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MEDICINAL CHEMISTRY RESEARCH

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# Ganoderic acid modulating TNF and its receptors: in silico and in vitro study

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**Abstract** Cancer is a multifactorial disease with a network of genes causing genetic alterations. The sophisticated techniques in molecular biology revealed different cancer pathway, but their mechanistic approach is still shrouded. Tumor necrosis factor and TNF-related apoptosis-inducing ligand receptors (DR5) emerged as potential target drug for the cancer therapy. Among natural products basidiomycete fungus, *Ganoderma lucidum* and its constituents endowed with a plethora of activities modulating signaling in cancer. Ganoderic acid, a triterpene with lanosteroidal skeleton play an inextricable role in modulating signaling cascades in various mitogenic pathways. In the present study, receptor-based molecular docking was performed to study the dynamic behavior of the docked complexes and the molecular interactions between ganoderic acid and its isoforms with tumor necrosis factor and its receptor (DR5). The top scoring compounds were compared with the already documented natural inhibitor of tumor necrosis factor, DR5-curcumin, catechin, bupropion, pentoxyphyllin for their binding affinity and other absorption, distribution, metabolism, excretion, and toxicity properties. Ganoderic acid A interact more promising as compared with other isoforms with GScore (−9.858 (kcal/mol), Lipophilic EvdW (−1.7), H Bond (−0.9), Glide emodel (−40.5) with the involvement of Tyr 151, Leu 120 and Gln 149 residues during binding with tumor necrosis factor. During docking of ganoderic acid with DR5, ganoderic acid A exhibits GScore (−8.7), HBond (−2.9), Glide emodel (−30.0) with the involvement of hydrogen bonding inMet99, Arg101, Pro97, Glu98 residues. Natural inhibitors already documented exhibit low-binding energy and other docking parameters, which have an edge of ganoderic acid A to tumor necrosis factor and DR5. Ganoderic acid A efficiently inhibits the proliferation, viability, and intracellular reactive oxygen species and messenger RNA expression of tumor necrosis factor and DR5 in the breast cancer cell lines.

**Keywords** Ganoderic acid · TNF · DR5 receptor · Apoptosis · Cancer

## Introduction

Cancer is an uncontrolled somatic cell proliferation by the accumulation of mutations in critical genes controlling growth and differentiation. These changes characterized by structural and morphological changes leading to the cell shrinkage, plasma membrane blebbing, mitochondrial welling, the release of cytochrome c, chromatin condensation, and DNA fragmentation resulting in cells death by aprocess of apoptosis (Gill et al. 2016a). The phenomenon of apoptosis is initiated by triggering intracellular apoptotic signals received by receptors in plasma membrane. Tumour necrosis factor (TNF) and its receptors initiate the process of apoptosis and control the expression of various proteins. The tumor necrosis factor (TNF) family comprises of TNF-alpha (TNF-α), Fas ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL) cytokines involved in

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physiological processes, systematic inflammation, tumor lysis, and apoptosis (Hersh et al. 1991). Upon stimulation by the ligand, TNF receptors form trimers by proper orientation of the monomers into the groove of monomers (Anand and Gill 2015). Ligand-mediated trimerization by the TNF family ligands causes recruitment of several intracellular adaptors. This causes a conformational change in the receptor, resulting in the dissociation of inhibitory protein silencer of death domain from the intracellular death domain. Subsequently, dissociation enables the adaptor protein TNFR-associated death domain protein (TRADD) to bind to the death domain that activates multiple signal transduction pathways. TRADD initiates several DNA-binding sites for the transcription factor importantly nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinases (JNK), and caspases (Fig. 1). The exact mechanism of NF-κB activation is not clear, but some studies demonstrated that death receptors (DR4, DR5, FADD, TRADD) are interrupted by the dominant negative form of FADD and FLIP, which are responsible for their activation (Schneider et al. 1997). TNF also triggers activation of the Ikappa B (IκB) kinase (IKK)/NF-κB and MAPK/AP-1 pathways, indispensable for the expression of pro-inflammatory cytokines. TNF induces the activation of the JNK that evokes the moderate response of the p38-MAPK which is accountable for minimal activation of AP-1. TNF has emerged as an important risk factor for tumorigenesis, tumor progression, invasion, and metastasis (Wajant et al. 2003). Agents are targeting TRAIL receptors DR5 undergone both preclinical models and phase I clinical trials, and considered possible targets for the development of antitumor agents (Herbst et al. 2010). Many trials verified their safety, tolerability and therapeutic efficacy of TRAIL in patients. Several studies have demonstrated that DR5 expressed in the primary tumor and cancer cell lines (Spierings et al. 2003).

Anti-TNF therapy or agents based on a chemical synthesis of drugs are beneficial, but some side effects have been reported. Therefore, to minimize the side effects of therapy, natural products have enormous power to curb this without detrimental effect (Negi and Gill 2013). The strategies developed to block TNF and DR5 receptor signaling by anti-TNF from natural products like curcumin, catechin, bupropion, pentoxyphyllin have a promising effect (Siddiqui et al. 2006) (Negi et al. 2014). In this study, one of triterpene of *Ganoderma lucidum* is ganoderic acid (GA) and its 11 isoforms were docked against TNF and death domain receptor DR5. The results were analyzed, and best one docked isoform of GA was checked for the biological activity in cancer cell lines. It was well documented that triterpenes act on receptor tyrosine kinase, and it related downstream signaling (Gill and Kumar 2016) (Yadav et al. 2010). GA is having lanosteroidal skeleton portray diverse therapeutic spectrum ranging from molecular immunity functionality modifier to anticancer activity significantly (Gill et al. 2016b). Our previous study disclosed the

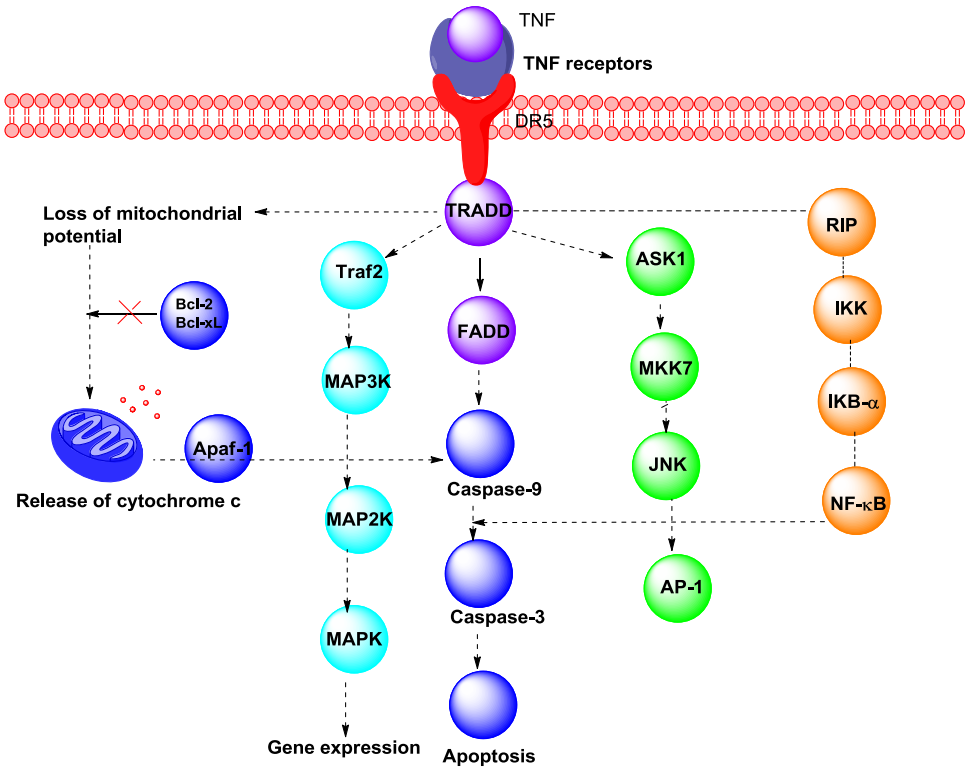



**Fig. 1** Signaling cascade initiated by TNF, and TRAIL receptor DR5, which on stimulus brings conformational changes in the receptor and leads to activation of downstream signaling. TNF activates NF-κB, AP-1, caspase, MAPK, and others adapter molecules engaged in the process of apoptosis

