# 4
# Myricetin-Assisted Proteasomal Degradation Suppresses Misfolded Proteins Aggregation and Cytotoxicity

In previous studies, neuroprotective roles of Myricetin was thought to be due to its antioxidant properties, which helps in reduction of oxidative stress generated during multiple brain pathologies [Barzegar, 2016; Dajas *et al.*, 2003]. Here, in the present study, it has been reported that Myricetin treatment induces the cellular levels of Hsp70 chaperone and QC E3 ubiquitin ligase E6-AP and most importantly it also decreases the aggregation of crucial misfolded proteins. Additionally, the study also suggests that Myricetin treatment enhances the clearance of neurodegeneration-linked proteins that could be reversed by the addition of proteasome inhibitor. Interestingly, use of Myricetin also stabilizes the endogenous levels of E6-AP protein. These results suggest that small molecules based modulation of PQC mechanism at different levels can improve the survival of cells under proteotoxic-stress conditions engendered by the accumulation of aggregate-prone proteins.

## 4.1 RESULTS

Cytoprotective effect of Myricetin has been observed by various experiments that are explained in further sections.

### 4.1.1 Myricetin Treatment Enhances the Endogenous Levels of Protein Quality Control Components

The mechanisms underlying misfolded proteins-related proteotoxicity and the interrelationships between the aggregation of abnormal proteins, their degradation, and the establishment of neurodegeneration remain poorly understood. How intervention of small molecules in these mechanisms can improve the overall functionality of PQC mechanism linked with the refolding, ubiquitination and degradation of misfolded proteins is a challenging problem. To answer this question, in preliminary experiments, cells were treated with Myricetin and then the endogenous levels of various molecular chaperones and QC-E3 ubiquitin ligases were checked. As shown in immunofluorescence analysis, it has been found that endogenous levels of E6-AP, a putative QC E3 ubiquitin ligase and molecular chaperone Hsp70 get affected in response to Myricetin treatment in cells, Figure. 4.1.

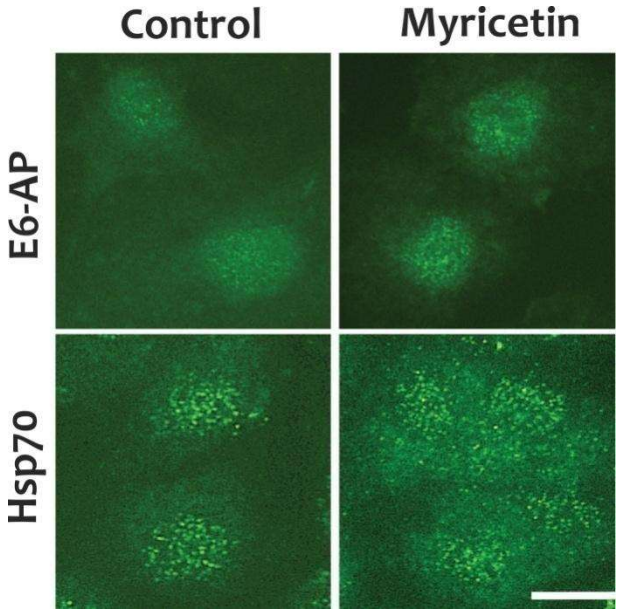


**Figure 4.1 :** *Myricetin affects endogenous levels of QC E3 ubiquitin ligase E6-AP, and chaperone Hsp70:* Cos-7 cells were seeded into chamber slides (two-well) and processed for immunofluorescence staining with primary antibodies of E6-AP and Hsp70 in presence or absence of Myricetin 20 µM for 24 Hrs. Scale bar 20 µm. Submitted as Joshi and Mishra et al., 2019.

The difference in expression of Hsp70 and E6-AP bound us to check the endogenous levels of HSF-1 transcription factor, as it has previously been observed that the 5′-untranslated region of E6-AP gene retains numerous consensus heat shock factor 1 (HSF-1) binding sequences [Mishra *et al.*, 2009]. Immunofluorescence analysis of HSF-1, after the treatment of Myricetin, may probably have an effect on the endogenously present HSF-1 as could be seen in Figure 4.2.

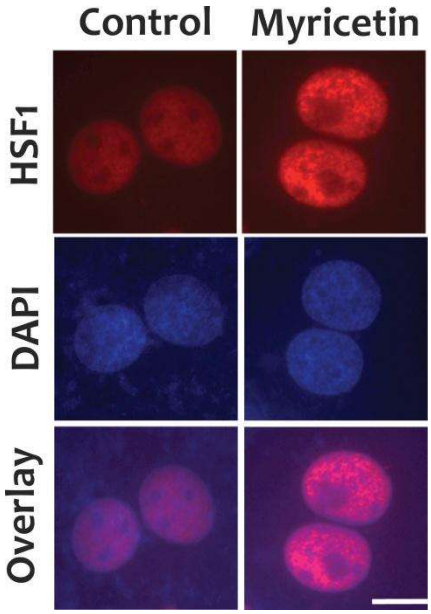


**Figure 4.2 :** *Affect on HSF1 by Myricetin treatment:* Upstream regulator of heat-shock proteins, HSF1, was probed with the anti-HSF1 antibody. The nucleus was stained with antifade DAPI. Scale bar 20 µm.

To further confirm these preliminary results, cells were again exposed with Myricetin in both concentration, Figure 4.3 and time dependent manner, Figure 4.4. As depicted in Figure 4.3 and 4.4, immunoblot analysis represents the increased levels of E6-AP, Hsp70, and HSF-1 under the concentration and time dependent effect of Myricetin treatment. These data indicate that treatment of Myricetin can induce the expression of HSF-1, Hsp70, and E6-AP inside the cells.

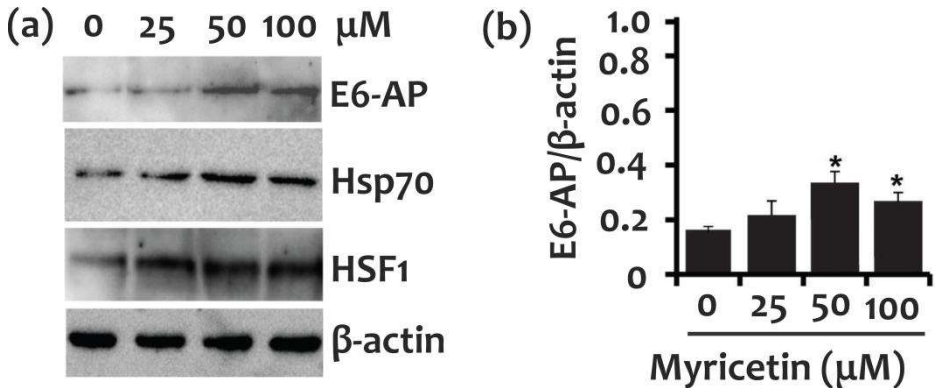


**Figure 4.3 :** *Effect of Myricetin concentration dependent treatment:* (a) Cells seeded into six-well tissue culture plates were exposed with Myricetin in concentration dependent manner as 0, 25, 50 and 100 µM for 24 Hrs. Expression levels of E6-AP, Hsp70, and HSF1 were analyzed by western blotting using whole cell lysates, collected from these cells using β-actin as loading control. (b) Quantification of bands in figure (a) was performed using NIH image analysis software. *p<0.05 in comparison with the control.

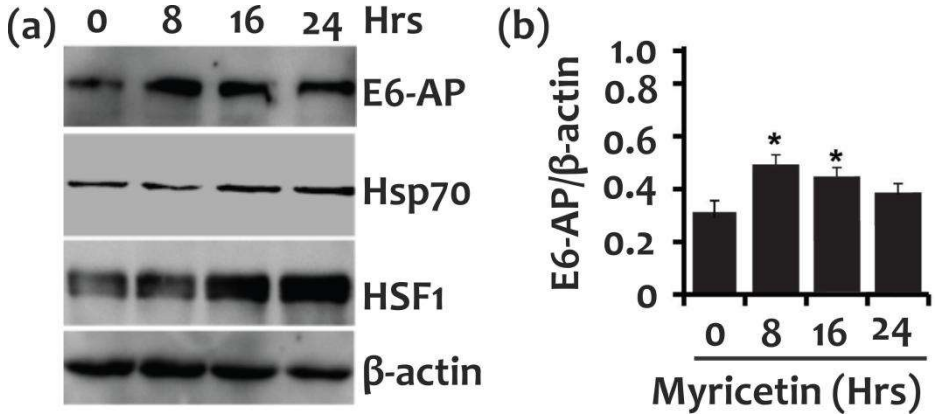


**Figure 4.4 :** *Effect of Myricetin time dependent treatment:* (a) Myricetin (20 µM) was given to Cos-7 cells at different time intervals of 0, 8, 16 and 24 Hrs and cell lysates were used for E6-AP, Hsp70 and HSF1 expression level analysis by western blotting. (b) NIH image analysis software was used for quantification of bands in figure (a). *p<0.05 in comparison with the control.

