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FROM CELLS TO SIMULATIONS: THERAPEUTIC INSIGHTS INTO BONE CADENCE MODULATION IN OSTEOPOROTIC WOMEN

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ABSTRACT
It is common hypothesis that postmenopausal osteoporosis starts when there is a low level of estrogen after menopause. Estrogen deficiency interacts with cytokines and upregulates osteoclast formation.It has been indicated that an increase of proinflammatory cytokines IL-1, IL-6, and TNF-a are linked to ovarian dysfunction. RANKL and MCS-F are the other key cytokines that control the growth and activation of osteoclasts by regulating an intricate signaling mechanism. This study describes how oxidative stress contributes to bone loss under estrogen deficiency. The study estimated the oxidative stress pattern, inflammatory measures, hormonal pattern, and circulatory markers in the blood of 300postmenopausal osteoporotic women and 100 healthy normal females. The findings of the current study indicate that in females' postmenopausal osteoporosis depends on higher oxidative stress, Endothelial Dysfunction and lower estrogen levels. The resultant elevated proinflammatory cytokines, MMPs, deteriorated anti-oxidative defence system and a crammed activity of the glutathione reductase enzyme. The in-silico studies indicate that Epigallocatechin-3-gallate and Stigmasterol are possible therapeutic molecules, which influence osteoporosis cell proliferation and bone cadence expression.

KEYWORDS: Endothelial Dysfunction, Homocysteine, Interleukin 6, Uteroplacental insufficiency, Molecular docking,

INTRODUCTION

Bone is a very specialized form of connective tissue and is composed of organic as well as inorganic parts. It is rehabilitated frequently throughout life and consists of different types of functional cells and an extracellular matrix (ECM). Osteoblasts are bone building cells that grow and produce both the organic and inorganic building blocks of the ECM and osteoclasts are bone remodeling cells (Amarasekara et al., 2021). The balance between the creation and destruction of bone is needed so that the remodeling of bones occurs appropriately and, in that regard, osteoclasts make a significant contribution. This life span, differentiation, and activation of osteoclast and osteoblast play a vital role in the development of a number of bone-related diseases. Mesenchymal progenitor-originating osteoblast or stromal cells in bone marrow produce essential factors like macrophage colony stimulating factor (M-CSF) and a membrane-bound cytokine, receptor activator of NF-kB ligand (RANKL), for the formation of osteoclast from hematopoietic precursors (Almeida et al.,2017).

Pre-osteoclast is differentiated to mature osteoclast by interaction of RANKL with its receptor RANK at the surface of hematopoietic precursors. Skeletal disorder known as Osteoporosis, which is caused by the failure of healthy bones is one of the leading issues affecting the people globally. Osteoporosis liability is largely caused due to aging, which is more than ten million in the United States alone and close to two hundred million in the whole world, which comprises females only (Abdellatif et al., 2021). Developing countries are becoming victims to osteoporosis because the chances of developing the condition relates to how long the populations are living. The structural and material factors influence bone quality, and detail the density of bones, which affect bone strength.The factors which influence these constituents are bone turnover rate which is the old bone resorption by osteoclast and the new bone formation by osteoblast. High rates of bone turnover in old age females are potential sources of fracture and owing to post-menstrual decline in the amount of estrogens, bone resorption and regrowth have been linked to an increase in the latter (Boyce et al.,2021).

The lack of estrogen enhances bone resorption because it advances the life cycle of the osteoclast and suppresses the life cycle of the osteoblast which minimizes the making of bones. Estrogen mainly acts on the skeleton development and homeostasis mechanism by enhancing immune and oxidative stress. Flux of inflammation also results in a higher secretion of cytokines that include T cells, a modulator not only of the production and formation of osteoclasts, but also of loss of bones (Blumer et al., 2012). In post-menopausal women, reactive oxygen species (ROS) play a vital role in the formation and activation of osteoclast, leading to severe bone loss. ROS may result into serious damage of protein, lipids, and the DNA leading to progressive diseases such as osteoporosis, diabetes, post-menopause, arthritis, cancer, and aging processes. Osteoclasts can also make ROS which is a local significant

detector of osteoclasts differentiation, activation, and osteoclast bone resorption by up-regulating the levels of RANKL and M-CSF (Christensen and Shastri., 2015). The elevated production of ROS blocks the bone formation process and the fastest method of breaking down intracellular hydrogen peroxide is through the activity of glutathione peroxidase which is the main antioxidant enzyme expressed by osteoclast. The estrogen upregulates or performs the upregulation of this enzyme (Cheng et al., 2022).

MATERIALS AND METHODS

This study viewed at osteoporosis during the postmenopausal stage in 300 female patients with 100 age matched healthy females served as control of Teaching hospital of 'The Lahore University' ranging 45-60 years of age. The participants were randomly screened with extensive questionnaire, medical history, and physical examination. Postmenopausal status was defined as one year without menstrual cycle and was checked by assessing follicle stimulating hormone (FSH), luteinizing hormone (LH), and serum estrogen levels. This study was conducted on the institute of the molecular biology and biotechnology (IMBB), The University of Lahore under the approval of ethical review board (IMBB/UOL/M01/0117). Exclusion criteria were the history of using drugs, **Commented [u1]:** ERB antioxidants supplement, vegetarianism or veganism diet, use of any medication, hormone replacement therapy, and history of chronic infection or disease. The study used spectrophotometer and ELISA kits to measure serum concentrations of glutathione (GSH), superoxide dismutase (SOD),catalase (CAT), osteoprotegerin (OPG), serum RANKL, estrogen (17β-estradiol), malondialdehyde (MDA), nitric oxide (NO), advance oxidation protein products (AOPPs), advance glycation of end products (AGEs), matrix metalloproteinases (MMPs), TNF-α IL-1, 6,7,glutathione peroxidase (GPX), and vitamin A and E. Informed written consent was obtained from patients and healthy participants before inclusion in the study.

