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Alantolactone Inhibits the Expression of STAT3 Downstream Target Genes: An *In Silico* and *In Vitro* Study

Nabia Fayyaz[1], Abrar-ul-Haq[1], Haiqa Siddique[2], Sadia Zahoor[1], Zunaira Khalid[1], Muhammad Akhtar Ali[2]* and Muhammad Khan[1]*

[1]*Cancer Research Lab, Institute of Zoology, University of the Punjab, Quaid-e-Azam Campus Lahore-54590, Pakistan*
[2]*School of Biological Sciences, University of the Punjab, Quaid-e-Azam Campus Lahore-54590, Pakistan*

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MK and MAA designed and supervised the study. NF performed experimental work and wrote initial draft. AuH, HS, SZ and ZK assisted in experimental work. MK edited and approved the manuscript.

Key words
STAT3, c-MYC, Cyclin B1, Survivin, ALT, MCF-7, *In-silico*, Gene expression

ABSTRACT

One of the most significant transcription factors that contribute to nearly all forms of cancers is the signal transducer and activator of transcription 3 (STAT3). It amplifies the expression of several cell survival genes such as cyclin D1, bcl-2, c-MYC, and survivin and thereby prompts unrestricted cancerous growth. Sesquiterpene lactones are a very potent group of antitumor compounds which are extracted mainly from plants and have been reported to inhibit activation of STAT3 in different human cancers. The present study aimed to evaluate the effect of ALT, a potent sesquiterpene lactone, on STAT3 downstream target genes in MCF-7 breast cancer cells. Our qPCR data showed that ALT significantly reduced the mRNA expression of key STAT3 downstream target genes including c-MYC, survivin, and cyclin B1. Moreover, our computational docking study revealed that ALT could directly bind to c-MYC, survivin, and cyclin B1 through various binding interactions. Moreover, ALT exhibits drug-likeness and excellent pharmacokinetics properties as well.

INTRODUCTION

STAT (signal transducer and activator of transcription) is a major cluster of cytoplasmic proteins that regulate several vital functions of cells. This group comprises eight members, each of which plays a role in cell metabolism (Darnell *et al*., 1994). Among eight members, STAT3 is most extensively studied protein. It involves diverse physiological mechanisms such as cell differentiation, cell sustenance and propagation, angiogenesis, and metabolism in physiologically normal cells. It delivers the transcriptional signals from the growth factors (e.g., cytokines) at the cell membrane to the nucleus (Zou *et al*., 2020). Dysregulated STAT3 activity has been identified in numerous cancers. Watson and Miller (1995) first detected elevated levels of STAT3 in breast cancer; despite the presence of many potent repressors such as protein tyrosine phosphatases (PTPs) (Wake and Watson, 2015). Furthermore, the hyper-stimulation of STAT3 also leads to over-expression of its downstream target genes such as Bcl-2 and Cyclin B1 (Yang *et al*., 2022; Sun *et al*., 2019). To date, a large number of bioactive molecules have been identified and isolated from plants to inhibit STAT3 and its downstream target genes in tumors (Yang *et al*., 2022).

Sesquiterpene lactones (SLs) are an immense group of naturally occurring compounds with anticancer and anti-inflammatory properties. These compounds have been extracted from over 100 families of flowering plants; Asteraceae family being the predominant family. Numerous cell cultural and in situ studies have manifested that the SLs exert their anti-tumor effects by inducing apoptosis and inhibiting metastasis (Zhang *et al*., 2005). Alantolactone (ALT) is a potent member of the SL family. It is primarily sequestered from the roots of *Inula helenium,* a renowned accustomed Chinese therapeutic herb (Gierlikowska *et al*., 2020). It has been shown to suppress cancer cell viability through numerous mechanisms in different cancer types, for instance, lung adenocarcinoma (Maryam *et al*., 2017), cervical cancer (Sun *et al*., 2021), glioblastoma (Khan *et al*., 2012), hepatocellular carcinoma (Khan *et al*., 2013), pancreatic cancer (Zheng *et al*., 2019), breast cancer

* Corresponding author: mkhan.zoolo@pu.edu.pk, akhtaralimuhammad.sbs@pu.edu.pk
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(Naderi *et al*., 2022), and prostate cancer cells (Babaei *et al*., 2020).

