

The methanolic extract of *Bergenia ciliata* attenuates cancer by modulating Unfolded Protein Response in HeLa cells

Ozaira Qadri¹, Nazia Hilal¹, Khalid Majid Fazili^{1#}

¹Department of Biotechnology, University of Kashmir, India.

ABSTRACT

Cancer cells are subjected to different stressful conditions such as nutrient deprivation, hypoxia, reduced vascularization, or pH changes, which act as the growth-limiting factors and are responsible for activating the Unfolded Protein Response (UPR). Because of the tumor microenvironment, cancer cells critically depend on the UPR-signalling to survive the adverse circumstances. UPR therefore acts in a cytoprotective manner, enabling cancer cells to adapt and flourish in the severely stressed tumor environment. Targeting the UPR may therefore be advantageous in specifically eliminating the cancer cells. Since natural products, such as plant extracts provide unlimited opportunities for new drug discoveries, our studies aimed to explore the potential of targeting UPR pathways with the potent bioactive herbal extracts of *Bergenia ciliata*, an indigenous herb of Kashmir valley. The plant is known for its anti-inflammatory and anti-neoplastic properties which makes it a good candidate for the study. In the current study, the cytoprotective doses of *Bergenia ciliata* crude extract were evaluated and thereafter its effect on different UPR markers in the cervical cancer cell line (HeLa) was investigated. The IRE1 α and ATF6 arms of the UPR showed a significant decrease following the treatment with different doses of the extract in a concentration dependent manner while PERK arm of UPR was contrastingly affected showing the increase in expression with the increase in the concentration. These findings thus suggest that the extract is able to modulate UPR particularly by inhibiting the protective UPR, which is significant in terms of cancer regression.

Keywords : *Bergenia Ciliata*, Cancer, Endoplasmic Reticulum Stress, UPR.

I INTRODUCTION

Cancers are dependent on the number of molecular pathways and are known to develop diverse mechanisms for resistance to therapies. To cope up with this chemoresistant nature of the cancer cells it is pivotal to curb the centrally operating pathways like Unfolded Protein Response (UPR) which regulates the growth and sustainability of cancer cells [1]. In higher eukaryotes UPR is initiated by three main signaling proteins; Inositol requiring protein 1 (IRE1), double stranded RNA dependent Protein kinase-like ER kinase (PERK) and Activating transcription factor 6 (ATF6) [2]. The primary consequence of the UPR is to relieve cells from stress, but leads cell death in response to continued stress [4]. In cancers, the stressed micro-environment such as nutrient deprivation, hypoxia, reduced vascularization, or pH changes, which potentially act as growth-limiting factors lead to the activation of UPR. The role of UPR in tumorigenesis is to enhance the ER protein-

folding capacity and maintain ER protein homeostasis (or proteostasis), thereby counteracting apoptosis [5, 6]. Therefore, targeting the UPR may be advantageous to specifically eliminate cancer cells.

Drugs from natural extracts are found to have potential for long-term treatment with minimal side effects by targeting multiple pathways in the cancer cells. With the corroboration about the cardinal role of UPR in widespread cancer types, it becomes imperative to pharmacologically modulate the UPR, using natural products. Recently several of the naturally occurring compounds derived from plants like salicylic acid, the epigallocatechin-3-gallate (EGCG) and many others [5-11] have been reported to interfere with GRP78 expression or activity at pharmacological concentrations. Of special interest is to determine how these novel compounds modulate UPR. This could be achieved either by pharmacological inhibition of the UPR resulting in increased cell death. Conversely, pharmacological agents which hyperactivate UPR that is already activated by the tumour microenvironment likely surpasses the threshold and leads to cell death as well.

