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# COMPUTATIONAL PRIORITIZATION OF A WITHANOLIDE-VEGFA INTERACTION IN DUCHENNE MUSCULAR DYSTROPHY USING NETWORK PHARMACOLOGY, MOLECULAR DOCKING, AND MOLECULAR DYNAMICS SIMULATIONS

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**ABSTRACT**
Duchenne Muscular Dystrophy (DMD) is a devastating X-linked recessive genetic neuromuscular disorder causes the progressive myopathy with few available therapeutic options. This study used an integrated network pharmacology, molecular docking, 200-ns molecular dynamics (MD) simulations, MM-GBSA and ADMET workflow computationally prioritize phytocompounds binding vascular endothelial growth factor A (VEGFA).An integrated Protein-Protein Interaction Network (PPIN) construct around VEGFA and predicted targets of screened compounds showed that VEGFA among the most central nodes by betweenness centrality, and computational knockout indicated it contributes disproportionality to network connectivity. Docking against VEGFA (PDB: 3QTK) showed withanolide, a steroidal lactone from Withania somnifera, achieved the most favorable binding score among all compounds (-9.3 kcal/mol), more favorable than the FDA-approved drug givinostat (-8.7 kcal/mol) under the same protocol. Through 200-ns molecular dynamic simulations, the VEGFA-withanolide complex remained structurally stable and MM-GBSA analysis represented a favorable binding free energy of -30.82 ± 3.04 kcal/mol. Withanolide also showed a favorable predicted ADMET profile relatives to givinostat. Ten common hub genes (MDM2, CASP3, ABL1, MTOR, AKT1, GSK3B, AR, SRC, PTGS2, CDK2) were identified within the network. These findings computationally prioritize withanolide as a candidate VEGFA-binding phytocompound and provides a strong rationale for further experimental validation and therapeutic target.

**KEYWORDS:** Duchenne Muscular Dystrophy (DMD), VEGFA, withanolide, molecular docking, molecular dynamics, network pharmacology.

## 1) INTROSUCTION

Duchenne Muscular Dystrophy (DMD) is a rare severe X-linked recessive neuromuscular disorder caused by molecular alterations in the DMD gene, resulting in the loss of dystrophin potein production. The disease affects approximately 1 in 3,500-5,000 live male births worldwide and is usually diagnosed in early childhood. Its clinical progression is characterized by continuous muscle degeneration, respiratory complications, cardiomyopathy, cognitive impairment, and premature death characterizing as a highly fatal and debilitating disorder [1], [2]. Although substantial progress has been made in genetic and molecular therapeutic approaches such as corticosteroid treatment, exon-skipping, emerging gene therapies and adeno-associated virus (AAV)-mediated gene therapies. These treatments can slow disease progression and improves the functional well being, they are constrained by high costs, delivery challenges, restricted long term efficacy and safety concerns [3]. Therefore, there is an urgent need for more effective, accessible, and safer therapeutic options for DMD.

Drug repurposing provides a cost-effective alternative by identifying novel therapeutic applications for current existing drugs with pre-established safety and pharmacokinetic profiles [4] and this approach is useful particularly valuable for rare disorders such as DMD despite of several challenges [5]. In parallel, phytocompounds obtained from medicinal plants have gained more attention for their anti-oxidant, anti-inflammatory activity with low toxicity level and angiogenesis- modulating properties and their regulatory effects [6]. These features have motivated their evaluation using computational approaches for multi-target drug discovery and medicinal-plant-derived compounds in particular remain an underexplored resource for DMD.

Computational approaches such as network pharmacology, molecular docking, molecular dynamic (MD) simulations and pathway-based network analysis, have become widely used tools for identifying and prioritizing therapeutic targets and candidate compounds [4]. Molecular docking estimates the binding interaction at the atomic level, while MD simulations provide detailed evaluation of important understanding into stability, functional roles and potential involvement of protein-ligand complex [7] [8]. Both approaches generate predictive, hypothesis-level evidence: docking scores and MD stability metrics indicate binding compatibility, not a confirmed pharmacological mechanism.

Angiogenesis is significanly impaired in dystrophic muscle, contributing to ischemia, poor nutrient delivery, and accelerated muscle degeneration. Vascular Endothelial Growth Factor A (VEGFA) serve as a potential regulator of angiogenesis that increase blood vessel permeability, migration, endothelium proliferaration and survival. Several preclinical studies have shown that increasing VEGFA activity, whether genetically modified myoblasts or AAV mediated gene transfer can effectively activate angiogenic signaling and improve muscle perfusion under both ischemic and non-ischemic conditions [9]. Inhibition of VEGFA receptor Flt 1 has similarly been shown to elevate free VEGFA levels, promote vascularization, reduced fibrosis, and improve muscle function in mdx mice [10] [11]. Overall as central role in vascular remodeling and its identification as a high-centrality hub node in our previous DMD-Combinatorial network analysis [12], VEGFA was selected as a candidate node of potential therapeutic interest for the present study, a protein whose modulation is biologically plausible for DMD, warranting evaluation of compounds that interact with it.

In the present study, we used an integrated computatuional framework by combining network pharmacology, molecular docking, ADMET profiling, MD simulations, and in silico network robustness analysis to identify small molecules that bind VEGFA and to place VEGFA within a broader DMD-relevant interaction network. We screened FDA-approved DMD drugs, including Givinostat (Duvyzat), alongside ten selected phytocompounds, with a particular focus on withanolide from Withania somnifera. ADMET profiling was performed on the compounds with the most favorable docking scores to to characterize their predicted their pharmacokinetic behavior and safety profiles. To place these compounds in a broader molecular context, we predicted their putative protein targets and integrated these with the experimentally curated VEGFA interactome into a combined protein-protein interaction network (PPIN). Topological analysis of this network was used to identify common hub genes, which were further characterized through module detection and functional enrichment analysis. Furthermore, to evaluate the importance of identified common hub genes and VEGFA in this network, we additionally performed computational knockout analysis, consistent with the centrality-lethality principle, highly connected nodes are often essential for network integrity, and their removal can substantially perturb network topology [13]. Finally, molecular dynamic simulation and MM-GBSA binding free enrgy calculations were used to evaluate the structural stability and dynamic conformations of VEGFA-withanolide complex over 200-ns trajectory. This study addresses the limited exploration of small-molecule pharmacological modulators of VEGFA signaling and provides a systems-level rationale for developing multi-target therapeutic strategies in DMD.

## 2) MATERIALS AND METHOD

### 2.1) Selection and preparation of Ligands

U.S. FDA-approved drugs for DMD treatment (casimersen, deflazacort, golodirsen, givinostat, and vamorolone) were retrieved from the FDA database (https://www.fda.gov/drugs) and their chemical structures were obtained from PubChem [14] in SDF (Structured Data File) format. Phytocompounds with muscle-protective weakness, preventing muscle wasting, muscle-relax and anti-inflammatory properties were selected based on literature survey and the Indian Medicinal Plants, Phytochemistry, and Therapeutics (IMPATT) database (version 1.0) [15]. Ligand structures were converted from 2D to three-dimensional structures and energy minimized using Open Babel [16] integrated in PyRx software (version 0.8) [17] to obtain stable conformations for molecular docking analysis. An overview of the complete methodology is presented in Figure 1.

**Figure 1.** Schematic workflow of the integrated computational approach used in this study. The pipeline includes selection of FDA-approved drugs and phytocompounds, molecular docking with VEGFA, ADMET profiling, target prediction, construction of integrated protein-protein interaction network (PPIN), hub gene identification, in silico knockout analysis, MCODE-based module detection, GO and pathway enrichment analyses, and 200-ns molecular dynamics simulation with MM-GBSA binding free energy calculation.

