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In silico identification and *in vitro* validation of Alpha-hederin as a potent inhibitor of Wnt/β-catenin signaling pathway in breast cancer stem cells

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Abstract

Intriguing evidence demonstrates that breast cancer stem cells (bCSCs) play a vital role in tumor cell proliferation, metastasis, recurrence and chemoresistance in breast cancer. β-catenin is a Wnt signaling protein that is frequently overactivated or expressed in bCSCs. β-catenin interaction with T-cell factor (Tcf) DNA binding proteins plays a critical role in the activation of proliferative genes in response to upstream Wnt/β-catenin signaling. Thus, the purpose of this study was to identify a small molecule inhibitor that can effectively disrupt β-catenin/Tcf4 interaction thereby inhibiting the proliferation of bCSCs. Molecular docking studies were performed against the TCF binding hotspot on β-catenin using 100 natural or synthetic small molecules ligands. Protein ligand complexes having interaction energy better than -7 kcal/mol were investigated for binding interactions and the stability studies by molecular dynamics (MD) simulations. Alpha-hederin (AH) with binding energy -8.2 $kcal.mol^{-1}$ having stable MD profile was investigated for anti-proliferative and apoptotic effects *in vitro* using bCSCs isolated from triple negative breast cancer cell line (MDA-MB-231). Further, oral bioavailability and toxicity of AH was predicted using in silico tools. Interestingly AH significantly decreased the viability of bCSCs. In addition, AH suppressed the transcription of Wnt/β-catenin downstream target genes *Cyclin D1* and *CD44* while up-regulating the transcription of the tumor suppressor gene *p53*. AH was predicted to have acceptable overall drug likeness. Although AH is currently known to inhibit the growth of various cancer cells in vitro, present study demonstrated first time that AH is a potent inhibitor of Wnt/β-catenin signaling pathway and induce apoptosis in bCSCs.

1.Introduction

Despite the recent advancements in the detection and treatment options, breast cancer remains a leading cause of cancer related deaths among women worldwide (Siegel et al., 2016). Cancer recurrence and drug resistance have been considered as serious challenges in the treatments and clinical management of breast cancer (Touil et al., 2014). Cancer stem cells (CSCs) have been identified as the most probable cause for tumor recurrence, metastasis, and chemo-resistance (Reya et al., 2001; Al- Hajj et al., 2003; Korkaya et al., 2009; Liu et al., 2005). CSCs are immortal tumor-initiating cells that have unique capacity to self-renew and give rise to heterogeneous lineage of cancer cells which eventually leads formation of metastatic tumors (Reya et al., 2001; Visvader et al., 2008; Dean et al., 2005; Li et al., 2012; Yu et al., 2012a). CSCs population is a small population (0.05-1%) present within a tumor mass (Li et al., 2012;Yu et al., 2012a;Yu et al., 2012b) and CSCs have been identified in multiple malignancies such as acute myeloid leukaemia (AML), breast, brain cancer and lung cancers (Vermeulen et al., 2008; Al- Hajj et al., 2003). As CSCs play a crucial role in tumor initiation, therapeutic response and progression (Valent et al., 2012), complete eradication of CSCs from the tumor would effectively improve the overall clinical outcome of cancer therapies.

Wnt/β-catenin signaling pathway plays a key role in stem cell self-renewal, differentiation, cell polarity, proliferation, and migration (Polakis, 1999; Reya et al., 2003; Niehrs, 2012). Abnormal activation of Wnt signaling pathway has been reported in several CSCs types (Lin et al., 2000; Malanchi et al., 2008; Vermeulen et al., 2010). For the functioning of Wnt/ β-catenin signaling pathway, interaction between cytosolic β-catenin and T-cell factor/lymphoid enhancer factor (Tcf/Lef) (Gan et al., 2008) is necessary and β-catenin – TCF/LEF complex then translocate to the nucleus stimulating the transcription of several Wnt target genes such as c-Myc, Cyclin D1, CD44, and ALDH1, which are essential in cell

proliferation, survival or migration (Al-Hajj et al., 2003; Ginestier et al., 2007; Reya and Clevers, 2005; Zeilstra et al., 2008). Although, several β-catenin/Tcf4 inhibitors such as OMP-18R5, JW55, OMP-54F28, PRI-724, and LGK974 are in clinical trials, no small-molecule inhibitor has been approved for human use (Tai et al, 2015, Yu et al, 2021). Therefore, identification and development of novel inhibitors which can target β-catenin/ Tcf4 interaction is urgently needed. Hence, taking in consideration above mentioned facts, the present study was planned to identify promising drug leads which can target Wnt signaling pathway, using *in silico* and in vitro studies.

