

Allosteric modulation on specific gene mutated Vitamin D receptor by essential PUFAs and its distinct molecular effects on type 2 diabetes mellitus

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A serious health threat affecting the T2DM group is evident more cases T2DM are diagnosed. In this research, we choose to research into all of this possible mechanism of 3T3-L1 Cell lines and Molecular Docking studies Schrodinger software identified Vitamin D, *Omega-3*, and 6 PUFAs (EPA DHA & AA) Compounds of hydrophilic and hydrophobic pocket throughout molecular modeling besides T2DM. A group of three analog VDRs is being developed for discovery treatment with T2DM. Its use as it was agreed to run a molecular cell culture and docking study. Recognize the binding method involving the compound in T2DM through ADME prediction. The molecular dynamics simulation was enhanced by confirmation of the strength of the possible composite binding. Based on the computational results, the *Omega-3* and 6 PUFAs compound encourages energy interaction. The composite contains an *in vitro* anti-diabetic activity; the compounds have clearly shown that they are active on T2DM. Our studies provide vital information on the findings of the bimolecular T2DM inhibitors.

Keywords: Arachidonic Acid, Docosahexaenoic Acid, Eicosapentaenoic Acid, Polyunsaturated fatty acids, Type-2 Diabetes Mellitus, Vitamin D Receptor, Vitamin D, 3T3-L1

The amounts of individuals with T2DM rise significantly in 2000. T2DM occurrence in individuals throughout the world is estimated at around 10.5% (536.6 million people), rising to 12.2% (783.2 million) in 2045, 4.4% of the inhabitants might have been affected by diabetes. As more than just a consequence, 366 million T2D anguishes in the globe are being described throughout the last fifteen years 2004¹. T2DM was related through insulin resistance during skeletal muscle adipocyte tissue, with hepatic gluconeogenesis dysregulation also uninhibited insulin secretion in reestablishing insulin sensitivity². In Asian countries, they could increase insulin sensitivity during the response to *Omega-3* and *Omega-6* PUFAs supplementation although encouraging the evaluation with patients to ethnicity³. Previously, there will be an increasing indication that perhaps acute phase inflammatory reaction produced through cytokines directly associated within identification in insulin resistance and T2DM. Even though important genetic factors, it is necessary to consider the description that diabetes is a multi-factorial disorder⁴. VD3 is a few compounds of both the metabolite 1, 25 (OH) D3, as well as the transcription factor VDR, which provide a direct

then influence control of gene expression. In the same case as other endocrine nuclear receptors, as such alternative significant conformation changes that have taken place from outside Ligand binding domain, in this influences it is the receptor's protein-protein interaction profile. Through Adaptor proteins in the nucleus, while the corepressors co-activate the intermediate compounds. VDR ligand-dependent transcriptional regulator adheres to a super family with nuclear receptors (NRSF)⁵. In the specific instance of PUFAs in VDR of T2DM, a metabolic disorder with distinct etiology is described by typical lipid, carbohydrate, protein combination arising Insulin action were affected due to a deficiency in insulin secretion. Otherwise, associated T2DM typically appears in middle age and seems to affect insulin resistance and relative insulin sensitivity. Individuals with T2DM contain a difficult threat, in recent decades, T2DM has become extremely prevalent.

Materials and Methods

Selected Polyunsaturated Fatty Acids (PUFAs)

a. Eicosapentaenoic Acid (EPA): (C₂₀H₃₀O₂) (Mol. Wt. 302.5g/mol) and *Omega-3* PUFAs have 5 Cis-double bond positions (5, 8, 11, 14 and 17) EPAs⁶ (Fig. 1A & B).

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b. Docosahexaenoic acid (DHA): ($C_{22}H_{32}O_2$) (Mol.Wt.328.488g/mol) is *Omega-3* PUFAs Containing 6 Cis-double bonds at positions (4, 7, 10, 13, 16, and 19)⁷ (Fig. 2A & B).

c. Arachidonic acid (AA): is *Omega-6* long-chain PUFAs ($C_{20}H_{32}O_2$) have 4 (Z)-double bonds on positions (5, 8, 11, and 14). Synthesized as dietary Linoleic acid (LA) Arachidonic acid⁸ (Fig. 3A & B).

Vitamin D receptor

Hyperglycemia induces expression and triggers VDR and RXR, which is counteracted by *Omega-3* and 6 PUFAs through the (VDR/RXR) pathway-mediated

up-regulation of VD in adipose pre-adipocyte. VDR is separated through various domains LBD, DNA binding domain, N-terminus, HINGE region which could be used for dimerization. Among the various domains, LBD plays an active role in the binding of 12 helical receptors and when bonded to a ligand, it forms a compact three-dimensional structure. The ligand-binding pocket extremely covered by the receptor that enables special interactions with ligands found natural, especially important VD. The VDR receptors interact symmetrically through their ligand-binding domain together in the Helix 12 activate their configuration, as such DNA Binding Domain (DBD) is regressive,

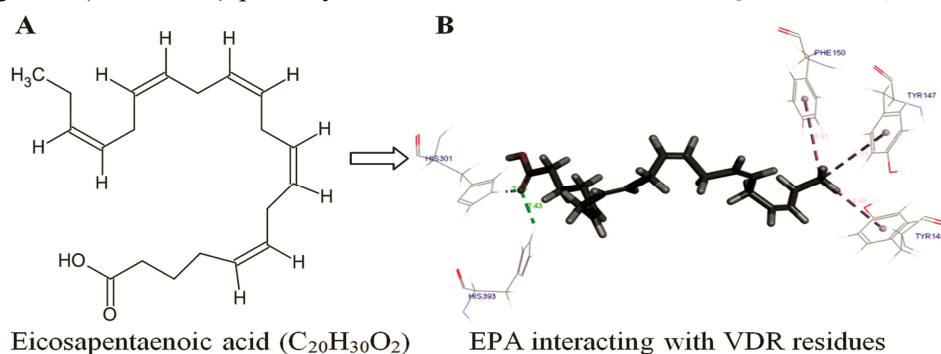


Fig. 1 — (A) Eicosapentaenoic acid ($C_{20}H_{30}O_2$); and (B) EPA interacting with VDR residues

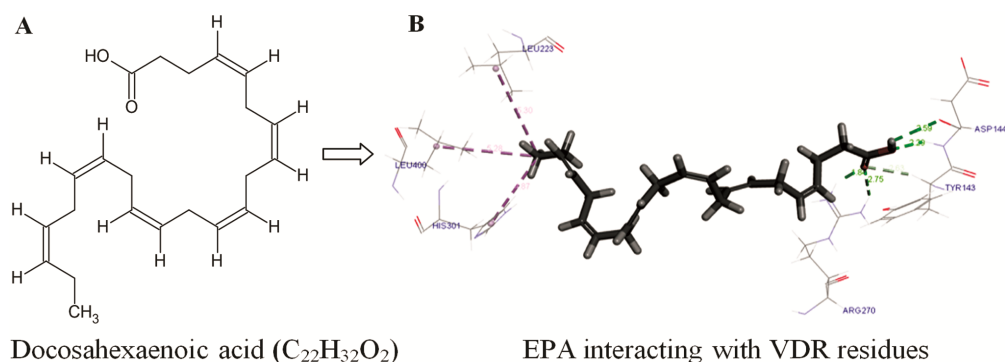


Fig. 2 — (A) Docosahexaenoic acid ($C_{22}H_{32}O_2$); and (B) EPA interacting with VDR residues

