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Department of Cellular and Molecular Biology

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**Specialty: Applied Biochemistry**

**Theme**

# Study of risk factors, prognostic value of some biochemical, hematological and oxidative stress markers and evaluation of the effect of *Proso millet* on osteoporosis in postmenopausal women from Guemar (El Oued, Algeria) region

**Presented by:**

**HADDIG Nour El-Houda**

**ZERZOUR Aicha**

**Examining Committee:**

| | | | |
|---|---|---|---|
| **President :** | **Dr. TOUMI Ikram** | **M.C.A,** | **El-Oued University** |
| **Examiner :** | **Mrs. MEHELLOU Zineb** | **M.A.B,** | **El-Oued University** |
| **Supervisor :** | **Dr. DEROUICHE Samir** | **M.C.A,** | **El-Oued University** |

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# *Dedications*

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**Abstract**

The main purpose of this work was to assess some socioeconomic and clinic risk factors of osteoporosis disease, to analysis some biological and oxidative stress markers to predict and diagnose this disease and to evaluate the effects of *Proso millet* aqueous extract against osteoporosis in postmenopausal women of Guemar El-Oued region. Our socioeconomic and clinic risk factors study was conducted on 241 postmenopausal volunteers divided into 100 healthy women and 141 women patients with osteoporosis. For biological analysis of osteoporosis we have chosen 16 healthy women as control and 16 menopausal women have osteoporosis, we used this second group for *Proso millet* aqueous extract treatment during four weeks. The results of risk factors study illustrate the high relationship between some clinical factors such as hormonal alternatives, menstrual perturbations and vitamin D deficiency before menopause and thyroid problems are shown to be significant risk factors, but bone problems before menopause and family history are the most dangerous risk factors (OR = 65.646; OR= 41.152) respectively, for osteoporosis in postmenopausal women. While, milk, fish diet, legumes, fresh vegetables and fruits, sunshine exposure, long distance walks and menopause after 50 years old are protective factors against this disease. Our results of biological study demonstrate a significant variations ($P<0.05$) in biochemical parameters, hematological markers and oxidative stress markers which some of them represented an important significant specificity ($P<0.05$) such as MDA in leukocyte (AUC=70.7%), in serum (AUC=99.6%) and sedimentation rate in both $1^{st}$ (AUC=69.9%) and $2^{nd}$ hour (AUC=60.2%). About phytotherapeutic study, the results of the qualitative and quantitative analysis revealed that the aqueous extract of the plant is rich in alkaloids, tannins, flavonoids, saponins, reducing sugars and terpenoids, with a considerable levels of phenols and flavonoids. The results show also that the plant extract significantly reverse ($P<0.05$) the change of calcium, GSH, SOD and ORAC levels induced by osteoporosis which represent the efficiency of the plant in reducing its severity.

In conclusion, several clinical factors contributed to the evolution of the osteoporosis in postmenopausal women in Guemar El-Oued region. MDA level in leukocyte and in serum and the sedimentation rate in both $1^{st}$ and $2^{nd}$ hour represent very important predictive factors for the studied disease. The phytotherapy have beneficial effects on the oxidative stress and calcium status in case of osteoporosis in postmenopausal women.

**Key Words:** Osteoporosis; Post-menopause; Risk factors; Oxidative stress; Calcium; *Proso millet*.

## الملخص

الهدف الأساسي من هذه الدراسة هو تقدير بعض عوامل الخطر الاجتماعية و السريرية لمرض هشاشة العظام، تحليل بعض المؤشرات البيولوجية و معايير الإجهاد التأكسدي لتنبؤ و تشخيص هذا المرض و كذلك تقييم التأثير العلاجي للمستخلص المائي لحبيبات نبات الدرع *Proso millet* ضد هشاشة العظام عند نساء في مرحلة ما بعد انقطاع الطمث من منطقة قمار ولاية الوادي. تم إجراء دراسة عوامل الخطر الاجتماعية و السريرية على 241 متطوعة في مرحلة ما بعد انقطاع الطمث موزعات على قسمين 100 امرأة سليمة و 141 امرأة تعاني من هشاشة العظام. من أجل الدراسة البيولوجية لهشاشة العظام اخترنا 16 امرأة شاهدة و 16 امرأة في مرحلة ما بعد انقطاع الطمث تعاني من هشاشة العظام، استعملنا المجموعة الثانية لتقييم العلاج بالمستخلص المائي لنبات *Proso millet* لمدة أربعة أسابيع. النتائج المتحصل عليها من خلال دراسة عوامل الخطر توضح العلاقة القوية بين بعض العوامل السريرية مثل البدائل الهرمونية، اضطرابات الدورة الشهرية و نقص فيتامين D قبل انقطاع الطمث و كذلك مشاكل الغدة الدرقية وبين الإصابة بهشاشة العظام عند النساء، لكن تبقى مشاكل العظام قبل انقطاع الطمث و التاريخ العائلي هما العاملان الأكثر خطورة بقيم (OR=65.646;OR=41.152) على التوالي، للإصابة بهشاشة العظام عند النساء. في حين وجدنا أن استهلاك الحليب، الأسماك، البقوليات، الخضراوات و الفواكه الطازجة، التعرض لأشعة الشمس، المشي لمسافات طويلة و انقطاع الطمث بعد سن الخمسين هي عوامل وقائية ضد هذا المرض. نتائج الدراسة البيولوجية أظهرت تغيرات كبيرة في المعايير البيو كيميائية و مكونات الدم و معايير الإجهاد التأكسدي مما أظهر وجود معايير مهمة للتنبؤ بالمرض مثل MDA في كريات الدم البيضاء(AUC=70.7%);MDAفي مصل الدم(AUC=99.6%); معدل الترسيب للساعة الأولى (AUC=69.9%) و معدل الترسيب للساعة الثانية (AUC=60.2%). بالنسبة لدراسة النبات أظهرت نتائج التحليل الكيفي للنبتة أن مستخلصها المائي غني بالفلويدات، التانينات، الفلافونويدات، الصابونين، السكريات المرجعة، التربينويدات، أما التحليل الكمي فلقد أظهر أن نبات الدرع يحتوي على كميات مهمة و معتبرة من الفينولات و الفلافونويدات. النتائج أظهرت أيضا أن استعمال مستخلص النبتة حسن بشكل كبير ($P<0.05$) التغيرات في مستويات الكالسيوم، GSH، SOD و ORAC الناجمة عن هشاشة العظام مما يعكس كفاءة النبتة في تخفيض شدة المرض عند النساء.

و ختاما، هناك العديد من العوامل السريرية التي ساهمت في تطور هشاشة العظام المصاحب لمرحلة ما بعد انقطاع الطمث عند النساء في منطقة قمار ولاية الوادي. كما أن مستويات MDA في كريات الدم البيضاء و في مصل الدم و كذلك تحليل سرعة الترسيب تمثل معايير تنبؤية لهذا المرض. للعلاج النباتي تأثيرات مفيدة على الإجهاد التأكسدي و مستوى الكالسيوم في حالة مرض هشاشة العظام عند النساء اللاتي في مرحلة ما بعد انقطاع الطمث.

**الكلمات المفتاحية:** هشاشة العظام، ما بعد انقطاع الطمث، عوامل خطر، الإجهاد التأكسدي، كالسيوم، الدرع (*Proso millet*).

# Abbreviation list

**25(OH)D:** 25-hydroxyvitamin D.

**$AlCl_3$:** Aluminum chloride.

**APX:** Ascorbate peroxidase.

**ATP:** Adenosine triphosphate.

**AUC:** Area Under Curve.

**BMD:** Bone Mineral Density.

**BMI:** Body Mass Index.

**BPAL:** Bone-specific Alkaline phosphatase.

**$Ca^{2+}$:** Calcium.

**$CaBP_{9k}$:** 9KDa vitamin D-dependent calcium-binding protein.

**CAT:** Catalase.

**CI:** Confidence interval.

**COX:** Cyclo-oxygenase.

**CRP:** C-reactive protein.

**$CuSO_4$:** Copper sulfate.

**DEXA:** Dual-energy X-ray absorptiometry.

**DNA:** Deoxyribonucleic acid.

**DTNB:** 5,5′-Dithiobis-(2-nitrobenzoic acid).

**EDTA:** Ethylenediaminetetraacetic acid.

**ESR:** Erythrocyte sedimentation rate.

**FCR:** Folin-Ciocalteu reagent.

**$FeCl_3$:** Ferric chloride.

**FNS:** Hematological analysis.

**FSH:** Follicle-stimulating hormone.

**GAE:** Gallic acid equivalent.

**GI:** Glycemic index.

**GPx:** Glutathione peroxidase.

**GSH:** Reduced glutathione.

**GSSG:** Glutathione disulfide.

**GST:** Glutathione S-transferase.

**$H_2O_2$:** Hydrogen peroxyde.

**$H_2SO_4$:** Sulfuric acid.

**HCl:** Hydrochloric acid.

**HCT:** Hematocrit.

**HGB:** Hemoglobin.

**HSCs:** Hematopoietic stem cells.

**IFN-γ:** Interferon gamma.

**IGF-1:** Insulin-like Grouth Factor 1.

**IL-1:** Interleukin-1.

**$K_2HPO_4$:** Dipotassium phosphate.

**$KH_2PO_4$:** Monopotassium phosphate.

**LOX:** Lipoxygenase.

**LYM:** Lymphocytes.

**MDA:** Malondialdehyde.

**$Mg^+$:** Magnesium.

**MHC II:** Major histocompatibility complex class II.

**MHT:** Menopausal Hormone Therapy.

**MPO:** Myeloperoxidase.

**$Na_2CO_3$:** Sodium carbonate.

**$Na_3C_6H_5O_7$:** Sodium citrate.

**NaCl:** Sodium chloride.

**NADPH:** Nicotinamide adenine dinucleotide phosphate.

**NBT:** Nitro Blue Tetrazolium.

**NCX1:** Sodium-Calcium Exchanger.

**NF-$\kappa$B:** Nuclear factor-kappa B.

**NOS:** Nitric oxide synthase.

**$O^{2\cdot}$:** Superoxide anion.

**OD:** Optical Density.

**OPG:** Osteoprotegerin.

**OR:** Odds Ratio.

**ORAC:** Oxygen Radical Absorbance Capacity.

**p38MAPK:** p38 Mitogen-activated protein kinase.

**PAL:** Alkaline phosphatase.

**PGE2:** Prostaglandin E2.

**pH:** Potential hydrogen.

**PLT:** Platelets.

**PMCA1b:** Plasma membrane calcium pump.

**PTH:** Parathormone.

**QE:** Quercetin equivalent.

**RANK:** Receptor activator of nuclear factor- κB.

**RANKL:** Receptor activator of nuclear factor- κB Ligand.

**RBC:** Red Blood Cell.

**RNS:** Reactive Nitrogen Species.

**ROC:** Receiver Operating Characteristics.

**ROS:** Reactive Oxygen Species.

**SD:** Standard deviations.

**SE:** Standard error.

**SOD:** Superoxide dismutase.

**SPSS:** Statistical Package for Social Sciences.

**T3:** Triiodothyronine.

**TBA:** Thiobarbituric acid.

**TBS:** Tris buffer Saline.

**TCA:** Trichloroacetic acid.

**TNF-α:** Tumor necrosis factor- alpha.

**TRPV6:** Transient receptor potential vanilloid type 6.

**UV-VIS:** Ultraviolet-visible.

**WBC:** White Blood Cell.

**WHO:** World Health Organization.

**XO:** Xylo-oligosaccharides.

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# SUMMARY

# *Introduction*

# Introduction

Menopause signifies the permanent cessation of menstruation and the end of reproductive potential. It is the culmination of some 50 years of reproductive aging a process that unfolds as a continuum from birth through ovarian senescence to the menopausal transition and the postmenopause. A result of aging changes in the ovary and in hypothalamic-pituitary-ovarian axis function (Weiss et *al*., 2004), the menopausal transition encompasses a period of dynamic changes in reproductive and non-reproductive tissues. The menopausal transition is known to play a major role in the etiology of symptoms such as hot flashes, night sweats, uterine bleeding problems, and vulvovaginal atrophy (NIH, 2005). Mood changes, in addition to the osteoporosis, defined as a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture, is a major public health problem throughout the world (Genant et *al*., 1999 ; Schmidt, 2005). The social and economic burden of osteoporosis is increasing steadily because of the aging of the world population (Cummings & Melton, 2002). Currently affecting more than 10 million people in the United States, osteoporosis is projected to impact approximately 14 million adults over the age of 50 by the year 2020 (National Osteoporosis Foundation, 2002). Worldwide, approximately 200 million women have osteoporosis. Although the likelihood of developing osteoporosis currently is greatest in North America and Europe, it will increase in developing countries as population longevity in these countries continues to increase (Genant, 1999). Many of recent land marks in scientific research have shown that in human beings, oxidative stress is an important factor causing metabolic and physiological alterations and various diseases in the body (Derouiche et *al*., 2019).

Several *in vitro* and animal studies have shown that oxidative stress diminishes bone formation by reducing the differentiation and survival of osteoblasts .While ROS also activate osteoclasts and thus, enhance bone resorption (Baek, 2010). Clinical studies have also suggested that the involvement of ROS and/or antioxidant systems may play a role in the pathogenesis of bone loss (Abdollahi, 2005).

Minerals play an important role in the building of bones, clotting of blood, sending and receiving signals, keeping normal heart beat, cell energy production, transportation of oxygen, metabolize and synthesize fats and proteins, act as coenzymes, provide immunity to the body and help nervous system work properly (Soetan et *al*., 2010).

Calcium content of finger millet is about eight times higher than wheat and being the richest source of calcium (348 mg/100 g) it has the ability to prevent osteoporosis (Chandel et *al*., 2014).

In light of these data, the aim of our work is based on the realization of three following complementary aspects:

**The first part:** is to study the risk factors associated with osteoporosis in menopausal women that several types of factors are studied in this context: socioeconomic, environmental and clinical factors.

**The second part:** is a biological study concerns the determination of the variation and specificity of some biochemical, hematological and oxidative stress markers in the prediction and diagnosis following up on osteoporosis in menopausal women of Guemar El-Oued population.

**The third part:** is to study the effect of millet extract against osteoporosis in volunteer menopausal women.

# First part

## Bibliographic part

# Chapter I

## Calcium

# I. Calcium

## I.1. Definition

Calcium is an alkaline-earth metal with symbol Ca and atomic number 20, located in the fourth period of the periodic table in Group II (Perrone & Monteiro, 2016). In living cells, calcium is a divalent cation ($Ca^{2+}$), it is one of the essential minerals for conducting cell functions. In mammals, calcium is present in all cells and accounts for up to 4% of total body weight (Victor, 2016).

In humans, it ranks fifth after oxygen, carbon, hydrogen, and nitrogen and it makes up 1.9% of the body weight. Approximately 99% of calcium is contained in bones and teeth as calcium hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$), a mineral composed of different calcium salts, and the remainder is inside the cells (0.9%) and extracellular fluid (0.1%) (Victor, 2016 ; Perrone & Monteiro, 2016).

## I.2. Calcium metabolism

### I.2.1. Absorption

Most of calcium absorption occurs in the duodenum and jejunum, but minor amounts can also be absorbed in the colon. Intestinal calcium absorption occurs by two general mechanisms: passive (paracellular) and active (transcellular) transport (Robert, 2007):

**a. Passive transport**

The paracellular transfer (80%) occurs throughout the entire length of the intestine, it depends on the concentration gradient of calcium between the lumen and plasma, it is facilitated by proteins, called claudins, such as claudin-16 (paracellin-1) and claudin-19 (Banerjee, 2005 ; Shifrin, 2019). This pathway predominates at high intraluminal calcium concentrations (Domschke et *al*., 1999).

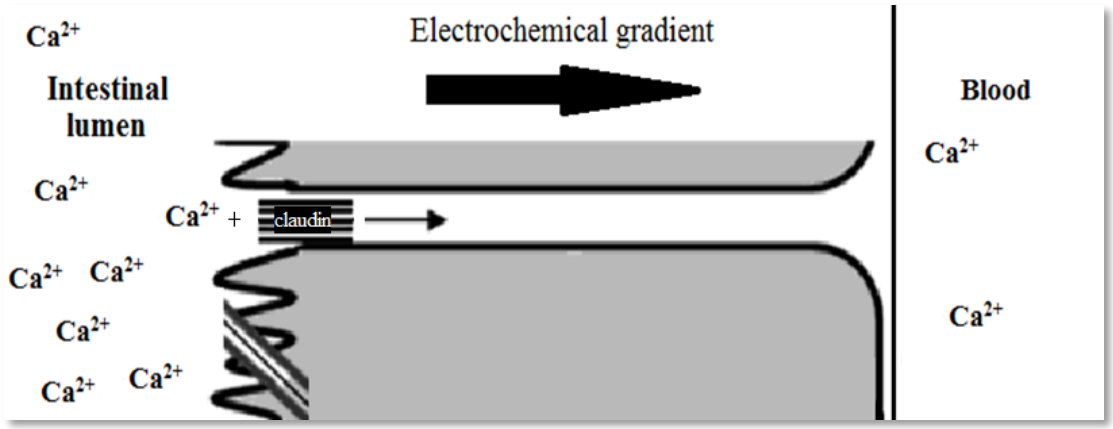



**Figure 01:** Paracellular pathway (Victor, 2016).

**b. Active transport**

The transcellular process (20%) is located largely in the duodenum and upper jejunum and regulated by vitamin D (Bronner, 2003 ; Banerjee, 2005).

Calcium entering the entyrocyte via the transient receptor potential vanilloid type 6 (TRPV6) channel becomes tightly associated with the calbindin 9k ($CaBP_{9k}$). The calbindin/calcium complex then diffuses across the cytosol to the basolateral membrane. Free calcium then dissociates into the low-cytosolic calcium environment maintained immediately subjacent to the basolateral membrane by high-affinity membrane calcium-stimulated magnesium-dependent ATPase (($Ca^{2+}$ + $Mg^{+}$)-ATPase) and sodium calcium exchanger (NCX1) that extrude $Ca^{2+}$out of the enterocyte to the bloodstream (Harrihar, 1980 ; Jameson & De Groot, 2015 ; De Barboza et *al.*, 2015).

Calcitriol is the principal mediator of transcellular $Ca^{2+}$ transport, inducing the expression of TRPV6, calbindin-$D_{9k}$, and the plasma membrane calcium pump (PMCA1b) (Martin et *al.*, 2013). The active transport is characterized as the main mechanism of calcium absorption when the intake of this nutrient is low (Dawson-Hughes et *al.*, 1995).

