Mol Biol Rep (2016) 43:1411–1422
DOI 10.1007/s11033-016-4074-z

ORIGINAL ARTICLE

Evaluating anti-oxidant potential of ganoderic acid A in STAT 3 pathway in prostate cancer

Balraj Singh Gill[1] · **Sanjeev Kumar**[1] · **Navgeet**[2]

Received: 15 June 2016 / Accepted: 6 September 2016 / Published online: 17 September 2016

Abstract Evaluating anti-oxidant potential of Ganoderic acid A in STAT 3 pathway in Prostate cancer. Molecular docking and ADMET activities of different isoforms of ganoderic acid on STAT 3 pathway were performed by Maestro 9.6 (Schrödinger Inc). The ganoderic acid A is best-docked among isoforms which analyses the expression level of antioxidant and STAT 3 pathway in PC-3 cells. The receptor-based molecular docking reveals the best binding interaction of SH2 domain of STAT3 and ganoderic acid A with GScore (−6.134), kcal/mol, Lipophilic EvdW (−1.83), Electro (−1.1), Glide emodel (−31.857), H bond (1.98), MM-GBSA (−69.555). The molecular docking QikProp analyzed the absorption, distribution, metabolism, excretion, and toxicity (ADME/T). The ganoderic acid A is best-docked among isoforms which downregulates the expression of STAT 3 in PC-3 cells. Moreover, ganoderic acid A inhibits proliferation, viability, ROS, DPPH, and analyzed the expression of SOD1, SOD2, and SOD3 by Real time PCR in a PC-3 cell in a dose-dependent manner. Molecular docking revealed the mechanistic binding of Ganoderic acid A in STAT3 signaling, which inhibits the proliferation, viability, and ROS in PC-3 cells.

✉ Navgeet
geet20capricorn@gmail.com

Balraj Singh Gill
gillsinghbalraj@gmail.com

Sanjeev Kumar
sanjeevpuchd@gmail.com

[1] Centre for Biosciences, Central University of Punjab, Bathinda, India

[2] Department of Biotechnology, Doaba college, Jalandhar, Punjab, India

Keywords STAT3 · Cancer · Ganoderic acid · Molecular docking · Antioxidant

Introduction

Signal transducer and activator of transcription (STAT) are the cytoplasmic proteins acting as transcription factors and signal messengers in the normal cellular processes. During stimulus from cytokines and growth factors, STAT gets phosphorylated at tyrosine 705 and translocated from cytoplasm to the nucleus [1]. STAT factors are recruited to phosphotyrosine-containing motifs of activated receptor chains via their SH2 domains. The noncatalytic domains (SH2) target to specific phosphotyrosyl peptide sequences thus controls numerous signaling events. STAT pathway has a key role in initiation and progression of complex diseases including diabetes and cancer by maintaining immune system homeostasis and hematopoiesis of the body [2]. Literature survey revealed that inhibition of STAT3 expression by small molecule decreased the tumor volume and tumor recurrence [3]. STAT3 is constitutively active in a variety of anomolies such as cancers, multiple myelomas, cancers, hepatocellular carcinoma, lymphomas, and leukemia [1–4], makes it hot spot target in the drug discovery and therapeutic intervention [5]. Aberrancy in STAT signaling revealed the link between defects in activation of Janus kinase (JAKs) which consequence in human immunodeficiency syndromes, human X-linked combined immunodeficiency (XCID) [6]. Activation of the STAT 3 result in the physiological response, but deregulation or hyperactivation leads to damage of tissue consequence in Crohn's disease, pleurisy, and psoriasis [7]. Several methods and approaches (nucleotide and non-nucleotide) were developed undergoing clinical evaluation for the treatment of various cancers [8]. However, due to some

detrimental effect of those products, there is need to develop a new compound which will be effective in cancer treatment. Therefore, any treatment counteracting the hyper-expression or activation of STAT3 has been considered as a new strategy to treat these world-widely increasing pathologies [9]. The present study focused to find natural products disrupting the process of translocation, dimerization of STAT3 and STAT3–DNA binding which improves the specific binding [10]. Literature survey exposed the role of different natural products such as curcumin [11], quercetin [12], withaferin A [13], betulinic acid [14], ursolic acid [15] to modulate STAT3 signaling cascade. One of the renowned natural product is *Ganoderma lucidum*, polypore family of mushrooms with myriad therapeutic indications [16], but its effect on STAT has yet to be explored. Our research group is presently engaged in analyzing the anticancer properties of *G. lucidum* and its constituents [16, 17]. *Ganoderma lucidum* mainly comprises of terpenes, proteins, and polysaccharides [18]. Among different bio-constituents in *Ganoderma lucidum*, ganoderic acid was explored for revealing mechanism in different cancer pathway [19]. Unfavorable condition (abiotic stress) changes the morphology and biochemical composition of *G. lucidum* to cope the prevailing stress conditions. Our previous study indicates the abiotic stress conditions, changes the biochemical composition of *G. lucidum*. Furthermore, it also gives the indication about the role of host plant of *G. lucidum* in their biochemical composition. *G. lucidum* which grows on host plant *Azadirachta* has elevated the level of biochemical composition.

In order to know more about the external morphology, chemical composition and orientation of the fruiting bodies of *G. lucidum* and element present in crude extract under stress condition, studied was conducted by Scanning electron microscopy (SEM). This study qualitatively or semi-quantitatively gives the chemical composition using Energy-dispersive X-ray spectroscopy (EDX) of the fruiting bodies. It was concluded that fruiting bodies in the stress condition upregulate the level Ca, K, Fe elements which may help *G. lucidum* to cope with abiotic stress (Table 1). In the present study, molecular docking was performed for the first time on SH2 domain of STAT3-DNA binding with 50 isoforms of ganoderic acid along with natural inhibitors to reveal the mechanistic binding of STAT 3 cancer signaling pathway. After molecular docking, best isoforms of ganoderic acid on the basis docking score was checked for the biological activity in the cell line. Biological activity includes proliferation, viability, ROS, DPPH were performed and the expression was analyzed for SOD1, SOD2, and SOD3 by real-time PCR in a PC-3 cell in a dose-dependent manner.