2. Materials and methods

2.1 Materials

MDA-MB-231 human triple negative breast cancer cell line, L15 medium, fetal bovine serum (FBS) and trypsin/EDTA were purchased from the American Type Culture Collection (Manassas, VA, USA). Cancer Stem cell media Premium media was purchased from the ProMab Biotechnologies, Inc., USA. Cell culture flasks (25cm^3 and 75 cm^3), cell culture ultra-low adherent flasks (25cm^3 and 75 cm^3) and cell culture ultra-low 96-well plates were purchased from the Corning Incorporated, Corning, NY, USA. Bovine serum albumin (BSA), LD columns, LS columns, MidiMAC separator or QuadroMACS separator, CD24$^-$ micro beads, CD24$^-$ anti-micro beads and CD44 micro beads were purchased from the Miltenyi Biotec, USA. TRIzol reagent used for RNA extractions was purchased from the Invitrogen Life Technologies, Carlsbad, CA, USA. All the reagents needed for complimentary DNA (c-DNA) synthesis were purchased from the Invitrogen life technologies, Carlsbad, CA, USA. Primers were purchased from Integrated DNA Technologies (IDT) USA. MESA GREEN qPCR Master MIX Plus for SYBR® assay Low Rox Kit was purchased from the Eurogentec, Liège, Belgium. For molecular docking iMac desktop computer with the following specifications was used; Processor: Intel (R) core (TM) i5-4200U, CPU: 2.30GHz, RAM:

4.00GB, System type: 64-bit operating system Windows, Linux, Virtual machine-Freebit Package. Molecular dynamics simulations were run on Ubuntu 64 bit operating system with the hardware configuration of GFORCE RTX GPU, Ryzen 9 (1.8 GHz) processor and 16GB random access memory.

2.2 Preparation of receptor and ligands for *in silico* screening

The crystal structure of β-catenin was prepared for docking by extracting chain A from the β-catenin/Tcf4 complex with a resolution of 2.5 Å (PDB entry 1JPW). The receptor file was prepared for docking and saved as a PDBQT file. Preparation of receptor files was accomplished using the MGLTools-1.5.6. The 2D structures of 100 small molecules, selected from the natural/synthetic compound library at the Institute of Biochemistry, Molecular Biology and Biotechnology (IBMBB), were generated using the ACD/ChemSketch software and the geometry optimization was carried out using MMFF94force field in Avogadro (Marcus et al., 2012). Optimized structures were saved as PDB and converted to PDBQT using MGLTools-1.5.6.

2.3 Virtual screening

The targeted ligand binding hot spot consisted the residues of β-catenin that form most critical polar interactions (Lys435, Arg469, Lys508) as well as residues that form important non-polar interactions (Pro 463, Cys 466 and Arg 386) with Tcf4. Vina search space dimensions were, Center X =101.876 Å, Y= 4.920Å, Z= 26.707 Å and the size X = 29.4, Y= 27.43 and Z= 30.28 which was defined to cover the whole groove of the target hotspot. All the ligands were docked using AutoDockVina (Trott and Olson, 2010) with the Lamarckian genetic algorithm as scoring function. Polar side chains in the ligand binding site was kept flexible and the other parameters were default values. Complexes thus formed by molecular docking with ligand binding energy better than -7 kcal/mol were characterized using protein–

ligand interaction profiler (PLIP) (Salentin et al., 2015) to identify protein residues that interact with ligands.

2.4 Molecular dynamics simulation

Complexes used for interaction analysis were subjected to molecular dynamics simulation using academic version of Maestro (Schrödinger, Inc). The OPLS_2005 force field and explicit solvent model with the TIP3P water molecules were used in this system. TIP3P solvent model a trio site solid water molecule containing charges were used to solvate the docked complexes in a cubic box of 0.5 Å. 10 Cl^- ions were added to neutralize the charge. To simulate the physiological environment 0.15 M NaCl was provided. Initially, the system was equilibrated using NPT ensemble for 100 ns to retrain over the complex using temperature of 300 k, relaxation period of 1.0 ps and pressure of 1 bar preserved throughout the simulations. The bond forces were computed for each trajectory using the RESPA integrator with a time step of 2 fs. The stability of complexes was monitored by analysing RMSD (Root Mean Square Deviation) and RMSF (Root Mean Square Fluctuation) of the ligand and protein atom positions in 100 ns time.

2.5 Analysis of drug likeness

The ADMET (absorption, distribution, metabolism, excretion and toxicity) properties of the compounds found to be stable during MD simulation was analyzed using FAFDrugs4 web server (Lagorce et al., 2017) and admetSAR server (Wang et al, 2018) to predict the oral bioavailability and possible toxic effects.

2.6 Culturing of MDA-MB-231 cells

For in vitro assays, MDA-MB-231 cells were cultured in L-15 medium supplemented with 10% fetal bovine serum according to the ATCC recommendations. Cells were maintained

without CO_2 at 37 °C. The growth medium was changed every 2-3 days. Upon reaching 80 % confluency cells were trypsinized and sub-cultured into new culture flasks.