Bergenia ciliata commonly called 'winter bergonia', is a traditional medicinal plant used for the treatment of various ailments ranging from wound healing to anti-inflammatory property. In the period 2000-2008 various studies such as antibacterial, anti-tussive, anti-oxidant and DNA protection abilities, isolation of natural antiviral agents have been made from this plant [12-15]. *Bergenia ciliata* rhizome extracted in methanolic and aqueous forms was found to have promising potential towards the development of drug that might be used to target tumours for chemoprevention/chemotherapy to check neoplastic growth and malignancy. According to the American national cancer institute, the IC₅₀ value to consider a crude extract promising for development of anticancer drugs is lower than a limit threshold (30µg/ml) [16]. Thus, being known for the anti-neoplastic activities the plant may have prospective clinical use as precursor for preventive medicine. Our study was emphasised on evaluating the effect of *Bergenia ciliata* extracts on unfolded protein response in cervical cancer cell lines. The finding from the study indicates that *Bergenia ciliata* significantly modulates the different markers of UPR, thus showing the potential in cancer regression.

II MATERIALS AND METHODS

2.1 Chemicals and plant material

2.1.1. Chemicals and Reagents

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was obtained from Sigma Aldrich, St. Louis, MO, USA. Tunicamycin (Tm) used was from Cal Biochem, USA. The primary antibodies used for western blotting were monoclonal anti-pIRE1 α (Abcam, Cambridge, MA), anti-Xbp-1 (Abcam, Cambridge, MA), anti-GRP78 (Cell Signaling Technologies Danvers, MA), anti-eIF2 α (Cell Signaling Technologies Danvers, MA), anti-ATF4 (Abcam, Cambridge, MA) and anti-GAPDH (Cell Signaling Technologies Danvers, MA). The secondary antibody used was goat anti-mouse or anti-rabbit obtained from LI-COR Biosciences, USA. All other chemicals and reagents including cell culture media, chemicals like Tris-Base, SDS, 2-ME and other solvents were procured in their purest form available commercially from Sigma-Aldrich, Invitrogen and Hi-media.

2.1.2. Plant Material

B. ciliata plant was collected from their natural habitat in the Himalayas at Gulmargh (34.0456° N, 74.3844° E), Jammu and Kashmir, India. Collected specimens were shade dried, powdered, sieved and stored until further use.

2.1.3 Extraction

100 g of *B. ciliata* powder was serially extracted with using methanol and water as solvents in a Soxhlet apparatus. The powder: solvent ratio was maintained as 1:2. The extracts obtained were evaporated to dryness at 40 °C. The dried extracts were weighed to determine the yield of soluble constituents and stored in a vacuum desiccator.

2.2 Cell Culture and treatments

Cervical cancer cell line HeLa was obtained from the National Centre for Cell Sciences, Pune, India and maintained on Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin and streptomycin (each 1% v/v), 10% FBS, and was maintained in proper growth environment of 37°C and humid environment containing 5% CO₂. The medium was refreshed approximately three times a week.

The cell viability was assessed upon reduction of tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Aldrich, St. Louis, MO, USA).

The HeLa cells were cultured at a concentration of 3*10⁴ cells/well in a 96-well tissue culture plate and treated with *Berginia ciliata* extracts at various concentrations ranging from 1mg/ml-1µg/ml. After overnight incubation, 50µL of the MTT solution (5 mg/mL in PBS as the stock solution) was added into each well, and further incubation was carried out at 37°C for 3 h. The supernatants were removed, and DMSO (200 µL) was then added as a MTT solvent to solubilize MTT. The plates were read at 560 nm to obtain the percentage of viable cells. The readings were analysed using GraphPad PRISM v6.03 statistical software (GraphPad Software, La Jolla, CA) which gave 61.07µg/ml as an IC₅₀ dose of the extract.

2.3 Protein extraction and estimation

Cells were washed twice with ice-cold PBS (pH 7.4), harvested with PBS-EDTA (1mM), and centrifuged at 3000rpm for three minutes and this was repeated thrice with PBS washing each time. The pellet so obtained was treated with RIPA cell lysis buffer containing; 150mM NaCl, 1% NP-40, 0.5%SDS, 0.5% Sodium Deoxycholate, and 50mM TrisCl (pH=7.5) which was supplemented by NaF (10mM), β-Glycerophosphate (17.5mM), and Protease Inhibitor Cocktail (PIC) (1X) . The samples were vortexed and incubated at 4°C for 45 minutes on ice. Homogenates were centrifuged at 14000rpm for 25 min at 4°C. The supernatant was transferred to chilled eppendorf tubes and stored at -80°C for further use.