### 4.1.2 Exposure of Myricetin Suppresses the Aggregation of Misfolded Proteins

To determine whether the induced expression of heat shock protein and QC-E3 ubiquitin ligase based on Myricetin treatment can suppress the aggregation of misfolded proteins, green fluorescence protein (GFP) fusions of wild-type chloramphenicol-acetyltransferase (wtCAT) and mutant GFP-Δ9CAT proteins were used in experiments [Arslan *et al.*, 2012; Chhangani *et al.*, 2014]. Mutant GFP-Δ9CAT forms intracellular misfolded proteinaceous inclusion bodies; however, wild type GFP-wtCAT remains diffusely distributed inside the cells. Transient transfection was performed with GFP-wtCAT and GFP-Δ9CAT plasmids, and post-transfected cells were treated with or without Myricetin in the presence or absence of MG132, a putative proteasome inhibitor. Treated cells were prepared for detailed

immunofluorescence analysis; and were probed with ubiquitin antibody. As shown in Figure 4.5, overexpression of wild type GFP-wtCAT diffusely localized in the cells and accumulated in the form of inclusions after MG132 treatment, Figure 4.6.

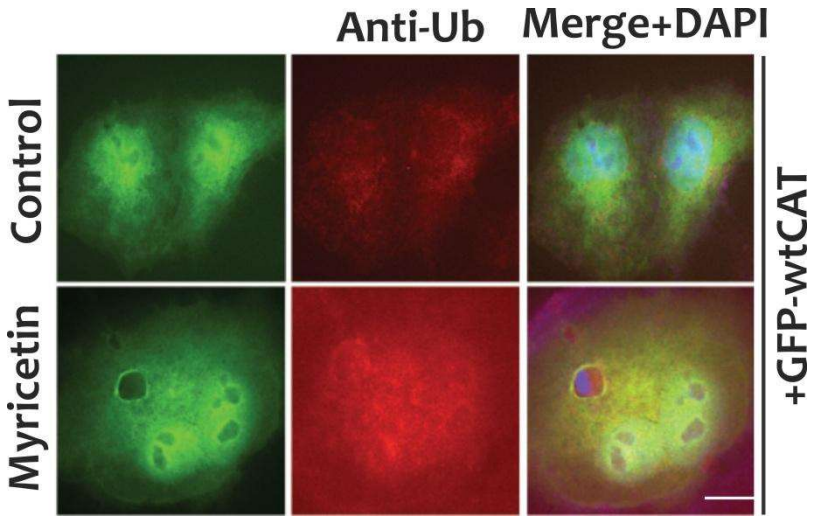


**Figure 4.5 :** *Observation of Myricetin treatment to GFP-wtCAT transfected cells:* Cos-7 cells grown in two-well chamber slides and subsequently transfected with the GFP-wtCAT construct. Post-transfection, cells were given a treatment of DMSO and Myricetin (10 µM for 48 Hrs) processed for immunocytochemistry with anti-ubiquitin antibody. Scale bar 20 µm.

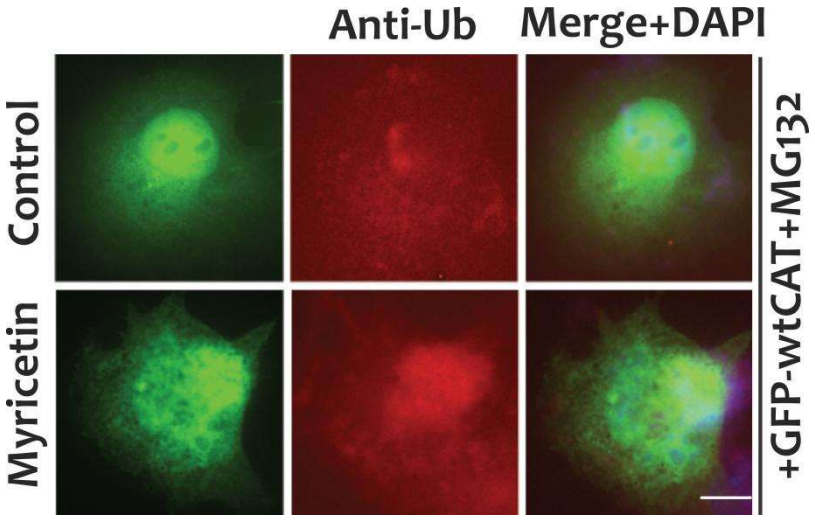


**Figure 4.6 :** *Myricetin treatment to GFP-wtCAT transfected cells with MG132:* Cos-7 cells grown in two-well chamber slides and subsequently transfected with the GFP-wtCAT construct. Post-transfection, cells were given a treatment of DMSO and Myricetin (10 µM for 48Hrs) with MG132 µM for 12 Hrs and processed for immunocytochemistry with anti-ubiquitin antibody. Scale bar 20 µm.

The effect of Myricetin on the aggregates of overexpressed mutant GFP-Δ9CAT proteins was shown in Figure 4.7. Treatment of Myricetin significantly suppresses the aggregation of misfolded GFP-Δ9CAT mutant protein, which can be reverted by the addition of proteasome inhibitor MG132, as represented in Figure 4.8. Analysis by immunofluorescence microscopy revealed that treatment of Myricetin was able to reduce the accumulation of large ubiquitin-positive perinuclear misfolded inclusions of mutant GFP-Δ9CAT protein and the effect of Myricetin can be overcome by the addition of MG132 proteasome inhibitor.

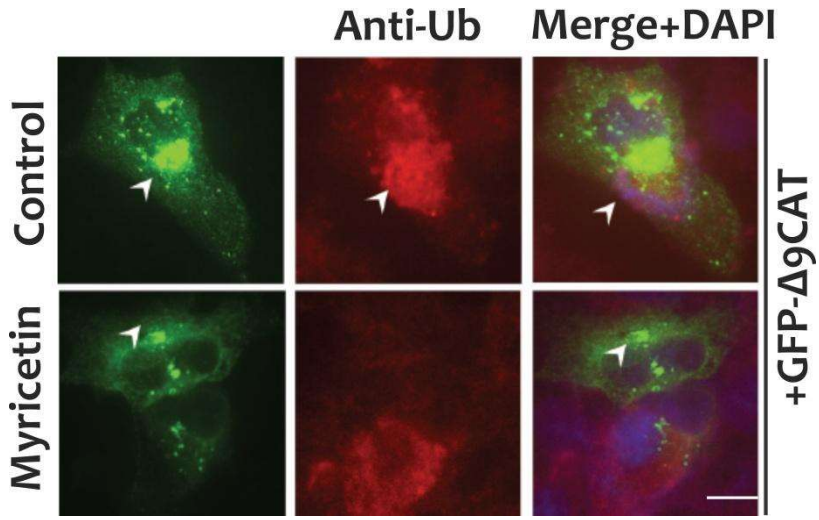


**Figure 4.7 :** *Effect of Myricetin treatment on mutant GFP-Δ9CAT inclusions:* Post treatment, immunostaining was done using ubiquitin as a primary antibody and with rhodamine-conjugated secondary antibody for microscopic analysis of cells. Fluorescence microscope was used to obtain images and DAPI was used for nuclear localization and overlay. Arrowheads indicate the co-localization of ubiquitin with GFP-Δ9CAT aggregates at perinuclear space in the cytoplasm. Scale bar 20 µm.