2.7 Isolation and culturing of bCSCs

Magnetic cell sorting (MACS) system was used to isolate bCSCs from MDA-MB-231 cells as described in a recent study published from our laboratory and, isolated bCSCs in this study were used for all the *in-vitro* experiments in the present investigation after characterization (Rajagopalan, 2018). Briefly, MDA-MB-231 cells were trypsinized and collected as cell pellets. Cell pellets were then rinsed with sterile MACS buffer and then incubated with $CD24^-$ biotin (10 µL) conjugated ferromagnetic beads for 15 min at 4˚C. Cells were then washed with 1 mL MACS buffer and cell suspensions were again centrifuged at 300 g for 10 min at 4 ^{0}C. The pellets were then re-suspended in 80 µL of MACS buffer and incubated for 15 min at 4˚C with 20 µL of anti-biotin. Following the incubation, cells were washed with 1.2 mL of MACS buffer and centrifuged at 300g for 10 min at 4 ^{0}C. Washed cell pellets were again re-suspended in 50 µL of MACS buffer and run through LD column with 1 mL of MACS buffer. Unlabeled cells ($CD24^-$) were depleted from the LD column and flow through of the unlabeled cells were collected and centrifuged at 300g for 10 min at 4 ^{0}C. The collected pellets were then re-suspended in 80 µL of MACS buffer and incubated for 15 min at 4˚C with 20 µL CD44 micro beads. Following incubation, cells were mixed with 1-2 mL of running buffer and centrifuged at 300g for 10 min at 4 ^{0}C. After mixing cell pellets with 500 µL of MACS buffer, cell suspensions were run through LS columns and the flow through was discarded. After removing LS columns from the magnetic stand, columns were flushed out with 5 mL of MACS buffer to collect cancer stem cell populations ($CD44^+/CD24^-$). Required number (approximately 2.5 x 10^5 cells/mL) of cells were counted and cultured with 6mL of cancer stem cell premium medium in ultra-low flasks incubated at 37 ^{0}C in a humidified atmosphere of 5 % CO_2.

2.8 WST-1 cell proliferation (cell viability) assay

Cells (bCSCs with a density of 5×10^3 cells/well) were plated in 96 well ultra-low attachment plates and incubated for 3 days at 37°C. After incubation, cells were treated with various concentrations of AH (0, 4, 8, 16, 32, and 64 μM) and paclitaxel (positive control) and incubated for 24 h. Following incubation, At the 24 h post incubation, cell viability was determined by the WST-1 assay. For the WST-1 assay, 10 μL of WST-1 reagent was added to each well and incubated at 37°C with 5% CO_2 for 4 h. Following incubation, absorbance was measured at 440 nm (reference wavelength 650 nm) using a micro plate reader (Synergy™ HT; Bio-Tek Instruments Inc., Winooski, VT, USA). Percentage cell viability was calculated using the formula: $[(A_T-A_B)/(A_C- A_B)] \times 100$ where A_T - absorbance of the treatment, A_C -absorbance of the untreated control and A_B -absorbance of the blank. All the experiments contained three technical and biological replicates and IC_{50} values of AH was calculated using the Graph pad Prism software.

2.9 Caspase-3/7 activity

Cells (bCSCs with a density of 2×10^4 cells/well) were plated in 96 well ultra-low attachment plates and incubated for 3 days. After incubation, cells were treated with different concentrations of AH (0, 4, 8, 16, 32 μM) and incubated for 24 h. Caspase 3/7 activation in AH treated bCSCs were then determined using the ApoTox-Glo™ triplex assay (Promega, Madison, WI, USA) kit as per manufacturer's instructions. Caspase 3/7 activation was expressed as percentage compared to untreated controls.

2.10 DNA fragmentation assay

Genomic DNA was isolated following the method described by Pathiranage et al., 2019. after 72h exposure of AH to bCSCs at different concentrations. Genomic DNA was isolated and

quantified. Equal quantity of DNA from test samples and the vehicle control was loaded in to the wells of 2 % agarose gel and 4h electrophoresis run.

2.11 Quantitative real time polymerase chain reaction (qPCR)

bCSCs ($2x10^5$ cells/mL) were cultured in T_{25} ultra-low flasks and incubated for 3 days. Following incubation flasks were treated with different concentrations of AH (10 µM and 20 µM) for 24 h. After 24 h post incubation, RNA was extracted using the TRIzol Reagent according to manufacturer's instructions. The reverse transcription of isolated RNA samples to c-DNA was performed as previously described. Quantitative Real-time PCR reactions were performed using MESA GREEN qPCR Master MIX Plus for SYBR® assay Low Rox Kit in MX-3000P real time PCR system (Stratagene, Basel, Switzerland). The primers used in real-time PCR are mentioned in the Table. 1 GAPDH gene was used as the internal reference housekeeping gene and comparative Ct ($2^{-\Delta\Delta Ct}$) method was used to analyze expression of genes (Livak and Schmittgen, 2001).

