

Rooibos tea flavonoids increase mineral content in human osteoblast-like cells

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Abstract

Some cross-sectional and prospective studies have demonstrated a positive correlation between habitual tea consumption and bone mineral density in post-menopausal women. Rooibos tea contains no caffeine and is a rich source of flavonoids such as rutin, orientin, hyperoside and luteolin. These flavonoids have similar structures to estradiol, and therefore may act as estrogen mimics to promote favourable outcomes in bone. The overall objective of this research was to identify flavonoids that could enhance mineral content in human osteoblast Saos2 cells. Mineral was quantified by alizarin red staining and characterized by quantifying alkaline phosphatase (ALP) activity, cell mitochondria activity and toxicity, in addition to changes in regulatory markers of osteoblastic activity. Rutin ($\geq 50\mu\text{M}$), hyperoside ($\geq 5.0\mu\text{M}$), orientin ($0.1\mu\text{M}$ - $1.0\mu\text{M}$, $15\mu\text{M}$ - $100\mu\text{M}$) and luteolin ($5.0\mu\text{M}$) enhanced mineral content. This was in part due to elevated ALP and mitochondrial activity, and lower toxicity, pro-inflammatory cytokines, and Wnt inhibitors.

Keywords: alkaline phosphatase, flavonoids, mineral, osteoblast, rooibos tea

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List of Symbols and Abbreviations

ALP – alkaline phosphatase

FRAX - Fracture risk assessment tool

BMD – bone mineral density

SERMs - Selective estrogen modulators

RANK – receptor activator of nuclear factor kappa-B

RANKL - receptor activator of nuclear factor kappa-B

NF κ B - nuclear factor kappa-B

OPN – osteopontin

M-CFS - macrophage colony stimulating factor (M-CFS)

HCL – hydrochloric acid

OPG – osteoprotegerin

EGCG - epigallocatechin gallate

AP1 – activator protein 1

RUNX – runt related transcription factor

siRNA - small interfering RNA

MTT – (2-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

DMSO – dimethyl sulfoxide

LDH – lactate dehydrogenase

SOST - sclerostin

PTH - parathyroid hormone (PTH)

IL6 - interleukin 6

TNF α - tumor necrosis factor alpha

Wnt – Wingless-related integration signalling

Chapter 1.0 - Introduction

The population of the developed world is aging. With an aging population, strategies for preventing chronic disease are essential for preserving quality of life and reducing health care costs. The aging “baby-boomer” generation in North America currently has an average age of 65 years, and thus is at high risk of developing chronic diseases. Osteoporosis is described by an individual having low bone mineral density (BMD) and weakened bone structure, thereby making the individual’s skeleton more prone to fragility fractures [1]. According to the International Osteoporosis Foundation, 1 in 5 men and 1 in 3 women will experience a fragility fracture within their lifetime [2]. Moreover, fragility fractures worldwide are frequent, occurring every 3 seconds [3]. Fragility fractures are more commonly seen in areas of impact such as the wrist, spine and hip. Fragility fractures often result in disfigurement, impaired mobility and loss of independence, that together result in an overall diminished quality of life [1]. Moreover, mortality rates are positively associated with all types of osteoporotic fractures, with the highest risk among men and women age 60-69 years [4,5]. Within a year of the first hip fracture, likelihood of death increases by approximately 30% for women and 40% for men [1]. The economic costs of treating fragility fractures and rehabilitation are substantive and a significant burden to the economy. Visits to the hospital due to osteoporotic fractures, occur more often than stroke, heart attack, and breast cancer combined [1]. Approximately 20 billion dollars a year are spent on osteoporotic related injuries within Canada [1] and the United States [6], and is expected to increase with the

aging population. Thus, there is a need to develop strategies that prevent degradation of bone tissue during aging, thereby preventing debilitating fractures.

Until recently, measurements of BMD at the spine and/or hip using dual energy x-ray absorptiometry were solely utilized to assess the risk of fragility fracture for an individual [1]. This measurement of BMD is described in terms of a T-score, in which the T score refers to how many standard deviations the BMD of the patient is from the BMD of the average, healthy individual. A score of -1.0 and higher is considered healthy; where -1.0 to -2.5 standard deviations away describes osteopenia and -2.5 or lower indicates osteoporosis [1]. All of the studies referenced in this thesis that measured risk of fragility fracture in humans (without measuring incidence of fracture directly) used BMD measures. However, it is important to note that clinicians are being encouraged to use the Fracture Risk Assessment tool (FRAX) to more accurately predict risk for osteoporotic fracture [7]. This assessment includes BMD as well as numerous risk factors including age, body mass index, medication history (i.e. previous fragility fractures, family history) and alcohol consumption [7]. Using these risk factors, FRAX provides an estimate of the 10-year risk of an osteoporotic fracture. There are many different pharmacological agents that are used to attenuate loss of bone mass. These include bisphosphonates, a monoclonal antibody that binds RANKL, and selective estrogen modulators. As is common for most drugs, there can be adverse effects. This provides a basis for considering potential dietary strategies that may provide a safe approach to maintain bone health or delay the age at which a pharmacological agent is warranted to prevent a fragility fracture [7,8].

1.1 Overview of Drug Treatment Strategies for Osteoporosis

Patients that are at low or moderate risk of fragility fracture are placed on a supplement regime of vitamin D, and calcium, while also being encouraged to start a regular exercise cycle [7]. There are a variety of drug treatments offered to assist with slowing bone loss in individuals who are at high risk for an osteoporotic fracture. As with most drug interventions, potential adverse effects can occur [7]. Bisphosphonates such as Fosamax and Aclasta are used to prevent bone resorption through inhibition of osteoclast activity. Bisphosphonates are a common treatment for osteoporosis treatment as they are easily accessible, and can be given either orally or intravenously. Oral medication is given for individuals at high risk but otherwise healthy, where intravenous injections are used to stabilize osteoporotic patients. Bisphosphonates have the ability to bind to osteoclasts and prevent their catabolic action. This allows for osteoblasts to achieve a higher level of efficiency, bringing bone turnover back to a more steady state, lowering the risk of fracture [9]. Adverse side effects due to bisphosphonates depend on the route of administration. These adverse effects include muscle and bone pain, ulcers, and esophageal cancer. Long-term effects of bisphosphonates are currently not understood and therefore are only recommended for short-term usage.

Selective estrogen modulators (SERMs), such as raloxifene, mimic estrogen through structure recognition. SERMs will bind to specific estrogen receptors (typically either alpha or beta estrogen receptors within a localized area) found within the bone. By binding to these receptors, they exert biological effects, similar to estrogen, and regulate target genes. This selectivity allows for interaction and up-regulation of genes by the

desired receptors while blocking these same effects within other tissues such as those found in the breast and uterus. However, complications such as blood clots, cancer and leg cramps have been associated with the use of SERMs [10].

An alternative option for treatment is hormone therapy, in which estrogen or progesterone can be administered to supplement the loss of estrogen after menopause [10]. The Women's Health Initiative has shown that estrogen supplementation for treatment of osteoporosis can decrease risk (~30%) in overall fracture, but due to health risks outweighing the benefits, the study ended early. It was through the Women's Health Initiative that the use of the steroidal hormone, estrogen, was discovered to increase a woman's risk for cardiovascular disease and stroke, in addition to a variety of cancers in the breast and endometrium tissues [11].

Additional hormone supplements include calcitonin and parathyroid hormones. Calcitonin is produced in the body naturally and is able to slow down osteoclasts, thereby increasing productivity of osteoblasts, and ultimately slowing bone resorption [12]. Recently, parathyroid hormone has shown success with upsetting the balance of osteoclasts to osteoblasts. This hormone works by increasing the rate of bone formation relative to destruction, thereby increasing BMD in areas across the body, resulting in a lower risk of fracture; unfortunately it is not covered in drug plans [13]. These hormone therapies are an effective way to slow down bone loss, preventing spine and hip fractures, but come with many risks such as cancer, blood clotting, elevated blood-calcium levels and kidney stones [1].

A newer pharmacological intervention is Prolia, an antibody that has been engineered to slow osteoclast activity by preventing the interaction of the RANK-RANKL bond. As the RANK-RANKL bond is required for osteoclast maturation, its inhibition allows for depletion in osteoclast activity. Although new, the human monoclonal antibody is currently being pushed as a treatment to allow for ‘drug vacations’ from osteoporosis medications in which long term effects have not been studied, such as bisphosphonates [12,14,15].

With all of the above treatments, there is currently no ideal strategy to effectively target osteoporosis, or prevent it. Therefore it is desirable to seek a natural, efficient means for deterring bone loss in which side effects are few or none. This leads scientists to seek potential therapeutics in nature that may prove to be proficient in their ability to treat or prevent osteoporosis, but also abundant and potentially less expensive, making it a more accessible treatment.

1.2 Bone Metabolism

While osteoporosis is recognized to be a disease that comes with aging, bone health is a life-long process. Throughout the life cycle, bones are consistently remodeling and are influenced by a variety of factors including genetics, nutrition and physical activity [1]. From infancy, our bones grow and reshape until adulthood when an imbalance of bone production and breakdown erupts due to a loss of estrogen during menopause [16]. Although estrogen is commonly recognized for its ability to provide women with secondary sex characteristics such as breasts, males also produce estrogen. Males develop estrogen through an enzymatic process in which testosterone is synthesized into estrogen through an enzyme known as p450 aromatase. This enzyme is

distributed across the body, but exists largely in the brain and in adipose tissue [17]. Men also experience a drop in testosterone levels as they age, but it is not as dramatic as females whom experience menopause [17,18].

Bone is a multifaceted tissue, largely made of mineral and matrix, while also containing smaller amounts of water and lipids [19]. Bone mineral, specifically hydroxyapatite is a semi-soluble complex composed of mainly calcium and phosphate [19]. The ability to solubilize hydroxyapatite via osteoclast action is appropriate to allow for bone turnover to maintain strong bones. To produce mineral, concentrations of phosphate and calcium will accumulate inside of vesicles within the bone matrix [19]. These extracellular vesicles provide the suitable environment for micronutrients (i.e. magnesium, and selenium) to ensure crystallization takes place. As phosphate and calcium begin to crystalize, the newly formed mineral will deposit in holes between the matrix, acting like cement [19]. Mineral gives the bone properties such as strength and hardness, where the matrix allows for the bones to be flexible and elastic. Maturation of matrix and mineral is typically marked by an increase in gene expression for: ALP, osteocalcin and osteopontin (OPN), bone sialoproteins, and non-collagen proteins, all of which are important to maintaining healthy bones [19].

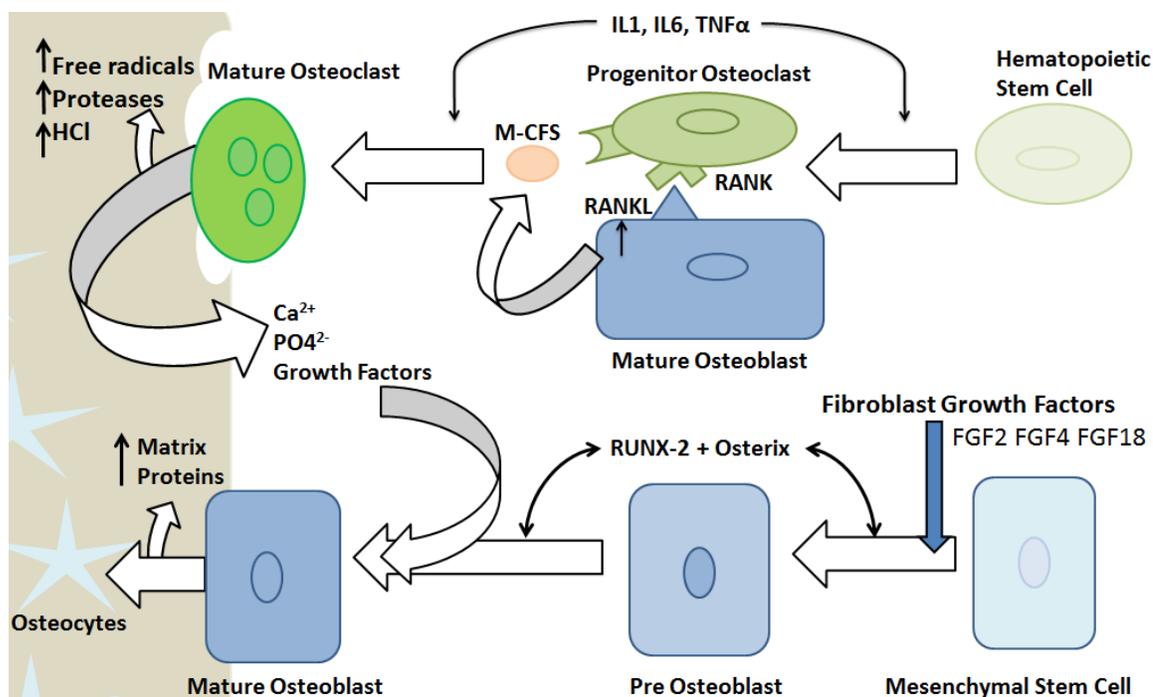


Figure 1.1 Bone Turnover. There are three main cell types which influence bone turnover; osteoblasts, cells that make bone; osteoclasts, cells that break down bone; osteocytes, mature osteoblasts buried in the bone matrix which are used to communicate between osteoclasts and osteoblasts.

Bone turnover, the process of remodeling bone, is acknowledged as the difference in action of bone making cells, osteoblasts, in comparison to the activity of bone breaking cells, osteoclasts (Figure 1.1). Maintaining a healthy rate of bone turnover involves a balance of osteoblast and osteoclast activity [16]. Osteoblasts are derived from mesenchymal stem cells that respond to growth factors and proteins, to stimulate their conversion to pre-osteoblast cells. Osteoblasts are not only important for producing bone, but for also providing the receptor activator of nuclear factor $\kappa\beta$ (NF $\kappa\beta$) ligand (RANKL). RANKL is responsible for binding to the RANK receptor on progenitor osteoclasts [20]. This interaction will promote the differentiation of progenitor osteoclasts

into mature osteoclasts. During this phase, macrophage colony stimulating factor (M-CFS) will be released to signal for osteoclast differentiation and recruitment [20]. Once mature, osteoclasts may bind to the matrix of the bone and release hydrochloric acid (HCl) and proteases that activate bone resorption, a process that can be ongoing for several weeks [21]. Osteoblasts are also capable of releasing a ligand known as osteoprotegerin (OPG) that blocks the RANKL binding site to prevent osteoclast maturation. After osteoblasts have produced mineral, a portion of these will become osteocytes in the bone matrix. Osteocytes serve to communicate between osteoblasts and osteoclasts to instruct bone development [22].

One of the main components of hydroxyapatite is calcium. Thus, ensuring an appropriate, regular intake of calcium is very important for obtaining and maintaining healthy bones. Currently, the daily recommended intake for calcium for adults is 1000mg and this further increases to 1200mg for women over the age of 50 [23,24]. There are plenty of food sources that supply the body with calcium such as milk, cheese, yogurt (1 serving of dairy is approximately 300mg of calcium), and chickpeas. In addition, many store bought juices are fortified with calcium. Women often find it difficult to achieve the recommended daily intake of calcium through food and therefore are many women are suggested to take supplements. Calcium found within supplements is poorly absorbed into the body, and is therefore recommended to be taken with vitamin D. Vitamin D will increase absorption of calcium, enhancing calcium's bioavailability [19]. Vitamin D can also stimulate ALP, an enzyme that will produce free phosphate for the manufacture of bone mineral, as well as promote the differentiation of osteoblasts. Vitamin D has also been shown to increase gene expression of osteocalcin and osteonectin bone matrix

proteins, which are important for bone growth, while controlling proliferation and cell death of osteoclasts and cytokines [19]. When vitamin D is ingested, a more useful form, 25-hydroxyvitamin D is synthesized by the liver, and this undergoes further modification in the kidneys to produce a very reactive species, 1,25-dihydroxyvitamin D. 1,25-hydroxyvitamin D has an incredibly short half-life (4-6hrs) making it incapable to be appropriately measured and therefore 25-hydroxyvitamin D (stable for approximately 3 weeks) is quantified to assess the vitamin D status of an individual [25]. Stabilization of vitamin D within the body takes approximately 3 months to occur. Therefore, to ensure an individual is consuming enough calcium and absorbing it into the body, healthy adults are recommended to intake a minimum of 600IU daily [23].

Food components other than calcium and vitamin D, can also be used to positively modulate bone physiology. In particular, flavonoids have been shown to have a positive influence on bone physiology. Flavonoids, in which their structures resemble estradiol, are desirable due to the potential of binding to estrogen receptors to exert biological effects. Furthermore, flavonoids have anti-oxidant potential, which help limit inflammation. Inflammation is known to negatively affect bone turnover by increasing osteoclast differentiation, activity, and decreasing osteoblast activity [26]. High levels of reactive oxygen species have been positively associated with pro-inflammatory cytokines and bone resorption [27]. Therefore, flavonoids offer multiple modes of action to help improve outcomes in bone metabolism.

1.3 Estradiol, Flavonoids and Osteoporosis

Estradiol (Figure 1.2) has been recognized as a key modulator for bone development throughout the lifecycle. Estradiol can limit cellular death of osteoblasts and prevent osteoclast proliferation [28]. *In vitro* experiments have demonstrated the addition of estradiol results in a significant increase in OPG levels [29], type 1 collagen and TGF- β [30], all of which are important for suppressing osteoclast activity and building mineral. Estradiol directly added to human osteoblasts also results in a reduction in pro-inflammatory markers IL1, IL6 and TNF α [31]. Pro-inflammatory markers increase osteoclast activity relative to osteoblast activity. However, when estradiol was added to human osteoblasts (1pM – 10pM), there was no significant increase in osteoblast proliferation or ALP [32]. In contrast, estradiol (10nM) added to Saos2 cells, resulted elevated nodule formation and ALP activity [33]. Therefore estradiol's benefits seem to stem from interacting with multiple pathways to help stimulate mineralization in osteoblasts, while indirectly suppressing the activity of osteoclasts.

When a substrate, such as a hormone binds to an estrogen receptor, the two will form a complex that results in a change in shape. This new complex will give access for binding of response elements using its activator protein 1 (AP1) site. Binding of the estrogen response elements allow for recruitment of proteins that may activate or repress the promoter region to influence gene expression [34]. It has been demonstrated that mutations within genes that express estrogen receptors and enzymes such as aromatase

(allowing for production of estrogen from testosterone) have led to low BMD, and skeletal problems [35,36]. Estrogen is also a key player in the immune system where it can regulate inflammation through a reduction of cytokine levels. Activation of cytokines has been shown to influence and increase levels of osteoclasts [37]. This reduction in cytokine concentration helps limit inflammation, and further reduce the likelihood of osteoclasts upsetting the balance of osteoclasts to osteoblasts. Therefore, this loss of estrogen creates an increased rate of bone loss; creating fracture thresholds that exist earlier in life [38].

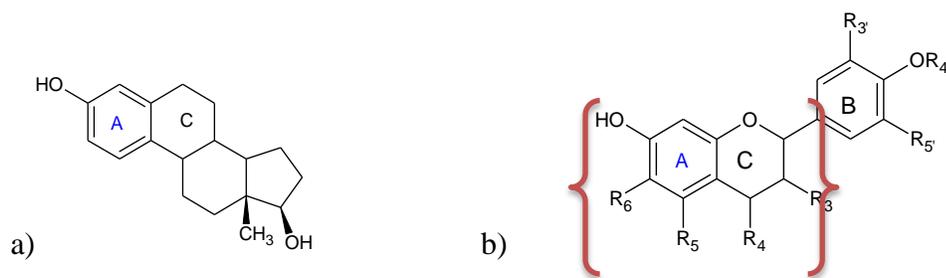


Figure 1.2. Structure of a) estradiol. b) a general backbone of a flavonoid.

Rings A and C of estrogen and the general backbone of a flavonoid are similar. R represents different chemical moieties that can be added to the structure where R' represents additional functional groups within subclasses

Flavonoids such as epigallocatechin gallate (EGCG) from green tea have been shown to increase mineral deposition by increasing the activity of ALP [39]. Lycopene, a flavonoid found in a variety of fruits such as tomatoes is recognized to be a powerful antioxidant and has shown the ability to increase osteoblast activity and mineral deposition at micromolar concentrations [40]. Naringin, a flavonoid found in high concentrations in oranges was shown to increase tibia bone strength and BMD when

ovariectomized rats consumed the flavonoid over a period of 6 weeks [41]. Rooibos tea is a rich source of flavonoids that may have effects on bone health. Therefore, this thesis will specifically study four flavonoids present in rooibos tea: rutin, orientin, luteolin, and hyperoside to test their ability to modulate mineral deposition in bone cells.

Tea, as well as many fruits and vegetables contain polycyclic systems known as flavonoids. These flavonoids represent a class of chemical compounds with multiple rings that are responsible for adding taste, smell and color to food and beverages. These compounds can be further broken down into classes such as: flavones, flavonols, flavanones, isoflavones, catechins, anthocyanidins and chalcones, which are all dependent on the functional groups that surround their ring systems [42]. Flavonoids have become increasingly important because of their structural similarity to hormones such as estrogen. For example, part of the structure of estradiol is seen within the flavonoid rings A and C, and these are important for maintaining the structural properties that allow for ligand recognition by the estrogen receptors. (Figure 1.2). Similarities such as these allow flavonoids to act as substrates and produce interactions with hormone receptors that may result in a biological response [42].

1.4 Tea

Only next to water, tea is considered the beverage of choice across the world. Tea represents a world-wide multi-billion dollar industry. Consumption of tea has increased more than 10% over the past 10 years and continues to rise [43]. In 2012, there were over 79 billion servings of tea consumed in the United States, resulting in sales of over \$2.25 billion dollars [43]. Tea, being made with numerous types of natural fruits and plants, is a rich source of flavonoids that are recognized for having antioxidant, antibacterial, and

antifungal activity [44-54]. Moreover, many, but not all, population studies are beginning to suggest that regular tea intake is linked to individuals with higher BMD (Table 1.1) than non-consumers. Also, there are a few studies that have also identified individuals with regular tea consumption had a reduced rate of fragility fracture than non-consumers (Table 1.2).

1.4.1 Cross-Sectional and Prospective Studies: Tea and BMD

Cross sectional and prospective studies have provided substantial data suggesting that tea is able to impact bone health (Summary in Table 1.1) in a variety of populations. Studies have been conducted in North America, Europe, Australia, and Asia. Postmenopausal women have been most thoroughly studied with a few studies also including men and/or premenopausal women.