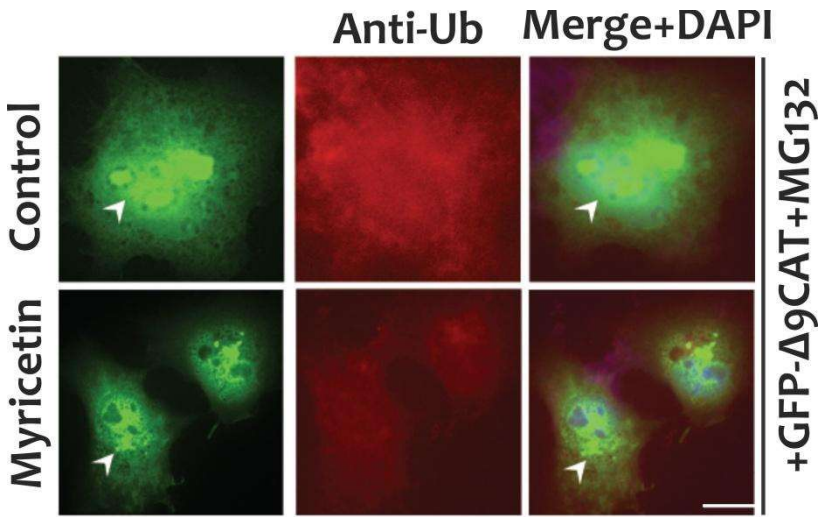


**Figure 4.8 :** *Effect of Myricetin on inclusion formation following proteasome inhibition:* Post treatment, immunostaining was done using ubiquitin as a primary antibody. DAPI was used for nuclear localization and overlay. Arrowheads indicate the co-localization of ubiquitin with GFP-Δ9CAT aggregates at perinuclear space in the cytoplasm. Scale bar 20 µm.

### 4.1.3 Myricetin Reduces Aggregation of Misfolded Luciferase Proteins and Protects from Stress-Induced Cell Death

In the present study, it has been observed that use of Myricetin reduces the aggregation of misfolded proteins and most likely mediates the aggregate clearance via proteasomal pathway. To further ascertain the effects of Myricetin on misfolded proteins, thermally denatured misfolded protein based study was used. After transfection of intracellularly expressing luciferase constructs, cells were exposed with Myricetin, kept at 43 °C for 30 minutes, and then provided a 2 Hrs recovery period at 37 °C. Thereafter, cells were subjected to immunofluorescence microscopy analysis to observe the colocalization of luciferase with E6-AP antibody, Figure 4.9 or Hsp70, Figure 4.10 in different experimental sets. The results obtained from immunofluorescence staining clarify that Myricetin reduces the aggregation of heat-denatured luciferase perinuclear bodies.

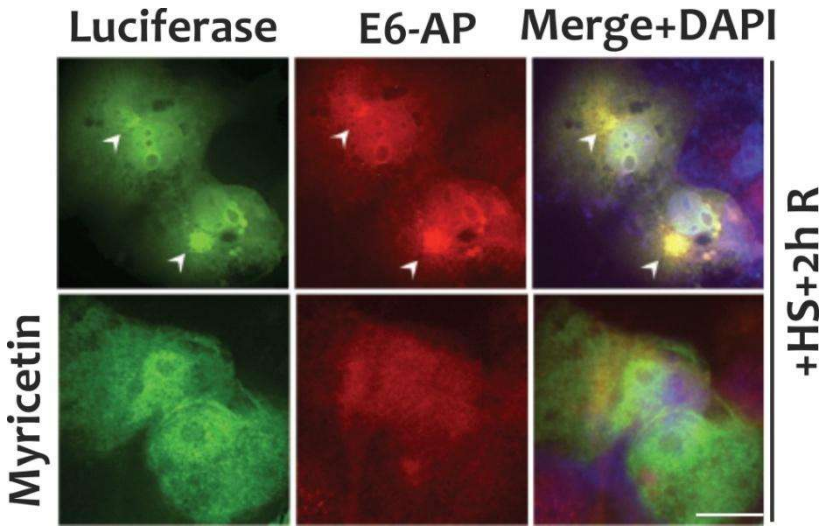


**Figure 4.9 :** *Analysis of E6-AP and luciferase co-localization:* Cells seeded in chamber slides were transfected with constructs of firefly luciferase and treated with or without Myricetin 10 µM for 48 Hrs. Post-treatment, cells were given 30 minutes heat shock (HS) at 43 °C, followed by a recovery period of 2 Hrs at 37 °C and then further used for immunofluorescence staining with primary antibodies of luciferase, and E6-AP. FITC conjugated secondary (green) antibody was used for luciferase, whereas, E6-AP was stained using rhodamine conjugated (red) secondary antibody. Arrowheads indicate the co-localization of E6-AP with luciferase aggregates. Scale bar 20 µm.

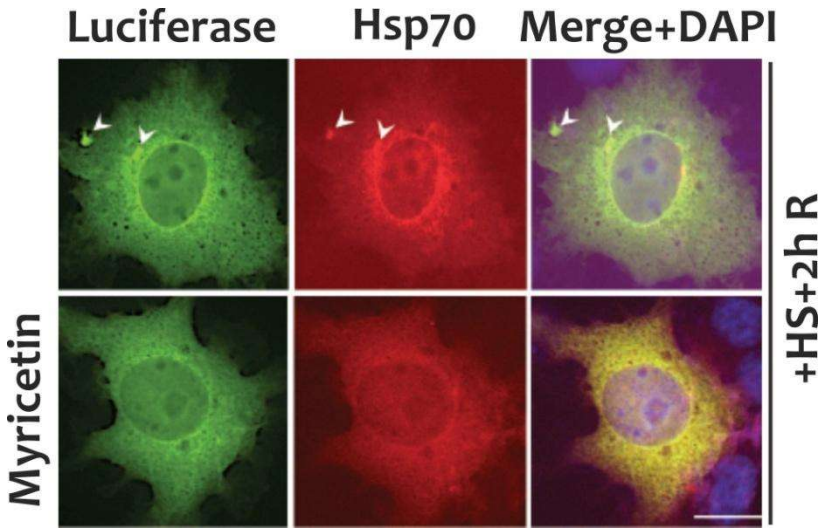


**Figure 4.10 :** *Monitoring the co-localization of luciferase and Hsp70:* Cells seeded in chamber slides were transfected with constructs of firefly luciferase and treated with or without Myricetin (10 µM for 48 Hrs). Post-treatment, cells were given 30 minutes heat shock at 43 °C, followed by a recovery period of 2 Hrs at 37 °C and then further used for immunofluorescence staining with primary antibodies of luciferase, and Hsp70. FITC conjugated secondary (green) antibody was used for luciferase, whereas, E6-AP was stained using rhodamine conjugated (red) secondary antibody. Arrowheads indicate the co-localization of Hsp70 with luciferase aggregates. Scale bar 20 µm.

Further, immunoblot analysis was performed on similar set of cells Figure 4.11 a and luciferase activity assays according to manufacturer's protocol Figure 4.11 b. Both results support the previous observations that most likely, Myricetin triggers a cytoprotective response in cells via inducing the intracellular degradation of various misfolded proteins. These above results likely present the cytoprotective proficiency of Myricetin against aberrant proteins.

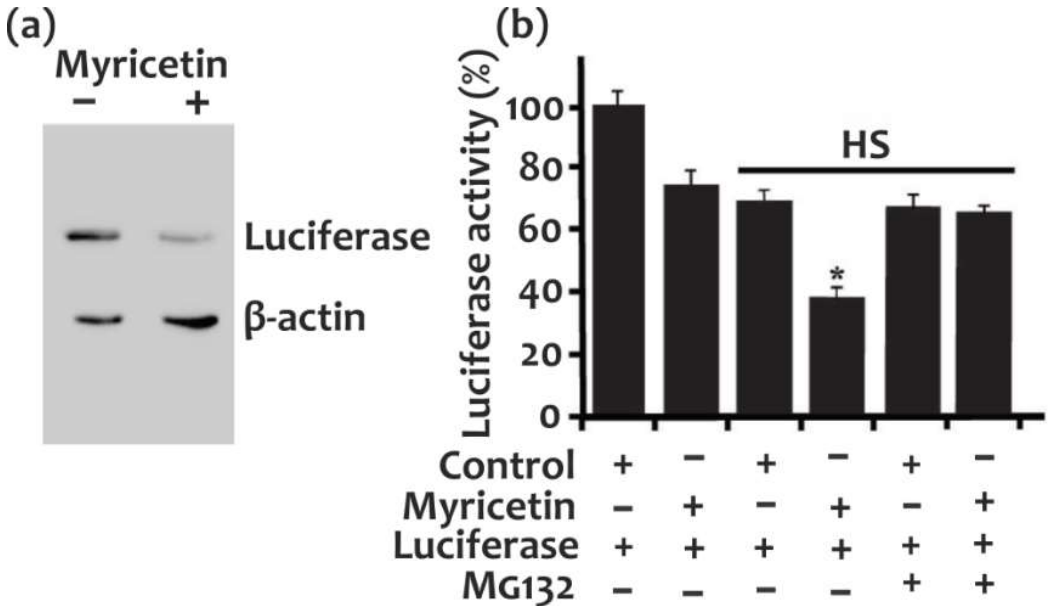


**Figure 4.11 :** *Luciferase activity assay and immunoblot analysis:* (a) Set of cells, transfected with luciferase and administered with or without Myricetin 30 µM for 24 Hrs, were collected and subjected for western blot analysis with luciferase antibody. β-actin was used as loading control. (b) Luciferase activity assay was also performed according to the given manufacturer's protocol in cells treated with DMSO and Myricetin. *p<0.05 in comparison with the control.

