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Research Article

In vitro and *in silico* anticancer activity of ammonium glycyrrhizinate isolated from roots of *Glycyrrhiza glabra* Linn

Padmaa M Paarakh*[*1], Dileep Chandra Sreeram[2], Shruthi SD[3], Sujan Ganapathy PS[4]
[1]*Dept of Pharmacognosy, The Oxford College of Pharmacy, Hongasandra, Bangalore, Karnataka, India*
[2]*R & D[Phytochemistry], Natural Remedies Private limited, Bangalore, India*
[3]*Microbiology and Cell Biology Department, Indian Institute of Science, Bangalore, Karnataka, India*
[4]*Centre for Advanced Studies in Biosciences, Jain University, Bangalore, India*

***Corresponding Author:**
Padmaa M Paarakh
Email: padmaparas@hotmail.com

Abstract: *Glycyrriza glabra* Linn.[Fabaceae], commonly known as yashtimadhu, mulhatti, is used as medicine for treatment of various aliments in Traditional System of Medicine fairly throughout the greater part of India. In the present study, we have evaluated anticancer activity of Ammonium glycyrrhizinate by *in vitro* and in silico method. Ammonium glycyrrhizinate was isolated from roots of *G.glabra* and was characterized by solubility, melting point, infrared (IR) and thin layer chromotography (TLC) pattern. *In vitro* anticancer activity was done using HeLa cell lines by MTT assay at different concentrations ranging from 100-500 µg/ml using microtitre plate assays by ELISA and in silico docking studies using enzyme EGFR tyrosine kinase. The IC_{50} value was found to be 282.45 µg/ml in *in vitro* anticancer activity in HeLa cell lines. Ammonium glycyrrhizinate was subjected to molecular docking studies for the inhibition of the enzyme EGFR tyrosine kinase, which is one of the targets for inhibition of cancer cells. It has shown -11.03 kJ mol^{-1} binding and -12.47 kJ mol^{-1} docking energy with five hydrogen bonds. We can conclude that ammonium glycyrrhizinate has shown to possess anticancer activity both *in vitro* and *in silico* studies.

Keywords: *In vitro* anticancer activity; In silico docking studies; Isolation; Ammonium glycyrrhizinate; *Glycyrriza glabra*

INTRODUCTION

Cancer is one of the highest impacting diseases worldwide with significant morbidity and mortality rates. The current known therapies are based on radio and chemotherapies and although in many cases, the patients have their health re-established, the treatment is very painful since their immunological system is severely compromised, because these procedures are not cells selective [1]. Substantial advances have been made in understanding the key roles of receptor tyrosine kinase (RTK) in the signalling pathways that govern fundamental cellular processes, such as proliferation, migration, metabolism, differentiation and survival. In the normal cells RTK activity is tightly controlled.When they are mutated or structurally altered, they become potent oncoproteins which leads to abnormal activation of RTKs in transformed cells has been shown to be causally involved in the development and progression of many human cancers[2,3]. The cost of treatment is very high and with lot of side effects. In order to find new natural sources that are biologically active substances from plants have acquired immense attention. A number of studies have been carried out on various plants, vegetables and fruits because they are rich sources of phytoconstituents which prevent free radical damage thereby reducing risk of chronic diseases viz., cancer, cardiovascular diseases etc. This beneficial role of plants has led to increase in the search for newer plant based sources for the treatment of diseases like cancer. One such plant is *Glycyrriza glabra* Linn.

Glycyrriza glabra Linn.[Fabaceae], commonly known as yashtimadhu, mulhatti, is used as medicine for treatment of various aliments in Traditional System of Medicine fairly throughout the greater part of India. Traditionally the plant has been recommended as a prophylaxis for gastric and duodenal ulcers and dyspepsia, as an anti-inflammatory agent during allergenic reactions. In folk medicine, it is used as a laxative, emmenagogue, contraceptive, galactagogue, anti-asthmatic drug and antiviral agent.It is scientifically proved to be used as antitissive, demulcent, antiulcer, antimicrobial, antiviral, antioxidant, anticancer, antidiabetic and hepatoprotective activities.

Liquorice has Glycyrrhizin as the major water-soluble constituent which is responsible for its sweet taste. Glycyrrhizin is a triterpenoid saponin that is present within a range of 2-14% in different species and

ammonium glycyrrhiziate is the pure form which is readily isolated[4].

The aim of the present study is to isolate ammonium glycyrrhizinate from roots of *G.glabra* and perform *in vitro* MTT assay and in silico activity to prove its anticancer activity.

MATERIAL AND METHODS

Drugs and Chemicals

DMEM medium (GIBCO), heat-inactivated fetal bovine serum (FBS), trypsin, ethylene-diaminetetraacetic acid (EDTA), PBS and MTT were purchased from Hi media and Sigma Chemicals. All chemicals and reagents used in this study were at least of analytical grade.