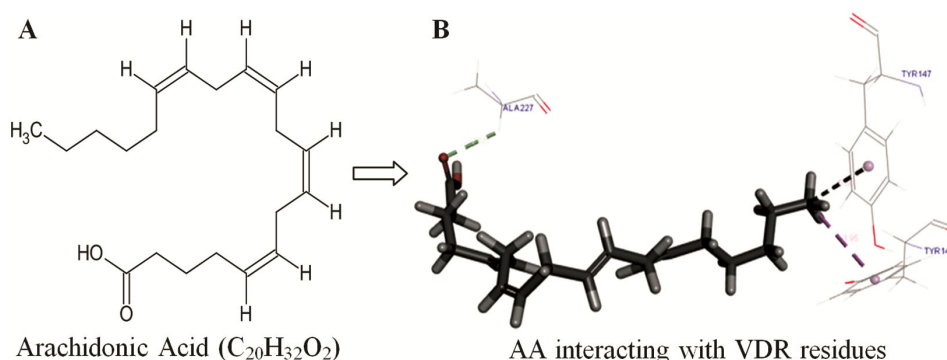


Fig. 3 — (A) Arachidonic Acid ($C_{20}H_{32}O_2$); and (B) AA interacting with VDR residues

with the Retinoic X Receptor because of additional compactness in addition to the VDR. During the wide-ranging configuration of both the LBD and DNA Binding Domains. Several VD control genes include a pair of VDEs within promoter, the chromatin immunoprecipitation study, the receptor complex binds to different VDR elements were exposed during repeated influencing factors^{9,10}. There is significant evidence that the cytokine-induced acute-phase inflammatory response is related to the development of insulin resistance and T2DM. IL-1, IL-6, TNF- α , as well as other inflammatory and immune system biomarkers were associated with these pathologies. Hypothesized that 1, 25(OH)D3 concentration that acts a role in the development of T2DM. Interleukin-1 is an Insulin resistance-associated cytokine. Insulin signaling is directly affected by IL-1 through the induction of cytokine-3 signaling suppressor (SOCS-3), it is primarily stimulated by TNF- α and catecholamine's and inhibited by glucocorticoids. It has multiple effects on different tissues, acting together with IL-6 as endogenous pyrogens, stimulating thermogenesis¹¹. It also regulates Protein C Reactive (PCR) hepatic production, and its presence in adipose tissue can stimulate Protein C Reactive synthesis (Fig. 4).

Research Methodology

Devices and materials

To study led *in silico* molecular through the use of bioinformatics tools. Several offline programming on the web will be used for exploration. Programming used for the examination is ACD/Labs' ChemSketch 12.01, study Collaboratory used Structural Bioinformatics Protein Data Bank (RCSB PDB). The materials used in this evaluation were information on the arrangement and 3D structure of selected VDRs. The drug relevance was checked using the mo inspiration toolkit (<http://www.molinspiration.com/>) This information is accessible online under European Molecular Biology Laboratory-EBI (<http://www.ebi.ac.uk/>) National Center of Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) PDB at the RCSB site (<http://www.rcsb.org/pdb/home/home.do>). In executing a molecular docking analysis of the synthesized compounds against by the three VDRs, the Schrodinger Software System Docker and Glide module were accomplished.

Preparation of protein

The information of three-dimensional structural macromolecules was retrieved as of website

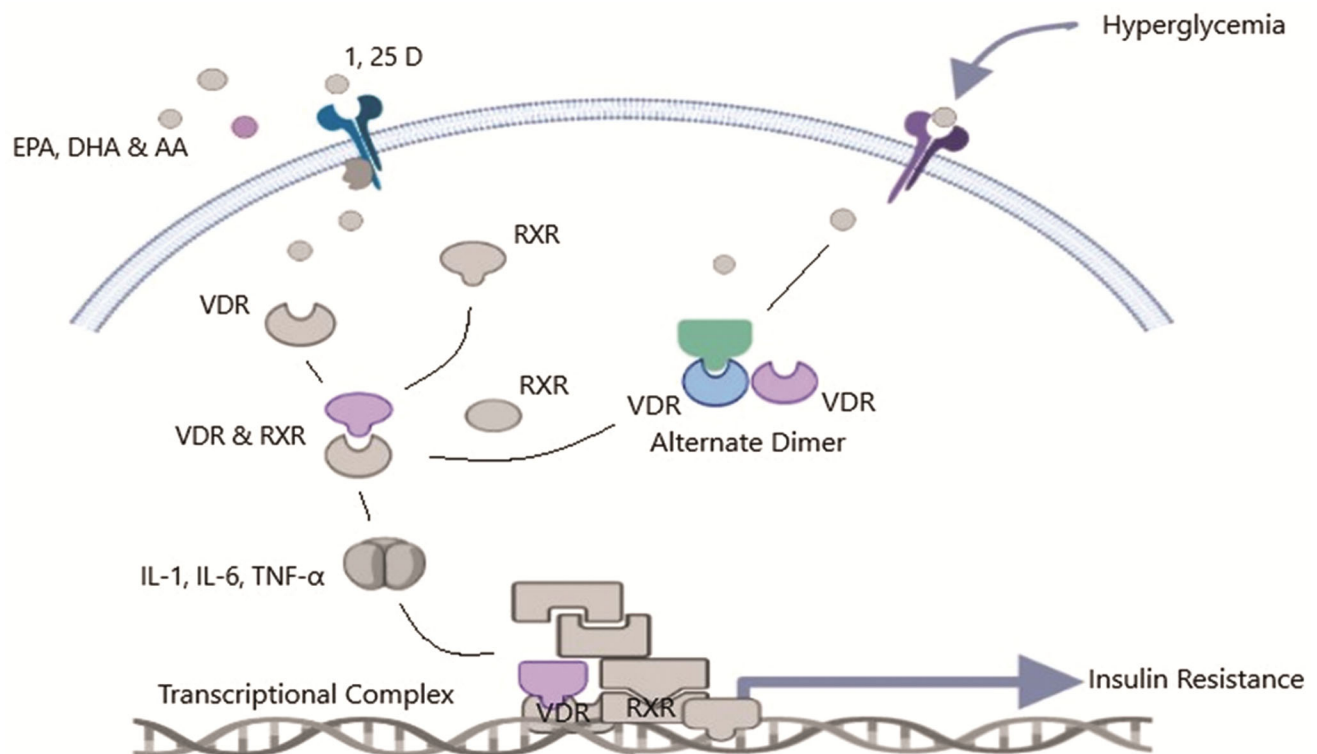


Fig. 4 — Schematic depiction of the role of endogenous Vitamin D3 and *Omega*-3 and 6 PUFAs, such as (EPA, DHA & AA) in hyperglycemia-induced insulin resistance in adipose tissues

(<http://www.rcsb.org/pdb>). From PDB, VDR Including the Protein Data Bank (PDB RCSB Id: 1RKH, 3M7R, 3VT3, 3VT7)¹². With a resolution of (2.1Å⁰) were obtained. The crystallographic water molecules were reduced as protein followed by the addition of missing hydrogen atoms. Alternate conformations and valence monitor options should be used for exact crystallographic disorder and untaken valence atoms. CHARMM (Chemistry at Harvard Macromolecular Mechanics). It was used to minimize protein energy. The prepared protein was validated by the Ramachandran plots (Fig. 5A-D)

Identification of active site

The receptor cavity method was used to predict the receptor protein's binding or active site, and even the inhibitory properties of the amino acid residues present in the binding sites¹³.