1.4.1.1 Cross Sectional Studies

A small study of 62 postmenopausal women in Canada measured BMD at the femur and spine. Analysis found a positive correlation between tea intake and BMD at both measured sites [45]. In England, BMD was measured in over a thousand postmenopausal women at several areas in the spine and femur [46]. Women who consumed tea had approximately 5% greater BMD measurements at the spine, greater trochanter and Ward's triangle. In addition to this, tea consumers were categorized on whether they consumed their tea with milk, as milk provides a good source of calcium, which aids in bone development. When BMD was compared between tea drinkers who use milk to those who did not, no statistical difference in BMD was noted, suggesting the small quantity of milk added to tea does not influence BMD. They also determined that those

who drank 1-3 cups versus more than 4 cups a day did not influence BMD. The differences in BMD (i.e. 5%) translate to a 10-20% lower risk of obtaining a fracture [46]. These results were also reflected in a cross-sectional study completed in Japan in which they investigated lumbar spine BMD in Japanese women over 60 and found that those who consumed green tea had higher BMD and better T-scores (-1.59 ± 2.70 versus -2.17 ± 2.08) than those who did not consume tea [47].

A study in Iran assessed both males and females between the ages of 20-76 and compared the relationship between the BMD of those who consumed tea and coffee and non-consumers. Habitual consumption of tea was considered to be more than 5 cups/day. Urine and plasma were analyzed for bone markers, while BMD was assessed at the spine and hip. Only women whom habitually consumed tea showed a lower frequency of osteoporosis (5.6% versus 12.1%) and osteopenia (24.5% versus 36.5%) as (rates of osteoporosis were determined by the Iranian Multicenter Osteoporosis study) compared to non-consumers. Significance was lost when assessing the male population. This trend was also seen in BMD measurements, in which women who habitually consumed tea in general had higher BMD at the spine, but was not shown to be significant. However BMD at the hip was significantly higher in these women, but again lost when assessing the male population [48]. Similar to the Iranian females, peri-menopausal women from the Danish Osteoporosis Prevention Study discovered a positive correlation existed between the BMD in the femur and the quantity of tea consumed a day [49].

In contrast to the studies above in which a positive correlation existed between women's intake of tea and their BMD, Greek men ages 18-30 in the armed forces had their diets analyzed and compared to their bone mineral content and BMD. Bone mineral

content and BMD were measured at two sites, the distal and ultradistal radius. No relationship was identified between tea consumption and BMC or BMD [50]. Assessing an older population of males ages 45-65 and characterizing them by their body types, no significance was identified between the intake of tea and BMD at the lumbar spine and femur [51]. This suggests that flavonoids in the tea may have an estrogenic effect, producing biological effects similar to that of estrogen, which could play a role in helping women to slow bone loss after menopause.

1.4.1.2 Prospective Studies

A prospective study followed 1027 women for 5 years and investigated their BMD in relation to their intake of tea using a food frequency questionnaire. The results showed that hip BMD was 2.8% higher in those who consumed tea compared to those who did not. Moreover, 164 of these women were followed for an additional 4 years in which bone loss was measured. Those who drank tea experienced an average loss of approximately 1.6% of their total BMD at the hip over the four years, whereas non-tea consumers lost approximately 4.0% [52]. This suggests that tea could provide a natural approach to slowing attenuate bone loss.

A study in Taiwan enrolled both males and females from the prospective survey of chronic disease in Tainan, to evaluate the relationship concerning customary tea consumption and BMD. Individuals who consumed tea once a week for a minimum of 6 months were deemed customary tea consumers. These tea consumers were then categorized by years of consumption. A positive, linear correlation was identified between consumption of tea and BMD for body, lumbar spine, hip neck and Ward

triangle. Individuals who consumed tea for +10 years had the highest levels of BMD amongst the groups. Individuals who consumed tea for longer than 6 years had significantly greater lumbar spine BMD than non-tea consumers. No significance was found between consumers and non-consumers when tea was consumed for less than 5 years, suggesting benefits may only be observed with longer-term consumption [53].

In comparison, a study done on Turkish postmenopausal women ($n = 724$) looked at population characteristics both at baseline and compared them a year later for osteoporosis and fracture. Groups included habitual consumers of tea, defined as +2 cups/day, while anything less classified the individual as a non-consumer. T-scores were determined by measuring BMD at three different phalanges and estimated for whole body. Habitual consumption of tea led to women with better T-scores (-1.51 ± 1.68 versus -1.09 ± 1.66), but this was not statistically significant. Osteoporosis prevalence was also lower in those who consumed tea regularly (29.1% versus 37.8%) but again, this was not statistically significant [54].

Positive outcomes in bone, as the result of habitual consumption of tea, are primarily seen in peri-menopausal women, in which women are beginning to lose (or already have lost) the ability to produce estrogen. Estrogen as mentioned above is important for building and maintaining strong, healthy bones and therefore it is likely that tea contains compounds that can mimic estrogen and influence estrogen receptors in a positive manner. Tea contains a high concentration of flavonoids, and due to the positive association between tea and bone health (see Table 1.1 and 1.2), women ages 45-54 had their BMD analyzed (lumbar spine and femoral neck) and their diet assessed for their flavonoid intake to identify if there was any association between flavonoid intake and

BMD. The diet analysis found that 57% of flavonoid intake was attributed to consumption of tea, in which there was an average intake of 596 ± 504 mL of tea/day. Furthermore, as the concentration of flavonoid increased in their system, so did BMD at the femoral neck and lumbar spine. Urine analysis also demonstrated that free deoxypyridinoline, an indicator of bone resorption was negatively correlated to flavonoid concentration. Overall, a negative correlation existed concerning flavonoid level and level of bone resorption. This recent data suggests that populations who consistently consume tea will have higher levels of flavonoids that may help lower bone turnover and sustain BMD [55].

1.4.1.3 Prospective Studies: Tea and Fracture

While BMD is a good measure of bone health, it does not tell the full story. Measuring the rate of fracture is important for determining how strong bones are, as the strength of bones can be determined by both the mineral content and the architecture. Ideally, fracture would be measured, as it is the fracture that makes osteoporosis most lethal and debilitating.

In the United States, the Women's Health Initiative found a positive connection between tea consumption and BMD at specific skeletal sites. Fractures were reported by participants on an annual basis by questionnaires, and included only confirmed hip fractures by medical records. Other fractures were included but only as self-report. Measurements for BMD included total body BMD, total hip BMD and posterior-anterior spine BMD. Herbal and decaffeinated tea was not included for tea consumption. Unlike the previous study, post-menopausal women were categorized by their habitual

consumption of tea. Individuals who drank ≥ 4 cups/day had greater total body BMD and total body BMD in comparison to the individuals who consumed less than 1 cup/day. Furthermore, a positive trend was identified for how much tea was consumed and BMD. Women consuming between 2-3 cups a day had higher spine BMD in comparison to individuals who drank less than 1 cup. Although drinking tea was positively associated with BMD, no association was found between quantity of tea consumed, as measured by cups, and the rate of fracture [56].

Findings from Women's Health Initiative were supported by findings from a study completed in Sweden. The Swedish population is recognized for their large consumption of caffeine, and black tea holds one of the largest concentrations of caffeine in tea. A longitudinal study examined the risk of bone fracture in women aged 40-76 years over 10 years and how fracture relates to their intake of black tea and coffee. Fractures were identified by personal hospital records and included if they were considered typical areas of osteoporotic fractures such as the femur, hip, spine, distal forearm, pelvis and proximal humerus. Tea was separated into the number of servings consumed and compared against the rate of fractures. Although greater coffee ingestion was linked to higher bone fracture, this significance was not found with an increased intake of caffeinated tea [57].

Unlike the study in Sweden, a cross-sectional study in India which paired 100 individuals (43 male and 57 female) with fractures and compared them to a control group found that tea consumption increased the risk of fracture [58]. However, it is noted that the study included individuals of all ages and therefore may suggest that the benefits of tea may be only seen during midlife. Another study conducted on a Chinese centurion

population in which all participants were over 90 years of age, no relationship was identified between tea intake and osteoporotic fractures in either gender [59].

In contrast to the previous fracture studies, the MEDOS study, a prospective study completed in Europe involving six countries, measured the incidence of hip fracture in both genders over the age of 50 [60]. Tea consumption was indicated as either never, sometimes, 1-2 cups/day or 3+ cups/day. When assessing males, the intake of tea was linked to a reduction in rate of fracture, regardless of dose [60]. Likewise, women were also found to have a lower rate of fracture when they consumed tea [61].

Both cross-sectional studies and prospective studies suggest a trend exists in which habitual consumption of tea can have effects on bone health by increasing BMD in women. Those who were considered regular habitual tea drinkers were shown to have higher BMD that could translate to a lower risk of fragility fracture. Females were the general focus of these studies, and where males were incorporated, tea consumption was generally not positively associated with BMD at any skeletal site. The general lack of effect within male populations may be attributed to the evidence that flavonoids are acting as estrogenic mimics and therefore have little benefit.

Unlike females after menopause, males produce testosterone throughout their lifetime, and therefore still have the ability to produce estrogen late in life (via aromatase) [62]. Therefore, this ability to produce estrogen means that flavonoids would have to compete with estrogen for binding to its receptor as estrogen will bind to estrogen receptors with a higher affinity than flavonoids. Moreover, flavonoids are present at low concentrations within plasma. Furthermore, the majority of cross-sectional and

prospective studies include consumption of many different tea types (black, oolong, green, rooibos) and due to the differing compositions (i.e. flavonoids, caffeine, etc.), certain types of tea may produce different biological effects. However, the study in Taiwan that reported a positive relationship amongst consumption of tea and BMD also analyzed BMD between individuals who consumed green tea or oolong tea *versus* those who consumed black tea. Analysis between these groups found that there was no difference in BMD across tea types [53]. The consumption of tea and how it relates to rate of fracture studies is controversial; as studies across six European countries have identified both men and women who consume tea on a regular basis had a lower rate of fracture [60,61]. Women who had participated from Sweden or the Women's Health Initiative did not show a negative correlation between tea consumption and fracture [56-57]. Therefore, with the current information, no conclusion can be made on how tea impacts rate of fracture.

Table 1.1 Summary of Cross-Sectional and Prospective Studies Investigating Tea Consumption and BMD

Study	Country of Study, Sample Size, Sex, Age	Tea Consumed*	Type of Study	Effects
Hoover and coworkers[45]	Canadian <i>n</i> = 62 F +57yrs	Tea consumers	Cross-sectional	Tea consumption was positively correlated to BMD at the lumbar spine ($p < 0.01$) and femoral neck ($p < 0.01$).
Hakim and coworkers[56]	American <i>n</i> = 91,465 F +50yrs	<1 cup/day 1 cup/day 2-3 cups/day >4 cups/day	Cross-sectional	Tea consumption was positively correlated to total body BMD ($p < 0.05$). There was no association between tea consumption and total hip BMD ($p = 0.22$) or lumbar spine and BMD ($p = 0.06$). Individuals who drank at least 4 cups/day had greater total body BMD than individuals who drank 1 cup/day ($p < 0.05$). Individuals had higher BMD at the spine when they consumed between 2-3cups/day than 1 cup/day ($p < 0.05$).
Devine and coworkers[52]	Australian <i>n</i> = 1,027 F +70yrs	Tea consumers	Cross-sectional	Tea consumers had a significantly higher BMD in comparison to non-consumers for total hip ($p < 0.05$) and trochanter ($p < 0.01$) BMD
Hegarty and coworkers[46]	English <i>n</i> = 1,256 F +65yrs	Tea consumers 1-3 cups of tea/day, 4+ cups of tea/day	Cross-sectional	Tea consumers had 5% greater BMD at lumbar spine ($p < 0.05$), greater trochanter ($p = 0.004$) and Ward's triangle ($p < 0.05$) than non-consumers. No difference in BMD when amount of tea consumed was compared. Addition of milk did not result in any differences
Muraki and coworkers[47]	Japanese <i>n</i> = 632	Green tea consumers	Cross-sectional	Consumers of Green Tea had higher BMD and better T-scores (-1.59 ± 2.70 versus -

	F +60yrs			2.17±2.08) at the lumbar spine than those who did not consume tea ($p<0.05$)
Larijani and coworkers[48]	Iranian $n = 830$ M, F +20yrs	Habitual tea consumers (≥ 5 cups of tea/day)	Cross-sectional	Women whom habitually consumed tea showed a lower frequency of osteoporosis (5.6% <i>versus</i> 12.1%) and osteopenia (24.5% <i>versus</i> 36.5%) compared to non-consumers. No significant difference in prevalence of osteoporosis when assessing the male population. Women who habitually consumed tea in general had higher BMD at the spine, but was not shown to be significant ($p < 0.07$), but did show greater BMD at the hip ($p = 0.01$). Among males, no differences in BMD of consumers and non-consumers.
Kyriazopoulos and coworkers[50]	Greek $n = 300$ M 18-30yrs	Tea consumption	Cross-sectional	No association amongst tea intake and BMD or tea intake and bone mineral content of the distal and ultra-distal radius ($p < 0.05$).
Saitoglu and coworkers[51]	Turkish $n = 70$ M 45-65yrs	Tea consumers	Cross-sectional	No association amongst tea consumption and BMD ($p > 0.05$).
Vestergaard and coworkers[49]	Danish $n = 2,016$ F 45-58yrs	Tea consumption	Cross-sectional	Tea consumption was positively associated with T scores above -0.75 for BMD at the femoral neck.
Ungan and coworkers[54]	Turkish $n = 727$ F +48yrs	> 2 cups of tea/day	Cross-sectional	No statistical difference between BMD of tea consumers and non-consumers when measured across three phalanges

Ungan and coworkers[54]	Turkish <i>n</i> = 727 F +48yrs	> 2 cups of tea/day	Prospective (1 year in duration)	No statistical difference between tea consumers and non-consumers in osteoporosis prevalence
Hardcastle and coworkers[55]	Scottish <i>n</i> = 3,226 F 45-54yrs	Flavonoid consumption	Prospective (5-9 years in duration)	57% of flavonoid intake was attributed to consumption of tea Total flavonoid levels were correlated with femoral neck ($p < 0.01$) and lumbar spine ($p < 0.05$) BMD. Catechins and Procyanidin levels were correlated with % annual change in BMD at the femoral neck and BMD at the lumbar spine ($p < 0.05$). Total flavonoids were negatively correlated to free deoxyypyridinolines ($p < 0.05$). A higher flavonoid intake relates to lower rate of bone resorption ($p = 0.001$).
Wu and coworkers[53]	Taiwan <i>n</i> = 1,037 M, F +30yrs	Habitual versus non-habitual tea consumption Categories: 1-5 years, 6-10years >10years	Prospective (2-4 years in duration)	Tea consumers (for 6 or more years) had greater BMD at the lumbar spine BMD ($p < 0.01$) and hip (at the neck and Ward triangle, $p < .05$) and total body BMD ($p < 0.001$) than non-consumers. There was no statistical significance in BMD at any site when tea was consumed less than 5yrs ($p > 0.05$). No change in BMD when individuals consumed green or oolong tea <i>versus</i> black tea.
Devine and coworkers[52]	Australian <i>n</i> = 164 F +70yrs	Tea consumers	Prospective (5 years in duration)	Tea consumers lost 1.4% of total hip BMD <i>versus</i> non-consumers who lost 4.7% in the fully adjusted model ($p < 0.01$). Tea consumers lost 2.0 % of trochanter BMD <i>versus</i> non-consumers who lost 6.3% ($p <$

				0.01). Tea consumers lost 1.0% of intertrochanter BMD <i>versus</i> non-consumers who lost 4.5% (p < 0.01).
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* “tea consumers” refers to whether individuals were tea consumers or not with no quantification of how much tea they habitually consumed. Tea consumed includes consumption of all tea (green, black, oolong, herbal tea) and type of tea consumed was not measured unless specified.

Table 1.2. Summary of Prospective Studies Investigating Tea Consumption and Fracture

Study	Nationality, Sex, Age	Tea Consumed	Duration of Study	Outcomes
Kanis and coworkers[60]	European $n = 1,762$ M +50yrs	Tea consumers or non-consumers	1 year	Tea consumers had a lower rate of hip fracture ($p < 0.001$) than non-consumers.
Johnell and coworkers[61]	European $n = 5,618$ F +50 yrs	Tea consumers or non-consumers	1 year	Tea consumers had a lower rate of hip fracture ($p < 0.001$) than non-consumers.
Hallstrom and coworkers[57]	Swedish $n = 31,527$ F +40yrs	Black tea <1 cup/day, 1 cup/day, 2-3 cups/day, ≥ 4 cups/day	10.3 years	No significant difference in incidence of fragility fracture between consumers and non-consumers.
Chen and coworkers[56]	American $n = 91,465$ F +50yrs	<1 cup/day, 1 cup/day, 2-3 cups/day, >4 cups/day	4.1 years	No significant difference in incidence of fragility fracture between consumers and non-consumers. Fractures were listed as hip fractures, forearm or wrist fractures and other fractures. <i>Herbal and decaffeinated tea was not included for tea consumption.</i>
Jha and coworkers[58]	Indian $n = 100$ M F 65 yrs#	≤ 1 cup/day, > 1 cup/day	Cross-sectional	Tea consumers had a higher fracture risk ($p < 0.001$)
Du and coworkers[59]	Chinese $n = 703$ M F 90+yrs	Tea consumers or non-consumers	Cross-sectional	No association between tea consumers and non-consumers with rate of fracture

* “tea consumers” refers to whether individuals were tea consumers or not with no quantification of how much tea they habitually consumed. Tea consumed includes consumption of all tea (green, black, oolong, herbal tea) and type of tea consumed was not measured unless specified.

M = male, F = female

the age listed is an average

1.4.2 Composition of Tea

Tea from the leaves of *Camellia sinensis* or herbal sources (*Aspalathus linearis*) contain an abundance of diverse flavonoids which help provide antioxidant, anti-cancer, anti-bacterial activity. Along with trace elements taken up from the soil, tea may also contain larger concentrations of caffeine, fluoride, and flavonoids, all of which can impact health [63]. A combination of caffeine, fluoride and flavonoid content could lead to different biological activities in a system, and these combinations can vary depending on season, location, the age of the plant and how it is processed [64].

1.4.2.1 Caffeine

Caffeine, a diuretic, is found in most tea, including green, oolong, and black tea, but not within rooibos tea. Black tea is recognized for containing the largest concentration of caffeine per cup (8oz) [65], but even this is considerably less than coffee [66]. High consumption (≥ 4 cups/day of coffee [67]) of caffeinated beverages can be a concern for bone health with its ability to limit calcium absorption into the blood stream through urinary loss [68], and this has been associated with accelerated bone loss in elderly women [69]. However, calcium absorption measured in pre-menopausal women whom were given 400mg/day of caffeine over 19 days, demonstrated no difference in calcium levels when compared to controls [70]. On the other hand, caffeine intake has been shown to have significant negative effects on fasting serum phosphorous levels and

bone turnover markers [70]. Furthermore, a study in Sweden which followed women ages 40-76 over 10 years found that excessive caffeine intake equating to over 300 mg of caffeine a day was related to a moderate increase in osteoporotic fracture, but attributed this to be partially due to lack of calcium intake [57]. A study that assessed BMD at 5 sites on the bodies of postmenopausal women across three different countries did not detect a relationship between caffeine intake and BMD [71]. More recently, a study that analyzed data from 38,984 women from the Swedish Mammography Cohort demonstrated that increased coffee consumption was not related to an increased rate of fracture. Although, a high intake of caffeine (≥ 4 cups/day) was linked to slightly lower spine (4%), proximal femur (2%) and total body (2%) BMD compared to women who consumed < 1 coffee/day [72]. Additionally, women who consumed below 745mg of calcium/day that also ingested high levels of caffeine (≥ 450 mg/day) had significantly lower spine and total BMD. When calcium levels were above 745mg/day, this effect of caffeine on spine and total BMD was lost [73].

1.4.2.2 Fluoride

Fluoride, is another common element found in tea, has been suggested to be able to manipulate bone metabolism. The levels of fluoride in tea depend on the drinking water used for boiling the tea leaves in. When postmenopausal women with osteoporosis were given 75 mg/day of fluoride and supplemented with 1500 mg of calcium per day, they experienced increased BMD at sites with high ratios of trabecular bone to cortical, but were reduced in sites of high cortical bone. Furthermore, there was an increase in

non-vertebrate fractures in the individuals consuming fluoride, suggesting that fluoride is not beneficial for bone health [74].

This heavy consumption of fluoride is now recognized as fluorosis, in which high, repeated exposure to fluoride can result in fluoride accumulating in the bone matrix. This new fluoride embedded in the mineral has not only suggested to be resistant to bone turnover but also possesses the ability to change the microarchitecture of the bone through the production of ionic bonds with nearby calcium atoms [75]. Although rare, there have been several reports published on individual cases where increased consumption of tea, high in fluoride, has resulted in consumption of fluoride levels that were orders of magnitude more than the average individual [76]. This new microarchitecture, referred to as fluorapatite, now represents a new crystalline structure from the previous hydroxyapatite. This fluoride-substituted crystal has been identified as having lost elasticity and tensile strength [77].

1.4.2.3 Flavonoids

Many of the potential benefits of flavonoids in bone metabolism may be due to effects that mimic estrogen, i.e. anti-oxidant activity and/or increasing apoptosis and decreasing activity in osteoclasts, thereby enhancing the activity and differentiation of osteoblasts. Therefore, a brief overview of how estrogen acts on bone tissue is provided. Studies showing a positive relationship between consumption of tea and BMD [45-48] may be due to its flavonoids having a role in regulating inflammation and cellular stress

via antioxidant activity. In addition, flavonoids may enhance the production and regulate bone markers by interacting with osteoblasts, while inhibiting activity and/or increasing apoptosis in osteoclasts [78,79,80]. With this wide range of biological influence, but lack of information regarding bone health (especially within human cell lines), these flavonoids are seemingly a promising step to finding a natural product to increase bone mineral density while offering benefits to other aspects of health.

As demonstrated through menopause, estrogen is a key player in the lifecycle of our bones. Produced primarily by the ovaries, estrogen binds to estrogen receptors ($ER\alpha$, $ER\beta$) found on various cell types in order to promote a biological change. A loss of estrogen (i.e. menopause, oophorectomy) has been linked to a large loss in BMD and strength, equating to a higher likelihood of a fragility fracture [81,82].

Mutations within genes that express estrogen receptors and enzymes such as aromatase have led to low BMD and skeletal problems [83,84]. Nevertheless, suppression of androgen production, or mutations within androgen receptors may also leave males with similar characteristics of extreme bone mineral loss and reduced bone strength [85,86]. When older men were given testosterone and/or estrogen inhibitors and followed by hormone replacement for either androgen or estrogen, estrogen replacement prevented 70% of the effects of bone turnover, while testosterone accounted for the other 30%. Bone turnover was measured by changes in bone creation and destruction markers such as ALP and deoxypyridinoline, respectively. In addition, estrogen was the only hormone significantly involved in both the creation and destruction of bone [87].