To test the influence of Myricetin on cellular protection under various stress conditions, cells were treated with different stress-inducing agents. Chloroquine (CQ) for autophagic-dysfunction, MG132 for proteasomal inhibition and tunicamycin for ER-dysfunctions and some cells were also exposed with Myricetin. As shown in Figure 4.12, use of Myricetin significantly improves the survival of cells as compared to control (DMSO treated) cells, under similar stress conditions. Together, these observations suggest that most probably, Myricetin alleviates cytotoxicity and provides protection due to the enhanced elimination of misfolded proteins, which could be over accumulated during different stress conditions.

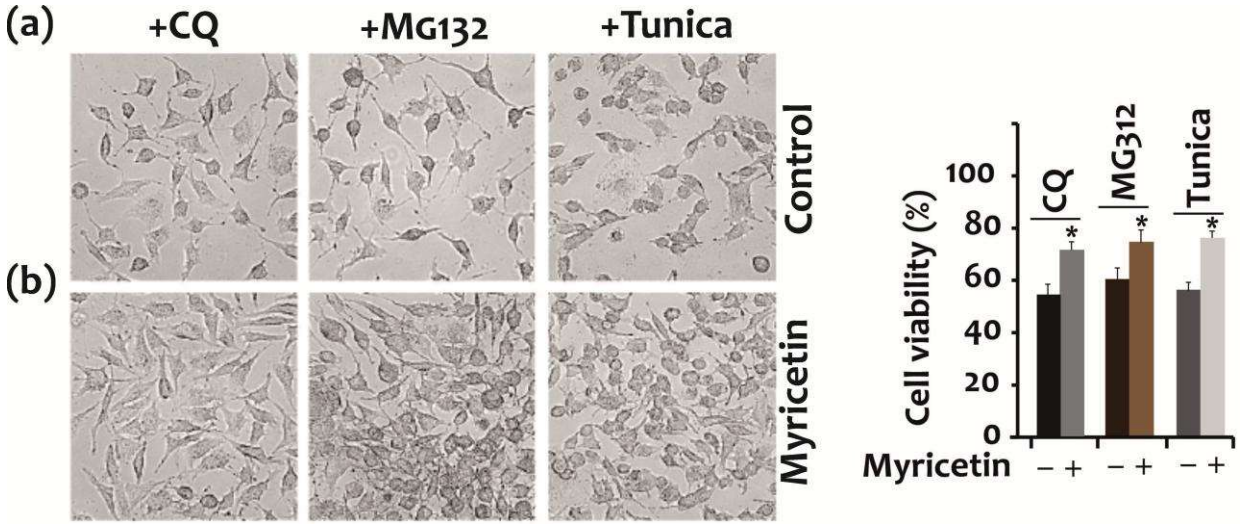


**Figure 4.12 :** *Myricetin provides cytoprotection from various stresses:* A549 cells were plated into six-well tissue culture plates, treated with chloroquine (CQ) (20 µM for 10 Hrs), MG132 (10 µM for 12 Hrs) and tunicamycin (Tunica) (10 µg/ml for 12 Hrs) with or without Myricetin 10 µM for 48 Hrs. Bright field images were obtained to visualize the effects of Myricetin on cellular morphology under stress conditions.

### 4.1.4 Aggregation Inhibitory Effects of Myricetin on Intracellular Mutant SOD-1 Inclusions

Although the precise mechanism, by which Myricetin can inhibit the accumulation of misfolded proteins is not clear till now, yet the current observations provide some intriguing clues linked with elevation of PQC components. To further determine the function of Myricetin against aberrant proteins accumulation, next, Amyotrophic lateral sclerosis (ALS) associated mutant superoxide dismutase-1 (SOD-1) constructs were used. Cells were transfected with both wild-type SOD1 (GFP-SOD1-WT) and mutant SOD1 (GFP-SOD1-G37R) plasmids as ALS cellular model in the absence or presence of Myricetin. To assess the efficacy of Myricetin in the clearance of neurodegeneration-linked protein, immunofluorescence staining was performed, as described in above experimental set of cells. In this cellular model, it has been observed that wild type SOD1 (GFP-SOD1-WT) does not exhibit profound aggregate formation Figure 4.13, but the inhibition of proteasome leads to the over-accumulation of expressed GFP-SOD1-WT proteins and produce perinuclear aggresome Figure 4.14.

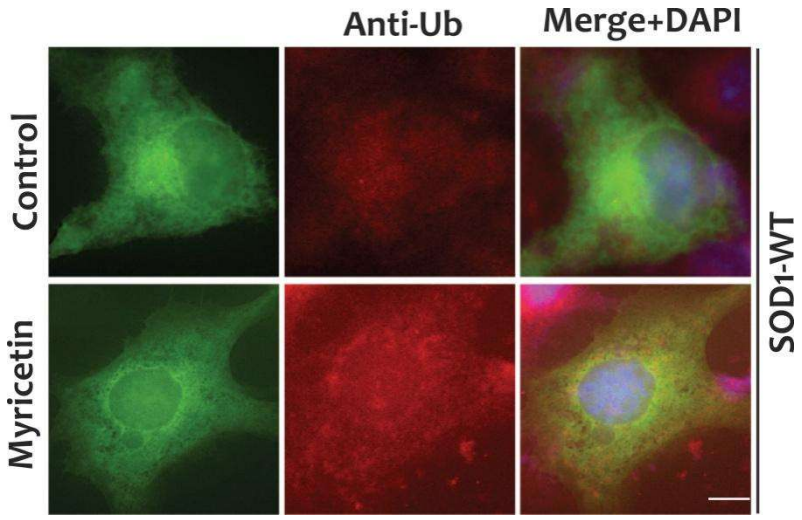


**Figure 4.13 :** *Myricetin-mediated ubiqutination of GFP-SOD1-WT:* Immunocytochemistry analysis of Cos-7 cells, transfected with GFP-SOD1-WT constructs with or without Myricetin 10 µM for 48 Hrs, was performed. Ubiquitin primary antibody was used with rhodamine conjugated secondary antibody and DAPI was used as a nuclear stain. Scale bar 20 µm.

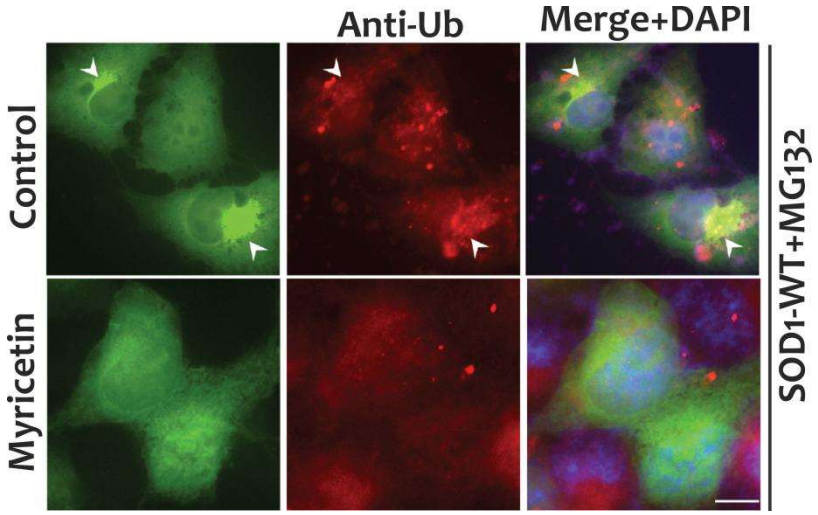


**Figure 4.14 :** *Myricetin-mediated ubiqutination of GFP-SOD1-WT in presence of MG132:* Immunocytochemistry analysis of Cos-7 cells, transfected with GFP-SOD1-WT constructs with or without Myricetin 10 µM for 48 Hrs, was performed. Few sets of cells were also given a treatment of MG132 10 µM for 12 Hrs. Ubiquitin primary antibody was used with rhodamine conjugated secondary antibody and DAPI was used as a nuclear stain. Scale bar 20 µm.