Recent study has shown that ALT suppresses malignant growth by downregulating STAT3. ALT stimulates STAT3 glutathionylation in the A549 lung adenocarcinoma cells, resulting in cancer suppression (Maryam *et al*., 2017). It hinders STAT3 activity in non-small cell lung cancer both *ex vivo* and *in vivo* (Fu *et al*., 2022). ALT subdues cervical tumor (HeLa cells) proliferation by declining the expression of BMI1 and p-STAT3 (Sun *et al*., 2021). It also stimulates mitochondrial apoptotic death by provoking reactive oxygen species (ROS) and impairing STAT3 in HepG2 cells (Khan *et al*., 2013). ALT treatment in combination with Erlotinib (as nanoparticles) diminishes the STAT3 signaling pathway in pancreatic tumors (Bao *et al*., 2021). It suppresses metastasis by reducing vimentin and N-cadherin through STAT3 attenuation in MDA-MB-231 cell line (Naderi *et al*., 2022). The negative regulation of the STAT3 pathway results in the suppression of its downstream-responsive genes as well, as Liu *et al*. (2017) identified that Afatinib enhances the inhibition of STAT3 in human osteosarcoma cells and causes a decline in the expression of BCL-2.

The main purpose of present study was to evaluate the effect of ALT on expression of three major STAT3 downstream target genes including c-MYC, survivin and cyclin B1 using MCF-7 breast cancer cell line. STAT3 stimulates these target genes through multiple mechanisms and once activated these genes participate in cancer formation, progression, and metastasis. Since ALT has been reported to inhibit STAT3 activation, it is hypothesized that it can also be used to subdue the expression of STAT3 target genes.

MATERIAL AND METHODS

Cell culture and drug treatment

MCF-7 breast cancer cells were cultured in recommended medium (DMEM + 10% FBS) in a CO_2 incubator overnight and treated with ALT using 10 and 20 µM drug concentrations. The cells were observed and photographed following drug treatment.

mRNA isolation and cDNA synthesis

The mRNA isolation was achieved using the FavorPrep Tissue Total RNA Purification Mini Kit as per manufacturer's instructions. The Thermo Scientific RevertAid First Strand cDNA Synthesis Kit was employed for cDNA synthesis as per the kit's instructions.

Real-time qPCR analysis

The sequences of primers used in the current analysis are provided in Table I. The stock solutions of primers were prepped by adding 250µl of distilled water into the primer (100 µM). Then, each primer's stock solution (10 µl) was added to distilled water (90 µl) to make working primer solutions in Eppendorf. These solutions were placed at -20 ºC until further use.

Table I. Primer sequences.

Genes	Primer sequence 5`→ 3`	Length
c-MYC	F GCTGCTTAGACGCTGGATTT	20
	R CTCCTCCTCGTCGCAGTAGA	
Cyclin B1	F TTGGGGACATTGGTAACAAAGTC	21
	R ATAGGCTCAGGCGAAAGTTTTT	
Survivin	F AGGACCACCGCATCTCTACAT	23
	R AAGTCTGGCTCGTTCTCAGTG	22
GAPDH	F ATGCCTCCTGCACCACCAACT	21
	R ATGGCATGGACTGTGGTCATGAGT	

The qPCR reaction mixture was prepped by following the instructions from Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X). GAPDH was used as a housekeeping gene in this procedure. The trial was designed in triplicate. The protocol and plate layout of the experiment were set up in Thermo Fisher Pico Real software and the protocol was initiated on it.

The Cq/CT values were analyzed to discover gene expression. The ΔΔCT technique was employed to identify the fold difference in varied gene expressions of cells treated with different doses of ALT compared to control cells.

In silico *analysis*

Extraction of proteins and ligand (ALT)

The proteins; c-MYC, cyclin B1, and survivin, (PMIDs: 1NKP, 2B9R, and, 1E31, respectively) were sourced from the protein data bank (PDB) in PDB format. Protein structures exhibit the resolution of 1.80 Å, 2.71 Å and 2.90 Å, respectively. Similarly, the ALT structure (3D-conformer) was acquired from PubChem in SDF format (PubChem CID: 72724).