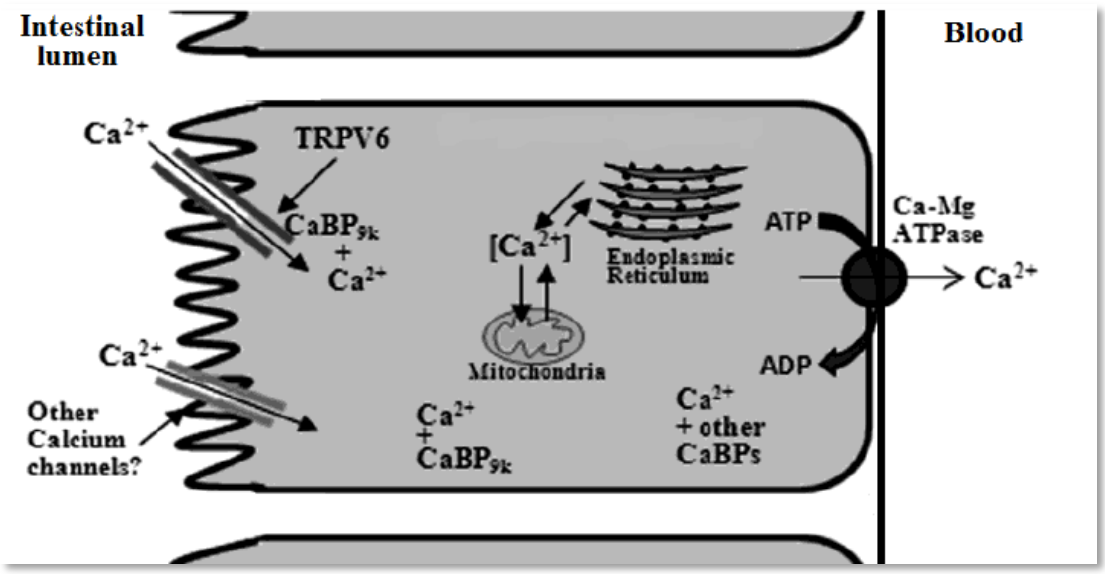



**Figure 02:** Transcellular pathway (Victor, 2016).

## Factors involved in calcium absorption

Several factors can enhance or inhibit the calcium absorption:

**a.** ***Enhancing factors*****:** Proteins enhance calcium absorption, such as casein that stimulates circulating IGF-1 which promotes bone formation (Hoppe et *al.*, 2009 ;

Gropper & Smith, 2012), IGF-1 stimulate the expression and activity of the 1-α-hydroxylase in the kidney, increasing in 1,25-dihydroxyvitamin D levels, that stimulates intestinal $Ca^{2+}$ absorption (Ameri et *al.*, 2013), therefore, low protein diets decrease this process (Kerstetter et *al.*, 2018). Also, the acidic environment of the upper digestive tract facilitates the solubility of $Ca^{2+}$ (Barasi, 2003). During pregnancy, $Ca^{2+}$ absorption is higher than normal because of the increased demand from the fetus for skeletal growth (Prentice, 2000).

**b.** ***Inhibitory factors*****:** A ratio of dietary calcium to phosphorus of at least 1 is desirable to maintain balance (Staggers et *al.*, 1974). If phosphorus intakes are very high it binds calcium in the intestine, limiting absorption and leading to increased urinary excretion of calcium (Mangels et *al*., 2004 ; Blackburn, 2007).

$Ca^{2+}$ absorption may be reduced by phytic acid, or by oxalic acid, owing to the formation of insoluble calcium phytate and calcium oxalate salt. Unabsorbed dietary fatty acids will combine with $Ca^{2+}$ to form soaps, removing it from the body (Barasi, 2003). Fibers such as cellulose bind calcium in the intestines and make it unavailable for absorption (Gropper et *al*., 2004). High sodium concentrations may also decrease $Ca^{2+}$ absorption (Mangels et *al*., 2004 ; Blackburn, 2007).

Alcohol, caffeine and smoking have been found to contribute to reduced endogenous $Ca^{2+}$ absorption (Heaney & Recker, 1982 ; Massey & Whiting, 1993).

## I.2.2. Distribution

The vast majority of total body calcium (>99%) is present in the skeleton primarily as hydroxyapatite (Peacock, 2010). Both quantitative and qualitative changes in bone tissue and in bones themselves occur not only during growth and development but also during aging (Kiebzak, 1991).

Nonbone calcium represents <1% of total body calcium (~10 g in an adult). However, it is responsible for a wide range of essential functions (Peacock, 2010). In plasma 45% of calcium is found in ionized form, 45% bound to proteins that occur predominantly in albumin, and 10% is complexed with anions such as citrate, sulfate and phosphate (Upadhyay, 2017). Serum calcium is tightly maintained within a physiologic range of ~8.8 to10.4 mg/dl (2.2 to 2.6 mM) in healthy subjects (Peacock, 2010).

## I.2.3. Elimination

Calcium leaves the body mainly in urine and feces, but also in other body tissues and fluids, such as sweat (Hoenderop et *al.*, 2000). The glomerular filtrate contains 9 to 10 g of calcium⁄24 hours and 98% of this is reabsorbed in the renal tubule (Walls, 2012). Approximately 60% to 70% of the filtered calcium is reabsorbed in the proximal convoluted tubule, 20% in the loop of Henle, 10% by the distal convoluted tubule, and 5% by the collecting duct (Blaine, 2014).

Calcium excretion is affected by a variety of hormones, ions, nutrients, and drugs. Among these, PTH is the principal physiologic regulator of renal tubular transport (Epstein, 1968 ; Torikai et *al.*, 1981) it increases the tubular reabsorption of calcium. In the other hand, increases in dietary sodium will cause an increase in calcium excretion (Walls, 2012). Also, diuretics such as thiazide diuretics act in the distal tubule, are associated with hypocalciuria (Felsenfeld et *al.*, 2013 ; Blaine, 2014), because it inhibits Na reabsorption (Edwards et *al.*, 1973), and enhance Ca reabsorption (Costanzo & Windhager, 1978).

## I.3. Regulation of calcium metabolism

Maintenance of normal calcium homeostasis is generally controlled by Parathyroid hormone (PTH), vitamin D and calcitonin, occurring at the levels of three major organ systems: intestine, bone, and kidney (Porat & Sherwood, 1986 ; Ferrari et *al.*, 2006).

**I.3.1. Parathormone:** The parathyroids gland produces parathormone (PTH), which has the key role in the regulation of serum calcium level (Mutschler & Derendorf, 1995 ; Summers & Macnab, 2017). It inhibits the reabsorption of phosphate in the proximal and distal tubule, and increases the reabsorption of calcium and magnesium in the distal tubule of the kidney. PTH also effect indirectly by facilitating the hydroxylation of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol, the active form of vitamin D3, in the kidney (Mutschler & Derendorf, 1995).

**I.3.2. Vitamin D:** 1,25-dihydroxyvitamin D (or calcitriol) directly stimulate intestinal calcium absorption throughout the entire length of the intestine (Garabedian et *al.*, 1972 ; Lips, 2006). Also it induces the formation and activation of the osteoclast to function in the mobilization of calcium from bone (Suda et *al.*, 1992 ; Yasuda et *al.*, 2005). In addition, calcitriol together with PTH stimulates the renal distal tubule reabsorption of calcium,

ensuring retention of calcium by the kidney when it is needed (Sutton et *al.*, 1976 ; Yamamoto et *al.*, 1984).

**I.3.3. Calcitonin:** Calcitonin is a hormone secreted by the parafollicular cells of the thyroid (C cells), it increase calcium absorption into bones and inhibit the active transport from the bone cells to the circulation to lower blood calcium in case of high calcemia (Sturkie, 1981).

## I.4. Physiological role of calcium

Calcium has many important functions in the body, it plays a key role in many fundamental biologic processes (Higgins, 2007 ; Upadhyay, 2017). It is the major structural element in vertebrate skeleton (bones and teeth) in the form of calcium phosphate ($Ca_{10}[PO_4]_6[OH]_2$) known as hydroxyapatite. Calcium also has important role in bone remodeling and tooth re-mineralization, where it provides hard tissue with its strength (Upadhyay, 2017). In addition, the calcium in bone is a reservoir of calcium that is important for maintaining plasma calcium concentration regardless of its intake (Power et *al*., 2000 ; Higgins, 2007).

However, calcium's role in health and performance extends beyond the skeleton (Fink et *al*., 2011), most of its physiologic functions carried out by ionized calcium (Williams & Wilkins, 2007), it plays a very imperative role in many biochemical reactions, which are essential for normal functioning of cells (Upadhyay, 2017) including:

- ✓ Blood clotting: Calcium serves as cofactor for blood-clotting factors involved in the production of fibrin, the protein responsible for the structure of blood clots (Higgins, 2007 ; Fink et *al*., 2011 ; Herndon, 2012).
- ✓ Nerve transmission: Calcium is required for proper nerve function, releasing neurotransmitters that facilitate the perpetuation of nerve signals and activation (Fink et *al*., 2011).
- ✓ Muscle contraction: Calcium is pumped into and out of muscle cells to initiate both muscle contraction and relaxation in smooth muscle, skeletal muscle, and the heart (Fink et *al*., 2011).
- ✓ Transients or local gradients in the $[Ca^{2+}]$ contribute to different events including nuclear envelope breakdown and reformation, cellular proliferation and synthesis of

DNA through the regulatory nature of this elemental mineral (Hepler, 1994 ; Micozzi, 2006).

- ✓ Membrane rigidity, permeability and viscosity are partly dependent on local calcium concentration (Upadhyay, 2017) and it is essential for the maintenance of the electrical potential difference across cellular membranes (Perrone & Monteiro, 2016).
- ✓ Calcium has a vital function as a second messenger transmitting signals between the plasma membrane and the intracellular machinery's cascades (Power et *al*., 2000), such as process that results in hormone secretion by endocrine tissue cells, and intracellular processes, including cell division, depend on calcium signaling (Higgins, 2007).

## I.5. Pathology associated with calcium

### I.5.1. Hypercalcemia

#### I.5.1.1. Definition

Hypercalcemia are a common and heterogeneous group of disorders, ranging from the occasional detection of a high level of serum calcium (> 13 mg/dL) (Francesca et *al*., 2012), during routine laboratory assessment to a life-treating condition (Pellitteri, 2010).

#### I.5.1.2. Etiology of hypercalcemia

##### I.5.1.2.1. Hypercalcemia PTH dependent

A severe symptomatic hypercalcemia with high-normal PTH level due to the coexistence of primary hyperparathyroidism and hypercalcemia (Francesca et *al*., 2012).

##### I.5.1.2.2. Hypercalcemia PTH independent

PTH-independent mechanisms include most cases of malignancy-associated hypercalcemia, vitamin D intoxication, granulomatous diseases, vitamin A intoxication, thyrotoxicosis, milk-alkali syndrome and immobilization (Shane & Dinaz, 2006).

#### I.5.1.3. Effect of Hypercalcemia

Excessive calcium ions are toxic to cells, and increased serum $Ca^{2+}$ concentration decreases cellular function by causing alterations in cell membrane permeability and cell membrane calcium pump activity (Schenck et *al.*, 2006).

### I.5.2. Hypocalcemia

#### I.5.2.1. Definition

Hypocalcemia is a common biochemical abnormality that can range in severity from being asymptomatic in mild cases to presenting as an acute life-threatening crisis. Serum calcium

levels are regulated within a narrow range (2.1 to 2.6 mmol/L) by 3 main calcium-regulating hormones—parathyroid hormone (PTH), vitamin D, and calcitonin—through their specific effects on the bowel, kidneys, and skeleton (Fong & Khan, 2012).

#### I.5.2.2. Etiology of hypocalcemia

##### I.5.2.2.1. Hyperphosphatemia

Phosphate modifies calcium efflux from bone in animal and human studies. In parathyroid-ectomized rats given a normal phosphate diet and a fixed replacement dose of PTH sufficient to maintain normal serum calcium and phosphorus values, changing to a high phosphate diet resulted in hypocalcemia and hyperphosphatemia (Levine et *al*., 2014).

##### I.5.2.2.2. Hypovitamin D

Lower levels of 25(OH)D can lead to severe hypocalcemia, which is considered one of the side effects of bisphosphonates (Elena et *al*., 2012).

##### I.5.2.2.3. Hypomagnesemia

Hypomagnesemia impairs the function of the parathyroid glands, which are small hormone-producing glands located in the neck. Normally, the parathyroid glands release a hormone that increases blood calcium levels when they are low (Genetics Home Reference, 2020).

## I.6. Calcium and menopausal women

### I.6.1. Definition of menopause

Menopause (as defined by the World Health Organization) is the permanent cessation of menstruation due to loss of ovarian follicular activity (World Health Organization, 1981). And as the last menstrual period followed by at least twelve months of amenorrhea (no menstrual bleeding) (Shailendra, 2011). Usually occurs between the ages of 45 and 55 years (Southern, 2019). There are three stages:

- **Perimenopause** is the transitional time that starts before menopause and includes the 12 months that follow a person's last period;
- **Menopause** starts either 12 months after the last period or when menstruation has stopped for a clinical reason, such as the removal of the ovaries;
- **Postmenopause** refers to the years after menopause, although it can be difficult to know when menopause finished and postmenopause starts (Carolyn, 2020).

### I.6.2. Symptoms

- ✓ Hot flushes (occurring in approximately 60% of women);
- ✓ Sweats (often at night);
- ✓ Tiredness;
- ✓ Joint and bone pain;
- ✓ Thinning hair;
- ✓ Dry skin;
- ✓ Unusual skin sensations;
- ✓ Vaginal dryness;
- ✓ Depression (Southern, 2019).

### I.6.3. Treatment

- **Menopausal Hormone Therapy (MHT)**

Involves replacing hormones previously produced by the ovaries and relieving the symptoms, also help to reduce the risk of osteoporosis following menopause (Southern, 2019).

- **Diet**

Women need a higher intake of calcium and vitamin D after menopause to help reduce the risk of osteoporosis (Southern, 2019).

- **Exercise**

Regular weight-bearing exercise such as walking, tennis, aerobics and golf helps in maintaining a healthy weight, fitness and general wellbeing (Southern, 2019).

### I.6.4. Relation between calcium and menopause

Calcium levels remains stable until menopause, when the bone resorption rate increases in association with the decrease in ovarian estrogen production that effect the intestinal calcium absorption (North American Menopause Society, 2006).

# Chapter II

## Osteoporosis

# II. Osteoporosis

## II.1. Definition

The National Institutes of Health has defined osteoporosis as "a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture." (Gullberg et *al.*, 1997). Osteoporosis is generally a disease of older adults because the cumulative effects of slow bone mineral loss take time to deplete the skeleton (Power et *al.*, 2000).

## II.2. Risk factors

Physical inactivity and low weight shows good evidence that, among both women and men, they are risk factors for osteoporosis as well as for fractures (Cummings, 1955 ; Brot et *al.*, 1977). Previous fractures are a major risk factor for new fractures (Kanis, 2004). Also, smoking decreases bone density and increases the risk of fractures (Forsen, 1994), and low exposure to sunlight leads to poor uptake of vitamin D (Johnell & Hertzman, 2006). Treatments with cortisone reduce bone density and increase the risk of fractures (Espallargues, 2001).

## II.3. Symptoms

There typically are no symptoms in the early stages of bone loss. But once the bones become weak by osteoporosis, signs and symptoms might appear, including:

- Back pain, caused by a fractured or collapsed vertebra;
- Loss of height over time;
- A stooped posture;
- Bones that breaks much more easily than expected (Clarke, 2019).

## II.4. Diagnostic

Osteoporosis is a silent disease without obvious symptoms and evidence until a fracture occurs, table (01) shows some diagnostic category for osteoporosis.

**Table 01:** Diagnostic category for osteoporosis according to BMD and DEXA values (Kanis, 1994).

| Diagnostic Category | DEXA BMD Values | Definition |
|---|---|---|
| **Normal bone mass** | T score >-1.0 S.D | BMD above 1 SD below the average young adult value. |
| **Osteopenia** | T scorebetween–1.0 and–2.5 SD | BMD between1.0 and 2.5SD below the average young adult value. |
| **Osteoporosis** | T score<-2.5 SD | BMD more than 2.5 SD below the average young adult value. |
| **Severe osteoporosis** | T score<-2.5 SD | BMD more than 2.5 SD below the average young adult value and at least 1 osteoporotic fracture. |

Bone mineral density (BMD), dual energy X-ray absorptiometry (DEXA).

## II.5. Calcium and osteoporosis

Calcium is essential in maintaining total body health (Micozzi, 2006). When blood $Ca^{2+}$ levels drop too low, it is borrowed from the bones and returned to the bones from $Ca^{2+}$ supply through the diet (Pravina et *al.*, 2013). Thus, the effects of calcium deficiency may escape notice for a considerable time, until they manifest as skeletal weakness or fractures (Power et *al.*, 2000). In fact, low dietary calcium intake is associated with low bone density, and calcium deficiency cause age-related bone loss, therefore, osteoporosis (Kim et *al.*, 2014).

## II.6. Pathophysiology

Once the peak bone mass is achieved, bone regulation takes place by local remodeling which is regulated by RANK (or receptor activator of nuclear factor-kb), RANKL (or RANK Ligand) and OPG (or Osteoprotegerin). RANK is present in the osteoclasts and causes the increase in their activity. RANKL is synthetized by osteoblasts and it binds with the RANK receptor on the osteoclasts. OPG is also synthetized by the osteoblasts and prevents the binding of the RANK to the RANK Ligand by itself binding to the RANKL. Hence, the activity of the osteoclasts and in turn the bone remodeling depends upon the interplay

between the RANK and the OPG. This interplay is controlled by hormonal and local factors. Imbalance in bone remodeling lead to decreased skeletal mass. In most individuals, bone mass peaks in the third decade, after which bone resorption exceeds bone formation. Failure to reach a normal peak bone mass or acceleration of bone loss can lead to osteoporosis (Varacallo & Fox, 2014 ; Vikram et *al*., 2017).

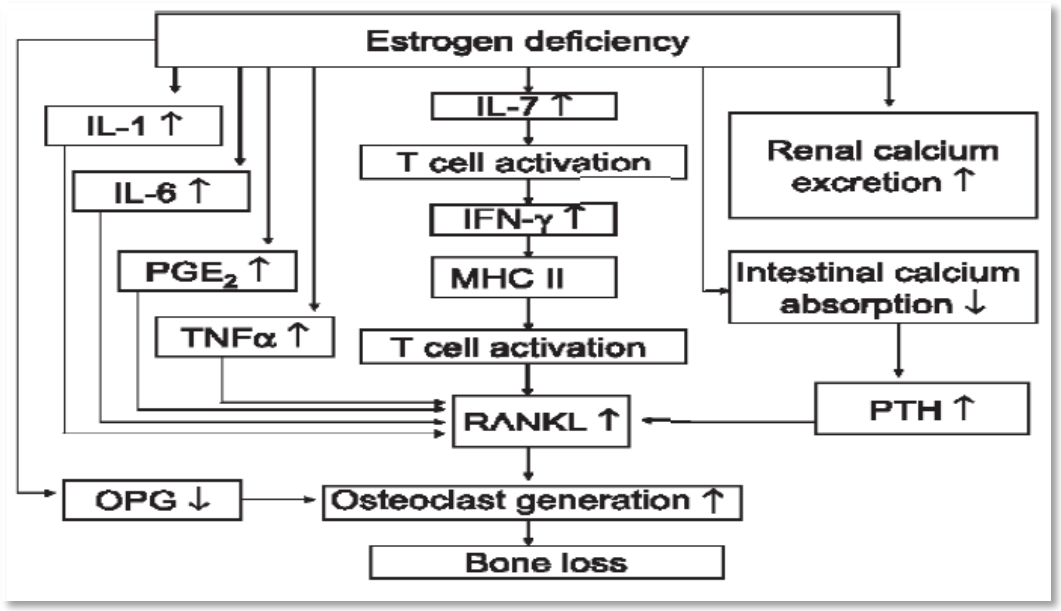



**Figure 03:** A model of the effects of estrogen deficiency on bone loss (Wolfgang et *al*., 2009).

## II.7. Complications

◆ **Hip and vertebral fracture:** Hip and vertebral fractures in osteoporotic individuals affect multiple aspects of daily life;

◆ **Functional limitations:** Functional limitations associated with osteoporosis can have an extensive impact on the daily life. When subsequent vertebral fractures or a hip fracture occur, the functional outlook changes substantially for osteoporotic patients;

◆ **Pain:** Pain from osteoporosis challenges the patient's coping skills, often in a way that causes a decline in physical and psychological well-being. Two types of osteoporotic pain exist: (1) acute pain that accompanies a fracture and (2) chronic pain that develops over time;

◆ **Psychological dysfunction:** The psychological impact on osteoporosis patients, although not visible, can be as serious and debilitating. Low self-esteem and depression are the two most frequent psychological disabilities resulting from osteoporosis and fractures (Gold et *al*., 1998).

## II.8. Treatment

♦ Bisphosphonates: Oral bisphosphonates inhibit osteoclastic activity and are potent antiresorptive agents (Black et *al.*, 1996).