Table 1 Elemental composition by percentage of weight and atomic by energy-dispersive X-ray spectroscopy (EDX) in SEM

S. no	Element	Weight (%)	Atomic (%)
1	Al K	13.19	17.35
2	Si K	20.97	26.50
3	K K	18.68	16.96
4	Ca K	36.84	32.63
5	Fe K	10.32	6.56

Methodology

Preparation of ligands and protein molecule

Methodology was taken from previous study in three-dimensional structure of protein crystallized structure was downloaded from PDB site with STAT3/DNA complex (PDB; 1BG1) [20] from which water molecules were removed to avoid unnecessary interaction [19]. Subsequently, polar hydrogen were incorporated to fulfill the inappropriate valency of the protein atoms, ensuing in increased polarizability of bonds. The increased polarizability increases the probability of interacligand-protein interactions [19]. Later, processed protein structure was checked for the stereochemical quality by residue-by-residue geometry as well as overall structural geometry. The preparation of different isoforms of ganoderic acid and others natural activators structures were carried out by software ChemBioDraw Office [19] (licensed @ Cambridge's soft). Literature survey highlighted that some of the natural activators such as curcumin [11], quercetin [12], withaferin A [13], betulinic acid [14], ursolic acid [15] target STAT3/DNA complex signaling. These compounds were subjected in the Ligprep for the ligand preparation by Maestro 9.3. Ligprep was corrected by addition and optimization of hydrogen bonds for correcting the valency, removal of bad contacts, creation of disulfide bonds, capping of protein terminals optimization of bond lengths and fixing of missing residues in order make protein ready for docking. The prepared structure was then minimization and afterwards, optimization in the optimized potential for the liquid simulations (OPLS 2005) force field to acquire an energetically stable geometry [21–23].

Receptor grid formation

Grid pre-calculates grid maps of interaction energies by scoring electrostatic, dispersive energies of surrounding target with a macromolecule prior to the docking [19]. Grid maps to determine the total interaction energy for a ligand with a macromolecule in the receptor based molecular docking [19]. Grid mapping computes the crucial coordinates of STAT3/DNA in the protein atomic data, important for targeting binding in the docking. In addition, grid mapping

gives an appropriate surface topology for the ligand atoms for interaction with the STAT3/DNA domain. Grid mapping is a pre-requisite to direct different isoforms of ganoderic acids to look for their region of the strong affinity of the STAT3/DNA domain (Fig. 1). Grid was generated for the search of favorable interaction and best post during docking which represent conformation, position, orientation about the receptors [24].

GLIDE molecular docking

The receptor based molecular docking was carried forward after preparing ligands, proteins, and preparation of grid formation of the active site of protein. GLIDE docking tools predict the systematic and computational simulation method for predicting best binding orientation to the protein target. GLIDE molecular docking output GScore (empirical scoring function) which is a combination of various parameters important for binding energy. The GScore is calculated by calculating ligand–protein interaction energies, root mean square deviation (RMSD), hydrogen bonds, hydrophobic interactions, internal energy, π–π stacking interactions and desolvation [19]. GLIDE module of the XP visualizer for analyzing the binding interaction of ligand–protein pattern. The ligands were docked with the X-ray crystal structure of STAT3/DNA (PDB; 1BG1), using GLIDE. The best possible fit compounds results after docking were analyzed for thermodynamic optimal energy value, the potential of bonding and conformations, types of interactions, residues involved in the interaction, and the distance between different residues [25, 26].

Prime/MM-GBSA simulation (free energy calculation)

The proper solvation facilitates in the process of molecular recognition. The solvation effect estimates the binding affinities by MM-GBSA scoring, where MM means molecular mechanics, GB-Generalized Born, SA-Solvent accessible surface area. In MM-GBSA simulation, electrostatics of ligand-receptor complex and solvation were analyzed which has an edge over prior methods of Coulomb-based terms. The Prime/MM-GBSA approach was used to calculate free energy and calculates the binding affinity of the receptor and the ligands [27]. The simulation was based on the structure obtained by receptor-based ligand molecular docking. The ligand poses were minimized by Prime, whereas energy of the complex was obtained by OPLS-2005 force field with VSGB2.0 solvent model in Generalized-Born/Surface Area continuum solvent model [28].

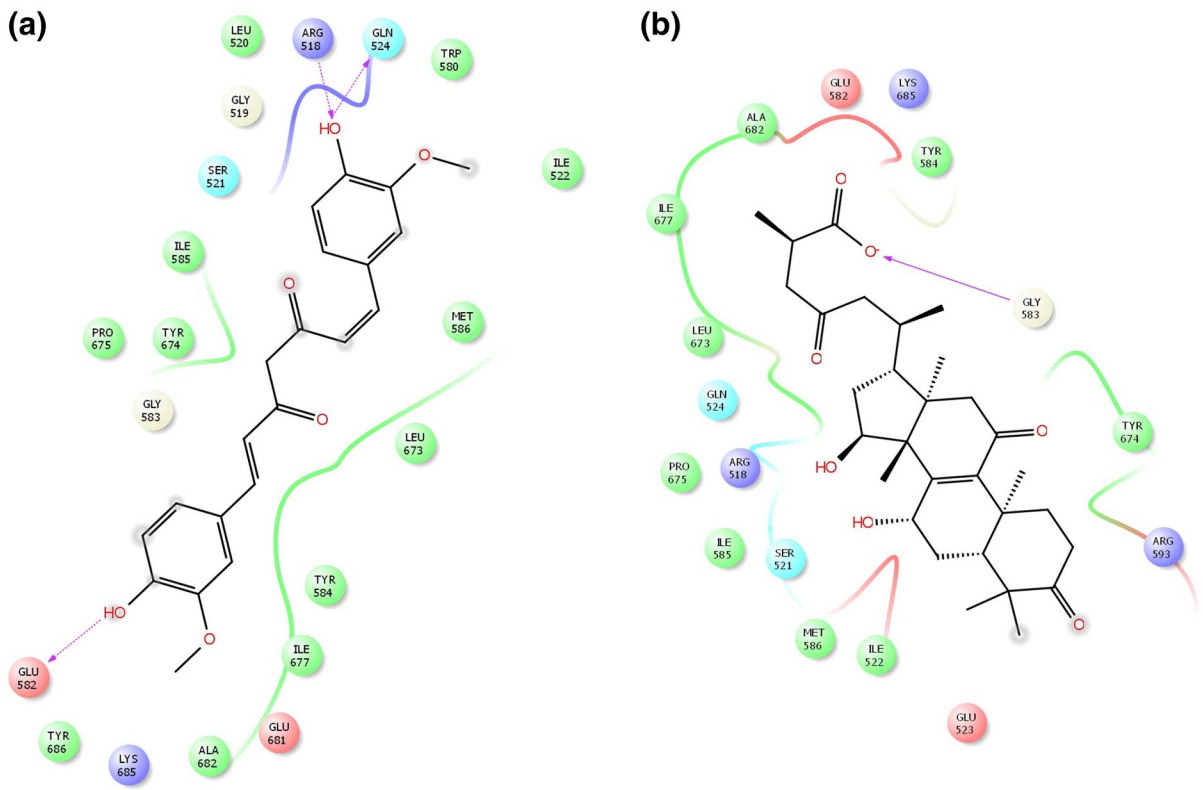

Fig. 1 Receptor-based molecular docking protein–ligands interactions profile of STAT3/DNA with ganoderic acid A and curcumin. Protein–ligand interactions revealed the involved in ganoderic acid A binding acid were Gly 583 and Glu 582, Gln 524, Arg 518 were involved in curcumin

