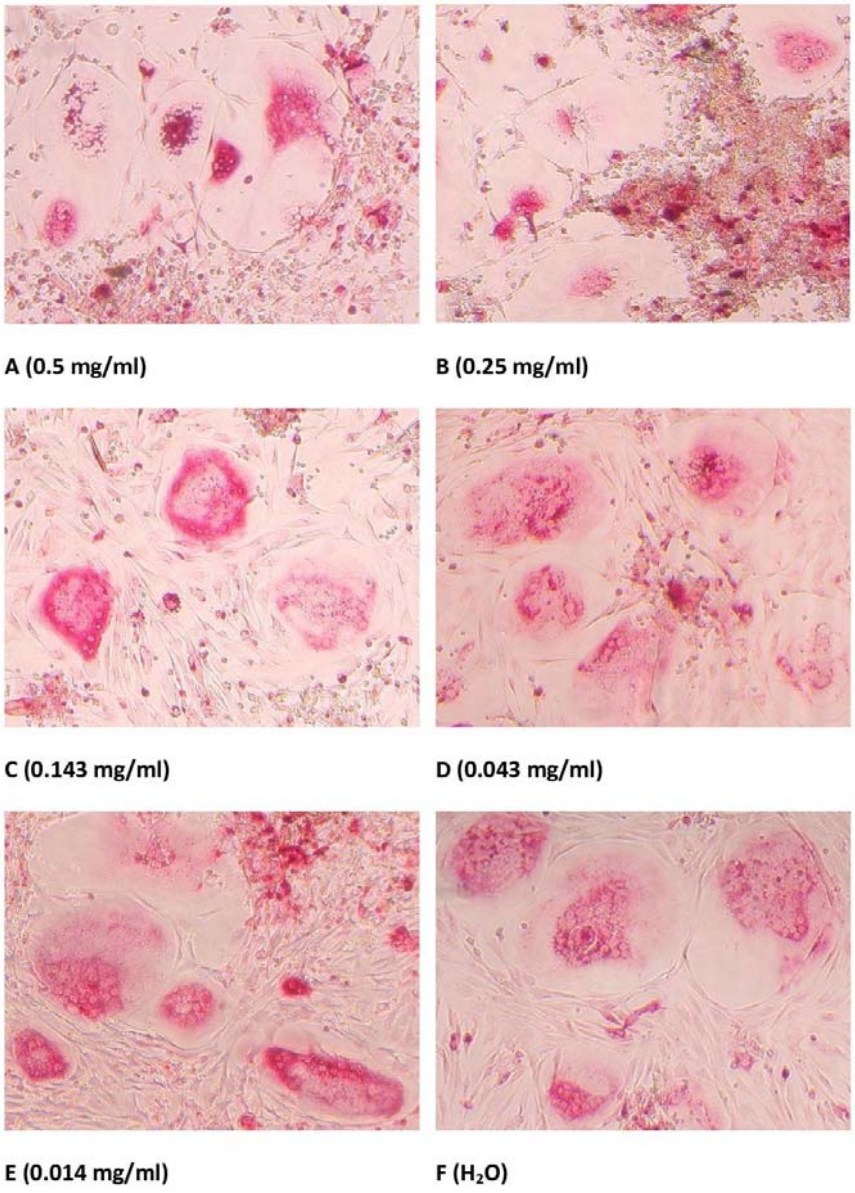


Figure 3.10: Stained TRAP-positive cells (PE 6) under the light microscope.

A – PE 6 (0.5 mg/ml)

B – PE 6 (0.25 mg/ml)

C – PE 6 (0.143 mg/ml)

D – PE 6 (0.043 mg/ml)

E – PE 6 (0.014 mg/ml)

F – Culture medium containing the solvent H_2O

A and B show small TRAP-positive cells, the nuclei are hard to detect, the cells are full of vacuoles. C, D and E show larger TRAP-positive cells, the nuclei are easier to detect, the vacuoles reduced. They look similar to the control group F, having large, round OCs.

3.2.3 Extr. Cichorie e rad. spir. sicc. (PE 7)

PE 7 decreased the number of TRAP-positive cells, especially in the treatment with the highest concentrated plant extract (0.143 mg/ml), where basically no TRAP-positive cells could be found. (Figure 3.11)

PE 7 in the concentration of 0.143 mg/ml decreased the number of TRAP-positive cells with a significance of $p<0.001$ compared to the control group with the solvent 70 % ethanol. The effect was much stronger than the effect caused by pamidronate.

In the concentration of 0.043 mg/ml, the number of TRAP-positive cells was as well decreased with a significance of $p<0.001$ compared to the control group with the solvent 70 % ethanol but the decrease was not as strong as the decrease caused by pamidronate.

PE 7 in the concentrations of 0.014, 0.004 and 0.001 mg/ml did not cause a significant decrease in the number of TRAP-positive cells compared to the control group with the solvent 70 % ethanol.

The results of the decrease in the number of TRAP-positive cells caused by PE 7 correlate with the result of the resorption assay (see figure 3.3), though PE 7 inhibited the number of osteoclasts more, especially in the treatment with a concentration of 0.043 mg/ml, than bone resorption.

The changes of the appearance of the osteoclasts by PE 7 can be seen in figure 3.12.

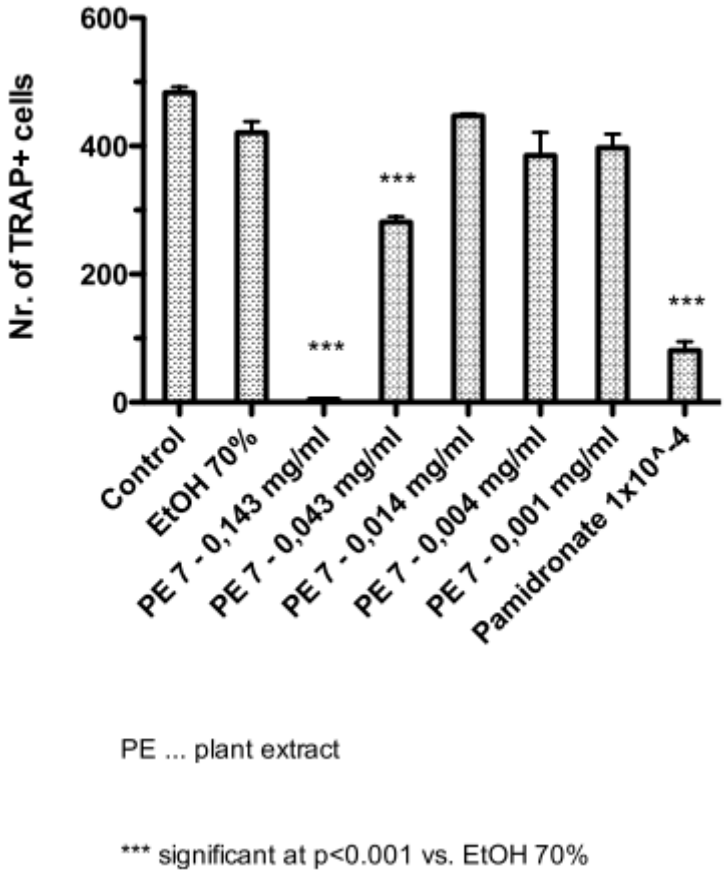


Figure 3.11: Effect of PE 7 on the number of TRAP-positive cells with three or more nuclei (osteoclasts).

Rabbit osteoclasts on well surface; time of treatment was 48 hours.