Expression of mutant SOD1 (GFP-SOD1-G37R) protein also forms clear aggresome-like structures in cells; and exposure of Myricetin decreases the intracellular aggregation of ubiquitin-positive mutant SOD1 (GFP-SOD1-G37R) proteins Figure 4.15. Addition of MG132 up to some extent reduces the anti-aggregation effect of Myricetin in the cells Figure 4.16. Overall, these results suggest that Myricetin improves the clearance of misfolded SOD1 aggregates from the cells, which may also contribute to the survival of cells under proteotoxic disturbances.

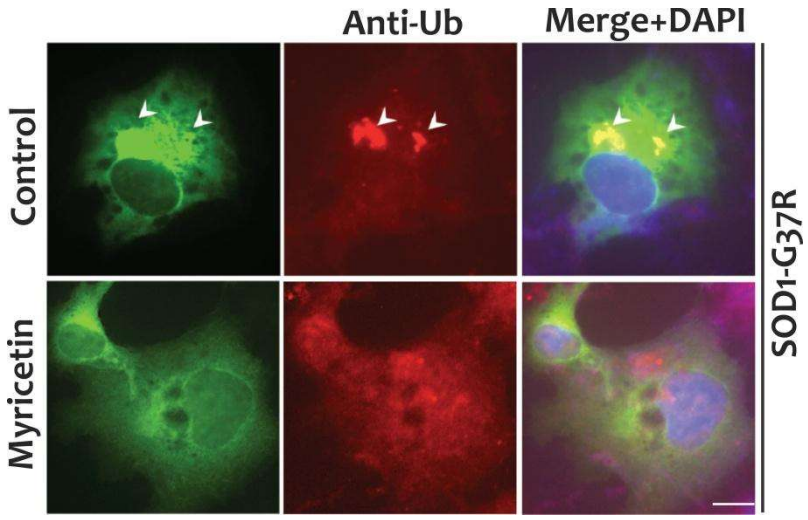


**Figure 4.15 :** *Myricetin-mediated ubiqutin localization with GFP-SOD1-G37R aggregates:* GFP-SOD1-G37R constructs transfected cells were analyzed after Myricetin treatment, and stained for visualization of ubiquitin co-localization with aggregates. Arrowheads show the presence of highly accumulated protein aggregates. Scale bar 20 µm.

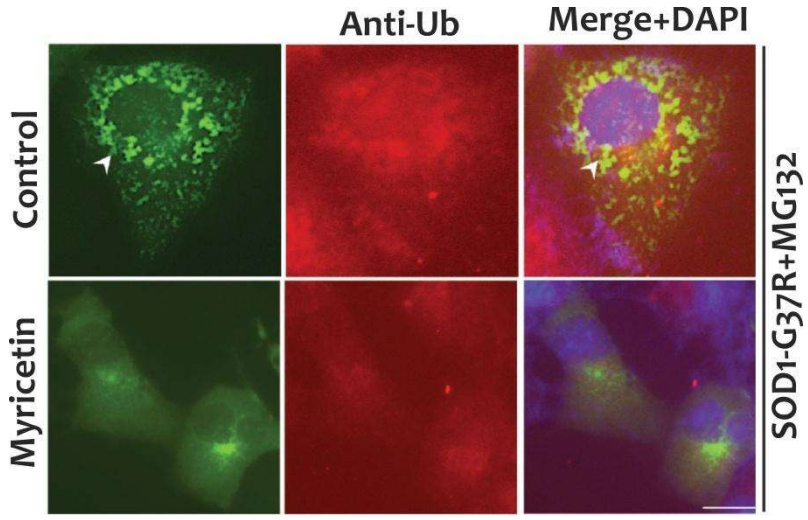


**Figure 4.16 :** *Myricetin-mediated localization of ubiqutin with GFP-SOD1-G37R in the presence of MG132:* GFP-SOD1-G37R transfected cells were also analyzed after Myricetin treatment with MG132 and stained for visualization of ubiquitin co-localization with aggregates. Arrowheads show the presence of highly accumulated protein aggregates. Scale bar 20 µm.

### 4.1.5 Inhibition of Mutant α-Synuclein Intracellular Aggregation by Myricetin

Interestingly, during the experiments with non-pathogenic modelled misfolded proteins, it has been observed that Myricetin suppresses their aggregation. Subsequently, it was also examined that treatment of Myricetin alleviates cytotoxic effects of various stress conditions and also reduces the aggregation of mutant SOD1 protein associated with ALS neurodegenerative disease. Next, the aggregation inhibitory effect of Myricetin was verified on another neurodegenerative disease (Parkinson's Diseases)-linked proteins. For this mutant α-Synuclein (S87A) plasmid constructs were used; and cells were transfected with both normal and mutant (S87A) constructs. Transfected cells were treated with Myricetin and subjected to immunofluorescence analysis by using α-Synuclein and ubiquitin antibodies. As shown in Figure 4.17, the expression of normal α-Synuclein represents an overall diffused pattern in cells;

whereas inhibition of proteasome with MG132 induces the over-accumulation of normal α-Synuclein proteins, which produces marginal ubiquitin positive aggregates in cells Figure 4.18.

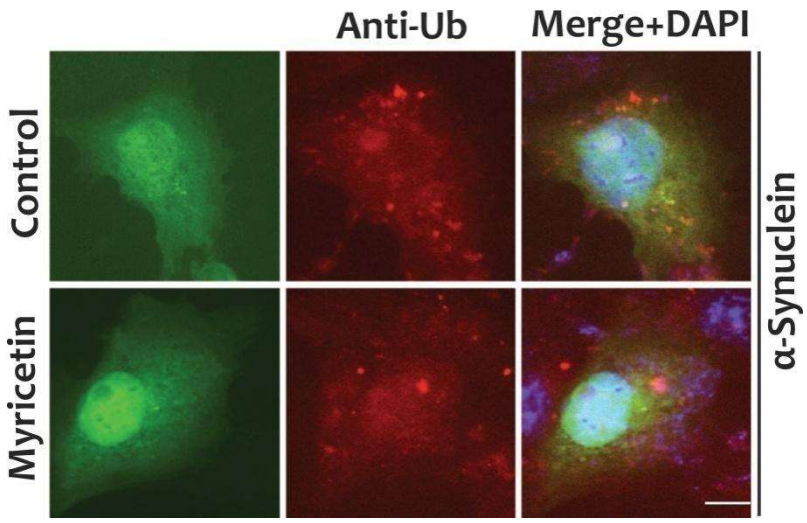


**Figure 4.17 :** *Effect of Myricetin on accumulation of α-Synuclein protein:* Cos-7 cells were grown in chamber slides, and transfected with α-Synuclein plasmid constructs with or without treatment of Myricetin (10 µM for 48 Hrs). Post-treatment, immunofluorescence staining was performed using ubiquitin and α-Synuclein antibody. DAPI was used for nuclear staining. Scale bar 20 µm.

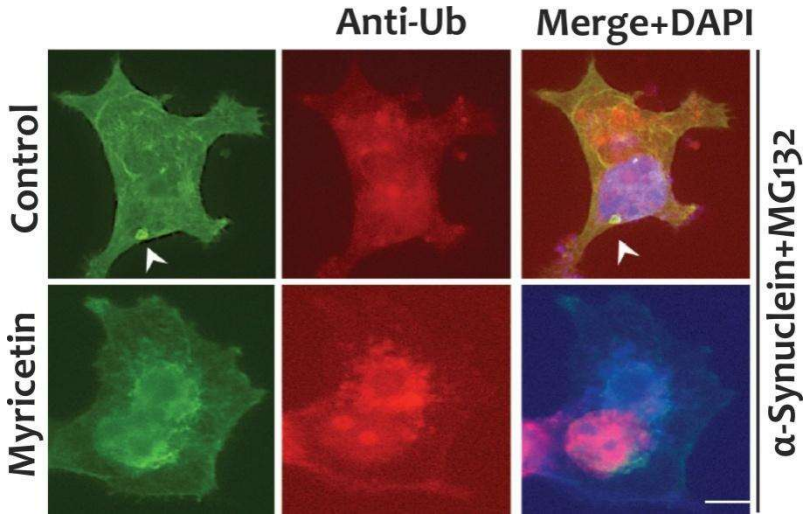


**Figure 4.18 :** *Effect of MG132 on Myricetin-mediated ubiqutination of α-Synuclein:* Cos-7 cells were grown in chamber slides, and transfected with α-Synuclein plasmid constructs with or without treatment of Myricetin (10 µM for 48 Hrs), whereas few sets of cells were then given a treatment of MG132, 10 µM for 12 Hrs. Post-treatment, immunofluorescence staining was performed using ubiquitin and α-Synuclein antibody to visualize the localization of ubiquitin with α-synuclein aggregates as shown by arrowheads. DAPI was used for nuclear staining. Scale bar 20 µm.

