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Computational Analysis for Prevention of Osteoporosis using Algal Extract

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Abstract

Osteoclasts are multinucleated cells that play a crucial role in bone resorption. The imbalance between bone resorption and bone formation results in osteoporosis. Therefore, substances that can suppress osteoclast formation are potential candidate materials for drug development or functional foods. There have been reports that extracts or purified compounds from marine micro- and macroalgae can suppress osteoclast differentiation. Symbioimine, isolated from the cultured dinoflagellate *Symbiodinium sp.*, had suppressive effects against osteoclast differentiation in osteoclastlike cells. Norzoanthamine, isolated from the colonial zoanthid *Zoanthas sp.*, has been shown to have anti-osteoporosis activity in ovariectomized mice. In response to marine extracts, the fucoxanthin- rich component from brown algae has been shown to have suppressive effects against osteoclast differentiation. An extract of *Sargassum fusiforme* has recently been shown to have anti-osteoporosis activity. This extract suppressed both osteoclast differentiation and accelerated osteoblast formation in separate in vitro experiments. In this study , we have undergone an *in-silico* interaction study of the each target proteins , namely TNFRSF11B, LRP5, RANKL, NOX4 , ER,PTH1R , sclerostin, NR3B1, HDAC with both reported anti-osteoporosis drugs (namely Calcitriol, Alendronate, Risedronate, Ibandronate, Zoledronate,)and phyto-chemical compounds (Symbioimine Norzoanthamine fucoxanthin, Largazole, dieckol, 1-(30,50-dihydroxyphenoxy)-7-(200,400,600-trihydroxyphenoxy) 2,4,9-trihydroxydibenzo-1,4,-dioxin , Biselyngbyaside, ikarisoside A, bolinaquinone,) obtained from algae. Interaction of phytochemical compound with target proteins shows better binding affinity as compared to drug molecules like Calcitriol and Alendronate. Thus, these marine algae and their extracts may be sources of marine medicinal foods for the prevention of osteoporosis.

Keywords: Alendronate, Alkaloids, Functional Food, Osteoporosis, *Insilico*.

1. INTRODUCTION

Osteoporosis is a disease where decreased bone strength increases the risk of a broken bone. It is the most common reason for a broken bone among the elderly [1]. Bones that commonly break include the back bones, the bones of the forearm, and the hip [2]. Until a broken bone occurs there are typically no symptoms. Bones may weaken to such a degree that a break may occur with minor stress or spontaneously. Chronic pain and a decreased ability to carry out normal activities may occur following a broken bone [1].

Osteoporosis may be due to lower than normal peak bone mass and greater than normal bone loss. Bone loss increases after menopause due to lower levels of estrogen. Osteoporosis may also occur due to a number of diseases or treatments including alcoholism, anorexia, hyperthyroidism, surgical removal of the ovaries, and kidney disease. Certain medications increase the rate of bone loss including some antiseizure medications, chemotherapy, proton pump inhibitors, selective serotonin reuptake inhibitors, and steroids. Not enough exercise and smoking are also risk factors [1]. Osteoporosis is defined as a bone density of 2.5 standard deviations below that of a young adult [3]. This is typically measured by dual-energy X-ray absorptiometry at the hip [3]. Prevention of osteoporosis includes a proper diet during childhood and efforts to avoid medications that cause the condition. Efforts to prevent broken bones in those with osteoporosis include a good diet, exercise, and fall prevention. Lifestyle changes such as stopping smoking

and not drinking alcohol may help [1]. Medication of the bisphosphonate type are useful in those with previous broken bones due to osteoporosis [4, 5]. In those with osteoporosis but no previous broken bones they are less effective [4-6]. A number of other medications may also be useful [1, 7]. Although there is no cure for osteoporosis, several medications approved by the U.S. Food and Drug Administration (FDA) can help stop or slow bone loss, or help form new bone, and reduce the risk of fractures. Currently, alendronate, raloxifene, risedronate, and ibandronate are approved for preventing and treating postmenopausal osteoporosis. Teriparatide is approved for treating the disease in postmenopausal women and men at high risk for fracture. Estrogen/hormone therapy (ET/HT) is approved for preventing postmenopausal osteoporosis, and calcitonin is approved for treatment. In addition, alendronate is approved for treating osteoporosis in men, and both alendronate and risedronate are approved for use by men and women with glucocorticoid-induced osteoporosis. Alendronate plus vitamin D is approved for the treatment of osteoporosis in postmenopausal women and in men. Risedronate with calcium is approved for the prevention and treatment of osteoporosis in postmenopausal women. Except these treatments, various types of marine extracts are also used to cure osteoporosis. The marine environment has proven to be a rich source of biological diversity. The marine organisms have high potency for the commercialization of interesting compounds and their applications towards food industry, cosmetic industry, neutraceuticals, pharmaceutical industries etc. Symbioimine, Norzoanthamine, Fucoxanthin, Largazole, Biselyngbyaside, Ikarisoside A, Bolinaquinone are some of the marine extracts which are used for treatment of osteoporosis. Symbiodinium sp., had suppressive effects against osteoclast differentiation in osteoclast-like cells. Norzoanthamine, isolated from the colonial zoanthid Zoanthas sp., has been shown to have antiosteoporosis activity in ovariectomized mice. With regard to marine extracts, the fucoxanthin-rich component from brown algae has been shown to have suppressive effects against osteoclast differentiation. An extract of Sargassum fusiforme has recently been shown to have antiosteoporosis activity. This extract suppressed both osteoclast differentiation and accelerated osteoblast formation in separate in vitro experiments. It also showed antiosteoporosis activity in ovariectomized mice by regulating the balance between bone resorption and bone formation. These marine algae and their extracts may be sources of marine medicinal foods for the prevention of osteoporosis. Osteoporosis becomes more common with age [1]. About 15% of white people in their 50s and 70% of those over 80 are affected [8]. It is more common in women than men [1]. In the developed world, depending on the method of diagnosis, 2% to 8% of males and 9% to 38% of females are affected [9]. Rates of disease in the developing world are unclear [10]. About 22 million women and 5.5 million men in the European Union had osteoporosis in 2010 [11]. In the United States in 2010 about eight million women and one to two million men had osteoporosis [9, 12]. White and Asian people are at greater risk [1]. The word osteoporosis is from the Greek terms for "porous bones" [13]. The human skeleton consists of both fused and individual bones supported and supplemented by ligaments, tendons, muscles and cartilage to serve as a rigid framework for the body. The skeleton supports organs, anchors muscles and facilitates movement and protects organs such as brain, lungs and heart [14].

