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RESEARCH ARTICLE

IN VITRO ANTIPROLIFERATIVE AND IN SILICO ACTIVITY OF RUBIADIN ISOLATED FROM ROOTS OF *Rubia Cordifolia*

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ABSTRACT

Background: *Rubia cordifolia Linn*.[Rubiaceae], commonly known as Indian Maddar and Manjistha, is used as medicine for treatment of various ailments in Traditional System of Medicine of India. **Purpose:** To isolate rubiadin and evaluate *in vitro* antiproliferative activity and in silico method. **Material and Methods:** Rubiadin was isolated from the roots of R.cordifolia. Rubiadin was characterized by IR, ^{1}H-NMR, ^{13}C-NMR and Mass spectrum. Standardization of rubiadin was done also by HPLC fingerprinting. *In vitro* antiproliferative activity was done using HeLa cell lines by MTT assay at different concentrations ranging from 20-100 µg/ml in triplicate and in silico docking studies using enzyme EGFR tyrosine kinase. **Results:** Fingerprinting of isolated rubiadin were done by HPLC method. The IC_{50} value was found to be 56.63 ± 0.025 µg/ml in *in vitro* antiproliferative activity in HeLa cell lines. Rubiadin 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 -7.07 kJ mol-1 binding and -7.12 kJ mol-1 docking energy with two hydrogen bonds. Conclusion: Rubiadin has shown to possess *in vitro* antiproliferative activity and in silico studies.

Key words: *In vitro* antiproliferative activity; in silico docking studies; Isolation; Rubiadin; *Rubia cordifolia.*

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 *Rubia cordifolia* Linn.

Rubia cordifolia Linn.[Rubiaceae], commonly known as Indian Maddar and Manjistha, is used as medicine for treatment of various ailments in Traditional System of Medicine fairly throughout the greater part of India. Traditionally the plant has been recommended as it is useful as bitter, acrid, astringent, thermogenic, antidysenteric, antiinflammatory, antipyretic, analgesic, anodyne, anthelmintic, antiseptic, constipating, diuretic, galactopurifier, febrifuge, rejuvenating and tonic [4]. It is scientifically proved to be used as antiacne, anticancer,antidiabetic, antimicrobial,anticonvulsant, anti-inflammatory,wound healing, antiulcer, antiviral, antioxidant, antistress, antiplatelet activating activity, gastroprotective, hepatoprotective, immunomodulatory and radioprotection activities[5].

The tyrosine kinases inhibitor activity of rubiadin is not studied till date.The aim of the present study is to isolate rubiadin from dried roots of *Rubia cordifolia* and perform in silico activity and *in vitro* MTT assay to prove its antiproliferative 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 *Rubia cordifolia* 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

Extract the dried roots of *R.cardifolia* (3 kg) with methanol (4 l) by refluxing for 1 h. Filter and repeat the process of reflux by adding methanol (4 l) to the marc and filter. Distill the combined filtered methanol extract to remove the solvent and dry the concentrate under vacuum to get a thick green paste (250 g). Carry out liquid-liquid partitioning with ethyl acetate and water (1:1) three times. Concentrate the ethyl acetate layer under vacuum and dissolve the extractive (110 g) in 100 ml methanol and adsorb over 100 g of silica gel (60-120 mesh grade). Dry the adsorbed material in vacuum oven at temperature not more than 70^{o} to remove solvent. Charge the adsorbed material on a silica column (~1 kg; 60-120 mesh grades). Elute the column by gradient elution using *n*-hexane with increasing percentage of ethyl acetate. Monitor the elution with TLC and combine the fractions eluted with 25-30 % ethyl acetate in *n*-hexane and concentrate under vacuum (35g). Adsorb the fraction over flash grade silica (230-400 mesh grade) and subject to flash chromatography by using flash grade silica column (40 g) with

mixture of *n*-hexane and ethyl acetate as the eluting solvents. Collect the fractions eluted with ethyl acetate:*n*-hexane (1.5:8.5) and concentrate under vacuum to get a enriched fraction of rubiadin (1.2 g). Crystallize the fraction in methanol to get rubiadin (350 mg). Store in airtight container in a cool and dry place.

Characterization of Rubiadin

The structure of rubiadin was characterized by UV, IR, NMR, Mass spectrum. HPLC fingerprinting was done to confirm the presence and purity of rubiadin.