Protein concentrations were determined using Bradford Assay (Marion M. Bradford, 1976). The assay was performed in cuvettes arranged in series. The dilutions of the sample were made in dd.H₂O and mixed with equal volume of Bradford reagent. The cuvettes were incubated at room temperature in dark, for 10min and absorbance was measured at 595 nm using spectrophotometer (Thermo Scientific). BSA of known concentration was used as a standard.

2.4 Western Blot Analysis

20–30µg of proteins extracted with RIPA buffer were analyzed by SDS PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 10%BSA in 1XTBS buffer (BioRad,Hercules, CA) and incubated with primary antibodies overnight at 4°C. The following primary antibodies were used at a 1:1000 dilution GRP78, eIF2α, (Cell Signaling Technologies Danvers, MA.), pIRE1α, Xbp-1, ATF4 and ATF6 (Abcam, Cambridge, MA). GAPDH (Cell Signaling Technologies Danvers, MA.) of 1:5000 dilution was used as loading control. The blot was then probed against the Mouse or Rabbit Secondary Antibody (LI-COR Odyssey, USA) with 1:5000 dilution. The blots were analysed using LI-COR imaging system (LI-COR Biosciences, USA).

2.5 Statistical Analysis

Cytotoxicity assay and densitometry analysis were performed in triplicates, and similar results were obtained in at least three separate studies. Statistical analysis was performed using the GraphPad PRISM v6.03 statistical software (GraphPad Software, La Jolla, CA). All the data is presented as mean ± standard error and range.

III RESULTS AND DISCUSSIONS

3.1 Determination of IC50 dose

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed in human cervical cancer cell lines HeLa which were treated with *Berginia ciliata* methanolic extracts with the range from 1µg/ml to 1mg/ml for 24 hours [Fig1A]. The results indicated that, with the increase in concentration the cell viability decreased. The half maximal inhibitory concentration (IC50) of extract was found to be around 61.07µg/ml [Fig1B]. According to this result, we investigated the effects of different levels of *Berginia ciliata* extracts ranging from 10µg/ml to 30µg/ml (considering the concentrations as least cytotoxic) on ER stress in HeLa cells.

3.2 Effect of *Berginia ciliata* extract on UPR markers

Unfolded Protein Response (UPR) acts as important survivor pathway in cancers. Thus, targeting UPR can be beneficial in combating with cancer. Besides, natural products with least side-effects provide unlimited opportunities for drug designing and therapeutics. Our study employed the methanolic extracts of *Berginia ciliata* of varying concentration ranging from 10µg/ml to 30µg/ml, in order to modulate the different markers of UPR. The expression of proteins from the three different arms of UPR viz. Inositol requiring protein 1 (IRE1), double stranded RNA dependent Protein kinase-like ER kinase (PERK) and Activating transcription factor 6 (ATF6) was analysed by western blotting where each of the arm was showing the difference in the protein expression with respect to the different doses of *Berginia ciliata* extract.

The data indicated that there was increase in the expression of GRP78 [Fig2A, C] while phosphorylated IRE1α and its downstream effector Xbp1 [Fig2B, D, E] showed the decrease with the increase in the concentration of *Berginia ciliata* extracts from 10µg/ml-30µg/ml. Besides, ATF-6 another sensor of UPR showed a significant decrease in its expression with the treatment of plant extract in a concentration independent manner [Fig3]. Since, IRE1α/Xbp1 and ATF6 arms of UPR are known for their role in adaptive UPR, where each of them are involved in enhancing the ER's protein folding capacity [17-20]. IRE1α leads to the splicing of Xbp1 thereby

leading to the transcription of all the genes involved in restoring proteostasis while ATF6 is itself an ER tethered transcription factor whose proteolytic cleavage and migration to nucleus leads to the activation of those genes involved in ER homeostasis [18-20]. Thus, our study implies that the extract is able to decrease the phosphorylation status of IRE1 α and the expression of Xbp1 as well that of the ATF6, therefore inhibiting adaptive UPR.