The risk of osteoporosis is influenced by many factors such as age, sex, diet, physical activity, medication use and menopausal status, but one of the most important clinical risk factors is a positive family history, underscoring the role of genetic factors in determining disease susceptibility [35]. Recently, we provided a comprehensive review of advances made over the past 15 years with respect to the discovery of osteoporosis causing genes. We noted an accelerating pace in identifying and validating osteoporosis susceptibility loci in the past 4 years, which was largely attributable to the use of genome-wide association studies (GWAS). Based upon a combined examination of the available data, we concluded that there are at least 15 confirmed genes (e.g., ESR1, LRP5, SOST, OPG, RANK and RANKL) and potentially another 30 genes or more that could be assigned as osteoporosis susceptibility genes. Notably, these genes are clustered into three biological pathways: the estrogen endocrine pathway, the Wnt/β-catenin signaling pathway and the RANK/RANKL/Osteoprotegerin (OPG) pathway [36]. Since the publication of our review, GWAS have identified two novel osteoporosis genes, *ALDH7A1* [37] and *JAG1*; [38] two further comprehensive reviews on the genetics of osteoporosis have also been published [39, 40].

2. MATERIALS AND METHODS

2.1 Materials and Methods of Wet Lab work

2.1.1 Sample Collection

Algal samples were collected from a depth of 100-150cm from the surface of water. They are so small that they can pass through the mesh of even finest net. Some species were collected through the net. The samples were washed for several times and allowed to have show time surface water, which was transferred to plastic bottle and were returned to laboratory. After several washing the samplings were observed under a microscope. Attempts were made to raise bacteria free cultures by repeated sub-cultivation and exposing to UV-radiation. Repeated washing and transfer to fresh media under asceptic condition to eliminate or reduce most of the bacteria and in some cases lead to the establishment of bacteria free culture.

2.1.2 Culture Preparation

(i) Culture vessel

Non-absorbent cotton wool plugged hard glass test-tubes, conical flasks and petriplates (all corning

glass makes) were used for culturing the algae. The volume of the medium was decreased in the vessel when the culture was to be shaken in order to avoid contamination by splashing the culture solution up to the cotton wool plug.

Fig. 1: Preparation of Culture Vessels

(Ii) Cleaning Methods

The glassware were cleaned first with tap water with detergent and then immersed in chromic acid (mixture 2.0 gm of $K_2Cr_2O_7$ in 100.0 ml of concentrated H_2SO_4 overnight). These were then thoroughly washed in running tap water and finally rinsed with all double distilled water and dried in hot air even before use.

(Iii) Culture Conditions

The cultures were incubated inside a culture room free from all types of contaminant. The pH of culture was maintained between 6 and 8 and temperature was 24± 2°C was given through whole day. The medium of the liquid culture was changed after 48 hours. Temperature, light intensity, pH was checked twice daily. The culture flasks were hand shaken twice daily throughout the observation period.

(iv) Inoculation

Inoculation was carried out in an inoculation chamber inside the culture room with aseptic conditions. Algal materials were inoculated into the sterile medium. The algal materials were examined under a microscope to see any damage caused to the cells. Always healthy materials were inoculated in the medium after thoroughly checked to avoid the contamination.

Fig. 2: Identification of Algae Using Compound Microscope

2.1.3 Growth Media

The microalgae were grown in the following media i.e. Provasoli's Enriched Seawater (PES) medium. To prepare the PES medium, 20 ml of the enrichment stock solution was added to 980 ml of filtered natural seawater pasteurize.

Enrichment Stock Solution

To prepare enrichment stock solution following components were added in 900 ml of sea water (Vitamins should be added last, after mixing other components) and the final volume was brought to 1 liter with distilled water and pasteurized (without autoclaved).

Table 1: Table Showing Enrichment Stock Solution's Composition

Component	***Stock solution***	***Quantity used***
$NaNO_3$	--	3.5 gm
Na β glycerophosphate H_2O	--	0.5 gm
Trisbase	--	5.0 gm
*Iron-EDTA Solution (see following table)	--	250 ml
*Trace metal solution (see following table)	--	25 ml
Thimaine HCl	--	0.500 mg
Biotin	0.005	1 ml
Tris buffer	--	1 ml

*** Iron-EDTA solution**

Into 950 ml of distilled H_2O, the EDTA and then the iron sulphate were added and were brought to the final volume to 1 litre Pasteurized and stored refrigerated.

Table 2: Table Showing Iron-EDTA Solution's Composition

Component	*Stock solution (gm /L) distilled water*	*Quantity used*
$Na_2EDTA\ 2H_2O$	--	0.841 gm
$Fe\ (NH_4)_2(SO_4)\ 6H_2O$	--	0.702 gm

*** Trace metal stock solution**

Into 950 ml of distilled H_2O, the EDTA was added and then individually the following components (The Boron is not necessary for enriching natural seawater and should be left out) were dissolved and brought the first volume to 1 litre and stored in refrigerator.

Table 3: Table Showing Trace Metal Stock Solution's Composition

Component	*Stock solution (gm /L) distilled water*	*Quantity used in gm*
$Na_2EDTA\ 2H_2O$	--	12.74 gm
$Fe\ Cl_3\ 6H_2O$	--	0.484 gm
H_3BO_3	--	11.439 gm
$MnSO_4.4H_2O$	--	1.624 gm
$ZnSO_4.7H_2O$	--	0.220 gm
$CuSO_4.\ 7H_2O$	--	0.048 gm

Fig. 3: Measuring the desired weight of NaNO3 by using weighing Machine

Fig. 4: Preparation of PES Medium

Fig. 5: Preparation of different stock concentration of solutions

2.1.4 Inoculation on solidified medium

Algal cells isolated by capillary pipette method were mixed with small volume of sterilized distilled water and a few drops were placed in the solidified agar medium. A bent glass was sterilized by dipping in alcohol and burning in the flame several times and then cooled. With the help of this glass rod, algal suspension was spread over the solidified medium, the inoculated plate was immediately covered, inverted and inoculated for 10-15 days under suitable condition. After 10-15 days desired algal cells free from impurities were picked up by sterilized wire needle and were resuspended separately in a small volume of sterilized distilled water. The process was repeated unless and until unialgal cells were developed. Finally a colony developed from single cell was transformed to the liquid medium for mass culture.

2.1.5 Streak plate method

For this method solidified medium of Provasoli enriched seawater medium (PES) with 1.5% agar-agar was prepared in a conical flask. Then the flask was autoclaved at 15lb for 30 minutes. The hot medium was poured in sterilized petriplates. Under laminar air flow the naturally collected samples were streaked on cold petriplates containing solidified PES medium. After 7 days isolated colonies were appeared on the plates and these isolated colonies were inoculated in a different test tube containing liquid PES medium. After seven days the algae inside the test tubes were examined under microscope for pure culture.

Fig. 6: Streaking plate on Solidified Media

2.2 Materials and Methods of Computational Analysis

2.2.1 Mining of Genes Associated with Osteoporosis from GWAS Catalog

The National Human Genome Research Institute (NHGRI) Catalog of Published Genome-Wide Association Studies (GWAS) Catalog which provides a publicly available manually curated collection of published GWAS assaying at least 1,00,000 single nucleotide polymorphisms (SNPs) and all SNP-trait associations with $P < 1X10^{-5}$, was used to mine the genes pertaining to Osteoporosis. Disease search for "**Osteoporosis"** with a p-value threshold of $p<10^{-5}$was performed to retrieve GWAS studies on Osteoporosis from GWAS Catalog (http://www.genome.gov/gwastudies/ currently https://www.ebi.ac.uk/gwas/). A total of 8 GWAS studies resulted in a total of 14 unique genes mapped to discrete genomic locations of human genome. The corresponding protein sequences encoded by these genes were obtained from UniProtKB database.