mechanistic binding of GA in Wnt signaling pathway (Gill et al. 2016c).

## Methodology

### Preparation of ligands, protein, and grid formation

The protein crystallized structure was retrieved from PDB site (PDB ID: 2AZ5) (Yuen and Gohel 2005), and methodology used remains same from previous study (Gill and Kumar 2015). The water molecules present in protein structure were removed to avoid hydrophobic interaction in the domain region. Furthermore, polar hydrogen was incorporated to complete valency to enhance polarizability of bonds. Enhancement in polarizability increases the effectivity of targeting the binding of ligand and proteins. The later processing involves stereochemistry by residue-by-residue geometry and structural geometry. Another major step involves the preparation of different isoforms of GA and TNF inhibitors structures with ChemBioDraw Office (licensed @ Cambridge's soft) software (Figs. 2 and 3). The Ligprep were used for ligand preparation and optimization by correcting valency of the hydrogen bond, disulfide bonds, removal of bad contacts, and fixing the missing residues (Gill and Kumar 2015). Minimization and optimization were performed with liquid simulations force field to form the stable geometry of prepared structures (Jorgensen et al. 1996; Jorgensen and Tirado-Rives 1988; Shivakumar et al. 2010).Grid pre-calculates grid maps of interaction energies for various atom types, such as aliphatic carbons, aromatic carbons, hydrogen bonding oxygen, and so on, with a macromolecule such as a protein, DNA, or RNA prior to docking. These grid maps are then used by GLIDE docking calculations to determine the total interaction energy for a ligand with a macromolecule. Grid mapping calculates the coordinates and atomic data in TNF and receptor DR5 for receptor-based molecular binding. Grid mapping provides the active area for the ligand for interaction with the TNF, and DR5 domain in which inhibitors were bonded. Grid mapping is a pre-requisite of GAs to have a strong affinity with TNF and DR5 domain. The grid allows the favorable interaction and best conformation, position, and orientation about the receptors (Repasky et al. 2007).

### GLIDE molecular docking

The receptor-based molecular docking was performed after preparing the ligand, protein and the grid preparation on the