In Silico Study

Different compounds obtained through the PubChem database and Protein Data bank were examined by the study such as Odanacatib, Risedronate, Ibandronate, Zoledronic acid, Squalene, CBD, Melanoxetin, THC, Sitosterol, and Stigmasterol **(www.pdb.org/pdb).The** undesired items present in the receptor proteins such as heat atoms, water molecules, and bounded ligands were removed using the Discovery Studio software 2021 (https://discover.3ds.com/discovery-studio-visualizer-download))and hydrogen atoms were added to them and saved in pdb format (Bhadra,2020b; Zahid et al., 2023). Ligands downloaded in the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) are in Sdf format, thus they were located in the open babel window of PyRx and converted to pdb file (Bhadra, 2020a; Zahid et al., 2023).

Molecular Docking

PyRx bioinformatics tool was used in the trial to gain access to the ligand-protein interaction of the phytocompound of choice with various proteins that functioned as ligands. The molecules of the ligands were singly uploaded and converted into macromolecules. Energy was minimized and conversion of the molecules to pdbqt were done using Open Babel window. The docking and grad box dimensions were observed with the use of Vina wizard. The results visualizing was done using the help of discovery studio 2021 where it has an opportunity to visualize 2D structures, hydrogen bonding and other type of bonding. The results showed that phytocompounds had a significant impact on various proteins, including Cathepsin k, COX-2, IL-1, IL-2, IL-7, RANKL, TNF-a, MMP13, MMP2, sclerostin, Avb3- Integrin, TGFB, and calcitonin. Swiss ADME online software was utilized for drug-likeness and ADME analysis, utilizing PubChem's SMILES (http://www.swissadme.ch/) of ligand compounds for a comprehensive analysis (Kakkar, Das, & Viswanathan, 1984). PubChem was used to collect Canonical SMILES (http://tox.charite.de/protox_II) of the ligands and drug compounds and copy it in Protox-ii online to check the toxicity of the drugs such as hepatotoxicity, mutagenicity and cytotoxicity. (Zahid et al., 2023).

Statistical Analysis

Data were analyzed using SPSS software version 20. The results were expressed as mean ±standard deviation (SD). Comparisons were made by one-way ANOVA (Analysis of variance).The correlation matrix between biomarkers was also assessed by Pearson's product-moment correlation coefficient. $P<0.05$ was considered statistically significant. Independent sample t-test was performed to analyse the results of all parameters.

RESULTS

Demographic and clinical characteristics were examined, and the occurrence of physiological differences between the postmenopausal osteoporotic women and healthy controls were studied **(Table** 1). The outcomes indicated that there were no cardinal variations between weights, age, systolic blood pressure, diastolic blood pressure, RBC count, WBC count, and Hemoglobin of both groups. BMI and neutrophil counts were significantly raised in osteoporotic subjects than controls. The results have indicated that systemic inflammation and obesity, as indicated by augmented BMI and neutrophilia, could be important factors in postmenopausal decrease in bone and the promotion of osteoporosis in this population.

Table 1: Demographic data of osteoporotic women's after menopausal

Variables	**Control (n=288)**	**Subjects (n=288)**	***P-value* (<0.05)**
WEIGHT (Kg)	85.26±5.29	81.26±4.29	0.361
AGE (Years)	56.36±5.26	57.26±4.16	0.521

SBP (mmHg)	121.26±3.26	126.25±3.19	0.162
DBP (mmHg)	81.33±4.26	83.26±7.26	0.071
BMI (kg/m^2)	22.33±1.26	31.26±3.99	0.011
RBC (M/mcl)	4.55±1.23	4.09±0.96	0.211
WBC (k/mcl)	7.88±2.09	8.66±1.29	0.311
Hb (g/dl)	14.19±3.29	12.25±2.66	0.161
NEUTROPHILS (%)	64.26±3.26	87.26±5.26	0.037

Lipid peroxidation biomarkers profile of postmenopausal osteoporotic patients e.g., MDA (malondialdehyde), Isoprostanes F2a (IsoP-F2a) (pg/ml), 4-Hydroxynonenol (4-HNNE) and 8-OHdG differed significantly between control and postmenopausal osteoporotic females. The serum levels of MDA, Isoprostanes F2α, 4-Hydroxynonenol (4-HNE) and 8-OHdG in postmenopausal osteoporotic subjects was increased (4.11±0.24nmol/ml), (14.29±1.27 pg/ml), (22.56±2.18µmol/l) and (1.08±0.023pg/ml) versus control group (0.95±0.009nmmol/ml), (1.29±0.0027 pg/ml), (7.29±2.18µmol/l) and (0.009±0.00052pg/ml) respectively **(Table 2).** Reactive oxygen species (ROS) and reactive nitrogen species (RNS) seems to play very crucial role for the progression of disease as reflected by enzymatic and non-enzymatic antioxidants profile precisely SOD (U/ml), GSH (µmol/L), CAT (U/L), AOPPs (mmol/L), AGEs (AU), NO (µmol/L), GPx (µmol/L) and GRx (µmol/L). The mean serum level of SOD, CAT, GSH and GRX decreases **(Table** 2) in postmenopausal osteoporotic patients (0.02±0.005,2.58±0.17,2.19±0.68 and 1.08±0.56) versus controls (0.14±0.02,9.65±1.99,6.29±1.23 and 2.58±0.65) respectively. The increased levels of GPx (7.25±1.08 Vs5.29±1.52),AOPPs(2.09±0.423 Vs 0.64±0.049),AGEs (3.19±0.19 Vs 0.95±0.11)and NO (29.65±4.29 Vs 10.29±2.08) in postmenopausal osteoporotic female were recorded as compared to control.