♦ Raloxifene (evista): a selective estrogen receptor modulator, it is approved for the treatment of postmenopausal osteoporosis (Sweet et *al.*, 2009).

♦ Calcitonin nasal spray (Miacalcin): is an antiresorptive agent approved for the treatment of postmenopausal osteoporosis at a dosage of 200 UI in alternating nostrils each day (Sweet et *al*., 2009).

♦ Teriparatide (Forteo): is a recombinant human parathyroid hormone with potent bone anabolic activity (Sweet et *al.*, 2009).

♦ Hormone therapy: The women's health initiative confirmed that estrogen, with or without progesterone, slightly reduces the risk of hip and vertebral fractures (Sweet et *al*., 2009).

## II.9. Prevention

✓ Ensure a healthy diet which includes enough calcium and protein, two key nutrients for bone health;

✓ Get enough vitamin D: made in the skin after exposure to sunlight, the average young adult needs about 15 minutes of daily sun exposure. Also, some foods like oily fish, eggs, mushrooms, and fortified dairy foods or juices can boost vitamin D intake;

✓ Maintain a healthy body weight: being too thin (BMI under 19) can damage bone health;

✓ Keep active: Take regular weight-bearing and muscle strengthening exercise;

✓ Stop smoking and heavy drinking (Cooper & Ferrari, 2019).

## II.10. Osteoporosis and menopausal women

A decline in estrogen has been shown to play a major role in the decreasing bone mass during the menopause, especially because it has a variety of protective effects on bone marrow and bone cells. This hormone allows for increased bone formation by reducing the

production and function of the osteoclasts as well as increasing osteoclast apoptosis. This effect on the osteoclastic cells of the bone is facilitated via estrogen's inhibition of a signaling molecule, RANKL, which is involved in osteoclast differentiation and survival. However, due to the estrogen deficiency during menopause, this beneficial effect on the bone is lost causing osteoporosis (Doshi & Agarwal, 2013).

Also, menopause has been linked to an increase in inflammatory cytokines within the serum specifically tumor necrosis factor (TNF-α), which negatively impacts the bone by contributing to increased osteoclast formation. Additionally, the high levels of FSH during menopause stimulate osteoclast differentiation and TNF-α production, both of which play an important role in osteoporotic bone loss (Doshi & Agarwal, 2013).

## II.11. Oxidative stress

### II.11.1. Definition

Oxidative stress is defined as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control" (Sies, 2018). The overproduction of free radicals can cause oxidative damage to biomolecules (lipids, proteins, DNA), eventually leading to many chronic diseases such as cancer, diabetics, cardiovascular diseases, chronic inflammation, aging and osteoporosis (Uttara et *al*., 2009 ; Tan et *al*., 2018).

### II.11.2. Free radicals

Free radicals can be defined as atoms or molecules or molecular fragments containing one or more unpaired electrons in their atomic or molecular orbitals (Qazi & Molvi, 2018). The free radicals, both the reactive oxygen species (ROS) and reactive nitrogen species (RNS), have central roles in various physiological conditions (Phaniendra et *al*., 2015).

### II.11.3. Sources of free radicals

Free radicals are generated from both endogenous and exogenous sources (Pizzino et *al*., 2017). Endogenous sources include mitochondria, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, myeloperoxidase (MPO), xanthine oxidases (Liguori et *al*., 2018 ; Ping et *al*., 2020), inflammation, ischemia, infection, cancer, excessive exercise, mental stress and aging (Pizzino et *al*., 2017).

However, exogenous sources of free radicals are air and water pollution, tobacco, alcohol, heavy or transition metals, drugs (e.g., cyclosporine, tacrolimus and gentamycin),

industrial solvents, cooking (e.g., smoked meat, waste oil, and fat), ultraviolet rays and radiation (Liguori et *al.*, 2018 ; Ping et *al.*, 2020).

## II.11.4. Antioxidants

An antioxidant is a molecule which has the ability to prevent or slow the oxidation of macromolecules. Their role is to lower or terminate these chain reactions by removing free radicals or inhibiting other oxidation reactions by being oxidized themselves. The antioxidant systems in the human body consist of powerful non-enzymatic and enzymatic antioxidants (Adwas et *al.*, 2019).

The enzymatic antioxidant defense systems consist of three major classes of antioxidant enzymes which are the catalases, superoxide dismutases (SOD), and glutathione peroxidases (GPx), all of these, play crucial roles in maintaining homeostasis into cells. The non-enzymatic antioxidant defense system, exist in many dietary natural sources such as vegetables, fruits, and beverages, include vitamins C (ascorbic acid), E (α-tocopherol) A, and glutathione. In addition, medicinal plants and herbs have an antioxidant effects due to the presence of flavonoids, alkaloids, carotenoids, glycosides, terpenoids, saponins, polyphenols and other essential dietary constituents (e.g., copper, iron, zinc, and magnesium) (Adwas et *al.*, 2019 ; Yeung et *al.*, 2019 ; Montoya-Estrada et *al*., 2020).

## II.11.5. Oxidative stress and osteoporosis

Oxidative stress activates the differentiation of osteoclasts which led to a significant increase in the number and activity of these cells. Also, ROS induce the apoptosis of osteoblasts and osteocytes, by activating numerous signaling pathways. The oxidative stress blocks the activation of osteoblasts and thus the production of OPG, under this condition, the action of RANKL prevails, and the differentiation and activity of osteoclasts are induced. Thereafter, the turnover of the bone remodeling process increases due to increased resorption rate without adequate and proper bone formation, this event has been related to osteoporosis (Domazetovic et *al*., 2017).

Antioxidants have opposing effects, they contribute to the differentiation of osteoblasts and bone formation, while reduce the osteoclast differentiation and their activity (Domazetovic et *al*., 2017).

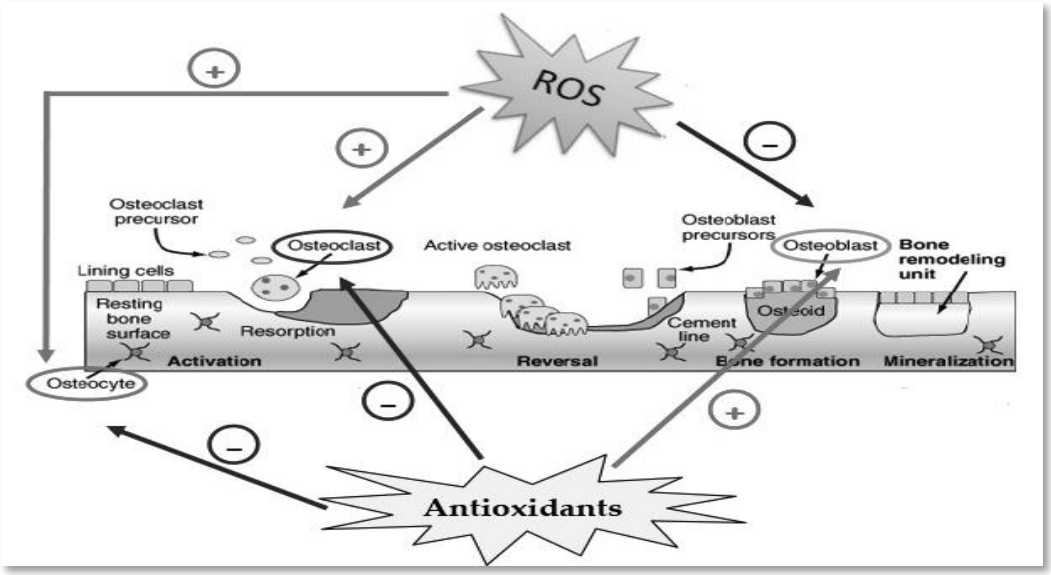



**Figure 04:** Effect of ROS and antioxidants on the activity of osteoclasts, osteoblasts and osteocytes in bone remodeling. ROS activate osteoclast differentiation and osteocyte apoptosis(+), while inhibit osteoblast activity(-) inducing bone resorption; antioxidants activate osteoblast differentiation(+) and inhibit osteoclast activity and osteocyte apoptosis(-) inducing bone formation (Domazetovic et *al.*, 2017).

## II.11.6. Oxidative stress and menopausal women

Menopause creates a redox imbalance and subsequent oxidative stress due to the decline of the natural antioxidant hormone estrogen (Sankar et *al.*, 2017). Specifically, at high concentrations, estrogen tends to have a beneficial antioxidant effect by inhibiting the 8-hydroxylation of guanine DNA bases (Amrita et *al*., 2016), also by increasing the activities of antioxidant enzymes, such as glutathione peroxidase, and causing an increase in antioxidant vitamin levels (Montoya-Estrada et *al*., 2020).

However, at low concentrations, this hormone has pro-oxidant like effects, including breaks in genetic material, formation of DNA adducts, oxidation of bases and changes in the lipid profile and the increase of lipoperoxidation (Amrita et *al*., 2016 ; Montoya-Estrada et *al*., 2020).

# Chapter III

## Proso millet

# III. *Proso millet* (*Panicum miliaceum L.*)

## III.1. Definition

*Proso millet* (*Panicum miliaceum L.*) is the oldest used cereal by humans besides wheat and barley. It was domesticated in Manchuria and introduced to Europe ~3000 years ago, followed by its introduction in the Near East and India. Because of its short growing seasons (60–90 days after planting), low water and nutrient requirements, it grows across wide environments up to 548 N/S latitude and also adapts well to plateau conditions and high elevations (Theisen et *al*., 1978).

## III.2. Botanical description and Taxonomy

*Proso millet* is a short-day plant and usually an erect annual, reaching to a height of 90 to 120 cm. with a shallow adventitious rooting system. The stems are slender, glabrous or slightly hairy, with hollow internodes. The plant has long, soft, narrow, pointed leaves expanded from leaf sheaths. The inflorescence is a drooping panicle varying in the degree of compactness, and often one-sided. The spikelets are pointed, about half a centimetre long and contain two flowers (Tonapi et *al*., 2015).

**Kingdom:** Plantae

***Clade*:** Angiosperms

***Clade*:** Monocots

***Clade*:** Commelinids

**Order:** Poales

**Family:** Poaceae

**Genus:** *Panicum*

**Species:** ***P. miliaceum*** (USDA & NRCS, 2006).

**Figure 05:** *Proso millet* (Dayakar et *al*., 2017).

## III.3. Distribution in the world and in Africa

Proso is produced throughout the central and northern Great Plains of the US including Nebraska, Kansas, Colorado, North Dakota, South Dakota, and Minnesota (Baltensperger, 2002). Asia (48%) and Africa (48%) dominate the production of millet compared to Europe (3%) and America (1%) (Saurav et *al*., 2019).

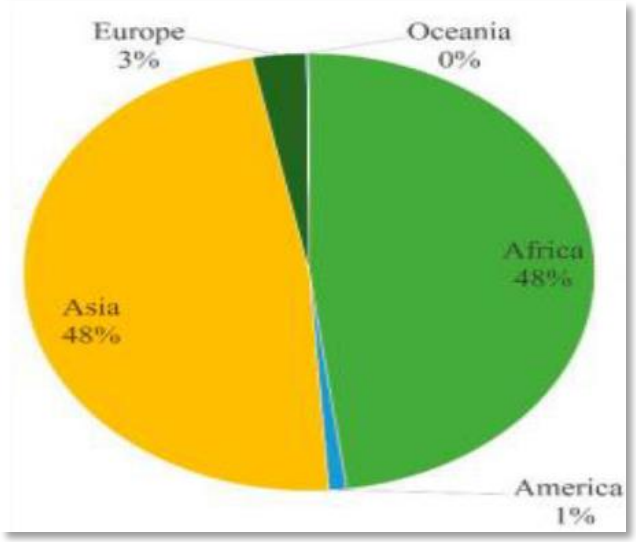



**Figure 06:** Distribution of *Proso millet* (Saurav et *al*., 2019).

## III.4. Biological interest of *Proso millet*

Nutritional quality is the key element that determines the dietary importance of a grain and its importance towards human health.

► *P. millet* is known for several health benefits. It has high nutritive value and is comparable to major cereal grains (Saleh et *al*., 2019).

► *P. millet* is a good source of minerals like calcium, phosphorus, potassium, sodium, magnesium, manganese, iron and zinc (Saleh et *al*., 2019).

► *P. millet* has all the essential amino acids such as methionine, phenylalanine, tryptophan, valine, etc. (Saleh et *al*., 2019). The limiting amino acid in *P. millet* is lysine, which is only 189 mg/g. The essential amino acid index was found to be higher (51%) in *P. millet* compared to wheat (Kalinova & Moudry, 2006). Major nutritional component protein, carbohydrate, and energy values are comparable to popular cereals like rice, wheat and barley (Devi et *al*., 2014).

► *P. millet* has 11% (which may range from 11.0% to 14.0%) of protein per 100 g of grains, compared to that of wheat (14.4%) and rice (7.5%) (Devi et *al*., 2014).

► *P. millet* has a low glycemic index (GI) compared to rice, wheat, and barley, which makes it an ideal food for people with type-2-diabetes mellitus and cardiovascular disease (Mcsweeney, 2014).

# Second Part

## Experimental Part

# *Materials*

# *&*

# *Methods*

# I. Materials and methods

## I.1. Materials

### I.1.1. Patients risk factors study

#### I.1.1.1. Population and period of study

Our study was organized over a period of 7 months (from the beginning of September 2019 to the end of March 2020). Faculty of Natural Sciences and Life at the University of Echahid Hamma Lakhdar El-Oued. In this study, we used the questionnaire (Annex 01) and asked 241 volunteers divided into 100 healthy menopausal women as a control and 141 menopausal women patients with osteoporosis from the origin of Guemar El-Oued region. Each volunteer submitted the questionnaire including social and clinical data that can give us different factors associated with osteoporosis.

#### I.1.1.2. Description of study region

The region of Oued-Souf is a valley located in the North east of the Algerian Sahara, approximately 700 km from south-east of Algiers and 350 km from west of Gabes (Tunisia). It is a group of discontinuous oases. The two main groups are EL-OUED–GUEMAR and ZGOUM-BEHIMA, accompanied by small secondary oases in the East and West (Khechana, 2014 ; Rosso, 2015).

Our study was conducted in Guemar, a commune located 16 km northwest of El Oued on the national road linking Oued-Souf to Biskra (Rosso, 2015) (figure 07).

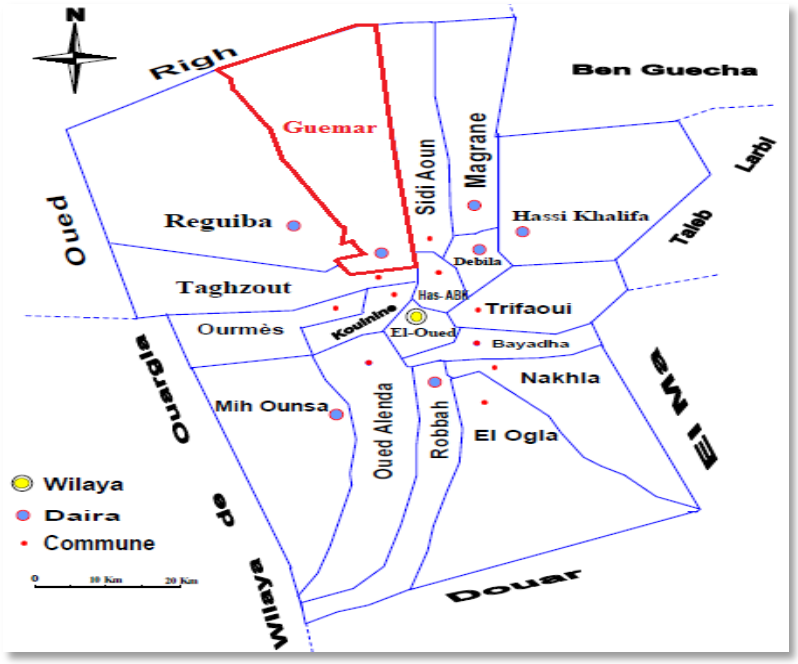



**Figure 07:** Map of Oued-Souf and Guemar region (Khechana, 2014).

### I.1.2. Prognostic and phytotherapeutic study

#### I.1.2.1. Plant material and aqueous extract preparation

In this study, the grains of *Proso millet* were obtained from the herbalist. These grains were powdered by mechanical grinder until a fine powder was obtained. The powders of *Proso millet* are stored at room temperature in airtight containers protected from bright light until the beginning of the experiment (figure 08). Aqueous extracts were prepared by putting 10 g of homogenized dried grains in 100 ml of water during 24h then the extract was filtered (Derouiche & Kaouachi, 2018).

**Figure 08:** The grains of *Proso millet* (original photo).

#### I.1.2.2. Patients study design

This work was applied on 32 volunteers women of age between 45-55 years, they were divided into two groups; a group of 16 healthy control women with mean age 49,234±0,341 year, a group of 16 menopausal women have osteoporosis with mean age 49,972±0,112 year, and after supplementing the group of women have osteoporosis with powdered grains of *Proso millet* for four weeks their results considered to be the third group.

With regard to the distribution of groups, it was according to the aim of the work, where in the prognostic study, we relied on a comparison between the results of the first and second groups, and about phytotherapeutic study, we relied on the comparison between the second and third group.

**Inclusion criteria**

- ✓ Voluntary women live in Guemar El-Oued region.
- ✓ Women suffering from osteoporosis aged from 45 to 55.
- ✓ Control women are not at menopause, in good health and do not have any pathology.

**Exclusion criteria**

- × Women are suffering from other acute or chronic pathology.
- × Women using drugs during menopausal periods.

#### I.1.2.3. Reagents

Mayer's reagent, Hydrochloric acid (HCl), Ferric chloride ($FeCl_3$), Sulfuric acid ($H_2SO_4$), Fehling's liquor, Chloroform, Sulfuric acid, Folin-Ciocalteu reagent (FCR), Sodium carbonate ($Na_2CO_3$), Methanol, Aluminum chloride ($AlCl_3$), Sodium citrate ($Na_3C_6H_5O_7$), Ethylenediaminetetraacetic acid (EDTA), Tris, Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), Hydrogen Peroxyde ($H_2O_2$), Phosphate-buffer ($KH_2PO_4$, $K_2HPO_4$), Salicylic acid, Sodium chloride (NaCl), DTNB (5,5′-Dithiobis (2-nitrobenzoic acid )), Copper sulfate ($CuSO_4$), Ascorbic acid, NBT, Riboflavin.

## I.2. Methods

### I.2.1. Plant in-vitro analysis

#### I.2.1.1. Phytochemical analysis

According to (Harborne, 1998 ; Evans, 2009 ; Wadood et *al.*, 2013) six phytochemical analysis assays were used to identify:

❖ **Phenolic compounds**

- **Alcaloids**

To 1 ml extract with few drops of Mayer's reagent were added, after acidifying the medium with a few drops of HCL. Formation of white precipitate indicates the presence of alkaloids.

- **Tannins**

To 5 ml extract, add 1 ml of 2% $FeCl_3$ solution. The presence of tannins is indicated by a greenish or bluish-blackish coloration.

- **Flavonoids**

To 5ml extract, add 5 ml of diluted ammonia and 1ml of $H_2SO_4$. The appearance of a yellow color indicates the presence of flavonoids.

❖ **Saponins**

5 ml of extract was mixed with 5ml of distilled water and shaken vigorously. The formation of stable foam was taken as an indication of the presence of saponins.

❖ **Reducing sugars**

To 2 ml of extract, add 0.5 ml of Fehling's liquor (1ml of reagent A and 1ml of reagent B) and incubate the whole in a boiling water bath. The appearance of a brick-red precipitate indicates the presence of sugar reducers.

❖ **Terpenoids**

To 5ml of extract, add 2 ml of chloroform and 3ml of concentrated sulfuric acid. The formation of a reddish brown color indicates the presence of terpenoids.

