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Original Article

Investigation of Anti-Cancer Potency of Sarsasapogenin: *In vitro* and *in silico* Insights against MDR Cell Line-KB-Chr-8-5

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ABSTRACT

Objectives: Cancer recurrence is a major global concern, contributing to the rising mortality rates in the most common types of cancer. To overcome these incidents, highly qualified herbal medicines are more targetable, thus this investigation demonstrates the treatment of a steroidal bioactive compound-Sarsasapogenin (SAR). **Materials and Methods:** To achieve the goal, Antioxidant capacity of SAR was studied by DPPH and ABTS assay. Cytotoxicity assessment was performed as first line confirmation about its anti-cancer potency through Tetrazolium assay and Trypan blue assay. Oxidative stress balance was examined by enzymatic assays (NO, LPO, SOD and CAT). Further, apoptosis was confirmed by fluorescence staining (DAPI, DCHFDA) and cell cycle analysis. *In silico* work was also performed to detect the interaction between ABCC1 protein and SAR using molecular docking studies. **Results:** Antioxidant assay confirmed SAR is a potent radical scavenger with cytotoxic potential at IC_{50} value of 600 µg/mL. Enzymatic assays, fluorescence staining and flow cytometry proved its anti-proliferative property which showed elevation of stress related enzymes and reversal in antioxidant enzymes. Nuclear damage was observed in DAPI, enormous free electron production in DCFDA assays and cell cycle arrest at late cell cycle phase respectively. **Conclusion:** In the point of conclusion, investigation performed with SAR against KB-ChR-8-5 showed remarkable anti-proliferative activity which paves the way for application of SAR as futuristic drug candidate especially in Multi Drug Resistant (MDR) cancer.

Keywords: Sarsasapogenin, Multi drug resistant, KB-ChR-8-5, Cancer recurrence, ABCC1, MRP 1.

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INTRODUCTION

Cancer, a non-communicable, life-threatening disease has become more dangerous in recent days due to its resistance to conventional chemotherapeutic drugs. Numerous cancers exhibit reversal mechanism for the chemotherapy and radiation, which makes the disease even more complicated. MDR- multidrug resistance, refers to the process of competition or reversal between oncogenes and cancer suppressor genes.[1] World Health Organization (WHO) and other reviews have indicated that between 50-70% of ovarian adenocarcinomas recur within a year following surgery, while between 30-55% of people with Non-Small Cell Lung Cancer (NSCLC) recur and cause death. Additionally, 20% of child instances of acute lymphoblastic leukaemia are thought to result in a malignant recurrence.[2]

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To prevent cancer recurrence due to the resistance of cancer drugs, it is crucial to enhance the specificity and precision of treatments by identifying drugs that can target specific molecular sites. There are several active studies looking into potentially beneficial anticancer drugs. Among these, medications with minimal toxicity and no adverse effects are being well received.

In this case, Phytochemicals/herbal extracts are playing tremendous role in drug formulations which has many marketable benefits such as low cost and simple purifications steps. Alkaloids, terpenoids, steroids, phenolic acids and many groups of phytochemicals being used in most of the drugs. Steroids, among other phytochemical groups, have excellent nuclear and membrane receptor binding capabilities and easily crosses cell membrane. A small modification in the steroid

portion can provoke a substantial biological reaction. Changing the structure will result in a wide range of pharmacological consequences. Many studies have explored and documented the use of steroid-based anticancer drugs for treating various types of cancer.[3] They have a basic structure known as the steroid nucleus, which is made up of four carbon rings. The addition of chemical groups at specific backbone sites leads to the formation of several natural saponins, including diosgenin, solasodine, glycyrrhizinic acid, boswellic acid, sarsasapogenin and guggulsterone.[4]

Sarsasapogenin-a steroidal saponin has been utilised extensively in both Japanese and Chinese traditional medicine. It has anti-diuretic, anti-platelet aggregation, and antidiabetic effects.[5] Despite indications that sarsasapogenin is effective in treating cancer resistance, its anti-tumour activity on MDR cell lines has not been studied or documented. So, the underlying mechanisms of sarsasapogenin against MDR cell line KB-ChR-8-5 was investigated to prove its anticancer potential by *in vitro* parameters such as cytotoxicity assessments, fluorescence assays and cell cycle analysis.

Generally, ABC transporters are the main players in the solute transport in cell membrane. The over expression of ABC transport causes the resistance against anti-cancer drugs. ABC transporters were classified into seven subfamilies from ABCA-ABCG. ABCC1 (multidrug resistance protein 1/MRP1) plays a vital function in mediating MDR in cancer cells and it is the main key player in fail of chemotherapy drugs in numerous malignant tumours, including many forms of cancer. Anthracyclines, vinca alkaloids, TKIs (including vandetanib), methotrexate, saquinavir, epipodophyllotoxins, camptothecins and other anticancer medications are all rendered ineffective by overexpressing the ABCC1 transporter. Thus, ABCC1 MRP's interaction with Sarsasapogenin drug was analysed using *in silico* applications by molecular docking software.[6]

MATERIALS AND METHODS

Antioxidant analysis of Sarsasapogenin

DPPH activity

Radical scavenging activity was inspected to understand the antioxidant potency of plant derived compounds, crude extracts etc., 1,1-Diphenyl-2-picrylhydrazyl radical, 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydroxyl was used here as free radicals and Ascorbic acid used as strong scavenger. In short, 500 µL of DPPH (0.1 mM) solution mixed with 500 µL of SAR solution (20-100 µg/mL) in methanol. Solution mixture incubated for 30 min in dark and read at 517 nm against blank consisting of the same amount of DPPH and methanol. Similar protocol was followed for Ascorbic acid solution of different concentration from 20-100 µg/mL instead of SAR sample and OD was taken. The IC_{50} value of antioxidant potency was calculated using,

$$\% \text{ scavenging of DPPH} = [(A0 - A1)/A0] \times 100$$

where A0 = absorbance of the control and A1 = absorbance of the test extracts.