The inflammatory biomarkers Profile of IL-1 (6.35±2.08Vs 3.55±1.28pg/ml), IL-2(5.99±1.04 Vs 4.28±1.08pg/ml), IL-6 (8.19±1.08 Vs 3.28±0.18pg/ml), IL-10 (4.66±1.08 Vs 3.99±1.08pg/ml), IL-11 (6.08±2.55 Vs 4.19±1.11 pg/ml), IL-13(7.18±1.09 Vs 4.77±1.23pg/ml), IL-14(4.88±0.18 Vs 3.88±0.99pg/ml) and TNF-a (45.29±6.35 Vs 27.59±4.19pg/ml) shows **(Table 2)** elevated level in postmenopausal osteoporotic subjects as compared to control. MMP-1 (ng/ml) in postmenopausal osteoporotic **(Table** 2) subjects elevated remarkably (65.29±5.12) in comparison to healthy control (34.29±3.99). MMP-2 (ng/ml) in postmenopausal osteoporotic subjects was higher (95.36±7.26) vs healthy controls (34.29±4.29). In postmenopausal osteoporotic females MMMP-8(ng/ml), MMP-9 (ng/ml) and MMP-13 (ng/ml) serum levels were observed elevated (66.36±5.26,155.29±16.28 and 102.26±6.58) vs controls (21.25±2.88,41.26±5.88 and 45.29±3.88) respectively. The serum level of vitamin-A (Table 2) decreases in postmenopausal osteoporotic subject (426.56±16.35 nmol/L) vs in controls (654.23±11.25 nmol/L). The lower level of vitamin-C in postmenopausal osteoporotic subject was recorded (0.42±0.056nmol/L) vs controls (0.66±0.043mol/L). The vitamin E (Vit-E) in postmenopausal osteoporotic subject was found lower (0.03±0.024nmol/L) versus healthy controls (0.29±0.014nmol/L). Lower level of vitamin D (Vit-D) observed in patients (11.32±3.26 ng/ml) vs controls (36.55±4.23 ng/ml). Lower level of follicle stimulating hormone (FSH) and Luteinizing hormone (LH) (6.23±2.08 mU/ml vs10.26±3.55 mU/ml and 8.16±2.08mU/ml vs 11.55±3.29mU/ml) were recorded than that of control with a significant value (P=0.039 andP=0.011) respectively. The level of cortisol was significantly elevated in postmenopausal osteoporotic females in contrast to control (21.26±4.26 µg/dL vs 12.54±2.44 µg/dL). The lower level of Thyrotropin releasing hormone (TRH) (16.35±3.25mU/ml) and Testosterone (16.35±3.99ng/dL) were observed in patients as compared to controls (21.55±3.66mU/ml) and (20.15±3.25ng/dL) respectively **(Table 2).**

The serum level of osteoprotegerin (OPG) decreases in postmenopausal osteoporotic subject (326.56±15.26pg/ml) vs in controls (565.56±12.22pg/ml). The higher level of hs-CRP in postmenopausal osteoporotic subject was recorded (1.88±0.51 mg/dl) vs controls (1.03±0.02mg/dl). The lower level of serum phosphates (1.88±0.56mg/dl vs 3.16±1.09 mg/dl), Progesterone (15.02±2.09ng/ml vs 21.26±4.16ng/ml) and estradiol (8.29±4.16pg/ml vs 18.26±2.11pg/ml) in diseased group were recorded as compare to control. The sRANKL in postmenopausal osteoporotic subject was found higher (4.26±1.99ng/dl) while in healthy controls (2.08±0.231 ng/dl). Lower levels of 17-β-Hydroxydehydrogenase-II observed in patients (0.15±0.009pg/mL) vs healthy controls (1.58±0.13pg/mL) whereas 17-β-Hydroxydehydrogenase-I is increased (4.16±1.22pg/mL) in patients as compared to control group (1.13±0.14pg/mL). Higher levels of serum COX-2, Cyclin-D1 and Epithelial-Cadherin (1.81±0.181ng/ml vs 0.63±0.031ng/ml,4.16±1.03ng/ml vs 0.93±0.049ng/ml and 3.26±1.02ng/m1 vs 1.08±0.43ng/ml) (6.23±2.08 mU/ml vs10.26±3.55 mU/ml and 8.16±2.08mU/ml vs 11.55±3.29mU/ml) were recorded than that of control with a significant value (P=0.029,P=0.029and P=0.003) respectively. Epidermal growth factor (EGF) (284.26±10.26pg/ml Vs 171.26±12.25pg/ml) and vascular endothelial growth factor (VEGF) (14.26±1.26pg/ml Vs 9.58±3.26pg/ml) shows elevated levels in postmenopausal osteoporotic subjects as compared with normal subjects **(Table 2).**

TABLE 2: levels of extrapolative variables of medical importance in osteoporotic women's after menopausal