### I.2.1.2. Quantitative analysis of total phenolic and flavonoids compounds

#### I.2.1.2.1. Total phenolic

The quantitative analysis of total phenols in phenolic extracts was carried out according to (Slinkard & Singleton, 1977): 125 µL of the extract are mixed with 500 µL of distilled water and 125 µL of Folin-Ciocalteu reagent (FCR), the mixture was stirred well. After 5 min, 1250 µL of sodium carbonate ($Na_2CO_3$; 7.5 g/l) are added and completed with distilled water to 3 ml then the stirred reaction mixture is kept in the dark and incubated for 2h at room temperature. The absorbance of each solution is measured at 765 nm using a UV-VIS spectrophotometer.

Calibration curve is carried out by gallic acid at different concentrations (50-100-150-200µg/ ml) under the same conditions and the same steps of the assay. The results are thus expressed in milligrams of gallic acid per gram of dry extract (mg of GAE/g). All measurements are repeated 3 times.

#### I.2.1.2.2. Total flavonoids

The determination of total flavonoids was carried out according to the method described by Ahn et al. (Ahn et *al.*, 2007). 1 ml of each sample and standard (prepared in methanol) is added to 1 ml of the AlCl3 solution (2% dissolved in methanol). After 10 minutes, the absorbance was measured relative to the blank prepared from reagent at $\lambda$ max = 430 nanometers.

The results are expressed in mg equivalent quercetin / g of dry vegetable material with reference to the quercetin calibration curve. The quercetin calibration curve is performed by quercetin at different concentrations (10-20-30-40 µg/ml) under the same conditions and the same steps of the assay.

### I.2.2. Patients study interventions

Performed blood sampling for both groups is done morning fasting. It is performed in the vein of the end of the elbow. After the blood sampling, the blood is collected in three types of tubes:

♦ In anticoagulant tube (EDTA), for hematological (FNS) and oxidative stress (MDA, GSH, SOD and CAT activity) parameters assays.

♦ In sodium citrate tube, used to detect inflammatory markers.

♦ In dry tubes, samples are centrifuged at 3000 rpm for 10 minutes and then recover the serum to achieve the dosage of biochemistry parameter: Glucose, calcium, iron, PAL, vitamin C and total antioxidant ORAC.

Urine samples are collected in sterile containers over a 24-hour period and stored in the freezer until the urinary creatinine dosage.

### I.2.3. Measurement of serum biochemical and hematological markers

Serum glucose, calcium, iron, PAL and urinary creatinine were determined by the *Semi-auto Analyzer Mindray BA-88A* and measured using commercial kits from Biomaghreb, (Biomaghreb: glucose-20121, calcium-20051, iron-20064, PAL-20015 and creatinine-20151). Hematological analysis (FNS) was performed by the hematology *Auto analyzer* (*Mindray*).

### I.2.4. Estimation of oxidative stress parameters

#### I.2.4.1. Preparation of erythrocyte samples

Blood EDTA tubes contents are centrifuged at 2000 rpm for 10 min and removed the plasma. The cap of EDTA tube was lysis with 50 ml of TBS buffer (EDTA 2.92M; tris 1.21M; pH=7) and incubated 30 min in Freezer. After incubation centrifuged at 2500 rpm for 10 min and the obtained supernatant (erythrocyte homogenate) was used for the determination of antioxidant activity (Miller et *al.*, 1988).

#### I.2.4.2. Leukocyte separation

After separation of erythrocytes, the rest of EDTA tube contents centrifuge at 2000 rpm for 10 min. Wash pellet with lysis buffer and shake incubate in Freezer for 30 min. After incubation centrifuged at 2500 rpm for 10 min. followed this step by washing with lysis buffer until the Leukocyte pairing and then recovered to make the dosage of stress tests (Miller et *al.*, 1988).

### I.2.4.3. Determination of malondialdehyde (MDA) level

MDA was measured according to the method described by Sastre et al. (Sastre et *al*., 2000). In brief, mix 100 µl of sample, 400 µl of TBA reagent into the glass and screw test tubes and seal. Heat the mixture in the Marie bath at 100 ° C for 15 minutes. Then cool in a cold-water bath for 30 minutes leaving the tubes open to allow evacuation of the gases formed during the reaction. Centrifuge at 3000 rpm for 5 minutes and read the absorbance of the supernatant at 532 nm using a spectrophotometer.

### I.2.4.4. Determination of Reduced glutathione (GSH) level

The level of reduced Glutathione is determined according the Weak and Cory (1988), by measuring the optical density results from the formation of 2-nitro-5-mercocapturic acid from the reduction of dithio-bis-2-nitrobenzoic acid, which is called Ellman reagent with SH groups exist in GSH briefly.

- 800µl of homogenate (or serum) samples are added to 200µl of salicylic acid (0.25%).
- The mixture was centrifuge at 1000 rpm for 5 min.
- Take 500 ml of supernatant and mixed with 1000µl of tris buffer (tris 0.4mol, 0.02mol NaCl, pH =8.9) and 25 µl of DTNB (0.01 mol/L).
- Read the absorbance at 412 nm after 5 min of incubation.

$$GSH(nM/mg\ of Hb) = \frac{(\mathrm{OD} \times 1 \times 1.525)}{13133 \times 0.8 \times 0.5 \times \mathrm{mg\ of\ pr}}$$

### I.2.4.5. Determination of catalase activity

The catalase activity consists in measuring the catalase-induced loss of $H_2O_2$ contained in the sample by measuring the absorbance of $H_2O_2$ at 560 nm using a UV/visible spectrophotometer according the method of Aebi (1984). Briefly in test tubes mix 1 ml of phosphate buffer ($KH_2PO_4$, 0.1 M, pH 7.2), 0.975 ml of freshly prepared $H_2O_2$ (0.091 M) and 0.025 ml of the enzyme source (homogenate). The absorbance is read at 560 nm each minute for 2 minutes.

$$CAT(UI/g) = \frac{\left(\left(\frac{2.3033}{\mathrm{T}}\right) \times \left(\frac{\mathrm{logA1}}{\mathrm{logA2}}\right)\right)}{\mathrm{g\ of\ protin}}$$

A1: Absorbance at the first minute.

A2: Absorbance at the second minute.

T: Time interval in minutes.

### I.2.4.6. Plasma vitamin C assay

The plasma vitamin C is measured according to the method of Jagota & Dani (1982) using the Folin reagent and a range of ascorbic acid. Briefly, add one ml of plasma to 0.5 ml of the TCA solution (10%). Vortex then places the tubes in an ice bath for 30 min. Centrifuge at 3000 rpm for 10 min. Take 0.75 ml of the supernatant to which 0.75 ml of distilled water and 150 µl of Folin (1/10) are added. Vortex and incubate for 15 min at room temperature. Read the OD using a blank spectrophotometer at 769 nm and determine the vitamin C concentration (µg / ml) from the standard curve.

### I.2.4.7. Determination of superoxide dismutase (SOD) activity assay

The assay method of SOD activity using the NBT by the superoxide anion (O2·), is used as a basis for detecting of presence of SOD by measuring the Spectrophotometrically absorbance at 560 nm (Beauchamp & Fridovich, 1971).

| **Collect in tubes** | **Blank** | **Sample** |
|---|---|---|
| **EDTA-Met** 0,1mM EDTA-13mM Met | 1000µL | 1000Ml |
| **Phosphate buffer** (50Mm) | 892,2µL | 892,2µL |
| **Sample** | 0 | 50 |
| **Phosphate buffer** | 1000µL | 950µL |
| **NBT** (75µM) | 85,2µL | 85,2µL |
| **Riboflavin (**2µM) | 22,6µL | 22,6µL |

**Expression of results:**

$$SOD = \frac{\text{DO blanc} - \text{DO sample}}{\text{DO blanc}}$$

### I.2.4.8. Measuring of total antioxidant capacities (ORAC)

**a. principle**

The total antioxidant power of the serum, its capacity to absorb free oxygen radicals (ORAC: Oxygen Radical Absorbance Capacity), is estimated by the ability of red blood cells to resist free radical-induced hemolysis in vitro in the presence of plasma according to the

method of Oyaizu (1986). This method is based on the time-dependent monitoring of red blood cell hemolysis induced by a free radical generator.

**b. Treatment of red blood cells**

• Centrifuge donor blood at 2000 rpm for 10 min and remove plasma.

• Wash gently 1 volume of the pellet with 2 volumes of physiological saline (without lysing the RBCs), then centrifuge again at 2000 rpm for 5 min.

**c. Operating mode**

◆ **Control tube**

• Add 1 ml of RC: 20 µl of CuSO4 (2 mM), 20 µl of $H_2O_2$ (30%) and 2 ml of physiological saline, then stir gently.

Incubate for 5 min at room temperature, centrifuge for 5 min at 2000 rpm.

• Read the OD at 450 nm from the supernatant and put it back into the tube and stir gently.

• Repeat this operation every 10 minutes for 1 hour.

◆ **Standard tube**

• To 1 ml of GR are added: 20 µl of $CuSO_4$ (2 mM), 20 µl of $H_2O_2$ (30%) and 2 ml of physiological saline, and 20 µl of vitamin C (400 µM) and then stir gently.

Incubate for 5 min at room temperature, centrifuge for 5 min at 2000 rpm.

• Read the OD at 450 nm from the supernatant and put it back into the tube and stir gently.

• Repeat this operation every 10 minutes for 1 hour.

◆ **Test tube**

• To 1 ml of RC are added: 20 µl of $CuSO_4$ (2 mM), 20 µl of $H_2O_2$ (30%) and 2 ml of physiological saline, and 20 µl of serum (400 µM) and then stir gently.

Incubate for 5 min at room temperature, centrifuge for 5 min at 2000 rpm.

• Read the OD at 450 nm from the supernatant and put it back into the tube and stir gently.

• Repeat this operation every 10 min for 1 hour (t0, t10, t20, t30, t40, t50, t60, and average the latter: ΣDO = Σ (t0, t10, t20, t30, t40, t50, t60) / 7

• To calculate the total antioxidant power using two methods.

**Calculate method:**

$$ORAC(UI) = \frac{\Sigma(\mathrm{ODcontrol} - \mathrm{ODsample})\Delta t}{\Sigma(\mathrm{ODcontrol} - \mathrm{ODstandard})\Delta t}$$

### I.2.5. Statistical analysis

Statistical analysis is performed by the SPSS20.0 software, results comparisons were carried out by the Student T test to compare means among the groups using Minitab 17, and the Excel 2007 (Microsoft) which helped us to do the tests and the histograms. The diagnostic model of osteoporosis with several factors was based on Logistic regression analysis. The statistical parameters, which are the area under curves (AUC) and the receiver operating characteristics (ROC) were used to show the potency of a biomarker in the diagnosis of osteoporosis. Specificity, sensitivity, AUC, and 95% confidence interval (CI) values were calculated. $P < 0.05$ indicates a statistically significant difference.

# *Results*

# *&*

# *Discussion*

# II. Results

## II.1. Study of predictors factors of osteoporosis

### II.1.1. Description of the study population

The general data of socioeconomic characteristics of the two groups of subjects include age, weight, height, number of children, social case, job, educational level, and blood group. These indicators do not have any statistically significant differences (as shown in table 02), at $P > 0.05$.

**Table 02:** Demographic description of control and osteoporosis patients.

| | | Control | Patients |
|---|---|---|---|
| **Age(ys)** | | 49,234±0,341 | 49,972±0,112 |
| **Body Weight(kg)** | | 66,596±0,243 | 70,390±0,500 |
| **Height(cm)** | | 160,76±0,164 | 160,38±0,130 |
| **BMI (Body mass index)** | | 25,52±0,121 | 27,46±0,124 |
| **Number of children** | | 5,333±0,183 | 6,064±0,183 |
| **Social Case** | Married% | 81 | 96 |
| | Single% | 7 | 2 |
| | Divorced% | 10 | 1 |
| | Widow% | 2 | 1 |
| **Job** | Worker% | 25 | 10 |
| | Housewife% | 75 | 90 |
| **Educational Level** | Illiterate% | 13 | 14 |
| | Primary% | 15 | 28 |
| | Junior high% | 26 | 24 |
| | High School% | 26 | 23 |
| | Higher education% | 20 | 11 |
| **Blood Group** | A% | 43.33 | 20 |
| | B% | 10 | 10 |
| | AB% | 6.67 | 13 |
| | O% | 40 | 57 |

### II.1.2. Study of socioeconomic and clinic factors

Odds Ratio (OR) values for socioeconomic factors (table 03) and clinic-pathological factors (table 04) show that thyroid problems, hormonal alternatives, menstrual perturbations and vitamin D deficiency before menopause are shown to be significant risk factors for osteoporosis (OR = 6.682; p = 0.005, OR = 5.688; p = 0.000, OR = 5.516; p = 0.000 and OR

= 5.394; p = 0.000), respectively. Menopause before 42 years old (OR = 4.846; p = 0.029), stress (OR = 3.841; p = 0.000) and calcium deficiency before menopause (OR = 3.305; p = 0.001) are also highly significant predictors of osteoporosis. In addition, obesity, hormonal perturbations before menopause, anemia, tap water, taking contraceptive pills, digestive problems and milk mixed with coffee are all predictive factors (OR ranging from 1.803 to 2.538, p<0.05). Also, bone problems before menopause and family history are considered to be very important risk factors in the study population, with the highest OR value (OR = 65.646; p = 0.000 and OR= 41.152; p=0.000). In contrast, milk, fish diet, legumes, fresh vegetables and fruits, sunshine exposure, long distance walks and menopause after 50 years old are protective factors for osteoporosis in the study population (OR ranging from 0,029 to 0.463, p<0.038). In addition, our results indicate that filtered water, pure milk, pure coffee, soft drinks, excessive thinness, standing up too long, ovarian cysts, kidney problems, liver problems, diabetes, hypertension, puberty before 12 years old, age of marriage after 30 years old, first birth after 30 years old, childbearing less than 2 years old, breastfeeding less than 1 year old, normal birth, caesarean birth, self-medication, medications for menstrual pain and herbal remedies are not considered as predictors of osteoporosis in our population since the OR values obtained are not significant.

**Table 03:** Comparison of the socioeconomic features of osteoporosis patients and control group (N=241).

| | **Control%** | **Patient%** | **OR** | **$CI_{95}$%** | ***P-value*** |
|---|---|---|---|---|---|
| **Tap water** | | | 2.220 | 0.945-5.214 | 0.048 |
| Positive | 3.73 | 10.37 | | | |
| Negative | 37.75 | 48.15 | | | |
| **Filtered water** | | | 0.800 | 0.316-2.024 | 0.407 |
| Positive | 37.75 | 51.87 | | | |
| Negative | 3.73 | 6.65 | | | |
| **Milk consomption** | | | 0,029 | 0.013-0.065 | 0,000 |
| Positive | 33.60 | 6.24 | | | |
| Negative | 7.88 | 52.28 | | | |
| **Pure milk** | | | 0.653 | 0.356-1.197 | 0.110 |
| Positive | 14.52 | 14.94 | | | |
| Negative | 26.97 | 43.57 | | | |
| **Milk mixed with coffee** | | | 1.803 | 0.969-3.355 | 0.043 |

| | | | | | |
|---|---|---|---|---|---|
| Positive | 26.97 | 44.81 | | | |
| Negative | 14.52 | 13.70 | | | |
| **Pure coffee** | | | 1 | 0.568-1.761 | 0.557 |
| Positive | 16.59 | 23.65 | | | |
| Negative | 24.89 | 34.87 | | | |
| **Fish diet** | | | 0.091 | 0.011-0.724 | 0.005 |
| Positive | 4.14 | 0.82 | | | |
| Negative | 37.34 | 57.70 | | | |
| **Legumes** | | | 0.124 | 0.035-0.431 | 0.000 |
| Positive | 40.24 | 46.88 | | | |
| Negative | 1.24 | 11.64 | | | |
| **Fresh vegetables and fruits** | | | 0.227 | 0.062-0.830 | 0.014 |
| Positive | 40.24 | 51.45 | | | |
| Negative | 1.24 | 7.07 | | | |
| **Sunshine exposure** | | | 0.463 | 0.263-0.815 | 0.005 |
| Positive | 24.06 | 22.82 | | | |
| Negative | 17.42 | 35.70 | | | |
| **Soft drinks** | | | 1.079 | 0.502-2.322 | 0.500 |
| Positive | 6.22 | 9.56 | | | |
| Negative | 35.26 | 48.96 | | | |
| **Obesity** | | | 2.538 | 1.133-5.687 | 0.016 |
| Positive | 4.14 | 12.86 | | | |
| Negative | 37.34 | 45.66 | | | |
| **Excessive thinness** | | | 0.792 | 0.206-3.039 | 0.500 |
| Positive | 2.07 | 2.07 | | | |
| Negative | 39.41 | 56.45 | | | |
| **Long distance walks** | | | 0.077 | 0.037-0.159 | 0.000 |
| Positive | 26.55 | 7.05 | | | |
| Negative | 14.93 | 51.47 | | | |
| **Standing up too long** | | | 0.920 | 0.522-1.620 | 0.443 |
| Positive | 16.18 | 34.45 | | | |
| Negative | 25.31 | 24.06 | | | |