FRAP reducing power assay

FRAP reducing power assay was performed to analyse about the antioxidant activity of the SAR. The ascorbic acid solution is used as the reference standard for the FRAP assay. The different concentrations of AA and SAR were used for the assessment such as 10, 20, 30, 40 and 50 µg/mL respectively. 2500 µL of phosphate buffer (pH 6.6) and 2500 µL of 1% $K_3Fe(CN)_6$ were added to the samples and reference standards. After 5 min of vertexing, the mixture was incubated at 50°C for 20 min. Following the incubation period, 2500 µL of 10% TCA was added to the mixture, and it was centrifuged for 10 min at 3000 rpm. After centrifugation, 2500 µL of the supernatant was collected, and 2.5 mL of deionised water was added. The mixture was thoroughly mixed. 500 µL of 0.1% ferric chloride was added to the mixture until the coloured solution was obtained. The UV-visible Spectrophotometer was used to determine the reference standard solutions and samples at 711 nm.

In vitro

Cytotoxicity studies

Cell culture procurement

KB-ChR-8-5 multi drug resistant cell line was procured from ATCC cell line, USA. Cells subculture using 10% DMEM containing fetal bovine serum, Penicillin-Streptomycin solution (1%) and incubated at 5% CO_2 incubator with appropriate humidity. Cells taken for the further assays when the confluency reaches 3/4th of the flask's surface.

Determination of cell cytotoxicity

Cytotoxicity potency of Sarsasapogenin (SAR) was evaluated using MTT assay prescribed by Mosmann *et al.*,1983[7] with slight modification. In brief, 5000 cells per wells were seeded in 96 well plate and incubated to achieve the 70% confluency. Then, the grown cells were treated with various concentration ranges of SAR such as 25, 50, 100, 200, 300, 400, 500, 600 and 700 µg/mL for 24 hr. The cells were observed for its respective dosage effect. The media in the wells were replaced with 200 µL of fresh 2% DMEM media containing 0.5% of MTT and incubated for 2-4 hr at 37°C dark. The MTT containing media was aspirated. 200 µL of DMSO was added to each well and formazan crystals were dissolved. The bluish-purple colored solution was read at 490 nm and 630 nm using ELISA reader (Biotek, ELX 800). The percentage inhibition was calculated as $(1-(A_{T490-630})/(A_{C490-630})) \times 100$.

Trypan Blue Exclusion Assay

Cytotoxicity potency of SAR was once again cross checked with TBE assay which is corresponding to the MTT assay. For this

assay the IC_{50} dosage concentrations of SAR such as 500, 600 and 700 μg/mL were added to the respective grown cells and untreated were kept as control. The cells treated and untreated were collected and pelleted through centrifugation. The pellet was resuspended with 1 mL of DMEM. Then, 50 μL of suspended cells mixed with 50 μL of Trypan blue dye and counted viable, non-viable cells using hemocytometer through phase contrast microscopy (Olympus, Italy).[8]

Cell Morphological observation

1 X 10^4 cells/well was seeded in 24 well plate and incubated to attain the maximum required confluency. The grown cells were treated with IC_{50} dosages of SAR and morphological observation was evaluated under light microscope at 200X. (Olympus, Italy) Untreated maintained as control.[9,31]

Enzymatic analysis

Nitric oxide estimation

Nitric oxide estimation was performed for treated and untreated groups as prescribed by Yaraee *et al*., 2011 with minor modification. Briefly, Griess A (sulphanilamide 1%) and Griess B (NED 0.1%) solutions each 50 μL was mixed with 100 μL of supernatant collected from treated and untreated groups, incubated at dark for 10 min, then read at 543 nm. The obtained absorbance values were fitted in sodium nitrate standard graph and calculated. NO release was expressed in μM.[10]

Reactive oxygen species release

1x10^5 cells were seeded and cultured for 24 hr in a 96-well plate. SAR at various concentrations were added to the cells for a duration of 24 hr. After washing the wells with 1X PBS, 0.1 mL of an NBT (0.1%) solution was introduced into the well, and they were then incubated for 1 hr. After the media was taken out of the wells, the cells were given three washes with 70% methanol and left to dry. 120 μL of Dimethyl Sulfoxide (DMSO) and Potassium Hydroxide (KOH) were added to each well. At 630 nm, the absorbance was measured.[11]

Super oxide dismutase analysis

SAR treated and untreated KB-ChR-8-5 cells were subjected to estimation of super oxide dismutase.[12] The treated and untreated cells were homogenized, centrifuged and 0.2 mL supernatant was collected in a fresh tube. To the supernatant 0.2 mL of 0.08 mM NBT, 0.2 mL of PMS and 0.4 mL of 0.25 mM NADH was added. The mixture was incubated at dark for 10 min at room temperature. Absorbance was read at 560 nm by UV spectrophotometer and estimated as follows,

$$\% \text{ SOD} = [(\text{ODSample} - \text{ODControl}) / \text{ODControl}] \times 100$$

Catalase estimation

Catalase activity was estimated for SAR treated and untreated groups.[13] 1X10^4 cells were seeded at 24 well plate and incubated. The grown cells were treated with 500, 600 and 700 μg/mL of SAR for 24 hr. Treated and untreated cells were homogenized and centrifuged. To the 0.5 mL supernatant, 0.01 mM Phosphate buffer (pH 7.4) 1 mL was added followed by 0.2 mL of 30% hydrogen peroxide, 0.4 mL of distilled water. The mixture was incubated at dark for 10 min at RT. 1 mL of 32.4 mM Ammonium molybdate was added to stop the reaction and read at 405 nm. Catalase activity was calculated as KU/L.

Fluorescence studies

DAPI staining

DAPI staining method was used to investigate the apoptotic potency of SAR towards KB-ChR-8-5 cell line.[14] Cells seeded in 24-well cell culture plate and treated with 500, 600 and 700 μg/mL of SAR for 24 hr. The treated and control groups were fixed with chilled methanol for 10 min and rinsed with chilled PBS buffer. The cells were then permeabilized with 4% formaldehyde and stained with DAPI dye. After staining, the cells were washed with PBS and photographed using fluorescence microscope.