Variables	Control (n=288)	Subject (n=288)	*P*-Value (0.05)	Variables	Control (n=288)	Subject (n=288)	*P*-value (0.05)
MDA (nmol/ml)	0.95±0.009	4.11±0.24	0.012	**Vit-C (nmol/L)**	0.66±0.043	0.42±0.056	0.034
Isoprostanes F2α (pg/ml)	1.29±0.0027	14.29±1.27	0.001	**Vit-E (nmol/L)**	0.29±0.014	0.03±0.024	0.014
4-Hydroxynonenol (µmol/l)	7.29±2.18	22.56±2.18	0.015	**Vitamin-D (ng/ml)**	36.55±4.23	11.32±3.26	0.03
8-OHdG (pg/ml)	0.009±0.00052	1.08±0.023	0.011	**NO (µmol/L)**	10.29±2.08	29.65±4.29	0.031
SOD (U/ml)	0.14±0.02	0.02±0.005	0.003	**GPx (µmol/L)**	5.29±1.52	7.25±1.08	0.012
GSH (µmol/L)	9.65±1.99	2.58±0.17	0.000	**GRx (µmol/L)**	2.58±0.65	1.08±0.56	0.016
CAT (U/L)	6.29±1.23	2.19±0.68	0.017	**Osteoprotegrin (pg/ml)**	565.56±12.22	326.56±15.26	0.033
hs-CRP (mg/dl)	1.03±0.02	1.88±0.51	0.035	**sRANKL (ng/dl)**	2.08±0.231	4.26±1.99	0.011
IL-1 (pg/ml)	3.55±1.28	6.35±2.08	0.029	**Calcium (Ca^{++}) (mg/dl)**	8.59±2.16	5.19±1.16	0.001
IL-2 (pg/ml)	4.28±1.08	5.99±1.04	0.028	**Serum Phosphates (mg/dl)**	3.16±1.09	1.88±0.56	0.032
IL-6 (pg/ml)	3.28±0.18	8.19±1.08	0.007	**Progesterone (ng/ml)**	21.26±4.16	15.02±2.09	0.019
IL-10 (pg/ml)	3.99±1.08	4.66±1.08	0.016	**Estradiol (pg/ml)**	18.26±2.11	8.29±4.16	0.008
IL-11 (pg/ml)	4.19±1.11	6.08±2.55	0.011	**FSH (mU/ml)**	10.26±3.55	6.23±2.08	0.039
IL-13 (pg/ml)	4.77±1.23	7.18±1.09	0.023	**LH (mU/ml)**	11.55±3.29	8.16±2.08	0.011
IL-4 (pg/ml)	3.88±0.99	4.88±0.18	0.031	**TRH (mU/ml)**	21.55±3.66	16.35±3.25	0.019
TNF-α (pg/ml)	27.59±4.19	45.29±6.35	0.032	**Cortisol (µg/dL)**	12.54±2.44	21.26±4.26	0.004
AOPPs (mmol/L)	0.64±0.049	2.09±0.423	0.021	**Testosterone (ng/dL)**	20.15±3.25	16.35±3.99	0.000
AGEs (AU)	0.95±0.11	3.19±0.19	0.033	**Serum COX-2(ng/ml)**	0.63±0.031	1.81±0.181	0.029
MMP-1 (ng/ml)	34.29±3.99	65.29±5.12	0.016	**17-β-Hydroxydehydrogenase-I (pg/mL)**	1.13±0.14	4.16±1.22	0.015
MMP-2 (ng/ml)	34.29±4.29	95.36±7.26	0.019	**17-β-Hydroxydehydrogenase-II (pg/mL)**	1.58±0.13	0.15±0.009	0.033
MMP-8 (ng/ml)	21.25±2.88	66.36±5.26	0.032	**Epidermal growth factor (EGF) (pg/ml)**	171.26±12.25	284.26±10.26	0.000
MMP-9 (ng/ml)	41.26±5.88	155.29±16.28	0.022	**Vascular endothelial growth factor (VEGF) (pg/ml)**	9.58±3.26	14.26±1.26	0.024
MMP-13 (ng/ml)	45.29±3.88	102.26±6.58	0.017	**Cyclin-D1 (ng/ml)**	0.93±0.049	4.16±1.03	0.029
Vit-A (nmol/L)	654.23±11.25	426.56±16.35	0.022	**Epithelial-Cadherin (ng/ml)**	1.08±0.43	3.26±1.02	0.003

The study carried out on postmenopausal women revealed intense positive correlation among the pro-inflammatory cytokines, markers of oxidative stress, regulators of bone remodelling, and antioxidant enzymes TNF-α and IL-6 were highly correlated, indicating the probability of increasing inflammatory activity to increase osteoclastogenic communication. The close links of IL-1 with IL-6 and iNOS have also been identified where all three are strongly correlated. IL-7 was positively correlated with TNF-α and sRANKL, which demonstrated its promotion of IRF-mediated inflammatory bone turnover. The negative relationships between TNF-α, IL-1, and osteoclastogenesis were present and sRANKL was shown to have a very strong negative correlation with osteoclastogenesis. Oxidative stress in turn was closely associated with reactive nitrogen species, lipid/DNA oxidative products, and iNOS, which indicates that the oxidative antioxidative balance might have been disrupted (Table **3).** The estrogen was significantly negatively correlated Commented [u2]: Rephrase and update with IL-1, TNF-alpha, NO, and 8OHdG showing its anti-inflammatory protective properties and antioxidants.

Table 3: Pearson S' correlation matrix coefficients of different variables in postmenopausal osteoporotic women

Variables	**r**	**P(<0.05)**	**Variables**	**r**	**P(<0.05)**
TNF-α Vs. IL-1	0.561	0.015	IL-1 Vs. MMP3	0.548	0.013
TNF-α Vs. PGE2	0.648	0.002	PGE2 Vs. sRANKL	0.618	0.032
TNF-α Vs. IL-6	0.848	0.031	TNF-α Vs. OPG	-0.485	0.019
IL-1 Vs. IL-6	0.719	0.000	IL-1 Vs. OPG	-0.625	0.032
IL-1 Vs. IL-6	0.651	0.008	sRANKL Vs. OPG	-0.856	0.016
IL-1 Vs. PGE2	0.549	0.016	PGE2 Vs. OPG	-0.745	0.032
TNF-α Vs. iNOS	0.648	0.007	NO Vs. MDA	0.435	0.011
IL-1 Vs. iNOS	0.745	0.015	NO Vs. 8OHdG	0.648	0.020
TNF-α Vs. NO	0.496	0.031	iNOS Vs. AOPPs	0.745	0.014
IL-1 Vs. NO	0.645	0.062	NO Vs. SOD	0.956	0.032
TNF-α Vs. MMP-9	0.668	0.025	iNOS Vs. SOD	-0.568	0.023
IL-1 Vs. MMP-9	0.748	0.014	iNOS Vs. AGEs	0.756	0.016
TNF-α Vs. sRANKL	0.649	0.024	Estrogen Vs. IL-1	-0.658	0.031
IL-6 Vs. sRANKL	0.813	0.019	Estrogen Vs.TNF-α	-0.745	0.009
IL-1 Vs. sRANKL	0.658	0.011	Estrogen Vs. NO	-0.659	0.034
IL-7 Vs. sRANKL	0.745	0.032	Estrogen Vs. 8OHdG	-0.802	0.017
IL-7 Vs. TNF-α	0.654	0.023	Estrogen Vs. SOD	0.738	0.021
IL-6 Vs. IL-1	0.659	0.011	Estrogen Vs. CAT	0.612	0.038
TNF-α Vs. MMP-3	0.654	0.044	sRANKL Vs. MMP-9	0.667	0.013