$$\Delta G_{bind} = \Delta_E + \Delta G_{solv} + \Delta G_{SA}$$

$$\Delta E = E_{complex} - E_{protein} - E_{ligand}$$

where ΔE is the minimized energy of complex, protein and ligand.

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

where ΔG is the salvation free energy of the complex protein and ligand.

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

where ΔGSA is surface area energy of the complex protein and ligand.

ADME properties

QikProp is an important tool that calculates properties of the significant descriptors and pharmaceutically relevant molecules by comparing their values with those of 95% of already known pharmaceutical drugs. Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADME/T) properties of the docked ligands molecules were subjected in QikProp tool. It analyzes and predicts different properties of drug about the use and aftermaths of drug intake i.e. absorption, distribution, metabolism, and excretion. It gives the information about QP log Po/w, QP log BB, overall CNS activity, Caco2, MDCK cell permeability, logKhsa for human serum albumin binding, and the percentage of human oral absorption [29].

Biochemical profiling of ganoderic acid

Measurement of DPPH free radical-scavenging activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is one of the reliable methods to measure free radical scavenging capacity. Free radicals scavenging property of Ganoderic acid A was assayed according to the method is given by Shimada [30] with some modifications. BHT (butylated hydroxytoluene) was used as a standard antioxidant. 100 mg of dried material was extracted with 5 mL of hot 80% ethanol. The ethanolic extract was filtered and dried using rotary vacuum evaporator. Extracts with different concentrations were added to 4 mL of 0.4 mM methanolic solution of DPPH. The mixture was subsequently incubated for 30 min in the dark. Absorbance was recorded using spectrophotometer at 517 nm [31].

RNA isolation, cDNA synthesis, and quantitative RT-PCR of SOD and STAT3

About 1 million cells were seeded and supplemented with culture media having 10% FBS and % penicillin/streptomycin, incubated at 37 °C. PC-3 cells were treated with 80 µM of ganoderic acid for 48 h and then, total RNA was isolated and extracted using Trizol (Life Technologies, Gaithersburg, MD). RNA was quantified and later, cDNA preparation was carried out. Approximately 1 µg of total RNA was reverse transcribed into cDNA using PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio Inc). Real-time quantitative PCR was performed with Assay-on-demand Applied Biosystems with primers listed in Table 2. Each data point was repeated in triplicates. Quantification values were obtained using threshold PCR cycle number (Ct), where the increase in signal results in increase in PCR product. The normalization of relative mRNA level in each sample was done with β-actin. The relative expression target gene levels equaled $\Delta Ct = Ct_{\text{target gene}} - Ct_{\beta-\text{actin}}$.

Cell culture

Prostate cancer cell line (PC-3 cells) used in the study was procured from National Centre for Cell Sciences, Pune, India. The cell line was carefully maintained in Ham-12 media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin (units/mL), streptomycin 100 mg/mL. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

MTT cell proliferation assay

Effect of different treatments of ganoderic acid on the growth of prostate cancer cells (PC-3) cells were assessed by using the MTT assay in triplicates. Approximately 10,000 cells were seeded in the 96-well plate in Ham-12 media

Table 2 Primers list

S. no.	Name	Forward primer	Reverse primer
1	SOD 1	ATTCTGTGATCTCACTCTCAGG	GCTAGCAGGATAACAGATGAGT
2	SOD 2	GTGACTTTGGTTCCTTTGAC	GAATAAGGCCTGTTGTTCCT
3	SOD3	TCTCCTCTGCTCCAACAG	GTGGGTCTCGGTATAGGG
4	STAT3	ACCCAACAGCCGCCGTAG	CAGACTGGTTGTTTCCATTCAGAT
5	GAPDH	ACGGATTTGGTCGTATTGGGCG	CTCCTGGAAGATGGTGATGG

containing 10% FBS and incubated overnight at 37 °C followed by serum starvation for 24 h. Cells were exposed to the different concentrations of ganoderic acid (5, 10, 20, 50, 80 μM/mL) in serum-free media which was incubated for 48 h. At the end of 48 h, the medium was removed and replaced with 100 μL of MTT (0.5 mg/mL in PBS) in 10% FBS containing media, and incubated at 37 °C for 4 h in the dark. The supernatant was removed from the wells and the reduced MTT, formazan complex, was solubilized in 200 μL/well dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using microplate reader.

Evaluation of cytotoxic/apoptotic effect

In another set of experiment, the rate of cell death in response to ganoderic acid with treatment was assessed by the trypan blue exclusion test. 2×10^5 PC-3 cells were seeded in six-well culture plates. The adhered cells were treated with ganoderic acid (5, 10, 20, 50, 80 μM/mL) for 48 h. After treatment, both floating cells in the medium and adhered cells on the plate were collected and concentrated by centrifugation. Cell viability was estimated by staining with 0.4% trypan blue for 15 min. Both live (unstained), and dead (stained) cells were counted in three replicates using Automated cell counter (Invitrogen). Percent data of dead cells was calculated and used as an indicator of the degree of cell death.

Effect of ganoderic acid ROS levels (H_2DCF-DA assay)

Intracellular ROS level was measured with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), which undergoes rapid oxidation into the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of intracellular ROS. PC-3 cells (2×10^5) were seeded in six-well culture plates and treated with ganoderic acid (5, 10, 20, 50, 80 μM/mL) for 24 h. Cells were washed with PBS and incubated in PBS containing 10 μM H_2DCFDA for 30 min at 37 °C. The cells were washed with PBS to remove excess dye, and the fluorescence was measured at 485 nm and 530 nm wavelength.