DCHFDA staining

The DCHFDA method was used to examine the amount of ROS release.[15] 24-well culture plate was used to seedKB-ChR-8-5 cells and exposed to different dosages of SAR as 500, 600 and 700 μg/mL for 24 hr. Then, the treated and untreated cells were rinsed with PBS and incubated for 30 min at 37°C with DCFHDA dye (10 μM). The excess dye was removed through washing and captured using a fluorescence microscope.

Cell cycle analysis

Flow cytometry analysis

Cell cycle phase distribution and cellular DNA content were analysed using Flow Cytometry (FACS). In short, 10000 cells/Flask were seeded and grown cells were exposed to different concentrations (500, 600 and 700 μg/mL) of SAR. Following treatment, the treated and control cells were trypsinized and rinsed with cold PBS. 70% ice cold ethanol was added to the cell pellet to fix the cells as a single cell suspension for an overnight period at 4°C. RNase A (0.1 mg/mL) was administered for RNA degradation since we need to detect only DNA.0.4 ml of PI dye (0.5 mg/mL) was used to stain the cells for 30 min at room temperature in the dark. Then, the PI dye fluorescence of individual nuclei was detected using a flow cytometer (BD FACS Calibur, Becton Dickinson, USA), the data was analysed using Cell Quest Pro V 3.2.1 software.[16]

In silico analysis

Acquisition of 3D Structures of Proteins and Ligands

The 3D structure of Sarsasapogenin was retrieved from the PubChem database in SDF format and converted to PDBQT format using MGL Tools. For the ABCC1 protein the 3D structure was downloaded from PDB and the PDB id is 2CBZ. The protein was cleaned and the water molecules were removed. These files were used for molecular docking analysis.

Molecular Docking

We determined the binding affinity of Sarsasapogenin with ABCC1 protein using PYRX software. The 3D structures of the active components were obtained from PubChem. Protein and ligand preparations were completed with MGL Auto dock tools and saved in PDBQT format. Docking was performed with grid dimensions of -12.7758x50.9065x14.914 Å. PYRX was used to obtain binding affinities. Binding configurations and interactions were visualized using Accelrys BIOVIA Discovery Studio 2024 R2.

ADMET Analysis

The pharmacokinetic parameters of Adsorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) were evaluated using the ADMET lab 2.0 online tool (https://admetmesh.scbdd.com). This included assessing Absorption, Distribution, Metabolism, Excretion, Toxicity, Physicochemical properties and Medicinal Chemistry.

Statistical Analysis

All the data obtained were statistically analysed using GraphPad Prism software. All the experiments were triplicated and expressed as Mean±SD. Results were subjected to one-way ANOVA and Tukey's multiple comparison test for the statistical analysis and $p<0.5$ is considered as statistically significant.

RESULTS

Antioxidant estimation of Sarsasapogenin

DPPH activity

Antioxidant property of plant extracts/plant compounds plays a vital role in the prevention or convalesce from cancer. In the process of investigating radical scavenging activity of SAR, the results indicated the presence of antioxidant potency which was depicted in Table 1. Ascorbic acid showed 50.28±2.039 of percentage inhibition at 60 µg/mL whereas SAR inhibited at 100 µg/mL with 50.10±0.771 percentage inhibition. Based on this analysis, we can confirm that SAR possesses antioxidant property compared with standard antioxidant.

FRAP reducing power assay

Further confirmation of antioxidant property of SAR was employed using highly sensitive assay called FRAP reducing power assay, where the antioxidants reduce Ferric to ferrous ion. Table 1 showed the IC_{50} of SAR at 40 µg/mL as 51.11±2.35% and for Ascorbic acid IC_{50} is 30 µg/mL as 52.40±1.047%. These results of both antioxidant assay indicate SAR possess strong free radical scavenging property which will be more helpful in the cancer treatments.

In vitro

Cytotoxicity studies

Determination of cell cytotoxicity

The investigation of interaction between bioactive compound towards the cell line whether it affects the cell structure and biological function termed as cytocompatibility of the drug which was assessed by MTT reagent. The results outcome was shown in Figure 1A. There was gradual increase in the percentage of inhibition. 25, 50 and 100 µg/mL shows no inhibition whereas from 200-700 µg/mL showed increasing percentage rate (in %) such as 500, 600, 700 µg/mL showed 33.819±3.25, 62.068±5.029 and 89.901±0.93 respectively. 600 µg/mL fixed as IC_{50} value and

Table 1: Drug likeliness and ADMET analysis.

Lipinski's Rule of five								
	Topological Polar Surface Area (Å)	c logP (<5)	MW (<500)	Heavy atom count (natoms)	Hydrogen bond donors (nOHNH) (≤5)	Hydrogen bond acceptors (nON) (≤10)	Rotatable bonds (nrotb) (≤10)	Lipinski Violations
	38.69	1.3219	416.33	30	1	3	0	1
ADMET								
Consensus Log Po/w	BBB	P-gp Substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor	Log Kp
5.24	Yes	No	No	No	No	No	No	-4.23 cm/s

Table 1 depicted the results obtained for Lipinski's rule and ADMET analysis.

500, 600, 700 µg/mL were the selected dosages for the further analysis.

Trypan Blue Exclusion Assay (TBE)

TBE assay correlated with the results of MTT, as discussed above 500, 600 and 700 µg/mL showed 70.25±0.41, 54.60±1.61 and 16.63±1.33 viability percentage respectively. Control/Untreated showed 98.74±0.54% (Figure 1B).

Cell Morphological observation

SAR treated and untreated cells observed for its morphological alterations shown in Figure 1C. A-denotes control showed regular and normal cell morphology with distinct cell wall and nucleus. B, C and D represented in treated groups such as 500, 600 and 700 µg/mL respectively showed the cell apoptosis, cell shrinkage, nucleus deterioration, cell wall damage, detachment of cells in dose dependent manner. This deterioration of cell morphometric strongly demonstrate the effective action of SAR towards KB-ChR-8-5 and firmly supports the results of MTT and TBE examination.