In silico study show that Epigallocatechin 3-gallate, and Stigmasterol may act as a potential therapeutic agent having top binding affinity with respect to COX-2 and MMP-13 to alter the mechanism and suppress or inhibit the growth of osteoporosis cells and control the expression of bone cadence based on the plant derived phytocompounds **(Table 4).**

Table 4: Docking analysis of potential therapeutic agent

Proteins	**Short Listed Phytocompounds**			
	Melanoxetin 15560442	Epigallooate-3-gallate 65064	Sitosterol 222284	Stigmasterol 50824669
Calcitonin	-7.7	-9.2	-7.2	-9.1
Cox-2	-9.4	-9.9	-8.8	-10
Cathepsin-k	-7.7	-7.4	-8.6	-9.1
MMP-13	-9.6	-9.4	-9.1	-9.4
Rankl	-8	-9.5	-7.8	-9.7

Molecular docking was conducted on selected compounds against various target proteins to evaluate their therapeutic potential. This method is crucial in identifying the best pose and binding sites of receptors for a ligand molecule. The docking analysis showed that stigmasterol, melanoxetin, epigallocate-3-gallate, and sitosterol were found to have the highest binding affinities compared to standard drug compounds. Interaction analysis of docked complexes revealed that TRP184 and TRP188 of TrkB, PRO154, GLN461, CYS41, CYS47, GLU465, CYS36, PRO153, HIS226, GLN374, GLY225, LEY224, LEU145, PHE142, ASN34, LYS468, ARG44, ARG469 of COX-2, GLN79, ALA127, VAL83 of IL-1, TYR45 and PRO65 of IL-2, LEU128, LYS68, PHE75 and HIS78 of IL-7, TRP193, TYR307, TYR217 and HIS167 of RANKL, GLU116, PRO100, GLN102, ARG103 and SER99 of TNF-a, GLU223, LEU218, LEU239, TYR244, HIS222, LEU185, VAL219, LEU218, LYS249, PRO255 and PHE252 of MMP13, VAL118, GLY81, TYR143, ALA140,LEU138, HIS121 and LEU83 of MMP2, GLU77, ARG62, ARG135, TYR114, VAL134, VAL137 and PRO111 of Calcitonin, ILE344, MET408, VAL98, SER279, ALA22,

ALA343 and VAL280 of Avb3- Integrin, and PHE8, TYR40 and VAL105 of TGFB interact with the selected drug candidates (**Figure 1)**

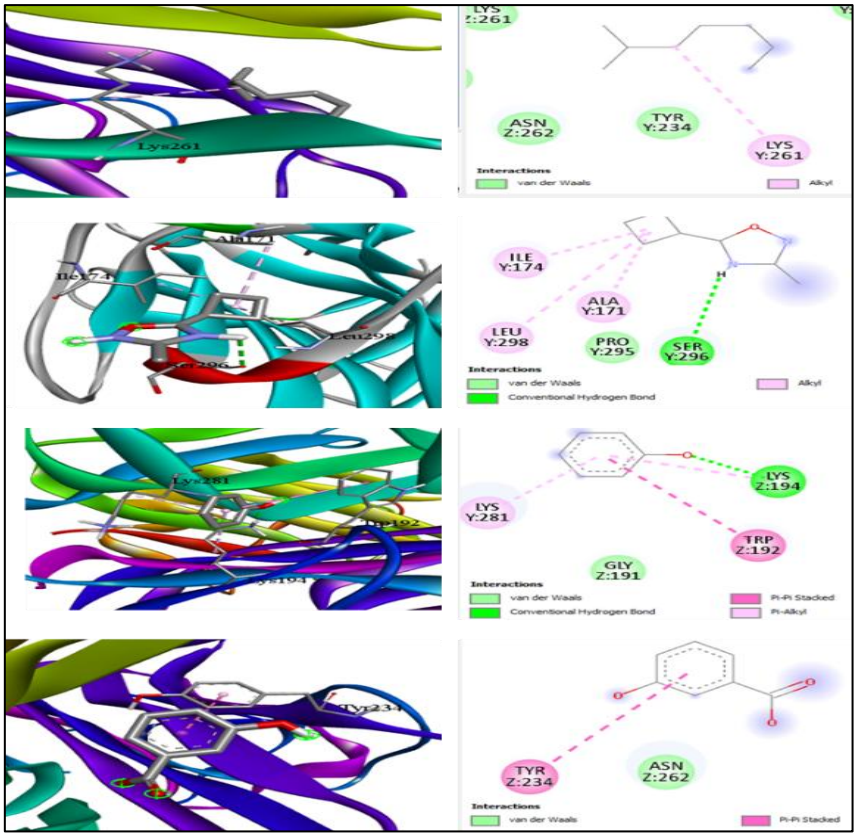

Figure 1: Rankle 2D and 3D interactions with melanoxetin, sitosterol, epigallocate-3-gallate and stigmasterol.

The study used SwissADME to analyze the physical properties, lipophilicity, Lipinski, and solubility of bioactive components. The Lipinski rule of five was followed for selected compounds to determine their potential as drugs. Results showed that all bioactive phytocompounds and standard drug compounds obeyed all five rules of Lipinski, with 0-2 violations. However, one violation was acceptable for a druggable compound (Table 5).

Table 5: ADME analysis of top binding affinity drug candidates and standard drug compounds.