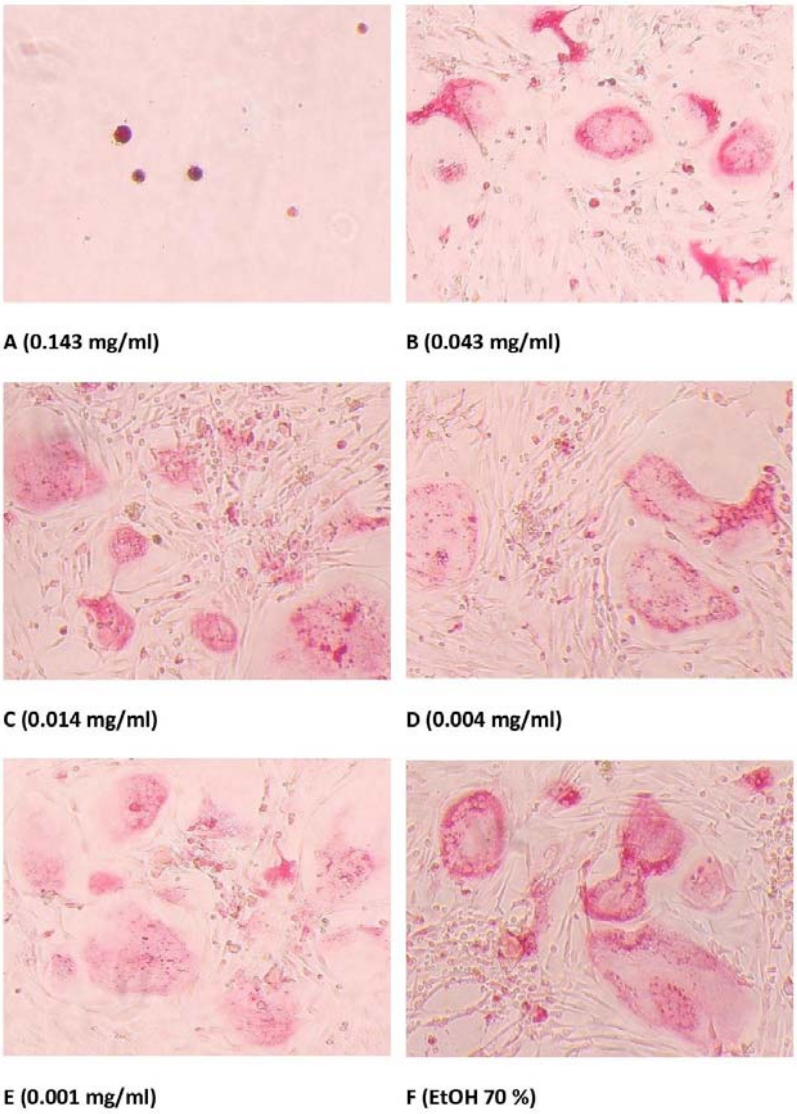


Figure 3.12: Stained TRAP-positive cells (PE 7) under the light microscope.

A – PE 7 (0.143 mg/ml)

B – PE 7 (0.043 mg/ml)

C – PE 7 (0.014 mg/ml)

D – PE 7 (0.004 mg/ml)

E – PE 7 (0.001 mg/ml)

F – Culture medium containing the solvent EtOH 70 %

A shows very small TRAP-positive cells, almost no cells with visible nuclei could be found. B shows larger TRAP-positive cells, but the nuclei are still hard to detect and the cells are full of vacuoles. C, D and E show large TRAP-positive cells without vacuoles, the nuclei are easy to detect. They are comparable to the large, round OCs of the control group F.

3.2.4 Extr. Wassabiae e herb. aquos. sicc. (PE 10)

PE 10 did not decrease the number of TRAP-positive cells. (Figure 3.13)

This result correlates with the result of the resorption assay. (Figure 3.4)

The appearance of the osteoclasts in culture medium with different concentrations of PE 10 can be seen in figure 3.14.

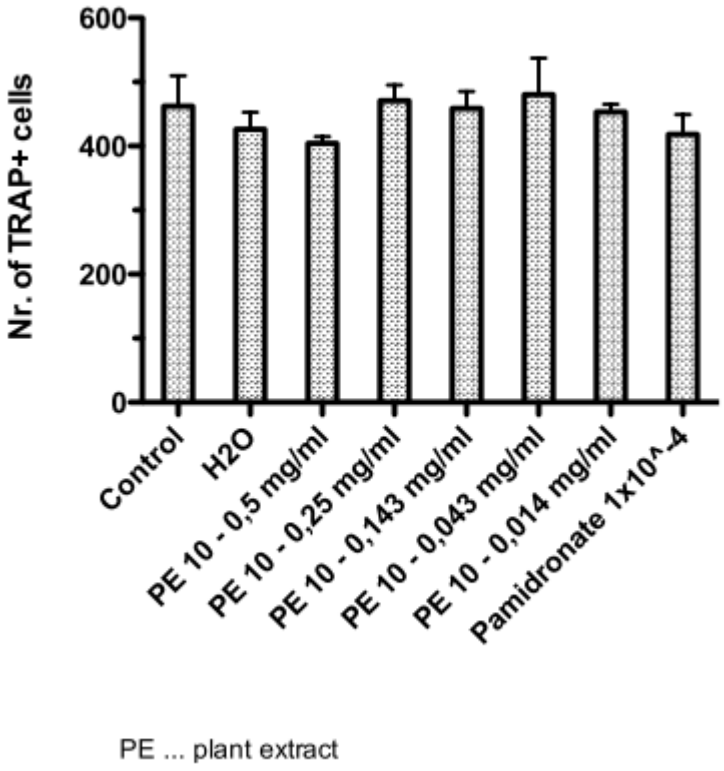


Figure 3.13: Effect of PE 10 on the number of TRAP-positive cells with three or more nuclei (osteoclasts).
Rabbit osteoclasts on well surface; time of treatment was 48 hours.

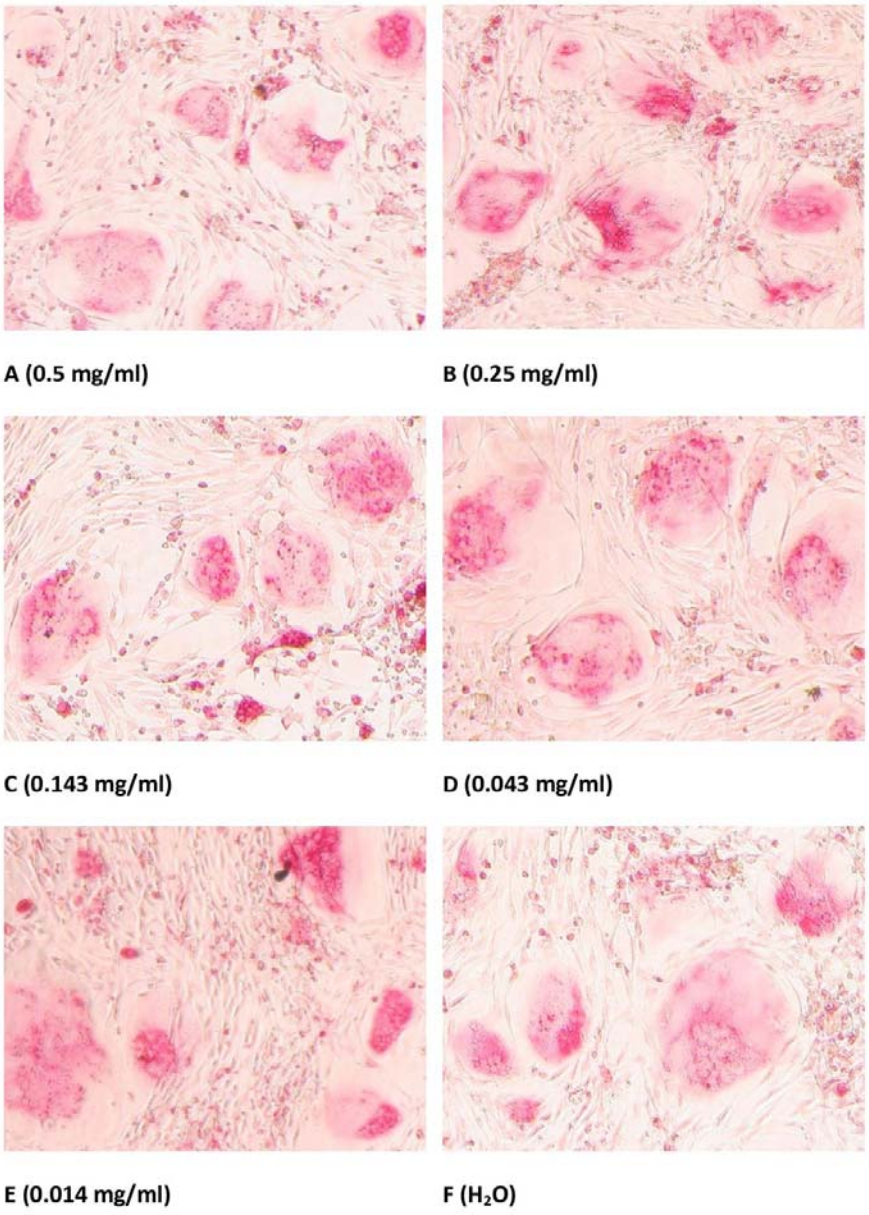


Figure 3.14: Stained TRAP-positive cells (PE 10) under the light microscope.