Table 1. Primers used for real time PCR experiments

Gene	Forward primer	Reverse primer	Size, bp
CD44	5'-TTGCTTGGGTGTGTCCTTCGCT-3	5'-TCAAATCGATCTGCGCCAGGCT-3'	299
P53	5'-TCTGGCCCCTCCTCAGCATCTT-3'	5'-TTGGGCAGTGCTCGCTTAGTGC-3'	369
Cyclin D1	5'-AGGAACAGAAGTGCGAGGAGG-3'	5'-GGATGGAGTTGTCGGTGTAGATG-3'	192

3. Results

3.1 Virtual screening

Out of the 100 small molecules screened, nine compounds had binding energy better than - 6 kcal/mol (table 2) and only four compounds (AH, AZ01, ASMW1 and ASMW2) had binding energy better than -7 kcal/mol. Out of four compounds, only AH was found to bind with the targeted binding site of the receptor by forming strong polar interactions with three key

residues important for TCF recognition (Lys 435, Arg 469 and Lys 508) (Guo et al, 2014, Wang et al ., 2021). Furthermore, AH was found to form interactions with other important residues i.e. Pro 463 and Arg 386 (Guo et al, 2014) of the hydrophobic cleft in the targeted site (Figure 1).

Table 2. Molecular binding energy values of β-catenin chain A with candidate compounds.

Compound Code	Compound Name	Binding Energy ($kcal.mol^{-1}$) of the best pose
AZ01	Betulinic acid	-7.1
AZ02	Ceanothic acid	-6.9
AZ03A	Zizyberenalic acid	-6.5
SCGP327	Caseamine	-6.2
A.SM.W1	Manoyloxide sesterterpenoid	-7.1
A.SM.W2	Salvimirzacolide	-7.1
SN-005	Eupafolin	-6.6
SN-58	1α,6αDihydroxyisocostic acid methyl ester	-5.4
AH	Alpha-hederin	-8.2

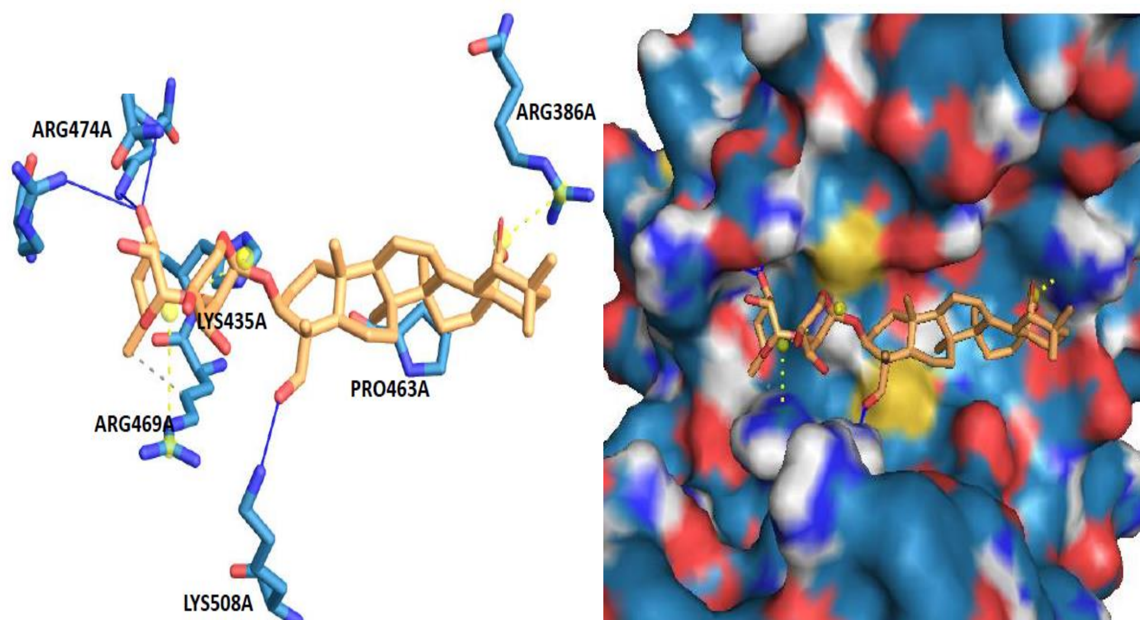

Figure 1. Predicted binding pose of alpha-hederin (AH) with the selected ligand binding site. (Charged centre, Hydrophobic interaction, Hydrogen bond , Cation л interaction, Salt bridge) The picture was generated by Autodock, PLIP and PyMol

3.2 Molecular dynamics simulation

Out of four compounds having binding energy better than -7 kcal/mol, AH formed the most stable protein-ligand complex with β-catenin during MD simulation (figure 2A). All key residues which were retained in the docked pose (Lys 508, Arg 386, Arg 469) showed interactions with the protein (figure 2C, 2D and 2E). Only AH showed the interactions with critical residues Lys508, Arg386 which did not deform during MD simulation. Relevant figures for other molecules are given in supplementary data section.