In addition, PERK which represents another sensor of UPR was found to be modulated using the *Berginia ciliata* extract in the same concentration range from 10 μ g/ml-30 μ g/ml. The data indicates that with the increase in the concentration there was an appreciable increase in the expression levels of the two downstream effectors of PERK viz eIF2 α and ATF4 [Fig4]. Basically, ATF4 which is activated downstream of the eIF2 α is responsible for the commencement of apoptosis through the PERK arm [21, 22]. As such its increase in expression indicates that cell would enter into apoptosis, implying that extract has the potential to induce apoptosis via PERK/eIF2 α /ATF4 pathway.

Since, cancer cells harness UPR to restrain the tumour microenvironment by bypassing ER stress induced cell death and maintaining the adaptive UPR. Therefore, our findings underlie the counter strategy to target cancer cells by disturbing the adaptive UPR while promoting the maladaptive UPR, thereby paving a way to combat with the disease using natural sources. However, the role of extract in the cancer regression needs to be further validated in other cell models as well in the animal models. Moreover, these are crude extracts with complexity in their composition, where multiple compounds within the extract might exert different effects. As such individual compound needs to be isolated and evaluated for the same.

IV FIGURES AND TABLES

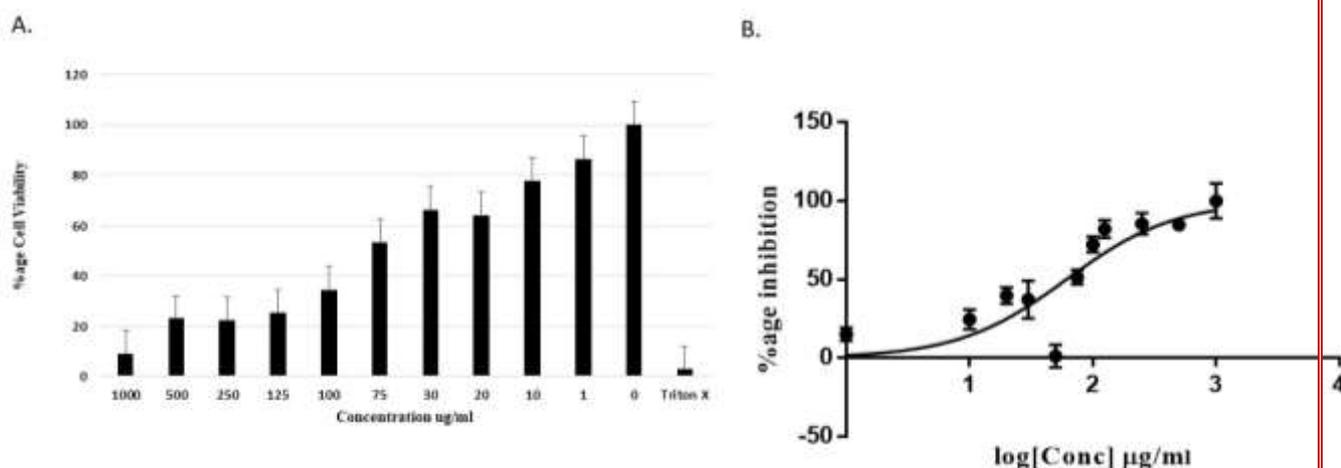


Figure 1 A. Cell Viability Assay: Viability of HeLa Cells following 24hr treatment of methanolic extracts of *Berginia ciliata* ranging from 1-1000ug/ml. The data was expressed as \pm SD (n=3). **B. Dose Response Curve:** Figure depicts the dose response cure of *Beginia ciliata* showing IC50 value of 61.07 μ g/ml (logIC50=1.7)