Target proteins preparation

The unwanted ligands, water molecules, or heteroatoms attached to the downloaded protein already might interfere in the molecular docking (MD) of ALT with these proteins. So, these unwanted compounds must be removed before MD (Haq *et al*., 2024). The proteins were organized through Autodock Vina by eradicating water molecules and introducing hydrogen atoms (polar only) and Kollman charges. Pdbqt format was utilized to save

the files, this format is required for MD in AutoDock Vina. PyMol software (3.0 version) was operated to transform the ligand's SDF format into pdb. AutoDock Vina was also used to change the ligand (pdb) file into pdbqt format.

Docking

A grid dimension file (the grid box chooses the borderline of the docking of the ligand with our chosen macromolecule) of each protein (pdbqt) was created through AutoDock. The material from the grid file was utilized to generate the protein(s) config file. A command was provided with the command prompt for making output files and revealing the binding affinities.

Visualization

The outcome was envisioned through Biovia Discovery Studio and LigPlot. The associations of protein (pdbqt) and the output file were envisioned through 2-D diagram of Discovery Studio. This software was also used to produce the protein-ligand complex. LigPlot was employed to depict the complex structure.

SwissADME screening

The physicochemical attributes of ALT were reviewed by PKCSM and ADMETlab 2.0 database servers. ALT's canonical smiles were derived from PubChem. The Swiss ADME server provided the boiled egg diagram.

Statistical analysis

Data were expressed as Mean ± SD (n=3). Students't-test was performed to determine the significant difference between control and treated groups using IBM SPSS Statistics. *P<0.05, **P<0.01, ***P<0.001

RESULTS

Effect of ALT on cell morphology

The effect of ALT on MCF-7 cell morphology has been shown in Figure 1. The control cells morphology was not distorted and their uniform distribution was also observed in culture media after 24 h. ALT treatment decreased the number of cells and exerted severe morphological changes in cells including cell shrinkage, loss of cellular geometry and detachment from the bottom of plate.

Effect of ALT on STAT3 downstream target genes

The effect of ALT on mRNA expression of some major STAT3 downstream target genes such as c-myc, cyclin B1 and survivin, qRT-PCR was measured by qPCR. ALT exhibited a suppressive effect on mRNA expression of all three studied STAT3 downstream target genes significantly at both doses (10 and 20 µM). However, it is important to mention that the suppressive effect of ALT on mRNA expression of all three studied genes was not dose-dependent as shown in Figure 2.

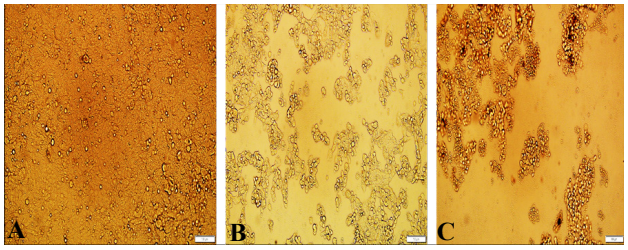

Fig. 1. Effect of ALT treatment on MCF-7 cells morphology. A, MCF-7 cells were treated with vehicle control (DMSO) for 24 h. B & C, MCF-7 cells were treated with 10 and 20 µM ALT for 24 h, respectively. Following treatment of cells with vehicle and ALT, images were captured (scale bar = 100 µm).

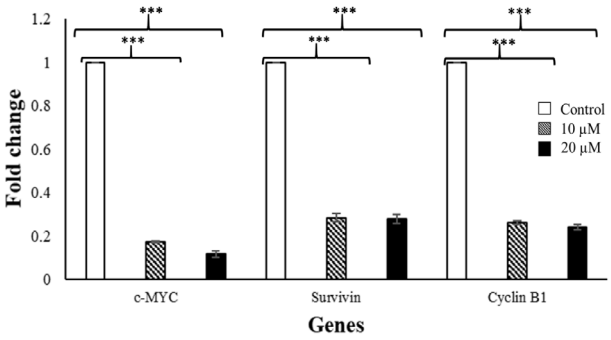

Fig. 2. Effect of ALT on expression of STAT3 downstream target genes. MCF-7 cells were treated with vehicle or ALT for 24 h. RNA was extracted using FavorPrep Tissue Total RNA Purification Mini Kit and was reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit. qPCR was performed as described in material and methods. ΔΔCT method was employed to measure the relative fold change in gene expression of c-MYC, survivin and cyclin B1. *p < 0.05, **p < 0.01 and ***p < 0.001 vs vehicle control group.