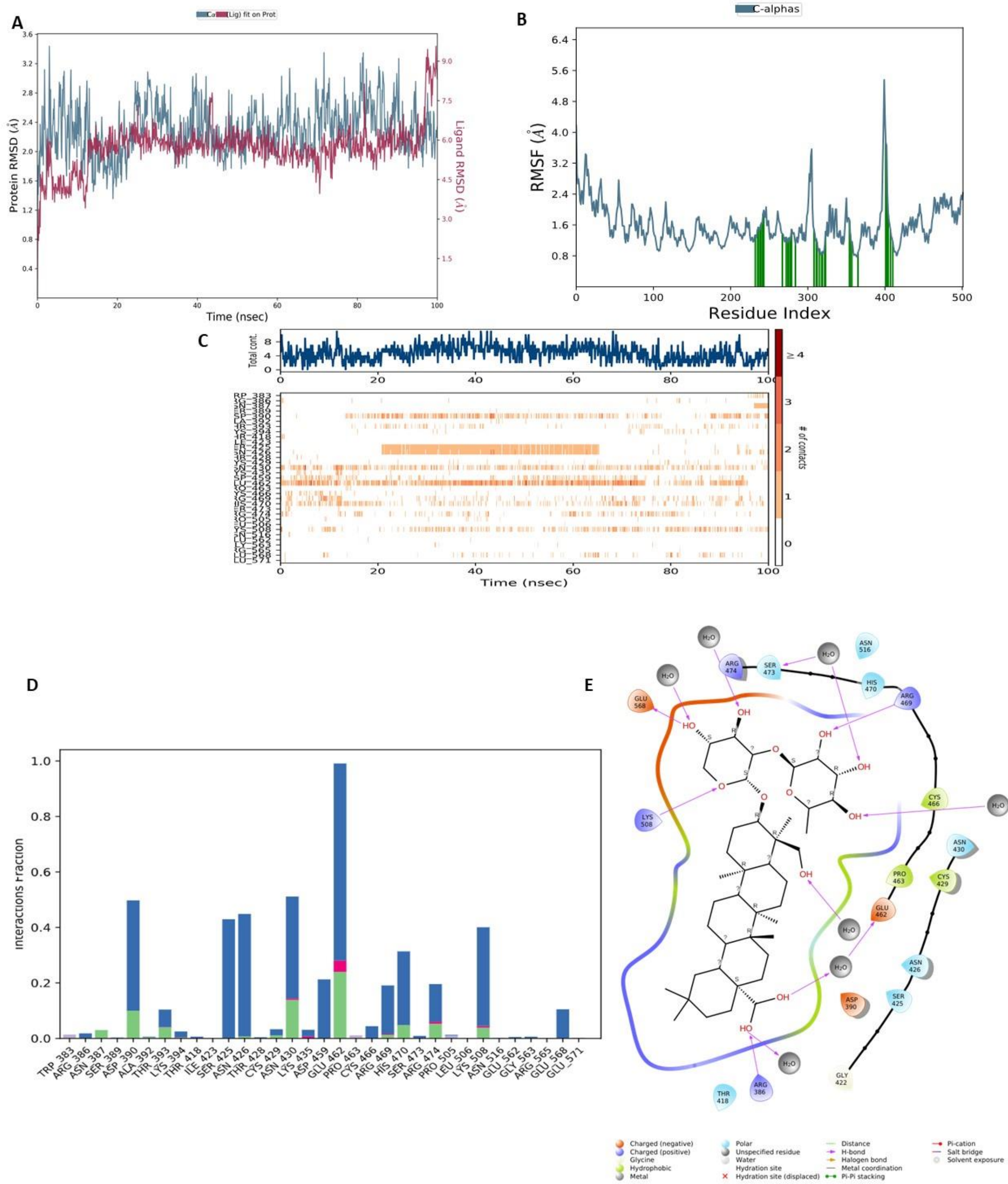

Figure 2. Conformational fluctuations and protein ligand contacts throughout the MD simulation trajectory. (A) RMSD of protein C-alpha backbone conformation and AH conformation. (B) RMSF of each amino acid residue in β-catenin protein. (C) Protein-ligand during in 100 ns simulation. (D) Interaction fraction between protein and AH. (E) 2D Plot of the protein ligand interaction.

3.3 Drug likeness

Result indicated that AH was within the limits of key rules of drug likeness and toxicity. Thus, AH have a potential of being used as drug for application in biological systems. The ADMET properties of AH obtained using FAFDrugs 4 and admetSAR filtering tools are given in Table 3 and Table 4 respectively.

Table 3. FAF-Drugs4 prediction results for AH

S/N	Property	AH
1	logP	3.64
2	logD	0..85
3	logSw	-6.39
4	Molecular Weight (g/mol)	750.96
5	H Bond donors	7
6	H Bond Acceptors	12
7	n_Lipinskiviolations	3
8	Solubility(mg/l)	1256.36
9	Solubility Forecastindex	Good Solubility
10	Veber Rule	Good
11	Egan Rule	Good
12	Phospo lipidosis	NonInducer
13	Status	Accepted

Table 4. Prediction results of AdmetSAR server for AH

Property	AH
Blood-Brain Barrier	BBB+
Human Ether-a-go-go- Re-lated Gene	Weak inhibitor
AMES Toxicity	Non AMES Toxic
Carcinogens	Non carcinogens
Fish Toxicity	High FHMT

Tetrahymena pyriformis Toxicity	High TPT
Biodegradation	Not readily Biodegradable
Acute Oral Toxicity	III