### 2.2) Preparation of the Target Protein

The crystallographic structure of human VEGFA (PDB ID: 3QTK) was downloaded from the Protein Data Bank. Water molecules and non-essential heteroatoms were excluded, and polar hydrogens and Gasteiger charges were added by using PyRx. Because VEGFA presents multiple potential binding pockets,a large grid box centered at coordinates (40.946, 12.445, 0.223) with dimensions (124 × 94 × 126 Å; x, y, z) and default spacing of 1 Å.

### 2.3) Molecular Docking

Molecular docking studies were carried out to analyze the binding potential of screened drugs and phytocompounds against VEGFA using AutoDock Vina (version 1.2.5) integrated into PyRx (version 0.8). The exhaustiveness parameter was set at its standard value of 8 to ensure thorough exploration of ligand binding conformations. The best binding pose for each ligand was selected based on the lowest binding energy (kcal/mol). Protein-ligand interactions were subsequently visualized and analyzed using BIOVIA Discovery Studio Visualizer [18].

### 2.4) ADMET Analysis

Potential compounds with the favorable docking scores (givinostat, withanolide and taraxestrol) were subjected to in silico ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) analysis. SwissADME

webserver [19] was used to calculate physicochemical properties (e.g., LogP, polar surface area, molecular weight) and initial pharmacokinetic parameters. Comprehensive ADMET predictions, including intestinal absorption, toxicity endpoints (e.g., hERG inhibition, hepatotoxicity), distribution (e.g., BBB penetration), metabolism (e.g., CYP450 inhibition) and excretion were performed using pkCSM [20]. All values reported are computational predictions and should not be interpreted as measured pharmacokinetic or toxicological data.

**2.5) Target Prediction**

Potential protein targets of givinostat, withanolide, and taraxasterol were predicted using SwissTargetPrediction (2019 version) [21]. Chemical structures were submitted in Simplified Molecular Input Line Entry System (SMILES) strings format with predictions restricted to Homo sapiens under default parameters. All predicted targets were considered, irrespective of probability score.

**2.6) Construction of Integrated PPIN and identification of common hub genes**

The PPINs of VEGFA and its experimentally validated interactiors was constructed using the IntAct App [22] integrated in Cytoscape software [23]. In addition, PPINs for the predicted targets of the selected compounds were created using the STRING database (Version 12.0) [24], with a confidence score cutoff 0.400. The networks were merged to create a single integrated PPIN using default Cytoscape merge settings (union of nodes and edges, with duplicate edges collapsed); no additional filtering for self-loops or isolated nodes beyond Cytoscape defaults was applied.

Topological analysis of integrated PPIN was performed using the Network Analyzer plugin and cytoHubba application [25] in Cytoscape using four centrality measures such as degree, betweenness centrality, closeness centrality, and maximal clique centrality (MCC), to identify key regulatory nodes (hub gene).

The topological properties help characterize the structural organisation of a network and give information about its global behaviour and functional features [26]. Hub genes are nodes having the highest number of connections, as determined by degree centrality [27].

**Degree centrality** represents the total no. of connections connected to a specific node; in directed networks it is calculated as the sum of indegree (incoming links) and outdegree (outgoing links), reflecting the overall connectivity and immediate importance of a node within the network [28].

**Betweenness centrality** defines how commonly a node appears along the minimal paths between other node pairs, reflecting its potential influence on information flow and its role in controlling communication within the network. Its role as a potential bottleneck or bridge act in information flow in network. For a node $v_i$, it is derived as the sum of the fraction of shortest paths between all nodes that pass through a node $v_i$:

$$C_B(v_i) = \sum_{s \neq v_i \neq t} \frac{\sigma_{st}(v_i)}{\sigma_{st}}$$

where $\sigma_{st}$ is the no. of shortest paths passing through node $s$ node $t$, and $\sigma_{st}(v_i)$ is the no. of shortest paths traversing through $v_i$ [29].

**Closeness centrality** quantifies a node's importance by considering its shortest-path distances to all other nodes and is : measured as the reciprocal of the total of these distances:

$$C_{clo}(u) = \frac{1}{\sum_{v \in V} dist(u, v)}$$

It is applicable only to strongly connected networks and is widely used to identify central nodes in complex networks [30].

**MCC** identifies essential proteins based on the tendency of critical nodes to cluster in network For a node v, MCC is represented as:

$$MCC(v) = \sum_{C \in S(v)} (|\ C\ | - 1)!,$$

where $S(v)$is the set of maximal cliques containing v. If v's neighbors are not connected, MCC equals its degree [25]. The top 20 genes ranked by each of the four parameters were compared, and genes present in all four top-20 lists were parameter were considered key hub genes and subjected to further study.

**2.7) In-Silico Network Robustness and Single-Node Knockout Analysis**

To further evaluate the structural importance of the identified hub genes and VEGFA within the integrated PPIN, a network robustness analysis was performed using computational knockout analysis. Each hub gene and VEGFA was individually removed from the network in Cytoscape, and the Network analyzer plugin was used to recalculate total degree (twice the number of edges in the resulting network) after each removal. The percentage decrease in total degree relative to the intact network was calculated as:

$$\%Decrease = \frac{\text{Original total degree} - \text{Post knockout total degree}}{\text{Original total degree}} \times 100$$

This metric quantifies the proportional loss of connections contributed by (or routed through the immediate neighborhood of) the removed node; it reflects the topological centrality of that node within the network as constructed, rather than a direct, general-purpose measure of overall network functional stability.

**2.8) Identification of Functional Modules**

Functional modules were obtained to detect highly interconnected groups of proteins within the PPIN were identified using Molecular Complex Detection (MCODE) [31] algorithm in Cytoscape with standard parameters like degree standard cutoff of 2, node score threshold 0.2, K-core of 2, and a max. search depth of 100. The MCODE analysis identified the various modules with their corresponding scores and density of intera-modular interactions.

**2.9) Gene Ontology and pathway enrichment analysis of common Hub Genes**

To understand the biological relevance of the commonly identified common hub genes, Gene ontology (GO) enrichment and pathway analysis were performed using ShinyGO (version0.85.1) [32], with a false discovery rate (FDR) cutoff of 0.05, restricted to Homo sapiens. GO enrichment analysis was performed to classify these hub genes into biological processes (BP), molecular functions (MF), and cellular components (CC) ontologies. Significantly, pathway enrichment analysis was also performed to understand the associated signaling pathways.

**2.10) Molecular Dynamics Simulation and binding free energy calculations**

MD simulation was conducted to find out the structural stability and conformational dynamics of VEGFA-ligand complexes were identified from docking and ADMET analyses of Withanolide D using GROMACS (version 2023.2) [33]. The VEGFA structure and the withanolide pose with the most favorable docking score were used as initial configurations. The protein-ligand complexes were prepared using the AMBERff14SB force field, while General AMBER Force Field (GAFF) to ensure that molecular interactions are accurately represented throughout the simulation. The complex was placed in a cubic simulation box with atleast distance of 1.0 nm between the box and solute edges, while it was then solvated with TIP3P water molecules [34]. The system was neutralized and adjusted to a salt concentration of 0.15 M by adding $Na^+$ and $Cl^-$ ions. Energy minimization was performed using the steepest descent method to clear the unfavourable steric interactions until the max. force fell below 1000 $kJ \cdot mol^{-1} \cdot nm^{-1}$. The system was equilibrated in two phases, an initial NVT canonical ensemble (constant no. of particles, volume, and temperature) followed by an NPT conditions with fixed no. of particles, pressure, and temperature followed by isothermic-isobaric ensembles for 200 ps at 300 K and 1 bar by applying the Berendsen thermostat and barostat. Production simulations for 200 ns was run in the NPT ensemble using a 2-fs integration time step, velocity-rescale thermostat, and Parrinello-Rahman barostat.