2.2.2 Gene-disease association study through WebGestalt

Web Gestalt (WEB-based Gene SeT AnaLysis Toolkit), one of the first software applications that integrate functional enrichment analysis and information visualization for the management, information retrieval, organization, visualization and statistical analysis of large sets of genes. In addition to significant data expansion, Web Gestalt has also improved user friendliness and added new visualization features that help users better understand the enrichment results.

Web Gestalt (http://bioinfo.vanderbilt.edu/webgestalt/) was used for further functional categorisation of 14 reported gene extracted from GWAS genes including gene–disease association and Drug association analysis. Further interactive phenotype ontology associated with Osteoporosis genes was elucidated. Organism Homo sapiens was selected against select organism of interest column, hsapiens_gene symbol was selected at Select gene ID type, and outcome of GWAS Catalog Reported gene list consisting of 14 genes was uploaded in the Upload gene list column. The following entries such as Statistical Method/test: hypergeometric, Multiple Test Adjustment: BH, Significance Level: Top 10 and .05, Minimum Number of Genes for a Category:2 was selected.

2.2.3 UNIPROT-(http://www.uniprot.org/)

The Universal Protein Resource (UniProt) is a comprehensive resource for protein sequence and annotation data. The corresponding protein sequences encoded by these genes were retrieved from UniProtKB database.

2.2.4 Retrieval of Drugs and proteins (corresponding targets)

The Structure Data Format (SDF) 3D structure of the reported drugs were retrieved from the NCBI PubChem database (http://www.ncbi.nlm.nih.gov/pccompound/) along with its PubChem ID, Molecular weight and Molecular formula. The compounds were converted into pdb format structure using the PyMol (academic version) tool, Discovery Studio v4.1 visulizer tools and online SMILES translator web server (https://cactus.nci.nih.gov/translate/) as per requirement.

The structures of the corresponding proteins of reported genes were retrieved from PDB Protein Data Bank (PDB). The unknown structures were predicted using various tools like Modeller 9.15 tool, web servers due to unavailability at PDB Protein Data Bank (PDB).

2.2.5 Protocol of Homology Modelling using Modeller tool

- Downloaded the folder named advance-example from Modeller tutorial website which contains all the files to model a protein.
- Copied the aa sequence of pf TS-DHFR retrieved from UniProt database for which modelling has to be done.
- Blast the protein sequence against PDB database and found the appropriate template which has the highest similarity and best e-value.
- The template structures was downloaded from PDB and saved in the advance-example folder.
- Opened the advance-example folder and the following changes were done over there.
- Opened TvLDH.ali and replace the existing sequence with the query sequence with 70 amino acids in a row with a star in the end of the file.
- Align2d_mult.py-rename the protein name in place of TvLDH
- Salign.py-Type /copy the names of the pdb files (templates). It are case-sensitive.
- Model_mult.py-Type/copy the names of PDB files(templates). The PDB file names are case-sensitive.
- Run Modeller and after specifying the exact path, type the following commands:
- “mod9.14 salign.py”. Press enter.
- “mod9.14 align2d_mult.py”. Press enter.
- “mod9.14 model_mult.py “. Press enter.

Now open the model_mult.log file and find the model with lowest mol pdf value out of assigned models.

2.2.6 Model Refinement, Evaluation and Structure Validation

The structure validation of protein**,** namely LRP5 was validated using various web servers like WhatIF used to refine the structure. Quality of the generated model was evaluated with Procheck by Ramachandran plot analysis.

2.2.7 Prediction of Binding Site

Structural and active site studies prediction of the proteins were done by using CASTP (Computed Atlas of Surface Topography of Proteins) at http://cast.engr.uic.edu

2.2.8 Docking approach

AutoDock 4.2 (autodock.scripps.edu/) was used for docking studies which is widely distributed public domain molecular docking software. The docking analysis was carried out for the reported drugs (can be said as ligands) with their corresponding targets (proteins) using AutoDock4.2 tool. The interactions of ligand and proteins were studied using LigPlot, Discovery Studio Visulizer and PyMol. The various bonding interactions of ligand and proteins were explored using the above tools.

3. RESULTS AND DISCUSSION

The GWAS reported 8 studies of dengue with a total of 14 unique genes, namely LRP5, SOX6,

TBC1D8, SCG2, RAP1A, OSBPL1A, MECOM, DOK6, LOC348751, FONG, LRRC4C, TNFRSF11B, SPP2, ALDH7A1 that were mapped to discrete genomic locations of human genome. The results are represented in Table 4. The effect of mutation/substitution on these genes can lead to osteoporosis.

Table 4: The GWAS studies with 8 studies of osteoporosis with a total of 14 unique genes

Reported Genes	***Full Name of the Protein***
LRP5	low density lipoprotein receptor-related protein 5
SOX6	SRY (sex determining region Y)-box 6
TBC1D8	TBC1 domain family, member 8 (with GRAM domain)
ALDH7A1	aldehyde dehydrogenase 7 family, member A1
RAP1A	RAP1A, member of RAS oncogene family
MECOM	MDS1 and EVI1 complex locus
OSBPL1A	oxysterol binding protein-like 1A
DOK6	docking protein 6
LOC348751	NULL
FONG	NULL
LRRC4C	leucine rich repeat containing 4C
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b
SPP2	secreted phosphoprotein 2, 24kDa
SCG2	secretogranin II

The Drug association analysis of Web Gestalt has reported 1 drug interacted with 2 genes and its corresponding proteins. The results of Web Gestalt pertaining drugs against osteoporosis and its corresponding genes/proteins were cross checked by literature survey (which are shown in Table 5), substantially presented in Table 5.

Table 5: The list of drugs and the corresponding targets of the drugs collected from Web Gestalt and various literatures

Sl. No	***Target***	***Drugs***	***Ref.***
1.	LRP5, TNFRSF11B	Calcitriol (Pucid 5280453)	GWAS/Webgestalt
2.	Activator of Nuclear Factor-Kb Ligand (RANKL) NADPH Oxidase 4 (NOX4)	Vitamin D3, Calcitonin, Ipriflavone, Bisphosphonates (Alendronate, Risedronate, Ibandronate, Zoledronate,)	Koyama T. Extracts of Marine Algae Show Inhibitory Activity Against Osteoclast Differentiation Adv Food Nutr Res. 2011;64: 443-54.
3	Estrogen Receptor (ER)/ NADPH Oxidase 4 (NOX4)	Raloxifene Hydrochloride, Cholecalciferol, Bazedoxifene, Lasofoxifene, Strontium, Ranelate, Calcitriol.	Bruce Ettinger *et al.,* Reduction of Vertebral Fracture Risk in Postmenopausal Women with Osteoporosis Treated with Raloxifene. JAMA. 1999;282(7):637645.
4	Receptor Activator of Nuclear Factor-Kb Ligand (RANKL)/ NADPH Oxidase 4 (NOX4)	Denosumab (Raloxifene)	Han Seok Choia *et al.,* Medical treatment of severe osteoporosis including new concept of advanced severe osteoporosis. Osteoporosis and Sarcopenia Volume 2, Issue 1, March 2016.
5	Parathyroid Hormone 1 Receptor (PTH1R)/ NADPH Oxidase 4 (NOX4)	Teriparatide	Inthrani Raja Indran *et al.,* Preclinical studies and clinical evaluation of compounds from the genus Epimedium for osteoporosis and bone health. Pharmacology and Therapeutics (2016)
6	NADPH Oxidase 4 (NOX4) Sclerostin	Bisphosphonates (Alendronate, Risedronate, Ibandronate, Zoledronate,)	Paula Hoff and Frank Buttgereit. NADPH oxidase 4 represents a potential target for the treatment of osteoporosis. Cellular & Molecular Immunology (2014) 11, 317–319.