Ligand Preparation

Structure on *n*-3 & *n*-6 PUFAs in VDR compounds Initial step for molecular docking studies. The advanced atomic model VDR, LBD omitted a map. H305Q is being used for fine ligand and electrons significantly contributed to exposure. With similar arrangements, the conformation of calcitriol is similar to that of a Wild-type VDR crystal structure. (A-, Seco-B-, C- and D-rings). Subsequently, crystal structures of human VDR LBD are identified during complex through VD3 and some ligands synthetic exposed in the existence of close to the surface, strongly bound water molecules form a channel C2 ligand pose, which might also take part in significant Protein stability plays a role¹⁴. Chemical structures of selected PUFAs are developed, modified, established, and optimized with ChemSketch software and transformed into a 3D structure. Subsequently, using the Ligprep module, the ligand structure will be arranged in favor of docking.

Docking analysis

Molecular docking studies have been carried out using the Schrodinger Software Protocol. Interaction with VD, selected PUFAs through a single protein are evaluated somewhere where the ligands remain flexible in addition to protein. To minimize the structures on selected PUFAs, its input ligand was used. Dock score and Glide score schemes for dock ligands were applied to the receptor-binding site. Both the docking score and glide score schemes would be applied on dock ligands even at the receptor binding sites as well. We are being generated by random ligand conformations-high-temperature MD. While random rigid-body rotations accompanied by simulated annealing will be used to translate the conformations at the binding site and shape the compound poses. Significant energy minimization is used to process ligand poses. DOCKER ENERGY (the sum of the internal ligand strain energy and the non-bonded interaction energy between the receptor and the ligand) and DOCKER INTERACTION ENERGY (non bonded interactions between ligand and receptor) are used for every pose. In any case, the lowest energy values were the most important favorable binding model.

The glide standard precision (SP) ligand docking

Standard Precision (SP) flexible ligand docking was accepted in Glide of (Schrodinger-Maestro v 10.1)¹⁵. Contained by which penalty be functional toward non-cis/trans amide bonds. Van der Waals scaling factor and partial charge cutoff be preferred to be (0.80 and 0.15), correspondingly used for ligand atoms. Final scoring is performed taking place energy-minimized poses and display as Glide score. Most excellent docked pose through lowest Glide score value be recorded designed for each ligand (Table 2).

ADME prediction via Lipinski's rule of five

The drug's molecular properties that are significant for its pharmacokinetics include absorption,

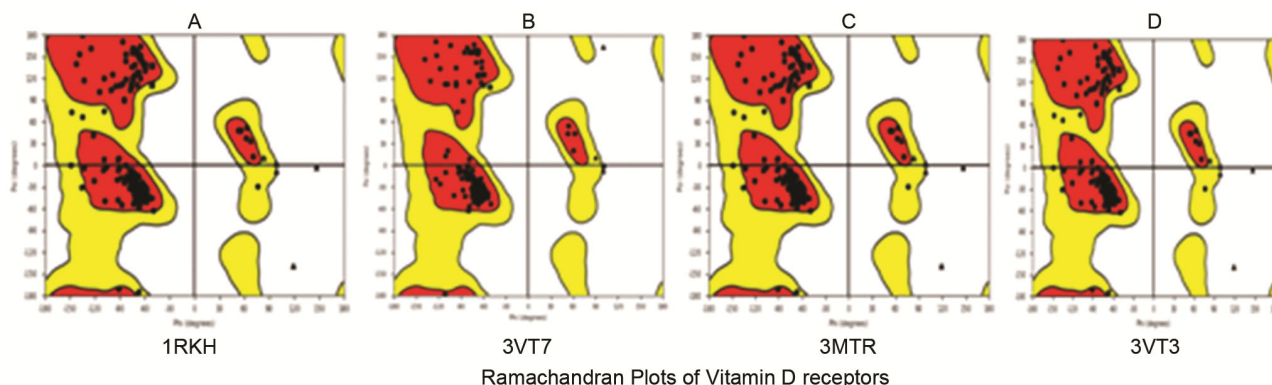


Fig. 5 — Represent of Ramachandran plots for Vitamin D receptor with PUFAs in EPA, DHA & AA

distribution, metabolism, and excretion (ADME). As a result, lead structures were sequentially optimized used for their efficient dynamics and kinetics. Alterations in the molecular composition regularly lead to drugs through higher molecular weight, additional rings, bonding, and efficient lipophilicity. The regulation states that in order to complete absorption otherwise access will become more likely while the ligand molecule violates Lipinski's rule of 5, so that it has added more than 5 H to the bond donor, to the M.Wt > 500, log P >5), addition N and O is more than 10^{16,17}. The similarity of the drug may be distinguished from the complex stability of the distinct morphological properties and structural features which are used to evaluate whether a molecule is related to the drugs that have also been identified. They contain protein content, transport properties, protein similarity, reactivity, toxicity, metabolic stability as well as several others, mainly hydrophobic properties, electronic distribution, hydrogen bonding properties, molecular size, and the occurrence of distinct pharmacophoric properties are all factors to consider to characteristics influencing the performance of a molecule during a living individual. Demonstrate methodology for the study of drug similarity to potential ligands when it influences the activities of molecules in a living individual, So many other factors were introduced, including pharmacokinetics, transport properties, protein affinity, reactivity, toxicity, and metabolic stability. They exhibited at the selected PUFAs under Lipinski's rule of 5 with Mol's inspiration. (Table 1), (<http://www.molinspiration.com/>).

Molecular dynamics stimulation of receptor-ligand complex

The ligand-target complex's stability was investigated using a molecular dynamic simulation¹⁸. The simulation is set to NVT ensemble with a temperature range of 300 K, Run 100, Time step: 0.002, and QT (the temperature response set in a Molecular Dynamics simulation which enforces constant temperature) for 0.2. The simulation took 12.55 h to complete.

In vitro studies on the cell lines

Chemicals and reagents

DMEM High glucose (#AL219A, Himedia), DMEM without glucose (#AL186, Himedia), Fetal

Bovine Serum (#RM10432, Himedia), D-PBS (#TL1006, Himedia), 2-NBDG (Invitrogen: Cat no. 13195), Cholecalciferol (#2936 2990, Himedia), DMSO (#PHR1309, Sigma). Oil Red O staining kit. (Abcam, #133102). Vitamin D3 (Cholecalciferol) (#CMS334, Himedia, India) PUFAs (EPA#E0441, DHA#D2226, & AA#A0781, TCL, Japan).

Cell culture

3T3-L1 adipocytes cells were purchased from National Center for Cell Science (NCCS, India) provided mouse embryo fibroblast cells. In a humidified chamber of 95% air and 5% CO₂ at 37°C, cells were preserved in Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal bovine serum and 1% penicillin and streptomycin (anti-anti).