HPLC Chromatogram of the Rubiadin

Weigh 10 mg of reference standard and isolated rubiadin were mixed with 50 ml of the mobile phase respectively. 1 ml of each solution was diluted with 200 ml of mobile phase and filter with syringe filter and kept ready for injection. The column used was ODS silica column[5 µm; 20cm X 4.6 mm]; mobile phase used was methanol: water :: 80:20 V/V with flow rate of 1.0 ml/min with UV detection at 300 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* antiproliferative 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 100U/ml and streptomycin 100µg/ml) at 37°C 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°C.

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 [20 to 100 µg/ml] were added and incubated for 48 h at 37°C in a CO_2 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-diphenyltetrazolium 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 [3 times x 3 wells each time /group] 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 Rubiadin [Fig 1]

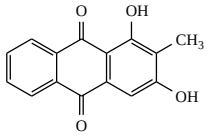

Fig 1: Structure of Rubiadin

Physical data

Rubiadin is Yellow needles, m.p. 303 -304 ºC (lit.6 302 ºC). Molecular formula: $C_{15}H_{10}O_4$. ; Molecular weight: 254

Spectral Data

UV-VIS spectrum shows absorption at nm: 245, 279, 412. The UV-VIS spectrum indicates the presence of chromophoric system in the molecule. The IR spectrum of rubiadin showed characteristic peaks at 3393(OH str.), 2361.9(alkyl CH str.), 1661.75 (unchelated C=O) and 1624 cm^{-1}(chelated C=O). Superimposible IR was done with reference standard and isolated compound and was found to be exactly the same as reference standard.

^{1}H NMR spectrum (DMSO-d_6-500 MHz): The signal at δ 13.25 (S, Cl-OH), 8.21-8.26 (2H, m, C5-Hand C8-H), 7.80-7.87 (2H, m, C6-H and C7-H), 7.30 (1H, s, C4-H). The δ values were comparable with that of reported ^{1}H NMR Rubiadin [10].

^{13}C NMR spectrum (CD_3OD -300 MHz): the signals at δ 109.6,116,126.9,127,133,133.4,133.9,

134,134.2,161.7,164.1,182 and 186.7. The δ values were comparable with that of reported ^{13}C NMR rubiadin [10].

Mass spectrum of rubiadin showed molecular ion peak of aglycone at (m/z) 254 which further confirmed the molecular formula as $C_{15}H_{10}O_4$.

The HPLC chromatogram showed peak with retention time of 25.07 minutes with 99.0 % purity which confirmed the presence of rubiadin in comparison with reference standard.

***In vitro* antiproliferative activity on HeLa Cell Lines**

The MTT values obtained demonstrated that rubiadin has antiproliferative effect as the IC_{50} value was found to be 56.63 ± 0.025 µg/ml. Microscopy images representing the cell death caused by the compounds are as seen in Fig No.2.& 3 It is very clear that it is antiproliferative agent when compared to control cell with vehicle alone.

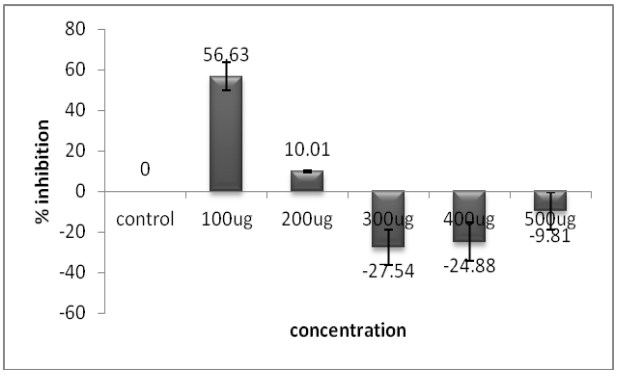

Fig 2: Percentage inhibition of cell growth at different concentrations of rubiadin compound. Data are Mean±SE (n=3).

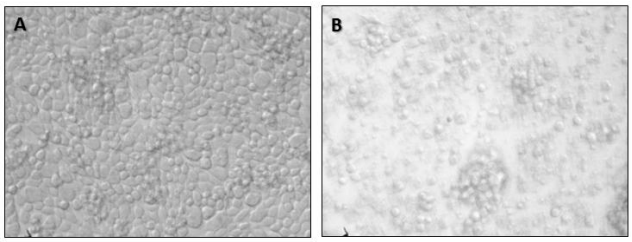

Fig 3: Anticancer activity of rubiadin showing cell death, A-control; B-treated.

