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RESEARCH ARTICLE

IN SILICO AND IN VITRO EVALUATION OF APOPTOTIC INDUCTION POTENTIAL OF ANDROGRAPHOLIDE IN BREAST CARCINOMA CELLS

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ABSTRACT

Andrographolide (AGL) is a labdane diterpenoid that has been isolated from the stem and leaves of Andrographis paniculata. In Ayurvedic tradition, Andrographis, commonly known as Kalmegh or "King of Bitters" has long been used as a bitter tonic, to promote digestion and appetite. Recently, there are many reports that have findings on anti-inflammatory and cytotoxic activites of AGL. This motivated us to carry out this study, in which we aimed at investigating the anti-apoptotic potential of AGL with special emphasis on breast cancer. One of the major anti-apoptotic proteins that are found to be over-expressed in cancers is Bcl-2 (B-cell lymphoma 2). We performed molecular docking analysis of AGL against Bcl-2 followed by prediction of ADME/Tox analysis. The docking results showed that AGL interacts potentially with BcL-2 with a docking score of -4.675 Kcal/mol. It also has significant pharmacokinetics property evaluated using preADMET tools. Following that the docking results have been evaluated in MCF-7 cell line. The cytotoxicity of AGL has been analysed using MTT assay. It was found that at a minimal concentration of 80µM, AGL was toxic to MCF-7 cells. To confirm the apoptotic induction in cells after AGL treatment, Ao/EtBr dual staining was performed. It was found that about 54% of cells were showing apoptotic induction. Further to support the same, DNA fragmentation analysis was performed in Agarose gel electrophoresis and it was found that intact genomic DNA was visualised in case of untreated cells whereas in AGL treated cells, fragments of DNA were visualised. Thus, the study provides clear indication that AGL possibly has an anti-cancer activity by inducing apoptosis in cancer cells.

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INTRODUCTION

Andrographolide (AGL) is a labdane diterpenoid that has been isolated from the stem and leaves of *Andrographis paniculata.* AGL is an extremely bitter substance (Nilavembu) which is used to treat dengue. The compound AGL has been studied for its effects on cell signalling, immunomodulation, and stroke (Yang *et al*., 2018; Wang *et al*., 2010). There are many studies on AGL which reports that it can bind to a spectrum of protein targets including NF-KB and actin by covalent modification. In this study, we attempt to keen into the binding or inhibition of Bcl-2 protein by AGL (Low *et al*., 2015; Zhao *et al*., 2018; Rajagopal *et al*., 2018; Richard *et al*., 2018). Since Bcl-2 family proteins are important integral membrane proteins located mainly on the outer membrane of mitochondria, which play a critical role in regulating and executing apoptosis. Bcl-2 proteins provide balance between the concentrations of pro-apoptotic and anti-apoptotic to maintain homeostasis in cells; any perturbations to this balance may lead to disease including restenosis, stroke, heart failure, HIV infection, and cancer (Yip and Reed, 2018). Specially, in breast cancer cells, Bcl-2 was found to be over-expressed and which causes the lack of apoptosis in these cells. Thus, if Bcl-2 is inhibited, pro-apoptotic proteins concentration will increase which may cause the cells to swift towards apoptosis (https://www.omicsonline .org/scientific-reports/srep458.php). In this study, the potential of AGL to inhibit Bcl-2 was studied using *in silico* approaches followed by the evaluation of apoptosis induction in Mcf7 cells.

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MATERIALS AND METHODS

Retrieval of Protein and Ligand 3D structures

The three dimensional structure of Bcl-2 protein was retrieved from PDB database. It was then energy minimized using swiss-pdb viewer. The modified residues were removed and optimized.

The ligand AGL structure was retrieved from pubchem compounds. The energy minimization of ligand was performed using Frog2 tool.

Molecular docking

Docking was performed using Autodock in Linux platform. This is most probably used to find binding site of protein and ligand, binding energy, binding affinity etc., The computer simulated docking work was performed using MGL Tools software 1.5.6 and Autodock 1.5.6 program. The first step was preparing protein by adding/editing colour, hydrogen (merge non-polar), charges (kollman charges), atoms (assign AD4type) etc., and saving it in pdbqt format. Ligand preparation was also done and grid was set with active site residues. The autogrid and autodock was run. The docking score and the docking interactions were then studied.

ADME/Tox analysis

To predict the pharmokinetic properties, preADMET tool was used online. DruLito was also checked to evaluate the drug likeliness properties of the compound AGL.