Plant Material

The dried roots of *Glycyrrhiza glabra* were collected, identified and authenticated by Botanist from Natural Remedies Private Limited, Bengaluru, Karnataka. A voucher specimen was deposited in the Herbarium of Department of Pharmacognosy, The Oxford College of Pharmacy, Bangalore. The roots were dried under normal environmental conditions. The dried roots were powdered and stored in a closed container for further use.

Extraction and Isolation Procedure

The dried roots of *G.glabra* (350 g) were mixed with 250 ml acetone and 20 ml of dilute HNO_3. Mix thoroughly, cork the flask and macerate for 2 hrs. Shake occasionally and filter the content. Add 100 ml of acetone to the marc and warm on water bath and filter again. Combine the filtrate and concentrate preferably under vaccum. To combined acetone extract add quantity of dilute ammonia solution for precipitation of Ammonium glycyrrhyzinate. The precipitates were separated by filtration, washed with 50 ml of acetone twice and recrystallized. The product is dried and weighed. The percentage yield was found to be 3.5 % w/w with reference to air dried plant.

Characterization of Ammonium Glycyrrhizinate

Standard Ammonium glycyrrhizinate was procured from Yucca Enterprises, Mumbai and subjected for characterization to check the purity of this marker compound. The marker was thus tested for solubility, melting point, infrared (IR) and thin layer chromotography (TLC) pattern by using the standard protocol mentioned in the literature along with isolated compound [5].

HPLC Chromatogram of the Ammonium glycyrrhyzinate

Weigh 50 mg of reference standard and isolated ammonium glycycrrhiiziate were mixed with 50 ml of the mobile phase respectively. 1 ml of each solution was diluted with 200 ml of mobile phase and filters with syringe filter and kept ready for injection. The column used was ODS silica column [5 µm; 20cm X 5 mm]; mobile phase used was glacial acetic acid: acetonitrile: water :: 6:380:614 V/V/V with flow rate of 1.2 ml/min with UV detection at 254 nm. 10 µl of the standard was injected and the chromatogram was run in triplicate to get retention time for standard. Similarly sample was also injected triplicate and run the chromatogram. The percentage purity of the sample was calculated.

In vitro anticancer activity using HeLA cell lines by MTT assay

Cell culture

HeLa cell line was maintained in DMEM medium (GIBCO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% antibiotic solution (penicillin 100 U/ml and streptomycin 100 µg/ml) at 37^0C in a humidified atmosphere of 95% air/5% CO_2. The medium was changed every second day, and cells were subcultured when confluency reach to 95% by 0.25% trypsin containing 0.02% ethylene-diaminetetraacetic acid (EDTA) in PBS for 3 min at 37^0C.

MTT Assay

The MTT assay was carried out as described previously to measure cell viability [6]. Ten thousand cells in 100µL of DMEM media were seeded in the wells of a 96-well plate. After 24 h, existing media was removed and 100 µL of various concentrations of compound was added and incubated for 48 h at37 °C in a CO2 incubator. Control cells were supplemented with 0.05% DMSO vehicle. At the 48th hour of incubation, MTT (3-(4,5-dimethylthaizol-2-yl)-2,5-diphenyl-tetrazolium bromide- supplied from Sigma, 10µL of 5 mg/mL) was added to the plate. The contents of the plate were pipetted out carefully, the formazan crystals formed were dissolved in 100 µL of DMSO, and the absorbance was measured at 550 nm in a microplate reader (Tecan, infinite F200 Pro). Experiments were performed in triplicate, and the results were expressed as mean of percentage inhibition. A graph of the concentration versus percentage growth inhibition was plotted, and the concentration at which 50% cell death occurred was considered as the IC_{50} value. Before adding MTT, bright field images (Olympus 1X81, cellSens Dimension software) were taken for visualizing the cell death.

In silico activity: Molecular Docking studies

The three dimensional structure of target protein EGFR tyrosine kinase (PDB ID:2J5F) was downloaded from PDB (www.rcsb.org/pdb) structural database. This file was then opened in SPDB viewer edited by removing the heteroatoms, adding C terminal oxygen. The active pockets on target protein molecule were found out using CASTp server [7]. The ligands were drawn using ChemDraw Ultra 6.0 and assigned with proper 2D orientation (ChemOffice package). 3D coordinates were prepared using PRODRG server [8].