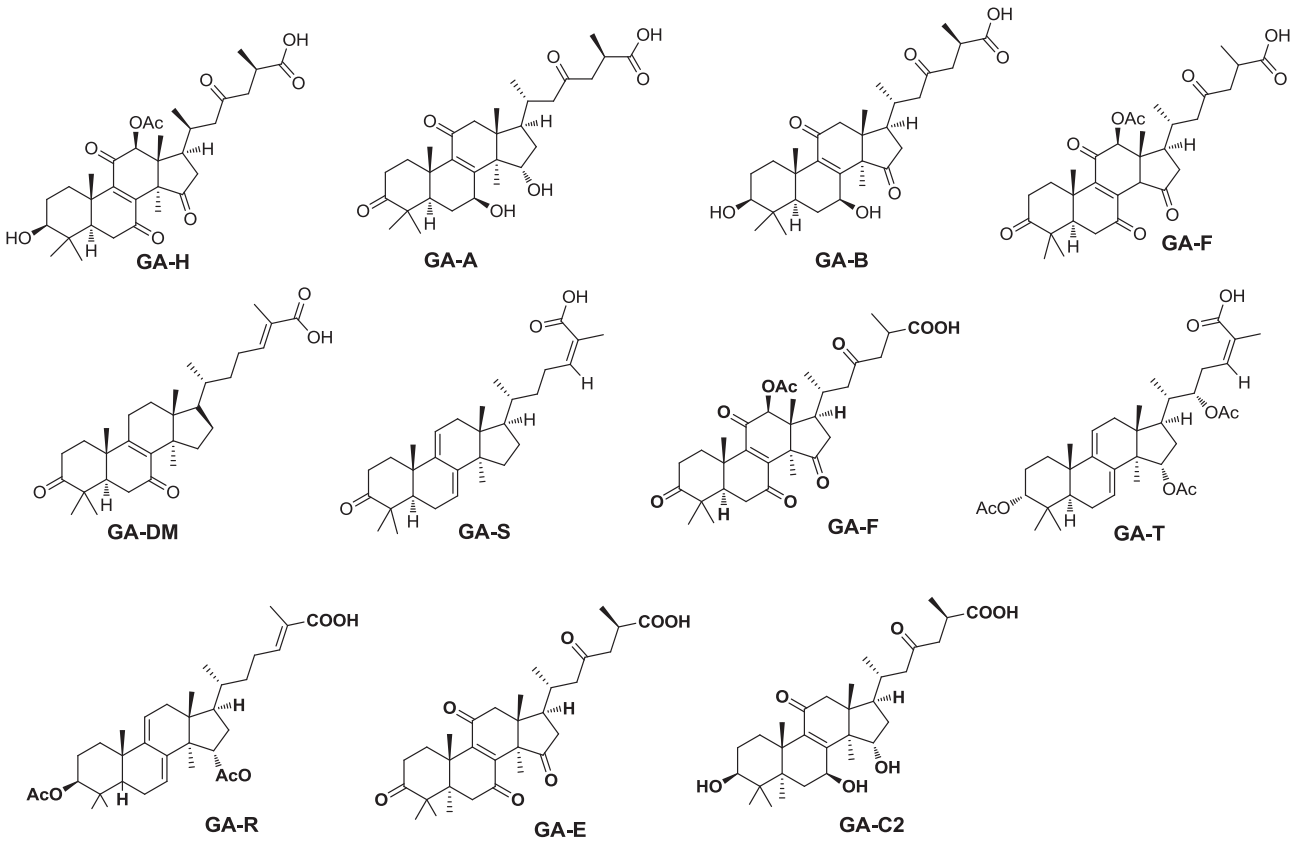



**Fig. 2** Depiction of the chemical structure of various ganoderic acids

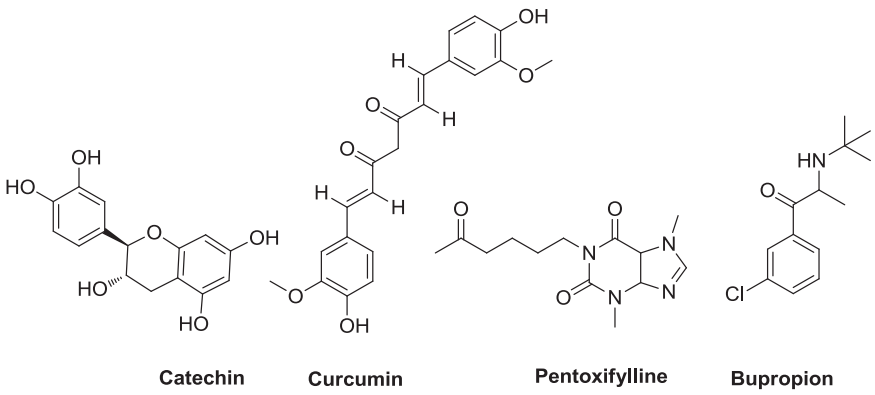



**Fig. 3** Chemical structure of anti-TNF natural compounds

active site of the protein. GLIDE docking method predicts the specific binding to the target and analysis the binding interaction. GLIDE calculates GScore (empirical scoring function), which is acombination of ligand–protein interaction energies, root mean square deviation, hydrogen bonds, hydrophobic interactions, internal energy, π–π stacking interactions, and desolvation. The docking module, GLIDE uses XP visualizer for analyzing binding interactions among ligand and protein. The different isoforms of GA were docked with the X-ray crystal structure of TNF (PDB; 2AZ5) and receptor DR5 (PDB; 2H9G). The docking predicts the best orientation of the ligand and proteins after analyzing types of interactions, amino acid involved, distances, bond length among different residues (Friesner et al. 2004, 2006).

## Absorption, distribution, metabolism, excretion, and toxicity (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. 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, the percentage of human oral absorption (Jorgensen and Duffy 2002).

## RNA isolation, complementary DNA (cDNA) synthesis, and quantitative reverse transcriptase PCR (RT-PCR)

GA-A isolated from *Ganoderma lucidum* in the previous study was used in this study and stored at −20 °C (Gill et al. 2016c; Tang et al. 2006). About one million cells were seeded and supplemented with culture media having 10% fetal bovine serum (FBS) and % penicillin/streptomycin, incubated at 37 °C. The cells were treated with 80 µM of GA 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 qPCR was performed with Assay-on-demand Applied Biosystems with primers**:** TNF forward primer 5′-GACGACAGCAAGGGACTAGC-3′ reverse primer 5′ GCTTT CTGTGCTCATGGTGC-3′ and DR5, forward primer 5′-CAGGTGTCAACATGTTGTCC-3′, reverse primer 5′-ATCGAAGCACTGTCTCAGAG-3′. Each data point was repeated in triplicates. Quantification values were obtained using threshold PCR cycle number (Ct), where the increase in signal results in an 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 $\Delta Ct = Ct_{targetgene} – Ct_{\beta\text{-actin}}$.

## Cell proliferation assay/MTT assay

Effect of different treatments of GA on the growth of breast cancer cells (T-47D, MDA-MB-231) was assessed by using the MTT assay in triplicates. Approximately 10,000 cells were seeded in the 96-well plate in media containing 10% FBS and incubated overnight at 37 °C followed by serum starvation for 24 h. Cells were exposed to the different concentrations of GA (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 phosphate-buffered saline (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. Absorbance was measured at 570 nm using microplate reader.

### Cytotoxic/apoptotic capacity

In another set of experiment, the rate of cell death in response to GA with treatment was assessed by the trypan blue exclusion test. In all, $2 \times 10^5$ (T-47D, MDA-MB-231) cells were seeded in six-well culture plates. The adhered cells were treated with GA (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 after 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 on reactive oxygen species (ROS) levels ($H_2$DCF-DA assay)

Intracellular ROS level was measured with 2′,7′-dichlorodihydrofluoresce in diacetate ($H_2$DCF-DA), which undergoes rapid oxidation into the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of intracellular ROS. T-47D, MDA-MB-231 cells ($2 \times 10^5$) were seeded in six-well culture plates and treated with GA (5, 10, 20, 50, 80 µM/mL) for 24 h. Cells were washed with PBS and incubated in PBS containing 10 µM $H_2$DCF-DA for 30 min at 37 °C. The cells were washed with PBS to remove excess dye, and the fluorescence was measured at 485 and 530 nm wavelength.

### Statistical analysis

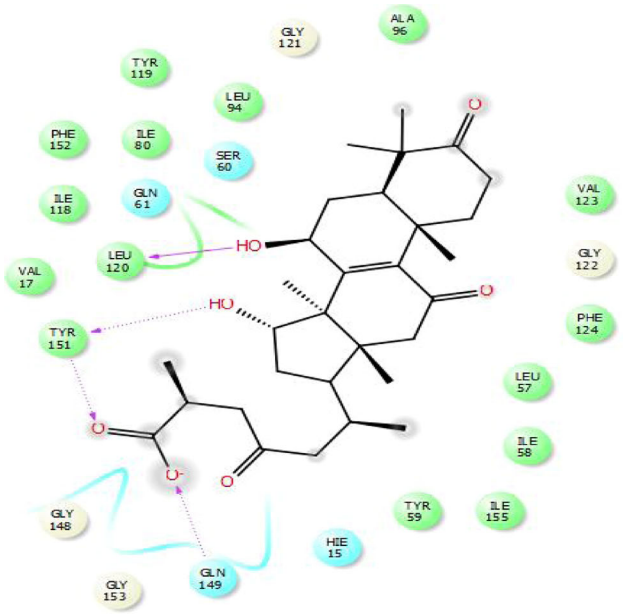
Results were expressed as the mean ± standard deviation of experiments performed in triplicates. Data obtained was subjected to one-way analysis of variance, and significant differences of the mean were determined statistically using Tukey's test using SigmaPlot 11.

## Results and discussion

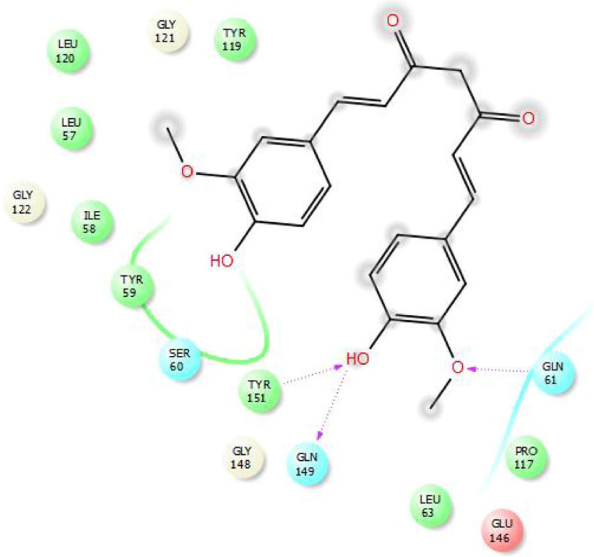
### In-silico study

TNF initiate and activates downstream signaling cascade of the different transcription factor involved in NF-κB, MAPK, and others factors involved in apoptosis pathway (Fig. 1). Dysregulation in the functioning of TNF and its receptors consequence in modulation in the expression of genes (Anand and Gill 2015). Current studies convey new perception into the biological and molecular mechanisms of TNF and its receptor with natural products involved in various abnormalities. In silico studies were carried for TNF-α and its DR5 receptor with triterpenes, which has enormous power to modulate cancer signaling. In this study, TNF and DR5 were docked with 11 isoforms of GA and other important natural inhibitor available in the literature. Molecular docking identifies the optimal energy, protein–ligand interactions involved, orientations and conformations best suited for drug preparation. The receptor-based molecular docking predicts the preferred orientation to form a stable complex and finds the strength of association of binding affinity by different docking parameters.