Anti-inflammatory analysis using HRBC assay

The Human Red Blood Cell (HRBC) method has been used as a method to study the in vitro anti-inflammatory activity (Anosike, *et al*., 2018). Blood was collected and stored at +4^{O}C and centrifuged at 3000 rpm for 10 min and the supernatant was removed. The HRBC suspension was washed with hyposaline (0.9% NaCl) and with the same amount of HRBC suspension PBS (P^{H} 7.4) was added. The different concentrations (0µM, 20µM, 40µM, 60µM, 80µM, 100µM, 120µM, 140µM) was prepared and then incubated for 30 min after incubation the samples were centrifuged at 3000 rpm for 20 min. Spectrophotometer reading was taken at 560 nm and the values were calculated using %protection = *(*Optical density of test sample/Optical density of control*)* × 100

Free radical scavenging estimation using DPPH assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical. On accepting hydrogen from a corresponding donor, its solutions lose the characteristic deep purple colour. DPPH is very popular for the study of natural antioxidants. The PubMed database shows that this radical has been employed in more than 850 studies since 1969 (Kedare and Singh, 2011). DPPH stock was prepared (4mg in 100ml ethanol) and different concentrations (0µM, 20µM, 40µM, 60µM, 80µM, 100µM, 120µM, 140µM) were prepared then stored at dark for 30 min at room temperature. Spectrophotometer reading was taken at 517 nm and the values were calculated using % of radical scavenging activity = *(*Optical density of test sample/Optical density of control*)* × 100

Cytotoxicity assay

MCF7 cell lines were used for the study and is cultured in Dulbecco's Modified Eagle's Medium (high glucose) supplemented with 10% FBS and 1% antibiotic solution. Cytotoxicity of AGL was determined by MTT assay. MTT (3-(4, 5- dimethylthiazol-yl)-2, 5-diphenyltetrazolium bromide), proliferation assay was utilized in MCF-7 cell lines to assess the dose-dependent effect of AGL. The culture was incubated with the test solution in DMEM at 37^{O}C for 18hrs in a 5% Co2 atmosphere. After an overnight incubation, cells were treated with varying concentrations (0µM, 20µM, 40µM, 60µM, 80µM, 100µM, 120µM, 140µM) and incubated for 18hrs. After incubation the readings were taken by ELISA reader at 570nm (Banerjee *et al*., 2016).

Dual staining

Dual staining was performed to differentiate cells in apoptotic phases. This was done using AO/EtBr (Acridine Orange and Ethidium Bromide) dyes (Prasad and Koch, 2014). Briefly, MCF7 cells were grown in DMEM media and washed with PBS. 1×10^6 cells/mL was treated with 80 µM of AGL for 12 h at 37˚C and 5% CO2. The cells were then fixed with absolute ethanol at −20˚C for 15 minutes. After fixation, cells were washed and stained with 1mg/mL propidium iodide (PI) at 37˚C for 15 minutes. The cells were washed again and 10 μL cell suspension was taken on a slide. Fluorescent images were scanned using fluorescence microscope (Accuscope EX31I) and the images were captured by a digital camera. Similarly, to investigate apoptosis or necrosis, acridine orange (AO) and ethidium bromide (EB) staining method was performed. Acridine orange permeates all the cells and makes the nuclei appear green. Ethidium bromide was only taken up by dead cells when cytoplasmic membrane integrity is lost and the nucleus stains yellowish orange. Therefore, live cells have a normal green nucleus; early apoptotic cells show bright green/yellowish nucleus with condensed or fragmented chromatin; late apoptotic cells display condensed and fragmented orange/red chromatin while the cells that have died from direct necrosis have a structurally normal deep orange nucleus

DNA fragmentation

DNA fragmentation was done to isolate the DNA from the cells (Kawabata *et al*., 1994). The cells were treated with the compound AGL (80 µM) and incubated for 12 h in 5% CO2 incubator. To perform DNA fragmentation the cells were trypsinized from the well pellet was washed with ice cold PBS and re-pellet the cells. The volume of cells was measured and then the same volume of digestion buffer was added into the vial then incubated at 50^{O} C for 18 hrs. After incubation cells were extracted with phenol/chloroform/isoamyl alcohol then centrifuged for 10 min at 1700 x g after centrifuging the cells two layers were formed in the vial from that layers the aqueous layer was taken out to a new vial and then treated with 100% ethanol in twice the volume. Following the extraction of DNA, it was run on Agarose electrophoresis and the bands were visulaised using UV-Transilluminator.

RESULTS AND DISCUSSION

Retrieval of structures

The protein Bcl-2 was retrieved from PDB and the protein ID was determined as 2W3L which was from homosapiens. The active sites of protein Bcl-2 are TRY67, GLU95, ARG142, ARG12, THR137, and ASN131 (Mohamad Rosdi *et al*., 2018). The ligand AGL structure was taken from PubChem which is an online tool and the Pubchem ID for ligand is 5318517. 2-D and 3-D structures of the ligand were retrieved to perform docking.

Pre ADMET

This was performed to determine the pharmacokinetic properties of the compound AGL. The results are given in

table 1 & 2. AGL obeys the Lipinski rule of five which shows the drug likeliness property of the compound.