NBT assay

8×10^3 cells/well were seeded into the 96-well plates in Ham-12 media containing 10% FBS and 1% penicillin/streptomycin and then incubated at 37 °C overnight followed by serum starvation for 24 h. After 24 h, the media was changed with fresh complete medium (200 μL) and exposed to different ganoderic acid 5, 10, 20, 40 and 80 μM concentrations. After 48 h, the medium were removed, and incubated with 100 μL 0.1% NBT, incubated for 4 h. The reduced NBT was solubilized with 100 μL 2 M KOH and 100 μL DMSO for 30 min and absorbance was taken at 570 nm.

Statistical analysis

Results were expressed as the mean ± standard deviation of experiments performed in triplicates. Data obtained was subjected to one-way analysis of variance (ANOVA), and significant differences of the mean were determined statistically using Tukey's test using SigmaPlot 11.

Results and discussion (PDB; 1BG1)

The STAT3 pathway is a key regulator acting as signals messenger in the process of growth and development. Abnormality in the functioning of STAT3 signaling pathway results in normal cellular processes to a transformed state. The noncatalytic domains (SH2) target the proteins to specific phosphotyrosyl peptide sequences which regulate the numerous signaling events. Thus, STAT3 acts as hot spot target in the cancer signaling. Presently, molecular docking study of SH2 domain of STAT3 against ganoderic acid along with its 50 isomers was carried out using receptor-based molecular docking by Maestro 9.6 (Schrödinger Inc). The molecular docking identifies the maximum energy, protein–ligand interactions, orientations and conformations best suited for effective drug design. Molecular docking predicts the best suitable fitted orientation to form a stable complex and finds the strength of association of binding affinity by different docking parameters [19]. The active site of the receptor predicts the optimal thermodynamic energy in term of scoring function during grid generation.

The present study was carried out by ganoderic acid, and it's 50 isoforms which were docked with X-ray crystal structures of STAT3/DNA retrieved (PDB; 1BG1) from Protein Data Bank. The crystal structure of the STAT3/DNA has been determined with 2.25 A° resolutions. The structure provides insight into the various steps by which SH2 domain of STAT proteins delivers a response signal directly from the cell membrane to their target genes in the nucleus. Triterpene of *Ganoderma lucidum*, ganoderic acid has lanosterol scaffold and variation in its isoforms varies to the functional group or in the side chain. The binding interaction between receptor complexed with various isoforms of ganoderic acid highlighted the lipophilic, salt bridges, electrostatic and hydrogen bonding interaction.

In molecular docking of STAT3, ganoderic acid A exhibits its best docking parameters as GScore (−6.134), Lipophilic Evdw (−1.83), Electro (−1.1), Glide emodel (−31.857), H bond (1.98), MM-GBSA (−69.555) (Table 3). Different residues involved in hydrogen bonding forms the stability of the complex. Also, the stability of complex depends on

Table 3 Different scores in receptor based molecular docking exhibiting GScore, Lipophilic, Glide emodel, H-Bond Length, MM-GBSA and residues involved in the protein–ligands interaction

S. no.	Ligand type	GScore (kcal/mol)	Lipophilic EvdW	Glide emodel (kcal/mol)	H-bond length (Å)	MM-GBSA (kcal/mol)	Protein-ligands interaction
1	Ganoderic acid A	−6.134	−1.83	−31.857	1.98	−69.555	Gly 583
2	Ganoderiol A	−5.662	−1.18	−47.455	2.16	−71.784	Gly 583
3	Ganodermatriol	−5.206	−2.22	−45.959	1.05 2.87	−69.456	Glu 582 Glu 523
4	Curcumin	−4.942	−2.64	−56.005	3.05 2.68 1.45	−52.618	Glu 582 Gln 524 Arg 518
5	Ganoderic acid alpha	−4.923	−3.01	−35.818	2.08	−48.724	Lys 601
6	Ganoderic acid G	−4.839	−1.7	−32.142	2.55 1.23	−62.734	Glu 582 Lys 601
7	Ganoderiol B	−4.661	−2.29	−47.406	2.01 2.48 2.77	−57.856	Gly 583 Arg 593 Glu 523
8	Ganodermanontriol	−4.612	−3.29	−40.918	1.92 2.78	−61.649	Arg 593 Glu 523
9	Ganoderic acid J	−4.371	−2.19	−30.708	2.12	−37.845	Lys 601
10	Ganoderiol F	−4.315	−2.52	−37.640	2.45 1.98	−42.107	Gly 583 Ser 521 Ile 585
11	Ganoderic acid X	−4.241	−1.54	−42.872	1.98 2.89	−48.753	Glu 582 Lys 601
12	Ganoderic acid C6	−4.188	−2.18	−34.777	3.51 1.63 1.98	−43.342	Gly 583 Ser 521 Glu 582
13	Quercetin	−4.156	−2.81	−32.590	0.76 3.56	−51.878	Ser 521 Ile 585
14	Ganoderic acid theta	−4.127	−3.91	−39.551	1.37 1.68	−39.556	Gly 583 Glu 582
15	Ganoderic acid K	−4.066	−1.29	−32.244	2.22	−38.798	Lys 601
16	Ganoderic acid C2	−4.026	−1.24	−35.942	3.76	−46.631	Gly 583
17	Ganoderic acid B methyl ester	−3.912	−2.73	−38.738	2.76 0.59	−33.873	Glu 582 Arg 593
18	Methyl ganoderate D	−3.912	−2.33	−35.732	2.87	−37.771	Gly 583
19	Ganoderic acid B	−3.819	−1.47	−36.244	2.76 1.56	−27.645	Glu 582 Ile 522
20	Ganoderic acid H	−3.775	−3.62	−41.833	1.78 2.87 1.39	−31.043	Gly 583 Glu 523 Lys 685
21	Ganolucidic acid B	−3.775	−2.08	−34.115	2.67 3.76 2.09	−29.101	Gly 583 Glu 582 Lys 601
22	Ganoderic acid D	−3.741	−1.85	−29.346	2.45	−26.227	Gly 583
23	Ganoderic acid beta	−3.736	−1.88	−29.059	2.98 3.61	−26.394	Glu 582 Lys 601
24	Ganoderol B	−3.730	−2.41	−36.268	3.97 2.33 1.98	−24.657	Glu 523 Glu 582 Arg 593
25	Lucidenic acid P	−3.696	−2.06	−28.115	1.69 2.31	−22.563	Glu 582 Lys 601
26	Ganolucidic acid A	−3.623	−1.55	−34.476	2.87	−27.444	Gly 583

Table 3 (continued)