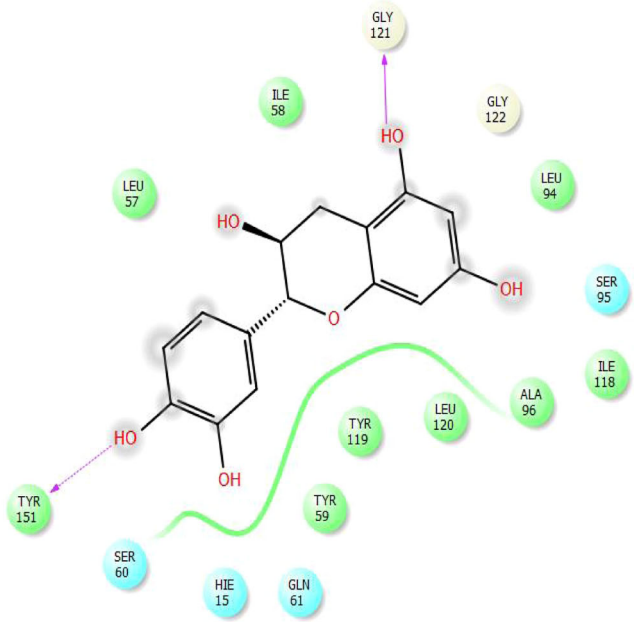
Different isoforms of GA, triterpenes of *Ganoderma lucidum* were docked with X-ray crystal structures retrieved TNF (PDB: 2AZ5) and TRAIL receptor i.e., DR5 (PDB: 2H9G) from of Protein Data Bank. The molecular docking was performed using Maestro 9.3 with natural inhibitors of TNF 969516, 9064, 444, 4740 (PubChem CID) with TNF (PDB: 2AZ5) and DR5 (PDB: 2H9G). Terpenoids of *Ganoderma lucidum*, the GA has lanosterol scaffold and variation in its isoforms varies to the functional group or in the side chain. The crystal structure of TNF with a small molecule inhibitor (PDB:2AZ5) that promotes subunit disassembly of this trimeric cytokine family member (He et al. 2005). The structure of TNF-α complexed with GA highlighted the lipophilic, electrostatic, and hydrogen-bonding interaction and considered to be the main contributor in protein–ligand interaction. GA-A interact more promising as compared with other isoforms of GA and found that residues Tyr 151, Leu 120, Gln 149 forms hydrogen bonding with other responsible forces during binding with TNF. Hydrogen of Tyr151 donates its electron to the oxygen of carboxyl group of GA-A and forms H-bonding with 2.60 A° bond length (side chain), whereas Tyr151 accept an electron from hydrogen from the hydroxyl group of GA-A with 1.83 A° bond length (side chain), thus Tyr151 show amphoteric nature. Gln149 forms H-bonding with GA-A by donating 2.13 A° bond length (side chain) electron from Gln149 to oxygen of GA-A (Fig. 4). The hydroxyl group of GA-A donates and forms H-bonding with the oxygen of Leu120 with 1.77 A° bond length. Lipophilic Evdw defines the hydrophobic grid potential and fraction of the total protein–ligand van der Waals energy. GA-A exhibits different parameters as Lipophilic Evdw (−1.7), Glide emodel (−40.5), H Bond (−0.9), and GScore (−9.858) (Table 1). On the other hand, GA-DM shows lowest of GScore (−7.381), Glide emodel (−26.8), H Bond (−0.6), and Lipophilic Evdw (−1.8) in which only Glu121 forms hydrogen bonding with 2.21 A° bond length. The receptor-based molecular docking poses disclosed the interaction with ligand and receptor in which best they were fitted. Lower docking energy in GA-DM results from the basic lanosterol structure that varies in functional or side chains as compared to GA-A. Other isoforms of GA range in between GA-A and GA-DM, and it was concluded that the



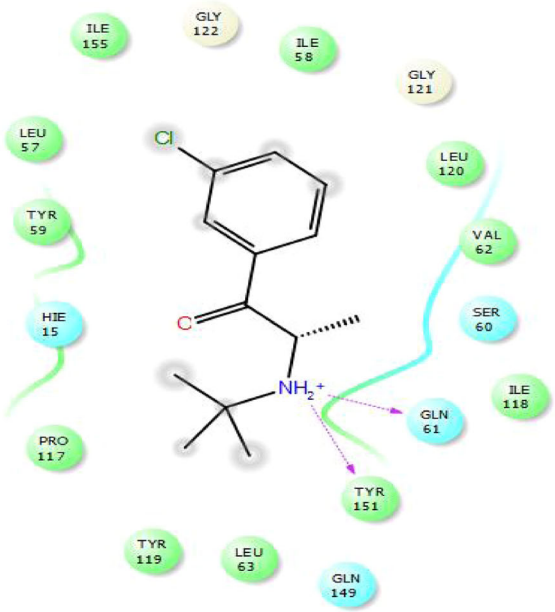

Ganoderic acid A




Curcumin




Catechin




Bupropion

**Fig. 4** Protein–ligands interactions profile of TNF (PDB: 2AZ5) with ganoderic acid A, curcumin, catechin, bupropion. Protein–ligands interactions profile revealed that residues involved in ganoderic acid A binding acid were Tyr151, Leu120, Gln120, whereas Tyr151, Gln149, Gln61 were involved in curcumin. Furthermore, amino-acid Tyr151, Gly121 in catechin, and Tyr151and Gln61 in bupropion forms the hydrogen bonding that forms the basis of strong interaction

modification in the basic structure lanosterol was not so appropriate for TNF binding. In the case of natural inhibitors of TNF, curcumin shows best results with GScore (−7.625), Glide emodel (−34.0), H Bond (−0.9), and Lipophilic Evdw (−0.1). In curcumin Tyr151, Gln61, and Gln 149 forms hydrogen bonding, in which Tyr151 donates electron from hydrogen to oxygen of hydroxyl group of curcumin with 2.25 A° distance. Gln149 on the same hydroxyl accept an electron from curcumin with 4.24 A° distance. Another hydrogen bonding takes place in which