1.5 Rooibos tea

Rooibos (*Aspalathus linearis*) tea contains a high level of flavonoids and is naturally located in the mountainous areas of Western Cape, South Africa. The plant contains very thin, needle-like leaves in which they can be used for fermented (green rooibos) or unfermented tea (red rooibos) [88,89]. Rooibos has attracted recent interest due to it containing a large concentration of flavonoids and lack of caffeine content, therefore potentially providing the benefits of green and black tea, without the detriments of a diuretic.

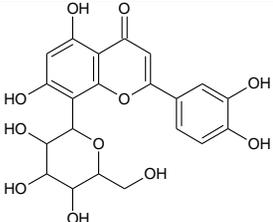
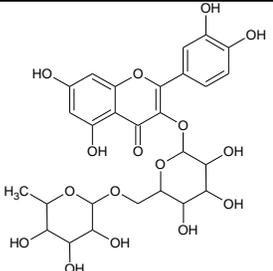
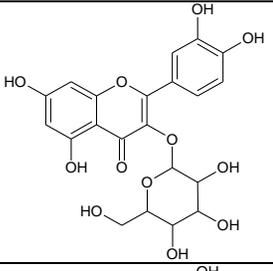
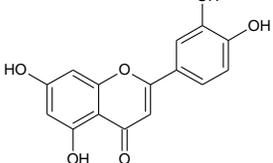
Rooibos tea is known to contain many flavonoids that possess the ability to scavenge free radicals [90]. Free radicals occur due to extra single electrons found on oxygen species (OH and O₂) formed during oxygen metabolism. If these free radical species are left and concentrations become too high, they will extract electrons from nearby macromolecules such as DNA and lipids, which result in cellular death [91]. Compounds that can extract and neutralize these free radicals are highly sought after by scientists, nutritionists and doctors, and take the form of multi-cyclic ring systems in which the free radical charge can be shared across the ring systems [90]. Flavonoids within rooibos tea contain multiple cyclic ring systems, making them the perfect free radical scavenging, macromolecular saving systems [89]. When antioxidant activity of green, oolong, black, unfermented rooibos, semi-fermented rooibos and fermented rooibos, were compared, green tea contained the best free-radical scavengers with an inhibition of 90.8% of the free radicals, while unfermented rooibos came in a close second with an inhibition success of 86.6% [92]. In contrast, a study in 2003 suggested

that black tea contained not only a higher concentration of flavonoids, but also out-competed rooibos tea, (fermented and unfermented) in antioxidant activity [93]. Rooibos' antioxidant power was further shown to be extensive when rooibos tea was able to reduce lipid peroxidation within rats when livers were introduced to fumonisin, a free radical agent that promotes liver cancer [94]. These results of rooibos' antioxidant potential were further fortified in a human study, where participants ingested rooibos tea daily (6 cups/day) for 6 weeks. Total plasma flavonoid concentrations of the subjects increased while lipid peroxidation decreased by. Additionally, reduced glutathione-to-oxidized glutathione ratio dramatically increased with consumption of rooibos tea and was maintained after completion of the study [95]. Therefore tea with high levels of flavonoids may be beneficial to limit reactive oxygen species and inflammation.

Flavonoid content varies in tea depending on the tea's grade. Rooibos production was examined from 2009-2011 with differing quality grades, A through D, with A being the highest quality. A positive trend was identified in which higher grades of tea were associated with higher flavonoid concentration. There were significantly higher concentrations of individual flavonoids in higher grades of rooibos tea such as: aspalathin, isoquercetin, rutin, hyperoside, quercetin-3-O-robinobioside, suggesting higher grades of rooibos tea would be more efficient food sources of flavonoids [96]. Whether the tea is unfermented or fermented can change the chemical composition of the tea extract in addition to its bioavailability and activity. A review of rooibos tea compared differing concentration of flavonoids from fermented and non-fermented rooibos tea in 100g of dried tea leaves from three different fermented and three different unfermented

plant sources. The flavonoids focused on from this study, found in rooibos tea, in both fermented and unfermented conditions are listed in Table 1.3 [44].

Table 1.3. Concentration of selected flavonoids in fermented and unfermented rooibos tea and other food sources

Flavonoid	Structure	Fermented (g/100g) [44]	Unfermented (g/100g) [44]	Average Weight in Rooibos tea (mg/500ml) [97]	Plasma Range after 3hr ingestion (nmol)[97]	Other food sources [98, 99] (mg/100g)
Orientin		0.263 ± 0.087	0.202 ± 0.026	17	0.0-0.7243	Buckwheat malt (80.9mg), Olives (0.22mg)
Rutin		0.245 ± 0.141	0.173 ± 0.016	7.9	0.0-0.4686	Capers (332.29mg), Buckwheat malt (102.1mg) Buckwheat whole grain flour (37.27mg), black raspberries (19.00mg), Olives (45.36mg), Asparagus (23.19mg)
Hyperoside		0.021 ± 0.012	0.016 ± 0.015	0.8	ND	American cranberry (10.81mg), Lingonberry (13.22mg), Black Chokeberry (46.46mg), Black tea (4.17mg)
Luteolin		0.007 ± 0.006	0.010 ± 0.005		Not tested	Mexican oregano (56.33mg), Thyme (39.50mg), Sage (33.40mg), artichoke (42.10mg)

\pm SD = \pm standard deviation, ND = not detected

Although the flavonoid content can differ by large concentrations, a study done by Serafini and coworkers [100] suggests that both the fermented and unfermented rooibos tea contain considerably large amounts of antioxidant activity. When consumed, the fermented tea resulted in a 6.6% higher antioxidant capacity within plasma. Unfermented tea had a 2.9% higher antioxidant capacity than the control. These findings suggest rooibos may be a source of nutritional antioxidants. In contrast to this, Marnewick [101] did not find an increase in plasma antioxidant status after volunteers drank rooibos tea daily for 6 weeks. But, a significant increase was noted in total flavonoid content within plasma. A large drop in lipid peroxidation, serum low-density-lipids was also noted, while a significant increase was found in reduced glutathione, high density lipids and triacylglycerols, all of which are beneficial to cardiovascular health. The ability for tea to change important biological characteristics such as antioxidant status, lipid peroxidation, and levels of low-density-lipids allow them to be characterized as bioactives. Bioactives are molecules that can exhibit a change in a biological system through their interaction with receptors and proteins at the molecular level.

Antioxidants play several roles within bone health, the first being that osteoclasts secrete free radicals to aid in the digestion of mineral by stimulation of cytokines which increases osteoclast differentiation and activity. Therefore the application of antioxidants such as vitamin E has been shown to help remove these free radicals thereby limiting bone resorption and optimizing growth [102]. It is currently understood that free radicals can increase production of $\text{NF-}\kappa\beta$ that activates bone resorption. Basu and coworkers [103] looked at the relationship between a biomarker for oxidative stress (8-iso-PGF2a) and BMD in a small group of men and women. They discovered a negative, linear

association with 8-iso-PGF2a and total BMD as well as 8-iso-PGF2a and BMD at the lumbar spine. Moreover, free radicals have been shown to inhibit osteoblast differentiation. These effects could then be counteracted by treatment with antioxidants [104]. Evidence has shown that during estrogen deficiency there is a loss of anti-oxidant defense systems producing a more suitable environment for osteoclast differentiation [105]. The administration of antioxidants to ovariectomized mice has prevented bone loss. Glutathione peroxidase, the dominant enzyme found within bone systems to deal with free radicals is up-regulated in the presence of estrogen, and therefore during estrogen deficiency, this defense is largely reduced [105,106]. This evidence suggests that flavonoids from rooibos tea may play a role in limiting osteoclast proliferation while aiding in the production of osteoblasts, allowing for the body to maintain healthy bones.

Anti-cancer, anti-fungal, anti-viral activities as well as potential effects on bone cell activity are all important characteristics of rooibos flavonoids [94,107,108,109,110]. The ability for these flavonoids to produce biological changes within both cells and organisms suggests that they can interact with cellular receptors and enzymes to up/down regulate different genes and therefore alter multiple mechanisms. The *in vivo* models in which flavonoids were detected in plasma suggests that these flavonoids can enter plasma after consumption and exert biological effects that are beneficial to the animal. This data suggests that flavonoids may possess the ability to not only interact with important receptors involved in bone metabolism, but also modulate them to improve bone health.

As these compounds show activity in biological systems, it is imperative to understand the metabolism of these compounds, the tissues they partition into, and at what concentrations. The bioavailability and antioxidant status of flavonoids found within

rooibos tea after human ingestion were therefore examined in 12 healthy males. Participants were provided a placebo, rooibos tea or the isolated flavonoid fraction. Flavonoid content was initially analyzed using 10g of dried leaves in 500ml of boiling water in which the compounds were detected using high performance liquid chromatography (summary in Table 1.3). Only Aspalathin, isoorientin, orientin, rutin, vitexin and isovitexin could be detected in plasma [97].

The *flavonoids of interest* within this study are: orientin, rutin, hyperoside and luteolin.

These flavonoids were chosen for the following reasons:

- i.** Some cross-sectional and prospective studies suggest that tea consumption may improve BMD, and within some studies, tea consumption is negatively correlated to fracture. Therefore, there is a strong possibility that the flavonoids in tea are mediating this biological effect. Flavonoids found in larger concentrations within tea, that are readily quantifiable in tea extracts are of more interest due to their likelihood of bypassing the first pass effect and entering plasma to produce a biological outcome.
- ii.** These four flavonoids feature antioxidant, antifungal, anticancer and antibacterial characteristics. This vast and increasing range of activity has increased the interest of these compounds, leading to a growth in production within chemical companies, providing research laboratories with more access to them.
- iii.** With this wide range of biological influence, but lack of information regarding bone health, especially within human cell lines, these flavonoids are

seemingly a promising step to finding a natural product to increase bone mineral density while offering benefits to other aspects of health.

- iv.** Due to the ability of orientin, and rutin, to enter plasma through consumption of rooibos tea, it is recognized that these compounds are the most physiologically relevant flavonoids when developing dietary strategies for disease prevention or management of a disease.
- v.** Other studies have reported the ability of hyperoside to enter plasma after consumption of other food and beverage [111,112,113]. In addition, luteolin is documented for its lack of ability to enter plasma through tea consumption, but has been identified in plasma of human and mice after oral consumption of luteolin (in its free form – not glycosylated) [114]. Therefore, it is not only important for scientists to understand the particular flavonoid of interest in a specific consumable (i.e. rooibos tea), but in addition, the flavonoid and its concentration found within other food or beverages.
- vi.** As many of these compounds resemble each other's structures with slight differences in position of hydroxyl or sugar components, changes in activity from one compound to the next will allow for a better understanding of how chemical structure may influence up or down regulation of important genes in bone turnover. Changes in positions of functional groups that result in different activity in bone cells will allow for a deeper knowledge of important functional positions. These positions will bring about information on possible steric hindrance that disallows interactions between substrates and receptors, while also identifying key bond relationships that may enhance or hinder

activity. With an idea of which enzyme or receptor a flavonoid may interact with, these interfaces can be modeled *in silico* to further develop our understanding of structure-function relationships which may help in decoding possible mechanisms.

1.5.1 Rooibos Flavonoids and Metabolism

When working with flavonoids, it is important to recognize the metabolic pathway in which the flavonoid is partitioned, broken down and removed from the body as not all flavonoids reach plasma in their ingested form. In addition, having a good understanding of the working metabolites of these flavonoids can present new ideas on the specific fragments of the structure that are important for improving bioavailability (Figures 1.3-1.6). With recent studies suggesting that tea flavonoids can influence bone turnover [115,116,117] and with sales of rooibos tea increasing, it is of great interest to understand potential influences that these specific flavonoids will have on bone cells.

1.5.1.1 Orientin

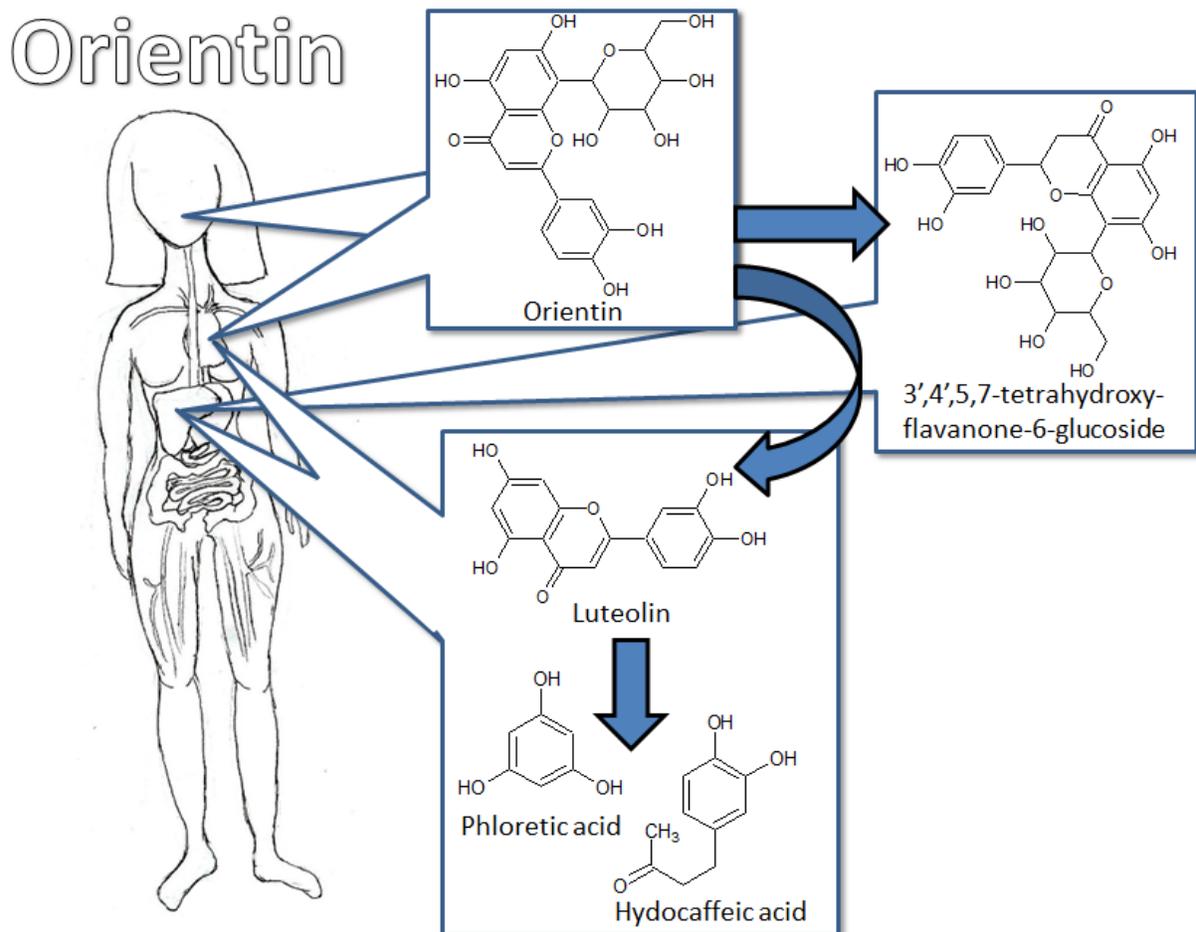


Figure 1.3. Metabolism of Orientin. The above diagram outlines the metabolites of orientin and the tissues that are responsible for metabolism. Information is adapted from references [97].

Orientin, another bioactive found within human plasma after consumption of Rooibos tea [97], will undergo hydrolysis to produce luteolin or can be hydrogenated into 3',4',5,7-tetrahydroxy-flavanone-6-glucoside which can be subsequently deglycosylated and undergo ring cleavage into smaller phenolic compounds such as hydrocaffeic acid and phloretic acid (Figure 1.3).

1.5.1.2 Rutin

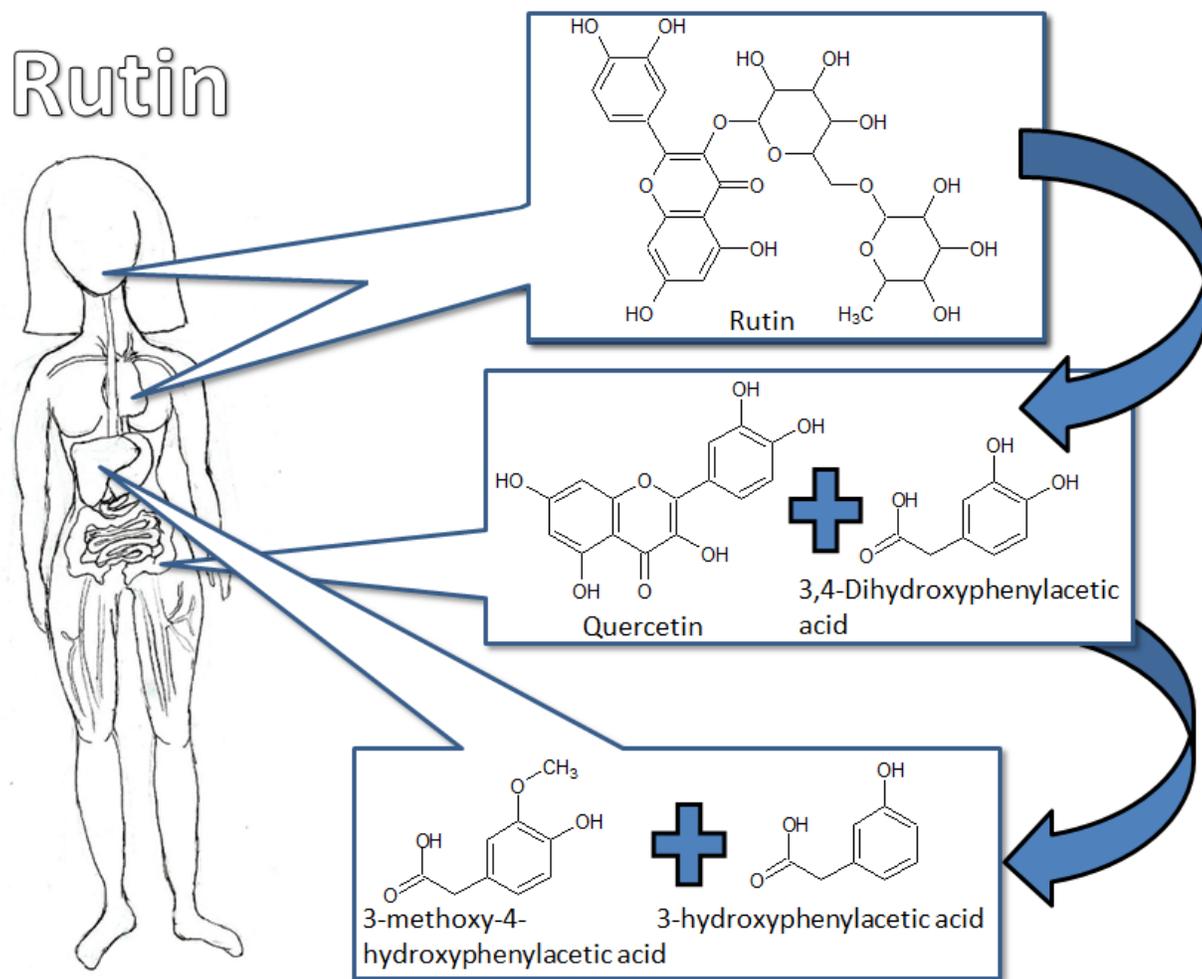


Figure 1.4. Metabolism of Rutin. The above diagram outlines the metabolites of rutin and the tissues that are responsible for metabolism [118].

Ingestion of rutin leads to production of quercetin and 3,4-dihydroxyphenylacetic acid in the large intestine which is further metabolized by the liver into 3-hydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid. Quercetin and rutin are both found in plasma [118].

1.5.1.3 Hyperoside

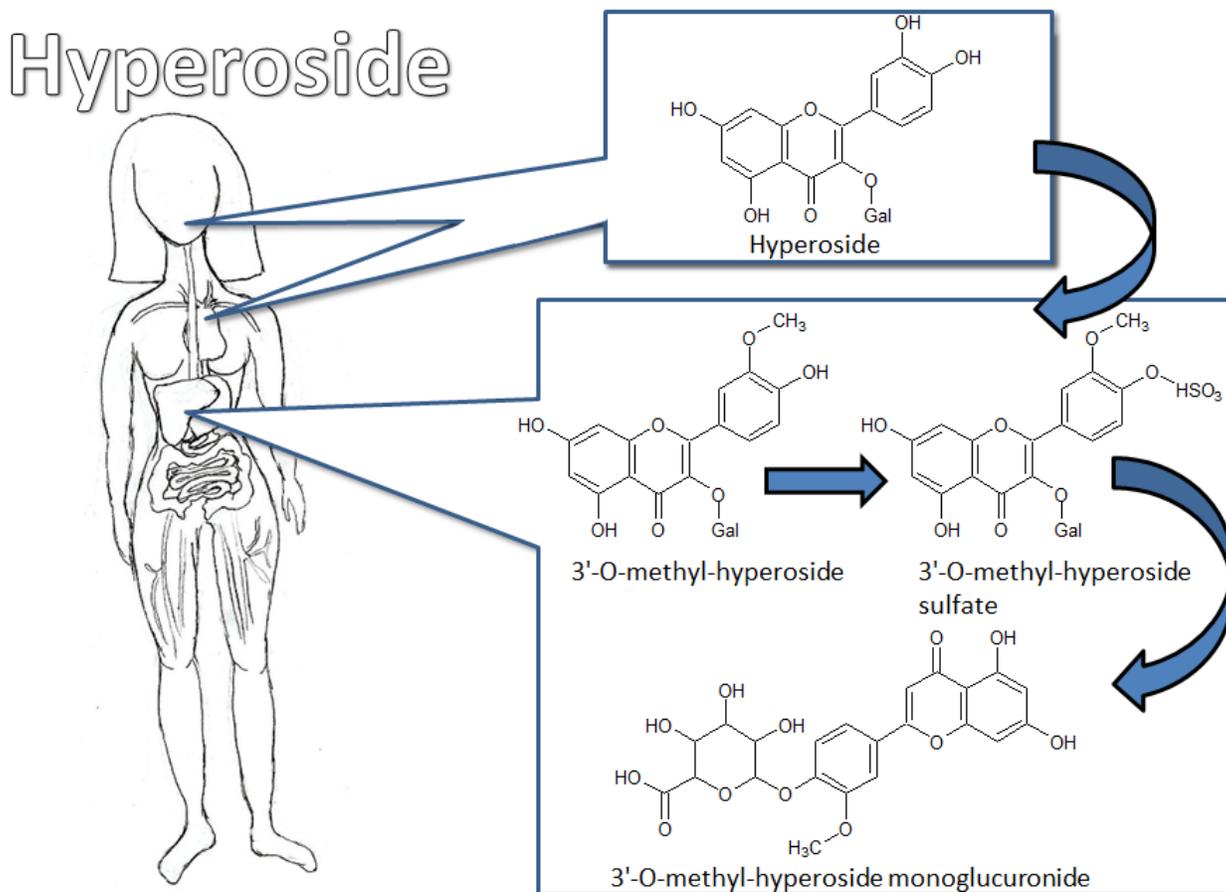


Figure 1.5. Metabolism of Hyperoside. The above diagram outlines the metabolites of hyperoside and the tissues that are responsible for metabolism [118, 119].