Immunofluorescence analysis of cells expressing mutant (S87A) α-Synuclein proteins revealed ubiquitin-positive aggregates formation inside the cells Figure 4.19. Exposure of Myricetin reduced the accumulation of mutant (S87A) α-Synuclein aggregates Figure 4.19. The anti-aggregation effect of Myricetin was reversed after the addition of MG132 Figure 4.20. These findings strongly suggest that Myricetin may serve as a useful compound against the aggregation of neurodegenerative disease associated toxic proteins in cells.

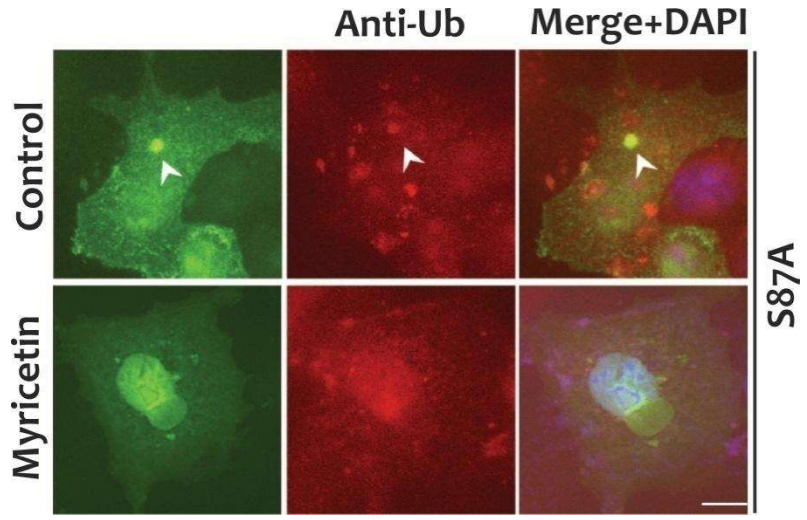


**Figure 4.19 :** *Myricetin-mediated suppression of aggregation propensity of α-Synuclein mutant S87A aggregates:* Cells were transfected with α-Synuclein mutant S87A constructs and treated with DMSO and Myricetin. Next day immunofluorescence was performed using ubiquitin and α-Synuclein antibody. Scale bar 20 µm.

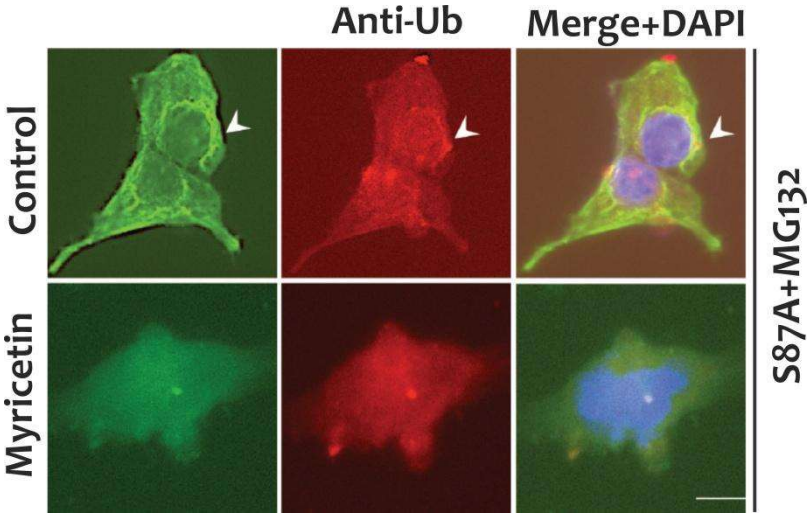


**Figure 4.20 :** *Effect of proteasomal inhibition on Myricetin mediated aggregate clearance:* Cells, transfected with α-Synuclein mutant S87A constructs were treated with DMSO and Myricetin along with MG132 to monitor the effects of Myricetin following proteasome inhibition. Scale bar 20 µm.

### 4.1.6 Myricetin Enhances the Stability of E3 Ubiquitin Ligase E6-AP

Results suggest that Myricetin increases the endogenous levels of E3 ubiquitin ligase E6-AP and previously, it has been observed that Hsp70 interaction with E6-AP assists the degradation of misfolded proteins [Mishra *et al.*, 2009]. Here, it has been observed that treatment of Myricetin reduces the intracellular aggregation of aberrant proteins. Therefore, it has been thought that there might be a possible interaction between E6-AP and Myricetin, which can provide an effective functional stability to E6-AP. To address this notion, detailed docking studies were performed. *In silico* analysis showed a −7.0 kcal/mol binding affinity between best docking conformations of Myricetin with E6-AP HECT catalytic domain. Two hydrogen bonds were obtained involving amino acid GLY545 and ASN822 of E6-AP HECT catalytic domain with Myricetin Figure 4.21.

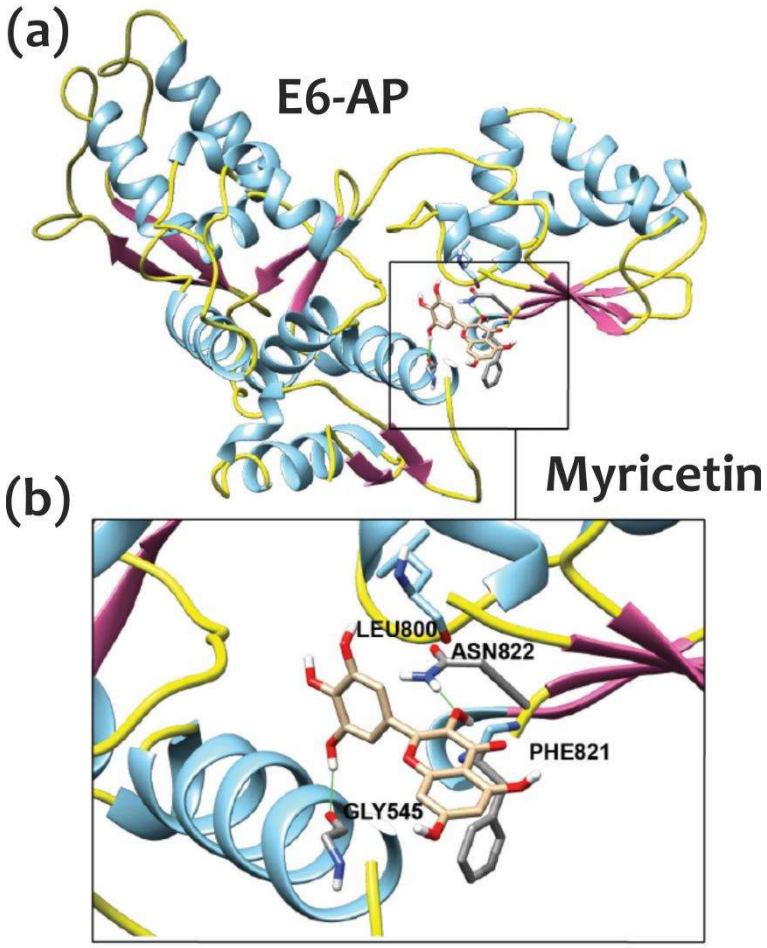


**Figure 4.21 :** *In-silico analysis of Myricetin interaction with E6-AP:* Myricetin-Ube3a HECT (PDB ID: 2O63 and PDB ID: 1D5F) catalytic domain interaction was obtained by *in-silico* docking approach along with a close-up view of the interacting regions marked with interacting amino acid residues.

Since, Myricetin influences the endogenous expression levels of E6-AP protein; therefore, it was important to examine the effects of Myricetin on the stability of E6-AP. Cells were exposed to control (DMSO), or Myricetin and post-treated cells were processed for cycloheximide chase experiment. As depicted in the Figure 4.22, administration of Myricetin slows down the rate of degradation of E6-AP as compared to control. Earlier observations and present results suggest that probably treatment of Myricetin not only upregulates the expression of E6-AP, but also stimulates the progressive intracellular stabilization of E6-AP Figure 4.22; which could serve as an additional contributing factor to affect the elimination of misfolded proteins from the cells.