Properties	**Features**	**ECGC**	**Melanoxetin**	**Beta-Sitosterol**	**Stigmasterol**
Physiochemical Properties	MW(g/mol)	458.37	302.24	414.71	412.69
	H.B Donor	8	5	1	1
	H.B Acceptor	11	7	1	1
	MR	112.06	78.03	133.23	132.75
	Arom. Heavy Atom	18	16	0	0
	Rotatable Bonds	4	1	6	5
	TPSA	197.37	131.36	20.23	20.23
Lipophilicity	logP(SILICOS-IT)	0.57	1.54	7.04	6.86
Water solubility	Logsw(SILICOS-IT)	-2.5	-3.24	-6.19	-5.47
Pharmacokinetics	GI Absorption	Low	High	Low	Low
Drug-likeness	Lipinski	2	0	1	1

ProTox-II Toxicity analysis

The ProTox-II online server was utilized for drug analysis, assessing cytotoxicity, mutagenicity, and hepatotoxicity. This method is crucial in drug design, as it reduces time, costs, and ethical challenges compared to traditional animal studies. All tested chemicals and medications are nontoxic **(Table 6).**

Table 6: Toxicity analysis of selected phytocompounds

Compounds	**Hepatotoxicity**	**Mutagenicity**	**Cytotoxicity**
Epigallocatechin 3-gallate	Inactive	Inactive	Inactive
Melanoxetin	Inactive	Inactive	Inactive
Beta-Sitosterol	Inactive	Inactive	Inactive
Stigmasterol	Inactive	Inactive	Inactive

Protein-ligand Dynamic Analysis

It studies the impact of a phytocompound on the recipient protein in terms of deformability,eigenvalues, variance, covariance maps and elastic networks. This study employs iMODS server in the investigations of the connections between COX-2 and stigmasterol, a plant-derived sterol, which may have anti-inflammatory effects **(Figure 2).**

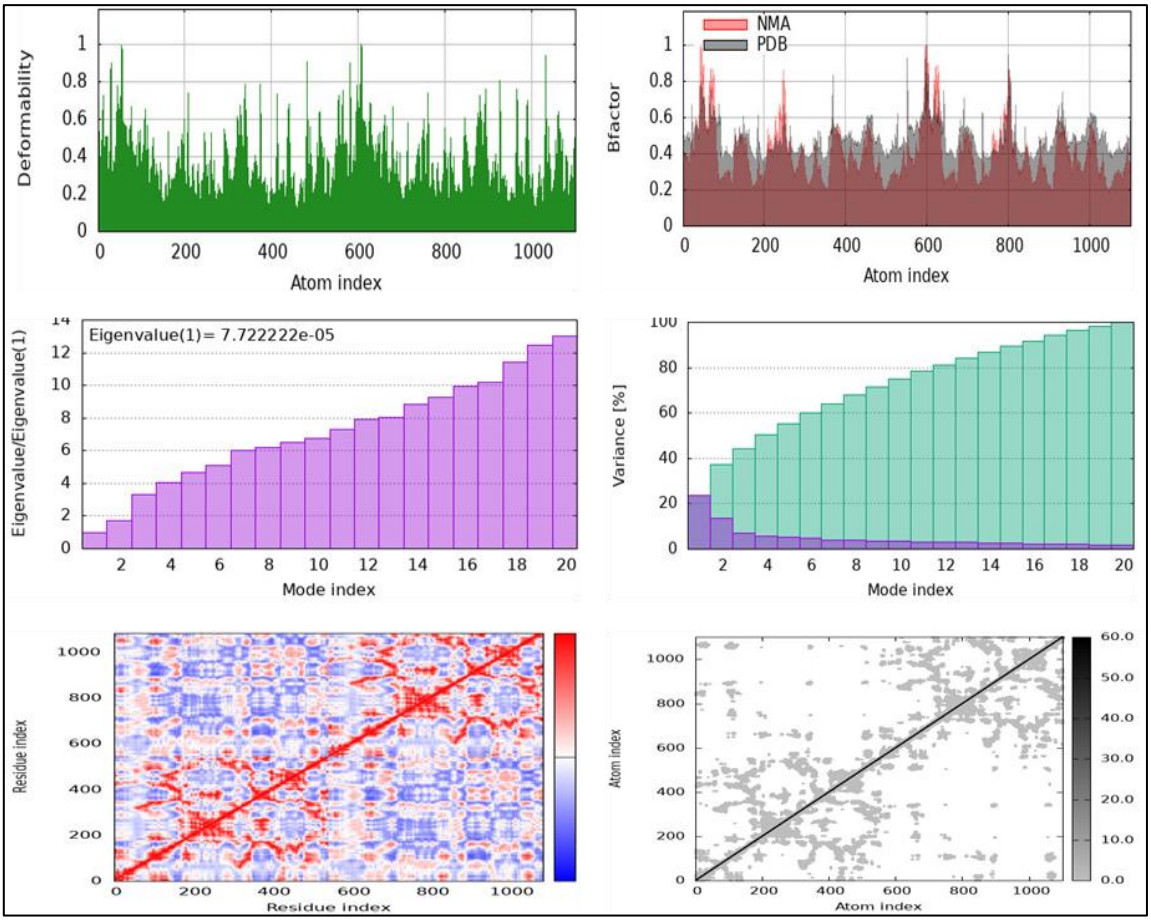

Figure 2: Normal Mode Analysis (NMA) of the protein-ligand complex

Stigmasterol integrates into the active site of COX-2, and it may interfere with its functions. Eigenvalue calculation indicates a decrease in stiffness after docking indicating that the stigmasterol fixes some of the flexible elements of the COX-2 enzyme locking the enzyme in the less active structure. Variance plot shows decreased mobility in key catalytic regions so it implies that stigmasterol restricts the flexibility necessary to carry out enzymatic reaction. Such studies give insight to the nature of mechanical and dynamical aspects of proteins. The covariance map provides insights into enzyme dynamics due to ligand binding, revealing new anti-correlated movements and increased stiffness in the elastic network model. Stigmasterol, a potential therapeutic for inflammation treatment, stabilizes COX-2 in a less flexible and active form, acting as an inhibitor.Using the IMODE server in bioinformatics, dynamic analyses can be conducted using proteins like Melanoxetin, Epigallooate-3-gallate, Stigmasterol, Cox-2, Cathepsin-k, MMP-13, and Rankl.Structural dynamics and mechanical behavior of the COX-2-stigmasterol complex. This study resulted in the localized flexibility around certain atomic indices with spiky peaks around such regions implying that they are prone to the changes of conformation. The regions that are involved in binding ligands were less flexible, as expected with an increase in structural stability which occurs when stigmasterol binds. The covariance matrix showed that there was a detailed pattern of correlated and anti-correlated motions between residues and large anti-correlated motions being present between distant regions. The elastic network model displayed the rigidity profile throughout the protein.