Hyperoside, when ingested has been reported both in and absent from plasma, produces major metabolites after going through the first pass effect in which the liver produces a methylated form. This methylated form is then sulfated and glucuronidated [119]. Hyperoside is also known to be a major metabolite of aspalathin [118].

1.5.1.4 Luteolin

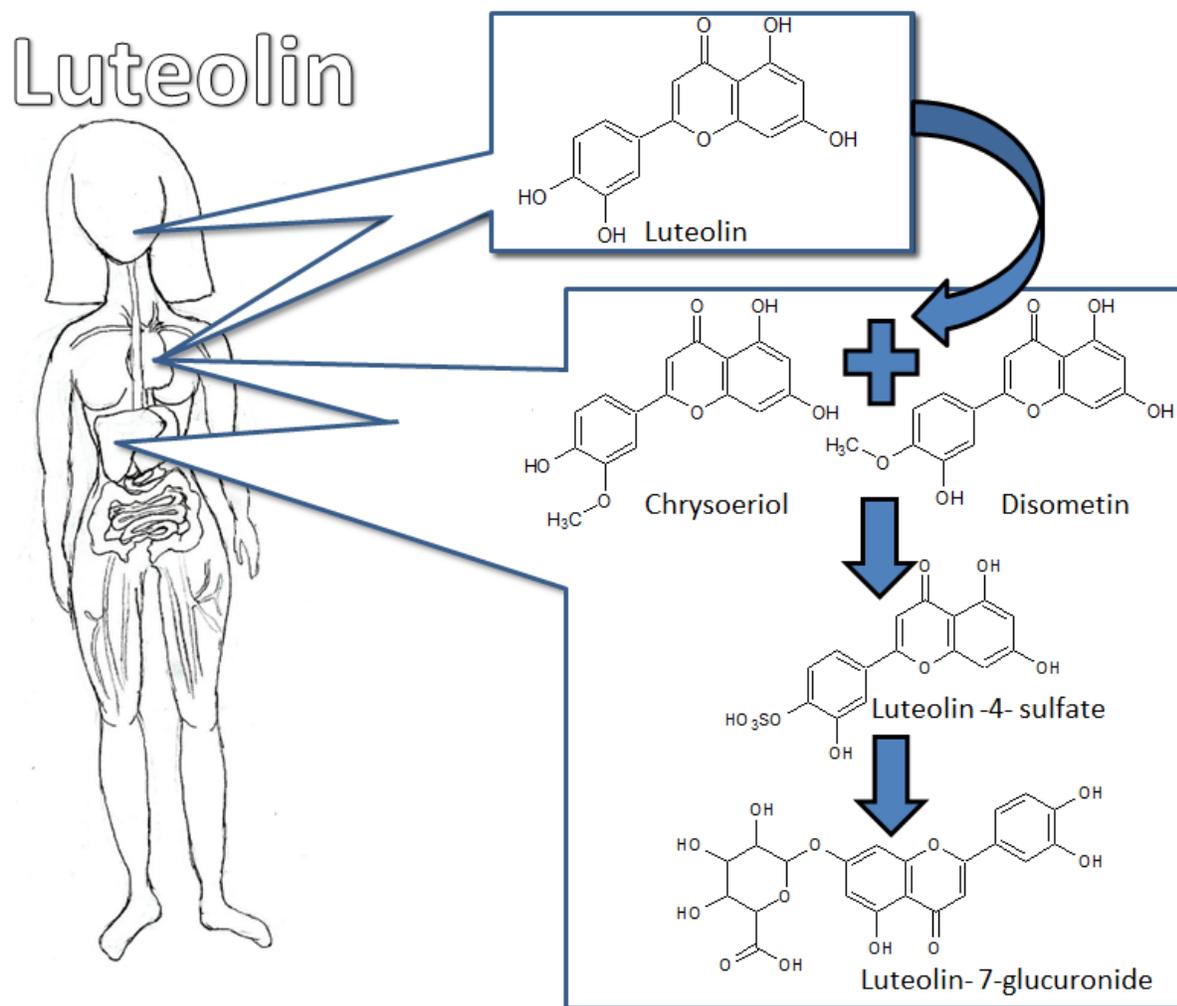


Figure 1.6 Metabolism of Luteolin. The above diagram outlines the metabolites of luteolin and the tissues that are responsible for metabolism [120,121].

Consumption of luteolin in its pure form will lead to methylation products (chrysoeriol 3'-O-methylation, and disomletin: 4'-O-methylation) within the liver produced through a transfer mechanism which involves a methyl group being transferred from S-adenosyl-L-methionine to a hydroxyl in the substrate via catechol-O-

methyltransferase. These can also be demethylated via cytochrome P450's (CYP1A2 and CYP3A4/5). Furthermore, luteolin is sulfated by uridine-5-diphosphate sulfotransferases and glucuronidated by glucuronosyl transferases [120,121].

1.6 Drug Discovery

While nutritional science generally first considers what flavonoids are present in plasma after ingestion of food, there is a rationale for studying flavonoids that are not present in high amounts or detectable amounts in plasma. Flavonoids with poor bioavailability can undergo alterations to improve pharmacokinetics such as modification to its structure or the encapsulation of the compound. For example, interestingly, luteolin has become of recent interest due to its inhibitory effects on β -hydroxyacyl-acyl carrier protein dehydratase of *Plasmodium falciparum*. *Plasmodium falciparum* is a bacterium responsible for malaria, and the dehydratase enzyme is responsible for allowing fatty acid biosynthesis within the infected host [122]. This compound was initially discovered after epigallocatechin gallate was shown to provide antibacterial and antifungal activity within many organisms that involve the inhibition of fatty acid synthase [123]. From there, a chemical library was developed in which flavonoid compounds were stored with information regarding their solubility, size, and biological information. Through screening methods, *in silico* experimentation and enzyme purification procedures, luteolin was discovered to be an efficient inhibitor for the dehydratase and thus a potential route for medicine [122]. If fatty acid biosynthesis cannot occur in the host,

successful transmission of the parasite is lost and is thus an important area of research for vaccine and drug development.

Looking at structure-function relationships using *in vitro* methods can positively lead to drug targets in which chemical synthesis can improve upon structure details to allow for increased bioavailability, longer/shorter half-lives and improved molecular interactions [124]. In Cragg's recent review (2013) on drug discovery, approximately 50% of new drugs are either natural products, natural products with synthetically modifications or synthetically produced drugs based on natural products. This percentage increases to 64% when considering synthetic compounds used to inhibit natural product substrates [125].

Natural health products have been recognized for millions of years for treatment of sores, rashes, fever, and are still largely within the pharmaceutical industry. Some of the most profitable and useful medications came from nature, such as morphine and ephedrine. Many compounds are also naturally derived but synthetically modified to improve pharmacokinetics. One example is quinine, which is isolated from the bark of the tree species *Cinchona*, which served as the basis for anti-malarial drugs [125]. Another compound known as digitalis that is derived from the foxglove plant has been chemically modified for medicinal treatment of heart failure [126]. Thus, it is important to understand and assess both compounds that enter plasma through tea consumption and those tea flavonoids that have the ability to enter plasma, but through other means, in their ability to manipulate bone mineralization.

1.7 Bone Culture Systems

Bone cell cultures are important for studying biochemical mechanisms in which a biological change is noted. They offer a faster, less costly and more controlled, detailed method for looking at protein and receptor interactions than working with *in vivo* models. Cells also allow for multiple replications in addition to studying multiple signaling pathways. Cell cultures are key for targeting pathways of interest and the development of drugs and vaccines for treatment of diseases such as osteoporosis.

When focusing on prevention of osteoporosis it is important to understand the affect the dietary component or drug has on both osteoblast actions as osteoblasts are important for the production of bone matrix and mineral. *In vitro* models utilizing human osteoblasts can help assess changes in the cell cycle and protein production that may favorably enhance bone production by osteoblasts. When studying new flavonoids, osteosarcoma cell lines (i.e. Saos2 cell line) make perfect candidates for analyzing changes in cell mechanisms due to their ability to differentiate into mature osteoblasts, continuously replicate while also containing similar properties to primary human osteoblasts. Common human osteosarcoma cell lines are: MG-63, U2 and Saos2 [127].

1.7.1 Saos2 Cells

First recognized by Fogh in the mid-70s, Saos2 cells, a human cell line from osteosarcoma, have been well characterized and established for studying how dietary

components such as flavonoids affect osteoblast activity [128]. For example, Saos2 cells are also well known for their high ALP activity ranging from 4-6 μ mol/min/mg protein which makes them great candidates for studying the effects of flavonoids on mineral deposition. Other osteosarcoma cell lines from human or mouse contains low ALP activity consistent around 2.5nmol/min/mg protein ultimately translating to less mineral being produced in a period of time [129]. Another advantage of using the Saos2 cell line is that it endlessly proliferates which allows for less variation when researchers use a specific population to target cell mechanisms. As a primary cell line cannot grow indefinitely, assessing different mechanisms within osteoblasts can be time consuming, and it is best to limit variation that can exist in passage number and population. Saos2 cells also increase its rate of proliferation as the passage number increases, this allows for larger population studies while keeping costs low [130]. Steroid receptors are important features for osteoblastic cell lines, especially because many flavonoids are believed to manipulate bone formation through mimicking the substrates for these receptors. Keenan et al., reported androgen (1605 sites/cell) and progesterone receptors (119 sites/cell) in the Saos-2 cell line, but failed to detect estrogen receptors. Concentrations of androgen and binding sites are approximately 21 times higher in Saos2 cell lines than rodent osteoblasts, and are a better representation of human primary osteoblasts [131]. In 2007, Sekeris and co-workers published their findings of both the α -ER and β -ER are found in the nucleus and cytoplasm, respectively [132]. Receptors for 1,25(OH)₂D₃, the active form of vitamin D, are also present within this cell line which are similar to those found in primary human cell lines, making them more representable as natural osteoblastic cells. This receptor has importance in its ability to enhance the production of a protein

known as osteocalcin [129]. Furthermore, these cells have become of great interest for their ability to differentiate into osteoblasts, allowing mechanistic studies on transformation that mimic those of primary cell lines. This suggests that Saos2 cell lines are good representatives of primary cell lines as they present similar receptor types, number of sites and differentiate in the same manner. These characteristics are helpful when looking at mechanistic studies involving changes in osteoblast activity as they are more likely to represent what is occurring in a primary cell line.

As with most model systems, there are some disadvantages to working with this cell line. These disadvantages include the lack of genes found in primary bone cells that could potentially change how compounds influence bone growth. The Saos2 cell line does not contain RUNX-1 or RUNX-3, only RUNX-2. RUNX-1 is responsible for differentiation of hematopoietic cells [133]. RUNX-2 coordinates the growth factors that allow for bone development and osteoblast maturation [134]. RUNX-3 controls the up-regulation and down-regulation of transcription factors and its removal is often associated with cancerous cells (i.e. Saos2 cells) as it is linked with the tumor suppressor factor [135,136]. Although this demonstrates a difference between primary cell lines and the Saos2 cell line, having only RUNX-2 does have its benefits. This availability of RUNX-2 and lack of RUNX-1 and -3 allow for mechanistic studies on this gene and its involvement with mineral deposition. In a study done by Jean-Christophe and Odile, Saos-2 cells were transfected with small interfering RNA (siRNA) to knock out RUNX-2 in order to evaluate the gene's involvement in proliferation and differentiation. This resulted in an extreme reduction in the cyclin inhibitor p21 which mediates anti-proliferation mechanisms within osteoblasts. There was also a decrease in Bax protein

expression, a protein that regulates apoptosis. Tumor necrosis factor (TNF), a cytokine that can naturally induce cellular death within osteoblasts was inhibited when RUNX-2 was introduced to siRNA, suggesting that RUNX-2 has an important role in maintaining the ability of the osteoblast to differentiate and proliferate [137]. Primary human osteoblasts and the osteosarcoma cell line MG-63 have been recognized for their ability to proliferate under mechanical stimuli, much like the human body experiences when exercising. Jones and coworkers tested this ability in other osteosarcoma cell lines including Saos2 cells and found that proliferation was not increased under the same stimuli. This was believed to be due to the lack of phospholipase C β 2, a protein believed to be expressed and up-regulated in cells sensitive to mechanical stimuli [138]. This is another representation of the lack of genes, or protein production that an osteosarcoma line such as Saos2 has in comparison to a primary cell line. Hausser reported the phenotypic instability of Saos-2 cells after recognizing the negative association between passage number and ALP activity. This pattern was also seen with Decorin, a gene suggested to control proliferation and mineralization. Decorin expression was significantly reduced after 20 passages, in which proliferation and mineral deposition dramatically increased [130]. This data is important for acknowledging that the use of the osteosarcoma cell line Saos2 requires experiments to contain controls of the same cell population and passage number to reduce variation.

With Saos2 cells closely resembling primary bone cells, these cells offer a method for clean replication of cell signaling analysis in a human cell line that is more likely to mimic those of primary cells than lines originating from other species. Saos2 provides a reasonable starting point for investigation into the effects of flavonoids on mineral

deposition in humans. Acknowledging the benefits of producing similar genes, receptors and protein interactions between Saos2 and primary human osteoblasts, gives reason to believe that results achieved using this osteosarcoma cell line will have a reasonable chance to be replicated in a human primary cell line. In saying so, results that achieve potential for enhanced mineral deposition should be investigated further within a human primary cell line to ensure accuracy and full creditability, and ultimately, tested in vivo.

Chapter 2.0 - Objectives and Hypotheses

The *overall objective* is to determine if the addition of purified rutin, hyperoside, orientin and luteolin to Saos2 cells, will result in greater mineral content. Secondary objectives are to determine potential mechanisms as to why the addition of rutin, hyperoside, orientin and luteolin results in greater mineral content, these are: osteoblast activity as defined by ALP, cell mitochondrial activity, cell toxicity, and changes in osteoblast regulatory protein production.

2.1 Specific objectives

To determine how tea flavonoids, specifically rutin, hyperoside, orientin and luteolin modulate:

- i. mineral content;
- ii. osteoblast activity, as indicated by ALP
- iii. cell mitochondrial activity;
- iv. cell toxicity;
- v. regulatory protein levels that affect osteoblast signaling: TNF α , interleukin 6 (IL6), sclerostin (SOST), OPG, and osteopontin (OPN).

- vi. Study a structure-function relationship, specifically, how a small change in structure can lead to changes in mineral deposition, osteoblast activity, cell mitochondrial activity, cell toxicity and the regulation of osteoblast proteins.

2.2 Hypotheses

Compounds that structurally resemble 17- β estradiol, have been shown to influence estrogen receptors through competitive binding. This competitive binding leads to biological responses in which genes are either up- or down-regulated. Furthermore, in estrogen deficient systems, flavonoids such as rutin, hyperoside, orientin and luteolin, may act as a substitute for estrogen and therefore promote those biological actions. As the majority of epidemiological studies involving tea consumption show a positive correlation with BMD, and a negative correlation with risk of osteoporosis, the **overall hypothesis** is that individual tea flavonoids rutin, hyperoside, orientin and luteolin will increase mineral content, partially by increasing osteoblast activity and favourable osteoblast proteins.

Specific hypotheses:

- i. Rutin, hyperoside, orientin and luteolin will increase mineral content by acting as estrogen mimics
- ii. An increase in mineral content will be in part due to an increase in ALP

- iii. Rutin, hyperoside, orientin and luteolin will improve cellular activity
- iv. Rutin, hyperoside, orientin and luteolin will decrease cell toxicity
- v. Rutin, hyperoside, orientin and luteolin will down regulate osteoblast inhibitor SOST, mineral inhibitor OPN and pro-inflammatory markers. Rutin, hyperoside, orientin and luteolin will up-regulate OPG, a favourable marker for bone formation.
- vi. Changes in rutin, hyperoside, orientin and luteolin structures, such as the addition of hydroxyl moieties or glycosylation will change the activity of osteoblast cells. As rutin, hyperoside, orientin and luteolin become increasingly different from estradiol; rutin, hyperoside, orientin and luteolin will have lower activity with respect to those that are closely related to estradiol's structure.

2.3 Scientific Approach

Multiple outcomes will be measured to comprehensively assess how flavonoids in tea modulate key aspects of osteoblast activity. The outcomes include:

a. Mineral content (primary outcome): Calcium deposits (i.e. bone nodules) will be measured using alizarin red staining assay.

b. ALP: This enzyme is up-regulated during the process of mineralization and is important for providing osteoblasts with the phosphate necessary to produce mineral. ALP can be measured using a p-nitrophenyl phosphate assay, where the hydrolysis

products (and thus the activity of the enzyme) can be monitored using visible spectroscopy.

c. Cell mitochondrial activity: Wherein a direct relationship exists between cellular enzyme activity and cellular viability, cell activity can be monitored by quantification of the enzyme: mitochondrial reductase. By adding a coloured tetrazolium complex to the cell lysate, a linear relationship between product colour and enzyme activity can be established, monitored by visible absorbance spectrometry.

d. Cell toxicity: Lactate dehydrogenase is measured as a release of cytotoxicity. Lactate dehydrogenase is kept within the cytosol, and is released into cellular media when the cell dies. Media will be extracted during flavonoid additions to measure the toxicity of the flavonoids on Saos2 cells.

e. Quantification of Regulatory Proteins involved in Osteoblast Activity: An extensive panel of proteins will be quantified that are important for osteoblast (and indirectly osteoclast) activity. These include: OPG, OPN, SOST, IL6 and TNF α .

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Chapter 3.0: Rutin or hyperoside, flavonoids present in tea, enhance mineral content in Saos2 cells

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Abstract

Osteoporosis is characterized by low bone mineral density that increases an individual's risk to a fragility fracture. Several cross-sectional and prospective studies have shown that habitual consumption of tea is associated with a higher bone mineral density in women. Tea contains a high concentration and wide-range of flavonoids with differing biological activities. Flavonoids are structurally similar to estrogen and therefore may act as an estrogen mimic to positively influence bone physiology. The present study analyzed two common flavonoids found in tea, rutin and hyperoside, to delineate their effect on mineralization in human osteoblast (Saos2) cells. Administration of rutin ($\geq 25 \mu\text{M}$) and hyperoside ($\geq 5 \mu\text{M}$) resulted in an increase in mineral as determined using the alizarin red assay. This increase in mineral was in part due to an increase in alkaline phosphatase activity, as determined by the hydrolysis of p-nitrophenyl phosphate. Rutin and hyperoside also increased cell mitochondrial activity as measured by the reduction of 3-(4,5-dimethylthiazolyl)-2,5-diphenyl-2H-tetrazolium. The expression of osteopontin, sclerostin, TNF α and IL6, known stimuli for increasing osteoclast activity and decreasing osteoblast activity, were reduced with the addition of rutin or hyperoside. Osteoprotegerin, a decoy for RANKL was also suppressed as the result of flavonoid supplementation. However, osteoprotegerin is highly expressed in transformed cell lines as a decoy for tumor necrosis factor related apoptosis ligand and has been positively associated with pro-inflammatory markers. Therefore changes in osteoprotegerin may be an artifact of the model chosen. Together, these findings demonstrate that rutin and hyperoside positively influence osteoblast activity, resulting in greater formation of mineral in Saos2 cells.

3.1 Introduction

Osteoporosis is defined by a low bone mineral density and compromised bone structure that leads to a high risk of painful and debilitating fragility fracture [1]. In women, the loss of bone mineral and weakening of skeletal sites often occurs most rapidly at the time of menopause as ovarian production of endogenous estrogen ceases [2]. Resultant fragility fractures are associated with diminished quality of life [3]. Furthermore, the risk of re-fracture is elevated if osteoporosis is not managed appropriately [4]. Osteoporosis is also a significant burden to the health care system. In the United States, over \$18 billion is spent on osteoporotic related fractures per year [5].

Although drug therapies can slow the deterioration of bone tissue and reduce the risk of fragility fracture, these strategies represent a treatment approach and can be associated with serious adverse effects [6]. Prevention of osteoporosis using dietary strategies should also be considered for achievement of peak bone mass by early adulthood and maintenance of healthy bone after the growth period [7]. With respect to nutrients, calcium and vitamin D have been studied extensively for bone health [8,9] while other dietary components such as flavonoids, that are present in large quantities in tea, are emerging as potential bioactives that may benefit bone health. Several epidemiological and prospective studies suggest higher BMD is associated with habitual tea consumption [10,11,12], and although less studied, some but not all of the few studies report lower rates of fracture [13, 14]. Most studies have not categorized the type of tea

of other food and beverage sources such as wine [21] and asparagus [22]. Flavonoids have the potential to act as strong antioxidants that can protect cells such as osteoblasts from damaging free radicals created as the result of inflammatory mediators and cell dysfunction. Pro-inflammatory cytokines such as TNF α and IL6 can stimulate differentiation and proliferation of osteoclasts and can thereby contribute to higher rates of bone resorption [23,24]. Higher production of pro-inflammatory cytokines and overall inflammation has been associated with a higher risk of fracture [25,26,27].

Both rutin and hyperoside are derivatives from quercetin, another flavonoid found within tea and a variety of foods [28], however unlike rutin and hyperoside, quercetin exists at some of its lowest levels in tea (Figure 3.1). Quercetin has also been shown to increase alkaline phosphatase activity (ALP) in osteoblasts *in vitro* [29], and BMD at the lumbar spine and femur in ovariectomized mice [30]. Due to the ability of quercetin to increase mineralization in bone cells, we hypothesized that rutin and hyperoside, would similarly enhance mineralization as they contain the core structure of quercetin. Rutin contains a disaccharide (glucose and rhamnose) on carbon 3 of quercetin, and hyperoside has a galactose ring on carbon 3. The presence of a disaccharide or a single galactose ring may result in different effects on osteoblast behavior – particularly since the chemical structure of hyperoside more closely resembles estradiol than the much larger and complex structure of rutin and therefore may increase mineral content at lower concentrations.

While both rutin and hyperoside are present in rooibos tea, only rutin is present in plasma at measurable levels after consumption of rooibos tea [31]. However, hyperoside has been detected in plasma of rats after consumption of hyperoside tablets [32]. Thus,

the bioavailability of these compounds is important to consider, as flavonoids need to be absorbed and enter the circulation to induce a biological change. Although hyperoside from rooibos tea may not enter plasma at measurable levels, compounds that may enhance biological activities *in vitro* can be enhanced by chemical modification or encapsulation to improve bioavailability. Moreover, identifying key moieties on the flavonoid structures that enhance activity helps identify bioactives or develop compounds that may favorably influence bone cell metabolism.