Table 6: Osteoporosis Drugs and their corresponding target genes/proteins from WebGestalt at significance level .05, Significance test Hypergeometric, MTC:BH

Sl. No	*Drug*	*Pubchem CID*	*Molecular Formula*	*Molecular Weight*	*Target*
1	Calcitrol	5280453	$C_{27}H_{44}O_3$	416.63646	TNFRSF11B
					LRP5
2	VitaminD3	5280795	$C_{27}H_{44}O$	384.63766	RANKL
					NOX4
3	Calcitonin	16220008	$C_{151}H_{226}N_{40}O_{45}S_3$	3417.84614	RANKL
					NOX4
4	Ipriflavone	3747	$C_{18}H_{16}O_3$	280.31784	RANKL
					NOX4
5	Alendronate	2088	$C_4H_{13}NO_7P_2$	249.096044	RANKL
					NOX4
					Sclerostin
6	Risedronate	5245	$C_7H_{11}NO_7P_2$	283.112264	RANKL
					NOX4
					Sclerostin
7	Ibandronate	60852	$C_9H_{23}NO_7P_2$	319.228944	RANKL
					NOX4
					Sclerostin
8	Zoledronate	68740	$C_5H_{10}N_2O_7P_2$	272.089624	RANKL
					NOX4
					Sclerostin
9	Raloxifene Hydrochloride	54900	$C_{28}H_{28}ClNO_4S$	510.04422	NOX4
					Estrogen receptor (ER)
10	Cholecalciferol	10883523	$C_{27}H_{44}O$	384.63766	NOX4
					Estrogen receptor (ER)
11	Bazedoxifene	154257	$C_{30}H_{34}N_2O_3$	470.60256	NOX4
					Estrogen receptor (ER)
12	Lasofoxifene	216416	$C_{28}H_{31}NO_2$	413.55124	NOX4
					Estrogen receptor (ER)
13	Strontium	5359327	Sr	87.62	NOX4
					Estrogen receptor (ER)
14	Strontium Ranelate	24871329	$C_{12}H_{20}N_2O_{15}SSr_2$	639.5966	NOX4
					Estrogen receptor (ER)
15	Calcitriol	5280453	$C_{27}H_{44}O_3$	416.63646	NOX4
					Estrogen receptor (ER)
16	Denosumab (Raloxifene)	No	No	No	RANKL
					NOX4
17	Teriparatide	No	No	No	RANKL
					NOX4
Sl. No	*Algal Extracts*	*Pubchem Cid*	*Molecular Formula*	*Molecular Weight*	*Target*
1	Symbioimine	101727402	$C_{19}H_{24}NO_5S^+$	378.46256	RANKL
					NOX4
					ERRα / NR3B1
					Sclerostin
					LRP5
					TNFRSF11B
2	Norzoanthamine	24939455	$C_{29}H_{39}NO_5$	481.62366	RANKL
					NOX4
					ERRα / NR3B1
					Sclerostin
					LRP5
					TNFRSF11B
					HDAC
3	Fucoxanthin	5281239	$C_{42}H_{58}O_6$	658.90632	RANKL
					NOX4
					ERRα / NR3B1
					Sclerostin
					LRP5
					TNFRSF11B
4	Biselyngbyaside	44140287	$C_{34}H_{52}O_9$	604.77128	RANKL
					NOX4
					ERRα / NR3B1

					Sclerostin
					LRP5
					TNFRSF11B
5	Ikarisoside A	5481982	$C_{26}H_{28}O_{10}$	500.49452	RANKL
					NOX4
					ERRα / NR3B1
					Sclerostin
					LRP5
					TNFRSF11B
6	Bolinaquinone	10066979	$C_{22}H_{30}O_4$	358.4712	RANKL
					NOX4
					ERRα / NR3B1
					Sclerostin
					LRP5
					TNFRSF11B
7	Largazole	24757913	$C_{29}H_{42}N_4O_5S_3$	622.862580	HDAC
					NOX4
					ERRα / NR3B1
					Sclerostin
					LRP5
					TNFRSF11B

Table 7: Potential targets of Osteoporosis disease with their PDB ID and region of interest

Sl. No	*Target*	*UNIPROT Id*	*PDB Id*	*Short Name*	*Full Name*	*Position*
1	TNFRSF11B	O00300	3URF	TNFRSF11B	Tumor necrosis factor receptor superfamily member 11B	22-186
2	RANKL	O14788	3URF	TNFSF11	Receptor activator of nuclear factor kappa-B ligand	162-317
3	Estrogen	P03372	1XPC	ER	Estrogen receptor	307-554

The structure of the protein, namely LRP5 whose structure was not available in PDB was generated using Modeller 9.15. The PDB ids 3S2K, 3S94, 4DG6 were used as the templates by the Modeller 9.15 for the generation of 3D structure. The detail of the structure predictions about the protein is reported at Table 8.