**Table 04:** Comparison of the clinico-pathological features of osteoporosis patients and control group (N=241).

| | **Control%** | **Patient%** | **OR** | **$CI_{95}$%** | ***P-value*** |
|---|---|---|---|---|---|
| **Calcium deficiency before menopause** | | | 3.305 | 1.544-7.073 | 0.001 |
| Positive | 4.56 | 17.03 | | | |
| Negative | 36.92 | 41.49 | | | |
| **Vitamin D deficiency before menopause** | | | 5.394 | 2.565-11.342 | 0.000 |
| Positive | 4.56 | 23.26 | | | |
| Negative | 36.92 | 35.26 | | | |
| **Menstrual perturbations** | | | 5.516 | 2.559-11.890 | 0.000 |
| Positive | 25.72 | 52.69 | | | |
| Negative | 15.76 | 5.83 | | | |
| **Ovarian cysts** | | | 1.949 | 0.901-4.217 | 0.063 |
| Positive | 4.97 | 12.44 | | | |
| Negative | 36.51 | 46.08 | | | |
| **Thyroid problems** | | | 6.682 | 1.455-30.685 | 0.005 |
| Positive | 0.82 | 7.05 | | | |
| Negative | 40.66 | 51.47 | | | |
| **Kidney problems** | | | 1.570 | 0.729-3.382 | 0.167 |
| Positive | 5.39 | 11.21 | | | |
| Negative | 36.09 | 47.31 | | | |
| **Digestive problems** | | | 2.005 | 1.138-3.533 | 0.011 |
| Positive | 19.50 | 37.34 | | | |
| Negative | 21.99 | 21.17 | | | |
| **Liver problems** | | | 2.087 | 0.608-7.167 | 0.186 |
| Positive | 1.65 | 4.56 | | | |
| Negative | 39.83 | 53.96 | | | |
| **Diabetes** | | | 1.673 | 0.781-3.583 | 0.126 |
| Positive | 5.39 | 11.64 | | | |
| Negative | 36.09 | 46.88 | | | |
| **Hypertension** | | | 1.577 | 0.810-3.071 | 0.120 |
| Positive | 7.88 | 15.76 | | | |
| Negative | 33.60 | 42.76 | | | |
| **Anemia** | | | 2.444 | 1.150-5.196 | 0.014 |
| Positive | 4.97 | 14.54 | | | |
| Negative | 36.51 | 43.98 | | | |
| **Stress** | | | 3.841 | 2.077-7.102 | 0.000 |

| | | | | | |
|---|---|---|---|---|---|
| Positive | 19.91 | 45.64 | | | |
| Negative | 21.57 | 12.88 | | | |
| **Bone problems before menopause** | | | 65.646 | 19.338-222.852 | 0.000 |
| Positive | 1.24 | 39.02 | | | |
| Negative | 40.24 | 19.50 | | | |
| **Family History** | | | 41.152 | 12.211-138.679 | 0.000 |
| Positive | 1.24 | 32.78 | | | |
| Negative | 40.24 | 25.74 | | | |
| **Hormonal perturbations before menopause** | | | 2.448 | 1.366-4.387 | 0.002 |
| Positive | 12.03 | 29.05 | | | |
| Negative | 29.46 | 29.46 | | | |
| **Hormonal alternatives** | | | 5.688 | 2.930-11.039 | 0.000 |
| Positive | 6.63 | 30.70 | | | |
| Negative | 34.85 | 27.82 | | | |
| **Puberty before 12 years old** | | | 0.960 | 0.922-0.999 | 0.061 |
| Positive | 0 | 2.07 | | | |
| Negative | 41.49 | 56.44 | | | |
| **Menopause after 50 years old** | | | 0.395 | 0.155-1.007 | 0.037 |
| Positive | 6.63 | 4.14 | | | |
| Negative | 34.85 | 54.38 | | | |
| **Menopause before 42 years old** | | | 4.846 | 1.020-23.028 | 0.029 |
| Positive | 0.82 | 5.39 | | | |
| Negative | 40.66 | 53.13 | | | |
| **Age of marriage after 30 years old** | | | 0.563 | 0.251-1.263 | 0.114 |
| Positive | 7.46 | 6.24 | | | |
| Negative | 34.02 | 52.28 | | | |
| **First birth after 30 years old** | | | 0.638 | 0.314-1.296 | 0.142 |
| Positive | 9.54 | 9.54 | | | |
| Negative | 31.95 | 48.97 | | | |
| **Childbearing Less than 2 years** | | | .0474 | 0.156-1.440 | 0.141 |
| Positive | 4.15 | 2.90 | | | |
| Negative | 37.34 | 55.61 | | | |
| **Breastfeeding Less than 1 year** | | | 0.468 | 0.168-1.301 | .0108 |

| | | | | | |
|---|---|---|---|---|---|
| Positive | 4.97 | 3.34 | | | |
| Negative | 36.51 | 55.18 | | | |
| **Normal birth** | | | 0.388 | 0.073-2.047 | 0.222 |
| Positive | 40.66 | 55.60 | | | |
| Negative | 0.82 | 2.92 | | | |
| **Caesarean birth** | | | 1.441 | 0.678-3.063 | 0.223 |
| Positive | 5.80 | 11.20 | | | |
| Negative | 35.68 | 47.32 | | | |
| **Contraceptive pills** | | | 2.071 | 1.179-3.640 | 0.008 |
| Positive | 17.42 | 34.85 | | | |
| Negative | 24.06 | 23.67 | | | |
| **Self-medication** | | | 3.128 | 0.616-15.886 | 0.140 |
| Positive | 0.82 | 3.75 | | | |
| Negative | 40.66 | 54.77 | | | |
| **Medications for menstrual pain** | | | 1.222 | 0.701-2.128 | 0.286 |
| Positive | 19.91 | 31.14 | | | |
| Negative | 21.57 | 27.38 | | | |
| **Herbal remedies** | | | 1.464 | 0.826-2.596 | 0.122 |
| Positive | 14.10 | 25.33 | | | |
| Negative | 27.38 | 33.19 | | | |

OR>1 and P<0.05 indicate a risk factor
OR<1 and P<0.05 indicate a protective factor

## II.2. In vitro essays of *Proso millet*

### II.2.1. Phytochemical study

#### II.2.1.1. Qualitative phytochemical analysis of *Proso millet*

Results of phytochemical essays shows that aqueous extract of *Proso millet* rich on different important chemical compounds such as alkaloids, tannins, flavonoids, saponins, reducing sugars and terpenoids (table 05).

**Table 05:** Phytochemical essays of *Proso millet* aqueous extract.

| Compounds | Alkaloids | Tannins | Flavonoids | Saponins | Reducing sugars | Terpenoids |
|---|---|---|---|---|---|---|
| **Aquatic Extract of *P. millet*** | +++ | +++ | +++ | +++ | +++ | +++ |

(+): Present

### II.2.1.2. Total phenols and flavonoids concentration

**Table 06:** Total phenols and flavonoids concentration in *Proso millet* aqueous extract.

| Compounds | Polyphenols (mg of GAE/g of Powder) | Flavonoids (mg QE/g of Powder) |
|---|---|---|
| **Aqueous Extract of *Proso millet*** | 7,613±0,542 | 0,700±0,0115 |

## II.3. Biological markers study

### II.3.1. Hematological parameters

The illustrated results of the hematological parameters in (figure 09) show that, for osteoporosis patients group there is a significant increase ($P<0.05$) in WBC and a very high significant increase ($P<0.001$) in HCT and PLT in the same group, also a very high significant increase ($P<0.001$) in PLT; a high significant increase ($P<0.01$) in HGB and a significant increase ($P<0.05$) in HCT for osteoporosis group after the treatment as compared to the control group. The results show that in WBC of osteoporosis after the treatment group a very high significant decrease ($P<0.001$) and a high significant increase ($P<0.01$) in HGB as compared to the osteoporosis patients group. While in HCT for osteoporosis after the treatment group is a high significant decrease ($P<0.01$) as compared to the osteoporosis patients group.

Our results of erythrocyte sedimentation rate (or ESR) show that, for osteoporosis patient group ESR of the 1st hour is significant increase ($P<0.05$), and of the 2nd hour is a high significant increase ($P<0.01$) as compared to the control group, also, for osteoporosis after treatment group it showed that ESR of the 1st hour is a significant decrease ($P<0.05$) as compared to the osteoporosis patient group.

Also, the results showed that there is no significant changes ($P>0.05$) in WBC, ESR of the 1st hour and ESR of the 2nd hour of osteoporosis after treatment group as compared to that in the control, in PLT and ESR of the 2nd hour of the same group as compared to the osteoporosis patient group, in HGB of the osteoporosis patient group as compared to that in controls, and in LYM and in RBC in all groups.

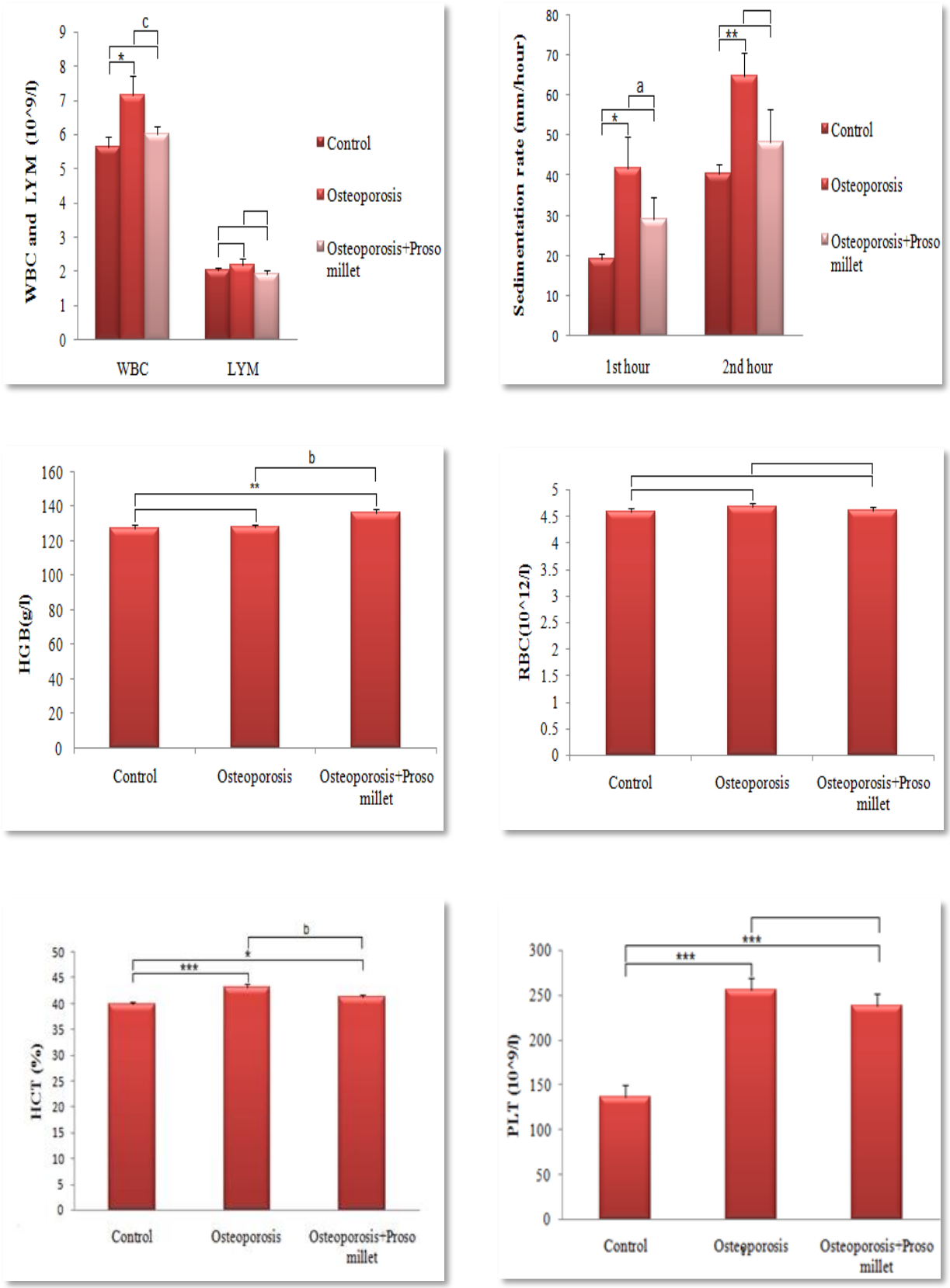
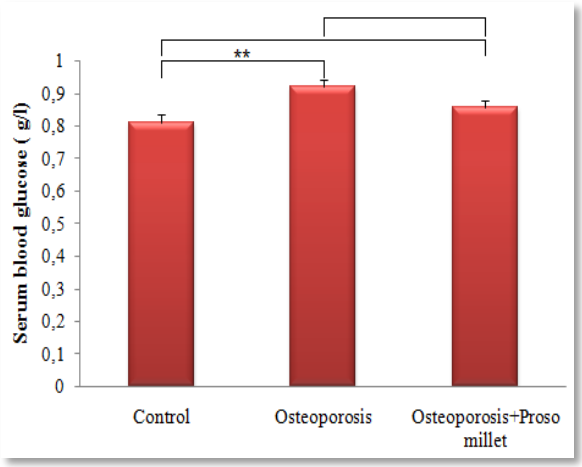


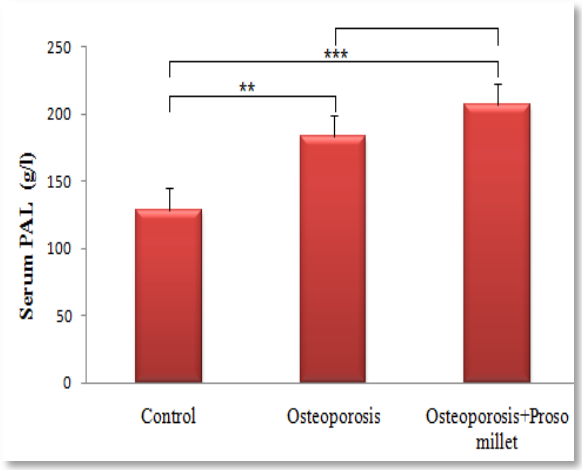

**Figure 09:** Hematological parameters levels of control and experimental groups.

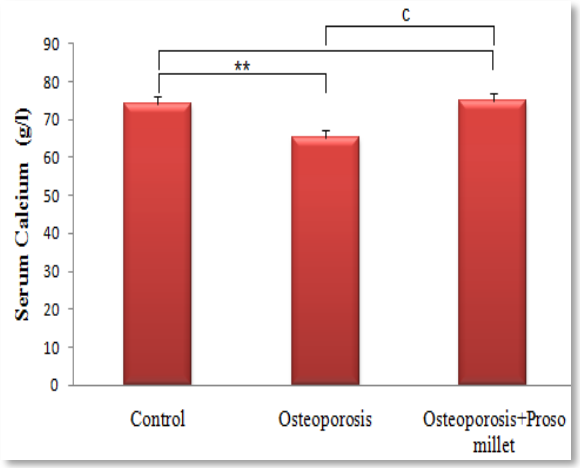
### II.3.2. Biochemical markers

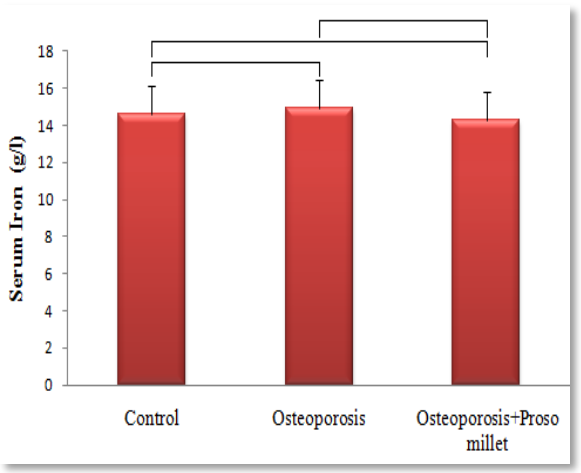
Concerning biochemical markers, our results in (figure 10) show that for osteoporosis patients group a high significant increase (P<0.01) in serum blood glucose and PAL levels, and a high significant decrease (P<0.01) in serum calcium as compared to the control group and the reference values, also that in serum PAL level for osteoporosis after treatment group is very high significant increase (P<0.001) as compared to control group, and in urinary creatinine of osteoporosis patients group and of osteoporosis after treatment group is very high significant decrease (P<0.001) as compared to that in the control group. While in serum calcium for osteoporosis after treatment group show a very high significant increase (P<0.001) as compared to osteoporosis patients group.

Also, the results show that there is no significant changes (P>0.05) in serum glucose and calcium of osteoporosis after treatment group as compared to the controls, in serum glucose, PAL and urinary creatinine of the same group as compared to osteoporosis patients group, and in iron in all groups.



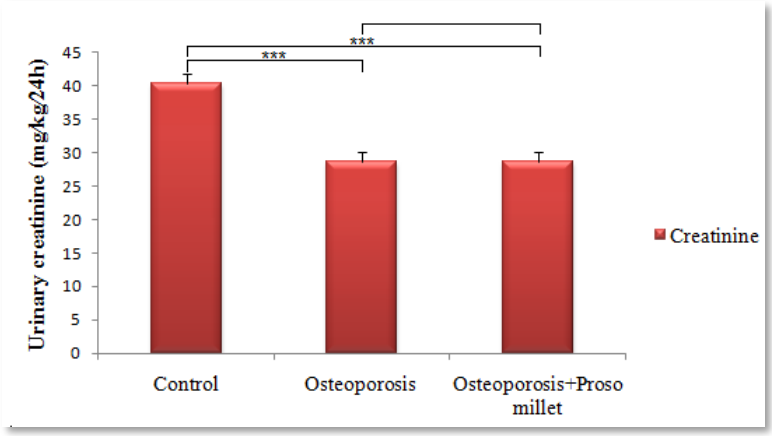



**Figure 10:** Serum blood glucose, PAL, calcium, iron and urinary creatinine levels of control and experimental groups.

### II.3.3. Oxidative stress parameters

#### II.3.3.1. Malondialdehyde (MDA) level

The results in (figure 11) show that for osteoporosis after treatment group, a very high significant decrease in erythrocyte MDA level ($P<0.001$) and a very high significant increase in serum MDA ($P<0.001$) as compared to that in the control, and a high significant decrease ($P<0.01$) in erythrocyte MDA as compared to that in the osteoporosis patients group. For osteoporosis patients group, the results obtained show a significant increase in leukocyte MDA level ($P<0.05$) and a very high significant increase in serum MDA level ($P<0.001$) as compared to the control, and a very high significant decrease in leukocyte and in serum MDA ($P<0.001$) for osteoporosis after treatment group as compared to that in the osteoporosis patients group. While there is no significant change ($P>0.05$) in erythrocyte MDA level for osteoporosis patients group, and in leukocyte MDA level for osteoporosis after treatment group as compared to the controls.

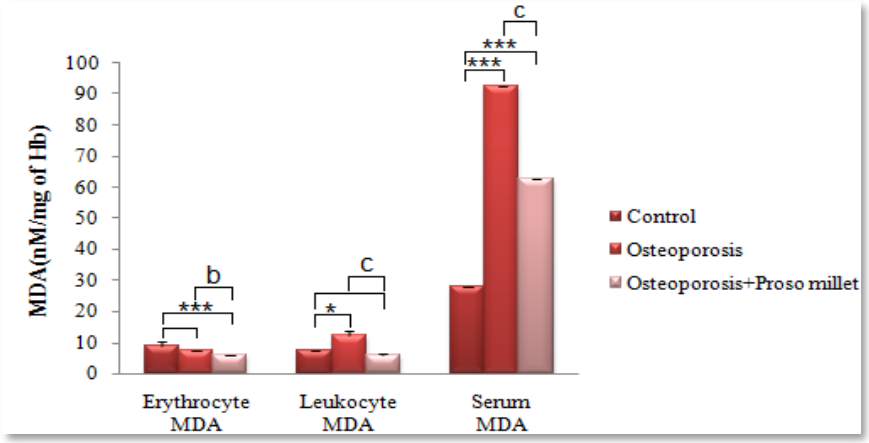



**Figure 11:** Lipid peroxidation level of control and experimental groups.

### II.3.3.2. Reduced glutathione (GSH) level

The results in (figure 12) show that for osteoporosis patients group, a very high significant decrease in erythrocyte and leukocyte GSH level ($P<0.001$) as compared to that in the control, also a high significant increase ($P<0.01$) for osteoporosis after treatment group as compared to that in the osteoporosis patients group, and for osteoporosis patients group, the results obtained show a significant decrease in serum GSH level ($P<0.05$) as compared to that in the control, and a significant increase ($P<0.05$) for osteoporosis after treatment group as compared to that in the osteoporosis patients group. Also, the results showed that there is no significant changes ($P>0.05$) in erythrocyte, leukocyte and serum GSH of osteoporosis after treatment group as compared to that in the control.

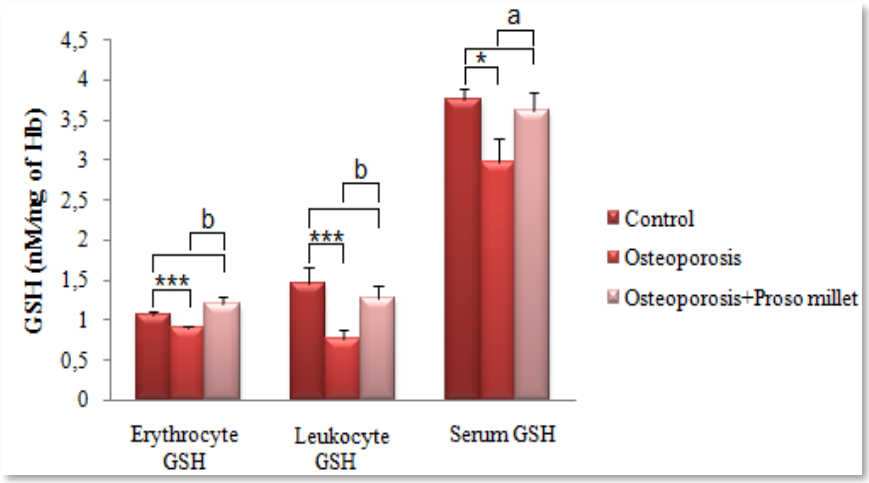



**Figure 12:** Reduced glutathione (GSH) level of control and experimental groups.

### II.3.3.3. Catalase

In the (figure 13), for osteoporosis patients group, the results obtained show a very high significant decrease in leukocyte catalase level ($P<0.001$) and a significant decrease in serum catalase ($P<0.05$) as compared to that in the control, also show a significant decrease in leukocyte catalase ($P<0.05$) for osteoporosis after treatment group as compared to that in the control. While there is no significant change ($P>0.05$) in serum catalase of osteoporosis after treatment group as compared to that in the control, and in leukocyte and serum catalase of the same group as compared to osteoporosis patients group.