S. no.	Ligand type	GScore (kcal/mol)	Lipophilic EvdW	Glide emodel (kcal/mol)	H-bond length (Å)	MM-GBSA (kcal/mol)	Protein-ligands interaction
27	Ganoderic acid Am1	−3.613	−3.19	−36.146	2.42 0.64	−25.109	Glu 582 Ile 522
28	Ganoderic acid Y	−3.612	−1.37	−30.710	0.89 1.98	−31.756	Glu 582 Lys 601
29	Ganoderic acid TR1	−3.565	−2.2	−30.676	2.08 2.75	−22.458	Gly 583 Lys 685
30	Ganoderic acid R	−3.563	−2.21	−41.518	1.84	−26.735	Lys 601
31	Ganodermic acid S	−3.563	−0.29	−41.518	1.29	−33.558	Lys 601
32	Ganoderol B	−3.520	−1.81	−38.081	2.83 1.05 0.69	−28.563	Glu 582 Arg 593 Glu 523
33	Ganoderic acid Me	−3.499	−1.81	−37.152	3.34	−19.641	Lys 601
34	Withaferin A	−3.436	−1.78	−42.900	1.99 3.76	−21.745	Glu 582 Ile 585
35	Ganoderic acid DF	−3.426	−2.88	−32.149	3.03	−26.328	Gly 583
36	Ganoderic acid Mk	−3.423	−2.71	−42.658	1.49 2.54	−35.664	Gly 583 Ile 522
37	Lucialdehyde C	−3.276	−1.25	−31.314	0.65 1.93	−22.521	Glu 582 Lys 601
38	Ganoderic acid C1	−3.246	−0.39	−34.041	1.63	−25.731	Gly 583
39	Ganoderic acid T	−3.238	−2.37	−36.884	2.72 1.08	−20.666	Gly 583 Arg 518
40	Lucidenic acid C	−3.104	−1.38	−29.592	1.96 2.57	−31.764	Gly 583 Ser 521
41	Ganoderic acid T-Q	−3.069	−1.68	−35.865	0.68	−24.453	Gly 583
42	Ganoderic acid TR	−2.908	−0.35	−35.236	2.31 2.05	−27.754	Gly 583 Ile 522
43	Betulinic acid	−2.899	−1.33	−37.797	2.65	−38.682	Glu 681
44	Ganoderic acid S	−2.833	−0.89	−28.720	3.78	−16.438	Gly 583
45	Pristimerin	−2.814	−1.95	−41.399	2.79	−31.023	Glu 582
46	Ganosporeric acid A	−2.778	−1.82	−38.778	2.09	−21.059	Ile 522
47	Ursolic acid	−2.673	−2.11	−30.194	1.39	−27.742	Glu 582
48	Ganoderic acid E	−2.625	−1.73	−28.937	2.37 3.98	−19.109	Gly 583 Arg 593
49	Ganoderic acid Sz	−2.452	−0.79	−29.060	1.81	−18.865	Gly 583
50	Ganolucidic acid E	−2.448	−0.66	−31.524	2.23	−21.643	Lys 685
51	Lucialdehyde B	−2.431	−1.84	−31.416	1.73	20.554	Arg 593
52	Tirucallol	−2.422	−1.33	−29.085	2.52	−33.799	Glu 681
53	Ganoderic acid F	−2.335	−0.63	−36.683	1.05	−18.865	Gly 583
54	Lucidenic acid A	−1.612	−2.65	−25.405	2.97 1.75	−22.861	Arg 518 Gln 524
55	Celastrol	−0.397	−1.67	−29.395	2.07	−29.309	Ser 521
56	Ganoderic acid Lm2	0.466	−1.65	−36.670	2.95	−17.666	Gly 583

activation of other covalent and non-covalent bindings as shown in (Fig. 2). The binding affinity was calculated by MM-GBSA, which assesses the orientation of ligands to the receptors using scoring and thereby predicts binding interaction (Table 3).

Ganoderic acid A scavenge free radical (92 %) by DPPH assay and expression was analysis in SOD1, SOD2, and SOD3 by real-time PCR (Fig. 3).

The structure of ganoderic acid A have a tetracyclic ring with one double bond at a different position, and the

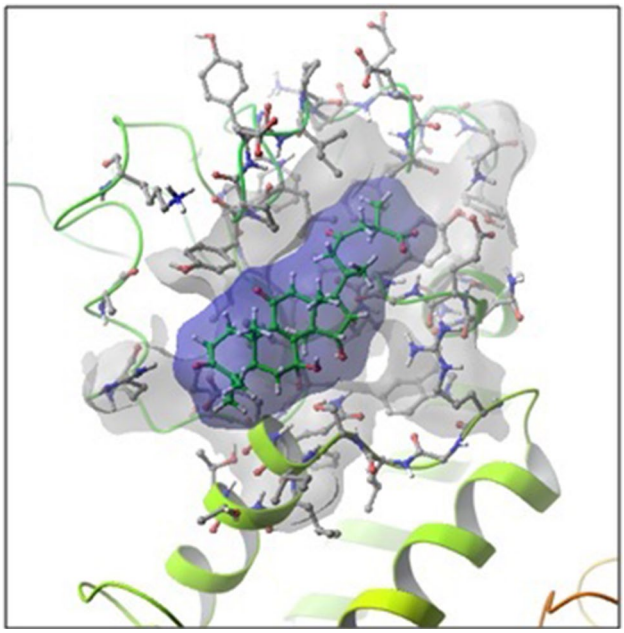

Fig. 2 Ribbon presentation of STAT3 (PDB; 1BG1) protein molecule with ganoderic acid A

branch ends with a carboxyl group while others with some modification. In molecular docking of ganoderic acid and its isoforms, –COOH group have a crucial role in different activities such as inhibitory effects whereas –C=O or –OH group also have a role in signaling through different receptors. The lanosterol moieties in the ganoderic acid A have involvement of cysteine residue which leads to the activation and translocation of STAT3 to the cytoplasm to the nucleus.

Among different natural activators, curcumin exhibits best-docked docking parameters with GScore (−4.9), Lipophilic Evdw (−2.64), Glide emodel (56.005), and Glu 582, Gln 524, Arg 518 residues participate in hydrogen bonding (Fig. 2). The binding interaction of STAT3/DNA with curcumin exhibits participation of both phenyl and aromatic moiety during docking process which makes it vital to designing an effective drug. Different parameters of other isoforms and natural activators were presented in Table 3. The active participation of particulars residues during molecular docking assist in translocation and activation of different antioxidant genes in STAT3 signaling pathway.