Enzymatic analysis

Nitric oxide estimation

Nitric oxide estimation is one of the main factors to assess the oxidative stress level of treated cells. Figure 2A showed the increasing trend of nitric oxide release as related to the dosage range, control group showed 10.91±1.82 and treated groups 500, 600 and 700 µg/mL showed 12.02±0.61, 16.29±0.4 and 17.29±0.29 respectively. These values 600 and 700 µg/mL showed less significance as $p<0.1$ when compared to control whereas 500 µg/mL showed non-significant.

Reactive oxygen species release

Free radical release was estimated in SAR treated and untreated groups shown in Figure 2B. Treated groups showed 31.52±1.41, 38.60±5.48 and 44.78±11.80 percentage for ROS release for 500, 600 and 700 µg/mL respectively. ROS release was gradually increased upon treatment with SAR in dose dependent manner. 500 and 600 µg/mL showed less significance compared with control and 700 µg/mL showed moderate significant. (* $p<0.1$, ** $p<0.01$).

Super oxide dismutase analysis

Antioxidant enzyme level-Super Oxide Dismutase (SOD) was investigated in SAR treated and untreated groups (Figure 2C). Treated groups showed 80.55±7.84, 73.67±2.28 and 49.60±4.11 percentage of SOD release for 500, 600 and 700 µg/mL respectively. SAR treated group showed significant decrease SOD release based on the increasing in dosage level. 500 and 600 µg/mL showed less significant ($p<0.1$) compared to control. 700 µg/mL showed moderate significant compared with control as $p<0.01$.

Catalase estimation

Catalase is one of the main antioxidant enzymes need to be investigated to analyse the antioxidant potentiality of SAR. Control group showed 19.18±2.04 KU/L Catalase release. Treated group showed 15.16±1.55, 7.00±1.94 and 4.09±0.48 for 500, 600 and 700 µg/mL respectively (Figure 2D). 600 and 700 µg/mL showed moderate significant as $p<0.01$ compared with control whereas 500 µg/mL showed non-significant.

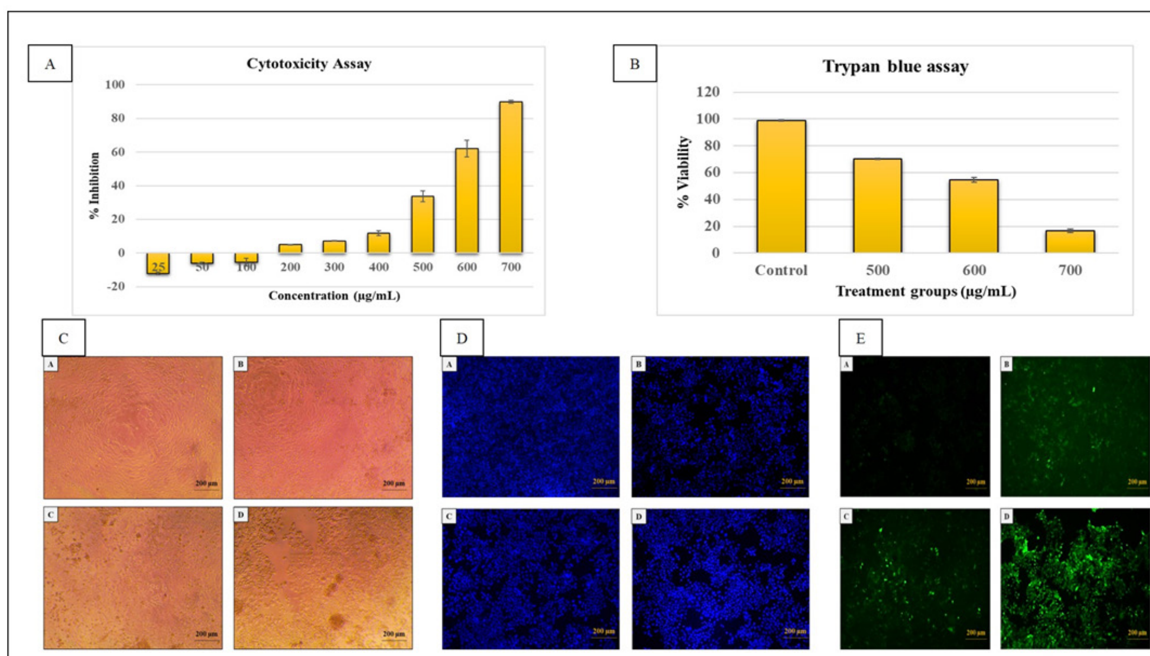

Figure 1: A) MTT assay, B) TBE assay, C) Morphometric Analysis, D) DAPI staining, and E) DCHFDA staining.

Fluorescence studies

DAPI staining

DAPI staining was performed for SAR treated and control cells. Figure 1D showed the fluorescence images of DAPI stained cells taken by fluorescence microscope, A, B, C, and D represents control,500 µg/mL, 600 µg/mL and 700 µg/mL respectively. From the images it can be understood that treated group showed increased blue fluorescent cells which indicates the nuclear condensation, cell deformation and cell apoptosis. The count of cells consisting condensed nuclei was increased as dose related manner. Untreated group showed no fluorescent cells denotes there was no such sufferings occurred. These results demonstrated the anti-cancer potentiality of SAR. Yellow arrows pointed the cells with damaged nucleus.

DCHFDA staining

ROS release was the important indicator to confirm the deterioration of cell morphology. In current study, level of ROS release in SAR treated cell group was considered as imperative factor. In Figure 1E, the treated groups showed increased green fluorescent emission indicating the excess reactive oxygen species liberated in dose dependent manner. Control group doesn't show green fluorescent confirming less or negligible amount of ROS levels.

Flow cytometry

Cell cycle analysis was performed to understand the apoptotic initiation point. The results obtained in the FACS analysis was given in Figure 3. Based on the research, the accumulation of fragmented DNA stained with PI was found to be 26.17% at a higher dose of 700 µg/mL. At 500 and 600 µg/mL, the percentages were 15.10% and 16.98% respectively. Control showed 14.27%. The results revealed that the cells cycle was terminated at late cell cycle phase G2/M in cell cycle.