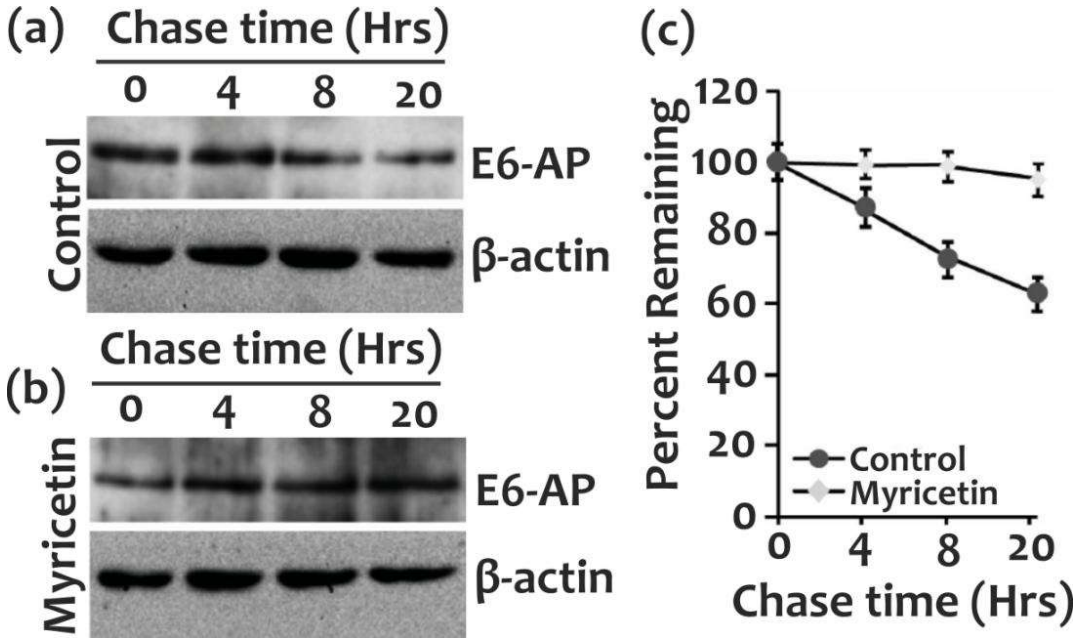


**Figure 4.22 :** *Myricetin stabilizes E6-AP in cells:* (a-b) Cos-7 cells were seeded on six-well tissue culture plates and treated with control and Myricetin 10 μM for 48 Hrs on a subsequent day and chased for 20 Hrs by cyclohexemide (15 μg/ml) treatment at different time intervals. E6-AP and β-actin were used for band detection in western blotting. (c) Quantification of bands obtained in a-b was done by NIH image analysis software.

### 4.1.7 Myricetin Reduces the Formation of Insoluble Expanded Polyglutamine Proteins Inclusion Bodies

Intracellular expression of expanded polyglutamine stretches generates misfolded protein aggregates and such CAG repeats/polyglutamine-linked inclusion bodies were earlier reported in Huntington's disease (HD) [Chen *et al.*, 2002]. Previously, it has also been shown that E6-AP promotes the degradation of expanded polyglutamine proteins [Mishra *et al.*, 2008]. Current observations indicate that Myricetin provides intracellular stability to E6-AP; therefore, next it was tried to understand whether Myricetin could decrease the load of misfolded inclusions formation in cells and alleviate cytotoxicity associated with another neurodegenerative disease (HD). Cellular model of HD was used in next set of experiments. Cells were transfected with normal (EGFP-HDQ23) Figure 4.23 and expanded (EGFP-HDQ74) polyglutamine expression constructs and some set of cells were also treated with Myricetin in the presence or absence of MG132. As shown in Figure 4.24, expressed normal glutamine repeats EGFP-HDQ23 does not exhibit significant aggregate formation in comparison with expanded EGFP-HDQ74 polyglutamine proteins.

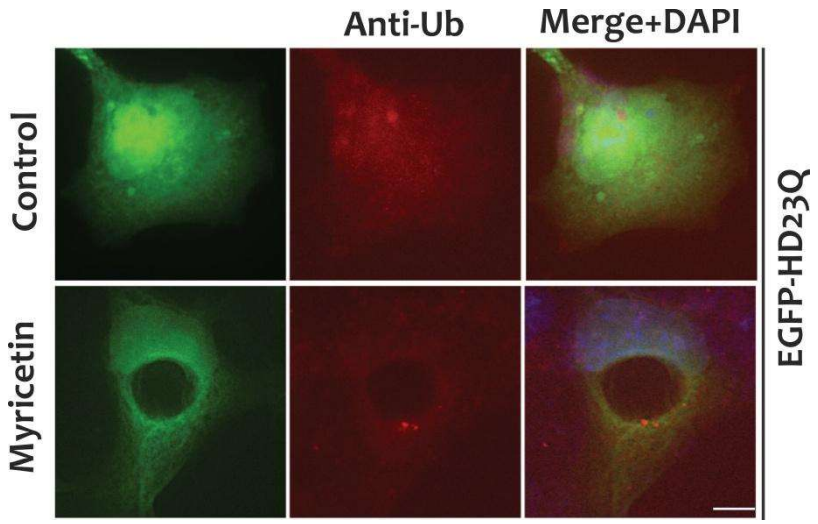


**Figure 4.23 :** *Myricetin treatment to cells expressing EGFP-HD23Q:* Cells were transfected with wild type EGFP-tagged huntingtin (23Q) plasmids and were exposed with control and Myricetin (10 µM for 48 Hrs). Immunofluorescence staining was done by ubiquitin primary antibody with rhodamine conjugated secondary antibody; and cells were analyzed under fluorescence microscope. Scale bar 20 µm.

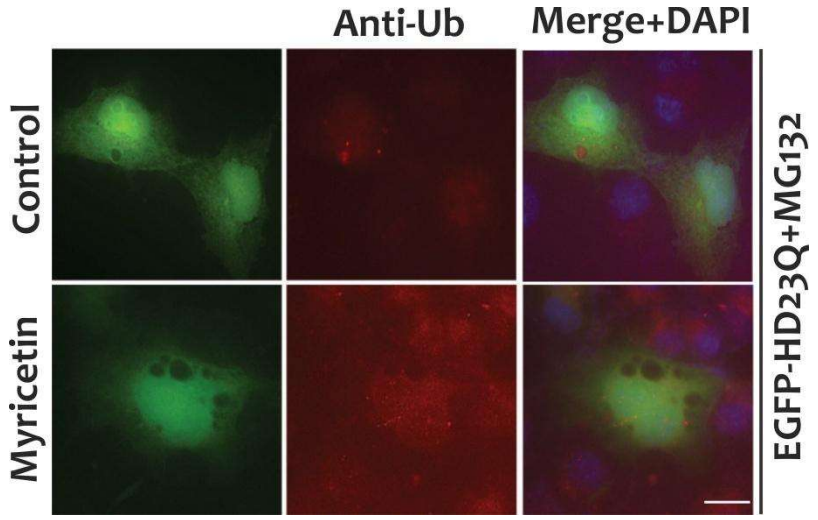


**Figure 4.24 :** *Monitoring the effect of MG132 treatment on Myricetin treated EGFP-HD23Q expressing cells:* Cells were transfected with EGFP-tagged huntingtin (23Q) plasmids and were exposed with control and Myricetin (10 µM for 48 Hrs) followed by treatment with proteasome inhibitor MG132, (10 µM for 12 Hrs). Immunofluorescence staining was done by ubiquitin primary antibody with rhodamine conjugated secondary antibody and cells were analyzed under fluorescence microscope. Scale bar 20 µm.

Addition of Myricetin dramatically reduces the toxic aggregates of expanded polyglutamine proteins Figure 4.25. Proteasome inhibitor (MG132) prevents the Myricetin-mediated clearance of EGFP-HDQ74 expanded polyglutamine proteins Figure 4.26. These observations suggest that probably Myricetin accelerates clearance of expanded polyglutamine protein aggregates via proteasome pathway.