Particle Mesh Ewald (PME) strategy wad used to find the long-range electrostatics forces and a standard distance of 10 Å used to short-range non-bonded interactions. The LINCS algorithm was used to limit all covalent bonds that contained hydrogen atoms. The GROMACS analysis tools were used to process the trajectories data to calculate the root-mean-square deviation (RMSD) of the protein backbone and ligand, root-mean-square fluctuation (RMSF) of protein Cα atoms and radius of gyration (Rg). All these quantities represent the overall structural deviation (RMSD), per-residue flexibility (RMSF), and protein compactness, respectively. Solvent-accessible surface area (SASA) was studied using a 1.4 Å probe, and hydrogen bonds were identified using a donor-acceptor distance ≤ 3.5 Å and angle ≥ 120°. These metrics evaluated the conformational stability, protein compactness, residue flexibility (particularly in functional loops), and the durability of ligand binding during the simulation period.

**Binding free energies** (ΔG binding) were computed for all systems using the Molecular Mechanics Generalized Born Surface Area (MM-GBSA) approach as used in gmx MMPBSA v1.6.4 package [35], [36]. The binding free energy was calculated as:

$$\Delta G_{binding} = G_{complex} - (G_{protein} + G_{ligand})$$

The total free energy (G) was decomposed as:

$$G = E_{MM} + G_{solv}$$

where the molecular mechanics energy term (E MM) consists of van der Waals (E vdW) and electrostatic (E elec) interactions:

$E_{MM} = E_{vdW} + E_{elec}$

The solvation free energy (G solv) was separated into polar and nonpolar contributions:

$G_{solv} = G_{polar} + G_{nonpolar}$

Polar solvation energy was computed using the Generalized Born (GB) implicit solvent model, whereas the nonpolar term was derived from solvent-accessible surface area (SASA) using the LCPO method.

## 3) RESULTS

### 3.1) Selection and Preparation of the Target Protein

Human VEGFA (PDB ID: 3QTK) X-ray crystal structure was selected as main receptor for molecular docking studies. This structure has a high-resolution value of 1.85 Å, with clear atomic details, which make it highly suitable to study molecular interactions. Due to its high resolution and well-defined structure, the 3QTK structure was considered most suitable to study the protein-ligand interactions.

### 3.2) Selection and Screening of ligands

To evaluate interactions with VEGFA, clinically relevant and FDA-approved DMD drugs were selected, including vamorolone, casimersen, givinostat, deflazacort and golodirsen. These drugs molecular structures were downloaded from the PubChem repository, ensuring standardized, reliable chemical information for docking preparation. All ligands were subjected to energy minimization, that allow optimal geometry and improved accuracy during docking calculations (Table 1). Givinostat and Deflazacort showed the highest binding energy with VEGFA.

**Table 1: Interaction score between protein and ligands**

| **Ligands** | **PubChem id** | **Binding Energy** |
|---|---|---|
| Vamorolone | 3035000 | -7.8 |
| Casimersen | 131842141 | -7.6 |
| **Givinostat** | **9804992** | **-8.7** |
| **Deflazacort** | **189821** | **-8.7** |
| Golodirsen | 135564824 | -7.4 |

In addition to clinically approved drugs, ten potential therapeutic phytocompounds were selected such as Withanolide (from Withania somnifera**),** Taraxasterol, Gitogenin, Tomatidine, Daucosterol, Cycleanine, Agrimol B, a Curcuma longa derivative (β-Bisabolene-related), Macamide B and Sulforaphane**.** The 3D structures of the selected phytocompounds were downloaded in SDF format and subsequently prepared for molecular docking studies using standard energy minimization and optimization protocols. Their corresponding chemical sources and primary bioactivities are summarized in Table 2.

**Table 2: Interaction score between protein and phytocompounds ligands.**

| **Sr. No.** | **Phytocompounds** | **PubChem id** | **Binding Energy** | **References** |
|---|---|---|---|---|
| 1 | Withanolide | 161671 | -9.3 | [37], [38], [39], [40], [41], [42], [43] |
| 2 | Taraxestrol | 115250 | -9.2 | [44], [45], [46] |
| 3 | Gitogenin | 441887 | -9.2 | [47], [48], [49] |
| 4 | Tomatidine | 65576 | -9.1 | [50], [51], [52] |
| 5 | Daucosterol | 5742590 | -8.8 | [53], [54] |
| 6 | Cycleanine | 121313 | -8.8 | [55] |
| 7 | Agrimol B | 194000 | -7.8 | [56] |
| 8 | Curcuma longa | 10104370 | -6.4 | [57], [58], [59] |
| 9 | Macamide B | 11198769 | -5.4 | [60], [61], [62] |
| 10 | Sulphoraphane | 5350 | -3.5 | [63], [64], [65], [66] |

The interaction of protein and ligand study showed different binding affinities of the two compounds in the active site (Figure 2). The number of hydrogen bond interactions (D:27) between Withanalide and ASP was one, which is a stable polar interaction. The hydrophobic interaction was observed with VAL (A:26), and several residues such as GLY (D:52), LEU (D:59), GLU (D:57), PHE (A:29), PHE (A:40), TYR (A:30), and LYS (D:100) were observed to interact through van der Waals forces implying an overall stabilization of the ligand in the binding pocket. Likewise, Givinostat formed a single hydrogen bond with ARG (A:16), and hydrophobic interaction with VAL (D:45) and LEU (D:47). Some of the residues included THR (D:24), LEU (D:25), GLU (D:23), MET (D:71), SER (A:17) and VAL (A:13) were involved in van der Waals interactions which lead to the stability of the protein-ligand complex (Table 3).

**Figure 2: 3D and 2D interaction analysis of the protein-ligand complex.** (A) 3D representation of the protein-ligand complex shown in ribbon structure. (B) 2D interaction diagram of the complex illustrating key binding interactions.

**Table 3: Detailed residue-level interactions of withanolide and givinostat with VEGFA on the basis of top-scoring docking pose for each compound.**

| S. No | Compound | No. of H-bonds | Hydrogen Bonding Residues | Hydrophobic Interactions | Van der Waals Interactions |
|---|---|---|---|---|---|
| 1 | Withanolide | 1 | ASP D:27 | VAL A:26 | GLY D:52, LEU D:59, GLU D:57, PHE A:29, PHE A:40, TYR A:30, LYS D:100 |
| 2 | Givinostat | 1 | ARG A:16 | VAL D:45, LEU D:47 | THR D:24, LEU D:25, GLU D:23, MET D:71, SER A:17, VAL A:13 |

### 3.3) ADMET analysis of Givinostat, Withanolide, and Taraxasterol

ADMET analysis was conducted on givinostat, withanolide and taraxestrol to evaluate their drug-likeness, pharmacokinetic behavior and safety profiles in the context of DMD therapy (Table 4). Withanolide, showed the most favorable safety profile with no predicted AMES toxicity or hepatotoxicity and complying with Lipinski rule of five. Its stability and binding compatibility are enhanced by these physicochemical features, including six hydrogen-bond acceptors. Whereas, givinostat demonstarted favorable overall pharmacokinetic properties, following the Lipinski's rule, without predicted AMES toxicity, and acceptable predicted blood brain barrier (BBB) penetration but the hepatotoxicity prediction was positive. Despite this, its strong binding affinity to VEGFA confirms its relevance as a potent therapeutic molecule for DMD.