Results and Discussion

Vitamin D and polyunsaturated fatty acid treatment

25(OH)D3 (Himedia, India) and PUFAs, EPA, DHA & AA were purchased from (TCI, Japan), To the PUFAs stock solutions were made in VD and then diluted in DMEM with 1.5% fatty acid-free bovine serum albumin (BSA). After 1 h incubation time at 37°C with constant shaking, supplement in the medium and VD and PUFAs (EPA, DHA & AA) On Day 0, 3T3-L1 adipocytes were given a vehicle-control on day 3 and day 5, cells were harvested (Fig. 6).

3T3-L1 cell viability assay

Preadipocytes from 3T3-L1 cells, Passage No.10 were given 1, 25(OH)D3 (200 and 400 µM) and PUFAs from Day 0, vehicle-control for 24 or 72 h. A commercial kit was used to evaluate cell viability using calcein-AM/propidium iodide staining (Dojindo Molecular Technologies). In a 200 µL phosphate buffer saline (PBS) solution containing 1 mM calcein-AM and 1.5 mM propidium iodide, cells were suspended. Cells were viewed immediately after 45 min of incubation at 37°C. From Day 0, Vehicle-control for 24 or 72 h. The spatial distribution of living cells in green (calcein staining) and a dead cell in red was assessed to use a Nikon TS100-F inverted microscope attached to a Shot S31S. (Propidium iodide staining).

At least three separate random fields were studied in each well. To be used to count, live and dead cells in each field.

Table 1 — Prediction of Molecular Properties *via* Lipinski's Rule

Compounds	No. of H Acceptors	No. of H Donors	A Log P	Molecular Wt.
EPA	02	01	5.6	302.5g/mol
DHA	02	01	6.2	328.5g/mol
AA	02	01	6.3	304.5g/mol

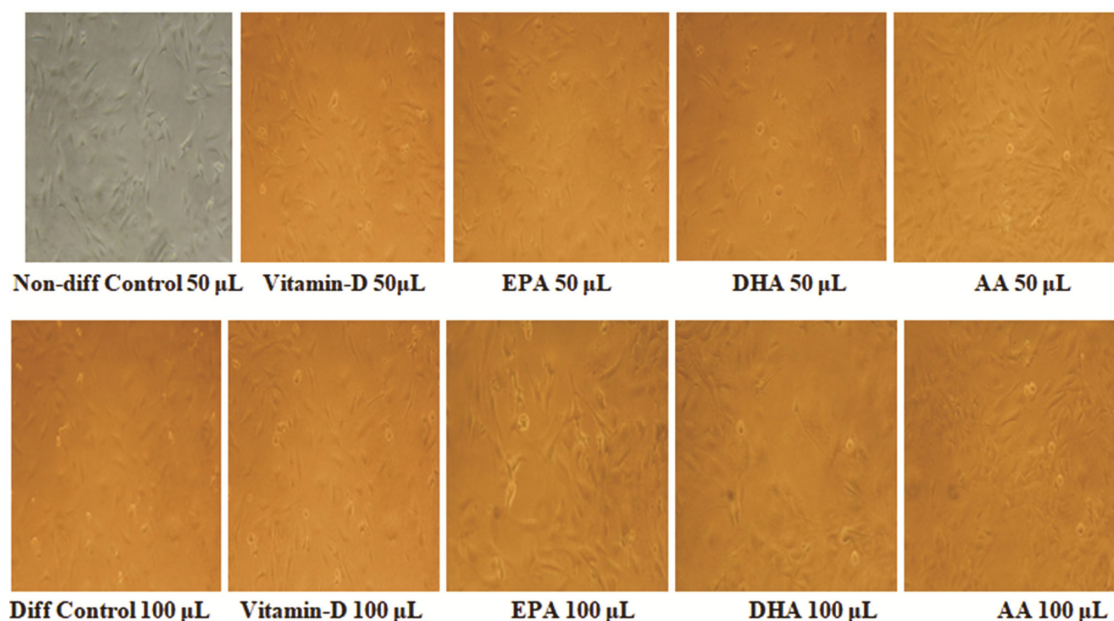


Fig. 6 — Effect of Vitamin D, *Omega-3* & *Omega-6* Polyunsaturated Fatty acids, EPA, DHA, AA differentiation on adipocyte 3T3-L1 cell lines

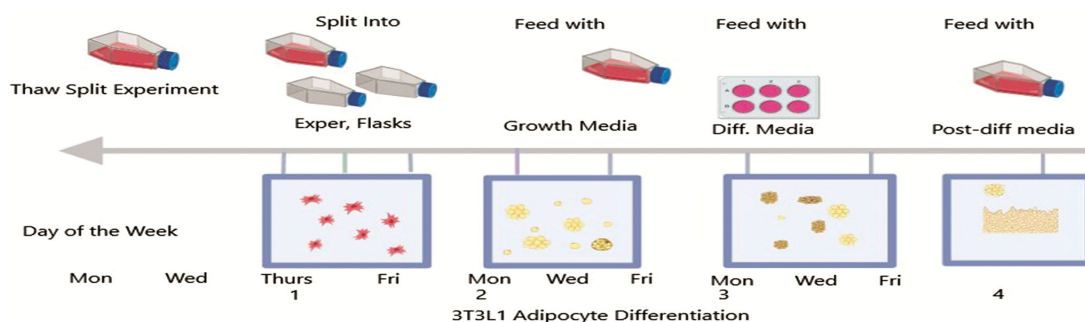


Fig. 7 — Diagram represents the major cellular process that leads to Differentiation on 3T3-L1 Adipocytes cells

Adipocyte differentiation

3T3-L1 Pre-adipocyte was used for 14 days and 3T3-L1 adipogenesis cells. were in support of 5 days after observing adipocyte differentiation, cells were grown in DMEM with 10% FBS, 2% horse serum until confluence have been reached, two days after confluence, Pre-adipocyte is simulated to differentiate in the presence of DMEM, 10% FBS, 0.5% VD and 0.5% PUFAs 10 µg/mL Insulin for two days after that, the cells were cultured in DMEM, 10% FBS, and 10% g/mL I M insulin for another 2 days before being cultured in DMEM, 10% FBS for another 4 days. Many of the media have 100 IU/mL Penicillin and 100 mg/mL Streptomycin in them. In DMSO, VD (Cholecalciferol) was dissolved on DMSO (final conc. 0.1% in media) used for the aim of co-culture studies. The pre-adipocytes taking place in the cell culture insert (pore size 1.0 M) were washed in PBS before being transferred to a culture plate containing

differentiated and wash 3T3-L1 adipocytes cells . In serum-free DMEM/F12 supplement within 0.1% BSA, 3T3-L1 cells were co-cultured with 3T3-L1 cells (BSA). To the Co-culture conducted for 24 h. For the entire culture era, the cultures were treated with test samples (day 0 to day 5) (Figs 7 and 8).