A – PE 10 (0.5 mg/ml)

B – PE 10 (0.25 mg/ml)

C – PE 10 (0.143 mg/ml)

D – PE 10 (0.043 mg/ml)

E – PE 10 (0.014 mg/ml)

F – Culture medium containing the solvent H_2O

A, B, C, D and E show large, round TRAP-positive cells without vacuoles and nuclei easy to detect. The cells are similar to the OCs of the control group F.

3.2.5 Extr. Humuli lupuli sicc. (PE 16)

PE 16 decreased the number of TRAP-positive cells, especially in the treatment with the highest concentration of the plant extract (0.25 mg/ml). (Figure 3.15)

PE 16 in the concentration of 0.25 mg/ml decreased the number of TRAP-positive cells with a significance of $p<0.001$ compared to the control group with the solvent 40 % ethanol. The effect was stronger than the effect caused by pamidronate.

The concentrations of 0.143, 0.043, 0.014 and 0.004 mg/ml did not cause a significant decrease in the number of TRAP-positive cells compared to the control group with the solvent 40 % ethanol. PE 16 in the concentration of 0.014 mg/ml even showed, with the significance of $p<0.05$, a larger amount of TRAP-positive cells compared to the control group with the solvent 40 % ethanol.

The results of the decrease in the number of TRAP-positive cells caused by PE 16 do not correlate well with the dose-dependent result of the resorption assay (see figure 3.5) as the experiments of the other plant extracts do.

The changes of the appearance of the osteoclasts by PE 16 can be seen in figure 3.16.

Comparing the resorption assay of PE 16 and the images obtained of the TRAP-positive cells of PE 16 (figure 3.5 and figure 3.16), it can be assumed, that the osteoclasts, though their nuclei are clearly detectable, are not able to resorb bone properly.

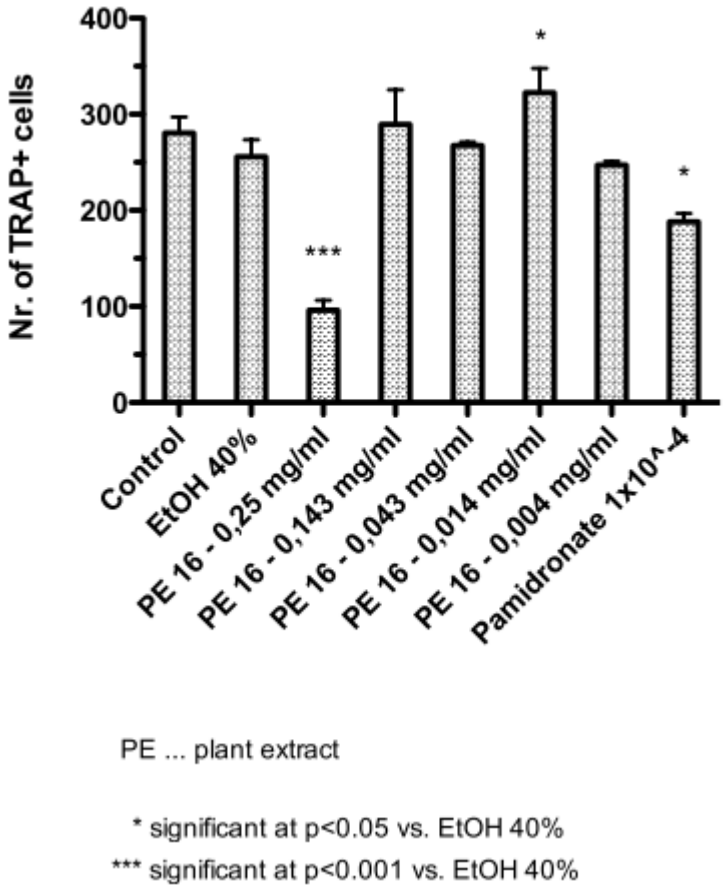


Figure 3.15: Effect of PE 16 on the number of TRAP-positive cells with three or more nuclei (osteoclasts).

Rabbit osteoclasts on well surface; time of treatment was 48 hours.

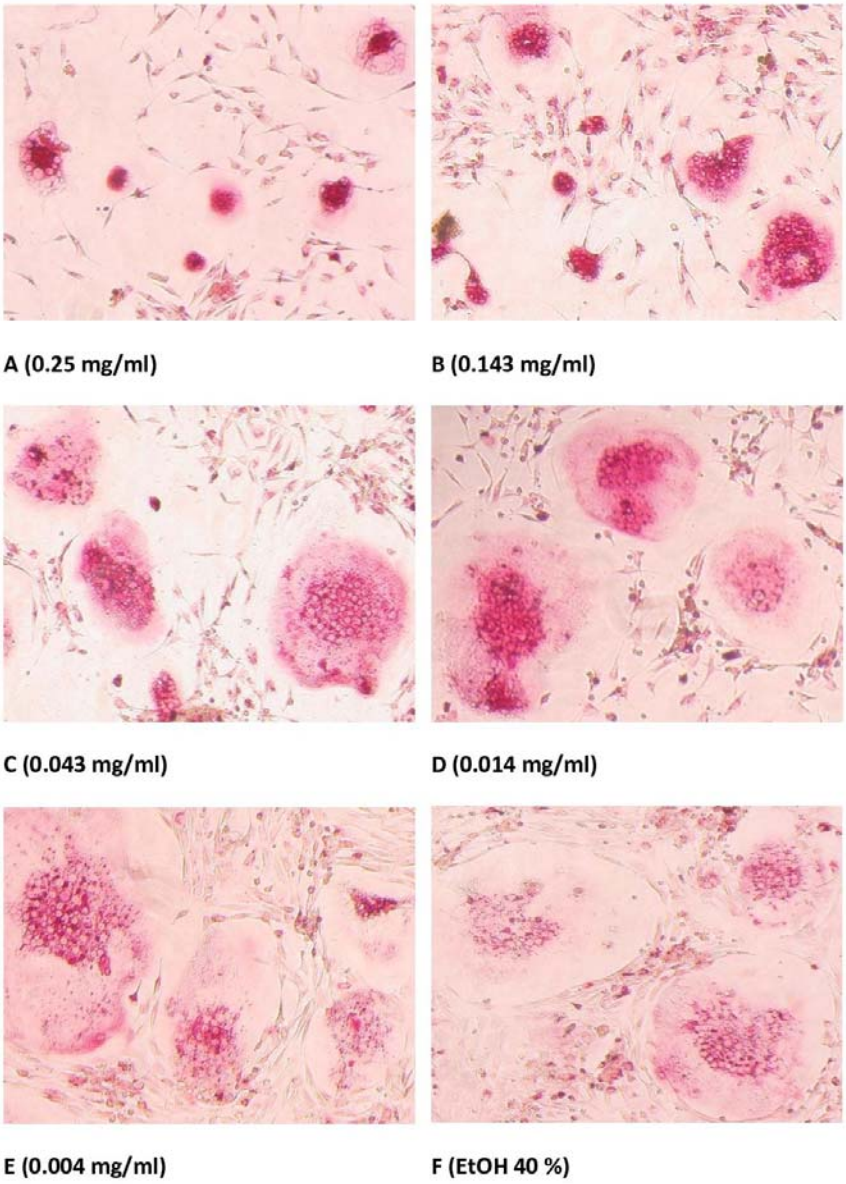


Figure 3.16: Stained TRAP-positive cells (PE 16) under the light microscope.

A – PE 16 (of 0.25 mg/ml)

B – PE 16 (0.143 mg/ml)

C – PE 16 (0.043 mg/ml)

D – PE 16 (0.014 mg/ml)

E – PE 16 (0.004 mg/ml)

F – Culture medium containing the solvent EtOH 40 %

A shows small TRAP-positive cells with nuclei hard to detect and many vacuoles. B shows slightly larger TRAP-positive cells, the nuclei are better detectable but there are still a lot of vacuoles. C, D and E show large, round TRAP-positive cells, comparable to the OCs of the control group F.