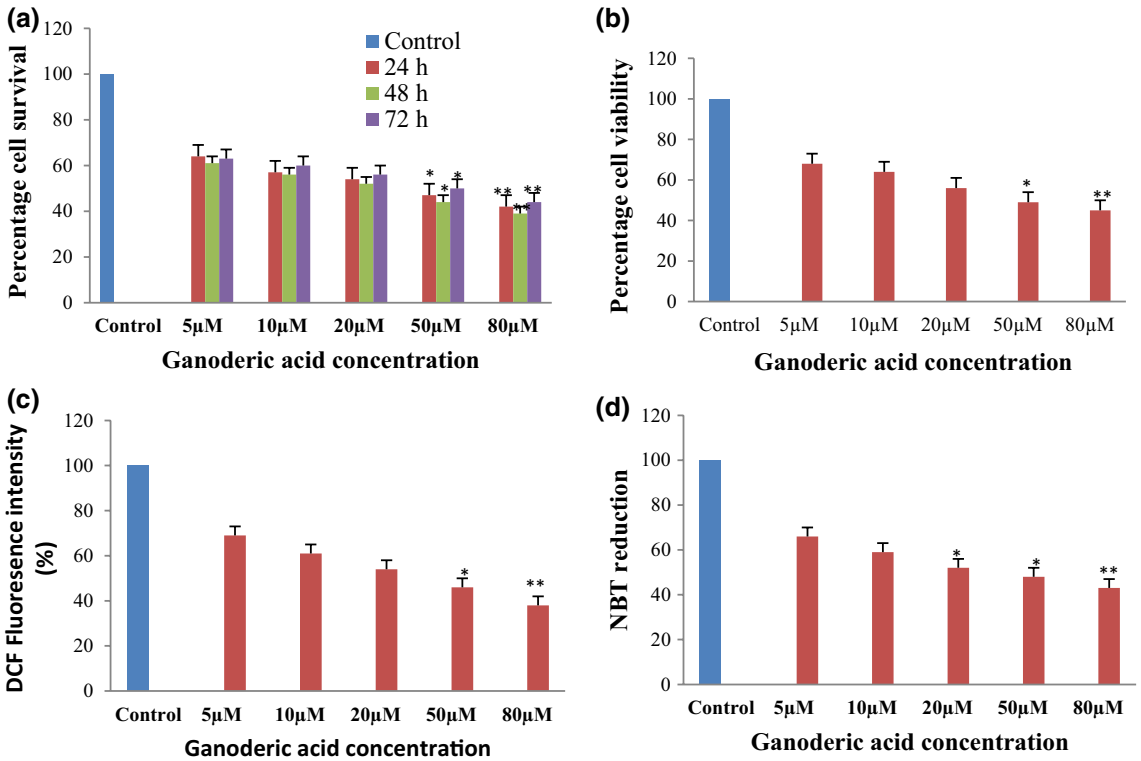

Fig. 3 Effect of ganoderic acid A on PC-3 cells. **a** Ganoderic acid A reduces the cell viability and growth in a dose-dependent manner determined by the MTT assay. **b** Ganoderic acid A reduces the cell number of PC-3 cells in a dose-dependent manner determined the trypan blue exclusion test of the cell. **c** DCF Fluorescence intensity (%) in PC-3 cells in a dose-dependent manner cells in response to ganoderic acid A. **d** Ganoderic acid A significantly reduces the cell number of PC-3 cells in a dose-dependent manner determined the NBT reduction assay. *P = 0.05 and **P = 0.01 vs. control

Table 4 Evaluation of drug-like properties of the different isoforms of ganoderic acid and other natural inhibitors of STAT3/DNA by Qikprop

Molecule	M.W	Dipole	Q P log Po/w (−2.0 to 6.5)	Q P log HERG (acceptable range: above-5.0)	QPP Caco (nm/s) \25—poor [500—great	Q P log BB (−3 to 1.2)	QPP MDCK (nm/s)	Q Plog Kp (−8.0 to −0.1)
Ganoderic acid A	516.673	6.123	4.761	−4.822	398.851	−1.541	212.862	−3.961
Ganoderiol A	474.723	2.542	4.905	−4.536	401.237	−1.484	184.363	−3.162
Ganodermatriol	456.707	4.69	5.328	−4.774	737.555	−1.183	356.001	−2.704
969,516 (Curcumin)	368.385	7.679	2.79	−5.997	246.014	−1.917	108.655	−2.565
Ganoderic acid alpha	574.71	6.129	2.885	−2.663	13.778	−2.352	6.13	−5.048
Ganoderic acid G	532.673	6.132	2.09	−2.568	9.059	−2.506	3.896	−5.402
Ganoderiol B	470.691	3.053	4.468	−4.644	271.611	−1.617	120.924	−3.558
Ganodermanontriol	472.707	3.13	4.979	−4.547	368.378	−1.466	168.099	−3.332
Ganoderic acid J	514.658	6.119	2.796	−2.159	18.299	−1.94	8.331	−5.001
Ganoderiol F	454.692	2.693	5.465	−4.646	618.726	−1.179	294.432	−2.947
Ganoderic acid X	512.728	4.0	6.121	−2.716	52.364	−1.588	25.955	−3.977
Ganoderic acid C6	530.657	4.34	1.865	−2.308	7.93	−2.408	3.374	−5.611
Quercetin	304.256	6.158	0.186	−4.887	23.071	−2.274	8.414	−5.42
Ganoderic acid theta	530.657	3.689	1.811	−2.327	6.507	−2.487	2.725	−5.75
Ganoderic acid K	574.71	3.157	2.678	−2.509	9.182	−2.478	3.953	−5.391
Ganoderic acid C2	518.689	7.298	2.879	−2.485	12.182	−2.343	5.366	−5.152
Ganoderic acid B methyl ester	528.684	4.787	3.152	−4.847	115.336	−2.05	47.912	−4.468
Methyl ganoderate D	528.684	6.979	2.732	−4.393	83.817	−2.009	33.931	−4.875
Ganoderic acid B	516.673	6.669	2.862	−2.201	20.918	−1.956	9.627	−4.792
Ganoderic acid H	572.694	7.241	2.639	−2.538	11.875	−2.313	5.22	−5.27
Ganolucidic acid B	502.69	2.79	3.82	−2.448	28.875	−1.889	13.639	−4.52
Ganoderic acid D	514.658	2.14	2.826	−2.584	12.638	−2.242	5.584	−5.313
Ganoderic acid beta	484.675	3.905	4.52	−2.371	38.138	−1.615	18.425	−4.449
Ganoderic acid A	516.673	4.425	2.669	−2.109	12.909	−2.132	5.713	−5.199
Ganoderol B	440.708	2.803	6.382	−4.48	1412.684	−0.723	718.672	−2.355
Lucidenic acid P	518.646	5.581	2.547	−2.299	10.714	−2.214	4.671	−5.453
Ganoderic acid J	514.658	4.47	2.717	−2.258	13.418	−2.104	5.957	−5.263
Ganolucidic acid A	500.674	12.383	3.78	−2.595	23.671	−1.963	11.003	−4.784
Ganoderic acid Am1	514.658	8.868	2.831	−2.601	16.201	−2.136	7.303	−5.104
Ganoderic acid Y	454.692	4.398	6.691	−3.195	114.228	−1.282	60.304	−3.41
Ganoderic acid TR1	468.675	7.273	5.541	−2.724	66.464	−1.432	33.585	−3.899
Ganoderic acid R	554.765	4.277	6.913	−3.287	74.619	−1.574	38.06	−3.682
Ganodermic acid S	554.765	4.277	6.913	−3.287	74.619	−1.574	38.06	−3.682
Ganoderol B	440.708	3.439	6.391	−4.629	1515.513	−0.711	775.378	−2.295
Ganoderic ACID Me	554.765	5.317	7.029	−3.153	52.498	−1.731	26.027	−4.039
Withaferin A	470.605	8.475	3.057	−4.45	274.688	−1.279	122.406	−3.788
Ganoderic acid DF	516.673	4.748	3.243	−2.847	17.786	−2.239	8.078	−4.929
Ganoderic acid Mk	556.738	3.742	5.836	−3.579	18.73	−2.423	8.543	−4.754
Lucialdehyde C	468.718	7.936	5.631	−4.261	621.983	−1.057	296.107	−3.084
Ganoderic acid C1	514.658	4.762	2.662	−2.514	11.391	−2.263	4.991	−5.401
Ganoderic acid T	612.802	5.91	6.547	−2.98	33.038	−1.945	15.777	−4.299
Lucidenic acid C	476.609	5.157	1.966	−1.97	14.333	−1.985	6.397	−5.207
Ganoderic acid T-Q	510.712	6.333	6.436	−3.061	68.528	−1.508	34.714	−3.887
Ganoderic acid DM	468.675	1.813	5.53	−2.403	102.311	−1.133	53.534	−3.711