ORO staining-measurement of lipid

3T3-L1 adipocytes cells were differentiated using 5 days in the presence or absence of VD and PUFAs washed in PBS and stained with ORO according to the instructions of a commercial kit (Abcam, #133102). Cells were washed in PBS before being fixed in formalin solution for 15 min. After that, the fixed lipid droplets were stained for 30 min at room temperature with ORO solution for 30 min at room temperature. To visualize Oil Red O staining in differentiated cells Images are taken into the Microscope (Figs 9 and 10).

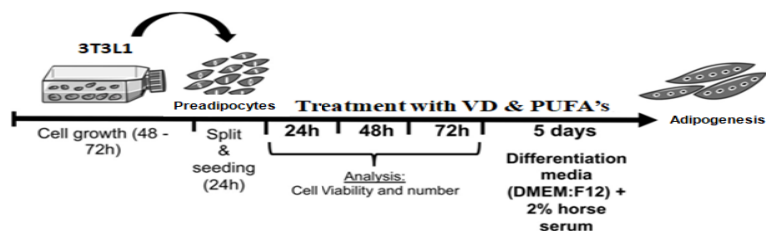


Fig. 8 — Representations of the new proposed 3T3-L1 cells. Pre-adipocytes was where developed with culture meant for 24 h, 48 h, 72 h or 5 days (differentiation procedure to form Adipogenesis) absence of presence 100 mM VD *i.e.*, $1\alpha, 25(\text{OH})_2\text{D}_3$). The analysis is performed within every time Interval. (Karina Romeu Montenegro *et al.*, 2019)²⁷

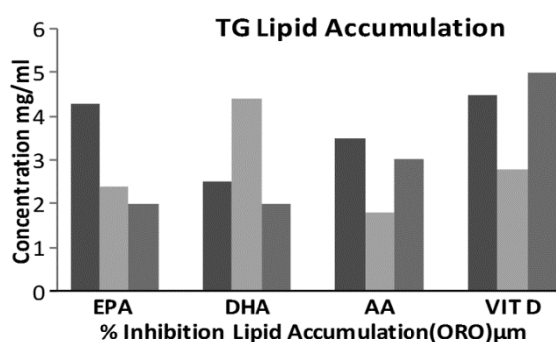


Fig. 9 — Represent the crystalline structure of Vitamin-D Receptor (VDR)

Crystal structure of proteins

To crystal structures, five VDRs into the ligand-binding domain of PUFAs, as well as VDR ternary complex through peptide contain the target sequence of PUFAs in addition to the ligands determined by the ligand individuals LBD in VDRs in a ternary complex within the peptide contain target sequence in nuclear receptor co-activators and mutations The structure reveals that the LCA and its derivatives bind on similar LBP to the VDR, that VD binds toward, however within opposing orientation¹⁹ (Fig. 11).

Molecular docking analysis

Responsibility for computational analysis, glide docking, is used to investigate binding mode on α - amylase enzyme. Both the glide standard (SP) extra precision (XP) mode have introduce wherever additional precision mode is used to support cross-validation²⁰. Grid-based docking study should be used toward analyzing how molecules are bound by AA's to active pocket protein. In control recognize specific antidiabetic straight molecule, were also subject to a docking.

Analysis to active PUFAs compound taking place even at active VDR site. To study the interaction with its compound through the VD receptor Binding Pockets (PDB id: 1RKH, 3M7R, 3VT3, 3VT7). While glide docking analysis through Schrodinger Suite v10.1, wherever VD along with the PUFAs, compounds. The

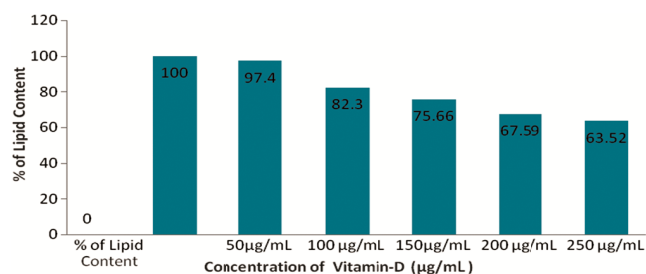


Fig. 10 — Molecular architecture of the Vitamin D and PUFAs: (a) PDB.ID: IRKH; (b).1RKH-AA; (c).1RKH-DHA; (d).1RKH-EPA; (e).3M7R; (f).3M7R-EPA;(g).3M7R-DHA; (h).3M7R-AA; (I).3VT3; (j).3VT3-EPA; (k). 3VT3- DHA; (l). 3VT3-AA; (m). 3VT7; (n). 3VT3-EPA; (o).3VT7- DHA; (p). 3VT7-AA

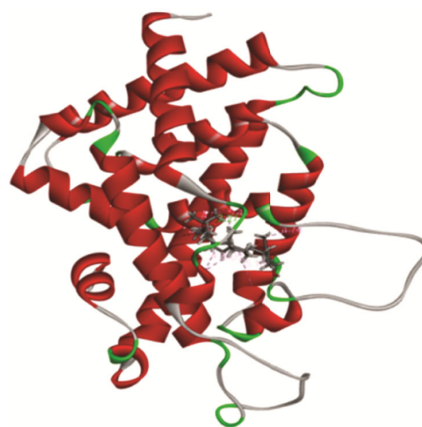


Fig. 11 — Expression of VD & PUFAs EPA, DHA, AA, in the percentage inhibition Lipid accumulation Oil Red O Staining

maximum docking score against the associated enzymes is also shown. The docking score recommended in such a case that the PUFAs have the maximum affinity to the VDR equivalent in the other compounds. The conclusion of docking square described in (Table 2) of docking information is shown. Interaction between best pose initiate to chosen compounds through VDRs in PUFAs was considered as a compound for future plan efforts and gave an establishment to investigate T2DM. We further wanted to analyze the hydrogen bonds, hydrophobic and hydrophilic interactions formed by

T2DM, and these compounds (PDB ID: 1RKH) (Fig. a) protein-ligand domain complex made resolution 2. PDB ID: 1RKH (2.8\AA), Mutation (0), Two Hydrogen bonds is 301A, (PDB ID: 1RKH- AA) (Fig. b). Protein-ligand domain complex made two hydrogen bonds distance (2.1\AA) Hydrophobic (1.89\AA) Hydrophobic bonds (Val 296A) Hydrophilic bonds (PDB ID: 1RKH- DHA), (Fig. c) the favorable interactions revealed that VDR analogs are modulating T2DM protein at the LBD. The hydrophobic interactions were predominant which reveal that the designed molecules were perfectly binding to the hydrophobic Space, Hydrophilic bonds (PDB ID: 1RKH- EPA), (Fig. d). complex made one hydrogen bonds (Arg270A) Hydrophobic, Hydrophilic (PDB ID:3M7R), (Fig. e) Resolution (1.80\AA), Mutation (1), Sequence length - 253, Hydrophobic, Green-Hydrophilic Polar bonds, (PDB.ID: 3M7R- AA), (Fig. f) protein ligand domain complex made Hydrophobic, Hydrophilic, polar bonds PDB ID:3M7R- DHA), (Fig. g) Protein-ligand domain complex made) Hydrophobic bonds, Hydrophilic,(PDB ID: 3M7R- EPA), (Fig. h) protein-ligand domain complex made Hydrophobic and Hydrophilic,(PDB ID: 3VT3- AA), (Fig. i): protein ligand domain complex made Hydrophobic bonds and Hydrophilic Bond, Resolution (1.80\AA) (PDB ID: 3VT3-DHA), (Fig. j) protein ligand domain complex made Mutation (1), Hydrophobic Bonds, Hydrophilic. Blue-Polar (PDB ID: 3VT3-EPA), (Fig. k) protein ligand domain complex made (Fig. l). (PDB ID:3VT7), (Fig. m). Protein ligand domain complex made Resolution (1.65\AA), Mutation (1), Sequence length -271, Hydrophobic, Hydrophilic, Blue-Polar (PDB ID: 3VT7- AA) (Fig. n) protein-ligand domain complex made Hydrophobic, Hydrophilic and Polar molecules PDB ID: 3VT7- DHA), (Fig. o); PDB ID:3VT7- EPA), (Fig. p). Hydrophobic, Hydrophilic