The primary objective of this research was to identify if the addition of rutin and hyperoside, flavonoids found in high concentrations in green, black and rooibos tea, results in greater mineral content in Saos2 cells as mineral content is an important indicator of osteoblast activity. Secondary objectives were to identify if rutin, a disaccharide of quercetin, and hyperoside, the galactoside of quercetin, modulate osteoblast activity, cell mitochondrial activity, cell toxicity and proteins that modulate osteoblast activity (i.e. TNF α , IL6, OPG, SOST, OPN). Because rutin and hyperoside have quercetin for their backbone but different glycosides branching from carbon 3, the structure-function relationships between these two flavonoids were also investigated to provide insight into potential preferential changes in flavonoid structure for enhancing mineral content in Saos2 osteosarcoma cells.

3.2 Methods and Materials

Human osteosarcoma cells (Saos2, [33]) were purchased from Cedarlane (catalogue # HTB-85, supplier ATCC). HAM-F12, 10X Trypsin, Phosphate buffer saline solutions (PBS), fetal bovine serum (FBS) albumin, antibiotic-antimycotic and flasks were purchased from Lonza. Rutin trihydrate and 3-(4,5-dimethylthiazolyl)-2,5-

diphenyl-2H-tetrazolium bromide was purchased from Alfa Aesar and hyperoside from Aktin Chemicals. Epigallocatechin gallate >95%, alizarin red S, paraformaldehyde, sodium thiosulfate, p-nitrophenyl phosphate, p-nitrophenol, calcium chloride, HEPES, glutamine, dimethylsulfoxide (DMSO) cell culture grade, dexamethasone, β -glycerophosphate, ascorbic acid and the lactate dehydrogenase activity kit were purchased from Sigma-Aldrich.

Cells were plated and sustained in HAM F-12 medium supplemented with 10% FBS, 0.028M HEPES buffer pH 7.4, 0.0014M CaCl_2 , 0.002M glutamine and 1% antibiotic-antimycotic. Media was changed three times a week and cells were passaged weekly. Differentiation occurred by promoting matrix secretion using media supplemented with 0.01M dexamethasone and 50 $\mu\text{g}/\text{ml}$ of ascorbic acid for one week. The following day, cell media was supplemented with the matrix media in addition to 0.01M β -glycerophosphate until day 21. During days 8-21, flavonoids were dissolved in DMSO and added at a final volume of 0.1%. Epigallocatechin gallate [34] and DMSO were used as a positive control and negative control, respectively. DMSO control was compared to cells with no vehicle to ensure no changes in cell morphology and cell death.

3.2.1 Mineral content

Mineral content was quantitatively assessed using alizarin red assay. Cells were fixed in 4% paraformaldehyde on day 21 and kept at 4°C for 24 hours. Following, cells were washed with PBS and replaced with 0.04M alizarin red S stain for 20 minutes on a plate shaker at room temperature. Cells were washed with PBS and analyzed using a FluorChem imaging system. Extraction required the addition of a 10% cetylpyridinium

chloride solution for 1 hour at room temperature. Mineral was quantified by measuring visible absorbance spectroscopy at 550nm and compared to controls.

3.2.2 ALP Activity

Cells were grown in well plates and lysed with 0.05% Triton X-100 solution dissolved in 0.05M Tris-HCl. Cell lysates were introduced to p-nitrophenyl phosphate and p-nitrophenol production was monitored over 5 minutes by absorbance spectroscopy at 410nm and normalized by protein concentration using the Bradford assay [34]. Cells were tested on days 3, 7 and 10 after the addition of β -glycerophosphate and flavonoid.

3.2.3 Cell mitochondrial Activity

Cell activity was assessed by measuring the ability of mitochondrial reductase to reduce 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). Cells were plated, and allowed to adhere over 24 hours. Every 24 hours the flavonoid supplemented media was replaced. Cells were harvested after 24, 48 and 72 hours by supplementing the media with 5mg/ml MTT dissolved in PBS for 4 hours. Extraction required 0.04M HCl-isopropanol and was measured at 570nm with a reference measurement at 610nm. Measurements were normalized to cell count, indicated by trypan blue.

3.2.4 Cell Toxicity

Lactate dehydrogenase (LDH) present in cell media was quantified as a measure of cellular death. LDH concentration was determined by an *in vitro* toxicology assay kit from Sigma-Aldrich (Tox7) and normalized to cell count. Cells were allowed to adhere to

plates over 24 hours. Media with flavonoid was replaced every 24 hours and cells were harvested at 12, 24 and 48 hours.

3.2.5 Quantification of Regulatory Proteins in Osteoblasts

Cells were harvested after 3 days into mineralization, after the addition of β -glycerophosphate and flavonoid. Cell media was analyzed for TNF α , IL6, SOST, OPG and OPN using a magnetic ELISA-based commercially-available kit from Millipore (HBNMAG-51K).

3.2.6 Statistics

Measurements of mineral content and ALP activity consisted of six plates (24 wells) per flavonoid. Each plate consisted of three wells per treatment, in which each well was measured three times by absorbance spectroscopy. MTT and LDH assays were measured in 96 well plates using six plates per flavonoid, with each plate containing three wells per treatment. Protein measurements were completed using four (75cm²) flasks per flavonoid. Each flask was measured three times. Measurements are reported as an average \pm standard error mean.

Data analysis was completed by IBM SPSS Statistics 21 utilizing 1 way-ANOVA with vehicle (rutin, hyperoside, or control) as the independent variable (IV). The dependent variable (DV) was expressed as per cent DMSO control and was a function of mineral content, ALP activity, MTT reduced, and LDH released. For protein measurements, the IV was rutin, hyperoside or control. The DV was the protein concentration of individual osteoblast markers. If the 1 way-ANOVA was statistically significant ($p < 0.05$), post-HOC analysis allowed for comparison of the IV to determine

which groups were statistically different from one another ($p < 0.05$). Data was checked for homogeneity of variance. When homogeneity of variance was not statistically different ($p > 0.05$), post-HOC analysis was completed by Bonferroni t-test. Where homogeneity of variance was statistically different across groups ($p < 0.05$), Games Howell t-test was utilized for post-HOC analysis.

3.3 Results

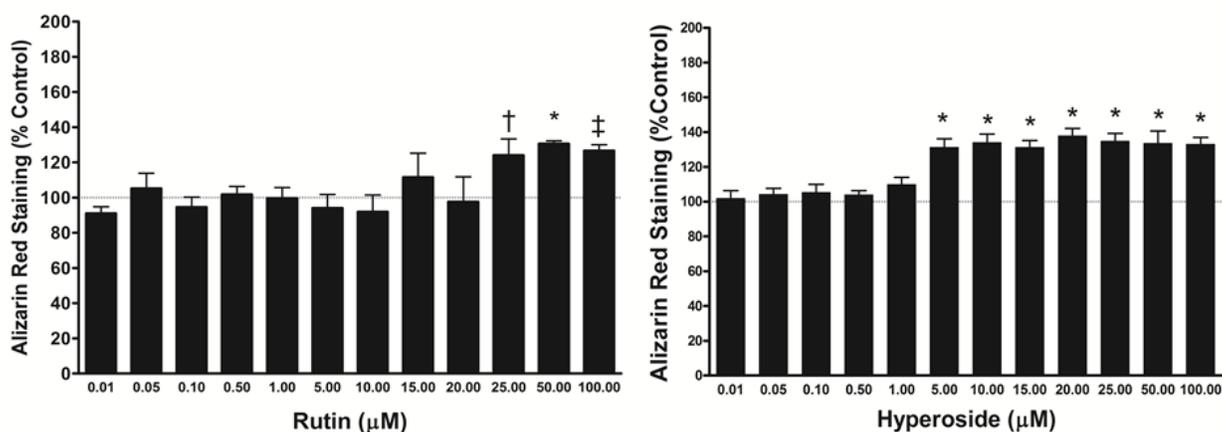


Fig. 2. Mineral content produced by addition of (a) rutin or (b) hyperoside. Mineral content was measured by alizarin red staining. † $p < 0.05$, ‡ $p < 0.01$, * $p < 0.001$ compared to control

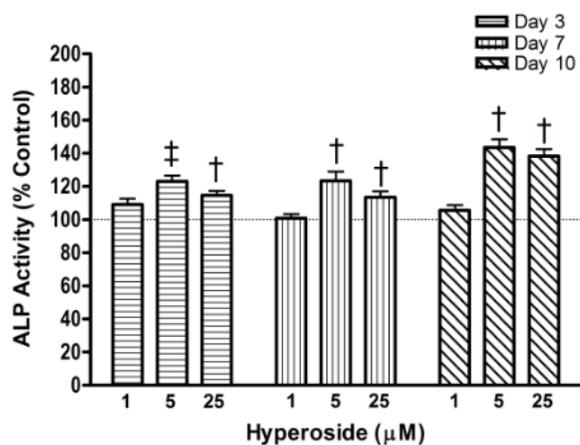
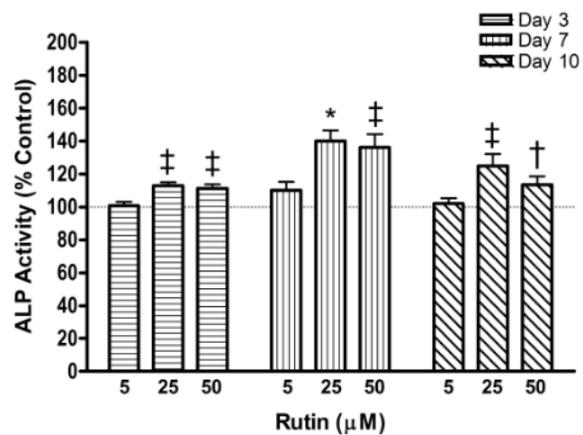


Figure 3.3. ALP activity with addition of (a) rutin or (b) hyperoside. Activity was measured by the rate of hydrolysis of p-nitrophenyl phosphate. † $p < 0.05$, ‡ $p < 0.01$, * $p < 0.001$ compared to control within a day.

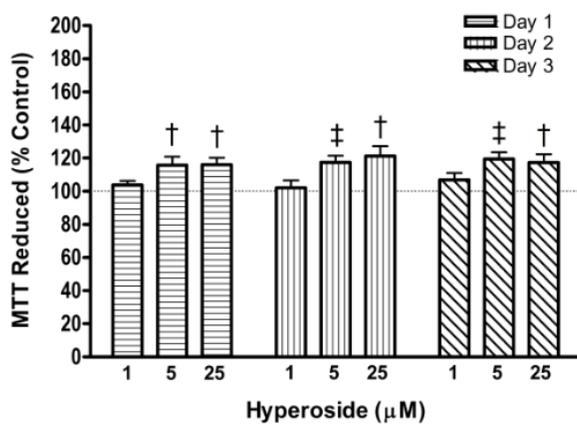
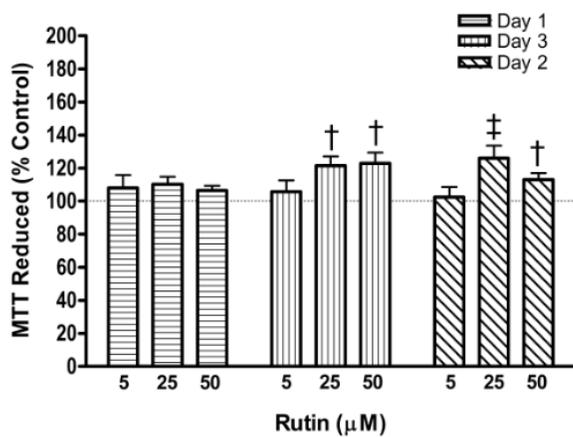


Figure 3.4. Cell mitochondrial activity with addition of (a) rutin or (b) hyperoside. Cell mitochondrial activity was measured by reduction of MTT. † $p < 0.05$, ‡ $p < 0.01$, * $p < 0.001$ compared to control within that day

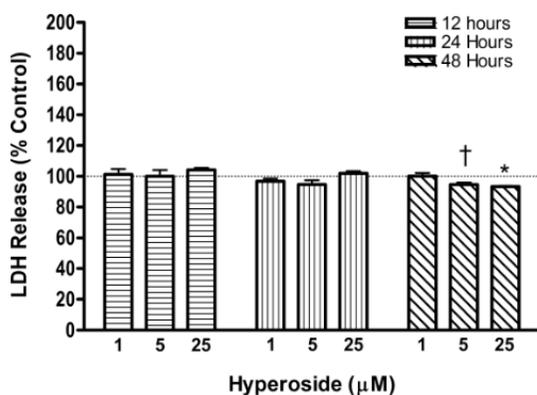
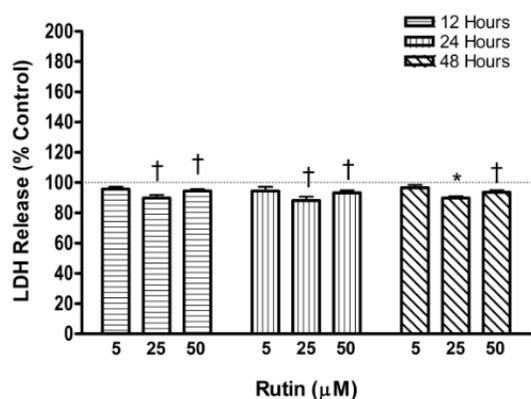


Figure 3.5. LDH release by addition of (a) rutin or (b) hyperoside

LDH activity was measured by the reduction of NAD⁺ to aid in the conversion of a tetrazolium dye to a formazan crystal. † $p < 0.05$, ‡ $p < 0.01$, * $p < 0.001$ compared to control within that day

Table 3.1 Concentrations of regulatory proteins involved in osteoblast activity

	Protein Concentration (pg/mL)					
	TNF α	IL6	PTH	OPN	OPG	SOST
Control	0.43 \pm 0.01	3.24 \pm 0.08	17.48 \pm 1.00	247.50 \pm 10.71	1659.25 \pm 107.78	810.00 \pm 22.15
Rutin (50 μ M)	0.20 \pm 0.02a*	1.36 \pm 0.09a*	13.60 \pm 1.78	126.21 \pm 10.78*	431.25 \pm 11.37a‡	572.00 \pm 19.07*
Hyperoside (25 μ M)	0.25 \pm 0.01b*	1.90 \pm 0.06b*	11.70 \pm 0.78†	145.75 \pm 2.75*	709.25 \pm 25.28b‡	542.75 \pm 10.01*

Significant differences in values, from control, are indicated by,

† $p < 0.05$, ‡ $p < 0.01$, * $p < 0.001$

Significant differences ($p < 0.05$) between rutin and hyperoside are indicated by differences in letters for each column. Data are expressed as mean \pm SEM

3.3.1 Mineral content

Levels of rutin and hyperoside added to Saos2 cells ranged from 0.01-100 μ M to determine the minimum and maximum effective concentrations that could induce a significant increase in mineral content (Figure 3.2). Rutin, when added at levels of 25 μ M, 50 μ M and 100 μ M resulted in higher mineral content ($p < 0.05$, $p < 0.001$, $p < 0.01$, respectively) compared to control. Hyperoside resulted in significantly higher mineral content at lower doses (5 μ M through 100 μ M, $p < 0.001$) compared to control. DMSO control was considered safe as it was not different from cells without vehicle (Data not shown).

3.3.2 ALP activity

ALP activity was assessed at 3, 7 and 10 days into mineralization to examine at when rutin and hyperoside increase ALP activity compared to control (Figure 3.3). Two doses in which the flavonoid resulted in higher mineral content were chosen along with one dose that did not result in higher mineral content (served as a negative control). Rutin (25 μ M, 50 μ M) resulted in higher ALP activity than control at day 3 ($p < 0.01$, $p < 0.01$, respectively), and this effect was also observed at day 7 ($p < 0.01$, $p < 0.01$, respectively) and day 10 ($p < 0.01$, $p < 0.05$, respectively). Exposure to hyperoside (5 μ M, 25 μ M) resulted in higher ALP activity than control at day 3 ($p < 0.01$, $p < 0.05$, respectively) and this persisted at days 7 ($p < 0.05$, $p < 0.05$, respectively) and 10 ($p < 0.05$, $p < 0.05$, respectively).

3.3.3 Cell Mitochondrial Activity

Mitochondrial reductase activity was measured at three time points - 24, 48 and 72 hours after addition rutin or hyperoside - to assess how single and multiple doses change mitochondrial activity (Figure 3.4). These time points were chosen to assess immediate and long term effects of flavonoid addition to Saos2 cells. The same doses were carried over from ALP activity measurements to measure mitochondrial reductase activity. Mitochondrial activity with rutin did not differ from control at any of the doses studied (5 μ M, 25 μ M, 50 μ M) within the first 24 hours ($p > 0.05$). Rutin (25 μ M, 50 μ M) significantly increased mitochondrial activity after 48 hours ($p < 0.05$, $p < 0.05$, respectively) and 72 hours ($p < 0.01$, $p < 0.05$, respectively) compared to control. At 24 hours, hyperoside (5.0 μ M, 25.0 μ M) increased mitochondrial activity ($p < 0.05$, $p < 0.05$, respectively) compared to control. This heightened mitochondrial activity was maintained for 48 hours ($p < 0.01$, $p < 0.05$, respectively) and 72 hours ($p < 0.01$, $p < 0.05$, respectively) compared to control.

3.3.4 Cell Toxicity

For consistency, doses of rutin and hyperoside were kept the same as previous assays for measuring LDH. Cell toxicity was measured by analyzing LDH released into cell media at three different time points (12, 24 and 48 hours) after flavonoid supplementation (Figure 3.5). Time points were chosen to assess immediate and additive effects of flavonoid addition on cellular toxicity. After 12 hours of the initial flavonoid addition, rutin (25.0 μ M 50.0 μ M) resulted in lower levels of LDH ($p < 0.05$, $p < 0.05$, respectively) compared to control. This lower level of LDH was maintained at 24 hours ($p < 0.05$, $p < 0.05$, respectively) and 48 hours ($p < 0.001$, $p < 0.05$, respectively). After

12 and 24 hours of supplementation with hyperoside (1.0 μM -25.0 μM), levels of LDH did not differ from control. After 48 hours, hyperoside (5.0 μM , 25.0 μM) had significantly lower levels of LDH ($p < 0.05$, $p < 0.001$, respectively) than control.

3.3.5 Quantification of Regulatory Proteins in Osteoblasts

For protein measurements, 50 μM of rutin or 25 μM of hyperoside was added to cells (Table 3.1). These levels of flavonoids were selected because they had been shown to increase mineral content, ALP activity, as well as cell activity without increasing cell toxicity. Rutin and hyperoside resulted in lower levels of IL6 ($p < 0.001$, $p < 0.001$), TNF α ($p < 0.001$, $p < 0.001$), OPG ($p < 0.01$, $p < 0.01$), OPN ($p < 0.001$, $p < 0.001$) and SOST ($p < 0.001$, $p < 0.001$) than control. Direct comparison of the effect of rutin (50 μM) and hyperoside (25 μM) showed that rutin resulted in a greater reduction in TNF α ($p = 0.035$), IL6 ($p < 0.002$) and OPG ($p < 0.001$) compared to hyperoside. However, when the change in regulatory proteins is normalized to the amount of flavonoid added (per mol) using the following equation: $(\text{Protein}_{\text{Flavonoid}} - \text{Protein}_{\text{Control}})/(\text{moles}_{\text{Flavonoid}})$, hyperoside was shown to induce greater changes than rutin (see Table 3.1).

3.4 Discussion

Mineral content was enhanced with both rutin ($\geq 25 \mu\text{M}$) and hyperoside ($\geq 5 \mu\text{M}$) in this human cell line. Increases in ALP are identified early into mineralization, in addition to higher mitochondrial activity, lower levels of LDH and lower expression of TNF α , IL6, SOST, OPN can at least partially explain how rutin and hyperoside enhanced mineral content. Importantly, the addition of rutin ($\geq 25 \mu\text{M}$) and hyperoside ($\geq 5 \mu\text{M}$) was shown to be non-toxic as it resulted in an increase in cell mitochondrial activity and a decrease in LDH release. Our finding that rutin ($\geq 25 \mu\text{M}$) demonstrated a greater

reduction in MTT by 48 hours and lower levels of LDH released by 12 hours, are similar to those from a study in mouse bone marrow mesenchymal stem cells which reported enhanced mitochondrial activity with the addition of rutin [35]. Likewise, the addition of hyperoside ($\geq 5 \mu\text{M}$) demonstrated higher levels of MTT and lower levels of LDH in comparison to the control, suggesting that both rutin and hyperoside stimulate osteoblast activity.

Rutin ($50 \mu\text{M}$) decreased the pro-inflammatory cytokines TNF α and IL6 by 53.5% and 58%, respectively, and hyperoside ($25 \mu\text{M}$) demonstrated a similar response, with a reduction of TNF α by 42% and IL6 by 41%. Similar to these tea flavonoids, previous work demonstrated that the addition of other tea flavonoids, epigallocatechin gallate or theaflavin-3,3'-digallate to human gingival fibroblasts attenuated the production of IL6 by down regulation of nuclear factor $\kappa\beta$ [36]. Earlier work has also identified that osteoblasts, when placed in the presence of high oxidative stress, experienced inhibition of differentiation, marked by a decrease in RUNX2, ALP expression and collagen formation [37]. High levels of TNF α have been linked to bone loss through increased production of pro-inflammatory cytokines and osteoclast activity partnered to lower osteoblast activity [38]. This inhibition of differentiation and promotion of apoptosis in osteoblasts has shown to be preventable through pretreatment with antioxidants [39]. Moreover, when rats with chronic inflammation were given 0.5% green tea polyphenols in their drinking water, the green tea polyphenols were able to prevent significant bone loss in part by decreasing the pro-inflammatory cytokines TNF α and cyclooxygenase-2, in comparison to control [40]. Thus, findings from previous studies and the present study suggest that flavonoids commonly present in tea provide a

good source of anti-oxidants that may promote mineralization and suppress bone resorption.