3.3 Morphological analysis

Polarization of osteoclasts is essential for bone resorption and actin rings are a marker of this polarization. The phalloidin-labelled actin rings can be recognized as bright red-stained belts surrounding the cells. The cytoskeleton was stained with Alexa Fluor®546 phalloidin and appears in red, the nuclei were stained with DAPI and appear in blue.

The plant extracts induced a variety of morphological changes in the osteoclasts: Disruption of the actin ring, spreading of F-actins throughout the cytoplasm of the cells and presented typical signs of apoptosis including condensation of chromatin, degradation of DNA into fragments and formation of plasma and nuclear vesicles.

Images were taken of three different treatments of every plant extract under a fluorescence microscope. (Table 3.1) Treatments where very few, respectively disrupted, osteoclasts were expected, were excluded from the Alexa Fluor/Phalloidin and DAPI staining. Images can be found in the corresponding chapters. Two images of each treatment are shown to provide a better impression and to achieve a higher comparability of the changes caused by the extracts.

Plant extract	**Treatments for images with fluorescent staining**
PE 2	0.25 – 0.143 – 0.014
PE 6	0.25 – 0.143 – 0.014
PE 7	0.143 – 0.043 – 0.004
PE 10	0.5 – 0.143 – 0.014
PE 16	0.25 – 0.143 – 0.014

Table 3.1: Treatments used for Alexa Fluor/Phalloidin and DAPI staining

See below the images of the control group (culture medium alone), the vehicle control groups (culture medium containing the specific solvent of the plant extracts) and the control group regarding the inhibition (culture medium containing pamidronate in a concentration of 1 x 10^{-4} M). Two images of the pamidronate group are shown because the appearance of the cells damaged by pamidronate was not the same in all areas. (Figure 3.17)

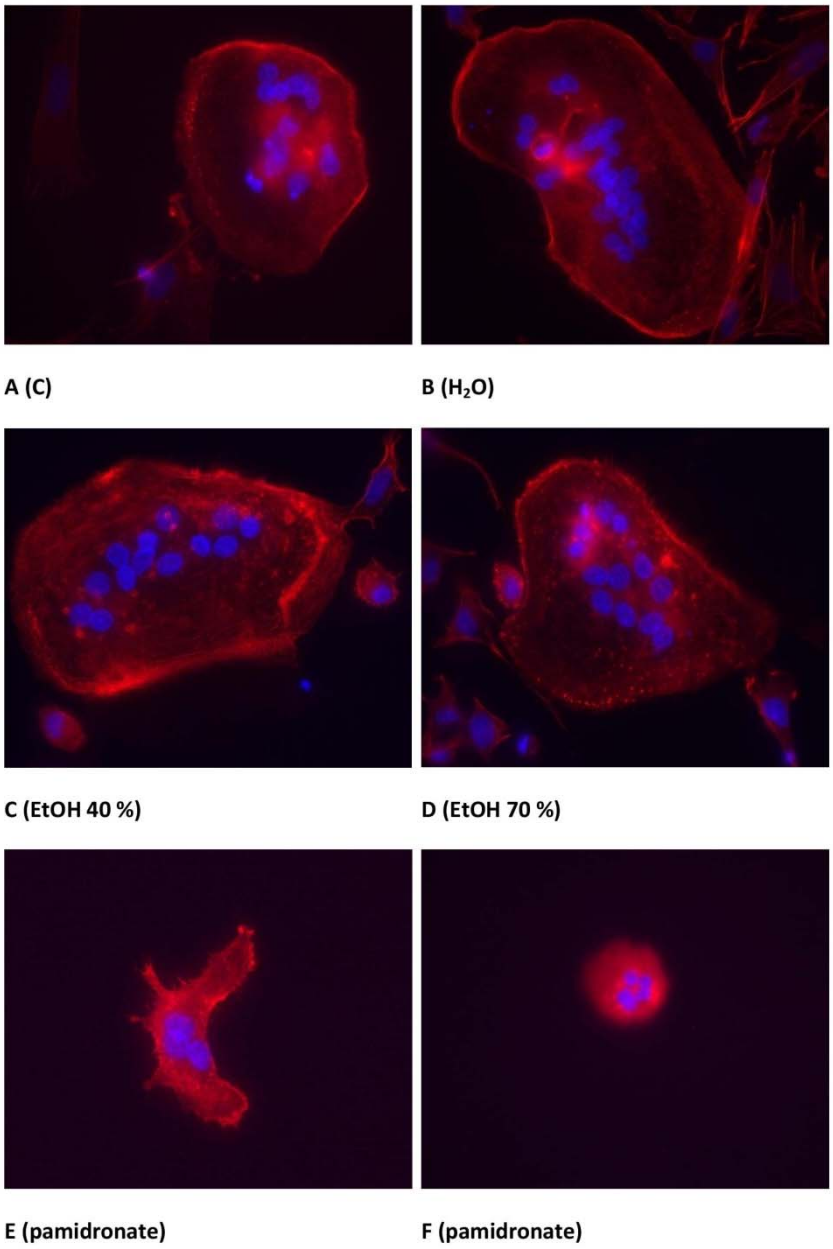


Figure 3.17: Stained OCs (control groups) under the fluorescence microscope.

A (C) – Culture medium

B (H_2O) – Culture medium containing the solvent H_2O

C (EtOH 40 %) – Culture medium containing the solvent EtOH 40 %

D (EtOH 70 %) – Culture medium containing the solvent EtOH 70 %

E (pamidronate) and **F (pamidronate)** – Culture medium containing pamidronate in a concentration of 1×10^{-4} M

A, B, C and D show large multinucleated OCs, actin rings and no signs of apoptosis. E and F show the effect of pamidronate on OCs – small cells with signs of apoptosis, diffuse cytoplasm, without smooth cell periphery.

3.3.1 Extr. Leuzeae e rad. aquos. sicc. (PE 2)

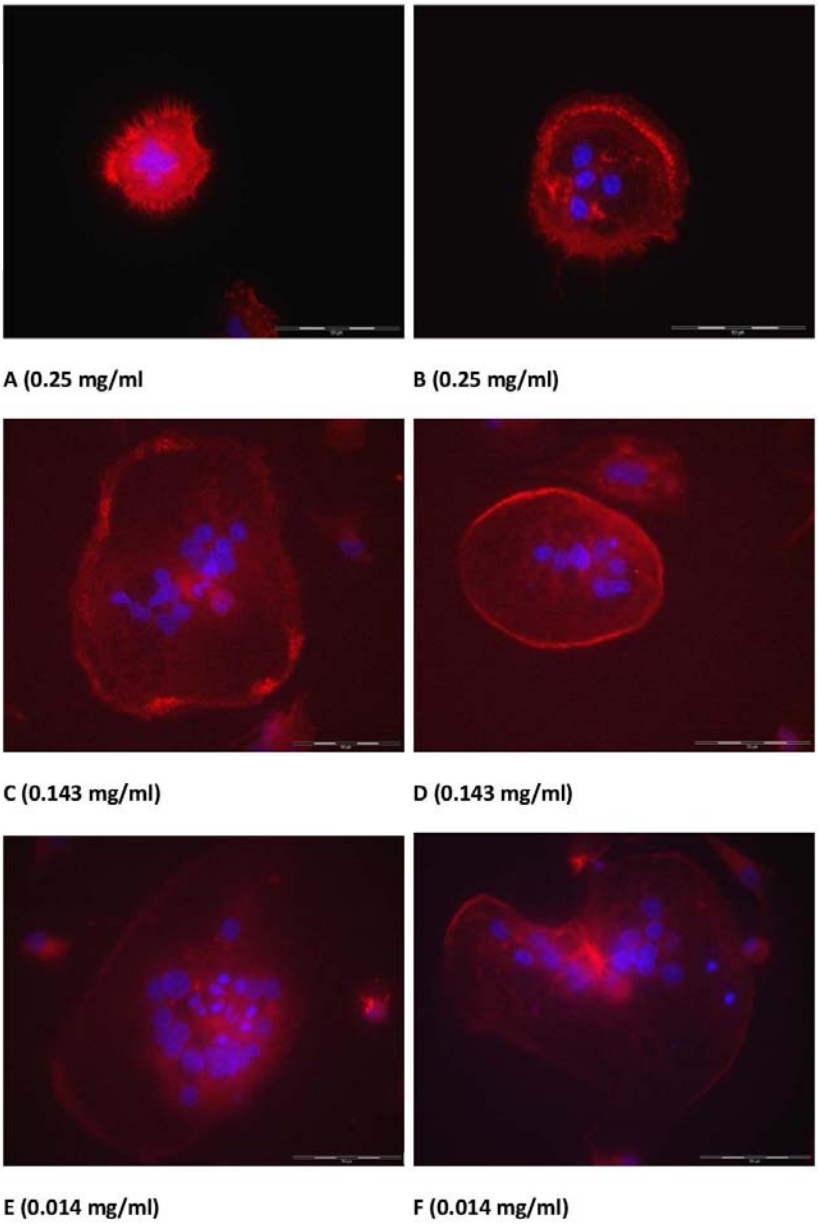


Figure 3.18: Stained OCs (PE 2) under the fluorescence microscope.