Taraxestrol also significantly demonstrated major ADMET parameters, such as non-toxicity predictions (no AMES toxicity or hepatotoxicity) and favorable metabolic stability. However, it exhibited slightly lower predicted intestinal permeability and lower BBB penetration potential compared with givinostat and withanolide. Although its metabolic stability and safety margins remain favorable, these pharmacokinetic constraints may reduce its suitability as a standalone systemic agent. Nevertheless, Taraxestrol may still be explored through formulation approaches or as part of combinatorial multi-target DMD therapeutic strategies.

pkCSM analysis predicted good oral absorption for all three molecules, with intestinal uptake ranging from 83-97% (Table 5). Givinostat and withanolide were identified as P-glycoprotein substrates, whereas Taraxestrol was not. BBB penetration remained low for givinostat and withanolide but was higher for Taraxestrol. All compounds were predicted CYP3A4 substrates, with Givinostat additionally inhibiting CYP1A2, CYP2C19, and CYP3A4. Hepatotoxicity and hERG II risk were predicted only for Givinostat, while Withanolide exhibited the most favorable overall safety margin.

SwissADME radar plots (Figure 3A) and the boiled egg analysis (Figure 3B) further supported the superior pharmacokinetic profile of withanolide. Givinostat and withanolide localized within the egg-white region, indicating high gastrointestinal absorption, whereas Taraxestrol fell outside this zone, consistent with its poor solubility and high lipophilicity. None of the compounds were predicted to efficiently penetrate the BBB. Collectively, integration of docking, pkCSM, and SwissADME analyses identifies Givinostat as a high-affinity, pharmacokinetically robust drug and withanolide exhibited the most favorable overall predicted safety profile, whereas taraxestrol may require formulation-based optimization prior to MD simulation and network pharmacology validation.

**Table 4: ADMET properties of Identified 3 compounds.**

| S.No. | Parameters | Givinostat | Withanolide | Taraxestrol |
|---|---|---|---|---|
| 1 | AMES toxicity | No | No | No |
| 2 | BBB Penetration | No | No | No |
| 3 | Lipinski Rule | Yes | Yes | Yes |
| 4 | H-bond donors | 3 | 2 | 1 |

| | | | | |
|---|---|---|---|---|
| 5 | H-bond acceptors | 5 | 6 | 1 |
| 6 | Hepatotoxicity | Yes | No | No |

**Table 5: pkCSM-Predicted ADMET Profiles of Givinostat, Withanolide, and Taraxestrol.**

| Sr. No. | Parameter | Givinostat | Withanolide | Taraxestrol |
|---|---|---|---|---|
| 1 | Intestinal absorption (%) | 84.4 | 83.6 | 96.5 |
| 2 | Water solubility (log mol/L) | -4.95 | -4.59 | -8.24 |
| 3 | Caco-2 permeability (log Papp) | 0.64 | 0.32 | 0.11 |
| 4 | P-glycoprotein substrate | Yes | Yes | No |
| 5 | BBB permeability (log BB) | -0.99 | -0.001 | 0.72 |
| 6 | VDss (log L/kg) | 0.24 | 0.06 | -0.30 |
| 7 | Fraction unbound (Fu) | 0.00 | 0.097 | 0.00 |
| 8 | CYP3A4 substrate | Yes | Yes | Yes |
| 9 | CYP inhibition | CYP1A2, CYP2C19, CYP3A4 | None | None |
| 10 | Total clearance (log ml/min/kg) | 0.87 | 0.45 | 0.61 |
| 11 | Renal OCT2 substrate | No | Yes | No |
| 12 | AMES toxicity | No | No | No |
| 13 | hERG II inhibition | Yes | No | No |
| 14 | Hepatotoxicity | Yes | No | No |
| 15 | Oral acute toxicity (LD50, mol/kg) | 2.85 | 2.97 | 3.11 |

**Figure 3.** In silico ADMET and pharmacokinetic profiling of Giovinostat, Withanolide, and Taraxestrol. **(A)** Radar plots comparing normalized ADMET parameters, including gastrointestinal absorption, lipophilicity, aqueous solubility, BBB permeability, CYP inhibition risk, and synthetic accessibility. **(B)** BOILED-Egg model illustrating predicted intestinal absorption (white region) and BBB permeability (yellow region) based on SwissADME. Marker color indicates P-glycoprotein substrate status. Together, these analyses provide a comparative assessment of the pharmacokinetic suitability of the three compounds for DMD therapy.

### 3.4) Target Prediction of Givinostat, Withanolide, and Taraxestrol

Target prediction was performed using the SwissTargetPrediction identified 100 potential protein targets based on 2D and 3D chemical similarity to characterized bioactive compounds and assigns a probability score to each predicted interaction (Supplementary file 1). For Givinostat, the predicted targeted were found in various functional protein categories such as kinases, erasers, proteases, G protein-coupled receptors and transporters, which shows that it has a wide range of polypharmacological profile. All these predicted targtes were used to construct pie charts which showed the distribution of functional classes. Top predicted targets were majorly belong to the histone deacetylase (HDAC) family, such as HDAC1 to HDAC9. This showed that the known pharmacological action of Givinostat as a pan-histone deacetylase inhibitor.

For withanolide, the predicted targets showed strong enrichment in kinases, nuclear receptors, phosphatases, and inflammatory signaling proteins High-confidence predicted targets included protein kinase C isoforms (PRKCA, PRKCD, PRKCE), mitogen-activated protein kinases (MAPK8, MAPK9, MAPK14), cyclin-dependent kinases, JAK family kinases, and nuclear receptors such as androgen and glucocorticoid receptors. This predominance of signaling and regulatory proteins associated with cell proliferation, inflammation, and angiogenesis supported the selection of withanolide for subsequent molecular dynamics simulation and network-based analyses. Whille, for taraxestrol, the predicted targets were distributed across enzymes, nuclear receptors, kinases, G protein-coupled receptors, and transporters, indicating broad polypharmacological. High-probability targets included 11β-hydroxysteroid dehydrogenase 1 (HSD11B1), cyclooxygenase-1 (PTGS1), protein tyrosine phosphatase 1B (PTPN1), and nuclear receptors such as androgen and estrogen receptors. These targets are implicated in inflammation, hormone regulation, and metabolic signaling, suggesting multi-target therapeutic potential.

### 3.5) Construction of integrated Protein-Protein Interaction Network (PPIN) and Identification of common hub genes

A PPIN for VEGFA and its experimentally validated interactors was constructed using IntAct, and a separate PPINs for the predicted protein targets of Givinostat, Withanaloid, Taraxestrol were constructed (FigureA-D). These networks were merged into an single integrated PPIN (Figure E) and analyzed using degree, closeness centrality, betweenness centrality and Maximal Clique Centrality. The top 20 nodes in each parameter were compared and identified common ten hub genes (MDM2, CASP3, ABL1, MTOR, AKT1, GSK3B, AR, SRC, PTGS2, and CDK2) as the most significant nodes and termed as “common hub genes" of integrated network (Figure 5, E). It is important to clarify that VEGFA itself was not among these ten common hub genes when ranked by degree, closeness, and MCC within this integrated network; VEGFA appeared prominently only in the

betweenness centrality ranking (rank 4, Table 6). VEGFA was analyzed throughout this study as the seed protein of primary biological interest, the therapeutic target around which the network was built rather than as one of the ten topologically "common" hub genes identified by the four-parameter overlap. Several of the common hub genes such as AKT1, MTOR, GSK3B, SRC, and PTGS2 are independently reported to participate in angiogenesis, inflammatory regulation, and muscle remodeling, which further supports the biological significance of the VEGFA-centered network. These genes functions as key regulators of signal transduction, cell cycle control, apoptosis, and angiogenic process, and were therefore selected for further functional enrichment, module detection, and pathway analyses.