The addition of rutin and hyperoside also resulted in lower levels of OPG. This may seem counter-intuitive, as an increase in OPG levels are typically desired when administering an agent to prevent osteoporosis [41]. Nonetheless, high levels of OPG are also recognized as a marker for cancer. OPG acts as a decoy ligand for tumor necrosis factor related apoptosis ligand (TRAIL) [42]. Because TRAIL is important for inducing apoptosis primarily in cancerous cells, high levels of OPG are produced by cells such as Saos2 cells to prevent apoptosis [42]. Interestingly, previous work has shown that the addition of rutin ($\geq 50 \mu\text{M}$) to mouse osteoblasts resulted in an increase in OPG [35]. Therefore the difference seen between these two studies may be due to differences in OPG machinery found within the particular cell line (human osteosarcoma cell line versus healthy mouse cell line). Other work has identified that high OPG levels in a human osteosarcoma cell line (MG63) are correlated with $\text{TNF}\alpha$ [43]. This suggests that inflammatory cytokines may aid in tumor proliferation by stimulating the production of OPG. With this knowledge, it is interesting that rutin resulted in significantly lower levels of $\text{TNF}\alpha$ and IL6 in comparison to hyperoside (similarly to the findings for OPG). As the addition of rutin and hyperoside reduced both $\text{TNF}\alpha$ and IL6, and previous work has shown that levels of $\text{TNF}\alpha$ are related to levels of OPG in cancer [43], OPG may have decreased as a result of lower levels of $\text{TNF}\alpha$ and IL6.

Rutin and hyperoside also decreased the release of OPN by 49% and 41%, respectively. One study has shown that rutin ($\geq 25 \mu\text{M}$) increased in OPN in mouse osteoblast cells [35] while another study showed that carnosol from rosemary reduced

OPN and IL6 in osteoblasts [44]. OPN is a protein produced by osteoblasts that is recognized to aid in bone turnover by anchoring osteoclasts to the surface of bone by binding to CD44 on the osteoclast membrane. OPN knock-out mice were shown to have reduced osteoclast motility, bone resorption and CD44 expression. Furthermore, these knock-out mice also had higher trabecular bone area, less trabecular spacing, higher trabecular thickness and mechanical strength [45]. OPN has also been identified to be sensitive to changes in redox status of the cell [46], and therefore may also be influenced by the presence of antioxidants such as rutin and hyperoside.

SOST, a marker of osteoblast inhibition, was reduced by the addition of rutin or hyperoside to Saos2 cells by 21% and 29%, respectively. SOST is produced by both osteoblasts and osteocytes and is an important mediator of osteoblast activity as it can inhibit the Wnt pathway. This pathway regulates bone formation, and therefore its inhibition is undesirable in individuals experiencing high bone resorption. Stimulation of the Wnt pathway allows for the expression of important proteins involved in building the matrix and mineral of bone [47]. SOST can bind to the lipoprotein receptor-related protein-5 and 6 (LRP5 and LRP6) that subsequently inhibits Wnt from binding to the receptors, thereby preventing gene transcription [47]. Previous work has shown that mutations in the SOST gene, causing it to be non-functional, are associated with Van Buchem disease in which there is excessive accumulation of bone [48]. Therefore, rutin and hyperoside may decrease levels of Wnt inhibitors and thus enhance osteoblast activity.

Similar to the favorable effects of rutin observed in the present study, rutin administration has been shown to be protective *in vivo*. While there are no studies in

humans that have investigated direct effects of rutin on bone health, a study using ovariectomized rats – a well-established model of deterioration of bone tissue due to estrogen withdrawal, showed a preservation of BMD in both total and distal femur [49]. Higher femur peak load and a reduction in deoxypyridinoline, a marker of bone resorption was also observed [49]. These findings are also supported by a study using mouse bone marrow stem cells. Rutin ($\geq 25\mu\text{M}$) enhanced mineral content but a significant increase in ALP activity was only observed at concentrations equal to or greater than $50\ \mu\text{M}$ [35]. Although mineral was not studied as an outcome, the addition of hyperoside ($1\text{-}100\mu\text{M}$) to UMR-106 cells (rat osteosarcoma cell line), has shown a significant increase in ALP activity [50] but hyperoside has not been studied using human bone cells.

Assessment of flavonoid structures can help predict bioactivity within various biological systems. Hyperoside is able to induce a significant increase in mineral at lower concentrations in comparison to rutin ($5\mu\text{M}$ versus $25\mu\text{M}$). At carbon 3, hyperoside has a galactose ring, and rutin contains a disaccharide. The difference in effective concentrations between hyperoside and rutin may be the result of the differences between the structures of the two flavonoids. This leads to the question of structure-function relationships. In general, hyperoside more closely resembles estradiol or androgens. In contrast, the large structure of rutin is more representative of an antioxidant. With the addition of hyperoside and rutin existing at different concentrations, levels of protein markers were normalized to flavonoid concentrations on a per mol basis (Table 3.1). Normalizing the protein measurements suggests that although the addition of the disaccharide results in lower levels of $\text{TNF}\alpha$, IL6, sclerostin, and OPN in comparison to

hyperoside, hyperoside is added at half the level. On a per mole basis, hyperoside demonstrates a greater reduction in these regulatory proteins compared to rutin. Both flavonoids have the potential to act as antioxidants, which would aid in bone formation, however, hyperoside resembles estrogen more closely than rutin and therefore may be more effective at binding and inducing changes in estrogen pathways at a lower concentration. Binding to estrogen receptors can be further investigated by using molecular modeling of rutin and hyperoside in the presence of estrogen receptors.

When assessing bioavailability, rutin has been detected in human plasma after consuming tea [31] and pure rutin tablets [51] in the low to mid nM range. Consumption of rutin by ovariectomized Wistar rats has also translated to doubling of estrogen levels, while also significantly increasing levels of prolactin, growth hormone and estrogen receptor alpha in plasma and gland tissues [52]. This demonstrates that rutin has the potential to influence biological receptors and change gene expression of important regulatory hormones and receptors, which are important for regulation of bone turnover. In contrast, hyperoside was not detectable in plasma after consumption of tea [31]. However, hyperoside has been detected in rat plasma in a dose-dependent manner after consumption of hyperoside tablets, but was quickly cleared by the hepatic system [53]. Although hyperoside is effective at lower concentrations than rutin, this is also an *in vitro* model and does not involve metabolism after oral ingestion. Therefore hyperoside may be a good candidate for encapsulation or chemical enhancement to improve its bioavailability in humans.

Improving bioavailability through encapsulation or chemical modification would allow higher levels of flavonoid to reach plasma, and may enhance the ability of a

flavonoid to modulate osteoblast activity and mineral formation. Previous work improved the oral bioavailability of rutin by 4.5 times in humans when rutin was conjugated to 2-hydroxy-propyl- β -cyclodextrin [54]. Therefore to improve bioavailability of hyperoside, like previous studies have shown with rutin [54], hyperoside could be encapsulated or chemically modified to ensure a more adequate level reaches plasma. In addition, bioavailability of flavonoids can be improved when in the presence of other bioactives. For example, hypericin has shown to increase its bioavailability by 58% in the presence of procyanidin B2 and by 34% in the presence of hyperoside [55]. When multiple bioactives are present, interactions can be made to increase solubility and stability during absorption. Due to the bioavailability of these flavonoids, to achieve these levels of rutin (25 μ M) and hyperoside (5 μ M) in plasma, supplemental levels above those attained through consumption of tea intake would be needed. However, as these flavonoids are found in multiple sources of food, and are consumed throughout the day, it is hard to know what the plasma levels of these flavonoids would be. Furthermore, the higher BMD associated with consumption of tea may be due to the long term consumption of multiple flavonoids, leading to potential synergistic and additive effects.

In summary, rutin and hyperoside enhanced mineral content in Saos2 cells, and this was associated with an increase in ALP and cellular activity as well as reduced levels of TNF α , IL6, SOST and OPN. Hyperoside's smaller structure of a single glycoside in comparison to rutin's disaccharide, resulted in a lower effective concentration for increasing mineral content and lowering pro-inflammatory cytokines, SOST and OPN. Further study is required to determine if increasing bioavailability of these compounds benefits bone health, as attaining these levels of rutin (25 μ M) and hyperoside (5 μ M)

would be likely only attained through supplementation. Findings from this study provide a rationale for future research investigating benefits of flavonoid consumption to promote bone health.

3.5 References

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Chapter 4.0: Rooibos flavonoids, orientin and luteolin, stimulate mineralization in human osteoblasts through the Wnt pathway

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Abstract

Several epidemiological studies have shown that tea consumption is associated with higher bone mineral density in women. Flavonoids in tea are recognized as potential estrogen mimics and may positively influence bone metabolism in estrogen deficient women. Luteolin and orientin, flavonoids from rooibos tea, are of particular interest as rooibos tea contains no caffeine that can be detrimental to bone health. This study analyzed changes in mineral content when luteolin or orientin was added to a human osteoblast cell line and the potential mechanisms involved. Measurements included alkaline phosphatase (ALP) activity, cell mitochondrial activity, toxicity, and changes in regulatory proteins involved in osteoblast metabolism. Mineral was significantly elevated in Saos2 cells treated with orientin (0.1-1.0 μ M, 15-100 μ M) or luteolin (5.0 μ M) and was associated with increased ALP activity and mitochondrial activity, as determined by the production of p-nitrophenol and the reduction of 2-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, respectively. Greater mineral content was also associated with lower toxicity as determined by lactate dehydrogenase activity and lower expression of TNF α , IL6, sclerostin, osteopontin and osteoprotegerin. Rooibos tea contains flavonoids that enhance mineral content in human osteoblasts and this is associated with higher osteoblast activity and reduced concentrations of TNF α , IL6, osteopontin and sclerostin.

4.1 Introduction

Peak bone mass, is attained by early adulthood and may set an individual on a trajectory for better bone health. Dietary strategies that promote bone development and mineralization are of particular interest as they provide a preventive strategy against fragility fractures [1]. It is generally accepted that individuals who achieve a higher peak bone mass will be at a lower risk of fragility fracture as they age [2]. Estrogen, established as a positive modulator of bone, promotes growth by balancing the activity of osteoblasts and osteoclasts [3]. The loss of estrogen is associated with a decrease in osteoblast differentiation, activation and proliferation, in addition to an increase in osteoclastogenesis, osteoclast activity and inflammation [3]. Pro-inflammatory markers also play a vital role in bone turnover, aiding in resorption [4,5] and are significantly increased in post-menopausal women [6,7].

With the increasing age of the general population, preventative measures are needed more than ever. It is currently predicted that osteoporosis will cost the United States over \$25 billion in 2025 due to more than 3 million fragility fractures per year [8]. While the majority of pharmaceuticals focus on inhibition of osteoclasts to slow bone resorption as a treatment for osteoporosis, it is also important to consider preventive strategies. Dietary strategies may ensure an individual reaches their peak bone mass and support bone health during later stages of life.

Many population studies that have been conducted throughout the world suggest a positive association between habitual consumption of tea and a lower risk of osteoporosis [9,10]. As the majority of the benefits are seen in women, estrogen-mediated pathways may play an important role in the ability of tea to mediate bone turnover. Tea is rich in

of either of these flavonoids would enhance mineral content in osteoblasts. Thus, the objective of this study was to determine if and at what concentration luteolin and orientin enhanced mineral content in a human osteoblast cell line. Secondary objectives were to determine how other aspects that influence mineral formation were altered – this involved measurements of ALP activity, cell mitochondrial activity, cell toxicity, and protein concentration of regulatory osteoblast markers including tumor necrosis factor alpha (TNF α), IL6, osteoprotegerin (OPG), osteopontin (OPN), and sclerostin (SOST).

4.2 Methods and Materials

Saos2 cells were purchased from Cedarlane. Luteolin and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Alfa Aesar. Orientin was purchased from Aktin chemicals. HAM-F12, PBS solution, antibiotic-antimycotic, fetal bovine serum (FBS), and trypsin were purchased from Lonza. Human Milliplex bone magnetic bead panels for bone metabolism were purchased from EMD Millipore. Remaining chemicals including Tox7 kit and epigallocatechin gallate (EGCG) >95% were purchased from Sigma-Aldrich.

Saos2 cells were cultured using HAM F-12 media with 10% FBS, 28mM HEPES buffer, 1.4mM CaCl₂, 2.0mM glutamine, and 1% antibiotic-antimycotic which was adjusted to pH 7.4 using 10.0M NaOH. Stimulation media (media 2) contained 10.0nM dexamethasone and 50 μ g/ml of ascorbic acid and seeded in 24 well plates. On day 8 media 2 was supplemented with 10mM β -glycerophosphate and treatment (media 3). Plated cells were grown until day 21, in which media was changed every other day. Flavonoids were dissolved in DMSO and diluted to a final concentration of 0.1%.

Controls consisted of cells in regular media, in addition to cells with 0.1% DMSO. On day 21 the cells were fixed in 4% paraformaldehyde and kept at 4°C.

4.2.1 Mineral content

Orientin (0.01µM - 100µM) or luteolin (5µM – 25µM) concentrations were tested to determine minimum and maximum effective concentrations. If the flavonoid demonstrated potential toxicity, further concentrations were not tested. Mineral was quantified using the alizarin red colorimetric assay on day 21. Paraformaldehyde was removed from the wells and cells were washed with PBS and dH₂O. Cells were stained with alizarin red (40mM, pH 4.2) for 20 minutes at room temperature while shaking. Cells were then washed and precipitated alizarin red was extracted over 1 hour at room temperature using a 10% cetylpyridinium chloride solution in 10mM sodium phosphate and quantified with absorbance spectrometry at 550nm [18].

4.2.2 ALP Activity

Cells were grown in 24 well-plates and were lysed at days 3, 7 and 10 into mineralization to assess ALP activity. Cells were washed and lysed using a 50mM Tris-HCl solution with 0.05% Triton X-100. Cells were then centrifuged at 1000g, 4°C for 15min. Lysates were introduced to p-nitrophenyl phosphate and monitored for 5 minutes in which 0.5M NaOH was used to stop the reaction. The OD was measured at 410nm and normalized to protein concentration [19].

4.2.3 Cell Mitochondrial Activity

Cell activity was measured by the reduction of MTT by cell mitochondrial reductase enzymes. Cells were plated in 96 well plates with media for 24 hours, and then

supplemented with flavonoid. Media was replaced every 24 hours and assessed every 24 hours for 3 days. Examination of cells involved media replacement with 150 μ l of MTT (5mg/ml) in PBS and placed in the incubator at 37°C for 4 hours. Crystallized formazan was dissolved with 40mM HCl-isopropanol and the OD was measured at 570nm and normalized to cell count using trypan blue [20].

4.2.4 Cell toxicity

Cellular toxicity was measured by quantifying the LDH present within the media using a Sigma-Aldrich LDH based toxicology kit (Tox7). Cells are seeded in 96 well plates and given 24 hours to adhere to plates which were followed by treatment. Media was assessed at 12, 24 and 48 hours using kit instructions. OD measurements are standardized to cell count using trypan blue.

4.2.5 Quantification of Regulatory Proteins in Osteoblasts

Protein expression of TNF α , IL6, SOST, OPG and osteopontin (OPN) were quantified using an ELISA based Millipore Magnetic Multiplex kit (HBNMAG-51). Cells were seeded and media was harvested at day 3 and 7 following the addition of media 3 and used for protein quantification. One concentration of orientin (20 μ M) and luteolin (5.0 μ M) was studied. The concentration that resulted in a significant increase in mineral content, ALP and cell activity, with no increase in cell toxicity was selected.

4.2.6 Statistics

Measurements of mineral content, ALP activity, cell activity and toxicity consisted of six plates per flavonoid. Each plate consisted of three wells per treatment, and each well was measured three times. Protein measurements were completed using

four flasks per flavonoid. Each flask was measured three times. Measurements are reported as mean \pm SEM.

Data analysis was completed by IBM SPSS Statistics 21 utilizing 1 way-ANOVA with treatment (rutin, hyperoside, control) as the independent variable for all measurements except protein. The dependent variable was expressed as a percent of control for mineral content, ALP activity, MTT reduced, and LDH released. For protein measurements, a 2-way ANOVA was utilized with treatment (orientin, luteolin and control) and time as main factors. Level of significance was determined by $p < 0.05$ and post-HOC analysis allowed for comparison between flavonoids ($p < 0.05$). All data was checked for homogeneity of variance. When homogeneity of variance was met ($p > 0.05$), post-HOC analysis was completed by Bonferroni t-test. Where homogeneity of variance was statistically different across groups ($p < 0.05$), post-HOC analysis utilized Games Howell t-test.

4.3. Results

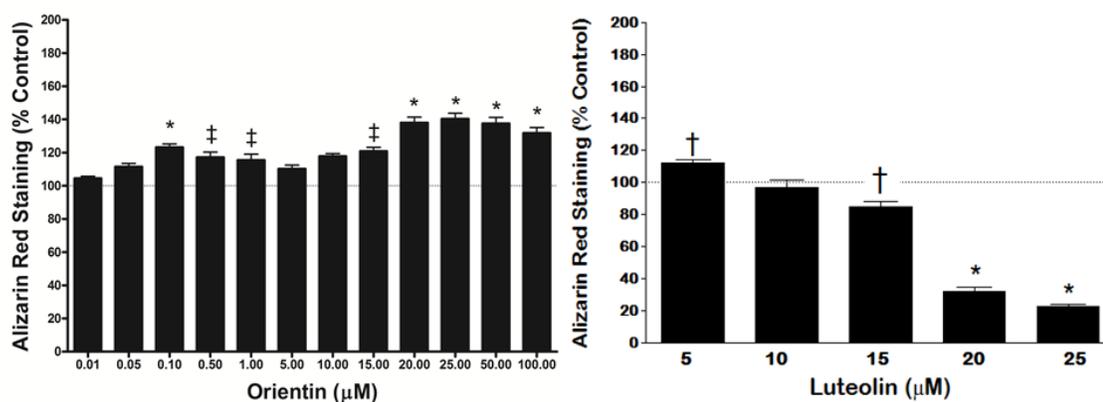


Figure 4.2. Mineral content produced by Saos2 cells with orientin and luteolin

Mineral was determined using the alizarin red assay. Mineral production as the result of

treatment with flavonoid was expressed as per cent control. † $p < 0.05$, ‡ $p < 0.01$, * $p < 0.001$ compared to control.

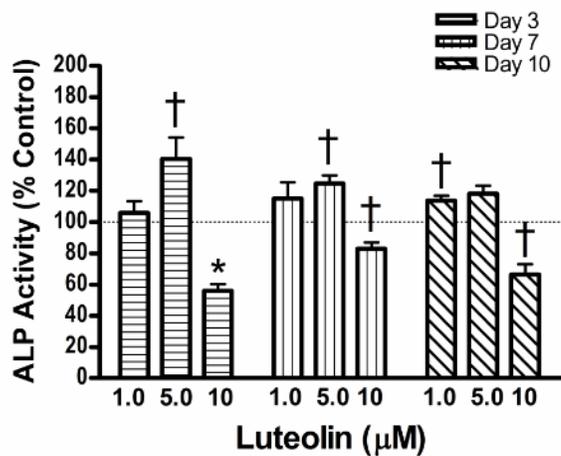
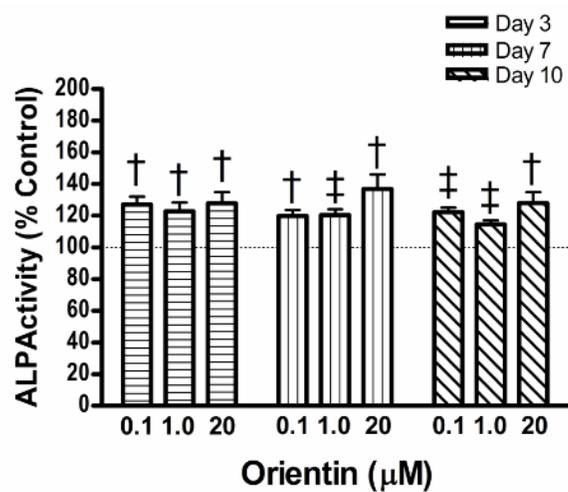


Figure 4.3. ALP Activity of Saos2 cells with Orientin and Luteolin

ALP activity was indicated by hydrolysis of p-nitrophenyl phosphate into p-nitrophenol and expressed as per cent control. † $p < 0.05$, ‡ $p < 0.01$, * $p < 0.001$ compared to control within that day.

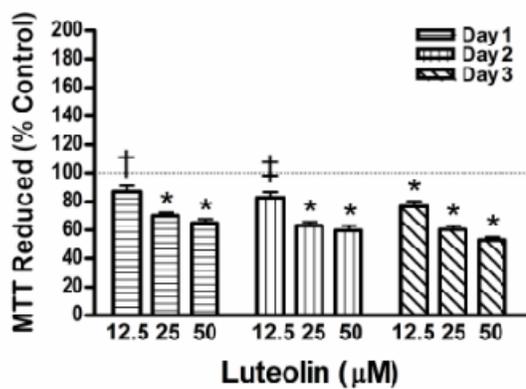
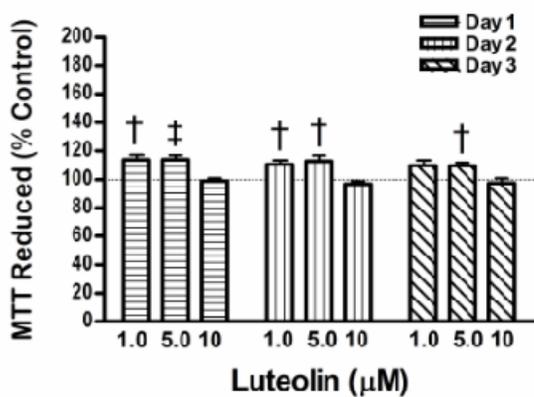
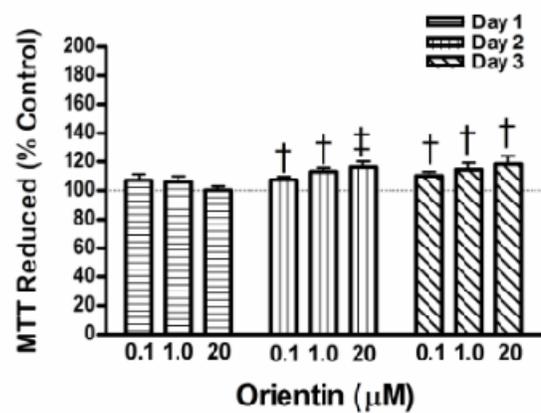


Figure 4.4. Cell activity of Saos2 cells with orientin and luteolin

Cell activity was measured by the reduction of MTT and expressed as per cent control. † $p < 0.05$, ‡ $p < 0.01$, * $p < 0.001$ compared to control within that day.