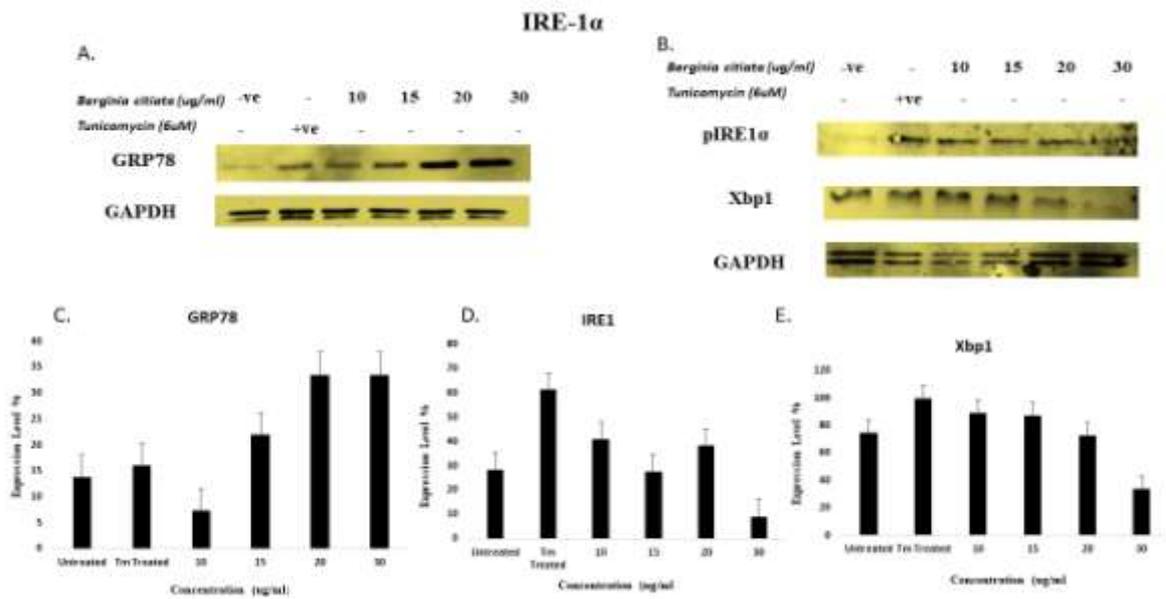


Figure 2: Effect of *Berginia ciliata* extracts on IRE1 arm of the UPR **A.** Western Blot analysis of GRP78 in HeLa cell lines, treated with 10µg/ml-30µg/ml of the *Berginia ciliata* extracts. **B.** Western blot analysis showing the effect of *Berginia ciliata* extracts ranging from 10µg/ml-30µg/ml on phosphorylated IRE1 α and Xbp1. The untreated cells and Tunicamycin treated cells were used as -ve and +ve controls respectively. As loading control GAPDH was used. **C., D., E.** Depicts the band densitometry graphs with standard deviation used to asses data depression.