**Figure 4:** Protein-protein interaction networks (PPINs) of VEGFA and its experimentally validated interactors (IntAct) along with the predicted targets of Givinostat, Taraxestrol, and Withanolide (STRING, confidence ≥0.400) were constructed. Cytoscape was used to visualize the network and identify common 10 hub genes using Degree, Betweenness, Closeness, and Maximal Clique Centrality analyses.

**Table 6: Top 20 genes identified using Degree, Betweenness Centrality, Closeness Centrality, and Maximal Clique Centrality (MCC) analyses. Ten common hub genes were present among all four parameters are highlighted.**

| Sr. No. | Name | Betweenness Centrality | Name | Closeness Centrality | Name | Degree | MCC | Score |
|---|---|---|---|---|---|---|---|---|
| 1 | **GSK3B** | 0.16374 | **GSK3B** | 0.5186 | **GSK3B** | 64 | **GSK3B** | 1460133 |
| 2 | **AKT1** | 0.15248 | MAPK3 | 0.50503 | MAPK3 | 58 | **CASP3** | 1435180 |
| 3 | MAPK3 | 0.13501 | **PTGS2** | 0.47092 | **SRC** | 56 | PARP1 | 1375009 |
| 4 | VEGFA | 0.10865 | **SRC** | 0.46654 | **AKT1** | 50 | **MDM2** | 1329921 |
| 5 | **SRC** | 0.10684 | **ABL1** | 0.46568 | **CASP3** | 49 | JUN | 1235416 |
| 6 | **PTGS2** | 0.09593 | **CASP3** | 0.46055 | **ABL1** | 47 | **AR** | 1213548 |
| 7 | **ABL1** | 0.053 | **AKT1** | 0.45887 | **PTGS2** | 47 | **ABL1** | 1148660 |
| 8 | PPARG | 0.0445 | **CDK2** | 0.44982 | **MDM2** | 39 | **CDK2** | 1050120 |
| 9 | CYP19A1 | 0.04356 | **MDM2** | 0.44504 | JUN | 39 | CDK4 | 795628 |
| 10 | HMGCR | 0.04255 | JUN | 0.44346 | **MTOR** | 38 | AURKA | 780721 |
| 11 | **CASP3** | 0.04111 | MAPK14 | 0.4419 | **CDK2** | 37 | **AKT1** | 530964 |
| 12 | **MDM2** | 0.04055 | **AR** | 0.43958 | MAPK14 | 35 | CDK1 | 501434 |
| 13 | **CDK2** | 0.03663 | CDKN1B | 0.43501 | ERBB2 | 35 | **MTOR** | 460575 |
| 14 | ESR1 | 0.03068 | PPARG | 0.43501 | PRKCD | 35 | **SRC** | 391415 |
| 15 | AGTR1 | 0.03029 | ESR1 | 0.43351 | PPARG | 35 | ERBB2 | 369942 |
| 16 | MAPK14 | 0.02473 | **MTOR** | 0.43351 | CYP19A1 | 31 | **PTGS2** | 329032 |
| 17 | SLC6A4 | 0.02304 | ERBB2 | 0.42906 | **AR** | 30 | AKT3 | 300480 |
| 18 | **AR** | 0.02262 | PARP1 | 0.4276 | HMGCR | 30 | AKT2 | 295320 |
| 19 | PRKCD | 0.02109 | HMGCR | 0.42687 | PARP1 | 29 | FYN | 242318 |
| 20 | **MTOR** | 0.02026 | PRKCD | 0.42399 | ESR1 | 29 | PGR | 221310 |

**3.6) In Silico Network Robustness and Single-Node Knockout Analysis**

An integrated PPIN consist of 255 nodes and 2804 edges, resulting in an average degree of 10.9961, indicating a highly interconnected and stable network. To assess the structural importance of hub genes, a computational knockout analysis was performed by individually removing each gene and recalculating network parameters (Figure 5). Following the removal of each node, the network size decreased to 254 nodes, accompanied by a substantial decline in connectivity. Notably, knockout of VEGFA resulted in the most pronounced disruption, reducing the total degree to 560, corresponding to an approximate 80.03% decrease compared to the complete network. This significant reduction underscores the central role of VEGFA in maintaining network integrity. Similarly, the removal of other hub genes, including ABL1, GSK3B, AKT1, AR, CDK2, MDM2, MTOR, and PTGS2, led to comparable decreases in total degree (~79.10-79.17%), indicating their critical contribution to network connectivity. In addition, CASP3 and SRC also exhibited strong effects, with reductions of approximately 79.03%. Overall, the consistently high reduction in total degree and average connectivity across all knockout conditions highlights the essential structural role of these hub genes in sustaining network stability. Among them, VEGFA demonstrated the greatest impact, suggesting its potential as a key regulatory and central node within the PPIN.

**Figure 5:** Knockout experuiment of VEGFA and the ten significant hub genes illustrated decrease in total degree leads to perturbation in network stability compared to the complete network without knockout.

**3.7) Gene Ontology enrichment analysis of 10 hub genes**

GO enrichment analysis was performed on 10 common hub genes identified as key regulators (Figure 6). BP enrichment analysis showed strong involvement of hub genes in pathways regulating cell migration, apoptosis, and intracellular signaling. The identified hub genes, including PTGS2, AKT1, GSK3B, ABL1, SRC, MTOR, MDM2, CDK2, CASP3, and AR showed enrichment in processes like positive regulation of epithelial cell migration, suggesting important roles in cellular motility and angiogenic regulation. Various processes such as intrinsic apoptotic signaling pathway, apoptotic signaling pathway and regulation of programmed cell death were prominently enriched, indicating coordinated control of cell survival and death mechanisms. Furthermore, the enhancement of the negative regulation of apoptosis and intracellular signal transduction highlights the involvement of important signaling mediators, including AKT1, SRC, MTOR, and GSK3B in fine-tuning cell fate decisions. The significant enrichment in peptidyl-serine phosphorylation and protein phosphorylation-related processes emphasizes the main role of kinase-mediated post-translational regulation. Moreover, the hub genes were strongly associated with cellular responses to organic cyclic compounds, nitrogen-containing compounds, hormones, and oxygen-containing compounds, thereby broadening adaptability to diverse biochemical stimuli. Collectively, these observations suggest that the hub genes act as central coordinators of signaling cascades, apoptotic balance, and cellular responsiveness, functions that are highly relevant to inflammation, angiogenesis, and several disease-associated pathways.