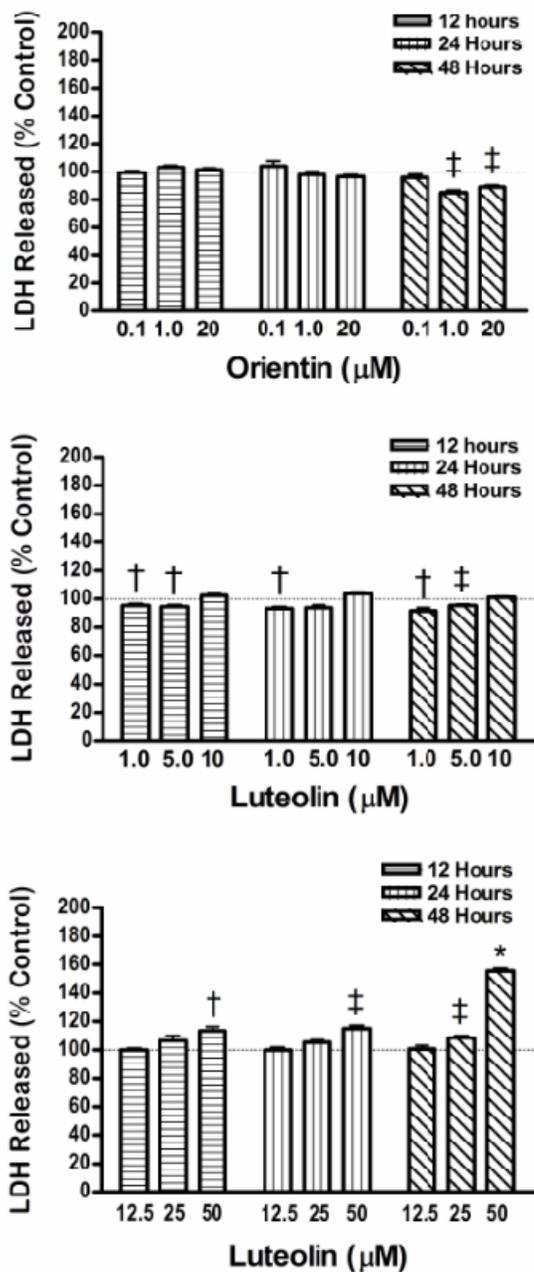


Figure 4.5. LDH released by Saos2 cells with orientin and luteolin

LDH was measured in cell media by Tox7 kits and expressed as per cent control. † $p < 0.05$, ‡ $p < 0.01$, * $p < 0.001$ compared to control within that day.

Table 4.1. Concentrations of regulatory proteins involved in osteoblast activity

	Average Protein Concentration									
	TNF α		IL6		OPN		OPG		SOST	
	Day 3	Day 7	Day 3	Day 7	Day 3	Day 7	Day 3	Day 7	Day 3	Day 7
Control	0.43 \pm 0.01	0.20 \pm 0.03a	3.24 \pm 0.08a	3.01 \pm 0.13	247.50 \pm 10.71	109.00 \pm 1.22	1659.25 \pm 107.78	1250.75 \pm 32.05	810.00 \pm 22.15	1287.25 \pm 29.25
20.0 μ M Orientin (pg/mL)	0.09 \pm 0.00*	0.17 \pm 0.00 \ddagger	0.50 \pm 0.02*	2.27 \pm 0.12 \ddagger	54.89 \pm 8.58*	87.01 \pm 1.94 \ddagger	166.25 \pm 4.70*	789.50 \pm 19.16*	358.00 \pm 10.46*	739.75 \pm 5.98*
pg/mL/mol ^a	-170	-15	-1,370	-370	-96,300	-10,995	-746,500	-230,625	-226,000	-273,750
5.0 μ M Luteolin (pg/mL)	0.13 \pm 0.00*	0.21 \pm 0.01	1.25 \pm 0.04*	2.59 \pm 0.09	137.50 \pm 7.10*	177.00 \pm 12.83*	1096.25b \pm 53.13 \ddagger	1172.50 \pm 48.33	599.00 \pm 56.73*	1887.00 \pm 55.52*
pg/mL/mol ^a	-600	+20	-3,980	-840	-219,000	+136,000	-1,126,000	-156,500	-422,000	+1,199,500

Data is expressed as mean \pm SEM

Significant differences in from control, are indicated by, \ddagger $p < 0.05$, \ddagger $p < 0.01$, * $p < 0.001$ within a column.

^a Indicates change in protein expression from control, normalized to the flavonoid treatment

4.3.1 Mineral content

Orientin treatment (0.10 μ M, 0.50 μ M, 1.0 μ M, 15.0 μ M, 20.0 μ M, 25.0 μ M, 50.0 μ M, 100 μ M) resulted in greater mineral content (Figure 4.2) than control ($p < 0.001$, $p < 0.01$, $p < 0.01$, $p < 0.01$, $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, respectively). Exposure to luteolin ranged from 5-25 μ M; concentrations greater than 25 μ M were not studied due to cellular death. Greater mineral content was seen at 5 μ M luteolin in comparison to control ($p < 0.05$). Luteolin at concentrations of 15 μ M, 20 μ M and 25 μ M

had less mineral in comparison to controls ($p < 0.05$, $p < 0.001$, $p < 0.001$, respectively) and demonstrated a dose-dependent decrease.

4.3.2 ALP Activity

To determine the effect of orientin and luteolin on ALP activity, concentrations that had significantly increased mineral content were studied (Figure 4.3). The addition of orientin (0.1 μ M, 1.0 μ M, 20.0 μ M) resulted in higher ALP activity ($p < 0.05$, $p < 0.05$, $p < 0.05$, respectively) at day 3, and was maintained at day 7 ($p < 0.05$, $p < 0.01$, $p < 0.05$, respectively) and day 10 ($p < 0.01$, $p < 0.01$, $p < 0.05$, respectively). 1 μ M luteolin was not different compared to control at days 3 and 7 ($p > 0.05$) but was higher at day 10 ($p < 0.05$). 5 μ M luteolin demonstrated higher ALP activity at day 3 and 7 ($p < 0.05$, $p < 0.05$, respectively) but not at day 10 ($p > 0.05$). A higher concentration of luteolin (10 μ M) had lower ALP activity at days 3, 7 and 10 ($p < 0.001$, $p < 0.05$, $p < 0.05$, respectively).

4.3.3 Cell Mitochondrial Activity

At 48 hours, orientin (0.1 μ M, 1.0 μ M, 20 μ M) resulted in higher levels of MTT reduced ($p < 0.001$, $p < 0.05$, $p < 0.01$, respectively) in comparison to control, and this was maintained at 72 hours ($p < 0.01$, $p < 0.01$, $p < 0.05$, respectively). After 24 hours of the initial flavonoid addition, luteolin (1.0 μ M, 5.0 μ M) had higher cell mitochondrial activity ($p < 0.05$, $p < 0.01$, respectively) in comparison to control, which was maintained until 48 hours ($p < 0.05$, $p < 0.05$, respectively). At 72 hours, only the 5.0 μ M dose of luteolin was higher than control ($p < 0.05$). Mitochondrial activity was lower in comparison to control when cells were treated with higher concentrations of luteolin (12.5 μ M, 25 μ M, 50 μ M) at 24 hours ($p < 0.05$, $p < 0.01$, $p < 0.01$, respectively), 48 hours

($p < 0.01$, $p < 0.001$, $p < 0.001$, respectively) and 72 hours ($p < 0.001$, $p < 0.001$, $p < 0.001$, respectively).

4.3.4 Cell Toxicity

After 12 and 24 hours of the initial flavonoid addition, orientin (0.1 μ M, 1.0 μ M, 20 μ M) did not demonstrate different levels of LDH ($p > 0.05$) in comparison to control (Figure 4.5). After 48 hours, orientin (1.0 μ M and 20 μ M) resulted in lower LDH release ($p < 0.01$, $p < 0.01$, respectively). After 12 hours of the initial dose of luteolin (1.0 μ M, 5.0 μ M), there was a decrease in LDH ($p < 0.05$, $p < 0.05$, respectively) in comparison to control. 24 hours after the initial addition of flavonoid, 1.0 μ M luteolin had lower levels of LDH ($p < 0.05$) than control. However, the second dose of luteolin (1.0 μ M, 5.0 μ M) resulted in lower levels of LDH ($p < 0.05$, $p < 0.01$, respectively) in comparison to control. Higher doses of luteolin (50 μ M) demonstrated an increase in LDH at 12 hours, and this was maintained at 24 hours ($p < 0.05$, $p < 0.01$, respectively) in comparison to control. After 48 hours, luteolin (25 μ M and 50 μ M) demonstrated a dose-dependent increase in LDH ($p < 0.01$, $p < 0.01$, respectively) relative to the control with 25 μ M of luteolin resulting in lower levels of LDH released from cells than 50 μ M luteolin ($p < 0.001$).

4.3.5 Quantification of Regulatory Proteins in Osteoblasts

20 μ M orientin and 5.0 μ M luteolin were used to assess concentrations of inflammatory and regulatory proteins involved in osteoblast metabolism (Table 4.1). 20 μ M orientin was used because it was not found to be statistically different from any of the higher doses of orientin ($\geq 25\mu$ M) in its ability to produce mineral nor was it toxic to

cells. 5.0 μ M of luteolin was used for these analyses as it was the only concentration of luteolin that resulted in greater mineral content compared to control.

IL6 ($p < 0.0001$), TNF α ($p < 0.0001$), OPG ($p < 0.0001$), OPN ($p < 0.0001$) and SOST ($p < 0.0001$), all demonstrated a significant interaction between treatment and time. There was a main effect of time for IL6 ($p < 0.0001$), TNF α ($p < 0.0001$), OPG ($p < 0.0001$), OPN ($p = 0.004$) and SOST ($p < 0.0001$). There was also a main effect of treatment for IL6 ($p < 0.0001$), TNF α ($p < 0.0001$), OPG ($p < 0.0001$), OPN ($p < 0.0001$) and SOST ($p < 0.0001$).

At day 3 into mineralization, orientin had lower levels of IL6 ($p < 0.001$), TNF α ($p < 0.001$), OPG ($p < 0.001$), SOST ($p < 0.001$) and OPN ($p < 0.01$) in comparison to control. Luteolin (5 μ M) had lower levels of IL6 ($p < 0.001$), TNF α ($p < 0.001$), OPG ($p < 0.01$), SOST ($p < 0.001$), and OPN ($p < 0.001$) in comparison to control. At day 7 into mineralization, orientin had lower levels of IL6 ($p < 0.01$), TNF α ($p < 0.05$), OPG ($p < 0.001$), SOST ($p < 0.001$) and OPN ($p < 0.01$) while treatment with luteolin resulted in no differences in expression of TNF α , IL6, and OPG ($p > 0.05$) compared to control. Luteolin had higher levels of OPN ($p < 0.001$) and SOST ($p < 0.001$) in comparison to control at day 7 of mineralization.

When mineral content at 20 μ M of orientin was compared to 5.0 μ M luteolin, orientin was shown to have higher ($p < 0.0001$) levels of mineral ($143.17\% \pm 2.27$ vs $112.17\% \pm 3.63$, expressed as per cent relative to control). Therefore, as mineral is the primary outcome of this study, and the dose of orientin is higher than the dose of luteolin, direct comparisons of protein measurements between the two bioactives may be skewed

because of this. To understand which flavonoid resulted in a greater change in protein expression in comparison to the control, protein concentration (denoted as C) were normalized (see equation 1) by calculating the change in protein concentration from control as a function of the flavonoid dose (per mol of flavonoid).

$$\frac{[C_{control}(\frac{pg}{ml}) - C_{flavonoid}(\frac{pg}{ml})]}{\text{moles of flavonoid}} = \Delta \text{protein expression} \left(\frac{pg}{ml * mol} \right)$$

Eq. [1]

This calculation demonstrates that the addition of luteolin by day 3 was shown to suppress expression of TNF α , IL6, OPN, OPG and SOST to a greater extent than orientin (Table 4.1). By day 7, treatment with orientin was shown to suppress the expression of TNF α , OPN, OPG and SOST to a greater extent than luteolin.

4.4 Discussion

We have shown that mineral content is enhanced through the addition of orientin (0.1-1.0 μ M, 15-100 μ M) and luteolin (5.0 μ M) to Saos2 cells over 21 days. However, the lower mineral content with higher doses of luteolin (15-25 μ M) relative to control suggests potentially harmful effects of luteolin at elevated levels. Increased levels of mineral were also the result of the ability of orientin and luteolin to increase ALP activity and cell mitochondrial activity, reduce cell toxicity, and decrease TNF α , IL6, OPN and SOST. Luteolin at concentrations >10 μ M reduced ALP activity and mitochondrial activity while increasing cell toxicity, all of which complemented the lower mineral content.

Orientin has not been previously studied in any bone cell culture systems while luteolin has been studied in mouse osteoblasts but not in a human bone cells. In mouse

osteoblasts (Mc3t3-E1 cells), 0.1 μ M luteolin increased ALP activity and collagen formation through interactions with estrogen receptors [21]. Furthermore, administration of luteolin (0.3-40 μ M) to murine bone marrow inhibited the process of resorption of osteoclasts with an EC_{50} value of 2.5 μ M [22]. Thus, combined findings of our study and previous studies in mouse bone cells suggest that luteolin may benefit bone turnover by increasing osteoblast activity and also limiting the activity of osteoclasts.

Saos2 cells with orientin (0.1 μ M – 20 μ M) or low concentrations of luteolin (1.0 μ M, 5.0 μ M) had greater mitochondrial activity and lower LDH released. Higher doses of luteolin (12.5-50 μ M) resulted in a decrease in cell activity immediately and this effect was sustained. This lower cell activity was accompanied with an increase in LDH release, suggesting that high doses of luteolin are toxic to Saos2 cells. Prior research has shown that kaempferol, a flavonoid found within tea, when added to rabbit osteoclasts would increase apoptosis in a dose dependent manner [23], although, when added to human osteoblasts, kaempferol stimulated mineralization and ALP activity, without changing cellular activity [24]. But, MTT measurements were only assessed over a single dose of flavonoid and therefore changes may only be evident after exposure to multiple doses. Similar to findings of the present study, icariine (20 μ M), a diglucoside of kaempferol, was found to increase mineral content and mitochondrial activity in human osteoblast cells [25].

There was a significant interaction between time and treatment for $TNF\alpha$, IL6, OPG, OPN and SOST, showing that flavonoids have different effects depending on the stage of mineralization. At day 3 into mineralization, both orientin (20 μ M) and luteolin (5.0 μ M) suppressed expression of pro-inflammatory cytokines $TNF\alpha$ and IL6. Orientin

reduced TNF α and IL6 by approximately by 79% and 85%. Luteolin decreased TNF α by 70% and IL6 by 61%. By day 7, TNF α and IL6 remained lower in orientin treated cells, whereas cells treated with luteolin had levels of TNF α and IL6 equivalent to control. This finding suggests that luteolin may initially have a greater effect than orientin. Yet, orientin may demonstrate a cumulative effect and be able to suppress the expression of pro-inflammatory markers for longer periods of time. Both luteolin and orientin may act as antioxidants and thereby may control reactive oxygen species that are produced as the result of cellular stress and damage as they can translate to an increase in pro-inflammatory markers [26]. Both estrogen and flavonoids are recognized for their ability to scavenge reactive oxygen species and limit the production of inflammatory markers [27]. Inflammatory markers can increase osteoclastogenesis [28], osteoclast activity [29] and inhibit osteoblast differentiation and activity [30,31]. Previous research in which rooibos tea was given to seven-week old Wistar rats for 1 month demonstrated an increase in superoxide dismutase, an enzyme important for controlling oxidative stress, and a decrease in DNA oxidative damage as determined by urine 8-hydroxy-2'-deoxyguanosine [32]. Work with rat and mouse macrophages has shown that pretreatment with luteolin lowers TNF α and IL6 expression when cells were exposed to LPS [33]. This suggests that luteolin can down-regulate inflammatory markers in both normal and challenged situations, and that the benefits seen within the study presented here with suppressing inflammatory cytokines using dietary components could be beneficial in limiting osteoclasts and improving osteoblast activity.

Orientin and luteolin were found to down regulate the production of OPG in Saos2 cells early into mineralization. However, by day 7, the concentration of OPG with

exposure to orientin remained lower whereas there was no difference in concentration of OPG with luteolin compared to control. The lower concentration of OPG early in mineralization at day 3 may be due to the model used as the Saos2 system is a transformed cell line [34]. Osteosarcomas naturally exhibit extremely high levels of OPG due to its ability to inhibit apoptosis in cancer [35,36]. Moreover, previous work has shown that OPG levels in cancerous cells are positively linked to levels of pro-inflammatory cytokines such as TNF α [37]. Therefore changes in OPG expression may be the result of changes in pro-inflammatory markers. We observed that both orientin and luteolin down-regulated TNF α and IL6 at day 3 into mineralization and thus it follows that OPG was also lower. This relationship is demonstrated again at day 7 when concentrations of pro-inflammatory markers (IL-6, TNF α) are similar to control with luteolin whereas orientin maintains significantly lower levels of TNF α , IL6 and OPG.

Orientin and luteolin reduced OPN by 78% and 44% and SOST by 56% and 26%, in comparison to control at day 3 into mineralization. By day 7, orientin maintained lower expression of OPN and SOST by 20% and 43%, respectively. In contrast, luteolin treatment by day 7 had higher levels of OPN and SOST (62% and 47%, respectively) in comparison to control. OPN functions to increase osteoclast adhesion to bone matrices, acts as a chemo-attractant to attract pro-inflammatory cytokines and stimulates proliferation and motility of osteoclasts [38]. Once bound to bone, OPN can also prevent further production of hydroxyapatite [38]. SOST inhibits bone formation by disallowing binding of the glycoprotein Wnt to the frizzled protein receptors and low density lipoprotein related receptors 5/6 to stimulate the Wnt conical pathway. If Wnt cannot bind to the receptors, β -catenin is tagged for destruction and gene expression of favorable

osteoblast proteins is suppressed [39]. Similar to orientin and luteolin, earlier work with a citrus flavonoid, hesperetin, has shown that the addition of this flavonoid to rat osteoblasts results in lower levels of OPN, while also increasing differentiation of osteoblasts via the bone morphogenetic protein pathway [40]. Conversely, the addition of ipriflavone, a synthetic isoflavone, to human osteoblasts increased mineral content and ALP activity while also increasing OPN mRNA [41]. However it is not currently known whether the addition of ipriflavone translated to an increase in OPN protein. Down-regulation of OPN and SOST by orientin and luteolin, as observed in this study, is a beneficial strategy for preventing bone loss as it aids in lowering osteoclast function while also increasing the activity of osteoblasts and overall mineral formation.

Structure-function relationships are important to determine how slight structural changes in a flavonoid may result in an increase in osteoblast activity. Orientin is an 8C-glucoside of luteolin. Our findings suggest that the presence of the glucose affords orientin with a wider range of effective concentrations at increasing mineral content without increasing cellular toxicity. This change in structure also allows for orientin to remain an effective stimulator for osteoblast activity over a longer duration in comparison to luteolin. Although the parent compound, luteolin, exhibits an increase in mineral at 5.0 μ M, the mineral content is lower than with orientin. Luteolin also results in lower mineral content at high (>10 μ M) concentrations and an increase in cell toxicity. However, when the concentrations of regulatory proteins were normalized to the dose of orientin and luteolin used, luteolin has a more potent effect early into mineralization (day 3). But, orientin maintains lower levels of pro-inflammatory markers, SOST and OPN by day 7 while luteolin does not. This highlights the importance of examining time course

effects, and may contribute to why orientin successfully produces more mineral in comparison to luteolin. This measurement, indicated by difference in protein concentration from control, highlights that although luteolin is potentially toxic at higher concentrations, the smaller structure of luteolin may bind more effectively to estrogen receptors to initiate a change at lower doses in the short term while orientin has longer-term stability.

With regards to bioavailability, orientin exists at higher concentrations than luteolin (0.02mg/g *versus* 2.34 mg/g) in unfermented tea and is detectable in plasma after consumption of rooibos tea but luteolin was not measured in this study [42]. Luteolin glucosides have been detected in human plasma after ingesting extracts containing mono- and di-caffeoylquinic acids and caffeic acid combined with luteolin glycosides [43]. Rats given tablets of luteolin or luteolin 7-O- β -glucoside had measurable levels of both free and metabolite (methyl and sulfate metabolites) forms of luteolin [44]. The bioavailability of luteolin has also been enhanced through conjugation to propyleneglycol [44]. For these reasons, it may be possible for luteolin to enter plasma with supplementation, and have its bioavailability improved upon via chemical modification. It is currently unknown how the bioavailability of these flavonoids changes with the consumption of food, as ingestion of food may enhance bioavailability through synergistic effects with other bioactives. Moreover, both flavonoids are found in various food and beverages (such as olives, artichokes, nuts and tea) [45,46,47] and therefore may be accumulated in plasma after ingestion of a variety of different foods.

In conclusion, orientin (0.1 μ M -1.0 μ M, 15 μ M -100 μ M) and luteolin (5.0 μ M) increase mineral content in Saos2 cells after 21 days in culture. High concentrations of

luteolin (>10 μ M) resulted in reduced mineral content, lower mitochondrial activity and higher levels of cellular toxicity. Higher mineral content with addition of orientin (20 μ M) or luteolin (5.0 μ M) was associated with higher ALP and mitochondrial activity, lower toxicity, lower pro-inflammatory cytokines and lower SOST and OPN. The lack of a sugar moiety on luteolin demonstrated a better ability to down-regulate levels of OPN, SOST, and pro-inflammatory markers in comparison to orientin early into mineralization. However, by day 7, only treatment with orientin maintained lower levels of TNF α , IL6, SOST and OPN. Higher concentrations of luteolin were also determined to be toxic to Saos2 cells. This research suggests that orientin and luteolin, flavonoids that are abundant in rooibos tea, have the ability to stimulate osteoblast activity and increase mineralization and may thereby be useful for supporting bone health.

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Chapter 5.0: General Discussion

The addition of rutin ($\geq 25 \mu\text{M}$), hyperoside ($\geq 5.0 \mu\text{M}$), orientin ($\geq 0.1-1.0, 15.0-100 \mu\text{M}$) and luteolin ($5.0 \mu\text{M}$) – all flavonoids in rooibos tea - were shown to increase mineral content in Saos2 osteoblast-like cells. This increase in mineral content was partially due to an increase in ALP activity and cell mitochondrial activity, with no toxic effects as determined by release of LDH into cell media. Protein measurements indicated that the addition of these rooibos flavonoids resulted in a significant decrease in markers that promote bone resorption (SOST, OPN), pro-inflammatory markers (TNF α and IL6). Reduction in OPG is believed to be an artifact of the model chosen. Therefore, the flavonoids found in rooibos tea may provide a beneficial dietary strategy for prevention of osteoporosis in post-menopausal women. Although levels of flavonoids tested in this cell model would be likely best achieved through supplementation, there may be potential synergistic effects found within tea that promote the higher BMD observed in epidemiology studies.