3.4 Alpha-hederin decreases bCSCs viability

Anti-proliferative effect of AH on bCSCs was evaluated by the WST-1 assay. AH effectively induce anti-proliferative effect (IC_{50} (AH)-27.74 µM) on bCSCs in a dose dependent manner when compared with the positive control paclitaxel (IC_{50} 64.67 µM) at 24 h post incubation period (Figures 3). These results suggest that interruption of β-catenin/Tcf4-mediated signaling pathway caused by AH resulted in the induction of bCSCs cell death

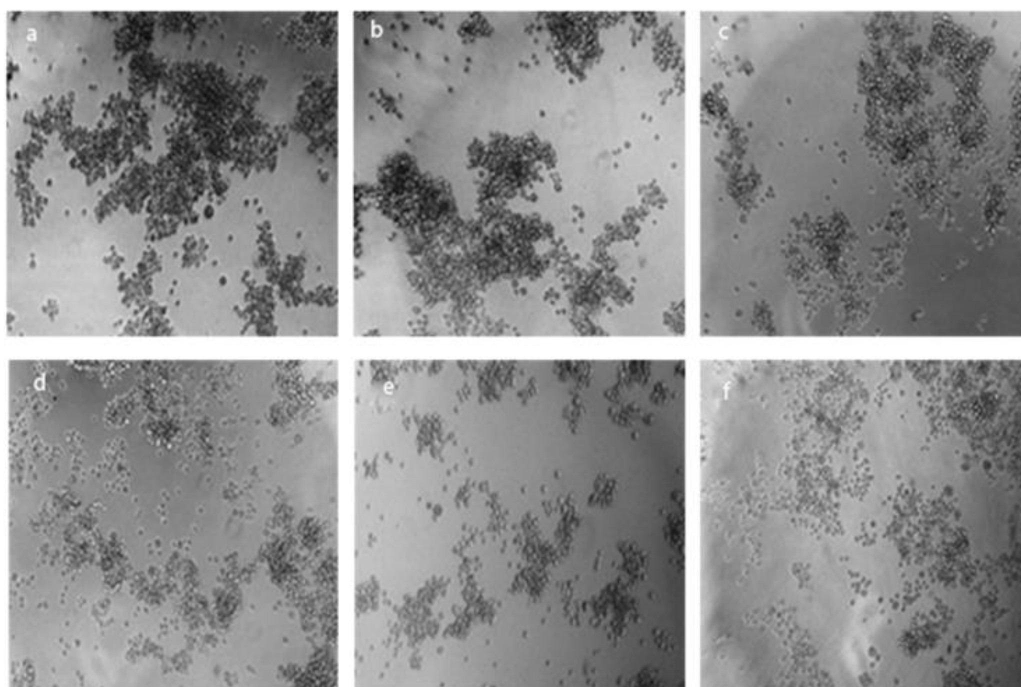

Figure 3. Phase contrast images of bCSCs after 24h of treatment with AH. a. control; b. 4. µM; c. 8 µM; d.16 µM; e. 32 µM and f. 64 µM. Magnification -200X.

3.5 Alpha-hederin activate apoptosis in bCSCs

As shown in Figure 4, treatment of bCSCs with AH induces a significant increase in caspase 3/7 activity in a dose-dependent manner. We found a significant increase in caspase 3/7 activity was only evident at last three doses tested in AH (8, 16 and 32 µM; **P <0.05 and ***P <0.001) treated bCSCs when compared to the untreated bCSCs.

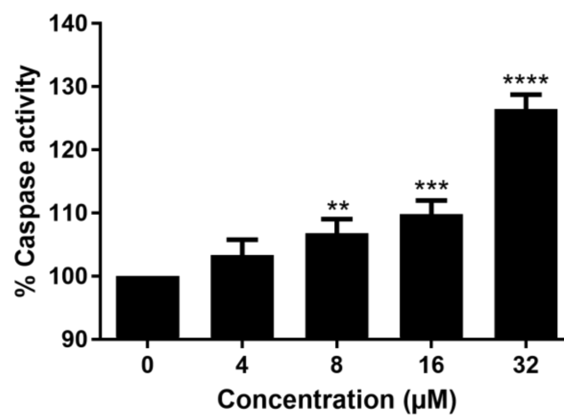

Figure 4: AH treatment triggers the activation of Caspase 3/7 in bCSCs **P <0.05 and ***P <0.001. Data are representative of three independent experiments (mean ± SD of three replicates)

3.6. DNA fragmentation assay

After 72 h exposure of AH to bCSCs, fragmentation of nuclear DNA was observed in 8 µM, 16µM and 32 µM concentrations (Figure 5.)

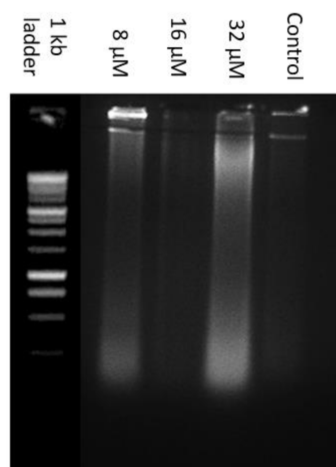

Figure 5. DNA fragmentation of bCSCs exposed to AH (8µM,16 µM 32 µM) for 72h.