Bonds Marked lines between ligand atoms and protein residues denote interactions with the protein: H-bonds to the protein backbone, dotted pink-H-Bonds to the protein side chains, green-Pi-Pi Stacking Interactions, orange-Pi-Cation Interactions. Gray spheres represent ligand atoms that have been exposed to solvent. A line is drawn around the ligand in the protein "pocket," which is color with the same color as the ligand. The favorable interactions revealed that EPA, DHA & AA analogs modulate VDR at the ligand-binding domain. The hydrophobic interactions were predominant which reveal that the designed molecules were perfectly binding to the hydrophobic space (EPA, DHA) of the Ligand-binding domain of T2DM protein. Interestingly we have observed that the designed ligands showed more hydrogen bonding interactions towards the crystal structure of human VDRs, LBD bound to VD solved at (1.8\AA) resolution when compared to the standard PUFAs moreover the AA has the least binding energy when compared to the standard EPA, DHA So we assume that the compound EPA, DHA has a prominent binding affinity towards the VDR protein than the standard PUFAs (Fig. 12).

Effects of 25(OH) D3 and PUFAs on 3T3-L1 cell viability

In our experiments, use $200\text{ }\mu\text{M}$ of PUFAs, Previous Study has shown that $200\text{ }\mu\text{M}$ is a healthy concentration for PUFAs treatment in 3T3-L1 cells²¹⁻²³. To determine the $200\text{ }\mu\text{M}$ of VD and PUFAs were safe for 3T3-L1 adipocyte Cells, cell viability examine were performed several of cells (blue), dead cells (dark) show on control cells treat by $200\text{ }\mu\text{M}$ of 25(OH)D3 and PUFAs no matter for the 24 h or 72 h. In contrast, $400\text{ }\mu\text{M}$ of 25(OH)D3 and PUFAs significantly reduce the cell viability of 8% (24 h) and 5% (72 h) of the control groups. These results signify

Table 2 — Pi-Pi stacking interaction parameters for selected PUFAs (EPA, DHA & AA) Compounds and VDR residues (PDB id: 1RKH, 3M7R, 3VT3, 3VT7)

S. No.	VDR Compound Names	Compound Id	Glide Scores	Docking Scores
1.	PDB ID: 1RKH	DHA	-10.681	-10.681
		AA	-9.415	-9.415
		EPA	-10.598	-10.598
2.	PDB ID: 3M7R	DHA	-12.09	-12.09
		AA	-11.59	-11.59
		EPA	-11.449	-11.449
3.	PDB ID: 3VT3	DHA	-10.66	-10.66
		AA	-10.574	-10.574
		EPA	-10.361	-10.361
4.	PDB ID: 3VT7	DHA	-2.291	-10.66
		AA	-7.69	-7.69
		EPA	-7.632	-7.632

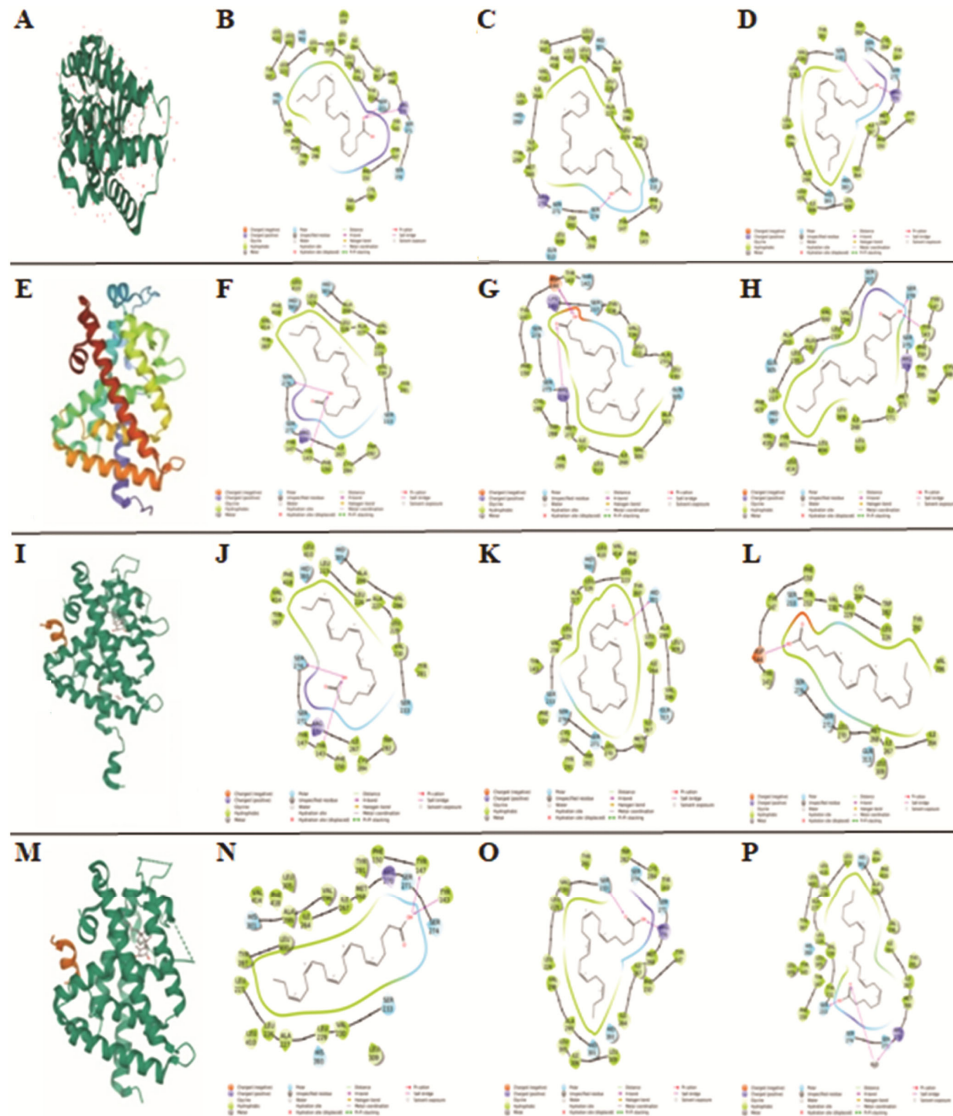


Fig. 12 — Expression of Lipid content in 3T3-L1 cells results are represented as mean ± SD (n ¼ 3). a-c, Different letters above the bars for a given extract Vitamin-D and PUFAs EPA, DHA & AA (µg/mL) are significantly different (*P <0.05) from each, other

to 3T3-L1 cells are not viable. Effected in 200 µM of 25(OH)D3 and PUFAs and, on adipocytes Non-specific cell toxicity did not affect differentiation and lipid aggregation in this sample (Fig. 13).