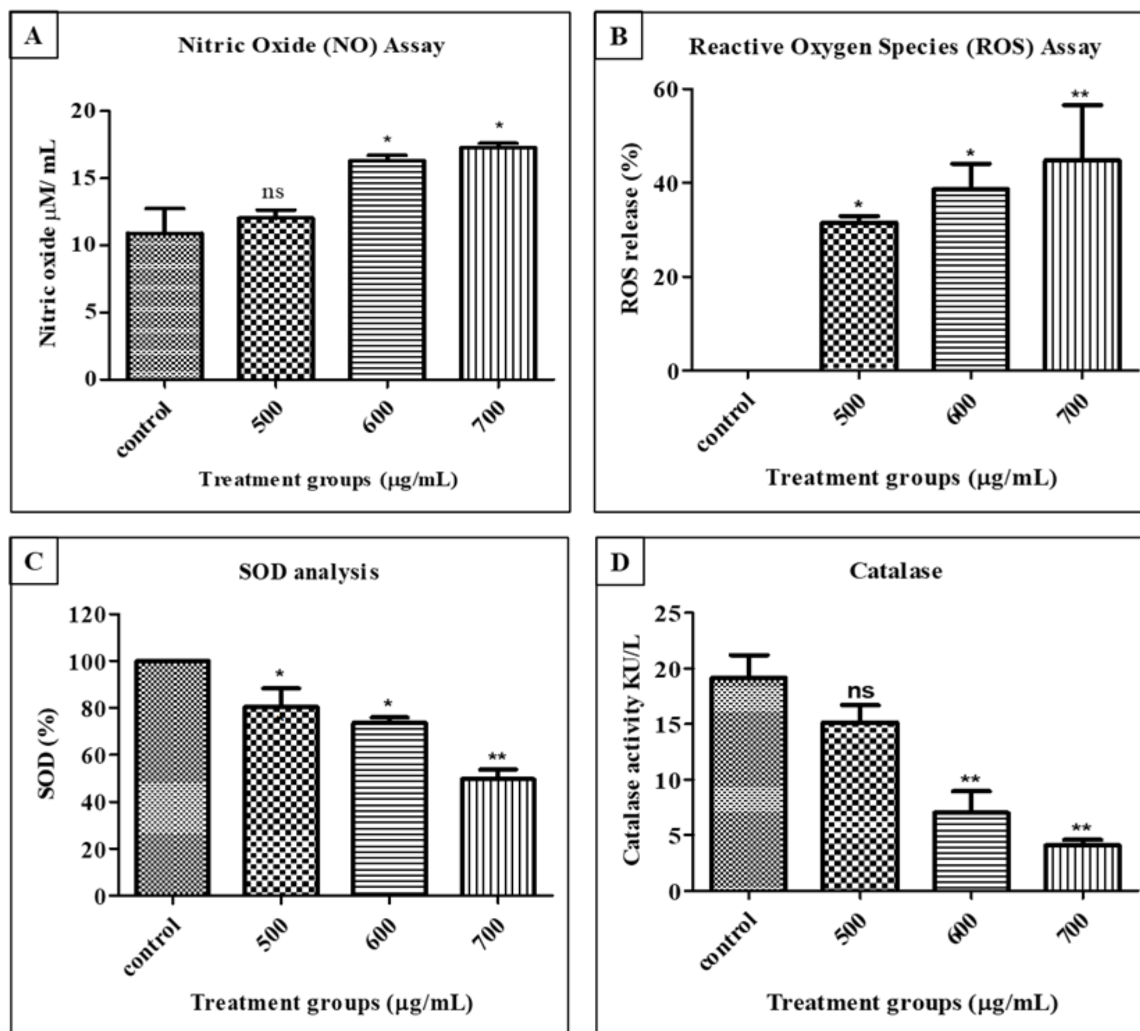

Figure 2: Enzymatic assays. 2A-Nitric oxide estimation, 2B-Reactive oxygen species analysis. NO and ROS assay showed significant increase related to the dose increases. 2C-Super oxide estimation, 2D-Catalase. SOD and catalase showed the gradual increase as dose dependent manner. All the results were statistically analysed.