3.7 Effect of Alpha-hederin on the expression of Wnt/ β-catenin target genes and the tumor suppressor gene *p53*

Real-time PCR was carried out to evaluate the effects of AH on the expression of the Wnt/ β-catenin target genes (*Cyclin D1* and *CD44*). Significant downregulation of *Cyclin D1* and *CD44* was observed at both the doses tested (10 µM and 20 µM) in AH treated bCSCs at 24h post incubation. AH also caused up-regulation of *p53* in a dose-dependent manner.

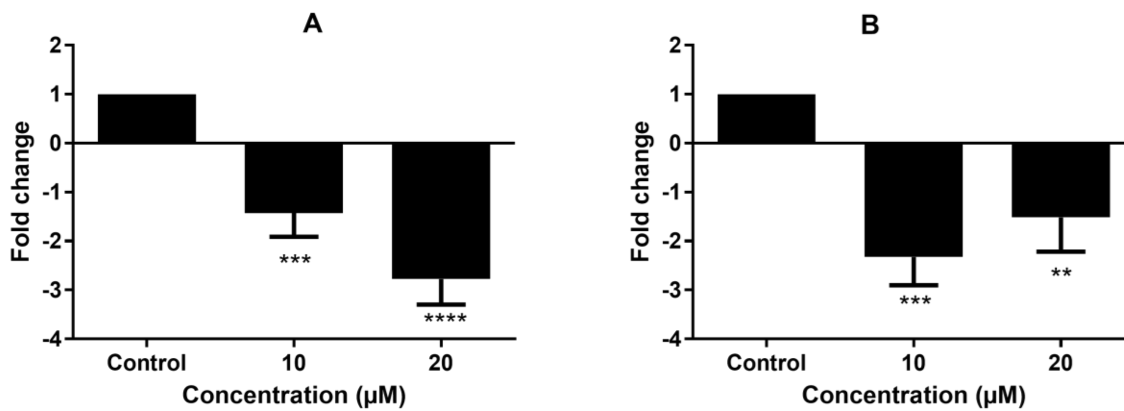

Figure 6. Effect of AH on the mRNA level of the Wnt/β-catenin target gene. The bCSCs were treated with the indicated concentrations of AH. Total RNA was prepared after 24 h (for the 10 µM, 20 µM treatment). The cDNA was synthesized and quantified by real-time PCR and normalized against a nontreatment control (A) *Cyclin D1 gene expression*; (B) *CD44 gene expression*. **P <0.05, ***P <0.001 and ****P <0.0001 when compared to untreated controls.

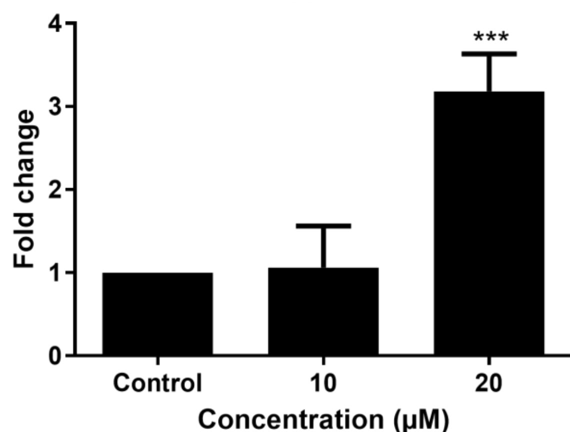

Figure 7. AH upregulate the expression of *p53*. ***P <0.001 when compared to untreated controls.

4. Discussion

Irregularities in the Wnt/β-catenin signaling pathway have been associated with CSCs renewal, making this pathway as one of the important signaling pathways for therapeutic intervention in anti-cancer treatments (Chen et al., 2013; and Weinberg, 2014; Mayan et al., 2016, Wang et al, 2021). Despite the recent pre-clinical and clinical advances made in target-based anti-cancer drug discovery, there is no FDA approved small-molecule inhibitor available which can target Wnt/β-catenin signaling. Potential Wnt/β-catenin signaling inhibitors such as XAV939, OMP-18R5, JW55, OMP-54F28, PRI-724, and LGK974 are still being investigated in clinical studies (Jang et al., 2015, Wang et al, 2021). In the present study, a small molecule named α-hederin (AH) was identified as potential inhibitor of Wnt/β-catenin signaling pathway by molecular docking and molecular dynamics analysis. Results were confirmed by further *in vitro* studies using bCSCs. AH is a triterpenoid saponin compound found in *Nigella sativa* seeds (Kumara and Huat, 2001). *N. sativa* has been traditionally used in many parts of the world for the treatment of various types of cancer (Dabeer et al ,2022). Previous studies have demonstrated the *in vitro* anticancer activity of relatively polar extracts of *N. sativa* and the AH has been identified to as a major active ingredient in those extracts (Kamara and Huat., 2001). To date, the ability of AH to modulate the Wnt/β-catenin signaling pathway and its anticancer activity against bCSCs is not reported.