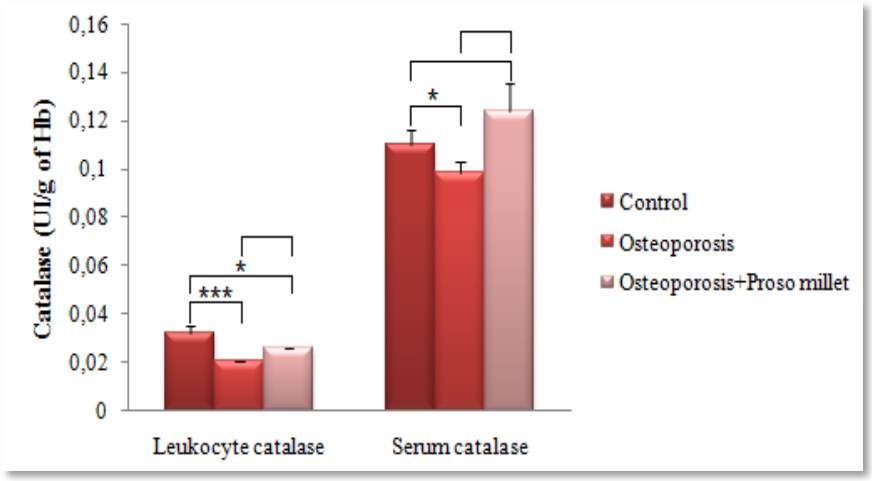



**Figure 13:** Catalase activity of control and experimental groups.

### II.3.3.4. Vitamin C

The results in (figure 14) show that for osteoporosis after treatment group, a very high significant decrease in serum vitamin C level (P<0.001) as compared to that in the control group, also as compared to that in the osteoporosis patients group. While there is no significant change (P>0.05) in the serum vitamin C for osteoporosis patients group as compared to the control group.

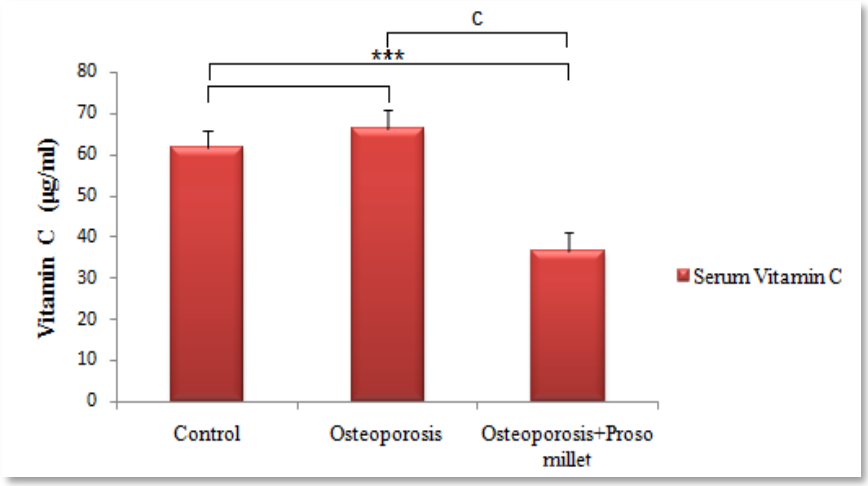



**Figure 14:** Vitamin C level of control and experimental groups.

### II.3.3.5. Superoxide dismutase (SOD)

The results in (figure 15) show that for osteoporosis patients group, a very high significant decrease in leukocyte and serum SOD level (P<0.001) as compared to that in the control, and for osteoporosis after treatment group, a very high significant increase in leukocyte SOD level (P<0.001) as compared to that in the osteoporosis patients group, a very high significant decrease in serum SOD level (P<0.001) as compared to that in the control,

and a significant decrease in serum SOD (P<0.05) as compared to that in the osteoporosis patients group. While there is no significant change (P>0.05) in leukocyte SOD of osteoporosis after treatment group as compared to the control group.

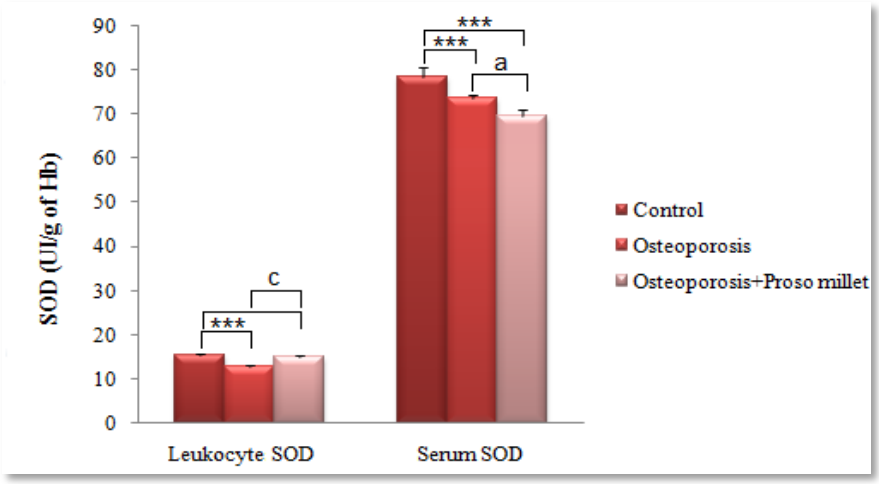



**Figure 15:** Superoxide dismutase activity of control and experimental groups.

### II.3.3.6. Oxygen Radical Absorbance Capacity (ORAC)

The results in (figure 16) show that for osteoporosis patients group, a high significant decrease in serum ORAC level (P<0.01) as compared to that in the control, and a very high significant increase (P<0.001) for osteoporosis after treatment group as compared to that in the osteoporosis patients group. Also, there is no significant changes (P>0.05) in serum ORAC level of osteoporosis after treatment group as compared to that in the control.

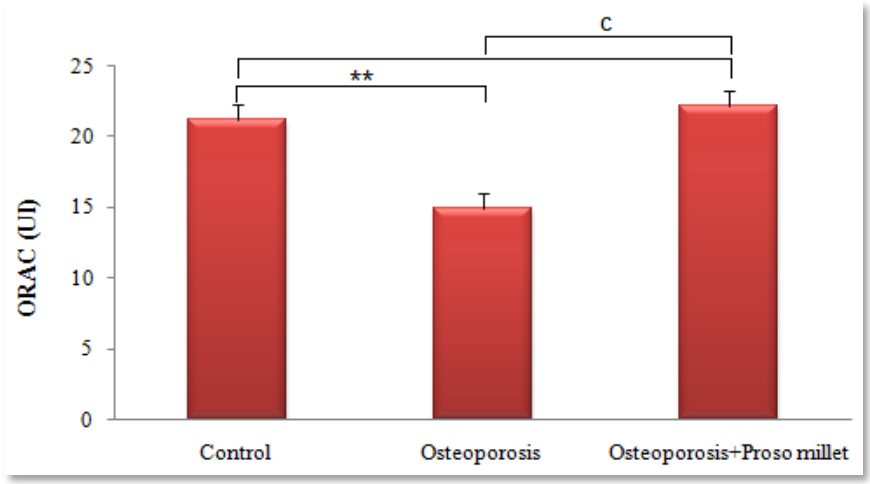



**Figure 16:** ORAC level of control and experimental groups.

### II.4. Predictive factors study

The results obtained (figure 17 and table 07) show that the MDA level in leukocyte and serum and sedimentation rate in both 1st and 2nd hour as the highest percentage of specificity (100%) and important percentage of sensitivity (43.8, 50.0, 37.5, 6.3%), respectively for women with osteoporosis.

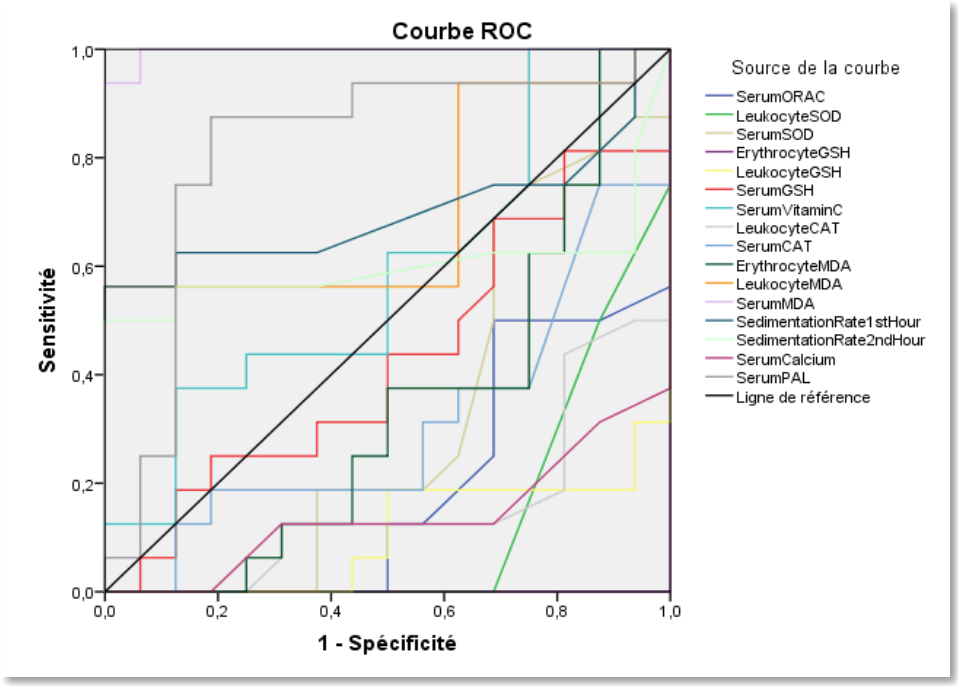



**Figure 17:** ROC Curve for biological markers in women with osteoporosis.

**Table 07:** Sensitivity, specificity and AUC values of biological markers for women with osteoporosis.

| Test Result Variable(s) | Sensitivity % | Specificity % | AUC % | SE | *P value* | Asymptotic 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| **Serum ORAC** | 50.0 | 25 | 19.1 | 0.076 | **0.003** | 0.043 | 0.340 |
| **Leukocyte SOD** | 75.0 | 00 | 12.5 | 0.063 | **0.000** | 0.001 | 0.249 |
| **Serum SOD** | 68.8 | 31.2 | 32.2 | 0.099 | 0.086 | 0.129 | 0.516 |
| **Erythrocyte GSH** | 43.8 | 00 | 00 | 0.000 | **0.000** | 0.000 | 0.000 |
| **Leukocyte GSH** | 18.8 | 25 | 10.5 | 0.058 | **0.000** | 0.000 | 0.219 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Serum GSH** | | 81.3 | 12.5 | 42.8 | 0.103 | 0.486 | 0.226 | 0.630 |
| **Serum vitamin C** | | 43.8 | 62.5 | 58.6 | 0.103 | 0.407 | 0.384 | 0.788 |
| **Leukocyte catalase** | | 50.0 | 6.2 | 15.8 | 0.071 | **0.001** | 0.019 | 0.298 |
| **Serum catalase** | | 31.3 | 43.7 | 30.9 | 0.096 | 0.065 | 0.121 | 0.496 |
| **Erythrocyte MDA** | | 43.8 | 25 | 34.0 | 0.100 | 0.122 | 0.145 | 0.535 |
| **Leukocyte MDA** | | 43.8 | 100 | 70.7 | 0.098 | 0.064 | 0.515 | 0.899 |
| **Serum MDA** | | 50.0 | 100 | 99.6 | 0.006 | **0.000** | 0.983 | 1.000 |
| **Sedimentation rate** | **1st hour** | 37.5 | 100 | 69.9 | 0.102 | 0.055 | 0.499 | 0.900 |
| | **2nd hour** | 6.3 | 100 | 60.2 | 0.115 | 0.327 | 0.376 | 0.827 |
| **Serum Calcium** | | 56.3 | 00 | 13.9 | 0.069 | **0.000** | 0.004 | 0.273 |
| **Serum PAL** | | 43.8 | 87.5 | 81.6 | 0.085 | **0.002** | 0.651 | 0.982 |

### II.5. Correlation between biological markers

The results (table 08) represent the correlation between oxidative stress parameters (serum ORAC, leukocyte SOD, erythrocyte GSH, leukocyte GSH, leukocyte catalase and serum MDA), hematological parameters (WBC, HGB, RBC, HCT, PLT and sedimentation rate) and biochemical parameters (glucose, calcium, PAL and urinary creatinine) in group patients of women with osteoporosis. There was a positive correlation ($P<0.05$) between erythrocyte GSH and calcium (P=0,005 and R=0,381), erythrocyte GSH and PAL (P=0,047 and R=0,393), leukocyte catalase and sedimentation rate of the 2nd hour (P=0,046 and R=0,395), serum calcium and WBC (P=0,008 and R=0,364), serum PAL and sedimentation rate of the 1st hour (P=0.040 and R=0.286) and of the 2nd hour (P=0.035 and R=0.294),

There was a negative correlation ($P<0.05$) between erythrocyte GSH and sedimentation rate of the 1st hour (P=0,018 and R= -0,461) and of the 2nd hour (P=0,020 and R= -0,455) and serum PAL and HGB (P=0,017 and R= -0,330). There was no correlation ($P>0.05$) between the rests of correlation test in patients groups.

**Table 08:** Correlation between biological markers for women with osteoporosis.

| | | WBC | HGB | RBC | HCT | PLT | Sedimentation rate | | Serum blood glucose | Serum calcium | Serum PAL | Urinary creatinine |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 1st hour | 2nd hour | | | | |
| **Serum ORAC** | **P** | 0,926 | 0.565 | 0,729 | 0,642 | 0,429 | 0,983 | 0,818 | 0,961 | 0,447 | 0,734 | 0,384 |
| | **R** | 0,019 | 0.118 | 0,071 | 0,096 | 0,162 | -0,004 | 0,047 | -0,010 | -0,108 | 0,070 | 0,123 |
| **Leukocyte SOD** | **P** | 0,580 | 0,879 | 0,582 | 0,420 | 0,644 | 0,569 | 0,379 | 1,000 | 0,435 | 0,146 | 0,122 |
| | **R** | -0,114 | -0,031 | -0,113 | -0,165 | -0,095 | -0,117 | -0,180 | -0,000 | 0,111 | -0,293 | 0,217 |
| **Erythrocyte GSH** | **P** | 0,624 | 0,221 | 0,665 | 0,303 | 0,617 | **0,018** | **0,020** | 0,959 | **0,005** | **0,047** | 0,926 |
| | **R** | -0,101 | -0,248 | -0,089 | -0,210 | 0,103 | -0,461 | -0,455 | -0,011 | 0,381 | 0,393 | 0,013 |
| **Leukocyte GSH** | **P** | 0,458 | 0,463 | 0,929 | 0,700 | 0,320 | 0,954 | 0,815 | 0,158 | 0,086 | 0,955 | 0,780 |
| | **R** | 0,152 | -0,151 | -0,018 | -0,079 | 0,203 | -0,012 | -0,048 | 0,285 | 0,240 | -0,012 | -0,040 |
| **Leukocyte catalase** | **P** | 0,318 | 0,379 | 0,962 | 0,722 | 0,761 | 0,077 | **0,046** | 0,824 | 0,375 | 0,115 | 0,630 |
| | **R** | -0,204 | -0,180 | -0,010 | -0,073 | -0,063 | 0,353 | 0,395 | -0,046 | -0,126 | 0,317 | -0,068 |
| **Serum MDA** | **P** | 0,938 | 0,870 | 0,715 | 0,619 | 0,285 | 0,445 | 0,499 | 0,501 | 0,582 | 0,521 | 0,432 |
| | **R** | 0,016 | 0,034 | -0,075 | 0,102 | -0,218 | 0,157 | 0,139 | 0,138 | 0,078 | -0,132 | 0,111 |
| **Serum calcium** | **P** | **0,008** | 0,664 | 0,244 | 0,952 | 0,982 | 0,872 | 0,798 | 0,920 | * | 0,759 | 0,825 |
| | **R** | 0,364 | -0,062 | -0,165 | -0,009 | -0,003 | -0,023 | -0,036 | 0,014 | 1 | 0,044 | -0,031 |
| **Serum PAL** | **P** | 0,187 | **0,017** | 0,629 | 0,560 | 0,109 | **0,040** | **0,035** | 0,398 | 0,759 | * | 0,749 |
| | **R** | -0,186 | -0,330 | 0,069 | -0,083 | 0,225 | 0,286 | 0,294 | 0,120 | 0,044 | 1 | -0,045 |

### I.6. Study of Odds ratio values of biochemical and oxidative stress markers

Odds ratio (OR) values for some oxidative stress parameters and biochemical markers of controls and experimental group (table 09) show that decreased serum ORAC, leukocyte SOD, erythrocyte GSH, leukocyte GSH, leukocyte catalase and serum calcium are shown to be significant risk factors for osteoporosis OR (6.303-106.375) with P<0.05. In contrast, decreased serum MDA and serum PAL are protective factors against osteoporosis in the study population (OR=0.032; P=0.000, OR=0.084; P=0.000), respectively.

**Table 09:** Comparison of some oxidative stress parameters and biochemical markers of controls and experimental group (N=32).

| | **Control%** | **Patient%** | **OR** | **$CI_{95}$%** | ***P-value*** |
|---|---|---|---|---|---|
| **Serum ORAC** | | | 6.303 | 2.604-15.255 | 0.000 |
| Positive | 32 | 11 | | | |
| Negative | 18 | 39 | | | |
| **Leukocyte SOD** | | | 106.375 | 13.475-839.745 | 0.000 |
| Positive | 37 | 01 | | | |
| Negative | 16 | 46 | | | |
| **Erythrocyte GSH** | | | 37.161 | 4.796-287.936 | 0.000 |
| Positive | 32 | 01 | | | |
| Negative | 31 | 36 | | | |
| **Leukocyte GSH** | | | 18.951 | 2.428-147.909 | 0.000 |
| Positive | 21 | 01 | | | |
| Negative | 41 | 37 | | | |
| **Leukocyte catalase** | | | 8.957 | 2.424-33.103 | 0.000 |
| Positive | 17 | 3 | | | |
| Negative | 31 | 49 | | | |
| **Serum MDA** | | | 0.032 | 0.004-0.248 | 0.000 |
| Positive | 30 | 50 | | | |
| Negative | 19 | 1 | | | |
| **Serum calcium** | | | 18.222 | 5.035-65.946 | 0.000 |
| Positive | 32 | 3 | | | |
| Negative | 24 | 41 | | | |
| **Serum PAL** | | | 0.084 | 0.030-0.233 | 0.000 |
| Positive | 19 | 44 | | | |
| Negative | 31 | 6 | | | |

### II.7. Multivariate analysis with several predictors (risk factors for osteoporosis) and oxidative status

In this analysis (table 10), the dependent variables are the markers of significantly increased oxidative status in cases of osteoporosis (serum ORAC, leukocyte SOD, erythrocyte GSH, leukocyte GSH, leukocyte catalase and serum MDA).

The results show that menstrual perturbations before menopause is a factor positively correlated with serum MDA (p=0.010) and it is responsible for 44.2% of the variation in this parameter in the study population. Also, bone problems before menopause is a factor significantly associated with leukocyte catalase levels (p=0.023), and explain part of their variation within the study population 33.9%. Hormonal alternatives before menopause, vitamin D deficiency before menopause, family history and thyroid problems do not appear to be factors that can influence markers of oxidative status in the study population.