3T3-L1 Adipocytes, VIT-D, PUFAs enhanced lipid accumulation

Oil Red O staining was used to visualize the effect of VD & PUFAs on intracellular lipid storage in mature 3T3-L1 adipocytes as shown in (Fig. 14A-E). In 3T3-L1 adipocytes, VD, PUFAs reduced the amount of lipid. The TG quantification assay verified the observed reduction in lipid accumulation. As shown in (Table 3), (Fig. 14F-I), 50 M treatments of VD, PUFAs significantly reduced TG content in

3T3-L1 adipocytes by (15%, 28%, 32%, and 35%), respectively, as compared to differentiated control cells. When compared to control cells, 200 M treatments of VD and PUFAs (EPA, DHA, and AA) significantly reduced TG content by (46%, 54%, 57%, and 61%), respectively. Here no significant effect on lipid accumulation in cells treated through VD and PUFAs against control cells (Fig. 11).

3T3-L1 Adipocyte, Vitamin D improves PUFAs levels

In 3T3-L1 cells differentiated in the presence or absence of *Omega-3* & 6 PUFAs, the metabolic fate of VD was investigated. VD is efficiently converted to EPA and, to a lesser extent, DHA, AA according to previous studies VD²⁴. As shown in (Fig. 15A-D) as

compared to vehicle-control, 100 M treatment of Vitamin D, PUFAs significantly increased cellular content of EPA by 103%, 345%, and 987%, respectively. VD treated cells would have an EPA content it was around a third of untreated cells as effective as (EPA). VD treatment resulted in a 3.5-fold increase in EPA enrichment in 3T3-L1

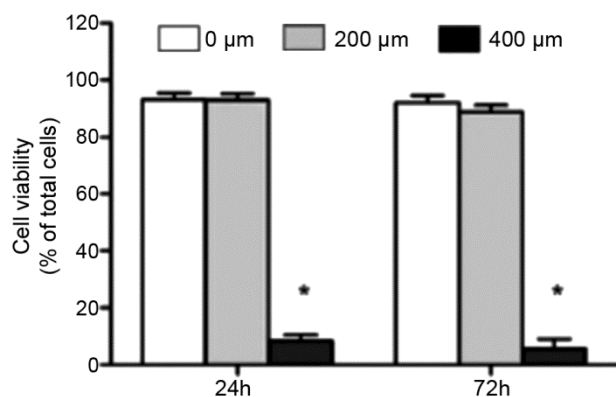


Fig. 13 — Expression of 3T3L1 200 mM and 400 mM of 25(OH)D₃, PUFAs (EPA, DHA & AA), on the matter for the 24 h and 72 h

adipocytes and compared to DHA, AA. For DHA and AA enhancement, 100 μM treatments of VD and PUFAs. As compared to the vehicle control group, DHA content in adipocytes increased by 10, 28, and 1967%, respectively, (Fig. 15E-H). VD levels have risen. DHA from adipocytes was around 0.5% as successful as DHA. The DHA level was unaffected by EPA procedure. The enrichment addition of AA did not affect DHA. To determine the dosage effect of VD on EPA, DHA, and AA enrichment, 3T3-L1 cells were differentiated in the presence of 0–200 M Vitamin D. As shown in (Fig. 15I-L), in 3T3-L1 cells, VD increased EPA content in a dose-dependent manner. VD treatment had a minimal but significant effect on DHA content, with a small but significant increase at 100 mM VD (Fig. 15M-P). The regular observation indicates low VD to DHA conversion²⁵. Together, these results reported that VD was a better surrogate for EPA than AA and that VD cellular effects are often mediated by conversion to EPA (Fig. 15).

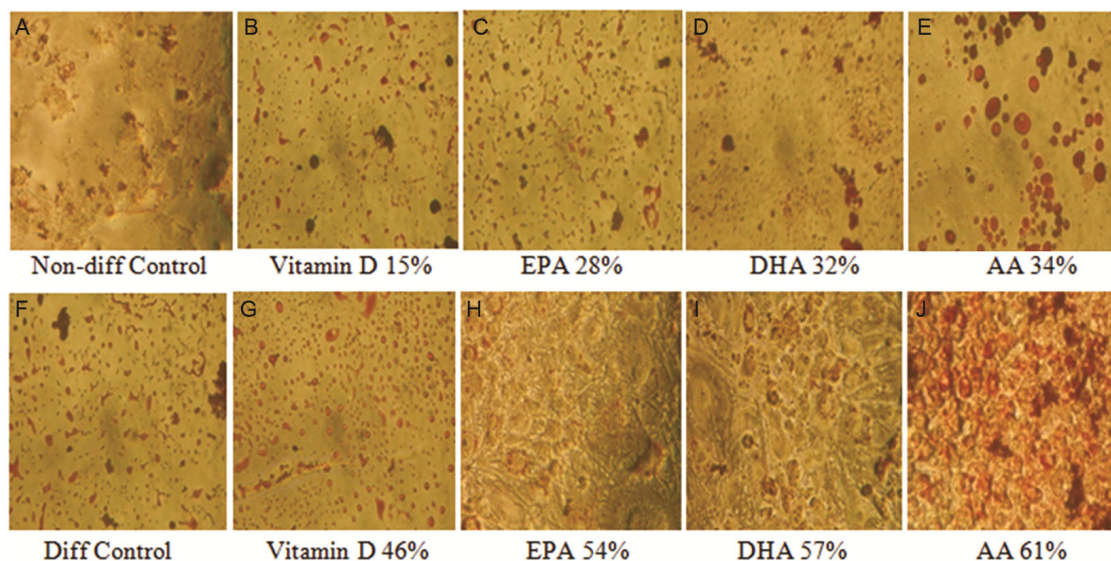


Fig. 14 — Expression of 3T3-L1 Adipocytes, Vit-D, PUFAs enhanced lipid accumulation

Table 3 — Evaluations of 3T3-L1 adipocytes in Vitamin D and PUFAs in the TG Quantification assay

Test Samples	Concentration	% Inhibition Lipid accumulation (ORO)	TG Accumulation
EPA	1.0 mg/mL	200 μM	46%
	0.5 mg/mL	50 μM	15%
DHA	1.0 mg/mL	200 μM	54%
	0.5 mg/mL	50 μM	28%
AA	1.0 mg/mL	200 μM	57%
	0.5 mg/mL	50 μM	32%
Vit-D3	1.0 mg/mL	200 μM	61%
	0.5 mg/mL	50 μM	35%