DISCUSSION

Menopause is a process during which there is a decline in the productions of the hormones within the ovary and fertility, especially inage in women falling in the category of 45-55 years old (Hall, 2015). Hormonal changes during menopause include increased levels of follicle stimulating hormone (FSH) and hormone (LH), which are influenced by the decline in inhibin B production. The lowering of FSH and LH results in the lowered amount of lipophilic hormone estrogen. Bone remodeling is an1 ongoing process which keeps the bone strong by the combined action of osteoclast and osteoblast with age (Robertson et al., 2021). Postmenopausal osteoporosis (PO) is a progressive disease caused by these metabolic changes, leading to reduced bone mineral density and increased

fracture risk. The lack and shortage of estrogen tends to create disproportion in the bone homeostasis, having high bone turnover and resulting in higher bone resorption than the formation process of new bones. The most significant sex steroid needed to mediate the development,maturation and maintenance of the skeleton is the estrogen. In bone remmodeling, resorption and formation of bone are coupled together by basic multicellular units (BMU). The frequency of newBMU activation per stage is more by estrogen deficiency at each of the stages and the rate of bone resorption is more compared to bone formation (Kawakita et al., 2023).

The pathophysiology of postmenopausal osteoporosis includes a higher level of involvement of the immune system of the body, in which more transmitting of the known regulators of T cell functioning and the immune system which include the interleukin (IL-1), IL-6 and TNF-a must be produced in order to induce the loss of bone (Kenkre and Bassett, 2018). This complex signal transduction involving various cytokines, including the bone marrow T lymphocytes, stimulates bone loss, enhancing the generation of TNF-α, an inflammation-related body signaling protein that regulates the process of bone turnover by increasing the production of osteoclast and reducing the osteogenic differentiation. Estrogen lack is also related to augmented osteoclast-mediated bone loss that involves the other cytokine known as IL-6. The presence of IL-6 receptor in osteoblasts results in control of osteoclast formation and activation by binding to the receptor and subsequent formation with gp130 to activate intracellular signaling cascades to produce RANKL. Pleotropic cytokine IL-1in collaboration with TNF-a results in bone resorption and activation of IL-6 (Puspitadewi *et al.,*2020 and Mills et al., 2021).

Deficiency of the estrogens may result in the development of the osteoclasts, porosity of bones,and resorption of the bone which consequently results in continuous loss of bones, severe bone resorption cavities and perforation of the bone. Bone loss can be partially compensated by higher production of osteoblast caused by an increase in the number of mesenchymal progenitors (Noirrit et al., 2021). But depletion of estrogen restrains the positive surplus of bone formation through upregulating the apoptosis of osteoblasts, which causes a derangement of the equilibrium of bone remodeling and loss of the bone. Among them, RANKL and M-CSF are produced by bone lining cell and osteoblast and OPG is produced by T-cells, macrophages, and fibroblasts. RANKL and M-CSF attach on membrane specific locations of the osteoclast precursors and promote its progression into an observer osteoclast (Sims and Martin 2014 and Almeida et al., 2017). There is a significant reduction in the expression of RANKL and M-CSF in estrogen-deficient post-menopausal osteoporotic women (Manolagas et al., 2013 and Uehara et al., 2020). Decreased serum levels of Osteoprotegerin (OPG) have also been observed in cases of postmenopausal bone loss, as OPG inhibits osteoclast differentiation by interacting with RANKL. Transforming growth factor beta (TGF-β), another growth factor responsible for recruiting and activating osteoblasts to initiate collagen synthesis, also shows decreased expression during postmenopausal osteoporosis. New research shows a decrease in the protection against oxidative stress because of the deficiency of estrogen (Foger-Samwald et al., 2020 and Zhang et al., 2022).

The oxidized formation of bone microenvironment by ROS and intracellular higher concentration of GSH results in postmenopausal bone loss. Estrogen protects bone against oxidative stress, but estrogen deficiency alters the formation of reactive oxygen species (ROS) and cell capacity to protect itself against oxidative stress, leading to an accumulation of ROS that upregulates the formation and resorptive activity of osteoclast (Marahleh et *al.,* 2019). Three key antioxidant enzymes, SOD, GPx, and CAT show considerable activities in reduction in postmenopausal women.High concentration of NO in women after menopause indicates their overall influence on bone turnover that leads to the loss of bone (Kim et al., 2017 and Boyce et al., 2023). Two large families of proteolytic enzymes are profound in the process of bone resorption, namely, cysteine protease and the matrix metalloproteinases. MMP-9 is essential in the process of bone turnover where collagen stacks on bone surface are removed and the process is followed by demineralization (Xu et al., 2023and Wadhwa et al., 2024). Tartrate-resistant acid phosphatase (TRAP) or type-5 acid phosphatase (ACP5) is involved in bone matrix degradation, and TRAP deficient mice show decreased osteoclast resorption activity and reduced endochondral ossification (Kitaura et al., 2020).