**Table 1** Binding affinities scores and energies of different compounds with TNF

| S. no. | Ligand type | PubChem CID | GScore (kcal/mol) | H Bond | Lipophilic EvdW | Glide emodel | Residues in H-bonding | H-bond length (Å) | Protein–ligands interaction |
|---|---|---|---|---|---|---|---|---|---|
| 1 | GA-A | 14109402 | −9.858 | −0.9 | −1.7 | −40.5 | Tyr151 | 2.60 | Tyr 151, Leu 120, Gln 149 |
| | | | | | | | Tyr151 | 1.83 | |
| | | | | | | | Leu120 | 1.77 | |
| | | | | | | | Gln149 | 2.13 | |
| 2 | GA-C2 | 57396771 | −9.750 | −1.5 | −0.7 | −42.1 | Tyr151 | 2.24 | Tyr 151, Leu 120, Gln 149 |
| | | | | | | | Tyr151 | 2.62 | |
| | | | | | | | Leu120 | 1.85 | |
| | | | | | | | Gln149 | 2.07 | |
| 3 | GA-R | 6449828 | −8.500 | −1.0 | −1.9 | −38.3 | Tyr151 | 2.05 | Tyr 151 |
| 4 | GA-H | 471005 | −8.465 | −0.9 | −0.1 | −35.6 | Tyr151 | 2.04 | Tyr 151, Gln 149 |
| | | | | | | | Gln149 | 1.01 | |
| 5 | GA-T | 21637704 | −8.241 | −0.3 | −1.5 | −38.1 | Gln149 | 1.98 | Gln 149 |
| 6 | GA-E | 23247894 | −8.157 | −1.2 | −1.6 | −29.8 | Tyr151 | 1.77 | Tyr 151, Gln 149 |
| | | | | | | | Gln149 | 2.10 | |
| 7 | GA-B | 471003 | −8.154 | −0.8 | −0.4 | −35.0 | Tyr119 | 2.63 | Tyr 151 |
| 8 | GA-S | 46888171 | −7.617 | −0.4 | −0.3 | −28.6 | Gln149 | 1.78 | Gln149 |
| 9 | GA-F | 23247895 | −7.402 | −0.8 | −0.6 | −37.6 | Tyr151 | 2.05 | Tyr 151, Gln 149 |
| 10 | GA-DM | 46888220 | −7.381 | −0.6 | −1.8 | −26.8 | Gln121 | 2.21 | Gly 121 |
| 11 | Curcumin | 969516 | −7.625 | −0.9 | −0.1 | −34.0 | Tyr151 | 2.25 | Tyr151, Gln149, Gln 61 |
| | | | | | | | Gln149 | 4.28 | |
| | | | | | | | Gln61 | 4.72 | |
| 12 | Catechin | 9064 | −6.922 | −0.7 | −0.5 | −31.4 | Tyr151 | 1.97 | Tyr 151, Gly 121 |
| | | | | | | | Gly121 | 7.27 | |
| 13 | Bupropion | 444 | −6.028 | −0.2 | −0.4 | −25.4 | Tyr151 | 1.78 | Tyr 151, Gln 61 |
| | | | | | | | Gln61 | 2.24 | |
| 14 | Pentoxyphylline | 4740 | | −0.04 | −0.2 | −28.0 | – | – | – |

Gln61 donates electron with 4.72 A° distance. On the other hand, bupropion has low GScore (−6.028) and forms hydrogen bonding with Tyr151 and Gln61 with 1.78 and 2.24 A° distance (Table 1). It was inferred from molecular docking that the hydrophobic interactions were the main force involved and matching the complementarity. The scoring function indicates the likelihood of the favorable binding interaction of the docking pose.

In another receptor DR5, the crystal structure of phage derived Fab BdF1 with human death receptor 5 (DR5) was retrieved from Protein Data Bank. Among different isoforms of GA, GA-A scores high among docked ligands with the DR5 receptor by computing complementarity simulation in the molecular recognition process by matching the complementarity surface between the ligand and the receptor. The prediction of best orientation in GA-A explains various parameters with GScore (−8.7), HBond (−2.9), Glide emodel (−30.0) (Table 2). Hydrogen bonding forms the major binding energy in which hydrogen of Met99 and Arg101 donates its electron to oxygen with 2.02 and 2.11 A° distance, respectively. On the other hand, lanosterol structure of GA-A donates its electron to the oxygen of Glu98 and Pro97 with 1.74, 1.96 A° distance, respectively (Fig. 5). GA-DM shows the lowest of docked energy as compared with other isoforms of GAs with GScore (−5.4), HBond (−2.5), Glide emodel (−15.4) and forms hydrogen bonding Arg101 with a distance of 1.86 A° in GA-DM.

In the case of natural inhibitors, curcumin results in best docked among different natural compounds. Curcumin accounts for GScore (−6.6), HBond (−1.1), Glide emodel (−43.6), and forms hydrogen bonding (Glu93 and Asp122) with the distance of 1.83 A° and 2.67, respectively. In this hydroxyl group of phenol ring of curcumin donate its electrons and forms hydrogen bonding with Glu93; whereas hydroxyl group of same phenol ring had involved in hydrogen bonding by donating electron from hydroxyl to oxide ion of Asp122. On the other hand, Arg 101 forms pi-

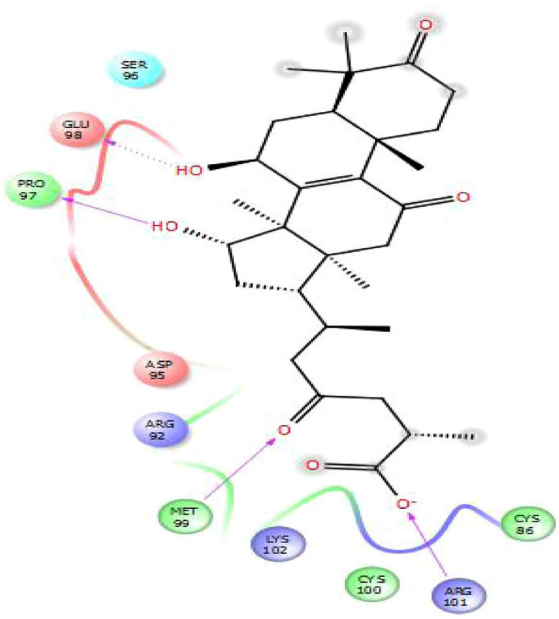
**Table 2** Binding affinities scores and energies of different compounds with DR5 receptor