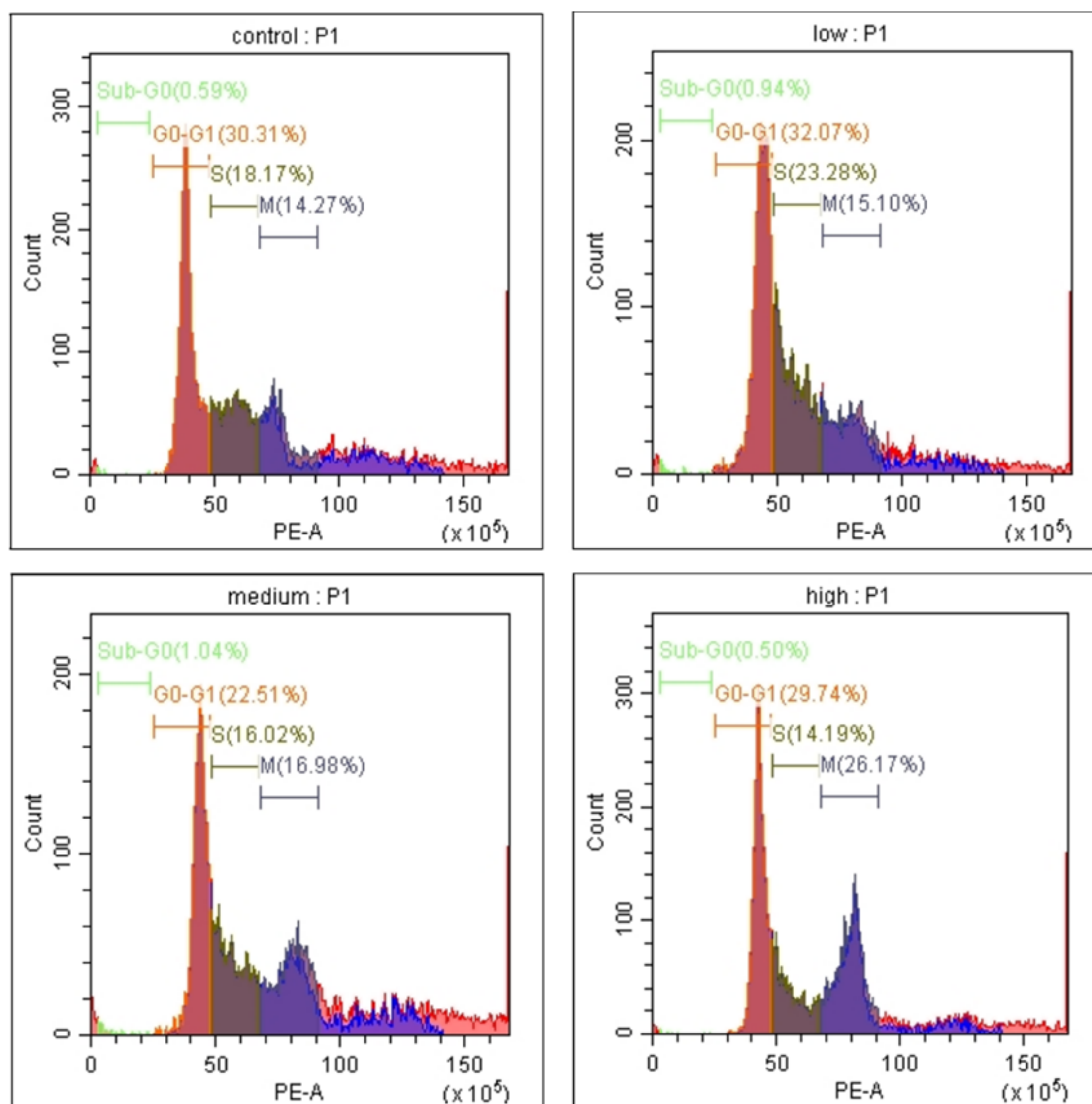

Figure 3: FACS analysis. Control-Untreated, Low-500 µg/mL, Medium-600 µg/mL and High-700 µg/mL. The figure demonstrated the percentage of DNA fragments availability in different cell cycle phase. G2/M phase showed gradually increased accumulation percentage of DNA fragments.

In silico

Molecular Docking

Molecular docking results showed that the compounds (Sarsasapogenin and ABCC1 Protein) have a strong Binding Affinity, as detailed in Figures 4 and 5. Visualization of the binding interactions confirmed that compounds formed both polar and non-polar bonds with the target proteins, supporting the potential of the Ligand compound towards the protein.

ADMET Analysis

The ADMET analysis shows the Sarsasapogenin as an Ideal compound as the TPSA (Topological Polar Surface Area) is 38, Lipinski rule with one Violation (logP is 6.3). The idealistic report is shown in Table 1.

DISCUSSION

Cancer is the second most deadly disease globally, contributing to a large number of deaths. Chemotherapy is the primary line of treatment for all cancer types; nevertheless, drug resistance makes chemotherapeutic therapies inefficient and leads to cancer recurrence. Ninety percent of recorded cancer-related deaths are due to treatment failure. Drug resistance condition in cancer treatment is caused by a variety of intricate processes, such as drug efflux by ATP-transporter, changing the anticancer medication's target, enhancing DNA repair pathways and evading apoptosis.[17] The overexpression of ATP transporters, which are crucial for expelling drugs from cells, is a major contributor to MDR. MDR refers to the ability of cancer cells to resist a variety of anticancer drugs.[6] The KB-ChR-8-5 and other cancer cell lines, known for its resistance to various drugs such as anthracyclines

(daunorubicin, doxorubicin), vinca alkaloids (vincristine, vinblastine), colchicine, taxanes, paclitaxeland cisplatin.[18,19]

Sarsasapogenin is recognized for its noticeable properties against many diseases including anti-inflammatory, anti-cancer, and antioxidant properties. In LPS-stimulated macrophages, sarsasapogenin significantly reduced IκBα phosphorylation, NF-κB, and MAPK activation. Anti-cancer property of SAR has been reported against various anti-cancer cell lines cervical, lung, colon etc. Steroidal bioactive compound-SAR, has many isomers formed by its molecular arrangements called as derivatives. Each derivative provides the solvation for many illnesses. Earlier researches have been demonstrated the effectiveness of its derivatives in reducing inflammation, protecting against neurodegeneration, and improving memory loss associated with ageing.[20,21]

In the process of assessing antioxidant potency of SAR, we obtained 50% free radical scavenging activity with reference of Vitamin C. Kashyap P *et al.*, 2020 analysed and reported that SAR exhibited63% scavenging activity at 50 μM by DPPH assay. So, it is clear that SAR possess commendable free radical quenching activity.[22,32]

SAR exhibits exemplary anti-cancer activity on KB-ChR-8-5 cell line. Cytotoxicity studies such as MTT, TBE and morphological assessment proved that SAR has notable impact on MDR cell line as IC_{50} value at 600 μg/mL. Similarly, earlier research reported that a steroid alkaloid called solanine and a few other plant-based active substances including ferulic acid, yohimbine, and reserpine were tested for their cytotoxicity against KB-ChR-8-5.[1,19,23,24]

Reactive Oxygen Species (ROS) generates mostly from the mitochondrial respiration. Under normal physiological conditions, normal cells maintain redox homeostasis, regulating the balance of ROS synthesis and their elimination with antioxidants. Excessive consumption of ROS and antioxidants can cause a disturbance in redox equilibrium, which can lead to oxidative stress. ROS, as mediators of intracellular signalling pathways, can break down the mitochondrial membrane, resulting in oxidative stress, cellular dysfunction, necrosis, and apoptosis.[25,26]