Table 8: Targets/Proteins and their protein sequences for structure prediction

Sl. no	*Target name*	*PDB –ID/ UniProt ID*	*Query Sequence*
1	LRP5	Homology modelling (MODELLER 9.15V) Query sequence from UniProt ID **O75197** (Sequence from1-335)	MEAAPPGPPWPLLLLLLLLALCGCPAPAAASPLLLFANRRDVRLVDAGGVKLESTIVV SGLEDAAAVDFQFSKGAVYWTDVSEEAIKQTYLNQTGAAVQNVVISGLVSPDGLACD WVGKKLYWTDSETNRIEVANLNGTSRKVLFWQDLDQPRAIALDPAHGYMYWTDWG ETPRIERAGMDGSTRKIIVDSDIYWPNGLTIDLEEQKLYWADAKLSFIHRANLDGSFRQ KVVEGSLTHPFALTLSGDTLYWTDWQTRSIHACNKRTGGKRKEILSALYSPMDIQVLS QERQPFFHTRCEEDNGGCSHLCLLSPSEPFYTCACPTGVQLQDNGRTCKAGAEEVLLLA RRTDLRRISLDTPDFTDIVLQVDDIRHAIAIDYDPLEGYVYWTDDEVRAIRRAYLDGSG AQTLVNTEINDPDGIAVDWVARNLYWTDTGTDRIEVTRLNGTSRKILVSEDLDEPRAIA LHPVMGLMYWTDWGENPKIECANLDGQERRVLVNASLGWPNGLALDLQEGKLYWG DAKTDKIEVINVDGTKRRTLLEDKLPHIFGFTLLGDFIYWTDWQRRSIERVHKVKASRD VIIDQLPDLMGLKAVNVAKVVGTNPCADRNGGCSHLCFFTPHATRCGCPIGLELLSDM KTCIVPEAFLVFTSRAAIHRISLETNNNDVAIPLTGVKEASALDFDVSNNHIYWTDVSLK TISRAFMNGSSVEHVVEFGLDYPEGMAVDWMGKNLYWADTGTNRIEVARLDGQFRQ VLVWRDLDNPRSLALDPTKGYIYWTEWGGKPRIVRAFMDGTNCMTLVDKVGRANDL TIDYADQRLYWTDLDTNMIESSNMLGQERVVIADDLPHPFGLTQYSDYIYWTDWNLH SIERADKTSGRNRTLIQGHLDFVMDILVFHSSRQDGLNDCMHNNGQCGQLCLAIPGGH RCGCASHYTLDPSSRNCSPPTTFLLFSQKSAISRMIPDDQHSPDLILPLHGLRNVKAIDYD PLDKFIYWVDGRQNIKRAKDDGTQPFVLTSLSQGQNPDRQPHDLSIDIYSRTLFWTCEA TNTINVHRLSGEAMGVVLRGDRDKPRAIVVNAERGYLYFTNMQDRAAKIERAALDGT EREVLFTTGLIRPVALVVDNTLGKLFWVDADLKRIESCDLSGANRLTLEDANIVQPLGL TILGKHLYWIDRQQQMIERVEKTTGDKRTRIQGRVAHLTGIHAVEEVSLEEFSAHPCAR DNGGCSHICIAKGDGTPRCSCPVHLVLLQNLLTCGEPPTCSPDQFACATGEIDCIPGAWR CDGFPECDDQSDEEGCPVCSAAQFPCARGQCVDLRLRCDGEADCQDRSDEADCDAICL PNQFRCASGQCVLIKQQCDSFPDCIDGSDELMCEITKPPSDDSPAHSSAIGPVIGIILSLFV MGGVYFVCQRVVCQRYAGANGPFPHEYVSGTPHVPLNFIAPGGSQHGPFTGIACGKSM MSSVSLMGGRGGVPLYDRNHVTGASSSSSSSTKATLYPPILNPPPSPATDPSLYNMDMF YSSNIPATARPYRPYIIRGMAPPTTPCSTDVCDSDYSASRWKASKYYLDLNSDSDPYPPP PTPHSQYLSAEDSCPPSPATERSYFHLFPPPPSPCTDSS

Validation of the generated structure using the online server PDBsum Generate suggests that the quality of the generated structure is good and can be used for protein-ligand studies which is shown in the following Fig. 7.

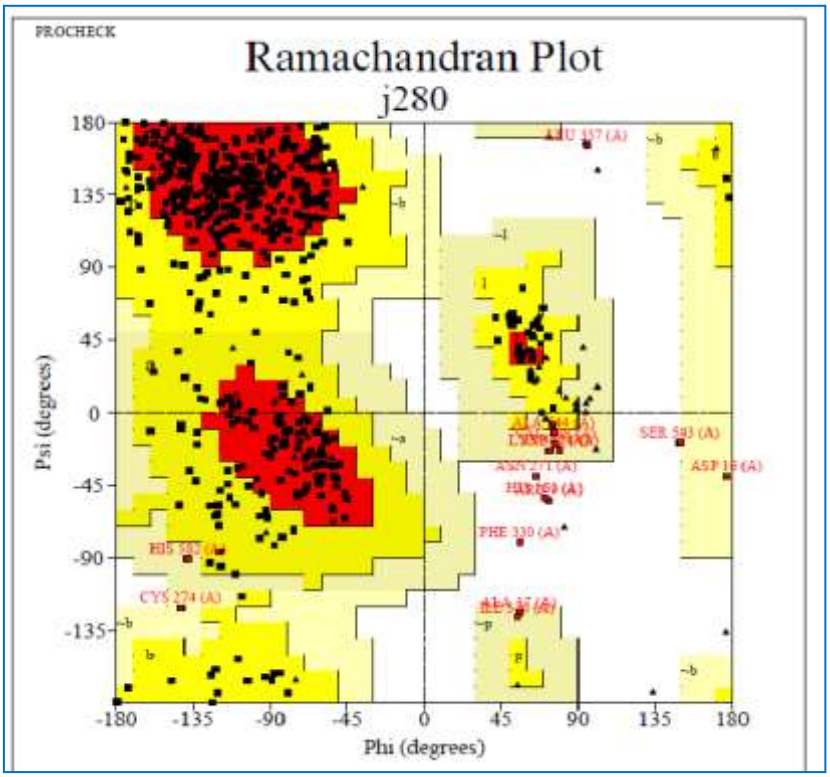


Fig. 7: Model Validation done by PDB_{sum} Generate in Ramachadran plot

Residues in most favoured regions 69.9%
Residues in additional allowed regions 148 27.4%
Residues in generously allowed regions 9 1.7%
Residues in disallowed regions 6 1.1%

Prediction of Binding Site

CASTp server was used to identify the active site binding of the 4 proteins named ER, RANKL, TNFRST11B & LRP5. The result is represented in Table 9.

Table 9: Active / binding site of the 4 proteins predicted by using CASTp

Sl No	*Target Name*	*Binding Site Region*
	ER	MET343,LEU346,THR347,LEU349,ALA350,ASP351,GLU353,LEU354,GLU380,TRP383,LEU384,LEU387,MET388,LEU391,ARG394,PHE404,MET421,ILE424,PHE425,LEU428,GLY521,MET522,HIS524,LEU525,TYR526,SER527,MET528,LYS529,CYS530,LYS531,ASN532,VAL534,LEU536,LEU539
	RANKL	SER179,HIS180,LYS181,GLN237,MET239,TYR241,SER294
	TNFRSF11B	PHE129,SER130,ASN131,GLU132,ALA137,PRO138,ARG140
	LRP5	MET1,SER2,GLU3,ALA4,ALA5,HIS6,VAL7,ILE9,THR10,ALA12,GLY14,GLN15,ILE16,GLY17,TYR18,ILE19,LEU20,SER21,HIS22,ILE24,LEU29,TYR30,GLY31,ASP32,ARG33,MET46,ASN47,ARG48,LEU49,PRO62,HIS63,LEU64,ALA65,GLY66,PHE67,VAL68,THR70,THR71,ASP72,PRO73,LYS74,ALA75,ALA76,PHE77,ASP79,ILE80,PHE84,LEU85,VAL86,ALA87,SER88,VAL96,ARG97,ALA98,ASP99,LEU100,ILE107,PHE108,LYS109,ASN110,THR111,GLY112,TYR114,LYS124,VAL125,LEU126,VAL127,ILE128,GLY129,ASN130,PRO131,ASP132,ASN133,THR134,ASN135,CYS36,GLU137,ILE138,ALA139,PHE151,SER152,LYS169,LEU170,ASN306,ASP307,TRP308,LEU309,ARG310,LYS318,ASP319,LEU320,PHE321,GLU323

Protein-ligand interaction studies were carried out using 4 genes whose active sites were predicted using Castp. AutoDock 4.2 (autodock.scripps.edu/) that was used for docking studies revealed docking score with energy minimization values, Binding energy, Ligand Efficiency, Inhibition Constant and Electrostatic energy

for 16 ligands/drugs-16 potential targets interactions are represented at Table 10.