Autodock V3.0 was used to perform Automated Molecular Docking in AMD Athlon (TM)2x2 215 at 2.70 GHz, with 1.75 GB of RAM. AutoDock 3.0 was compiled and run under Microsoft Windows XP service pack 3. For docking, grid map is required in AutoDock, the size of the grid box was set at 102, 126 and 118 Å (R, G, and B), and grid center -58.865, -8.115, -24.556 for x, y, and z-coordinates. All torsions were allowed to rotate during docking. The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization, using default parameters [9].

RESULTS

The Characterization of the Ammonium glycyrrhizinate [Fig-1]

Physical data:

Ammonium glycyrrhizinate is white or yellowish-white, hygroscopic powder; Soluble in water and hydro-alcohol solutions at recommended dosage levels; soluble in glycerine and propylene glycol. Molecular formula: $C_{42}H_{65}NO_{16}$; Molecular weight: 840

Spectral Data:

UV-VIS spectrum shows absorption at 254 nm ($\varepsilon = 1.8 \times 10^4$). The UV-VIS spectrum indicates the presence of chromophoric system in the molecule.The IR spectrum shows a broad band at 3309 cm^{-1} due to presence of hydroxyl group. A band at 1698 cm^{-1} indicates the carbonyl group in the molecule. The band at 1273 cm^{-1} can be assigned to a stretching at C–O–C. Superimposible IR was done with reference standard and isolated compound and was found to be exactly the same as reference standard.

The HPLC chromatogram showed peak with retention time of 21 minutes with 98.5 % purity which confirmed the presence of ammonium glycyrrhizinate in comparison with reference standard.

***In vitro* anticancer activity on HeLa Cell Lines:**

The MTT values obtained demonstrated that ammonium glycyrrhiziante has good cytotoxic effect. The IC_{50} value was found to be 282.45 µg/ml. Microscopy images representing the cell death caused by the compounds are as seen in Fig.2. It is very clear that it is very good cytotoxic agent.

In silico molecular docking studies:

The tyrosine kinase receptors have multidomain extracellular Ligands for specific Ligand, a signal pass transmembrane hydrophobic helix and tyrosine kinase domain. The receptor tyrosine kinases are not only cell surfaces transmembrane receptors, but are also enzymes having kinase activity [10]. In cancer, angiogenesis is an important step in which new capillaries develop and develop for supplying a vasculature to provide nutrient and removing waste material. So tyrosine kinase inhibitor as an anti-angiogenic agent is new cancer therapy. Developing natural drugs and prodrugs as inhibitor is today's trend. Low molecular weight substances, which inhibit tyrosine kinase phosphorylation block signaling pathway, initiated in the extracellular part of receptor [11].Since, the type I receptor tyrosine kinase is a major regulator of several distinct and diverse cellular pathways we have evaluated it as a target.

Ammonium glycyrrhizinate was taken and docked to get the best conformer. Results were compared for the binding energy, docking energy and number of hydrogen bonds formed. According to the docking results (Table 1), It has shown -11.03 kJ mol^{-1} binding and -12.47 kJ mol^{-1} docking energy with five hydrogen bonds. Molecular docking with EGFR tyrosine kinase domain revealed that, our compound have inhibitory capability and thereby exhibiting interactions with one or the other amino acids in the active pockets as shown in Fig.3b. The topology of the active site of EGFR tyrosine kinase was similar in all synthesized molecules, which is lined by interacting amino acids as predicted from the ligplot (Fig 3c).In *in vitro* studies the molecule emerged as more active against the cell line used.

Table-1: Molecular docking results of ammonium Glycyrrhizinate with EGFR tyrosine kinase.

Molecule	Binding energy	Docking energy	Inhibitory constant	Intermol energy	H-bonds	Bonding
GAH	-11.03	-12.47	8.28e-009	-12.58	5	GAH::DRG:HAV:TK:A:GLU762:OE1 GAH::DRG:OAU:TK:A:ASP855:HN GAH::DRG:HAU:TK:A:THR854:OG1 GAH::DRG:OCH:TK:A:ARG803:HH21 GAH::DRG:OAV:TK:A:LYS745:HZ3

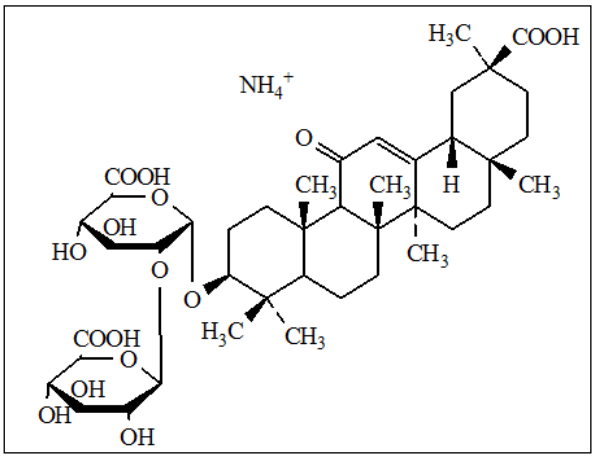

Fig-1: Structure of the compound ammonium glycyrrhizinate.