A, B – PE 2 (0.25 mg/ml)

C, D – PE 2 (0.143 mg/ml)

E, F – PE 2 (0.014 mg/ml)

A and B show small OCs with irregular cell periphery and partly diffuse cytoplasm. C and D show larger OCs and actin ring structures but also several vacuoles. E and F show even larger OCs with smooth periphery, actin ring structures and without vacuoles.

3.3.2 Extr. Cichorie aquos. sicc. (PE 6)

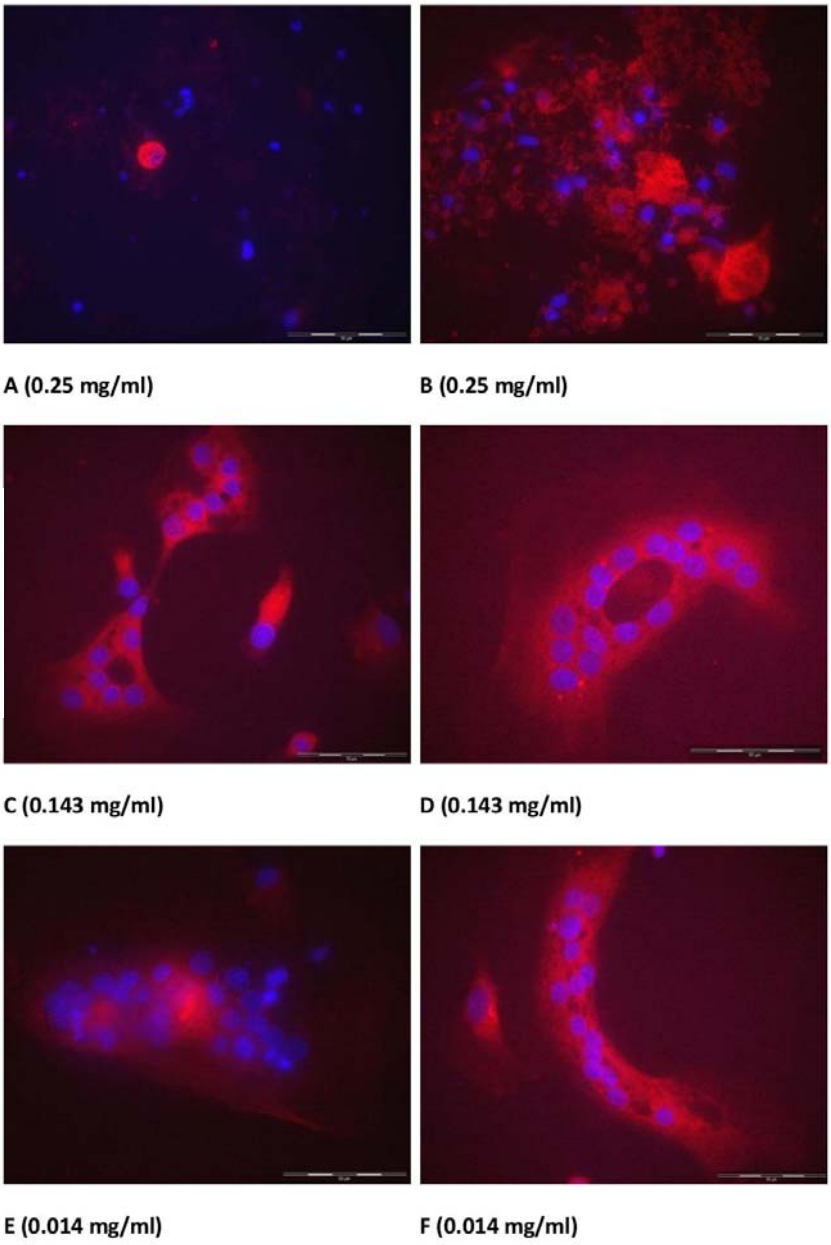


Figure 3.19: Stained OCs (PE 6) under the fluorescence microscope.

A, B – PE 6 (0.25 mg/ml)

C, D – PE 6 (0.143 mg/ml)

E, F – PE 6 (0.014 mg/ml)

A and B show completely disrupted cytoplasm and nuclei fragments. C and D show constricted OCs with very diffuse cytoplasm and vacuoles. E shows a large round OC with actin ring structures and without vacuoles. F looks similar to C and D but shows smaller vacuoles.

3.3.3 Extr. Cichorie e rad. spir. sicc. (PE 7)

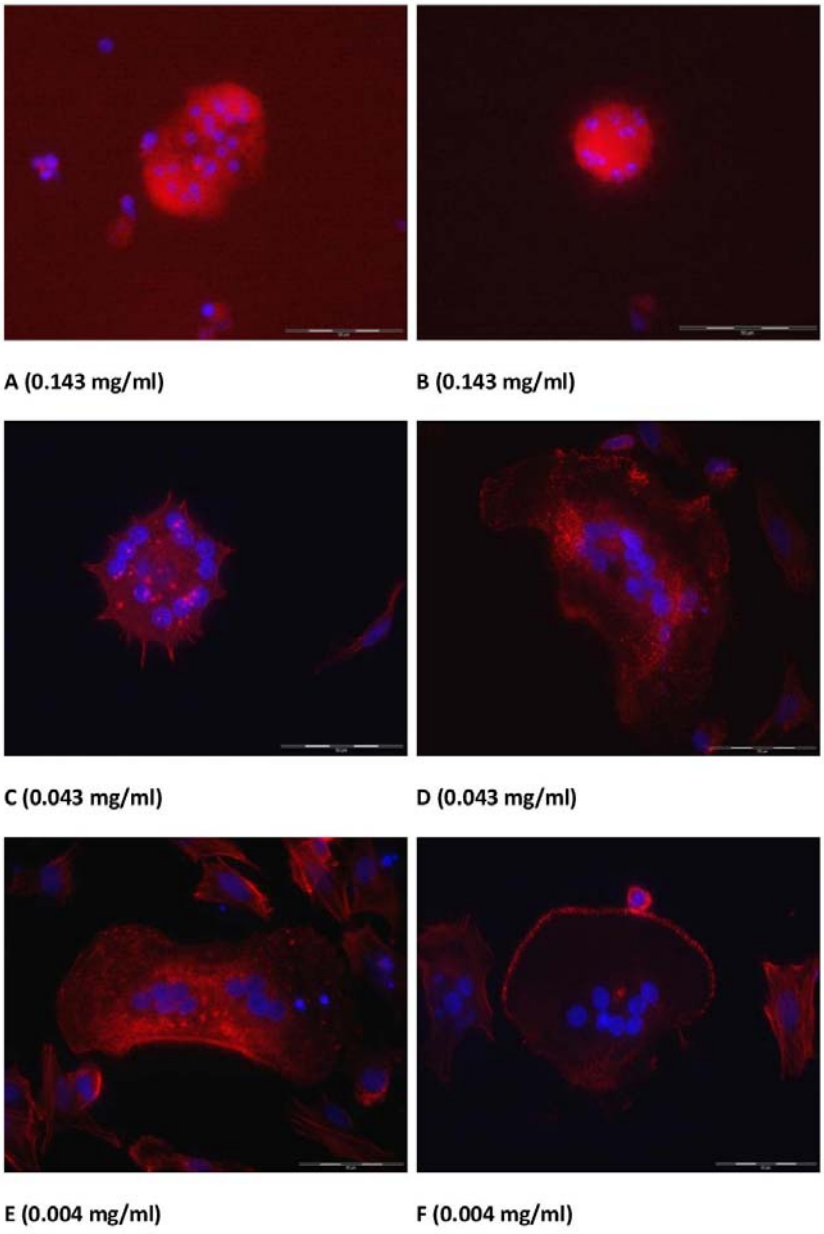


Figure 3.20: Stained OCs (PE 7) under the fluorescence microscope.