Plant derived compounds raise the oxidative stress levels beyond the tolerable limit of cancer cells which are selectively harmful and leads to ROS mediated apoptosis. This study investigated the level of oxidative stress enzymes NO, ROS and antioxidant enzymes SOD, catalase. Bharathi raja P *et al.*, 2025 reported that redox equilibrium was collapsed while treating KB-ChR-8-5 cell line with combined exposure of Solasodine and Dox showed catalase, Superoxide dismutase, glutathione and glutathione peroxidase levels were decrease but intracellular ROS generation and lipid peroxidation levels were significantly elevated.[27] The results supported the above statement that elevated range of stress enzymes and decreased level of antioxidant enzymes were obtained in KB-ChR-8-5 upon treated with SAR. DCFDA staining further confirmed ROS mediated apoptosis where the enormous liberation of ROS was observed in dose dependent manner. DAPI staining also demonstrated the dose dependent apoptosis with nuclear condensation, cell wall deterioration, cell blubbing, cell shrinkage and cell detachment. Same results were obtained for Lie *et al.*, 2023 and Bao *et al.*, 2007 for PANC1 and HepG2 cell lines respectively while treated with SAR demonstrated increased level of ROS release and nuclear condensation.[28]

Cell cycle analysis showed high DNA content in G2/M phase and gradual increased percentage was noticed in dose related manner which corroborated with the results of other assays and confirmed the apoptosis. Earlier studies of SAR against HepG2, MCF7 and HeLa cells stated that increased ROS liberation tends to disfunction of mitochondrial membrane by triggering ER

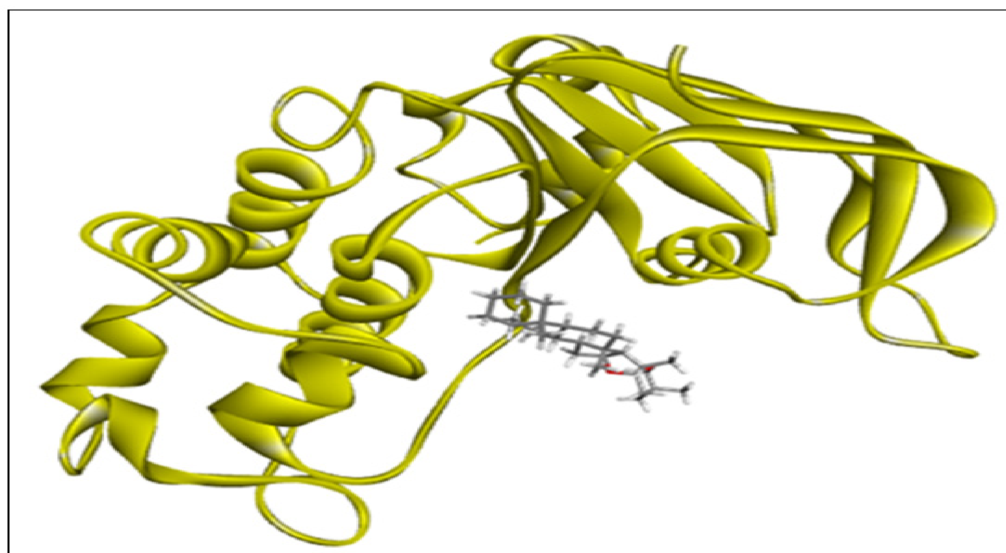

Figure 4: Docking analysis. Docked complex of ABCC1 with Sarsasapogenin with -7.6 as the Binding Affinity.

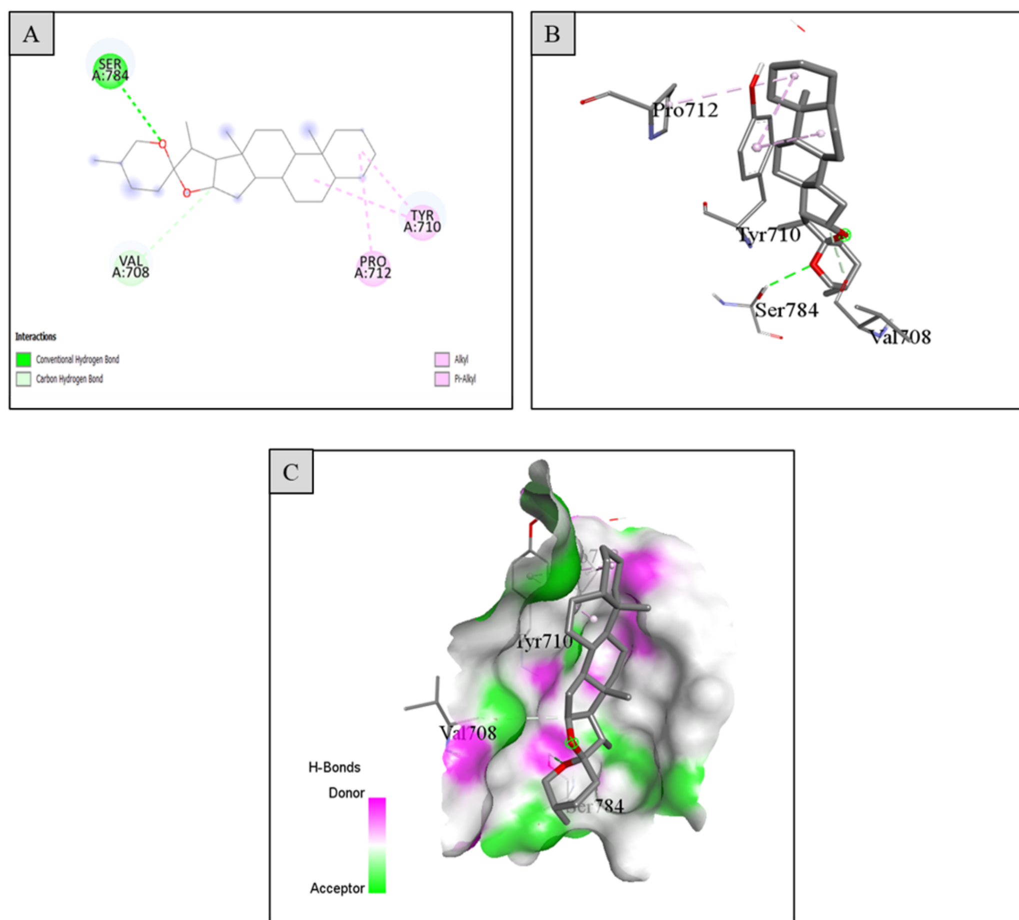

Figure 5: Ligand interaction. A: 2-D structure of the Ligand interacted complex. B: 3-D structure of Ligand Interaction of the complex. C: H-Bond Acceptor and Donor Regions of the Complex.