The interaction between β-catenin and Tcf4 derived peptide is essential for the activation of the Wnt/ β -catenin signaling pathway (Gan et al., 2008). β-catenin is a multifunctional protein, which possesses a long and shallow groove super helical structure. This long and shallow groove site on the β-catenin serves as the binding site for the linear Tcf4 derived peptide. Due to the large binding interface between β-catenin and Tcf4 derived peptide, it extremely difficult to find small molecules, which can occupy the whole binding pocket and thereby disrupting the binding of Tcf4 derived peptide (Florence et al., 2001). However, there are three major hot spots (sites A, B, C) on the β-catenin protein surface, which are important for the maintenance of binding affinity between Tcf4–β-catenin complexes (Fasolini et al., 2003). Out of these three hot spots, the hot spot A has been reported as the most important hot spot as it possesses essential polar residues such as; Lys 435, Arg 469 and Lys 508, which are critical for β-catenin-Tcf4 interaction (Fasolini et al., 2003).

As demonstrated in the present study, AH can disrupt the interaction between Tcf4 and β-catenin by blocking the key binding site (hot spot A) through the formation of hydrogen bonds with β-catenin Lys 435 and this interaction also prevents the formation of hydrogen bonds between Tcf4 Aspartic acid 16 and Lysine 435 (Florence et al., 2001). AH is also capable of forming hydrophobic interactions and a hydrogen bond with Arg 469 and Lys 508 respectively. Previous studies have also shown that the hydrophobic interactions between β-catenin residues Cys 466, Pro 463, and Arg 386 and Tcf4 residues; Ile19 and Phe 21 have significant functional contributions. It has been reported that loss of this interaction could lead to ~60% functional loss (Florence et al, 2001) and therefore blocking this interaction results a significant inhibitory activity. The carboxylate group of AH blocks this interaction by forming a salt bridge with Arginine 386. This interaction places AH in the hydrophobic cleft of β-catenin, which helps to interact with Ile 19 and Phe 21 of Tcf4.

According to drug likeness analysis (Table 3 and 4), AH have the potential to be used as drug. Although, based on the Lipinski rule of 5, there are four basic physicochemical parameters [molecular weight; log-P, H-bond donors and H-bond acceptors (MWT_500, logP_5, H-bond donors_5, and H-bond acceptors_10)] that a compound must fulfill to be used as a drug. AH violated 3 out of the five Lipinski rules. However, it does not nullify its potential use as a drug candidate. Of note, about 30 % of FDA-approved drugs violate the Lipinski rule of five (Wilkinson, 2007). Furthermore, AH obeyed the Veber's rule and passed the Egan's test (Table 3), indicating that two compounds have a good oral absorption

(Elipilla, 2015). In addition toxicity predicted by admetSAR (Table 4) showed that AH is non-carcinogenic and non-cytotoxic in nature and are safe to administer.

As the AH was found to inhibit Tcf4– β-catenin interaction *in-silico*, the compound was subjected for *in-vitro* evaluations to investigate its effects on proliferation, apoptosis and regulation of Wnt/β-catenin signaling pathway downstream genes in bCSCs. As expected, AH effectively decreased bCSCs cell proliferation at 24 h post-incubation. Furthermore, the caspase assay and DNA fragmentation assay, revealed that AH induce apoptosis in bCSCs in a dose dependent manner. This indicated that AH is a potential β-catenin/Tcf4 specific bCSC inhibitor.

Due to the dysregulation of Wnt/β-catenin signaling pathway, over-expression of downstream genes of the Wnt/β-catenin signaling pathway such as, *Cyclin D1* and *CD44* has been reported (Al-Hajj et al., 2003; Ginestier et al., 2007; Reya and Clevers, 2005; Zeilstra et al., 2008). *Cyclin D1* is a proto-oncogene, while *CD44* is a stem cell marker and a critical regulator of CSCs stemness (Shan et al., 2009; Wang et al., 2018). Gene expression analysis by quantitative RT PCR revealed that both AH and CHOL can significantly ($p<0.05$) down-regulate the expression of *Cyclin D1* and *CD44*. Moreover, AH significantly ($p<0.05$) up-regulate the tumor suppressor gene *p53*. Although the anti-cancer efficacies of AH have been reported in several cancer cells (Kumara and Huat, 2001; Adamska et al., 2019), this is the first report to show evidence that AH can inhibit the proliferation of bCSCs by targeting the Wnt/β-catenin signaling pathway.

5. Conclusion

Results of the present study indicated that, AH inhibits the growth of bCSCs and induced apoptosis through down regulation of Wnt/β-catenin signaling pathway leading to down regulate the Wnt/ β-catenin target genes. Results further validate the use of *N. Sativa,* a medicinal plant of which one of the main ingredients is AH, in the traditional systems of medicine for the management of various cancers.

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Disclosure

The authors report no conflicts of interest in this work.

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