A, B – PE 7 (0.143 mg/ml)

C, D – PE 7 (0.043 mg/ml)

E, F – PE 7 (0.004 mg/ml)

A and B show very small, round, partly disrupted cells with apoptotic nuclei. C and D show partly constricted OCs with F-actin distributed through the cytoplasm, irregular cell periphery and some vacuoles. E and F show large, round OCs with actin ring structures and without vacuoles.

3.3.4 Extr. Wassabiae e herb. aquos. sicc. (PE 10)

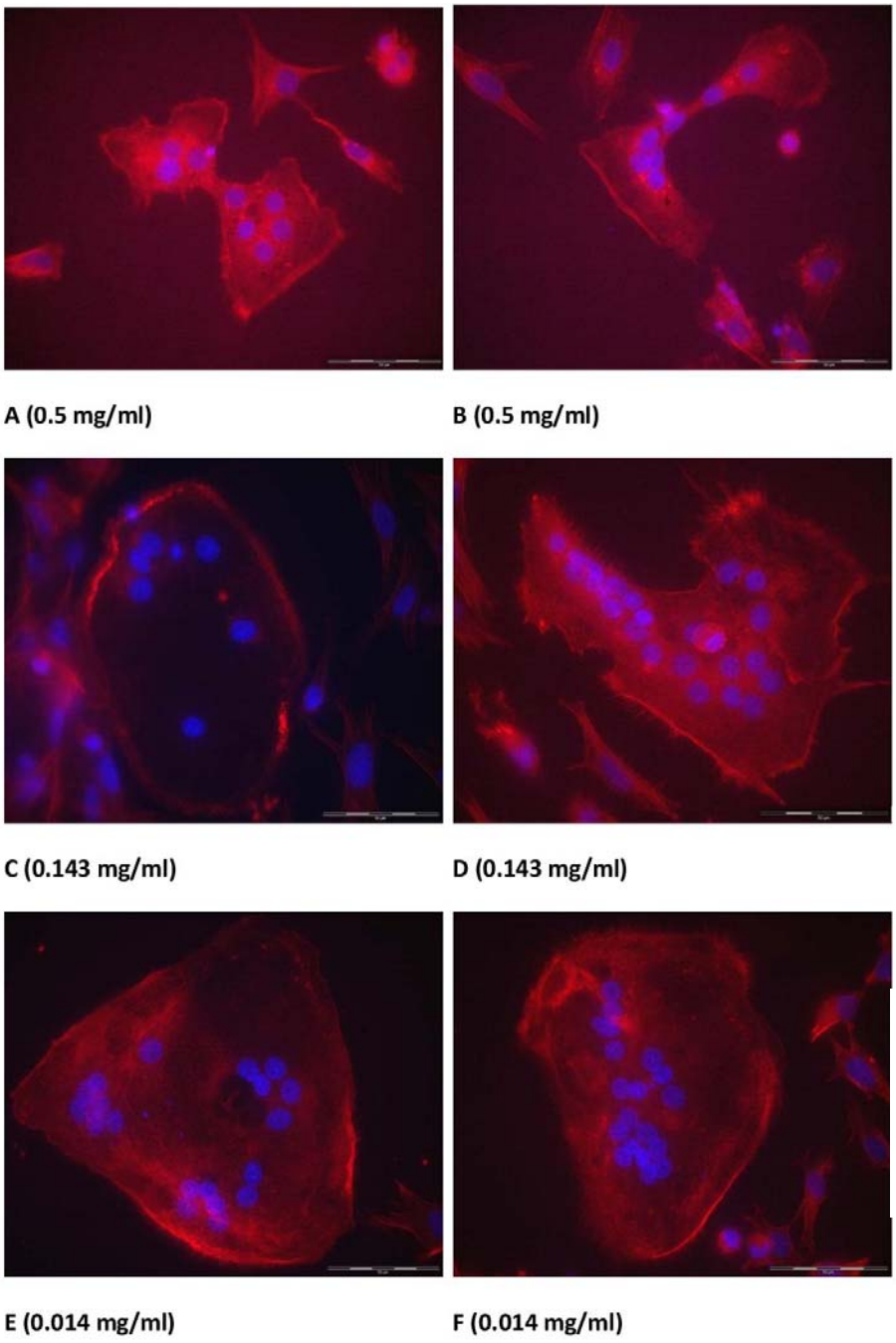


Figure 3.21: Stained OCs (PE 10) under the fluorescence microscope.

A, B – PE 10 (0.5 mg/ml)

C, D – PE 10 (0.143 mg/ml)

E, F – PE 10 (0.014 mg/ml)

A and B show small, constricted OCs with actin ring structures. C, D, E and F show large, round OCs with actin rings and smooth periphery.

3.3.5 Extr. Humuli lupuli sicc. (PE 16)

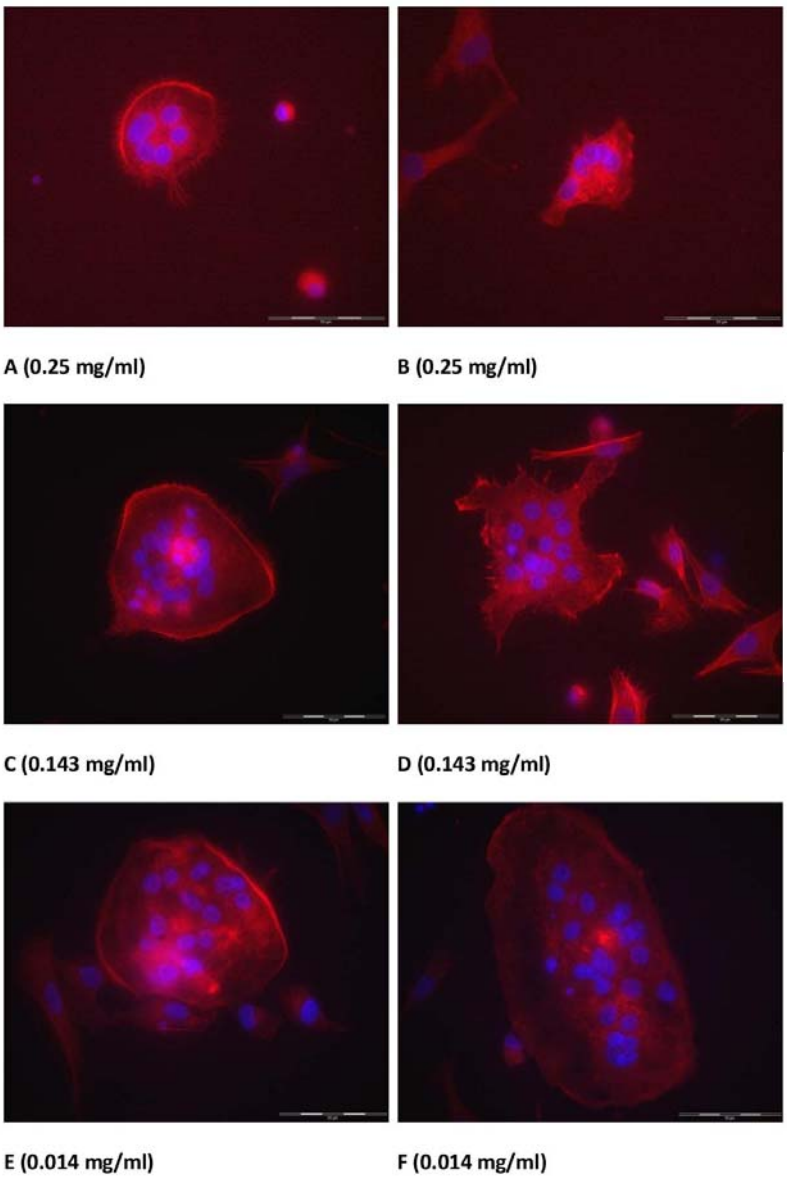


Figure 3.22: Stained OCs (PE 16) under the fluorescence microscope.