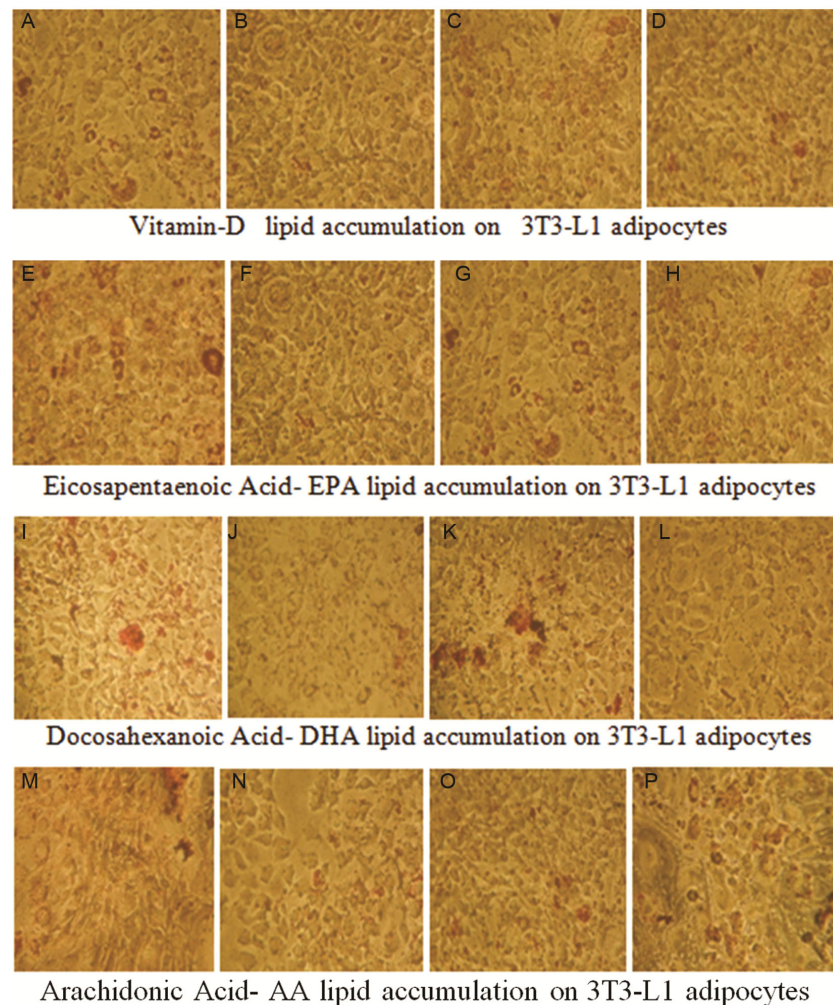


Fig. 15 — Expression of VD & PUFAs on TG accumulation in 3T3-L1 adipocytes. 2-day post-Confluency preadipocytes were incubated with differentiation medium in the presence of 5 days of a. Vit-D b. EPA c. DHA d. AA (0, 25, 50, and 200 μ M). A. Oil Red O Staining and Morphological Observation 3T3-L1 cells stained with ORO and photographed under a microscope (X200). The red dye was used to stain lipid droplets; (B) Measuring the amount of TG in the cells. Three separate tests yielded the results. Different from non-diff control cells: * $P < 0.05$; different from diff control cells

Adipocyte differentiation

Adipogenesis is the main cellular process mechanism that leads to T2DM. In addition, VD and PUFAs as lipid droplets 3T3-L1 cells indicate the degree to which adipocytes were differentiated. Our results showed that, Cholecalciferol (100 mg/mL) & *Omega-3* & 6PUFAs (100 mg/mL) can inhibit adipocyte differentiation through 67.59% when comparing with the control group (Fig. 16).

Discussion

Considerable view of the adverse effect of existing diabetes natural product-based drugs on VDR and PUFAs based on *in silico* molecular dynamics of studies on Adipogenesis has been identified as the main role to contain T2DM. Earlier studies on the

Omega-3 & 6 PUFAs and VD have shown their diabetes, Adipocytes, the fundamental process leading to diabetes is the major target of researchers to overcome T2DM. Agents, PUFAs that can attenuate hypertrophy or/and hyperplasia, and the modulators of PPAR γ (the master regulator of adipogenesis) and its upstream or/ and downstream genes could be targeted to develop novel anti-diabetic drugs/formulations. In cell culture studies with adipocytes, estimating lipid content and measurement of lipolysis reveals the extent of adipogenesis (Kim YS *et al.*, 2010)²⁶. Furthermore, in the presence of VD, enhanced lipolysis of 3T3-L1 cells (increased glycerol levels in the media) (Fig. 17), *Omega-3* & 6 PUFAs Its anti-adipogenic activity is supported by research, and their

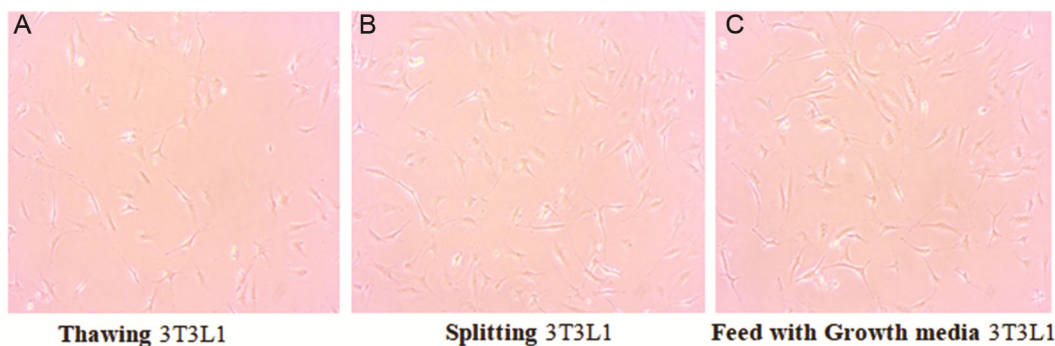


Fig. 16 — Expression of Vitamin D and on adipocyte differentiation on 3T3-L1 Cell lines

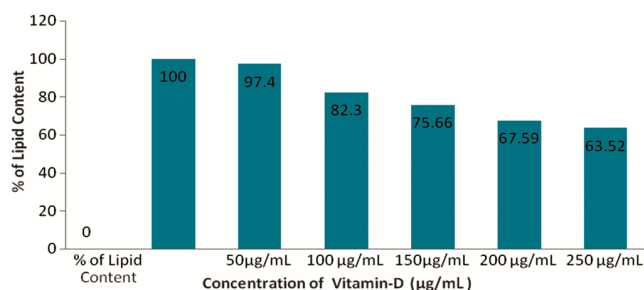


Fig. 17 — Lipid content in 3T3-L1 cells. Results are represented as mean \pm SD (n $\frac{1}{4}$ 3). a-c, Different letters above the bars for a given extract are significantly different ($*P < 0.05$) from each, other

in silico activity and molecular Tissue/cell culture were well to report in earlier *in vitro* studies.

Conclusion

Our *in silico* molecular docking studies and 3T3-L1 Cell lines revealed that selected *Omega*-3 and 6 PUFA's binding energies and interactions in VDR at LBD are being modulated. They were effective in mutated VDR. Based on this, we propose fatty acids and VDR as potential modulators of T2DM. These results deserve additional study to determine increased VD and PUFAs because the effective natural remedy for the treatment or prevention of Type-2 diabetic Mellitus. However, these results can be validated by further *in vitro* study analysis.

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Conflict of interest

All authors declare no conflict of interest.

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