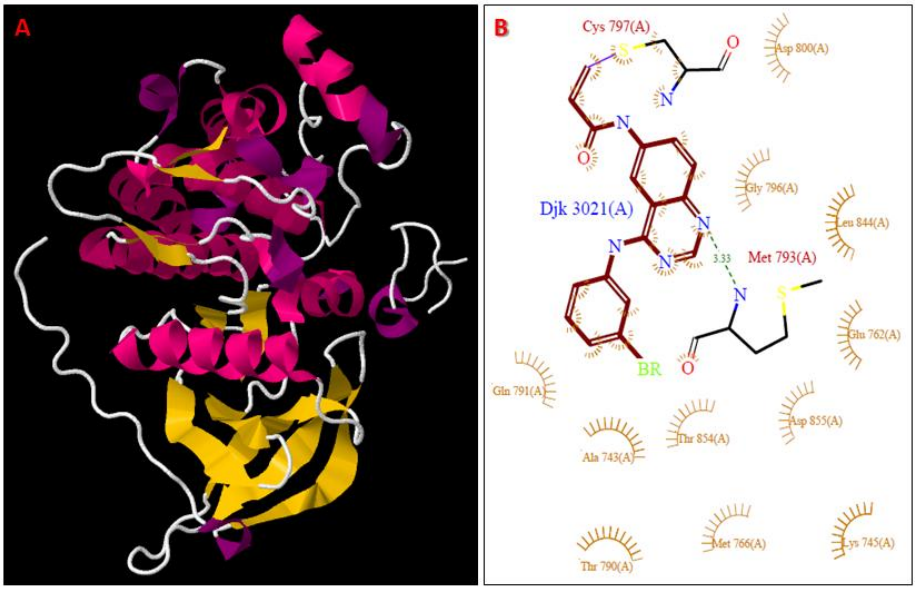

Fig 4: 3D structure of EGFR tyrosine kinase from PDB (A); Interacting amino acids as predicted from the ligplot (B).

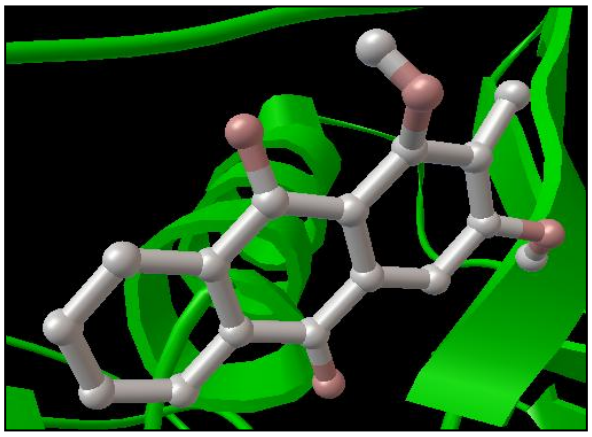

Fig 5: Enfolding of rubiadin in the active pocket.

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[11].In cancer, angiogenesis is an important step in which new capillaries 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[12,13].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.

Rubiadin 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 No.1), it has -7.07 kJ mol^{-1} binding and -7.12 kJ mol^{-1} docking energy with two hydrogen bonds. Molecular docking with EGFR tyrosine kinase domain revealed that, our compound has inhibitory capability and thereby exhibiting interactions with one or the other amino acids in the active pockets as shown in Fig No.5. 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 No.4.In *in vitro* studies the molecule emerged to be active against the cell line used in inhibiting the cell growth.

Table1: Molecular docking results of rubiadin with EGFR tyrosine kinase.

Molecule	Binding energy	Docking energy	Inhibitory constant	Intermol energy	H- bonds	Bonding
RD	-7.07	-7.12	6.54e-006	-7.07	2	RD::DRG:HAU:TK:A:LEU777:O RD::DRG:OAO:TK:A:LEU703:HN

CONCLUSION

Rubiadin has shown to possess *in vitro* antiproliferative activity and *in silico* studies. The IC50 value was found to be 56.63 ± 0.025 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.

ACKNOWLEDGEMENT

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ETHICAL ISSUES

There is none to be applied

CONFLICT OF INTEREST

None to be declared

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