mediated apoptosis pathway.[26] Recent studies conducted by Peijie *et al*., 2023 and T. Ahamed *et al*., 2024 stated that sarsasapogenin disturbs homeostasis status in PANC-1 and HT-29which led to blockage of Jak/stat3 pathway and EGFR/KRAS signalling pathways through necrosis. Both studies also reported that the cell cycle arrest was observed in late apoptosis phase G2/M.[29]

The primary factor influencing drug intake and efflux in the human body is the ABC transporter. Multidrug resistant protein 1 or ABCC1, are the main efflux pump, its overexpression causes chemotherapy to be ineffective. In this study, the binding interaction between ABCC1 and SAR was analysed using PYRX software confirmed the strong binding with the binding energy-7.6. According to Chen *et al*., 2016, steroidal saponins suppressed overexpressed MRP1 and p-gp in a dose related manner in K562 cells, which improved the retention ability of anti-cancer drug Adriamycin in cells with the underlying mechanism of P13K/AKT pathway.[30] The potential of this compound is vast as this study was conducted for the purpose of investigate its drug likeliness properties which showed one violation in the Lipinski rule, but still it can be considered for further studies as oral pills as it exhibited good bioactivity and good ADME properties. T. Ahmed *et al*., (2024) and Anand and Sukumaran (2022) reported that SAR is a potential compound that can be converted into oral pills and it has good ADME qualities with only one Lipinski rule of violation.[29,31] Overall, the data supports the compound with good scores as a lead molecule, but further extensive investigation will be needed to understand the underlie signalling pathways of SAR as drug for ABCC1 protein.[32,33]

CONCLUSION

Our study reveals that Sarsasapogenin (SAR) exhibited notable antioxidant property and effectively reduces proliferation of KB-ChR-8-5 cells. SAR treated MDR cells generates significantly increased level of Nitric oxide and Lipid peroxidase while reduction in Super oxide dismutase and catalase. The loss of haemostasis leads to enormous liberation of ROS induces apoptosis observed through fluorescence analysis using DCFDA and DAPI stains. Cell cycle analysis also confirming the late phase arrest (G2/M). *In silico* analysis reveals the strong binding between ABCC1 (Multi drug resistant protein 1/MRP 1) and SAR. Taken together, these findings strengthen our conclusion that SAR serves as a powerful anticancer agent by downregulating the efflux pumps over expression involved in the MDR process in cancer cells. Further investigation is necessary to gain a more profound comprehension of the fundamental signalling systems.

As a result, SAR shows as a promising drug candidate for future therapeutic option for cancer recurrence circumstances.

ABBREVIATIONS

SAR: Sarsasapogenin; **MDR:** Multi Drug Resistant; **KB-ChR-8-5:** A specific multi-drug resistant cell line; **DPPH:** 1,1-Diphenyl-2-picrylhydrazyl; **ABTS:** 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); **IC_{50}:** Half maximal inhibitory concentration; **NO:** Nitric Oxide; **LPO:** Lipid Peroxidation; **SOD:** Superoxide Dismutase; **CAT:** Catalase; **DAPI:** 4',6-Diamidino-2-phenylindole; **DCFHDA:** 2',7'-Dichlorofluorescin diacetate; **ABCC1:** ATP-Binding Cassette Subfamily C Member 1; **MRP1:** Multidrug Resistance Protein 1; **WHO:** World Health Organization; **NSCLC:** Non-Small Cell Lung Cancer; **FRAP:** Ferric Reducing Antioxidant Power; **AA:** Ascorbic Acid; **TCA:** Trichloroacetic Acid; **DMEM:** Dulbecco's Modified Eagle Medium; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **DMSO:** Dimethyl Sulfoxide; **TBE:** Trypan Blue Exclusion; **NBT:** Nitroblue Tetrazolium; **PMS:** Phenazine Methosulfate; **NADH:** Nicotinamide Adenine Dinucleotide; **PBS:** Phosphate-Buffered Saline; **FACS:** Fluorescence-Activated Cell Sorting; **PI:** Propidium Iodide; **ADMET:** Adsorption, Distribution, Metabolism, Excretion, and Toxicity; **TPSA:** Topological Polar Surface Area; **ROS:** Reactive Oxygen Species; **NF-κB:** Nuclear Factor kappa-light-chain-enhancer of activated B cells; **MAPK:** Mitogen-Activated Protein Kinase; **PI3K/Akt:** Phosphoinositide 3-Kinase/Protein Kinase B; **Jak/STAT3:** Janus Kinase/Signal Transducer and Activator of Transcription 3; **EGFR/KRAS:** Epidermal Growth Factor Receptor/Kirsten Rat Sarcoma Viral Oncogene Homolog; **P-gp:** P-glycoprotein.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

The study investigates the anti-cancer potential of Sarsasapogenin (SAR), a steroidal saponin, against the Multidrug-Resistant (MDR) KB-ChR-8-5 cell line, demonstrating its efficacy through *in vitro* and *in silico* approaches. SAR exhibited strong antioxidant activity and significant cytotoxicity with morphological changes indicating apoptosis. It disrupted redox balance by increasing nitric oxide and reactive oxygen species while reducing superoxide dismutase and catalase levels, as shown by enzymatic assays. Fluorescence staining revealed nuclear damage and high ROS production, and flow cytometry confirmed G2/M phase cell cycle arrest. *In silico* molecular docking showed strong binding affinity between SAR and the ABCC1 protein, suggesting inhibition of drug efflux, with ADMET analysis supporting SAR's drug-likeness despite one Lipinski rule violation. These findings position SAR as a promising candidate for combating MDR cancer, warranting further investigation into its signalling pathways.

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