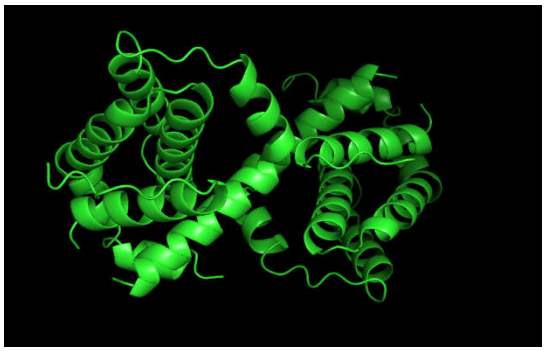


Fig. 1. The protein structure was viewed by PYMOL

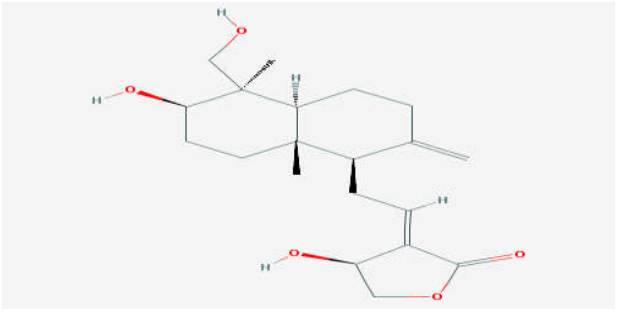


Fig. 2. The 2-D structure of Andrographolide

Table 1. Drug-Likeness Prediction which obeys Lipinski rule of five

S.no	Id	Acceptable Value	Obtained value
1.	Molecular weight ≤	500	350.21
2.	logP ≤	5	2.913
3.	H-bond donor	5	3
4.	H-bond acceptor	10	5

Table 2. ADME Prediction

S. no	ID	Value
1.	$CaCO_2$	19.1341
2.	MDCK	83.2465
3.	BBB	0.170359
4.	Plasma_Binding_Protein	94.873445
5.	Skin_permeability	-3.65236

Molecular Docking analysis

Molecular docking performed using Autodock showed that AGL bind to the protein well and forms hydrogen bond interaction. The docking score was -4.675Kcal/mol.

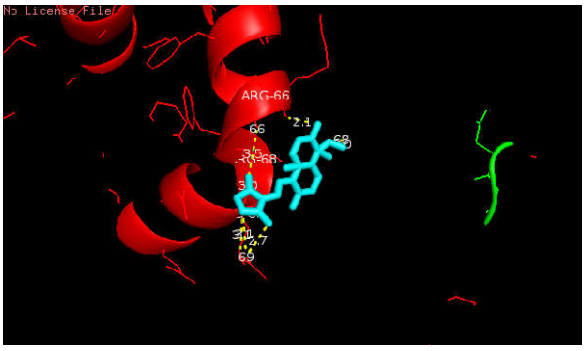


Fig. 3. Binding of AGL with the protein 2W3L. The red color ribbon shows the protein and blue color sticks represent the AGL molecule

Anti-inflammatory activity

The anti-inflammatory activity determined by HRBC assay showed that AGL has good anti-inflammatory activity. The percentage of membrane protection was increased with higher concentrations of AGL. It was maximum when AGL was supplied at 80µM concentration.

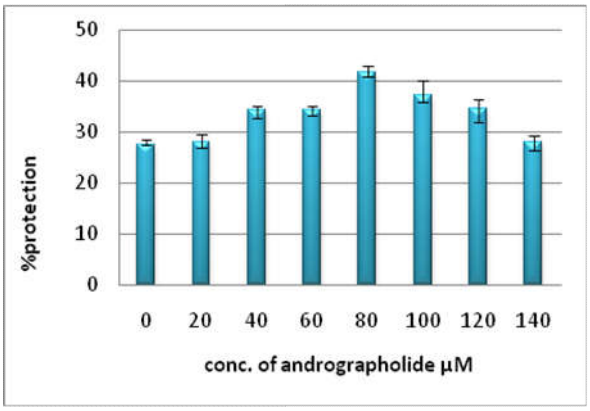


Fig. 4. The increase in anti-inflammatory activity of AGL up to 80µM concentration after-which the concentration gradually decreases

Free radical scavenging activity

The DPPH assay showed that the free radical scavenging activity augments with increasing concentration of AGL and after 80µM concentration it reduced gradually (Figure 5).