| S. no. | Ligand type | PubChem CID | GScore | H Bond | Lipophilic EvdW | Glide emodel | Residues in H-bond | H-bond length (Å) | Protein–ligands interaction |
|---|---|---|---|---|---|---|---|---|---|
| 1 | GA-A | 14109402 | −8.7 | −2.9 | −1.5 | −30.0 | Met99 | 2.02 | Met99, Arg101, Pro97, Glu98 |
| | | | | | | | Arg101 | 2.11 | |
| | | | | | | | Pro97 | 1.96 | |
| | | | | | | | Glu98 | 1.74 | |
| 2 | GA-C2 | 57396771 | −7.9 | −3.9 | −1.3 | −29.0 | Met99 | 2.05 | Met99, Met99 |
| | | | | | | | Met99 | 2.11 | |
| | | | | | | | Arg101 | 1.74 | |
| 3 | GA-H | 471005 | −5.3 | −0.8 | −1.6 | −26.6 | Met99 | 1.94 | Met99, Arg101 |
| | | | | | | | Arg101 | 3.54 | |
| 4 | GA-T | 21637704 | −6.9 | −2.3 | −0.9 | −22.3 | Arg101 | 1.92 | Arg101, Arg101, Asp112 |
| | | | | | | | Arg101 | 1.89 | |
| | | | | | | | Asp112 | 1.96 | |
| 5 | GA-E | 23247894 | −6.9 | −2.7 | −1.8 | −23.3 | Arg92 | 2.30 | Arg92, Arg92 |
| | | | | | | | Arg92 | 2.31 | |
| | | | | | | | Arg101 | 1.99 | |
| 6 | GA-B | 471003 | −8.9 | −3.9 | −0.5 | −25.6 | Arg101 | 1.99 | Arg101 |
| | | | | | | | Arg92 | 1.91 | |
| | | | | | | | Arg92 | 2.08 | |
| 7 | GA-S | 46888171 | −5.6 | −2.2 | −1.1 | −21.1 | Arg101 | 1.74 | Arg101 |
| 8 | GA-R | 6449828 | −4.5 | −2.8 | −1.2 | −19.8 | Arg101 | 2.87 | Arg101 |
| 9 | GA-F | 23247895 | −7.6 | −2.7 | −0.6 | −16.2 | Met99 | 3.74 | Met99, Arg101 |
| | | | | | | | Arg101 | 1.93 | |
| 10 | GA-DM | 46888220 | −5.4 | −2.5 | −0.7 | −15.4 | Arg101 | 1.86 | Arg101 |
| 11 | Curcumin | 969516 | −6.6 | −1.1 | −0.6 | −43.6 | Glu93 | 1.82 | Arg101, Asp112, Glu93 |
| | | | | | | | Asp122 | 2.67 | |
| 12 | Catechin | 9064 | −7.2 | −0.9 | −0.8 | −20.7 | Glu93 | 2.03 | Glu93, Glu93 |
| | | | | | | | Glu93 | 1.88 | |
| 13 | Bupropion | 444 | −5.8 | −1.5 | −0.6 | −21.0 | Met99 | 1.84 | Met99, Glu98 |
| | | | | | | | Glu98 | 1.89 | |
| 14 | Pentoxyphylline | 4740 | | −1.2 | −0.7 | – | – | – | – |

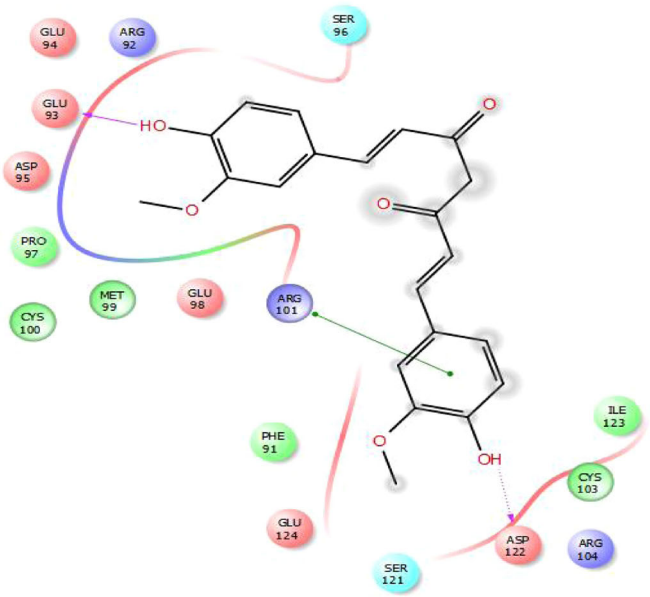
pi stacking between benzene electron cloud. Lowest docking energy was observed in bupropion with GScore (−5.8), HBond (−1.8), Glide emodel (−21.0), and forms hydrogen bonding in Met99 and Glu98 with a distance of 1.84 and 1.89 A°, respectively (Table 2). The backbone of Met 99 residue appears to be indulged in hydrogen bonding with the oxygen of bupropion. The amino group of bupropion donates its electron to the oxygen of Glu98 and forms hydrogen bonding. Molecular docking results disclose that the hydrophobic interactions form the basis of the strong binding interaction between the ligands and receptors. After docking, it concludes that docking predicted best binding orientation to the protein target and other activity of complex, and hence providing rationality in designing of drugs. The remarkable aspects of docking of compounds exhibiting Lipinski rule of five during pharmacokinetics and pharmacodynamics.

## ADME properties

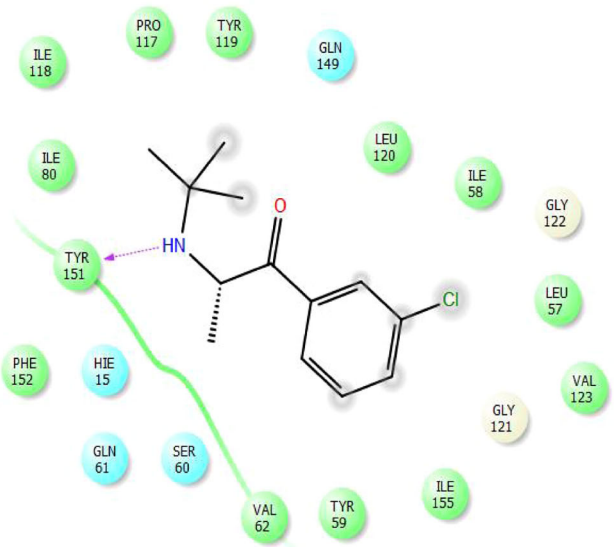
Binding affinity prediction is useful for analyzing the pharmacokinetic and pharmacodynamic properties. Qikprop is an important tool that calculates properties of the significant descriptors and pharmaceutically relevant molecules by comparing their values with those of 95% of already known pharmaceutical drugs. GA and its 11 isoforms were checked for ADME properties. Most interesting aspect of these compounds are their admirable QP log Po/w,



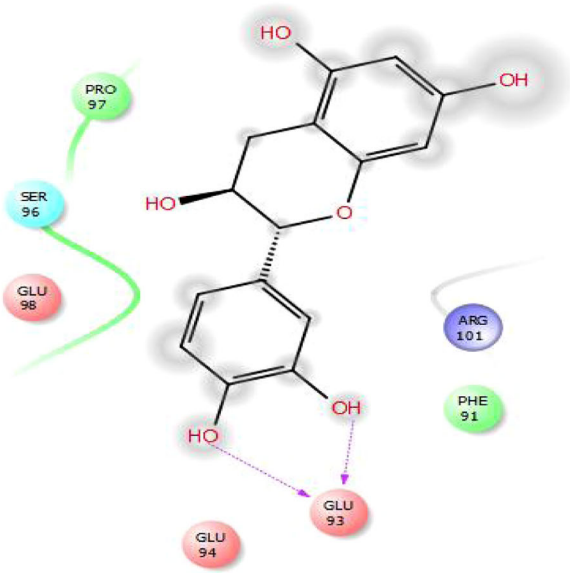

Ganoderic acid A




Curcumin




Bupropion




Catechin

**Fig. 5** Protein–ligands interactions profile of DR5 (PDB: 2H9G) with ganoderic acid A, curcumin, catechin, bupropion. Protein–ligands interactions profile revealed that residues involved in ganoderic acid A binding acid were Glu98, Pro97, Met97, Arg101, whereas Glu93, Arg101, Asp122 in curcumin. Furthermore, amino-acid Tyr151 in bupropion and Glu93 were involved in catechin and forms hydrogen bonding and pi–pi interaction, which forms the basis of strong bonding and interaction

QP log HERGK channels, QP log BB, QP log KP, and QP logKhsa values that satisfy the Lipinski's rule of five (Tables 3 and 4). GA proves to be best of all, whereas all other isoforms follow Lipinski's rule. GA follows QPPCaco (25.13), curcumin (108.43), catechin (50.64), pentoxyphylline (652.15), whereas bupropion (1803.05), pentoxyphylline (311.66) shows QPPMDCK activity that was not present in all other compounds with during TNF post-docking studies. In receptor DR5, QPPCaco activity was shown only by anti-TNF inhibitors like curcumin