Molecular Function (MF) enrichment analysis determined the strong overrepresentation of kinase and signaling-associated activities. There are various molecular functions that were determined, such as protein kinase activity, phosphotransferase activity and protein serine/threonine kinase activity predominantly influenced by GSK3B, ABL1, CDK2, AKT1, SRC, and MTOR. Several binding-related functions, such as protein kinase C binding, kinase binding, SH2 domain binding, and transcription factor binding were also significantly enriched. Furthermore, functions associated with enzyme binding, ATP & nucleotide binding, and purine ribonucleotide binding were represented, highlighting the catalytic and regulatory functions of the hub genes. Specefic molecular functions such as ephrin receptor binding, phospholipase/lipase activator activity, and receptor serine/threonine kinase binding further underscore the role of these genes in growth factor signaling, apoptosis, and inflammatory regulation.

Cellular Component (CC) analysis showed identified common hub genes were highly present across membrane, nuclear, cytoplasmic, and neuronal compartments. SRC and PTGS2 were involved in Caveola, which function as membrane signaling microdomains. These common hub genes were also significantly linked with glutamatergic synapse, postsynapse, dendrite, and neuron projection, indicating involvement in neuronal signaling and cellular communication between cells. Nuclear components, such as the nuclear envelope and nucleoplasm, show regulatory functions related to transcription and cell-cycle control. Furthermore, their presence in the cytoskeleton, cell junction, mitochondrion, and cellular projection indicate their roles in energy metabolism, structural organization, and cell motility (Supplementary file 2).

Figure 6: Ten common hub genes as predicted by ShinyGO were studied using Gene Ontology. A) Biological Process (BP), B) Cellular Component (CC) and C) Molecular Function (MF) enrichment terms highlight in the lollipop plot.

**Figure 6: Ten common hub genes as predicted by ShinyGO were studied using Gene Ontology. A)** Biological Process (BP), **B)** Cellular Component (CC) and **C)** Molecular Function (MF) enrichment terms highlight in the lollipop plot.

**3.8) Pathway Analysis**

Pathway analysis of ten common hub genes suggested their involvement in cancer-related and signal transduction pathways. Several important pathways in cancer, prostate cancer, PI3K-Akt signaling pathway, EGFR tyrosine kinase inhibitor resistance and ErbB signaling pathway highlighting the central role of the hub genes in oncogenic signaling networks. Additional, cancer-associated pathways, such as colorectal cancer, small cell lung cancer, proteoglycans in cancer, gastric cancer and glioma were also significantly enriched. Signaling pathways enriched in cell growth, survival, and angiogenesis, including the VEGF signaling pathway, prolactin signaling pathway and thyroid hormone signaling pathway were prominently represented (Figure 7A). All of these findings indicate that the common hub genes are highly integrated into pathways regulating cancer progression, cell survival, and therapeutic resistance

The VEGF signaling pathway was further investigated owing to its established role in angiogenesis and tumor progression (Figure 7B). Mapping of the hub genes onto this pathway identified VEGFA as a key upstream regulator interacting with several downstream signaling modules. Activation of VEGFR2 was related with downstream signaling through Src, PI3K-Akt, and MAPK pathways. The involvement of Src suggests a role in cytoskeletal remodeling and cell migration, whereas activation of the PI3K-Akt axis is associated to cell survival

signaling. Additionally, downstream mediators such as COX2 were mapped within the NFAT-mediated branch of the pathway, which is implicated in angiogenic regulation and inflammatory responses. Together, these pathway interactions suggest that VEGFA may contribute to the integration of survival, migration, and angiogenic processes through interconnected signaling cascades.

**Figure 7:** Pathway enrichment analysis of the ten common hub genes. **(A)** Bubble plot showing significantly enriched pathways identified from the common hub genes. Pathways are ranked based on fold enrichment; bubble size depicts the no. of associated genes, while color intensity showed statistical significance. **(B)** Mapping of common hub genes onto the VEGF signaling pathway highlighting VEGFA as a key regulator interacting with downstream signaling components such as Src, PI3K-Akt, MAPK, and NFAT-mediated modules associated with cell survival, migration, and angiogenesis.

### 3.9) Identification of Functional Modules

Functional module in the integrated PPIN were identified using the MCODE analysis. A total of 12 modules were identified using default parameters (Table 7). For further analysis, on the basis of MCODE score, top five modules were selected. The highest-scoring module (MCODE score = 7.846) comprised 27 nodes and 102 edges, followed by Module 2 (score = 6.000; 31 nodes, 90 edges) and Module 3 (score = 4.727; 34 nodes, 78 edges). Module 4 consisted of 4 nodes and 6 edges (score = 4.000), while Module 5 contained 6 nodes and 8 edges (score = 3.200) (Figure 8). These modules represent highly interconnected regions of the PPIN.

**Table 7: Summary of all 12 network modules with their corresponding MCODE scores, number of nodes and edges.**

| Module | Score | Nodes | Edges |
|---|---|---|---|
| Module 1 | 7.846 | 27 | 102 |
| Module 2 | 6 | 31 | 90 |
| Module 3 | 4.727 | 34 | 78 |
| Module 4 | 4 | 4 | 6 |
| Module 5 | 3.2 | 6 | 8 |
| Module 6 | 3 | 3 | 3 |
| Module 7 | 3 | 3 | 3 |
| Module 8 | 3 | 3 | 3 |
| Module 9 | 3 | 3 | 3 |
| Module 10 | 3 | 3 | 3 |
| Module 11 | 3 | 3 | 3 |
| Module 12 | 2.667 | 4 | 4 |

**Figure 8: Visualization of the top five modules identified by MCODE analysis in the integrated network.**

### 3.10) Comprehensive MD Simulation Analysis of VEGFA-Withanolide D Complex

A single 200-ns MD simulation of the VEGFA-withanolide complex to assess the structural stability. The simulation demonstrated stable dynamic behavior across all key structural parameters (Figure 9 A-E). The ligand RMSD showed a low mean value of 0.318 ± 0.061 nm (10-200 ns), confirming a highly stable binding pose duringthe simulation. After the first equilibration period (first 5-10 ns), the protein backbone RMSD remained within 0.12-0.22 nm and was stabalized. There were no significant deviations or upward drift were observed, suggesting that the protein maintained its structural integrity and show no signs of unfolding.

Protein Cα RMSF analysis further supported complex stability, which showed that residue fluctuations were minimal. A modest fluctuation (~0.42 nm) was observed at residues 88-96, which is a part of the receptor-binding loop. However, this level of flexibility is minimal and suggests that Withanolide helps stabilize the VEGFR-binding interface rather than of destabilizing it. The protein compactness also remained stable as showed by the radius of gyration (Rg), with an average of 1.995 ± 0.015 nm, demonstarate that the globular fold did not undergo any expansion.

The complex's Solvent Accessible Surface Area (SASA) was quite consistent, with a mean value of 121.8 ± 1.6 $nm^2$, showing no signs of surface expansion or hydrophobic core exposure during 200 ns trajectory. The hydrogen-bond analysis of the complex was found an average of 0.6-1.0 hydrogen bonds during the simulation. Although

modest, this is typical for natural-product inhibitors that rely predominantly on hydrophobic interactions supported by one or two key stabilizing H-bonds. Notably, the ligand RMSD was largely maintained between 0.35-0.50 nm throughout the simulation, with single transient spike (~0.80 nm at 140-150 ns), that rapidly returned to baseline indicating a brief conformational shift rather than dissociation. Every structural parameters such as RMSD, RMSF, SASA, Rg, and hydrogen-bonding consistently shows the structural stability of VEGFA-Withanolide complex. The protein backbone remained well-equilibrated, while the ligand remained continuously bound with normal internal flexibility, and no long-term unfolding is observed. All of these observation show that the VEGFA-withanolide complex maintained its structural stability during the simulation period, allowing for prolonged ligand interaction with the VEGFA binding pocket.