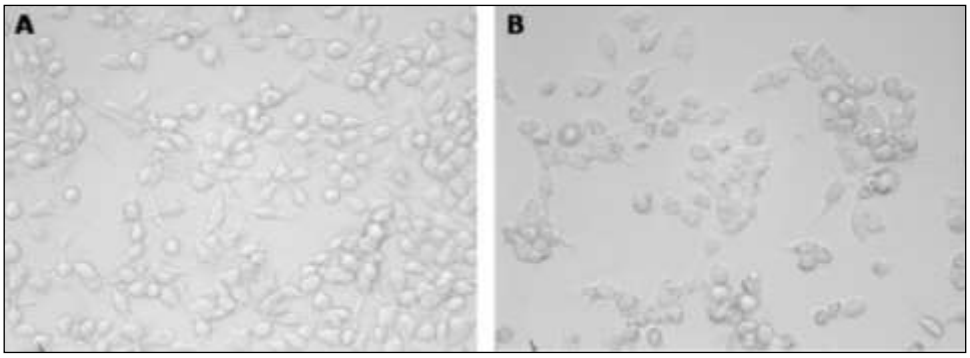

Fig-2: Anticancer activity of Ammonium glycyrrhizinate showing cell death; A-control; B-treated.

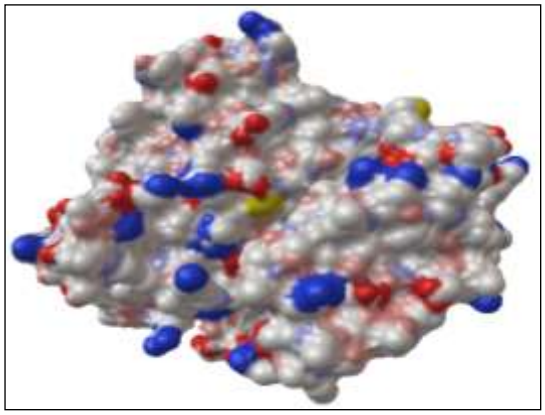

Fig-3a: 3D structure of EGFR tyrosine kinase from PDB

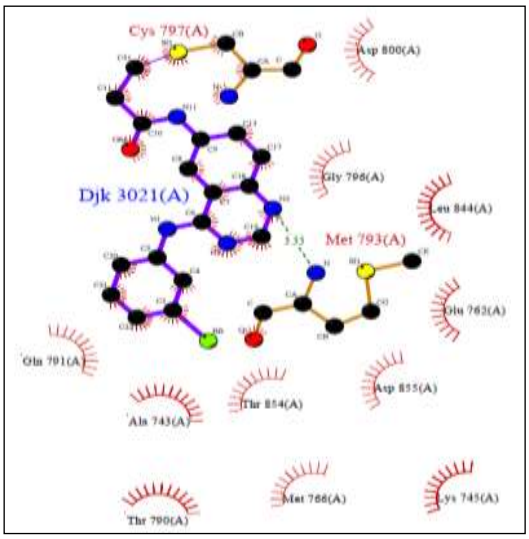

Fig-3b: Interacting amino acids as predicted from the ligplot

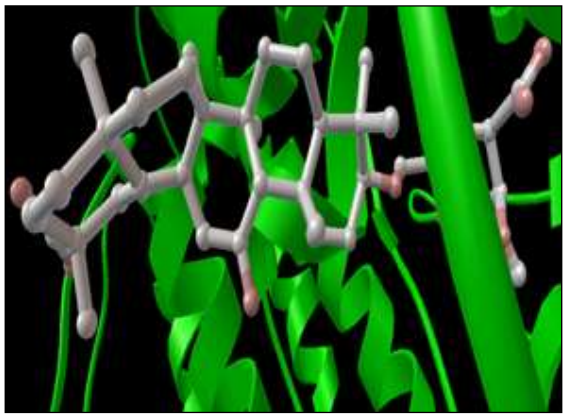

Fig-3c: Enfolding of Glycyrrhizin ammonical hydrate in the active pocket.

CONCLUSION

Ammonium glycyrrhizinate has shown to possess anticancer activity both *in vitro* and *in silico* studies. The IC_{50} value was found to be 282.45 µg/ml and *in silico* studies, it has more number of hydrogen bonds with minimum binding and docking energy and may be considered as inhibitor of EGFR tyrosine kinase. More experiments are required to understand the exact mechanism by which the cells are affected. It is important to correlate the structure of these compounds with their biological effect, which will be valuable to propose new lead compounds with better cytotoxic potential.

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