**Table 3** Evaluation of drug-like properties of the different isoforms of ganoderic acid and natural inhibitors of TNF by Qikprop

| Molecule | PubChem CID | QP log Po/w (−2.0 to 6.5) | QP log HERG (acceptable range: above −5.0) | QPP Caco (nm/s) \25-poor [500-great] | QP log BB (−3 to 1.2) | QPP MDCK (nm/s) | QP log Kp (−8.0 to −0.1) |
|---|---|---|---|---|---|---|---|
| GA-A | 14109402 | 2.888 | −2.418 | 25.13 | −2.085 | 11.15 | −4.912 |
| GA-C2 | 57396771 | 2.951 | −2.256 | 14.13 | −2.2 | 14.31 | −5.025 |
| GA-R | 6449828 | 6.278 | −3.314 | 17.05 | −1.817 | 13.125 | −4.095 |
| GA-H | 471005 | 2.494 | −2.409 | 9.8 | −2.352 | 11.242 | −5.432 |
| GA-T | 21637704 | 6.348 | −3.439 | 22.78 | −2.282 | 11.56 | −4.612 |
| GA-E | 23247894 | 2.75 | −2.46 | 13.291 | −2.115 | 11.896 | −5.367 |
| GA-B | 471003 | 2.772 | −2.665 | 10.965 | −2.396 | 14.789 | −5.337 |
| GA-S | 46888171 | 6.428 | −2.852 | 18.723 | −1.128 | 16.874 | −3.473 |
| GA-F | 23247895 | 2.286 | −2.577 | 18.415 | −2.422 | 13.598 | −5.656 |
| GA-DM | 46888220 | 5.581 | −2.584 | 15.703 | −1.304 | 13.658 | −3.961 |
| Curcumin | 969516 | 2.309 | −5.099 | 108.434 | −2.152 | 44.821 | −3.554 |
| Catechin | 9064 | 0.458 | −4.863 | 50.647 | −1.929 | 19.684 | −4.736 |
| Bupropion | 444 | 2.88 | −2.155 | 19.586 | 0.658 | 1803.052 | −3.259 |
| Pentoxyphylline | 4740 | 1.062 | −3.963 | 652.154 | −0.843 | 311.662 | −3.132 |

**Table 4** Evaluation of drug-like properties of the different isoforms of ganoderic acid and natural inhibitors of the DR5 receptor by Qikprop

| Molecule | PubChem CID | QP log Po/w (−2.0 to 6.5) | QP log HERG (acceptable range: above −5.0) | QPP Caco (nm/s) \25-poor [500-great] | QP log BB (−3 to 1.2) | QPP MDCK (nm/s) | QP log Kp (−8.0 to−0.1) |
|---|---|---|---|---|---|---|---|
| GA-A | 14109402 | 2.9 | −2.2 | 17.9 | −1.8 | 13.8 | −5.1 |
| GA-C2 | 57396771 | 3.0 | −2.4 | 12.7 | −2.3 | 11.6 | −5.1 |
| GA-R | 6449828 | 6.2 | −3.3 | 13.0 | −1.8 | 12.0 | −4.2 |
| GA-H | 471005 | 2.5 | −2.3 | 13.8 | −2.1 | 16.1 | −5.1 |
| GA-T | 21637704 | 6.0 | −3.4 | 17.2 | −2.2 | 12.8 | −4.5 |
| GA-E | 23247894 | 3.1 | −5.3 | 14.7 | −2.2 | 16.5 | −5.2 |
| GA-B | 471003 | 2.7 | −2.4 | 11.3 | −2.3 | 14.4 | −5.3 |
| GA-S | 46888171 | 6.6 | −2.7 | 11.4 | −1.1 | 13.8 | −3.4 |
| GA-F | 23247895 | 2.3 | −2.7 | 11.1 | −2.4 | 14.4 | −5.4 |
| GA-DM | 46888220 | 5.7 | −2.9 | 17.7 | −1.4 | 13.3 | −4.0 |
| Curcumin | 969516 | 2.9 | −6.3 | 326 | −1.8 | 148 | −2.2 |
| Catechin | 9064 | 0.4 | −4.7 | 53.3 | −1.8 | 20.8 | −4.7 |
| Bupropion | 444 | 3.1 | −4.9 | 1185 | 0.6 | 1622 | −3.3 |
| Pentoxyphylline | 4740 | 1.2 | −4.5 | 494 | −1.06 | 230 | −3.3 |

Predicted IC50 value for blockage of HERG K + channels; (Acceptable range: above −5.0) ; QPP Caco, predicted apparent Caco2 cell permeability in nm/s. Caco2 cells is a model for the gut-blood barrier; (nm/s) <25 poor >500 great; QP log BB, 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) <25 poor >500 great; QP log KP, predicted skin permeability; Percentage of human oral absorption; (<25% is poor and >80% is high)

(326), catechin (53.3), bupropion (1185), pentoxyphylline (494), and QPPMDCK activity by the bupropion (1622), pentoxyphylline (230) (Tables 3 and 4). GA and its 11 isoforms do not show these activities. It was fascinating to note that the activities like QPPCaco, QPPMDCK, and oral percentage absorption were not satisfying the level and needs some structural modification to modulate their activity. Drug designing requires some parameters that make the entry of drugs specific and efficient without any side effect. These requires polar surface area, high oral

bioavailability, H-bond donors, and acceptors for the development of therapeutic agents (Singh and Bast 2014). Lipophilic Evdw is defined from hydrophobic grid potential and fraction of the total protein-ligand Van der Waals energy.

### Effects of GA on cancer cells

To measure the cytotoxicity of GA in cancer cells, MTT assay were performed. Breast cancer cells (T47-D), (MDA-MB-231) were treated with different concentrations of GA-A (5, 10, 20, 50, 80 µM) at different time intervals (24, 48, and 72 h). GA-A exhibits the remarkable reduction in cell proliferation in cancer cells in a concentration-dependent manner (Fig. 6a). Moreover, trypan blue exclusion test of cell viability was performed to determine the number of the treatments of GA-A. The viable and dead cells were determined by trypan blue exclusion test. GA-A exhibited a pronounced decrease in cell viability (Fig. 6b). Overall, it can be inferred that GA-A reduced cell viability in a concentration-dependent manner.

To determine the intracellular ROS scavenging ability of GA-A, $H_2$DCF-DA assay was carried out with T47-D, MDA-MB-231 cells. The data obtained from the assay highlighted that GA-A potentially decreases the reactive oxygen species. GA-A inhibits upto 50% as compared to control (Fig. 6c). Therefore, it can be concluded that GA-A has the potential to decrease the ROS production.