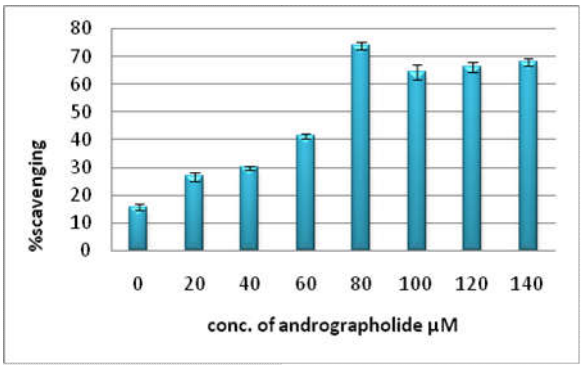


Fig. 5. The percentage of radical scavenging activity of DPPH assay

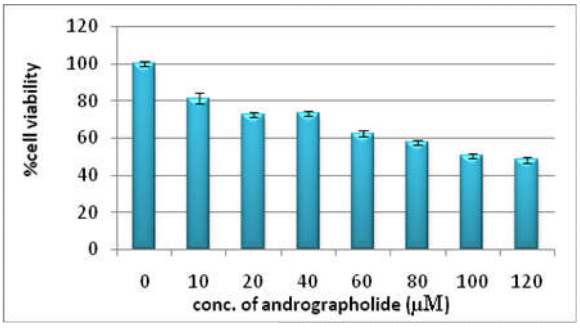


Figure 6. The trend in cytotoxicity with different concentration of AGL

Cytotoxicity assay

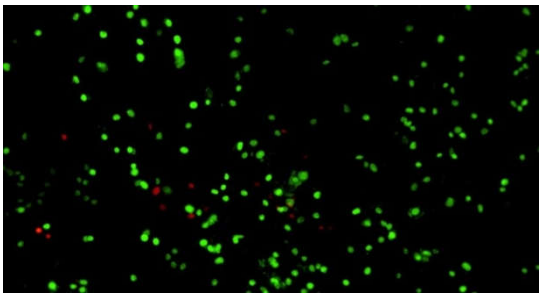
The MTT assay result showed that AGL controlled the proliferation of MCF7 cells at increasing concentrations.

At 80µM concentration, the cell viability decreased about half as that of control. Figure 6 shows the trend in cytotoxicity with different concentration of AGL.

Dual staining

The cells were stained with AO/EtBr and visualized using Fluroscence microscope. Around 300 cells were counted. The cells with green stains and red stains were counted and percentage was calculated. Figure 7 shows the representative picture of AGL treated (80µM) and untreated cells stained with AO/EtBr. Among untreated cells, less than 5% cells were apoptotic whereas in AGL treated cells, 18% of the cells were in early apoptotic stage and 36% cells were in late apoptotic stage.

Untreated cells



Treated cells

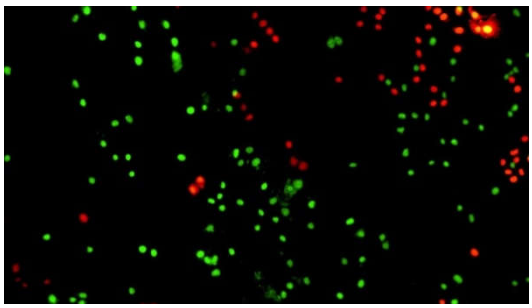


Figure 7. Apoptotic cell staining using AO/EtBr

DNA fragmentation analysis

The untreated cells and AGL treated (80µM) cells were collected and DNA was isolated.

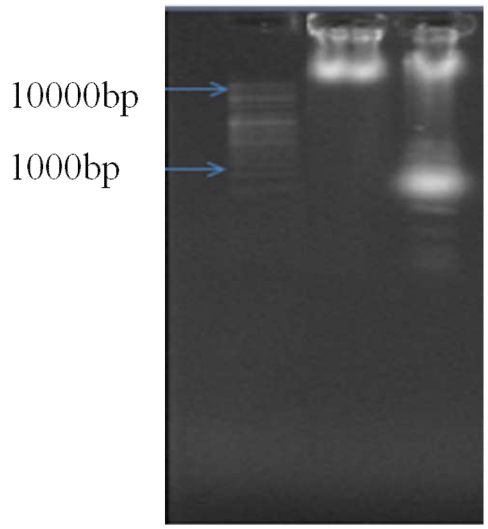


Figure 8. Shows the DNA fragmentation in AGL treated cells. Representative Lane: M - Marker lane, Lane U – Untreated cell's DNA; T - Treated cell's DNA

The DNA was loaded on agarose gel and visualised after electrophoresis. It was found that intact genomic DNA was visualised in case of untreated cells whereas in AGL treated cells, fragments of DNA were visualised.

Conclusion

Thus, this study shows that AGL possibly inhibits Bcl-2 protein which causes apoptosis in cancer cells. The computational results were also in good correlation with that of the biochemical and *in vitro* assays performed. Further, the insight into the molecular mechanism of the drug action shall be evaluated in cells by examining their gene or protein expression after treatment with AGL.

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