Table 10: Molecular docking analysis of 16 drugs against 4 target proteins using AutoDock4.2 tool

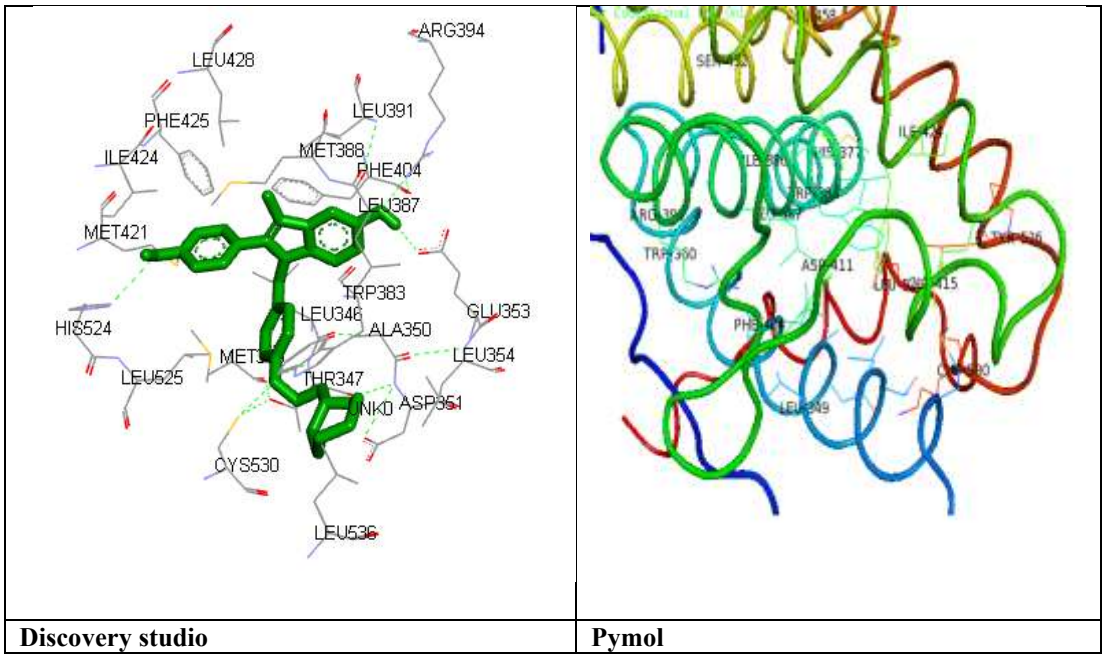
Target	*Drug*	*Binding Energy*	*Ligand Efficiency*	*Inhibition Constant*	*Hydrogen Bonding*	*Hydrophobic*
RANKL	ALENDRONATE	-3.04	-0.22	5.94	LYS181,GLN237,TYR 241	N/A
RANKL	IBANDRONATE	-3.15	-0.17	4.88	HIS180,LYS181,GLN2 37,TYR241,SER179	N/A
RANKL	IPRIFLAVONE	-6.1	-0.29	33.69	LYS181,SER294,GLN 237,ASN295	HIS180,LYS181,LEU23 6
RANKL	RISENDRONATE	-3.72	-0.22	1.89	THR233,TYR235,ASN 295,HIS180	SER179,HIS180
RANKL	VITAMIN D3	-7.7	-0.28	2.26	N/A	LYS181,PRO296,HIS18 0
RANKL	ZOLENDRONAT E	-3.2	-0.2	4.5	GLN237,ASN295,TYR 235,GLN237,HIS180	SER179,HIS180
RANKL	BISELYNGBYASI DE	-6.36	-0.15	21.66	ARG140,ASN131,SER 134	PHE129
RANKL	BOLINAQUINON E	-6.78	-0.26	10.79	GLN237,LEU236	MET239,LYS181,HIS1 80
RANKL	FUCOXANTHIN	-4.21	-0.09	819.38	SER297,ASN295	LYS257,LEU236,LEU2 98
RANKL	SYMBIOIMINE	-8.74	-0.34	390.02	HIS180,LYS181,GLN2 37,TYR235,ASN295	N/A
ER	BAZEDOXIFENE	-13.1	-0.37	251.18	CYS530,GLU353	PHE404,LEU346,THR3 47,LEU354,LEU356,M ET388,LEU391,MET42 1,LEU428,ILE424,TRP 383,LEU391,ALA350,L EU387,LEU525
ER	CHOLECALCIFE ROL	-9.86	-0.35	59.65	ARG140,GLU132	ARG140,PHE129
ER	LASOFOXIFENE	-13.14	-0.42	233.97	ARG394,CYS530,GL U353,ASP351	ALA350,LEU354,LEU3 87,MET388, LEU391,TRP383,LEU5 25,ILE424
ER	RANELATE	-3.4	-0.15	3.2	GLU419,LYS529,SER 341	N/A
ER	CALCITRIOL	-10.46	-0.35	21.46	LEU536,TYR537,GLU 380,ASP351	TRP383,LYS531,LEU5 36,PRO535,TYR526
TNFRS F11B	BISELYNGBYASI DE	-2.11	-0.05	28.58	ARG140,ASN131,SER 134	PHE129
TNFRS F11B	BOLINAQUINON E	-6.25	-0.24	26.36	ARG140,ASP161	PHE129,ARG140,LYS1 41
TNFRS F11B	CALCITRIOL	-4.19	-0.14	842.19	ARG14O,GLU132	ARG140,PHE129
TNFRS F11B	FUCOXANTHIN	-5.53	-0.12	87.99	N/A	ALA112,ALA137,PRO1 38,ARG140,HIS160
TNFRS F11B	SYMBIOIMINE	-6.43	-0.25	19.26	ASN131,ARG140,AS N131	PHE129,ARG140,LYS1 41
TNFRS F11B	NORZOANTHAM INE	-7.79	-0.22	1.95	N/A	ALA137,ARG140,PHE1 29
LRP5	FUCOXANTHIN	-8.16	-0.17	1.05	HIS22,SER21,MET46, PHE84	LYS74,ILE24,ARG48

The result obtained from docking studies revealed that Bazedoxifine, Lasofoxifene & Calcitrol may act as potential drug for treatment of Osteoporosis.