A, B – PE 16 (0.25 mg/ml)

C, D – PE 16 (0.143 mg/ml)

E, F – PE 16 (0.014 mg/ml)

A shows a small, round OC with actin ring structure but irregular periphery. B shows a small OC with diffuse cytoplasm and irregular periphery. C shows a larger, round OC with actin ring and smooth periphery. D shows a constricted OC with irregular cell periphery. E and F show large, round OCs with actin ring structures and smooth periphery.

4. Discussion

This thesis describes the effect of different plant extracts on osteoclast activity and bone resorption in vitro.

PE 2, derived from Leuzeae (Asteraceae), significantly inhibited the formation of pits and therefore bone resorption in a dose-dependent manner (figure 3.1). The number of TRAP-positive cells was also significantly decreased in a dose-dependent manner (figure 3.7). The inhibition of bone resorption of the treatments with the two highest concentrations was comparable with the effect of pamidronate (figure 3.1). The images obtained of the TRAP-positive cells show small cells with many vacuoles and difficultly detectable nuclei in the treatments with a high concentration of plant extract. With lower concentrations of plant extract, the cells became increasingly regular, the nuclei were easier to detect and the number of vacuoles decreased (figure 3.8). The same changes in appearance could be observed in the images with fluorescent staining. The cells in the treatments with a high concentration of plant extract showed an irregular cell periphery and a diffuse cytoplasm. The lower the concentration of plant extract, the larger and more regular the osteoclasts became and an increasing number of actin ring structures could be observed (figure 3.18).

The results of the resorption assay (figure 3.1) and the number of TRAP-positive cells with three or more nuclei (figure 3.7), strongly correlate. Also the images of the TRAP staining (figure 3.8) and the images with fluorescent staining (figure 3.18) show a similar picture. The images with fluorescent staining indicate that PE 2 completely or partly disrupts the osteoclast actin ring and thus inhibits bone resorption. In addition PE 2 decreases the overall number of osteoclasts.

PE 6, derived from Cichorie (Asteraceae), significantly inhibited the formation of pits and therefore bone resorption in a dose-dependent manner (figure 3.2). In addition the number of TRAP-positive cells was significantly decreased in a dose-dependent manner (figure 3.9). The images obtained of the TRAP-positive cells show small cells with many vacu-

oles and difficultly detectable nuclei treatments with a high concentration of plant extract. With a lower concentration of plant extract, the cells became larger and more regular. The nuclei were also easier to detect and the number of vacuoles decreased (figure 3.10). The change in appearance was more apparent in the images of cells with fluorescent staining. The cells in the treatments with a high concentration of plant extract were completely disrupted, only cytoplasm and nuclei fragments could be found. The images of cells with fluorescent staining of lower concentrations of PE 6, showed osteoclasts, but they were constricted and showed a very diffuse cytoplasm and vacuoles. Images of the treatment with the lowest concentration of plant extract showed partly regular, round osteoclasts with actin ring structures and without vacuoles (figure 3.19).

Comparing the dose-dependent inhibitory effect observed in the resorption assay (figure 3.2) and the dose-dependent decrease in the number of TRAP-positive cells with three or more nuclei (figure 3.9), PE 6 shows a strong and very similar effect in both analyses. Considering the images of the TRAP staining (figure 3.10) and the images with fluorescent staining (figure 3.19), it appears that PE 6 in high concentrations (0.5 and 0.25 mg/ml) and in lower concentration (0.143 mg/ml) disrupts the osteoclasts completely and partially, respectively. This was indicated as only cytoplasm and nuclei fragments could be found in the images with fluorescent staining. Hence bone resorption is inhibited and the number of osteoclasts decreased by PE 6.

PE 7, derived from Cichorie (Asteraceae), significantly inhibited the formation of pits and therefore bone resorption, especially in the treatment with the highest concentration of plant extract, where almost no pits could be found. Thus, the inhibition caused by PE 7 was even stronger than the inhibition caused by pamidronate (figure 3.3). The number of TRAP-positive cells was also significantly decreased, especially in the treatment with the highest concentration where almost no cells could be found (figure 3.11). The images obtained of the TRAP-positive cells showed very small cells and almost no cells with detectable nuclei in the treatment with the highest concentration. A treatment with lower concentration showed larger cells, but they were full of vacuoles and the nuclei were still hard to detect. The lower the concentration of plant extract, the larger the osteoclasts became. In addition the images showed cells without vacuoles and clearly visible nuclei (figure 3.12). The change of appearance could also be clearly observed in the images with

fluorescent staining. The cells of the treatment with a high concentration of plant extract were very small, partly disrupted and showed many apoptotic nuclei. The images with fluorescent staining of lower concentrated treatments showed partly constricted osteoclasts with F-actin distributed through the whole cytoplasm and some vacuoles. The treatment with the lowest concentration showed large, round osteoclasts with actin ring structures and without vacuoles (figure 3.20).

When the very strong inhibitory effect of the treatment with the highest concentrated plant extract in the resorption assay (figure 3.3) is compared with its effect on the number of TRAP-positive cells (figure 3.11), it appears that PE 7 in a concentration of 0.143 mg/ml completely disrupts the osteoclasts, as well as other cell types which were present in the cell culture. This is confirmed with the images of the TRAP staining (figure 3.12) and the images with fluorescent staining (figure 3.20), where very small cells, partly disrupted, with apoptotic nuclei could be observed. It can be assumed that PE 7, in high concentrations (0.143 and 0.043 mg/ml) significantly inhibits bone resorption and decreases the number of osteoclasts through inducing osteoclast apoptosis.

PE 6 and PE 7 are both derived from Cichorie. PE 7 was derived from Cichorie root; the origin of PE 6 was not further indicated. A known difference between the two extracts was the solvent. PE 6 was dissolved in water and PE 7 in 70 % ethanol. Thus the investigated concentrations were different. Despite that, both extracts showed a strong inhibitory effect on bone resorption and a severe disrupting effect on osteoclasts in the higher concentrated treatments.

PE 10, derived from Wassabiae (Brassicaceae), did neither inhibit the formation of pits, respectively bone resorption (figure 3.4), nor was the number of TRAP-positive cells decreased (figure 3.13). The images obtained of the TRAP-positive cells showed large, round cells without vacuoles and with nuclei that were easy to detect in all concentrations of plant extracts. All cells appeared similar to those of the control group (figure 3.14). The appearance in the images with fluorescent staining was slightly different to the appearance of the cells in the TRAP images. The cells of the treatment with a high concentration of plant extract were small and slightly constricted but showed actin ring struc-

tures. The cells of treatments with lower concentrations showed large, round osteoclasts with actin rings. Though the cells were smaller in the images with fluorescent staining of the treatment with the highest concentration, this did not seem to inhibit the formation of pits, respectively bone resorption (figure 3.21).

Based on these results, it is believed that PE 10 does not inhibit bone resorption or decrease the number of osteoclasts. Suzuki et al. (1997) showed that wasabi leafstalk extract has an anabolic effect on bone calcification in mouse calvaria tissue culture in vitro. Yamaguchi et al. (2003) found that wasabi leafstalk extract from a homogenate with 20 % ethanol, orally administered in rats, caused a significant increase in alkaline phosphatase activity as well as calcium content in bone tissues, and thus determined its anabolic effect on bone components in vivo. Furthermore, Yamaguchi (2006) mentions that wasabi leafstalk shows an inhibitory effect on PTH-induced osteoclast-like cell formation in a mouse marrow culture system and suggests that this component is inhibiting bone resorption (unpublished data). This cannot be confirmed with the results of the experiments with PE 10 of this thesis. PE 10 was dissolved in water, whereas Yamaguchi (2006) mentions a homogenate with 20 % ethanol. More information about Yamaguchis unpublished study would be needed to compare the results.

PE 16, derived from Humuli lupuli (Cannabaceae), significantly inhibited the formation of pits and therefore bone resorption in a dose dependant manner (figure 3.5). The number of TRAP-positive cells was also significantly decreased in the treatment with the highest concentrated plant extract (figure 3.15). The treatments with the two highest concentrations showed an even stronger inhibition of bone resorption than caused by pamidronate (figure 3.5). The images obtained of the TRAP-positive cells showed small cells with difficultly detectable nuclei and many vacuoles in the treatment with the highest concentration. A treatment with lower concentration of plant extract showed slightly larger cells with nuclei better to detect, but still with many vacuoles. The treatments with lower concentrations showed large, round cells with nuclei clearly visible and without vacuoles (figure 3.16). The images with fluorescent staining showed a heterogeneous picture. The cells of the treatments with a high concentration of plant extract were either small and with irregular periphery but round and with actin ring structure, or small, constricted, with diffuse cytoplasm and irregular periphery. The images of a treatment with

lower concentration showed either large, round osteoclasts with actin rings and smooth periphery, or constricted cells with irregular periphery. The treatment with the lowest concentration showed homogenously large, round osteoclasts with actin ring structures and smooth periphery (figure 3.22).