ALT is predicted to bind with STAT3 downstream target proteins

Since ALT suppressed the mRNA expression of STAT3 downstream target genes as evident from our qPCR data, we were interested to know if ALT could directly bind to these proteins in addition to downregulating mRNA expression. Our *in silico* investigation demonstrated that ALT shows binding interactions with these proteins through numerous types of interactions such as carbon-hydrogen bonds, hydrogen bonds, etc. The docking score of ALT with c-MYC, survivin, and cyclin B1 was -7.0, -7.1, and -9.4 kcal/ mol, respectively. The various bonds with cyclin B1, survivin, and c-MYC are shown in Figure 3.

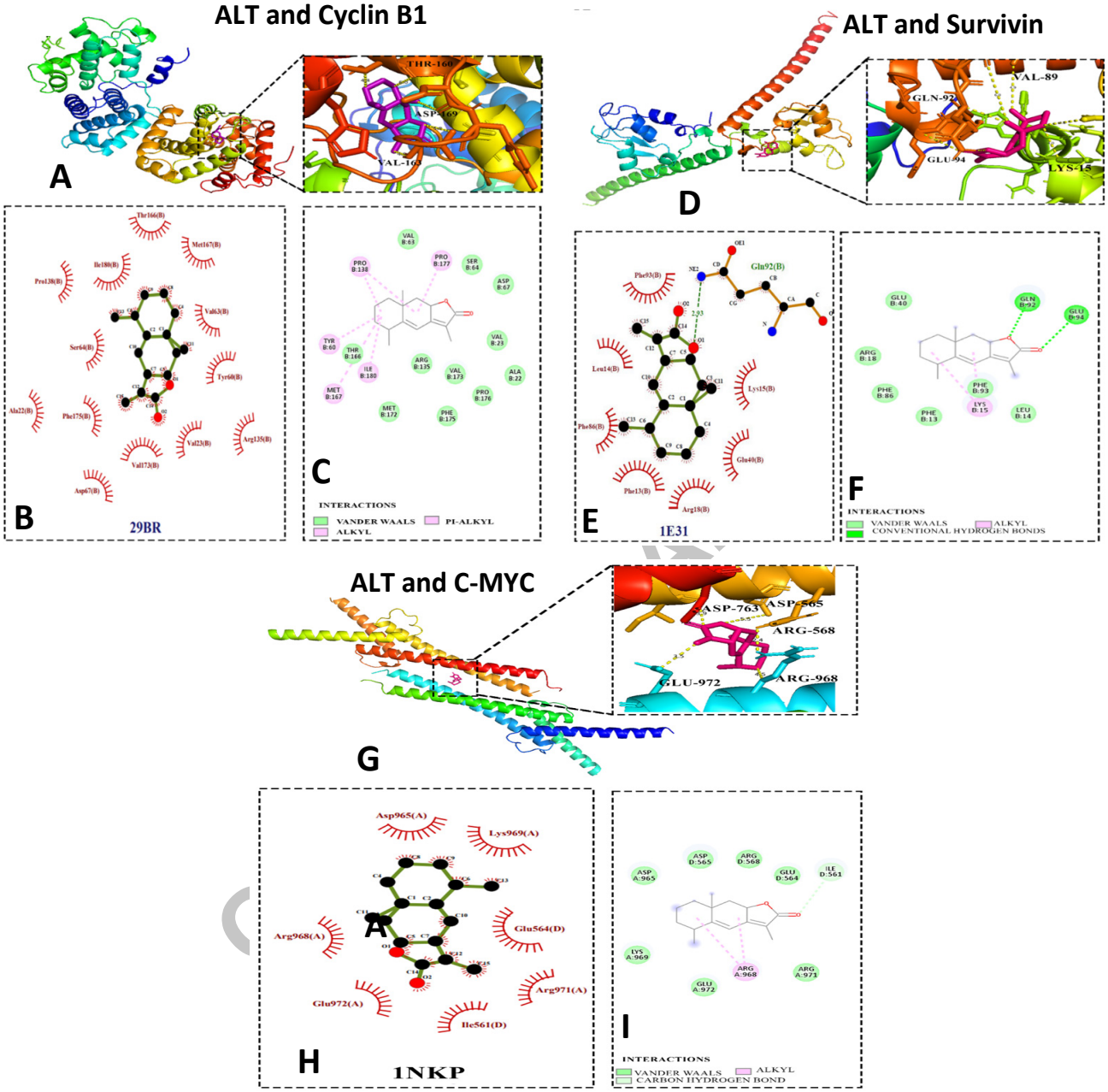

Fig. 3. Binding interactions between ALT and Cyclin B1 (A, B, C), ALT and Survivin (D, E, F) and ALT and C-MYC (G, H, I). A, D, G, visualization of 3D interaction of cyclin B1 (29BR) with ALT by using PyMol. B, E, H, 2D diagram obtained by LigPlot$^+$ (version 2.2) to visualize binding interactions. C, E, I, Discovery studio was used to visualize binding interactions (vander waals, pi-alkyl and alkyl bonds) of ALT with different amino acids of Cyclin B1, surviving and C-MYC.

Evaluation of physicochemical and pharmacokinetic properties of ALT

ALT examined through the SwissADME server manifested that it is readily immersed through the buccal cavity, has high permeability through the blood brain barrier (BBB) and intestine, and is not the P-glycoprotein substrate as shown in Figure 4. Rizwana *et al.* (2023) highlighted in their research the nonbelligerent nature of ALT as it aligns well with Lipinski's rule of five. The physicochemical attributes of ALT were contrasted with standard values in Table II.