- The molecular docking studies have reported the drug-target interactions of ER-Bazedoxifine was -13.1, ER-Lasofoxifene was -13.14, & ER-Calcitriol was -10.46.
- Interactions of ER-Lasofoxifene has been identified with the highest docking scores with energy minimization i.e; -13.14 and

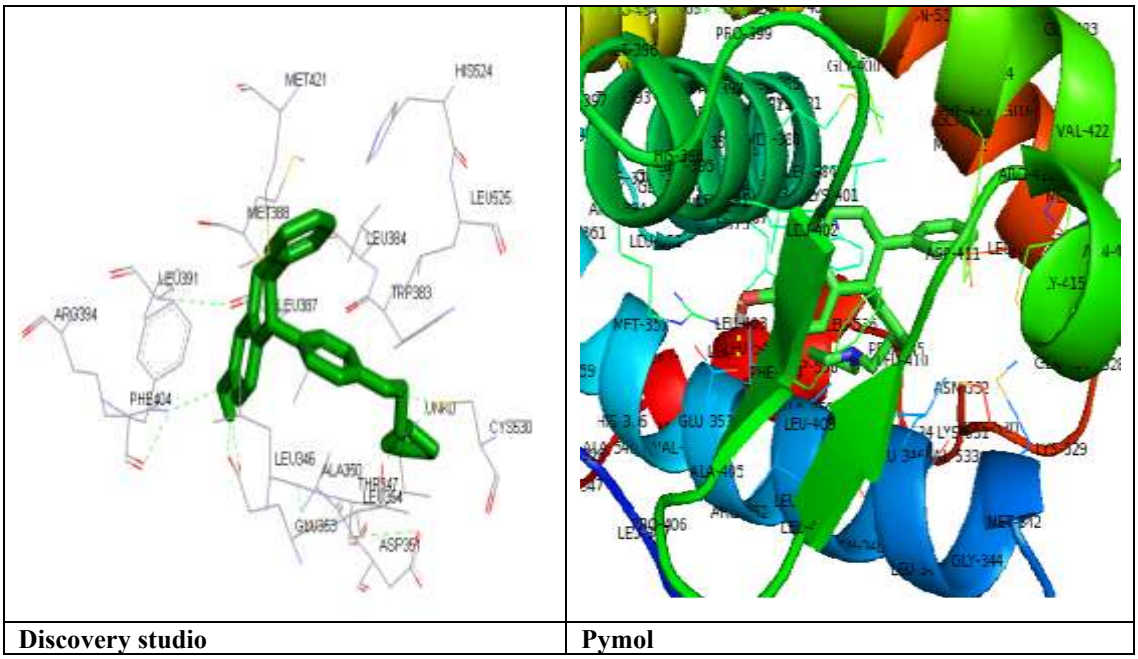
Interactions of TNFRSF11B-Biselyngbyaside is-2.11 which is identified as the lowest docking scores with energy minimization out of 22 ligand-protein interactions.

- ❖ Interaction of symbioimine, vitamind3, bolinaquinone, biselyngbyaside & ipriflavone with RANKL have docking score of-8.74,-7.7,-6.78,-6.36,-6.1 kcal/mol with energy minimization.
- ❖ Interaction of fucoxanthin with LRP5 has an average docking score of -8.16kcal/mol with energy minimization.
- ❖ Interaction of symbioimine, bolinaquinone & calcitrol with RANKL have docking score of -6.43,-6.25,-4.19 kcal/mol with energy minimization
- ❖ The ligand-target complex was performed for the best binding scores and its interaction studies was visualized in Discovery Studio Visualizer, and PyMol visualize depicted in the Fig.8, 9, 10, 11, 12 and 13.