Comparing these results, especially the dose-dependent inhibitory effect observed in the resorption assay (figure 3.5) and the effect on the number of TRAP-positive cells (figure 3.15), it appears that the resorbing osteoclasts could not be distinguished from the non-resorbing osteoclasts in the counting of the TRAP-positive cells with three or more nuclei. This can be explained with the images with fluorescent staining, as some images of the treatments with high concentration of plant extract showed regular osteoclasts with actin rings, while others showed constricted osteoclasts with diffuse cytoplasm and without actin ring structures (figure 3.22) which cannot be distinguished with the TRAP staining (figure 3.16). This suggests that PE 16 induces a disruption of the actin ring of the osteoclasts and thus inhibiting their bone resorbing activity.

This thesis and the performed experiments were based on a screening of twenty plant extracts; as such the experiments are preliminary results and should be repeated before significant statements can be made. In addition the effect of the different plant extracts on osteoblasts and the investigation of the effects in vivo should be part of further work.

5. Conclusion

The aim of this study was to investigate the effect of different plant extracts on the osteoclast activity, particularly bone resorption and the morphology of osteoclasts in vitro. This work was specifically focused on finding possible new therapeutic approaches for the treatment of bone diseases with bone loss, primarily osteoporosis. A pharmaceutical deriving from plant extracts may have the potential to provide treatment with less severe side effects than common pharmaceuticals, like for instance bisphosphonates, which are currently in use to treat bone disorders like osteoporosis.

PE 2 (Leuzea) was found out to inhibit bone resorption and to decrease the number of osteoclasts in a dose-dependent manner through completely or partly disrupting the actin ring of the osteoclasts. PE 6 was also found to inhibit bone resorption and to decrease the number of osteoclasts in a dose-dependent manner. The effect of PE 6 was due to complete disruption, leaving only cytoplasm and nuclei fragments, of the osteoclasts in treatments with high concentrations. PE 7, derived from the same plant as PE 6 – Cichorie, showed similarly to PE 6, a strong inhibitory effect on bone resorption, a severe decrease in the number of osteoclasts and a heavy disruption of osteoclasts in treatments with high concentrations. PE 7 caused disruption of cell membrane, a smaller cell size and induced osteoclast apoptosis. PE 10 (Wassabiae) did not show any inhibitory effect on bone resorption or a decreasing effect on the number of osteoclasts. PE 16 (Humuli lupuli) was found to inhibit bone resorption in a dose-dependent manner and to decrease the number of osteoclasts through disrupting the actin ring of the osteoclasts.

Because in this work only a screening was performed, further studies should include repeating the experiments and studies in vivo, before any of these substances can be considered as a pharmaceutical product used for treating bone disorders like osteoporosis.

6. Abstract

Bone remodelling is continuously performed by osteoblasts, which synthesize new bone matrix, as well as osteoclasts, which are responsible for the resorption of old bone. Any imbalance between these two types of cells can lead to bone disorders, primarily osteoporosis (bone loss). Osteoporosis is currently a major public health concern.

Plant extracts may provide a new therapeutical approach. Results of data analysis on pharmacological, chemical and toxicological characteristics of the different species indicate therapeutic properties and a high potential for use as effective herbal remedies. There is already evidence that one of the substances, PE 10, shows an anabolic effect on bone components in vivo (see Yamaguchi, 2006).

In this work I performed an investigation of five different plant extracts and their effect on osteoclast activity, primarily bone resorption in vitro. Furthermore, changes in morphological structure and induction of apoptosis were considered.

In the experiments, I used five different concentrations of each plant extract. The concentrations were calculated depending on their solvent, either water or ethanol at different concentrations.

PE 2, PE 6, PE 7 and PE 16 showed a significant inhibition of bone resorption and also significantly decreased the number of osteoclasts. PE 10 did not show an inhibitory effect on bone resorption or a decrease in the number of osteoclasts. The images with fluorescent staining of PE 2 and PE 16 showed that the inhibitory effect of the two plant extracts was caused by disrupting the actin ring of the osteoclasts, which is a marker of actively resorbing osteoclasts. PE 6 and PE 7 completely disrupted the whole osteoclasts at high concentrations. Additionally in the images with fluorescent staining of PE 7 many apoptotic nuclei could be found.

7. Zusammenfassung

Knochenumbau findet ständig im menschlichen Körper statt und wird von Osteoblasten, die neue Knochensubstanz aufbauen, und von Osteoklasten, die alte Knochensubstanz abbauen, durchgeführt. Ein Ungleichgewicht zwischen diesen beiden Zellarten kann zu Knochenkrankheiten, hauptsächlich Osteoporose (Knochenschwund), führen. Osteoporose ist heutzutage zu einem der weltweit größten Gesundheitsprobleme geworden.

Pflanzliche Extrakte könnten ein neuer Therapieansatz für Osteoporose sein. Die Ergebnisse von Datenanalysen der pharmakologischen, chemischen und toxikologischen Eigenschaften der verschiedenen Pflanzenarten zeigten ein hohes Potenzial für deren therapeutischen Einsatz. In der Literatur gibt es bereits Hinweise darauf, dass einer der Extrakte, PE 10, eine anabole Wirkung auf die Knochenbildung in vivo zeigt (siehe Yamaguchi, 2006).

In dieser Arbeit habe ich fünf verschiedene Pflanzenextrakte und ihre Wirkung auf die Osteoklastenaktivität, bzw. die Knochenresorption in vitro, untersucht. Zusätzlich wurden Veränderungen der morphologischen Struktur und der Einfluss auf die Apoptose berücksichtigt.

PE 2, PE 6, PE 7 und PE 16 zeigten eine signifikante Hemmung der Knochenresorption und verringerten ebenso die Anzahl der Osteoklasten signifikant. PE 10 zeigte weder eine hemmende Wirkung auf die Knochenresorption noch verringerte er die Anzahl der Osteoklasten. Bilder die mittels Fluoreszenzmikroskopie dargestellt wurden, zeigten, dass die inhibitorische Wirkung von PE 2 und PE 16 durch Schädigung des Aktinringes der Osteoklasten, ein Marker für aktive Osteoklasten, verursacht wurde. PE 6 sowie PE 7 wirkten in hohen Konzentrationen zytotoxisch auf Osteoklasten. Zusätzlich konnten auf den mittels Fluoreszenzmikroskopie dargestellten Bildern von PE 7 viele apoptotische Zellkernen identifiziert werden.

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Curriculum Vitae

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EDUCATION AND TRAINING

Since Oct. 2003	Studying Nutritional Sciences at the University of Vienna
Aug. 2007 – May 2008	Exchange student at the University of Kuopio, Finland, in the master program "Public Health Nutrition" at the Faculty of Medicine, Department of Public Health and Clinical Nutrition
Sep. 1995 – June 2003	High School: BRG Linz Hamerlingstraße, focus on natural sciences, school leaving exam passed with distinction

WORK EXPERIENCE

Since Oct. 2011	Board member for Finances at IAESTE (International Association for the Exchange of Students for Technical Experience), local committee BOKU
Apr. 2009 – July 2011	Marginally employed at P&R Marktservice, Vienna
Oct. 2005 – June 2011	Tutor within the practical course „Histology and Cytology" at the University of Vienna, Vienna
Since Jan. 2011	Vicepresident at IAESTE, local committee BOKU
Aug. 2010 – Oct. 2010	12-week internship at FAGE Dairy Industry S.A., Athens
Oct. 2009 – July 2010	Working on this master thesis at the Department of Pharmacology and Toxicology, University of Vienna, Vienna
June 2009	2-week internship within the „nutritionDay" at the general hospital of Vienna, Vienna
June 2008 – July 2008	8-week internship at a dietician's practice, Neufeld
July 2006	4-week internship at the „Brothers of Mercy" hospital within the nutrition team, Department of Internal Medicine, Linz
Aug. 2002	4-week internship at backaldrin corporation, Asten