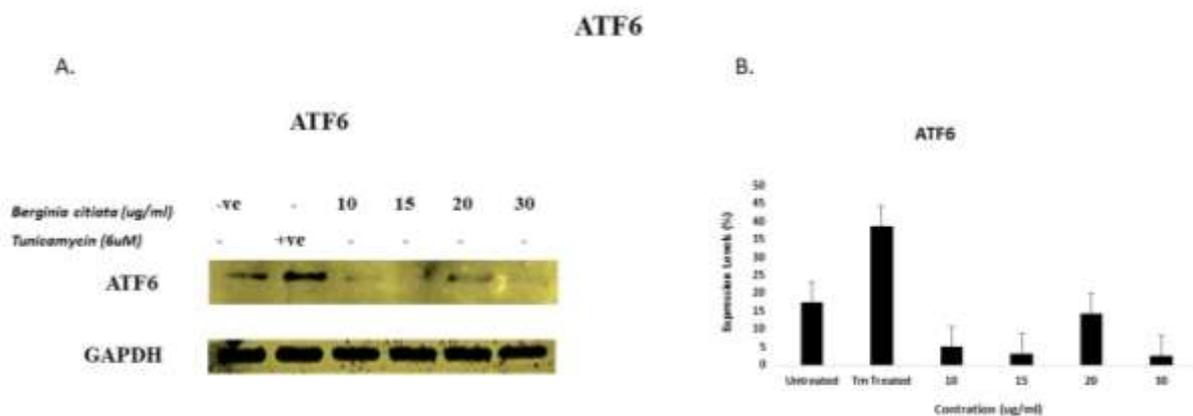


Figure3: Effect of *Berginia ciliata* extracts on ATF6 arm of the UPR **A.** Western Blot analysis of ATF6 in HeLa cell lines, treated with 10µg/ml-30µg/ml of the *Berginia ciliata* extracts. The untreated cells and Tunicamycin (Tm) treated cells were used as -ve and +ve controls respectively. GAPDH was used as loading control. **B.** Depicts the band densitometry graph with standard deviation used for estimating data depression.

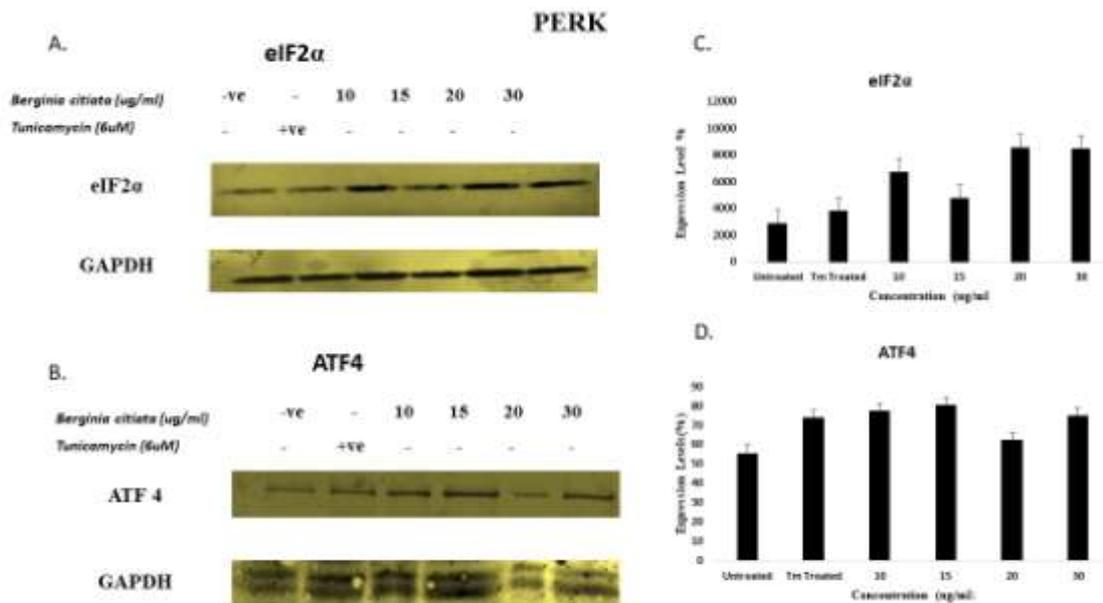


Figure 4: Effect of *Berginia ciliata* extracts on PERK arm of the UPR **A., B.** Western Blot analysis of eIF2 α and ATF4 in HeLa cell lines, treated with 10 μ g/ml-30 μ g/ml of the *Berginia ciliata* extracts. The untreated cells and Tunicamycin (Tm) treated cells were used as -ve and +ve controls respectively. GAPDH was used as loading control. **C., D.** Densitometric analysis with standard deviation used to evaluate data depression.

V CONCLUSION

This in vitro study puts forth that *Berginia ciliata* can act as a novel therapeutic agent for cancer regression, while targeting UPR. As the studies suggest, plant extract can substantially inhibit cytoprotective UPR and activate the reactive UPR. However, the studies need further substantiation in other cell models as well by using the defined compounds derived from the extract.

VI ACKNOWLEDGEMENTS

Our special thanks goes to Department of Botany, University of Kashmir for cordial help in the plant identification and collection. Our sincere thanks is also to Ministry Of Ayurveda, Yoga & Naturopathy, Unani, Siddha and Homoeopathy (AYUSH), Government of India for funding this project.

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