The effect of GA-A on TNF and DR5 receptors was determined by using semi-quantitative RT-PCR and quantitative real-time PCR (Q-PCR). After treatment of GA-A (48 h), mRNA expression in breast cancer lines (T-47D, MDA-MB-231) exhibits inhibition or downregulation in the expression. The RT-PCR densitometric bands analysis showed that GA-A reduction of mRNA expression in both TNF and DR5 receptors, whereas q-PCR quantitatively determine the downregulation of mRNA expression by 42% in T-47D cells and 32% in MDA-MB-231 cells in TNF. On the other hand, DR5 expression was downregulated to 31% in T-47D and 27% in MD-MBA-231 cells (Fig. 7). Thus, potentially inhibiting TNF and DR5 receptor and enhances the chances of using GA in cancer therapy. The previous

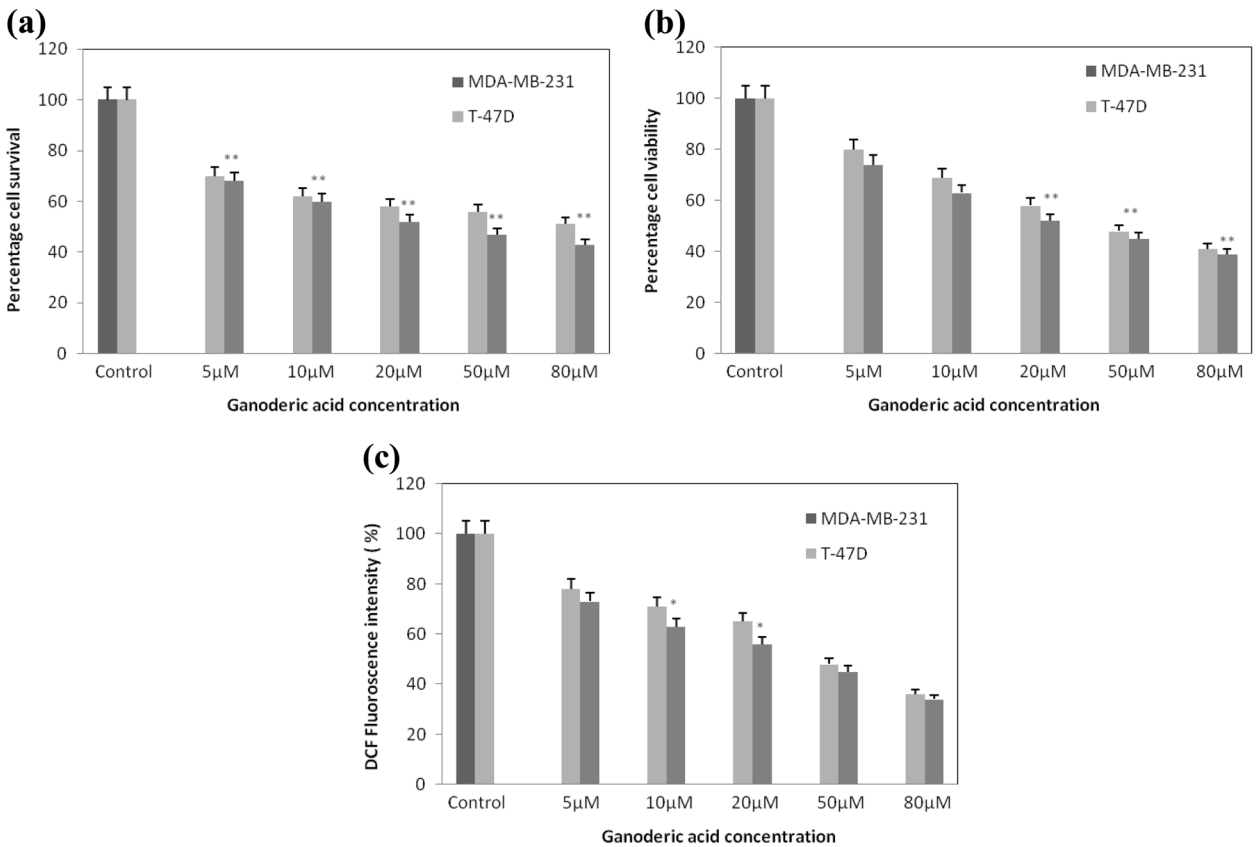



**Fig. 6** Effect of ganoderic acid A on MDA-MB-231, T-47D cells. **a** Ganoderic acid A reduces the cell survival in a dose-dependent manner determined by the MTT assay. **b** Ganoderic acid A reduces the cell viability of MDA-MB-231, T-47D cells in a dose-dependent manner determined the trypan blue exclusion test. **c** DCF fluorescence intensity (%) in MDA-MB-231, T-47D cells in a dose-dependent manner cells in response to ganoderic acid A. *P\0.05 and **P\0.01 vs. control

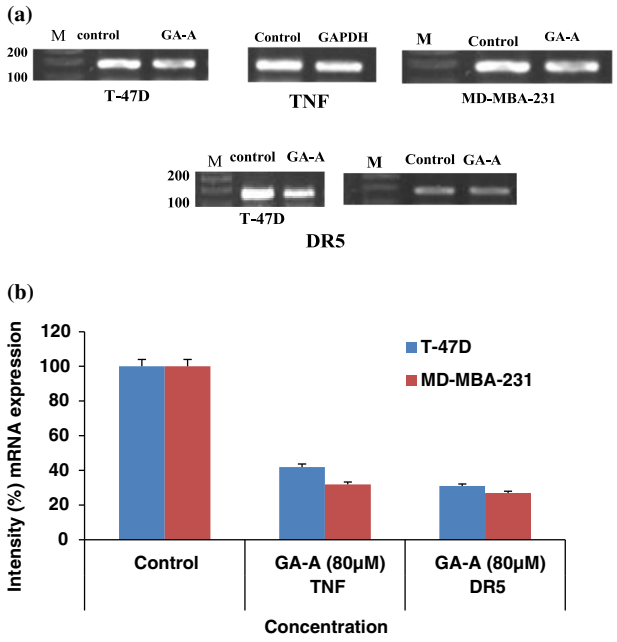



**Fig. 7** Effect of ganoderic acid A (GA-A) on TNF, and DR5 mRNA expression in breast cancer cells. **a** Densitometric analysis of band exhibits the downregulation of TNF and DR5 mRNA expression in MDA-MB-231 cells than T-47D cells, when treated with 80 μM ganoderic acid. **b** GA-A downregulated the expression of DR5 more effectively as compared to TNF in MDA-MB-231 than T47-D cells in q-PCR

study demonstrates that GA-A inhibits NF-κB effectively in breast cancer (Gill and Kumar 2015).

## Conclusion

Natural products are potentially accredited for targeting various signaling molecules in cancer pathway. In cancer, receptor tyrosine kinase plays a crucial role in downstream signaling by modulating the activity of the receptor and other transcription factor involved. The constitutive activation of TNF and its receptors results in aberrant activation of signaling. In the present study, we docked TNF and DR5 with its natural inhibitors (already documented for comparison) with GA and its 11 isoforms by using Maestro 9.6 molecular docking suite. The receptor-based molecular docking disclosed the binding efficiency of different isoforms of GA and highlighted the residues during the interaction. TNF and receptor DR5 signaling lead to activation of numerous downstream pathways mainly apoptosis, thus GA potentially downregulates the expression in the breast cancer. GA effectively inhibits proliferation, viability, and intracellular ROS and mRNA expression of TNF and DR5 in T47D and MD-MDA-231 cell lines. The present group is engaged in checking the anticancerous property of *Ganoderma lucidum* and its constituents growing in stress conditions.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no competing interests.

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