Table II. Physicochemical properties of alantolactone.

Properties	Standard values	Obtained values
Molecular weight (g/mol)	100-600	232.150
nHA	0-12	2
nHD	0-7	0
nRot	0-11	0
nRing	0-6	3
MaxRing	0-18	13
nHet	1-15	2
fChar	(-4) - (+4)	0
nRig	0-30	17
Stereo centers	≤ 2	4
TPSA (Å²)	<140	26.300
logS	-4–0.1	-3.518
logP	0-3	3.237
logD	1-3	2.952

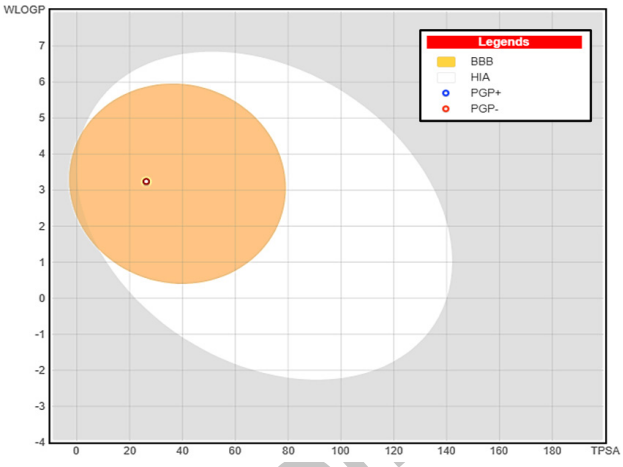

Fig. 4. Boiled egg plot. In this visual representation of a boiled egg, the grey region signifies a region of inadequate absorption; ALT has crossed the egg white indicating that it has elevated absorption via the digestive system, the yolk indicates the BBB (blood-brain barrier). As ALT is inside this area representing that it has elevated BBB permeability, the red dot indicates that ALT is not a substrate of P-glycoprotein.

DISCUSSION

In this research, we have shown that ALT, a natural bioactive anti-cancer compound, diminishes the expression of STAT3 downstream target genes. Interestingly, our computational data further demonstrated that ALT forms stable bonds with these proteins making hydrogen bonds only with survivin. However, the binding affinity with cyclin B1 is more as compared to other proteins showing only alkyl, van der Waals, and pi-alkyl interactions. This shows that the general binding affinity is considerably influenced by non-covalent interactions beyond hydrogen bonding, such as hydrophobic interactions, which can play a critical part in stabilizing the drug-protein complex. This observation is supported by studies showing that resilient binding affinities can be attained through other interactions, even in the absence of hydrogen bonds (Rizwana *et al*., 2023; Lee and Barron, 2017).