**Table 10:** Multivariate analysis with several predictors (risk factors for osteoporosis) and oxidative status in the study population.

| **Independant variable** | **Dependant variable** | | | | | |
|---|---|---|---|---|---|---|
| | Serum ORAC | Leukocyte SOD | Erythrocyte GSH | Leukocyte GSH | Leukocyte catalase | Serum MDA |
| Hormonal alternatives before menopause<br>**P**<br>**$R^2$%** | 0,757<br>0.8 | 0,305<br>8.7 | 0,488<br>8.3 | 0,573<br>4.8 | 0,475<br>4.0 | 0,633<br>2 |
| Menstrual perturbations before menopause<br>**P**<br>**$R^2$ %** | 0,451<br>4.8 | 0,703<br>1.3 | 0,081<br>42.2 | 0,253<br>18.1 | 0,279<br>9.0 | **0,010**<br>44.2 |
| Vitamin D deficiency before menopause<br>**P**<br>**$R^2$%** | 0,808<br>0.5 | 0,572<br>2.7 | 0,488<br>8.3 | 0,880<br>0.3 | 0,226<br>11.1 | 0,902<br>0.1 |
| Family history<br>**P**<br>**$R^2$%** | 0,653<br>1.7 | 0,572<br>2.7 | 0,246<br>21.6 | 0,560<br>5.1 | 0,226<br>11.1 | 0,961<br>0.0 |
| Thyroid problems<br>**P**<br>**$R^2$%** | 0,445<br>4.9 | 0,402<br>5.9 | 0,923<br>0.2 | 0,072<br>39.1 | 0,569<br>2.6 | 0,820<br>0.5 |
| Bone problems before menopause<br>**P**<br>**$R^2$%** | 0,666<br>1.6 | 0,118<br>19.1 | 0,515<br>7.4 | 0,599<br>4.1 | **0,023**<br>33.9 | 0,864<br>0.3 |

# III. Discussion

## III.1. Study of predictors factors of osteoporosis

In our study, we examined the association between some risk factors linked with menopause which may cause osteoporosis.

We found that family history is significantly associated with the risk of having osteoporosis in menopausal women of our region, the genetic predisposition to osteoporosis is the end result of multiple gene polymorphisms and gene-by-environment interactions, each contributing a small amount to BMD (bone mineral density) variance. There are few instances in which a single gene is responsible for a direct effect on BMD, and most of these genes have resulted in clinically apparent bone disease (Epstein, 2012). Besides, the results of our study shows that bone problems before menopause were significantly associated with osteoporosis risk, the unfavorable effects of low premenopausal bone mass and accelerated loss of bone after menopause are additive, and these individuals are the highest risk for fracture (Melmed et *al*., 2011). Bone mineral density measurements have shown that bone loss begins to increase in normal women before the menopause. Indeed, in a well designed longitudinal study, reported that more than 50% of all vertebral bone loss occurred before the menopause (Foa, 2012).

Also, our study shows that vitamin D deficiency and calcium deficiency are associated with osteoporosis risk, **v**itamin D plays an important role in the maintenance of calcium and phosphorus metabolism and thus in bone health so vitamin D deficiency have long-term detrimental effects such as increased risk of low bone mass and osteoporosis (Feizabad et *al*., 2017). Calcium gut by absorption and from bone by resorption, calcium leaves the extracellular fluid via the gastrointestinal tract, kidneys, and skin and enters into bone via bone formation. Osteoporosis is common in gastrointestinal diseases, particularly those associated with malabsorption and maldigestion (celiac disease, pancreatic insufficiency) (Katz & Weinerman, 2010). The rediscovery of earlier information that calcium deficiency led to the development of osteoporosis in experimental animals (WHO & FAO, 2004).

In addition, thyroid problems are associated with osteoporosis risk, the thyroid hormone is essential for normal bone maturation and it increases calcium release from fetal rat long bone cultures, and increases osteoclast number and activity (Mundy et *al*., 1976). In vivo, thyroid hormone also stimulates osteoblast activity (Mosekilde et *al*., 1977). T3 receptor has been demonstrated in osteoblasts, but not osteoclasts, suggesting that increased osteoclast

activity in bone cultures with T3 treatment is secondary to osteoblast activation. Impaired functioning of the thyroid gland has long been known to accelerate bone turnover and thus increase the risk for developing osteoporosis, especially in peri-and postmenopausal women (Kung, 1994).

In our study it was observed that obesity can possibly affects bone metabolism through several mechanisms. Because both adipocytes and osteoblasts are derived from a common multipotential mesenchymal stem cell, obesity may increase adipocyte differentiation and fat accumulation while decrease osteoblast differentiation and bone formation (Jay, 2011). The results of our study revealed that stress is related to osteoporosis, a new study demonstrates how anxiety levels are linked to an increased risk of bone fractures in postmenopausal women (Catalano et *al*., 2018). The increase in sympathetic nervous activity causes bone loss through increased bone resorption and decreased bone formation, associated with β2-adrenergic activity toward both osteoblastic and osteoclastic cells (Togari et *al*., 2012).

Additionally, in our study it was observed that taking contraceptive pills is associated with osteoporosis risk, the use of oral contraceptive within the perimenopausal years prevents the activation of bone metabolism and the decrease in bone mass. Recent data suggest that the oral contraception may impede the development of peak bone mass, particularly when started within the teen years. Initiation of oral contraceptive within the first 3 years after menarche would be a strong determinant of bone mass acquisition impairment (Trémollieres, 2013). Our study shows that early post menopause is associated with osteoporosis risk, early post menopausal women are more prone to osteoporosis due to reduction in estrogen which may further lead to elevation of oxidative stress and lipid accumulation which will promote osteoblasts apoptosis. Proinflammatory cytokines are elevated following estrogen deficiency. These cytokines are important determinants of osteoclasts differentiation and its bone resorption activity (Nazrun et *al*., 2012).

In our study, hormonal perturbations before menopause is associated with osteoporosis risk, sex hormone deficiency is a major factor contributing to postmenopausal osteoporosis. Oestrogen deficiency increases the rate of bone remodelling and leads to an imbalance between bone resorption and formation, resulting in a net bone loss and possibly osteoporosis (Manolagas & Jilka, 1995). Cytokines such as interleukin-1 (IL-1), tumour necrosis factor-a (TNF-α), interleukin-6, interleukin-11, and macrophage colony-stimulating factor may be involved in bone resorption by facilitating the recruited and maturation of osteoclast precursors (Rizzoli & Bonjour, 1997). Our study shows that anemia is related with

osteoporosis risk, it is associated with bone mass density scores by chronic hypoxia is likely to interfere with bone metabolism. Increased oxidative stress and extracellular acidification under hypoxic conditions are estimated to influence bone formation and remodeling (Oh et *al*., 2017).

Furthermore, our study shows that milk mixed with coffee is associated with osteoporosis risk, Hallstrom et *al*. found that a high coffee consumption significantly increased the risk of osteoporotic fractures. The results of the study indicate that a daily intake of 330 mg of caffeine, equivalent to 4 cups (600 ml) of coffee, or more, may be associated with a modestly increased risk of osteoporotic fractures, especially in menopausal women with a low intake of calcium (Hallström et *al*., 2006). Our results showed that healthy diet is a protective factor of osteoporosis, many studies that have used factor or principal component analysis have empirically derived a "healthy" or "prudent" food pattern that is high in fruits, vegetables, fish, low-fat foods, fiber, legumes, etc positively correlated with BMD (Park et *al*., 2012), and decreased the risk for osteoporosis increase potassium, calcium and vitamin D high intake (Prentice, 2004). Antioxidants may play an important role in the prevention of oxidative stress-related osteoclastogenesis and bone resorption (Park et *al*., 2012).

As well as, our result indicated that sunshine exposure is a protective factor of osteoporosis, there is a need for health education of these women in the prevention of osteoporosis and especially paying attention to the proper intake of calcium and more vitamin D in their diet. At the same time, adequate exposure to the sun is vital as it is the main source of vitamin D for the body coming not from the food consumed (Kopiczko, 2014). Also, our study revealed that long distance walks is a protective factor of osteoporosis, walking in combination with others forms of exercise (jogging, stair-climbing, stepping), can provide some protection against bone loss, by the benefits of walking on aerobic fitness on body composition and cardio-metabolic health (Daly et *al*., 2019).

### III.2. Qualitative and quantitative phytochemical analysis of *Proso millet*

The phytochemical analysis of the aqueous extract of *Proso millet* shows that it is rich on different important secondary metabolites such as alkaloids, tannins, flavonoids, saponins, reducing sugars and terpenoids. With high quantitative levels of total polyphenols and flavonoids.

These secondary metabolites have multiple properties such as anti-inflammatory, antibacterial, antioxidant, immune boosting, anticancer, anti-ageing and anti-diabetic

potentials (Kumar et *al.*, 2017). Phenolic compounds are classified into phenolic acids, flavonoids and tannins. Millets phenols are reported to have many biological and pharmacological properties such as antioxidant, anti-mutagenic, anti-inflammatory, antiviral effects and platelet aggregation inhibitory activity (Kumar et *al.*, 2018), these compounds have potential to protect us from various pathological conditions induced by free radicals such as cardiovascular disease, cancer, neurodegenerative disorders, diabetes, ischemia/reperfusion, and ageing (Sharma, 2014 ; Karabulut et *al.*, 2018) due to their chemical structure, especially the number and position of hydroxyl groups and the presence of an aromatic ring, working as efficient reactants of ROS (reactive oxygen species) with their ability to inactivate free radicals by transferring hydrogen atoms to these molecules or by donating an electron to the radical (De Lima Cherubim et *al.*, 2019). Alkaloids demonstrate a diverse array of pharmacological actions including analgesia, antibacterial, antiviral and allelopathic properties (Hussein & El-Anssary, 2018). Saponins also exhibit different biological activities including anti-inflammatory, hypocholesterolemic and immune-stimulating remedies (Kregiel et *al.*, 2017). Reducing sugars (D-glucose, D-arabinose, D-mannose) revealed a great antioxidant activity (Haghparast et *al.*, 2013). Also, terpenoids have various biological activities such as antitumor, anti-inflammatory, antibacterial, antiviral, preventing and treating cardiovascular diseases, lowering blood sugar, and immunomodulatory, antioxidant, antiaging and neuroprotective effects (Yang et *al.*, 2020).

Polyphenols exert antioxidant functions, scavenging ROS, as well as anti-inflammatory activities, altering the expression of genes like proinflammatory cytokines, lipoxygenase (LOX), nitric oxide synthase (NOS), and cyclo-oxygenase (COX). Flavonoids are the most common polyphenols which exert healthy effects in terms of metabolism, weight, chronic disease, and neuroendocrine immune control (Magrone et *al.*, 2019).

### III.3. Biological marker study

#### III.3.1 Biochemical markers

The obtained results show a significant increase in PAL level in patient group as compared to control. This result is in agreement with the study of (Tirtha et *al.*, 2014), show that the PAL level was slightly higher in the post-menopausal group. All bone mineral density (BMD) results were significantly decreased when PAL was increased. While bone-specific alkaline phosphatase, is a marker of bone formation and bone turnover and is used in the evaluation of skeletal status (Hailing et *al.*, 2018).

On the other hand, we found a significant decrease in calcium level in patient group as compared to control. This result is in agreement with the study of (Tirtha et *al.*, 2014), findings demonstrated that the serum calcium level was significantly lower in the post-menopausal group. Calcium plays a key role in human physiology. As a basic constituent of the mineral component providing stiffness to the collagen network of mature bone. Insufficient calcium accrual, leading to a sub-optimal bone mass peak and low bone mineralization, is an important factor favoring osteoporosis and fracture (DeLucia et *al*., 2003).

In our experimental study, the results show a significant increase in calcium levels in osteoporosis patients after treatment by *Proso millet* as compared to patient group. Millet(including: *Proso* (*Panicum miliaceum*)), contributes substantial amounts of energy and protein to the diets of people in many developing countries (Luis et *al*., 1981), but millets are similar to other cereals in calcium and phosphorus contents (Burton et *al*., 1972). In study of mineral components of puffed grains (mg/100 g) (Piłat et *al*., 2016), show that *Proso millet* composed 14.75(mg/100g) of calcium which may contribute to improve the level of calcium.

Our results showed that significant decrease of urinary creatinine level in patient group as compared to control. In two small studies, Yendt et *al*., (1991), showed a strong and positive correlation between creatinine clearance (based on three successive 24-hour urine specimens for creatinine) and bone mass at the radius and lumbar spine. In a second study, they compared 77 non-osteoporotic women to 37 women with primary osteoporosis and 25 osteoporotic patients with new vertebral crush fractures and a found a significant association between creatinine clearance and bone mineral density (BMD) at the radius and lumbar spine, independent of age and body stature (Yendt et *al.*, 1993); creatinine clearance was significantly lower in women with vertebral fractures than in age-matched women without fractures (Simerjot et *al*., 2010).

In addition, the results show a significant increase in blood glucose levels. 25(OH)D (vitamin D) was positively correlated with the presence of abnormal glycol-metabolism (Liu et *al.*, 2018). Previous studies showed that vitamin D may contribute to the development of type 2 diabetes mellitus. Insulin secretion may be influenced by vitamin D indirectly for its role in the regulation of calcium flux through the cell membrane combined with its role in the synthesis and regulation of calbindin, which is a vitamin D-dependent Ca-binding protein in pancreatic β cells (Liefde et *al.*, 2005).

### III.3.2 Hematological parameters

The results of hematological parameters showed a significant increase in WBC, HGB, HCT and PLT in osteoporosis patients group as compared to the control group. These findings support a possible linkage between bone metabolism and hematopoiesis. Hematopoiesis is the process by which immature blood cells develop into mature cells. An important role for osteoblasts in the regulation of multipotent hematopoietic stem cells (HSCs) was demonstrated by a series of *ex vivo* and *in vivo* studies, it has been provided by studies of targeted ablation of osteoblasts. Therefore, bone metabolism and hematopoiesis appear to be closely associated and directly linked by osteoblast activity in the elderly (Laudisio et *al.*, 2009 ; Kim et *al*., 2011 ; Paspaliaris & Kolios, 2019). According to Schyrr et *al*., (2018), differences in the osteoporotic bone microenvironment translate into altered dynamics upon hematopoietic stress. Also, Valderrábano et *al*., (2018) found that poor bone health would lead to increased cells of myeloid lineage such as neutrophils and monocytes. Cells of the osteoblastic lineage might affect differentiation of neutrophils and monocytes differently. The increase in neutrophils may be related to the chronic inflammation that occurs with aging.

The results of erythrocyte sedimentation rate (or ESR) show that, for osteoporosis patient group ESR of the 1$^{st}$ and 2$^{nd}$ hour are a significant increase as compared to the control group and that ESR of the 1$^{st}$ and of the 2$^{nd}$ hour have a high specificity in ROC statistic, which showed the importance of ESR in the prognostic of osteoporosis, which is confirmed by Chen et *al*., (2019), who found that elevated erythrocyte sedimentation rate was associated with low bone mineral density level. Therefore, elevated ESR might be an effective indicator in predicting osteoporosis. Chronic inflammation can inhibit the formation of osteoblast cells and stimulate osteoclast differentiation, and finally lead to systemic bone loss and secondary osteoporosis.

While in WBC of osteoporosis after the treatment group show a very high significant decrease, white blood cells are considered a reliable biomarker of inflammation, therefore, decreased WBC count indicate lower levels of inflammation (Wirth et *al.,* 2018). Also, a significant decreased ESR of the 1$^{st}$ hour for osteoporosis after treatment group as compared to the osteoporosis patient group may be due to its phenolic compounds such as niacin, riboflavin, folic acid and Hydroxycinnamic acid derivatives(such as p-Coumaric and ferulic acid) content (Kumar et *al.,* 2018). Si et *al*., (2014) studies suggest that niacin inhibits vascular inflammation *in vivo* and *in vitro* via downregulating nuclear factor kappa B (NF-κB) signaling pathway. There are also a number of studies describing the role of Vitamin B2

(riboflavin) and B9 (folic acid) in the regulation of immune responses and inflammation (Kennedy, 2016 ; Mikkelsen et *al.,* 2019). In addition, Hydroxycinnamic acid derivatives showed anti-aging and anti-inflammatory properties both *in vitro* and *in vivo,* ferulic acid prevented the production of TNF-α and inhibits NF-κB activation, and also, p-Coumaric acid decrease the expression of inflammatory mediator TNF-α (Pragasam et *al.,* 2013 ; Alam et *al.,* 2016 ; Taofiq et *al.,* 2017) and a high significant increase in HGB for osteoporosis after the treatment group as compared to the osteoporosis patients group, which is confirmed by several studies (Chandra et *al.,* 2016 ; Singh et *al.,* 2019 ; Durairaj et *al.,* 2019 ; Prakash, 2020) proving that aqueous extract of *Proso millet* have improvement on hemoglobin status.

### III.3.3. Oxidative stress markers

The results of the oxidative stress study showed for osteoporosis patients group a very high significant increase in serum MDA level as compared to the control women. In addition, the results showed that serum MDA has a high specificity in ROC statistic, which showed the importance of MDA in the prognostic of osteoporosis. The results found were similar to those observed in study of Sendur et *al*., (2009) which showed that serum MDA levels were significantly higher in postmenopausal women with osteoporosis than in the healthy controls. Kovachich & Mishra, (1980) ; Muthusami et *al*., (2005) and Altindag et *al*., (2008) studies reported that increased osteoclastic activity and decreased osteoblastic activity may be associated with an imbalance between oxidant and antioxidant status in postmenopausal osteoporosis and that may have been responsible for increased production of ROS in superoxide forms, which is evident by increased levels of serum MDA levels. Sontakki & Tare, (2002) reported that MDA had an osteoclastic activity.

Our results show that leukocyte MDA is significantly increased in osteoporosis patients group as compared to control women, with high specificity in ROC statistic test, which showed the importance of this parameter in the identification of the disease. These results are supported by Sheweita & Khoshhal, (2007) study. Raghavan et *al*., (2012) found that MDA could significantly induce key inflammatory cytokine expression in lymphocyte via oxidant stress, signaling pathways (p38MAPK), and transcriptional factors (NF-κB), which in turn enhance lymphocyte activation.

The results show also that for osteoporosis patients group, a very high significant decrease in leukocyte and serum SOD level ($P<0.001$) as compared to that in the control. Similarly, Rao & Rao, (2013) and Zhou et *al.,* (2016) whom demonstrated that decreased

SOD activity has been found in postmenopausal women compared with healthy controls, depressed activities of the antioxidant enzymes such as SOD illustrated a defense mechanism that may have been overwhelmed in mitigating the increased superoxide production by the osteoclasts represented by increased levels of MDA in the serum (Sontakke & Tare, 2002) and it might cause markedly increased bone demineralization and, as a result, may increase destructive free radical levels (Avitabile et *al.*, 1991).

The results in show that for osteoporosis patients group, a high significant decrease in serum ORAC level ($P<0.01$) as compared to that in the control. ORAC method is relevant to *in vivo* conditions because it uses a biologically relevant free radical source (peroxyl radical) which is the most prevalent free radical in human biology. It considers both inhibition time and degree of inhibition of free radical action caused by antioxidants (Prior, 2014).

The results of the oxidative stress study showed that there are a significant decrease in GSH, catalase, and SOD level in leukocyte; in GSH level in erythrocyte, and both of GSH and catalase level of serum in patients group compared to controls. Several studies show that anti oxidants has a fundamental role in preventing postmenopausal osteoporosis (Maggio et *al.*, 2003). To alleviate the cell damaging effects of ROS, aerobic organisms evolved by expressing numerous antioxidant defenses, including CAT and glutathione S-transferases (GST). The mechanisms by which cells sense $H_2O_2$ and $O^{2\bullet}$ are not understood, but a number of transcriptional factors that regulate the expression of antioxidant genes are modulated by oxidation and reduction reactions (Jamieson & Storz, 1997). Intracellular redox imbalance caused by SOD deficiency plays a pivotal role in the development and progress ion of bone fragility both *in vivo* and *in vitro* (Nojiri, 2011).