Table 4 (continued)

Molecule	M.W	Dipole	Q P log Po/w (−2.0 to 6.5)	Q P log HERG (acceptable range: above-5.0)	QPP Caco (nm/s) \25—poor [500—great	Q P log BB (−3 to 1.2)	QPP MDCK (nm/s)	Q Plog Kp (−8.0 to −0.1)
Ganoderic acid TR	468.675	6.264	5.539	−2.761	66.382	−1.435	33.54	−3.881
Withaferin A	470.605	4.492	3.069	−4.459	262.523	−1.3	116.557	−3.826
betulinic acid	456.707	2.763	6.191	−2.078	278.466	−0.523	157.998	−3.011
Ganoderic acid S	452.676	5.553	6.562	−2.694	153.158	−0.984	82.798	−3.266
Pristimerin	464.644	7.318	5.313	−4.188	542.907	−0.768	255.634	−3.541
Ganosporeric acid A	526.625	8.648	1.661	−2.131	14.057	−1.983	6.264	−5.319
Ganoderic acid theta	530.657	10.421	1.95	−2.312	10.065	−2.288	4.366	−5.371
Ursolic acid	456.707	3.999	5.848	−1.635	294.656	−0.389	167.95	−3.082
Ganoderic acid E	512.642	10.08	2.716	−2.418	19.089	−1.942	8.72	−5.061
Ganolucidic acid A	500.674	11.996	3.748	−2.447	32.865	−1.772	15.688	−4.507
Ganoderic acid Sz	452.676	5.732	6.585	−2.75	121.224	−1.1	64.307	−3.463
Ganolucidic acid E	484.675	7.625	4.562	−2.686	29.914	−1.809	14.171	−4.645
Lucialdehyde B	452.676	5.698	5.074	−4.291	473.062	−1.079	220.28	−3.574
101,257	426.724	1.881	7.438	−4.238	4425.242	−0.089	2469.059	−1.674
Ganoderic acid F	570.678	5.719	2.292	−2.689	5.788	−2.637	2.401	−5.972
Lucidenic acid A	458.594	6.041	2.603	−1.678	22.527	−1.599	10.429	−5.017
Celastrol	450.617	5.759	4.959	−1.981	73.439	−0.961	37.41	−4.121
Ganoderic acid Lm2	514.658	9.253	2.689	−2.405	11.34	−2.215	4.967	−5.38

ADME properties

Binding affinity prediction is useful for analyzing the pharmacokinetic and pharmacodynamic properties. ADMET predicts physicochemical, effective permeability in jejunum, blood–brain barrier permeation, pKa (ionization constants a multi-protic model, intrinsic clearance solubility, toxicity risks, optimal dose in human fraction absorbed in human, likely sites of metabolic attack, logD, logP, MDCK apparent permeability. QikProp is an important tool that calculates properties of the valid descriptors and pharmaceutically relevant molecules by comparing their values with those of 95 % of already known pharmaceutical drugs. Ganoderic acid and its 50 isoforms were checked for ADME properties. Most interesting aspect of these compounds are their admirable QPlogPo/w, QPlogHERGK channels, QPlogBB, QPlogKP and QPlogKhsa values that satisfy the Lipinski's rule of five (Table 4). In particular, ganoderic acid satisfies its toxicity or ADMET properties, which include Q P log Po/w (4.761) which predicts water partition coefficient, Q P log HERG (−4.822), predicts IC50 value for the blockage of HERG K^+ channel, QPP Caco (398.851), predicts apparent caco cell permeability acts as model for gut-blood carrier, Q P log BB (−1.541), predicts blood/brain partition coefficient, QPP MDCK (212.862), predicts the apparent MDCK cell permeability, and considered as good mimics for blood–brain barrier, Q Plog Kp (−3.961) predicts the skin permeability. Thus, ganoderic acid A satisfies various parameters in the ADMET. Some of the isomers in different receptors did not satisfy all aspect and needed some modifications in the basic lanosterol structure (Table 4). On the other hand, natural inhibitor of curcumin satisfies its toxicity or ADMET properties, which include Q P log Po/w (2.79) which predicts water partition coefficient, Q P log HERG (−5.997), predicts IC50 value for the blockage of HERG K^+ channel, QPP Caco (246.014), predicts apparent caco cell permeability acts as model for gut-blood carrier, Q P log BB (−1.917), predicts blood/brain partition coefficient, QPP MDCK (108.655), predicts the apparent MDCK cell permeability, and considered as good mimics for blood–brain barrier, Q Plog Kp (−2.565) predicts the skin permeability. Thus, curcumin also satisfies various parameters in the ADMET (Table 4).