Previous work with flavonoids has utilized cell systems to predict their ability to increase mineral content *in vivo*. *In vitro* studies are important for setting the ground work to understand how flavonoids and other bioactives interact with osteoblasts and osteoclasts at the molecular level. Epigallocatechin gallate (EGCG), a flavonoid found in green tea ($5 \mu\text{M}$), has demonstrated an ability to increase mineral content in Saos2 cells as the result of an increase in ALP activity, and a decrease in RUNX2 expression [1]. When mouse osteoclasts were pretreated with EGCG ($20 \mu\text{M}$), osteoclast formation was

significantly inhibited, even in the presence of pro-inflammatory stimulating factors such as IL1 and TNF α [2]. When the same level of EGCG (20 μ M) is added to mouse osteoblasts, RANKL nor OPG production changed [2]. However, studies have also shown that whole polyphenol extract from green tea induces apoptosis in Saos2 cells through activation of caspase-3 and caspase-8 [3]. This may be due to the addition of polyphenols at extremely high concentrations, leading to cell death activated pathways.

Other studies involving whole tea extract have shown that treatment of normal human osteoblasts with black or green tea polyphenol extract resulted in an increase in mineral content and ALP activity. Black tea polyphenol extract stimulated ALP activity at 5-50 μ g/ml, and prevented TNF α suppression of ALP activity. Green tea stimulated ALP activity only at low doses (5 μ g/ml). Therefore both black and green tea polyphenol extracts, both were able to increase mineral content and ALP activity [4]. This accumulation of work suggested that *in vitro*, green tea polyphenol extract and black tea polyphenol extract were both effective means for inducing mineralization by increasing osteoblast activity, while limiting the activity of bone resorption.

Work accomplished by *in vitro* studies is required to be tested *in vivo*, to ensure that the same, desired effect occurs in a living system, without any toxic effects. Although we know little about the *in vivo* effects of rooibos tea, we can learn from *in vivo* studies that have been done using green and black polyphenols. Previous work in which EGCG (3.4mg/kg/day) given to ovariectomized rats for 3 months was shown to be effective *in vivo* as it prevented bone loss as shown by maintaining a higher BMD, trabecular thickness, trabecular number, and bone volume [5]. Another study analyzed bone outcomes in ovariectomized and sham rats given green tea polyphenols (0.1% or

0.5%) for 4 months and found that bone mineral content and BMD were higher in sham rats given 0.5% green tea polyphenols in comparison to sham controls. Additionally, in the ovariectomized models, BMD was higher in rats given either dose of green tea in comparison to control [6]. This suggests that green tea polyphenols could play an active role in mitigating bone loss. As green tea polyphenols demonstrate an ability to increase favorable bone outcomes such as BMD, trabecular thickness, trabecular number, bone volume, ALP activity, tea is seen as a potential dietary strategy for prevention of osteoporosis. As rooibos tea contains comparable levels of flavonoids, there is good reason to suspect that the beneficial *in vitro* effects described in this study using rooibos tea will translate well into *in vivo* models. However, this will still need to be addressed in future animal studies.

But the question remains of how green tea polyphenols, along with flavonoids from other tea such as rooibos tea, translates to human studies, as humans have a more complex metabolism. Interestingly, a recent intervention involving postmenopausal osteopenic women has shown that oral ingestion of a green tea polyphenol supplement (500 mg/day) can increase ALP activity as early as 1 month [7]. This study suggests that green tea polyphenols can promote positive changes in bone physiology in clinical settings. Therefore, potentially through the action of polyphenols, the habitual consumption of tea may be a beneficial, proactive method for maintaining healthy, strong bones.

As potential estrogen mimics, flavonoids are currently believed to be the cause of these positive changes in post-menopausal models, due to the lack of endogenous estrogen. Therefore by acting as an estrogen mimic, these flavonoids can increase gene

expression of favorable bone outcomes that positively influence mineral content such as ALP activity and RUNX2. Due to the flavonoids having a structural identity that resembles estrogen, these flavonoids, like estrogen, can act as an antioxidant. Antioxidants help protect osteoblasts and limit the activity of osteoclasts, thereby reinforcing a healthy balance for bone turnover. Rats given 0.5% green tea polyphenols for 4 months have demonstrated a 1.3 fold increase in superoxide dismutase, an enzyme important for eliminating reactive oxygen species. This study also demonstrated that rats given the green tea polyphenols had approximately 1.4 fold decrease in catechol-O-methyltransferase, an enzyme which deactivates estrogens [8]. High levels of cellular stress lead to increased levels of reactive oxygen species, which have been linked to significantly lower BMD [8]. Therefore, identification of these molecular pathways, could lead to all-natural, dietary options that allow for prevention of osteoporosis.

5.1 Potential pathways for the mechanism of rooibos flavonoids effects

Rutin, hyperoside, orientin and luteolin demonstrate the ability to significantly decrease SOST, a Wnt pathway inhibitor (Figure 5.1). The Wnt pathway is important for regulation of bone markers such as RUNX2, a transcription factor that controls osteoblast differentiation and proliferation; and ALP expression, an enzyme important for mineralization. Previous studies have shown that stimulation of the Wnt pathway has been associated with an increase in osteoblast differentiation, proliferation and activity; while inhibiting the pathway with proteins such as SOST can substantially decrease these events [9]. SOST in particular, serves to prevent binding of Wnt to the frizzled receptors

and the LRP5/6. If the binding of Wnt does not occur, a protein complex consisting of GSK-3 β , APC, and Axin will signal for the degradation of β -catenin, thereby preventing β -catenin's translocation to the nucleus and halting gene transcription. As rutin, hyperoside, orientin and luteolin demonstrate an ability to significantly decrease SOST, we also see an increase in ALP activity, mineralization, and cellular activity.

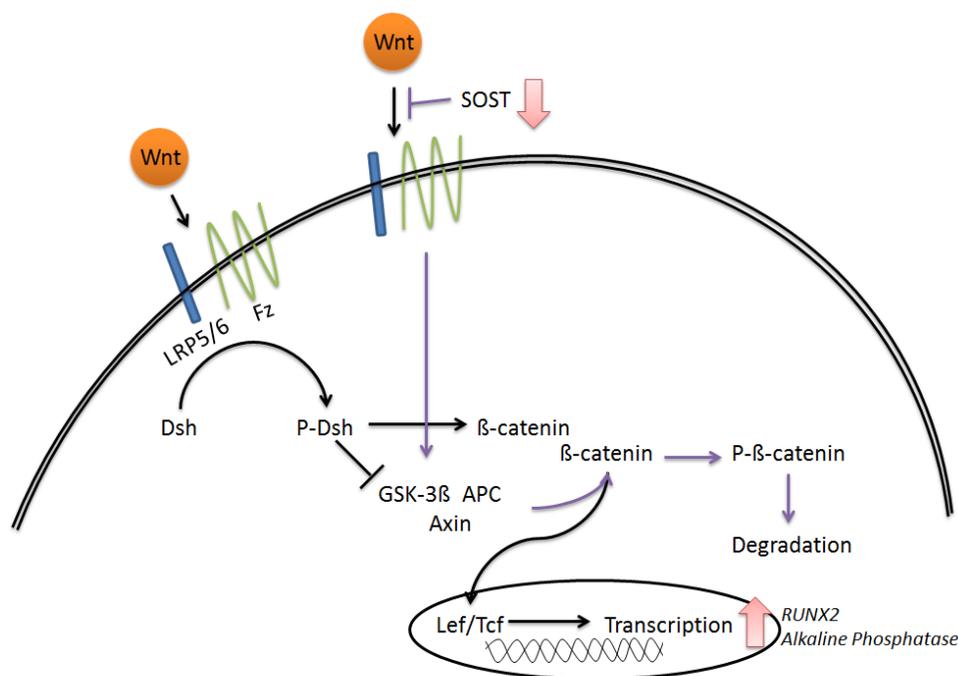


Figure 5.1 Changes in Wnt pathway by rooibos flavonoids. Pink arrows indicate changes in SOST and ALP expression by the addition of rooibos flavonoids.

At the same time that rooibos flavonoids rutin, hyperoside, orientin and luteolin had decreased SOST (aiding in the increase in ALP activity and cell mitochondrial activity), these flavonoids also significantly lowered the production of pro-inflammatory cytokines TNF α and IL6 (Figure 5.2). Reduction of these pro-inflammatory cytokines can aid in mineralization through the reduction of Wnt inhibitors such as SOST. Lower levels

of pro-inflammatory cytokines also aids in the reduction of RANKL, important for osteoclast activation. Previous work has shown that levels of TNF α and IL6 have been negatively linked to osteoblast differentiation and mineralization [10]. Pro-inflammatory cytokines are also recognized for their ability to increase osteoclast activity, by increasing the production of proteases [11] and tartrate resistant acid phosphatase [12], both of which aid in mineral degradation and release. Moreover, high levels of TNF α and IL6 have been associated with increased levels of osteoclastogenesis [13]. Therefore it is beneficial to identify dietary components such as flavonoids from rooibos tea, which reduce inflammatory markers and increase mineral content.

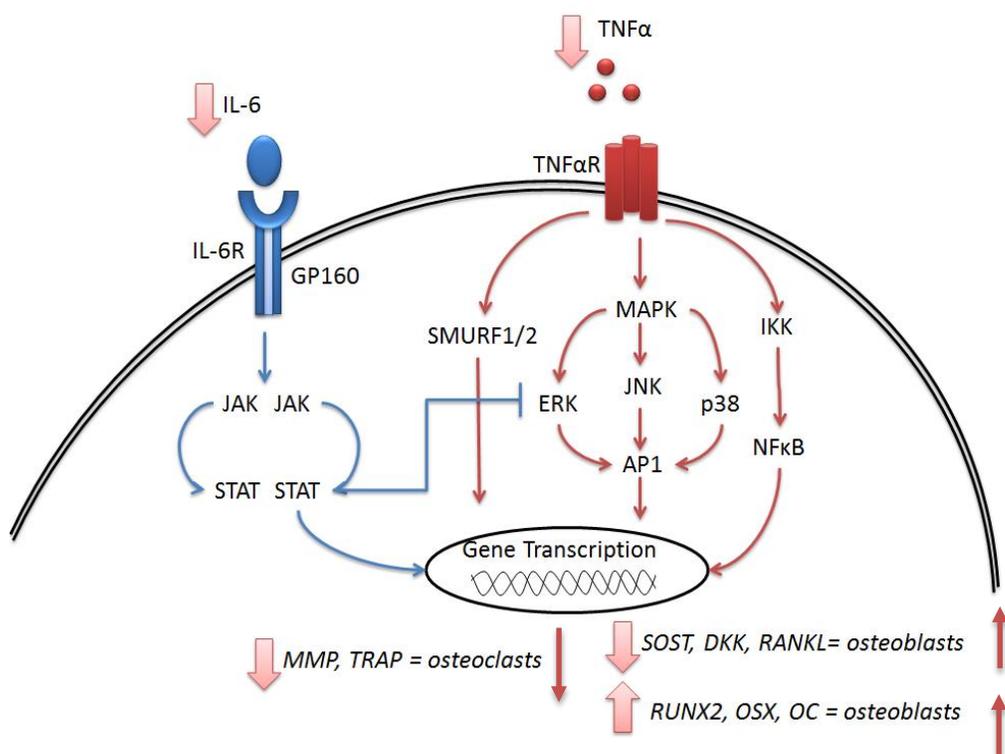


Figure 5.2 Changes in bone markers by decreased inflammation via rooibos

flavonoids. Pink arrows indicate changes in SOST and ALP expression by the addition of rooibos flavonoids.

5.2 Bioavailability of flavonoids

Bioavailability of flavonoids is important to induce biological changes through dietary means. Both rutin and orientin are found in plasma after consumption of rooibos tea, where hyperoside and luteolin was not tested for [14]. Hyperoside has been detected in plasma, but is eliminated quickly [15]. Luteolin has been found in plasma, both in its free form, and conjugated to glycosides [16]. Moreover, improvements have been reported on bioavailability through chemical modification of these flavonoids and encapsulation [16, 17]. It is likely that the consumption of rutin, hyperoside, orientin and luteolin would require supplements to achieve levels that were effective in inducing mineralization. However, these flavonoids also may be more readily available when consuming multiple flavonoids at once in food matrices, due to possible synergistic effects.

It is also important to recognize that bioavailability of flavonoids, among other polyphenols, varies depending on the microbiota that exists within an individual. Apigenin is a flavonoid found in many vegetables and beverages including tea and wine. When one of apigenin's glycosides, apigenin-7-glucoside, was incubated with fecal microbiota from humans, different metabolites were found in rats, and even more-so in comparison to germ-free rats [18]. Moreover, when black tea and red wine polyphenols were incubated with different human fecal microbiota samples, there were differences across each individual regarding the type of metabolites produced, the levels at which the metabolites were produced, and the time at which the metabolites were produced. This is believed to be due to the dissimilarity in microbial composition and variation in dietary lifestyles [19]. Microbiota largely dictates the distribution, bioavailability and bioactivity

of polyphenols such as flavonoids, and therefore plasma levels of flavonoids can fluctuate immensely from one individual to another. Thus the microbiota present and the species of the model of interest are both important considerations when testing the bioavailability of particular dietary components.

5.3 Structure-Function Relationships

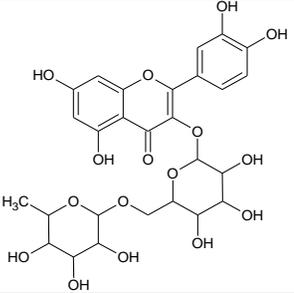
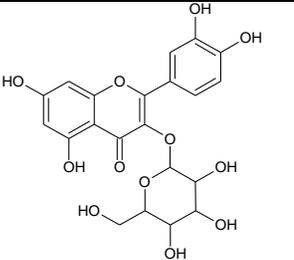
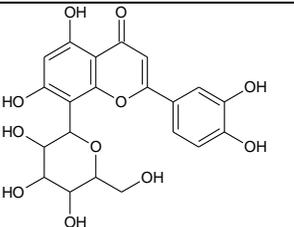
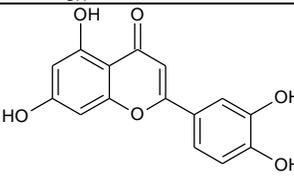
Rooibos flavonoids demonstrate an ability to increase mineral content at varying concentrations. When considering the flavonoids (rutin, hyperoside and orientin) that does not promote cellular death, the larger the molecular compound, the higher the minimum effective concentration. Rutin, the largest flavonoid, contains an extra 7 carbons, 10 hydrogens and 4-5 extra oxygens in comparison to hyperoside and orientin.

Rutin and hyperoside contain very similar structures, in which both rutin and hyperoside contain the quercetin backbone. The difference between rutin and hyperoside is the addition of either a disaccharide (rutin) or a galactoside (hyperoside) off of carbon 3 from the backbone. The benefit of this arrangement is that it allows both compounds to have the same side of the backbone to be exposed, and the same side to be crowded by their glycosides. When directly comparing these two flavonoids, the smaller flavonoid, hyperoside, contains a lower effective concentration.

Directly comparing the two quercetin metabolites, rutin and hyperoside, the smaller hyperoside demonstrates a more potent effect at lowering pro-inflammatory markers $\text{TNF}\alpha$ and IL6, when protein levels are normalized to the amount of flavonoid

used (per mole basis, see Chapter 3). When comparing all flavonoids that do not contribute to cell death, orientin also demonstrates the greatest potential at lowering pro-inflammatory cytokines. This may be due to the flipped position of orientin's backbone, exposing the ketone and hydroxyl subunits, allowing for more areas of interaction with the estrogen receptor. This flipped position, also seems to be beneficial for lowering OPN, SOST and OPG, perhaps suggesting that this position of the backbone is more effective at binding to receptors due to the increase in potential interactions with the newly freed electron acceptors and donators. However, using a flavonoid such as luteolin, which is not sterically hindered by the addition of a sugar moiety, is beneficial for increasing mineral content at low concentrations, in addition to lowering pro-inflammatory markers, OPN and SOST – potentially allowing it to more easily interact with the estrogen receptor. On the other hand, at slightly higher levels, luteolin prevents mineralization and is shown to be toxic to Saos2 cells, potentially through activation of cell death cascades. Therefore the addition of a sugar such as glucose is beneficial in that it allows for a larger range of effective concentrations at inducing positive influence in bone mineralization, but too large of a compound results in a decrease in activity.

Table 5.1 Rooibos flavonoids and their corresponding Saos2 regulatory protein measurements

Flavonoid & EC used	Structure	Formula & MW (g/mol)	Min. EC	TNF α & IL6 (pg/mL)	OPN (pg/mL)	OPG (pg/mL)	SOST (pg/mL)
Rutin (50.0 μ M)		C ₂₇ H ₃₀ O ₁₆ 610.52	25.0 μ M	0.20 \pm 0.02 1.36 \pm 0.09	126 \pm 10.78	431.25 \pm 11.37	572.00 \pm 19.07
Hyperoside (25.0 μ M)		C ₂₁ H ₂₀ O ₁₂ 464.38	5.0 μ M	0.25 \pm 0.01 1.90 \pm 0.06	146.0 \pm 2.75	709.25 \pm 25.28	542.75 \pm 10.01
Orientin (20.0 μ M)		C ₂₁ H ₂₀ O ₁₁ 448.38	0.1 μ M	0.09 \pm 0.00 0.50 \pm 0.02	54.9 \pm 8.58	166.25 \pm 4.70	358 \pm 10.46
Luteolin (5.0 μ M)		C ₁₅ H ₁₀ O ₆ 286.24	5.0 μ M*	0.13 \pm 0.00 1.25 \pm 0.04	138.0 \pm 7.10	1096 \pm 53.13	599 \pm 56.73

Values represented as mean \pm standard error mean. EC: effective concentration.

* lower concentrations were not tested due to cellular death

5.4 Study strengths and limitations

Strengths: There is strong evidence from epidemiological studies suggesting that habitual tea consumption improves BMD and some studies show that habitual consumption of tea is linked to a reduced risk of fracture. Tea has high concentrations of estrogen mimics known as flavonoids. These flavonoids carry large antioxidant capacity and are believed to be producing a biological effect through activation of estrogen receptors. Research presented in this study suggests that rooibos flavonoids rutin, orientin, hyperoside and luteolin have the potential to modulate osteoblast metabolism to increase mineral content in the Saos2 cell culture system.

The Saos2 cell system is frequently used to study the interactions that occur between dietary components and osteoblasts, in an effort to identify bioactives that can increase mineral content. Saos2 cells differentiate in a manner which resembles normal human osteoblasts [20], while also containing receptors found in normal human osteoblasts, such as parathyroid hormone, vitamin D, estrogen, androgen and progesterone receptors [21,22,23]. Additionally, the Saos2 cell system offers an inexpensive, well-controlled, *in vitro* model, which may be manipulated using methods such as siRNA to assess individual pathways. Saos2 cells are noted for its phenotypic instability [24], in which enzyme activity is altered with passage number. Still, this can be accounted for by using multiple controls at the same passages at which the experimental conditions exist. Due to its ability to endlessly proliferate, this cell system has allowed for larger sample sizes, while still using a human cell model. Another benefit to using this cell line includes the ability to measure the change in specific osteoblast markers as a

result of flavonoid addition, allowing the prediction of potential pathways in which tea flavonoids interact with.

Limitations: There are several limitations to using a cell model, and more so, an osteosarcoma cell model. Saos2 cells come from a human osteosarcoma cell line [25], and thus it is important to recognize changes in its machinery. For example, osteosarcomas have extremely high levels of OPG expressed [26]. OPG is an important measure in bone metabolism as it serves as a decoy for RANKL, however in cancer, it serves as a decoy for tumor necrosis factor related apoptosis ligand (TRAIL) [27]. Therefore it is imperative to reassess the changes in OPG as the result of the addition of a flavonoid in a normal human osteoblast model. Furthermore, this cell model represents only one half of what happens during bone turnover. Saos2 cells in this study are differentiated into osteoblasts, cells that build bone. Therefore it is important to measure the effects of these rooibos flavonoids in an osteoclast cell culture. This is necessary to ensure that the addition of rooibos flavonoids do not increase osteoclast activity. Lastly, as isolated system, cell models do not provide metabolism information that *in vivo* experiments would, and this is important as flavonoids can be modified as they cross the small intestine and as they enter plasma.

5.5 Study Knowledge Gaps and Future Research

Flavonoids found in larger concentrations within tea, that are easily quantified through analytical techniques are of more interest due to their likelihood of being detected in plasma. More work is required to identify how the bioavailability of

flavonoids changes with/without consumption of a meal, and how that varies between consumption from tea and as a supplement. Furthermore, it would be beneficial to test the flavonoids individually as well as produced in a mixture of other flavonoids to examine the potential for additive or synergistic effects in a biological system. Timing of the dose is important for understanding how often an individual may need to consume tea, or ingest supplements for them to increase BMD. Timing investigations should include, whether habitual consumption requires the consumption of more than one cup a day, how many times a week, and whether supplementation at increased levels of the desired flavonoids result in lower consumption requirements.

As many of these compounds resemble each other's structures with slight differences in position of hydroxyl or sugar components, changes in activity from one compound to the next will allow for a better understanding of how chemical structure may influence the regulation of important genes in bone physiology. Identification of important areas on a specific flavonoid structure may aid in finding other dietary components which could positively influence bone health. Moreover, it is important to understand that interactions between flavonoids and receptors occur at all areas of the body and not just the bone, therefore it is important to also assess the effects that these flavonoid concentrations have in other tissues.

Habitual consumption of tea is suggested to have beneficial effects on bone health, primarily in women who are post-menopausal. These potential benefits of habitual tea consumption are believed to be an increased BMD but it is largely unknown how tea consumption translates to fracture. More data is needed on how tea consumption relates to fracture incidence, and more specifically, which type of tea and what levels of

consumption are most beneficial. The effects of tea consumption are generally seen within women, and lost when consumed by men. This is believed to be due to the fact that men produce estrogen through an aromatization of testosterone, and thus do not necessarily go through the extreme estrogen deficiency as women do through menopause, but instead, have a slow decline in overall testosterone as age progresses. Therefore it may be beneficial to compare BMD at different age populations of males who habitually consume tea, those in their younger years around peak bone mass, and the elderly to test for age specific effects. It is evident that with an increase in tea sales and the mass availability of herbs to produce tea that any health benefits tea can provide will have world-wide impact and therefore is an important area to investigate when looking for dietary strategies to improve bone health.

6.0 Conclusions

The flavonoids rutin ($\geq 25\mu\text{M}$), hyperoside ($\geq 5\mu\text{M}$), orientin ($0.1\mu\text{M} - 1.0\mu\text{M}$, $15\mu\text{M} - 100\mu\text{M}$) and luteolin ($5\mu\text{M}$) enhance mineral content in Saos2 cells. This increase in mineral is in part due to the flavonoid's ability to increase ALP activity and cell mitochondrial activity in addition to reducing cell toxicity, inflammation (TNF α and IL6), SOST and OPN. Luteolin reduced mineral content at concentrations $\geq 15\mu\text{M}$. Higher levels of luteolin ($\geq 15\mu\text{M}$) were linked to significant levels of cell toxicity and a reduced level of cell mitochondrial activity.

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