The binding affinity of withanolide towards VEGFA was predicted using the MM-GBSA approach over 18,000 equilibrated frames obtainedfrom the MD trajectory at 298.15 K. The calculated binding free energy (ΔG binding) was -30.82 ± 3.04 kcal/mol (sample standard deviation), suggesting a thermodynamically favorable and stable interaction according to the model. Energy decomposition showed that binding is predominantly stabalized by gas-phase interactions, with van der Waals forces (ΔVD WAALS = -37.76 ± 1.52 kcal/mol) contributing most significantly and electrostatics (ΔE EL = -10.42 ± 1.70 kcal/mol), resulted in a strong favorable gas-phase energy (ΔG gas) of -48.18 ± 2.28 kcal/mol. These stabilizing interactions were partially offset by solvation effects where polar solvation contributed unfavorrly (ΔE GB = +21.37 ± 0.75 kcal/mol) while the nonpolar solvation provided a small stabalizing contribution (ΔE SURF = -4.02 ± 0.01 kcal/mol). Overall, the solvation energy remained positive (ΔG solv = +17.36 ± 2.26 kcal/mol), reflecting the expected desolvation cost during complex formation (Figure 9E).

Despite the significant desolvation penalty, the strong molecular mechanics interactions produced a net negative ΔG binding, supporting stable complex formation in the simulation. The MM-GBSA analysis showed the total binding free energy (ΔG binding) of -30.82 ± 2.40 kcal/mol. The interaction was predominantly guided by van der Waals (-37.76 kcal/mol) and electrostatic (-10.42 kcal/mol) contributions, partially offset by polar solvation energy (+21.37 kcal/mol) (Table 8). This showed that the Withanolide-VEGFA complex remains energetically stable during the simulation.

**Table 8: MM-GBSA Binding Free Energy Components for Withanolide-VEGFA Complex**

| Energy Component | Average (kcal/mol) | SD (kcal/mol) | SEM (kcal/mol) |
|---|---|---|---|
| ΔVD WAALS | -37.76 | 2.74 | 0.02 |
| ΔE EL | -10.42 | 2.44 | 0.02 |
| ΔE GB (Polar Solv.) | +21.37 | 2.35 | 0.02 |
| ΔE SURF (Nonpolar Solv.) | -4.02 | 0.27 | 0.00 |
| ΔG GAS | -48.18 | 3.96 | 0.03 |
| ΔG SOLV | +17.36 | 2.26 | 0.02 |
| ΔG binding (Total) | -30.82 | 3.04 | 0.02 |

**Figure 9**: Molecular dynamics stability analysis of the VEGFA-withanolide complex over 200 ns. (A) RMSD plots of protein backbone and ligand, (B) RMSF plot of protein Cα atoms, (C) Solvent-accessible surface area (SASA), (D) Radius of gyration (Rg), and (E) Number of hydrogen bonds..

**Figure 10:** MM-GBSA binding free energy decomposition of the VEGFA-withanolide complex. (A) Bar plot showing contribution of individual energy components. (B) Pie chart representing the percentage contribution of each energy term to the total binding free energy.

## 4) DISCUSSION

DMD is a devastating muscle-wasting recessive condition characterized as worsening muscle damage, chronic inflammation, fibrosis, impaired angiogenesis, and ultimately respiratory and heart failure**.** The absence of dystrophin disrupts sarcolemma integrity, resulting in calcium dysregulation, oxidative stress, initiation of inflammatory and fibrotic pathways [2], [67]. Despite the availability of several therapies based on exon-skipping, gene replacement approaches, and histone deacetylase (HDAC) inhibitors such as givinostat have broadened the

therapeutic options, but no single intervention fully eliminates the disease progression [3]. There is therefore a need to use multi-target therapies that will address muscle perfusion deficiencies, inflammation and regenerative failure simultaneously.

Impaired angiogenesis and vascular remodeling contributes to dystropic muscles ischemia, impairment in nutrient delivery and increased degeneration [9], [68], [69]. VEGFA is significant in enhancing the development of endothelial cells, migration, and formation of capillaries, which enhances the perfusion of tissues and the restoration of muscles. In preclinical studies in mdx mice, VEGFA signaling improves vascular density, reduces fibrosis, and enhances muscle function by direct delivery [70] [10]. Such findings position VEGFA as a promising yet underexplored therapeutic node in DMD [9], [69].In the present study, VEGFA had the most significant betweenness centrality in the integrated PPIN, supporting its therapeutic potential.

Through this integrated computational approach, withanolide was identified as the most promising VEGFA-targeting compound. It exhibited the strongest binding affinity (-9.3 kcal/mol) to VEGFA better than givinostat (-8.7 kcal/mol).

MD simulations confirmed high stability of VEGFA-withanolide complex over 200-ns supported by low RMSD, compact radius of gyration and stable SASA. In addition, the hydrogen bond occupancy remained high, supporting robust and stable ligand-receptor interactions. Further, MM-GBSA results reinforced these observations by showing a favorable binding free energy of -30.82 ± 3.04 kcal/mol primearily driven by van der and electrostatic interactions.

Relative to the FDA approved drugs, withanolide offers safer pharmacological profiles in the study. It follows Lipinski's rule of five and showed no predicted AMES toxicity or hepatotoxicity, along with favourable intestinal absorption and metabolic stability. In contrast, givinostat showed predicted hepatotoxicity and hERG II inhibition, indicating a comparatively narrower safety margin. Such properties highlight the potential suitability of withanolide for long-term therapeutic use, particularly in pediatric conditions such as DMD.

Network pharamacology analysis showed that withanolide targets multiple kinases (e.g., PKC isoforms, MAPK family & JAK families), nuclear receptors, and inflammatory signaling proteins aligning with the identified hub genes (AKT1, MTOR, GSK3B, SRC, and PTGS2) which have been reported to control cell survival, protein synthesis, and inflammatory responses. Gene ontology and pathway enrichment analyses further confirmed strong associations with PI3K-Akt signaling, VEGF signaling, ErbB signaling, and apoptotic regulation-processes that are dysregulated in DMD. The enrichment of the VEGF signaling pathway among the common hub genes suggests its potential involvement in the managing angiogenesis and tumor-associated cellular processes. Mapping of the hub genes onto this pathway identified VEGFA as a major upstream component linked to several downstream signaling modules. In particular, VEGFR2-mediated activation of Src, PI3K-Akt, and MAPK pathways suggests possible roles in cytoskeletal remodeling, cell survival, and proliferative signaling. The association of downstream mediators such as COX2 within the NFAT-regulated branch further supports a potential link to inflammatory and angiogenic mechanisms. Given the established role of VEGF signaling in coordinating vascular development and tumor progression, these findings suggest that VEGFA may contribute to the integration of survival, migration, and angiogenic responses through interconnected signaling cascades. However, as these observations are derived from computational pathway mapping, they indicate potential regulatory involvement rather than direct mechanistic validation.

Withanolides, particularly withaferin A and withanolide D from Withania somnifera (ashwagandha) have been extensively explored for the anti-inflammatory, antioxidant, and muscle-protective properties. Experimental and clinical studies showed that withania somnifera extracts and purified withanolides improves muscle strength, lower oxidative stress, inflammation, promotes mitochondrial function, activate autophagy, restore IGF-1/Akt/mTOR signaling, and suppress pro-inflammatory cytokines [71], [72], [73], [74], [75]. Although these geroprotective and myoprotective effects have not yet been validated in dystrophin-deficient systems, show strong mechanistic convergence strongly supports further investigation of withanolide-rich preparations as adjunctive candidates for DMD.