Previous literature has manifested that several drugs repress the expression of c-MYC, Cyclin B1 and survivin genes to restrict carcinogenesis as these genes significantly enhance cancer growth. c-MYC modulates various crucial functions in cells such as apoptosis, cell cycle progression, cell differentiation, and proliferation. It is highly expressed in about 70% of cancers and scientific literature suggests its attenuation ceases cancer growth (Yunta, 2016; Llombart and Mansour, 2022). ALT mitigates the expression of c-Myc in IM-9 cells (myeloma), in a dose-correlated fashion (Yao *et al*., 2015). Shi *et al*. (2011) reported that ALT treatment (1 µg/ml) decreased c-MYC expression 6-fold in comparison to control in HCT-8 cells, in a time-dependent fashion. ALT diminishes the tumor propagation in human osteosarcoma 143B cells, melanoma, and esophageal cancer cells by inhibiting the β-catenin/WNT signaling pathway. It halts the β-catenin expression leading to repression of c-MYC and GSK3β phosphorylation in a dose-related fashion in *in-vivo* study model (Yang *et al*., 2022; Zhang *et al*., 2023; Wang *et al*., 2021). In line with the previous reports, ALT remarkably reduced the mRNA expression of c-MYC in the MCF-7 cell line in the present study.

Cyclin B1 is a component of the essential contributors of the cyclin group that governs cell cycle continuity by binding with other cell cycle modulators. It primarily modulates the cell cycle transformation at the G2/M interface (Kang *et al*., 2024). ALT promotes ROS-sensitive apoptotic death in anaplastic thyroid leukemia (ATL) and hampers the G2/M checkpoint of the cell cycle. It attenuates the cyclin B1 and CDC2 expression (Hu *et al*., 2023). Yang *et al*. (2022) highlighted the similar impacts of ALT in osteosarcoma cell lines. It represses Bcl-2 expression to commence the mitochondria-mediated apoptotic death and conversely disturbs the cell cycle by inhibiting cyclin B1 expression at the G2/M interface in a dose-correlated fashion. ALT ceases colorectal cancer (CRC) development by interceding in several critical cellular signaling pathways such as MAPK and STAT3 pathways. It suspends the cell cycle at the G0/G1 interface by diminishing cyclin B1, D1, and E in a time- and dose-related manner in HCT-116 cells (Ren *et al*., 2021). As expected, ALT treatment attenuated the cyclin B1 expression in the current study. However, the

decrease in cyclin B1 expression in MCF-7 cells was not dose-dependent.

Survivin is a minuscule protein that safeguards the cells from the apoptotic process, monitors mitosis with the assistance of chromosomal passenger complex (CPC) and mitochondrial metabolic activities, initiates blood vessel formation and cellular migration, and is principally indicated by cancer stem cells (Wheatley and Altieri, 2019). STAT3 suppression in primary effusion lymphoma (PEL) cells causes a reduction in the expression of survivin and Bcl-2 family proteins (Aoki *et al*., 2003). Kanda *et al*. (2004) reported that elevated STAT3 gives rise to increased survivin expression in gastric cancer cell lines. ALT halts THP-1 cell development by hindering the STAT3 signaling pathway. It decreases survivin expression and incites apoptosis in this cell line (Ahmad *et al*., 2021). The diminution in survivin expression by ALT alone is more in contrast to the collaborative effect of ALT with cisplatin in the A549 cell line. However, ALT diminishes Bcl-2 family proteins and p-STAT3 as well to recover cisplatin anti-tumor activity in this cell line (Ahmad *et al*., 2020). Similar to cyclin B1, ALT inhibited the mRNA expression of survivin in MCF-7 cell line.

Plants derived bioactive molecules are multi-target molecules and exhibit their activity through multiple mechanisms (Khan *et al*., 2015). Since ALT inhibited the mRNA expression of cMYC, Cyclin B1 and Survivin, we were interested to know if ALT could directly interact with protein products of these genes. For this we performed molecular docking study and data demonstrated that ALT could bind with all three proteins through different binding interactions This set of data demonstrated that ALT could inhibit the mRNA as well as Protein expression of cMYC, Cyclin B1 and Survivin through different mechanism.

CONCLUSION

Collective data demonstrated that ALT exhibits cytotoxicity and effectively inhibits the mRNA expression of STAT3 downstream target genes (c-MYC, survivin, and cyclin B1) in the MCF-7 cancer cell line. The molecular docking study revealed that ALT could also interact with these three genes proteins through various binding interactions. Finally, ALT exhibits drug likeness and excellent pharmacokinetics properties which are of prime importance for any bioactive molecule to develop it into a drug.

DECLARATIONS

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Statement of conflict of interest

The authors have declared no conflict of interest.

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