The results of the effects of aqueous extract of *P. millet* on oxidative stress parameters appeared a very high significant decrease in erythrocyte, leukocyte and in serum MDA level and very high significant increase in leukocyte SOD, in serum ORAC, GSH in erythrocyte, leukocyte and serum for osteoporosis after treatment group as compared to the osteoporosis patients group. *P. millet* extract can reduce the oxidative stress status by its antioxidant activity and anti-proliferative properties (Awika & Rooney, 2004 ; Kamath et *al.*, 2004 ; Choi et *al.*, 2007 ; Kil et *al.*, 2009 ; Habiyaremye et *al.*, 2017). Kumar et *al.*, (2018) found that *Proso millet* contain a considerable quantities of hydroxybenzoic acid and derivatives (Protocatechuic acid, *p*-Hydroxybenzoic acid, Vanillic); hydroxycinnamic acid and derivatives (*p*-Coumaric acid, *Trans*-ferulic acid, *Cis*-ferulic acid, 5,5′-Di ferulic acid);

flavonoids and total polyphenols. *Proso millet* displayed varying free radical scavenging activities due to the phenolic and flavonoids constituents of this plant acting mainly as reducing agents or free radical terminators, metal chelating agents, hydrogen donors and singlet-oxygen quenchers during anti-oxidant mechanisms (Kim et *al.,* 2010). Millet possessed antioxidant properties such as phenolic acids, flavonoids, tannins, and carbohydrates (e.g., XOs and insoluble fibers, specific proteins, and peptides, as well as certain micronutrients such as vitamin E and carotenoids). Furthermore, millet could be enriched with antioxidants (i.e., phenolics and flavonoid) (Liang & Liang, 2019). Phenolic acids have the ability to donate hydrogen atoms via hydroxyl groups on benzene rings to electrondeficient free radicals and in turn form a resonance-stabilized and less reactive phenoxyl radical (Chandrasekara & Shahidi, 2012 ; Udeh et *al*., 2017). Various flavonoids, which included the flavones, luteolin, and tricin millet grains were shown to have antioxidant activity, it have multiple hydroxyl groups confer upon the molecule substantial antioxidant activity (Suma & Urooj, 2012 ; Bangoura et *al*., 2013). Ferulic acid, a type of hydroxycinnamic acid and a powerful antioxidant, exhibited high levels of free radical scavenging (Castelluccio et *al*., 1995). Besides the monomeric compounds, ferulate dimers exhibiting higher antioxidant activity, were present in the millet grains (Chandrasekara & Shahidi, 2011). Also, among the naturally occurring antioxidants, high-molecular weight tannins were reported to exhibit superior *in vitro* antioxidant activity (Bors et *al*., 2000). Carotenoids act as antioxidants by quenching single oxygen and free radicals (Viswanath et *al*., 2009). Biological activities of tocols are generally believed to be due to their antioxidant action by inhibiting lipid peroxidation in biological membranes, the lipidsoluble properties of these compounds could disrupt the peroxidation of polyunsaturated fatty acids, as well as other cell membrane-associated compounds (Christopher, 1981 ; Panfili et *al*., 2003 ; Asharani et *al*., 2010).

Also, for osteoporosis + *P. millet* group, show a significant decrease in serum vitamin C as compared to that in the osteoporosis patients group, this decrease may be due to the low levels of vitamin C in the matured grains of millets (Queroz, 1991 ; Siwela, 2009 ; Himanshu et *al*., 2018). Several epidemiological studies have reported a conflicting relationship between dietary vitamin C intake and bone mineral density (BMD) in postmenopausal women (Yano et *al*., 1985 ; Sahni et *al*., 2009 ; Sugiura et *al*., 2011). The Women's Health Initiative Study reported no independent association between dietary vitamin C and BMD (Wolf et *al*., 2005). By contrast, some studies have reported a positive relationship between dietary vitamin C and BMD, but this was significant only in women with a high calcium intake (Hall & Greendale,

1998 ; Nieves et *al,*. 1998 ; Morton et *al.*, 2001). Vitamin C is a well-known antioxidant and acts as a scavenger of superoxide anion and hydrogen peroxide. Administration of vitamin C *in vivo* prevented bone loss with decreased reactive oxygen species (Kim et *al.*, 2015).

### III.4.Correlation between biological markers

In our experimental study, the results show that there are a significant correlation between erythrocyte GSH and calcium. The endogenous antioxidant defense system in gut includes glutathione (GSH) and GSH-dependent enzymes as major components. When the reactive oxygen (ROS) and nitrogen (RNS) species production is exacerbated, oxidative stress occurs and the intestinal $Ca^{2+}$ absorption is inhibited (Gabriela et *al.*, 2017).

On the other hand, we found a positive correlation between leukocyte catalase and sedimentation rate of the 2$^{nd}$ hour. Free radicals are implicated in chronic inflammatory diseases including rheumatoid arthritis, they play an important role in the severity of rheumatoid arthritis and patients usually suffer high oxidative stress. Furthermore, antioxidants either synthetic or natural are potent scavengers of free radicals and have beneficial effects on human health and disease prevention (Mohamed & Al-Okbi, 2004).

In our experimental study, the results show a positive correlation between serum calcium and WBC .Calcium acts as a second messenger in many cell types, including lymphocytes. Resting lymphocytes maintain a low concentration of $Ca^{2+}$. However, engagement of antigen receptors induces calcium influx from the extracellular space by several routes (Peacock, 2010).

The obtained results show a positive correlation between serum PAL and sedimentation rate of the 1$^{st}$ hour. The concentration of serum alkaline phosphatase may be increased in patients with rheumatoid disease (Watson, 1940). In normal serum this enzyme is derived mainly from the liver and skeleton, although other organs, including the intestine, contribute. The increase in rheumatoid patients has been attributed to osteoblastic activity (Kendall et *al*., 1970).

When oxidative stress appears as a primary disorder, inflammation develops as a secondary disorder and promotes oxidative stress, which causes the body to need an increase in antioxidant production. Thus, these inflammatory processes increase the concentration of PAL in the blood in the presence of vasculitis (Biswas, 2016 ; Seo et *al*., 2019).

### III.5. Study of Odds ratio values of biochemical and oxidative stress markers

The results of Odds ratio (OR) values for some oxidative stress parameters and biochemical markers of controls and experimental group study showed that decreased serum ORAC, leukocyte SOD, erythrocyte GSH, leukocyte GSH and leukocyte catalase are shown to be significant risk factors for osteoporosis. Antioxidants have also been associated with bone health. When compared to matched controls, elderly osteoporotic women presented lower levels of dietary and endogenous antioxidants. In accordance, *in vitro* cell models demonstrate that ROS stimulate osteoclast differentiation (Suda et *al.*, 1993), and inhibit osteoblast differentiation (Mody et *al.*, 2001). Conversely, antioxidants may reduce the damaging effects of oxidative stress on bone mass by reducing the up regulated osteoclastic differentiation and enhancing the down regulated osteoblastic differentiation (Chuin et *al.*, 2009).

Our study show that decreased serum calcium is shown to be significant risk factor for osteoporosis. Adequate calcium nutrition increases bone mineral density during skeletal growth and prevents bone loss and osteoporotic fractures in the elderly (Tim & Murray, 1996).

The obtained results show that decreased serum PAL is a protective factor against osteoporosis in the study population. The two isoforms of tissue non-specific PAL, liver-specific PAL and bone-specific PAL (BPAL) exists in almost equal proportion in serum (Magnusson et *al.*, 1997). Physiologically, BPAL adheres to osteoblastic cell membrane with only small amount released in serum. Its concentration in serum rises only in cases of increased remodeling of bone (Jean et *al.*, 2012). The tissue mineralization stimulating effect of BPAL is achieved mainly through inactivation of pyrophosphate and osteopontin, which are themselves mineralization inhibitors (Sundus et *al.*, 2019).

Our study showed that decreased serum MDA is protective factor against osteoporosis in the study population. Oxidative stress, defined as an imbalance between antioxidants and prooxidants, in consideration of the former, potentially causing damage, generally indicates that antioxidants are low and markers of oxidative stress are increased (Polidori et *al.*, 2001). Lipid peroxidation is one of the most harmful effects of ROS, of which MDA is the end product (Eberhardt, 2001), and it was seen that serum levels of MDA increased in bone disorders (Abdollahi et *al.*, 2010).

### III.6. Multivariate analysis with several predictors (risk factors for osteoporosis) and oxidative status

The results of the Multivariate analysis with several predictors (risk factors for osteoporosis) and oxidative status study showed that menstrual perturbations before menopause is a factor positively correlated with serum MDA. These results were similar to those observed in the study of Umberto et *al.*, (2013) who showed that the peak oxidative stress phase occurs in the central phases of the cycle (late follicular phase and early luteal phase), or rather at the time of ovular maturation and possible implantation. This phase occurs with: a) an increase in the production of GPx; b) a reduction in the GSH; c) an increase in the GSSG; d) a substantial stability of the MDA.

Our study showed that bone problems before menopause is a factor significantly associated with leukocyte catalase levels. CAT plays a central role in the protection of different cell types against the deleterious effects of hydrogen peroxide. In human, CAT is implicated in many physiological and pathological conditions (Bousnane et *al.*, 2017).

# *Conclusion & prospects*

## Conclusion

Osteoporosis has emerged as a global public health problem, menopausal women in which characterized by compromised bone strength predisposing to an increased risk of fracture. In addition estrogen has been shown to play a major role in the decreasing bone mass during the menopause, especially because it has a variety of protective effects on bone marrow and bone cells.

The results of our study showed that thyroid problems, hormonal alternatives, menstrual perturbations and vitamin D deficiency before menopause, bone problems before menopause and family history are proven to be major risk factors for osteoporosis which indicates the importance of the clinical factor in causing osteoporosis. In contrast, milk, fish diet, legumes, fresh vegetables and fruits, sunshine exposure, long distance walks and menopause after 50 years old are protective factors for osteoporosis in the study population.

Our study showed that the results of biochemical markers exhibit high level of PAL, calcium, and blood glucose, and low level of urinary creatinine, have a significant association with osteoporosis, concerning hematological markers our results show that high level of WBC, HGB, HCT, PLT, and ESR which are indicated in osteoporosis. So hematological and biochemical parameters are an important markers of diagnosis and therapeutic follow-up of the disease.

The results of our study confirm the existence of a relationship between oxidative stress and osteoporosis in menopausal women through changes in oxidative stress parameters and also with the presence of high specificity in ROC analysis of serum MDA which become a new reliable marker for diagnostic and predictive against osteoporosis in menopausal women, suggesting to include antioxidants as a protective on the list of special drugs for this disease.

The phytochemical analysis of the aqueous extract of *Proso millet* shows that it is rich on different important secondary metabolites such as alkaloids, tannins, flavonoids, saponins, reducing sugars and terpenoids. With high quantitative levels of total polyphenols and flavonoids, which plays an important role in fighting inflammation and oxidative stress.

The result of the effect of the aqueous extract of *P. millet* on biochemical and oxidative stress parameters appeared a very low level of lipid peroxidation and very high levels of calcium and enzymatic and non-enzymatic antioxidants levels. Especially with a high correlation between calcium, PAL and oxidative stress represented by GSH in erythrocytes

and indicates that the plant has a beneficial effect on the disruption of calcium metabolism and oxidative stress the main cause of osteoporosis in menopausal women.

The results of the Multivariate analysis with several predictors (risk factors for osteoporosis) and oxidative status study showed that menstrual perturbations before menopause is a factor positively correlated with serum MDA, bone problems before menopause is a factor significantly associated with leukocyte catalase levels. This confirms that oxidative stress is the pathophysiological mechanism explanation that causes this disease through various risk socioeconomic and clinic factors.

**Prospects:**

Given the importance of these results, they open experimental perspectives and other in-depth studies that should allow us to clearly identify to:

- Determine other factors associated with osteoporosis risk.
- Evaluation of other diagnostic markers of osteoporosis in women.
- Determine the molecular and cellular mechanism of the effect of plant.
- Evaluation of the effect of antioxidant supplementation on osteoporosis in women.

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# Annexes

## Annex 01:

### دراسة أسباب هشاشة العظام لدى النساء في مرحلة ما بعد إنقطاع الطمث

**كلية العلوم الطبيعة و الحياة** **مستوى: ماستر بيوكيمياء تطبيقية**

**وصف العينات المدروسة:**

العمر:..... الوزن:..... الطول:..... الزمرة الدموية:.....

المستوى الدراسي:..... المهنة:.....

الحالة الاجتماعية:..... عدد الأطفال:..... نعم لا

| | | |
|---|---|---|
| هل تستهلكين ماء الحنفية؟ | | |
| هل تستهلكين ماء الفيلتر؟ | | |
| هل تستهلكين حليب طبيعي؟ ☐حليب المعزاة ☐حليب البقرة | | |
| هل تستهلكين حليب صناعي؟ | | |
| هل تستهلكين الحليب الصافي يوميا؟ | | |
| هل تستهلكين حليب ممزوج بالقهوة يوميا؟ | | |
| هل تستهلكين القهوة يوميا؟ | | |
| هل تتناولين الأسماك أسبوعيا؟ | | |
| هل تتناولين البقوليات (عدس, لوبيا ,بازلاء) بكثرة؟ | | |
| هل تستهلكين الخضر و الفواكه الطازجة يوميا؟ | | |
| هل تتعرض أطرافك يوميا للشمس؟ | | |
| هل كنت تعانين من نقص الكالسيوم قبل إنقطاع الدورة الشهرية؟ | | |
| هل كنت تعانين من نقص فيتامين د قبل إنقطاع الدورة الشهرية ؟ | | |
| في حالة المرض هل تشترين الدواء بدون وصفة طبية؟ | | |
| هل استعملتي حبوب منع الحمل قبل إنقطاع الدورة الشهرية؟ | | |
| هل كنت تستعملين دواء ضد آلام الدورة الشهرية؟ | | |
| هل صاحب انقطاع الدورة عدم انتظامها و اضطرابها؟ | | |
| هل عانيتي من مشاكل في المبايض أو تكيسها؟ | | |

| | | |
|---|---|---|
| هل تستعملين الأعشاب للتداوي بكثرة؟ | | |
| هل تشربين المشروبات الغازية بكثرة؟ | | |
| هل تعانين من مشاكل في الغدة الدرقية؟ | | |
| هل تعانين من مشاكل في الكلى؟ | | |
| هل تعانين من مشاكل في الجهاز الهضمي؟ | | |
| هل تعانين من مشاكل في الكبد؟ | | |
| هل تعانين من مرض السكري؟ ☐ نوع 1 ☐ نوع 2 | | |
| هل تعانين من ضغط الدم المرتفع؟ | | |
| هل تعانين من فقر الدم؟ | | |
| هل تعانين من السمنة؟ | | |
| هل تعانين من النحافة المفرطة؟ | | |
| هل تعانين من العصبية والتوتر؟ | | |
| هل تقومين بالمشي لمسافات طويلة يوميا؟ | | |
| هل تقفين بكثرة؟ وما هي مدة الوقوف:..... | | |
| هل كنت تعانين من مشاكل في العظام قبل إنقطاع الدورة الشهرية؟ | | |
| هل مشاكل العظام وراثي في العائلة؟ | | |
| هل كنت تعانين من اضطراب في الهرمونات قبل انقطاع الدورة؟ | | |
| هل تستهلكين بدائل هرمونية؟ | | |
| ما هو سن البلوغ؟..... | | |
| ما هو سن الزواج؟..... | | |
| ما هو سن أول مرة انجبتي؟..... | | |
| ما هو مقدار الفترة ما بين إنجاب ولد و الآخر؟..... | | |
| هل استعملتي الرضاعة الطبيعية و ما هي مدة الرضاعة؟..... | | |
| هل تنجبين طبيعيا؟ | | |
| هل اجريتي ولادة قيصرية؟ | | |

## Annex 02: Gallic acid calibration curve

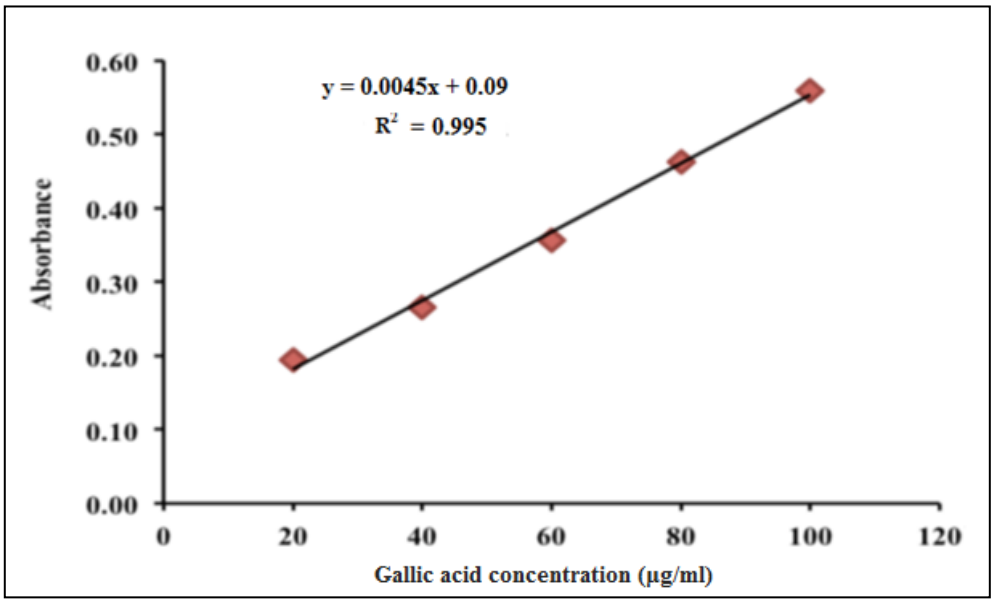


**Figure :** Calibration curve of Gallic acid for determination of total phenolic content.

## Annex 03: Quercetin calibration curve

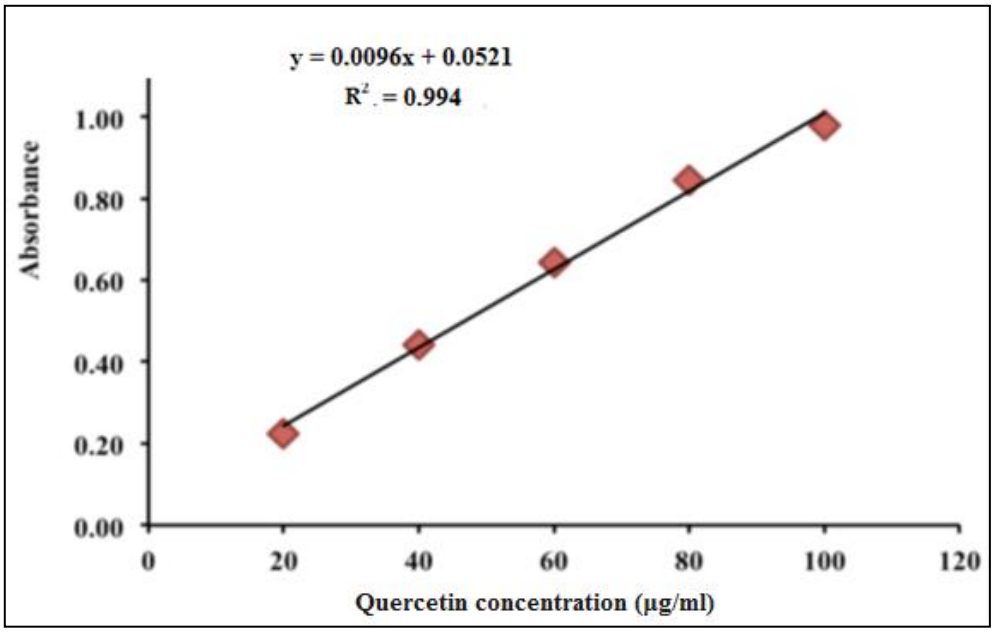


**Figure :** Calibration curve of Quercetin for determination of total flavonoids content.