In addition to withanolide, several other phytocompounds screened in this study exhibited promising VEGFA binding affinities and relevant pharmacological activities for DMD management. Taraxasterol exhibits anti-inflammatory and anti-fibrotic activities through inhibition of TGF-β1-induced extracellular matrix accumulation and suppression of p38/STAT3 and NF-κB signalling pathways [44], [45], [46], process central to dystrophic muscle remodelling. Although direct proof in dystrophic muscle has not yet been established, these characteristics imply potential utility as a supplementary treatment drug given the significant involvement of fibrosis and chronic inflammation in DMD.

Steroidal sapogenins from Tribulus terrestris, such as gitogenin, exhibit promising muscle-protective potential by enhancing antioxidant defences, lower lipid peroxidation, block NF-κB signalling, and regulate pro-inflammatory cytokines such TNF-α and IL-6 [47], [48]. Gitogenin also demonstrates cardiovascular regulatory effects as well as modulation of cardiac contractility [76]. Additionally, its activation of the AMPK pathway and modulation of ROS suggest implicate roles in maintaining cellular homeostasis and mitigating oxidative stress [77].

Structurally related compounds, such as diosgenin show similar mechanistic insights, demonstrating both antioxidant and anti-inflammatory activities in chronic tissue injury [78].

Tomatidine**,** a tomato-derived steroidal alkaloid, has shown potential to inhibit muscle atrophy in preclinical models by activating mTORC1 signaling, enhancing protein synthesis, mitochondrial biogenesis, muscle growth,

in vivo improves muscle mass, reduces atrophy, promotes hypertrophy, increase strength and exercise capacity [51]. It also suppresses IL-6/JAK/STAT3-mediated inflammation and limits extracellular matrix remodeling in aged muscle [50]. While, C. elegans studies show improved muscle function through activation of mitophagy and antioxidant pathway [79], supporting its evaluation as an adjunct candidate in DMD for preventing muscle atrophy in neurogenic and age-related contexts.

Daucosterol exerts anti-inflammatory, antioxidant, and anti-fibrotic effects in murine models of acute liver failure and myocardial infarction models via suppression of IL-6/STAT3 and ZBTB16/S100A8 signaling pathways, thereby reducing inflammatory cytokines, apoptosis, fibrosis, and improving tissue function [80], [81]. Additional modulation of PI3K/AKT, PTEN, caspase-3, and PPARα/NF-κB pathways, enhancing mitochondrial metabolism while limiting inflammation [53], [54]. The potential of Cycleanine, a bisbenzylisoquinoline alkaloid isolated from Stephania glabra has been reported to selectively inhibit vascular L-type $Ca^{2+}$ channels, more effectively reducing KCl-induced smooth muscle contraction than cardiac contraction, indicating its potential as a vascular-selective calcium antagonist [55]. While, Agrimol B, which has antioxidant and anti-inflammatory properties**,** modulate mitochondrial biogenesis via the PGC-1α/NRF1/TFAM pathway, suggesting that it may have potential impact on mitochondrial function in other tissues [82]. Furthermore, in animal models of tissue injury, it activates SIRT1/Nrf2 signaling**,** upregulate antioxidant defenses, and diminish oxidative stress, suggesting it may reduce ROS accumulation in muscle cells [83]. Furthermore, it suppress NF-κB-mediated inflammatory pathways, which may potentially reducing chronic inflammation implicated in DMD pathology [56]. Curcumin from Curcuma longa, has been widely reported due to its ability to support skeletal muscle health by attenuating inflammation, oxidative stress, and proteolysis under catabolic conditions, through modulation of calcium homeostasis and inhibition of NF-κB mediated signalling [57], [59], [84], [85]. Curcumin is a promising nutraceutical for muscle preservation and have favorable strong safety profile, low cost and because of its wide availability. Although direct evidence in disease-specific models like DMD is limited, its mechanistic effects suggest potential applicability in muscle-wasting disorders [57], [58].

Macamides from Lepidium meyenii (maca) have antioxidant, anti-inflammatory properties in skeletal muscles. It improve grip strength and exercise performance in animal model and humans in a dose-dependent manner [60], [61]. Additionally, macamides promote myogenic differentiation through AKT/p38 signaling and protects against dexamethasone-induced atrophy in vitro, [62]. These studies highlight their potential in supporting muscle strength and preventing muscle weakness under catabolic or aging-related conditions.

Furthermore, in dystrophin-deficient mdx mice, chronic sulforaphane administration activates Nrf2/HO-I signaling, increased phase II antioxidant enzyme expression, and significantly improved muscle mass, strength, endurance, histopathology and reduces oxidative markers of muscle damage and stress. It also attenuates skeletal and myocardial hypertrophy. [63], [64], [65]. Collectively, these findings provide strong preclinical evidence that pharmacological Nrf2 activation with sulforaphane mitigates dystrophic pathology and supports its potential as an adjunctive antioxidant strategy for DMD [65].

Collectively, these phytocompounds target multiple pathological hallmarks of DMD, including inflammation, oxidative stress, fibrosis, muscle atrophy, and impaired angiogenesis. The present findings, particularly the strong computational evidence for withanolide as a VEGFA modulator, provide a solid rationale for further experimental validation of these natural compounds as potential multi-target therapeutic or adjunctive agents for DMD.

**5) Limitations of study**

Although this study provides valuable insights through comprehensive computational analyses, several limitations should be acknowledged. All findings are based on in-silico approaches and therefore require experimental validation. These include biophysical binding assays (e.g., Surface Plasmon Resonance (SPR) and Isothermal Titration Calorimetry (ITC)), in vitro functional angiogenesis assays (e.g., HUVEC tube formation), and preclinical studies in mdx mouse models to confirm the effects on muscle perfusion, fibrosis, and functional improvement. Additionally, VEGFA signaling is tightly regulated and excessive or dysregulated activation may pose risks such as abnormal angiogenesis or vascular leakage. Therefore, careful evaluation of dose-dependent effects and long-term safety will be essential. Furthermore, MM-GBSA calculations, while widely used, rely on computational approximations and do not fully account for entropic contributions, which may result in overestimation of binding free energy. Hence, the predicted interactions and affinities must be experimentally verified.

**6) Future Perspectives and Conclusion**

Future studies should prioritize the experimentally validation of the VEGFA-withanolide interaction, through biophysical, cellular, and preclinical models. It will also be important to investigate potential synergistic effects when combining withanolide or other phytocompounds with existing FDA-approved DMD therapies.

In conclusion, this integrated computational study identifies VEGFA as a high-priority therapeutic target in DMD and positions withanolide from Withania somnifera as a promising multi-target phytocompound. The strong binding affinity, stable complex formation during 200-ns MD simulations, favorable MM-GBSA binding energy, and superior ADMET profile collectively highlight withanolide as an attractive lead candidate. These findings provide a robust systems-level rationale for further experimental validation and support the broader strategy of

integrating drug repurposing with phytocompound-based approaches to accelerate therapeutic development for DMD and other rare neuromuscular disorders.

**Ethical approval**
Not applicable

**CRediT authorship contribution statement**
Shashikala: Writing original draft, Visualization, Methodology, Editing, Data curation, Conceptualization. Vibha Rani: Supervision, Editing. Shazia Haider: Conceptualization, Supervision, Review and Editing, Methodology, Conceptualization.

**Declaration of competing interests**
I have nothing to declare.

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**Supplementary Material**
Supplementary material is available and can be provided upon request.

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