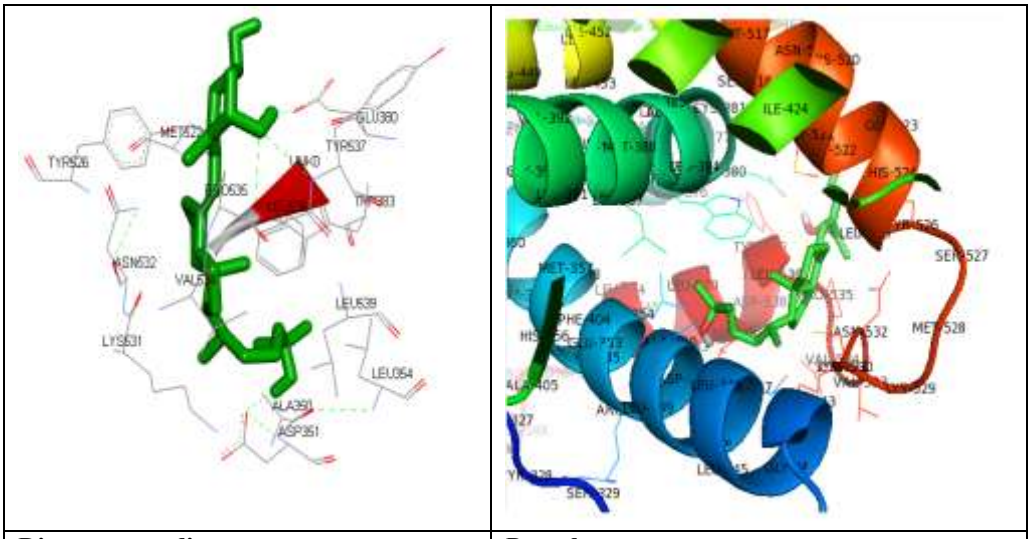
Discovery studio	Pymol

Fig. 8: Interactions of ER-Bazedoxifine



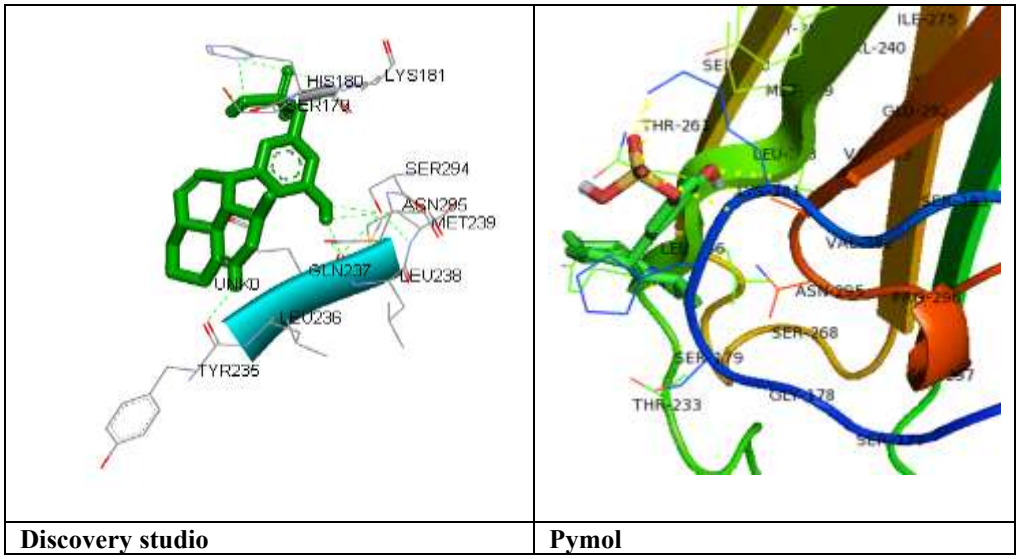
Discovery studio	Pymol

Fig. 9: Interactions of ER-Lasofoxifene



Discovery studio	Pymol

Fig. 10: Interactions of ER-Calcitrol



Discovery studio	Pymol

Fig. 11 Interactions of RANKL & Symbioimine

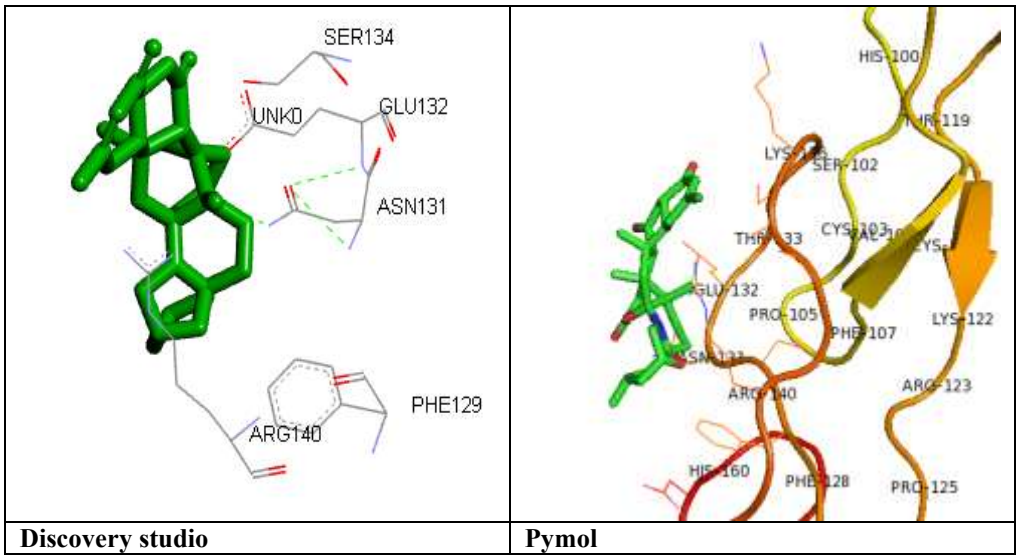


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Fig. 12: Interactions of TNFRSF11B & Norzoanthiamine

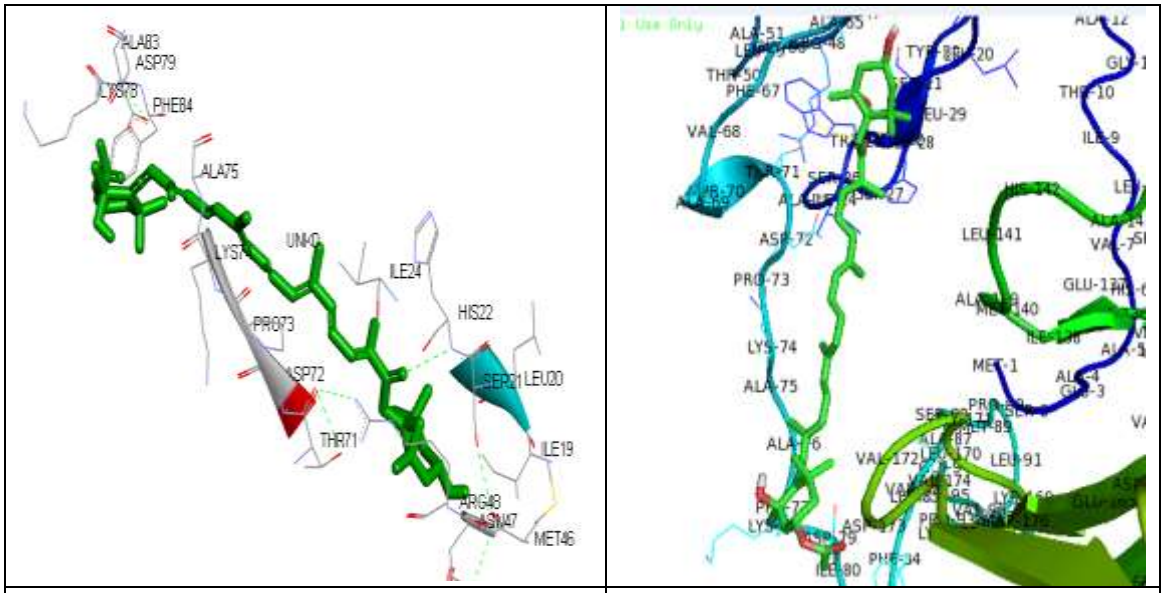

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Fig. 13: Interactions of LRP5 & Fucoxanthin

4. CONCLUSION

The present investigation was carried out to explore the activity of natural based drugs from algal extracts against osteoporosis through computational analysis using various bioinformatics tools and techniques. An *insilico* comparative study of activity of biosynthetic drugs and natural compounds (algal extracts) was carried out against osteoporosis.

Various genes or its corresponding proteins, drugs and algal extracts pertaining to osteoporosis were mined from various literature survey and GWAS studies. The GWAS reported 8 studies of Osteoporosis with a total no of 14 unique genes namely LRP5, SOX6, TBC1D8, ALDH7A1, RAP1A, MECOM, OSBPL1A, DOK6, LOC348751, FONG, LRRC4C, TNFRSF11B, SPP2 and SCG2. The Drug Association analysis of WebGestalt has reported only one Drug i.e Calcitriol against two potential targets i.e TNFRSF11B and LRP5.

The literature survey has reported a total of 25 drugs and Algal extracts against 8 potential targets, out of which only 5 Algal extracts namely Bolinaquinone, Fucoxanthin, Biselyngbyaside, Symbioimine and Norzoanthamine; 11 drugs namely alendronate, ibandronate, ipriflavone, risendronate, vitamin d3, zolendronate, bazedoxifene, cholecalciferol, lasofoxifene, ranelate and calcitriol; 4 targets namely ER, RANKL, TNFRSF11B and LRP5 were considered and undertaken for further *in silico* analysis as the details structural and functional analysis of the above said drugs, Algal extracts and potential targets are available. Protein-ligand interaction studies were carried out using only 4 proteins whose active sites were predicted using CastP. AutoDock 4.2 (autodock.scripps.edu/) that was used for docking studies revealed docking score with energy minimization values, Binding energy, Ligand Efficiency, Inhibition Constant and Electrostatic energy for 11 ligands/drugs and 5Algal extracts against 4 potential targets interactions.

The docking studies revealed that Bazedoxifene, Calcitriol and Lasofoxifene may act as potential drugs and the algal extracts namely Symbioimine, Norzoanthamine and Fucoxanthin could be used for treatment of Osteoporosis. The molecular docking studies have reported the drug-target interactions of ER-Bazedoxifene was -13.1, ER-Lasofoxifene was -13.14, ER-Calcitriol was-10.46 kcal/mol whereas the interactions of algal extracts-target represented RANKL-Symbioimine was -8.74, TNFRSF11B-Norzoanthamine was -7.79 and LRP5-Fucoxanthin was -8.16. was been identified with the highest docking scores with energy minimization. The interaction of TNFRSF11B-Biselyngbyaside was -2.11 which was identified as the lowest docking scores with energy minimization out of 10 ligand-protein interactions with algal extracts. This investigation could be validated by wet lab analysis which could provide promising results for osteoporosis.

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