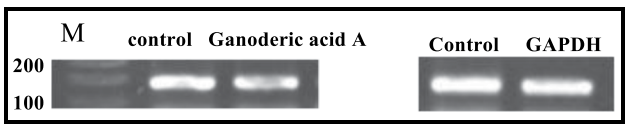

Fig. 4 mRNA expression after ganoderic acid A treatment in PC-3 cells in RT-PCR analysis

Predicted IC50 value for blockage of HERG K+ channels; (acceptable range: above −5.0); QPP Caco, predicted apparent Caco2 cell permeability in nm/s. Caco 2 cells is

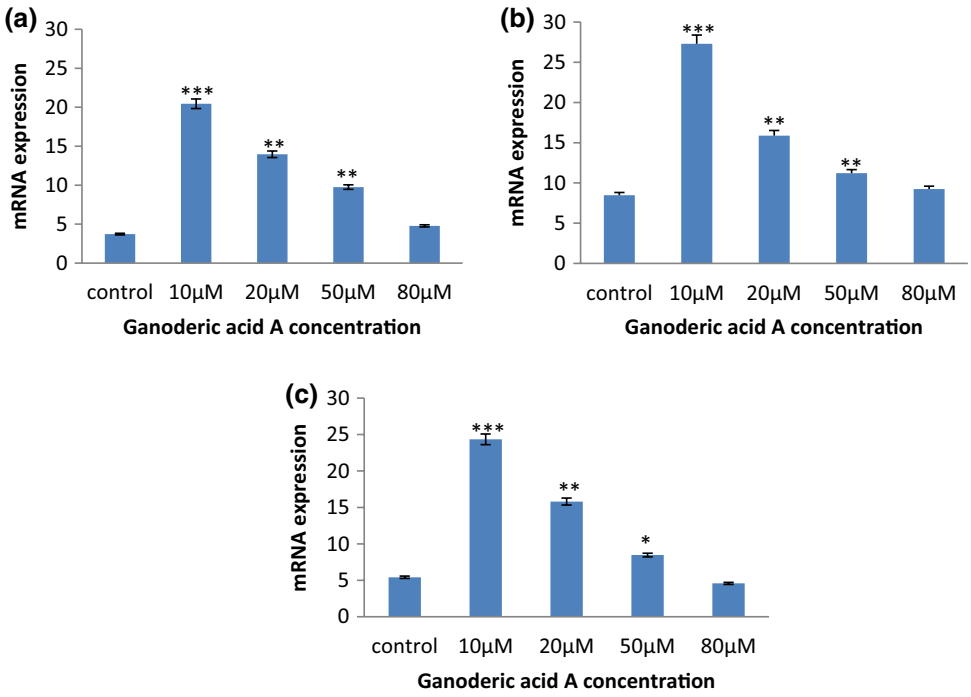

Fig. 5 Expression analysis of SOD1, SOD2, SOD3 mRNA expression after treatment of ganoderic acid A in a dose-dependent manner by real-time PCR

a model for the gut-blood barrier; (nm/s) <25poor >500-great; QPlogBB, predicted brain/blood partition coefficient; QPP MDCK, predicted apparent MDCK cell permeability in nm/s. MDCK cells are considered to be a good mimic for the blood–brain barrier; (nm/s) <25poor> 500great; QP log KP, predicted skin permeability; Percentage of human oral absorption; (<25 % is poor and >80 % is high) [32].

Effect of ganoderic acid A in mRNA expression in PC-3 cells

The effect of ganoderic acid A on mRNA expression was determined by using RT-PCR. The results show that ganoderic acid A has the potential to reduce the expression in PC-3 cells. The RT-PCR densitometric bands analysis showed that ganoderic acid A reduced the mRNA expression by 33 % in PC-3 cells at 80 µM concentration (Fig. 4).

Expression analysis of ganoderic acid A on SOD1, SOD2, and SOD3 by real-time PCR

The expression of ganoderic acid A on SOD1, SOD2, and SOD3 was represented in (Fig. 5). The analysis reveals that with an increase in the concentration of ganoderic acid A, mRNA expression decreases. SOD 1 activity was fivefolds higher as compared to control at 10 µM of ganoderic acid A concentration, which further decreases with increase in concentration. SOD2 activity was threefolds higher as compared to control at 10 µM of ganoderic acid A concentration. SOD3 activity was 4.5 folds higher as compared to control at 10 µM of ganoderic acid A concentration. Thus, it was concluded that SOD activity was high in lower concentration, but with an increase of ganoderic acid A concentration SOD activity decreases. With the increase in ganoderic acid A concentration, oxidative stress starts decreasing which decreases the mRNA expression of SOD. It can be inferred that at high ganoderic acid A concentration results in low oxidative stress, which ultimately results in a decrease in SOD expression.

Conclusion

Abiotic stress condition of *Ganoderma lucidum* increases the concentration of some of the particulars elements which might help the *G. lucidum* to survive in prevailing conditions. STAT, an important transcription factor critically important in the signaling relating cancer and diabetes. Receptor-based molecular docking revealed active residues involved STAT/DNA binding, which provides the platform to the researcher to work in molecular level of a ganoderic acid binding with STAT. SH2 domain of STAT3 was best docked with ganoderic acid A with GScore (−6.134), kcal/mol, Lipophilic EvdW (−1.83), Electro (−1.1), Glide emodel (−31.857), H bond (1.98), MM-GBSA (−69.555). The present docking study revealed the binding pattern, active residues involved which decides the docking score and others toxicity parameters. Ganoderic acid A proves better as compared to natural inhibitors in different parameters of receptor-based molecular docking and ADMET properties and thus checked for its biological activity. Ganoderic acid A downregulates the mRNA expression of STAT3 in

PC-3 cells. Moreover, ganoderic acid A inhibit cancer cell proliferation, viability, ROS, DPPH, and modulates the expression of SOD1, SOD2, and SOD3, STAT 3 in a PC-3 cell in a dose-dependent manner.

Acknowledgments Authors thanks, Central University of Punjab, Bathinda, for providing the necessary facilities to carry out the present work.

Compliance with ethical standards

Conflict of interest None.

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