This study explores the therapeutic relevance of phytocompounds, particularly epigallocatechin-3-gallate (EGCG) and stigmasterol, in modulating inflammatory and osteolytic pathways in osteoporosis. The docking outcomes demonstrated high affinities of stigmasterol and EGCG to the major regulators of bone turnover, i.e., COX-2, MMP-13, and RANKL. Stigmasterol demonstrated robust inhibitory effect on the inflammatory breakdowns and osteoclast differentiation whereas EGCG demonstrated impressive binding interactions with the MMP-13 and COX-2.Catalytically and structurally stabilizing residues of COX-2 were observed to interact with the compounds signifying ligand-receptor complementarity. The existence of cytotoxicity, mutagenicity,or hepatotoxicity was not observed in any of the phytochemicals tested in the study which further justified their non-toxicity pharmacological properties. The study finds that stigmasterol and EGCG have good therapeutic potential on the resorption of bones and inflammatory activity in osteoporotic cases with a multitargeted effect, good safety, and an appropriate drug-like profile.

Significant positive correlations between pro-inflammatory cytokines, oxidative stress biomarkers, bone remodelling regulators (sRANKL, OPG, MMPs), and antioxidant enzymes in postmenopausal osteoporotic women. These cytokines act in the process of osteoclastogenesis which is mediated by IL-6 as one of the principal cytokines of inflammation. IL-7, which is a main immune signaling protein is also associated with inflammatory bone loss. Interestingly,a negative correlation of sRANKL with OPG is so strong, which is linked to increased bone resorption and the development of osteoporosis in postmenopausal women. Augmentation of oxidative stress

markers including NO,8OHdG is positively correlated with iNOS and lipid peroxidation products indicating that the redox

imbalance has a role in degrading bone matrix. Estrogen constitutes a protective factor that shows a negative association with pro-inflammatory cytokines, NO, 8OHIdG, and a positive association with SOD and CAT. Below Figure 3 shows the metabolic pathway and highlight our findings.

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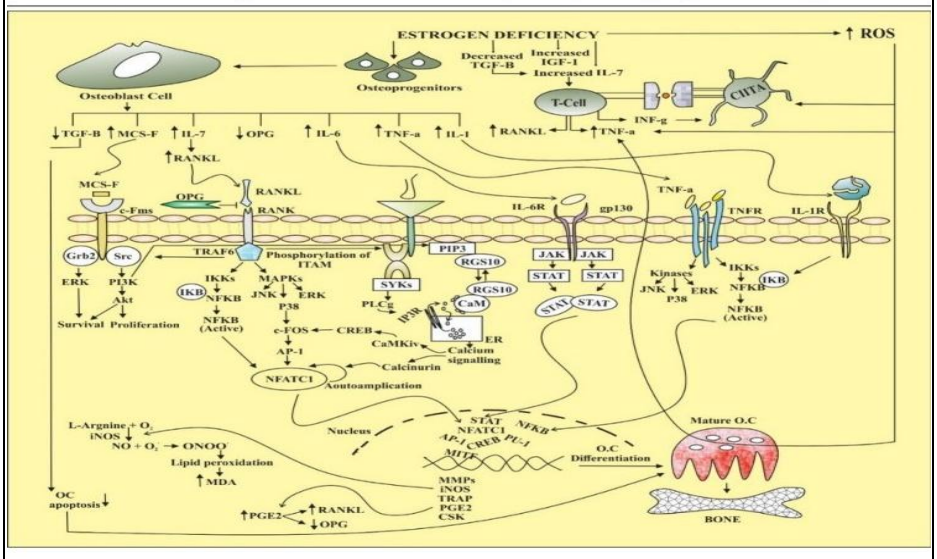

Figure 3: Estrogen deficiency triggers the formation of osteoclast by increasing the production of IL-7 and IGF-1 in target organs like bone. This leads to an initial wave of T-cell activation, producing IFNγ,which upregulates MHC classII expression through the transcription factor CIITA. Activated T-cells also release TNF-α and RANKL, which stimulate increased production of IL-1 and PGE2 expression via the NFkB signaling pathway. Osteoblasts are primarily affected by estrogen deficiency, with M-CSF, RANKL, TNF-α, and interleukins promoting osteoclast activity being increased while OPG and TGF-β are decreased.Ligand-induced activation of c-fms results in increased activity of its tyrosine kinase domains, which transphosphorylate specific residues in the cytoplasmic tail, thereby binding sites for signaling molecules.These pathways promote osteoclast precursor proliferation and survival. RANK activation results in TRAF-6 recruitment and phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) in DAP12and FcRy. ITAM phosphorylation recruit's spleen tyrosine kinase (SyKs) that activate calcium signalling through PLCy induced production of IP3R. Regulation of G-protein signalling-10 (RGS-10) determines calcium oscillation pattern through competitive binding of Ca2+/calmodulin and PIP3. TNF-α and IL-1share parallel signaling mechanisms within the cell, activating specific adaptor proteins and initiating downstream signaling cascades. Estrogen deficiency amplifies osteoclastogenesis by downregulating antioxidant defense pathways, leading to an upswing in ROS. Elevated ROS upregulate T-cell activation and stimulate the release of osteoclastogenic factors RANKL and TNF-a.

CONCLUSION

The present study suggests increased oxidative stress and estrogen deficiency as contributing factors for the postmenopausal osteoporosis in females. Due to oxidative stress different proinflammatory cytokines are released that play crucial role in disease progression. In such females perceived levels of MDA, AOPPs and AGEs were significantly higher as compared to controls. There was significantly reduced activity of anti-oxidant systems (GPx, GRx and SOD), but the activity of Glutathione Reductase (GRx) enzyme was observed to be most significantly compromised in the postmenopausal osteoporotic females. Therefore, it can be expressed as deficiency of estrogen in females is the contributing factor for osteoporosis and increased oxidative stress. In silico study show that Epigallocatechin 3-gallate, and Stigmasterol may act as a potential therapeutic agent having top binding affinity with respect to COX-2 and MMP-13 to alter the mechanism and suppress or inhibit the growth of osteoporosis cells and control the expression of bone cadence based on the plant derivedphytocompounds.

Acknowledgements

The authors are highly thankful for the valuable contribution of Prof. Dr. M Arslan Director Centre for Research in Molecular Medicine (CRiMM), The University of Lahore-Pakistan regarding support and critical review of the manuscript.

Funding

Not applicable

Conflict of Interest

Authors declares no conflict of interest

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