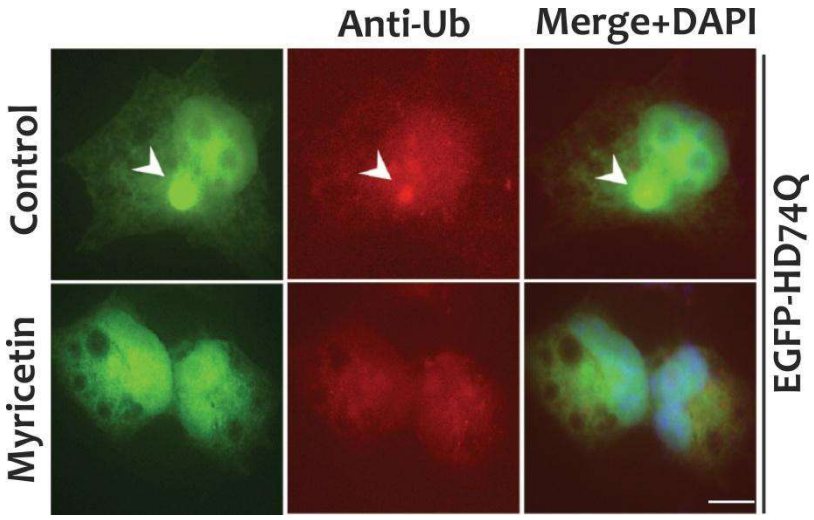


**Figure 4.25 :** *Myricetin reduces aggregates of EGFP-HD74Q:* Another set of cells were transfected with expanded polyglutamine huntingtin (74Q) plasmids and processed for immunofluorescence to observe ubiquitin co-localization with huntingtin polyglutamine aggregates, indicated by the arrowheads. Antifade with DAPI was used to stain nuclei in all sets of cells. Scale bar 20 µm.

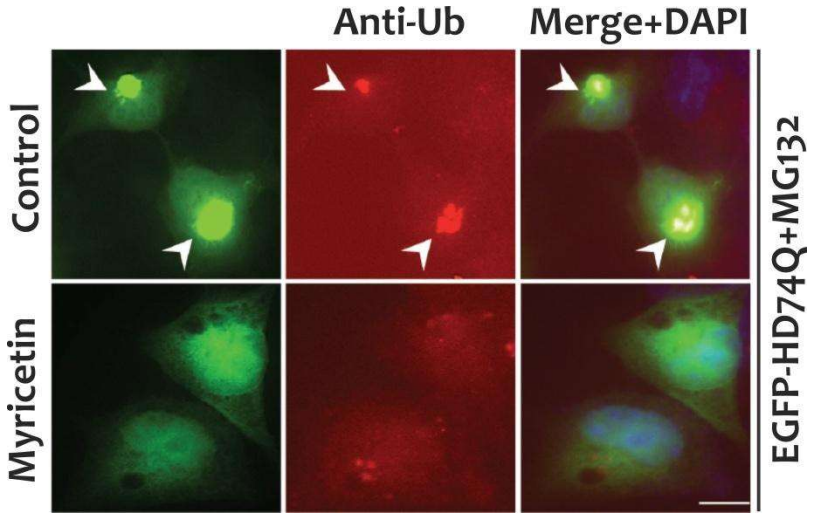


**Figure 4.26 :** *Myricetin reduces aggregates of EGFP-HD74Q in presence of MG132:* Another set of cells were transfected with extended polyglutamine huntingtin (74Q) plasmids and processed for immunofluorescence to observe ubiquitin co-localization with huntingtin polyglutamine aggregates, indicated by the arrowheads, in presence of MG132. Antifade with DAPI was used to stain nucleus in all sets of cells. Scale bar 20 µm.

### 4.1.8 Effect of Myricetin on Different Cellular Models of Neurodegenerative Diseases

Previously, it has been observed that the formation of intracellular inclusions sequester components of PQC pathways and further increases accumulation of aggregates which may lead to the impairment of proteasomal dysfunction and cell death [Schipper-Krom *et al.*, 2012; Zheng *et al.*, 2016]. In neurodegenerative diseases, including spinocerebellar ataxias, formation of similar intracellular inclusions serve as the major pathological hallmark [Koeppen, 2005; Koyano *et al.*, 1999]. To overcome these multifactorial complex pathogenic issues, it was important to find new molecules that can improve the clearance efficiency of PQC pathways against the aggravated deposition of misfolded proteinaceous inclusions. In the present study, it has been found that Myricetin treatment was effective against the aggregation of various

misfolded proteins. Therefore, it was crucial to understand the effects of Myricetin on the rate of formation of misfolded protein aggregate. Cells were transfected with EGFP-Ataxin-3(28Q) and expanded-polyglutamine EGFP-Ataxin-3(84Q) plasmids; and post-transfected cells were treated with DMSO or Myricetin. Few similar sets of cell lysates were collected and then subjected for filter trap assay as shown in Figure 4.27.

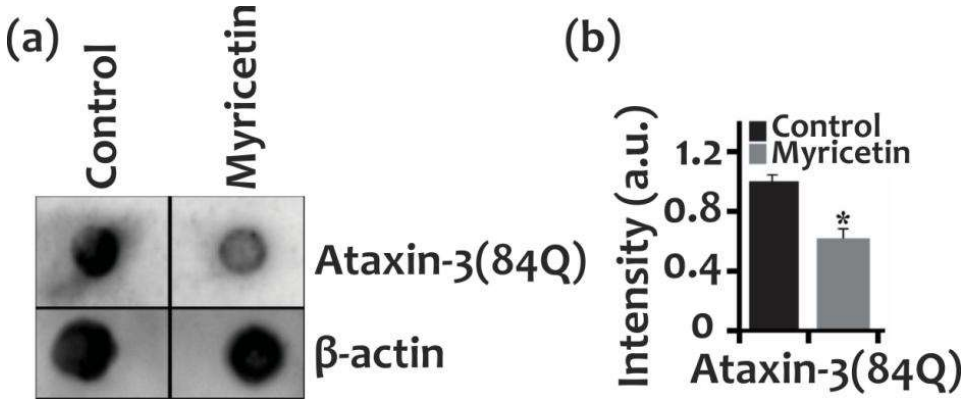


**Figure 4.27 :** *Myricetin treatment suppressed the aggregation of neurodegenerative diseases associated abnormal proteins:* (a-b) Cos-7 cells, seeded into six-well tissue culture dishes, transfected with EGFP-Ataxin-3 (84Q) plasmid with or without Myricetin (10 µM for 48 Hrs). Post-treatment samples were collected and subjected to filter trap assay with primary antibodies of anti-GFP and β-actin. Quantification of dots intensity was obtained via NIH image analysis software. In comparison with control *p<0.05.

It has been observed that use of Myricetin reduces the aggregation of misfolded inclusions of expanded polyglutamine proteins. Fluorescence microscopic analysis revealed that treatment of Myricetin suppresses the intracellular aggregation of EGFP-Ataxin-3(84Q) expanded polyglutamine aggregates Figure 4.28. Few sets of similar samples were processed for immunoblots analysis by using GFP and β-actin antibodies, as shown in Figure 4.29; and treatment of Myricetin enhances the degradation of expanded polyglutamine ataxin-3 repeats.

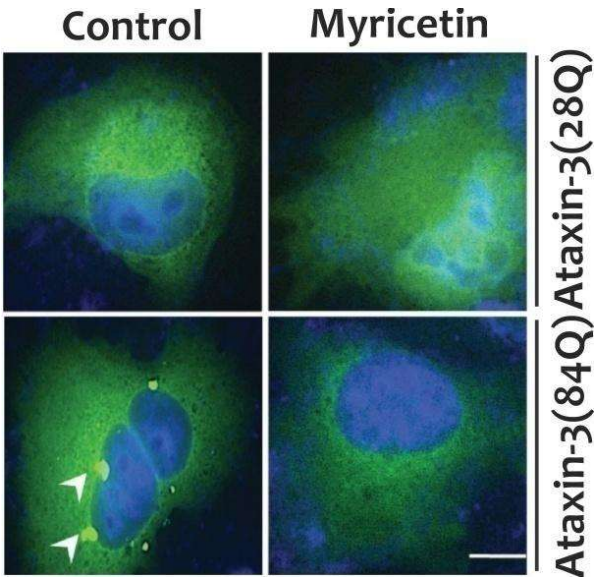


**Figure 4.28 :** *Myricetin treatment suppressed the aggregation of EGFP-Ataxin-3(84Q) proteins:* Cells transfected with plasmid constructs of normal polyglutamine repeats containing EGFP-Ataxin-3(28Q) and extended polyglutamine repeats containing EGFP-ataxin-3 (84Q) were subsequently treated with DMSO or Myricetin and were fixed to visualize under a fluorescence microscope. Scale bar 20 µm.

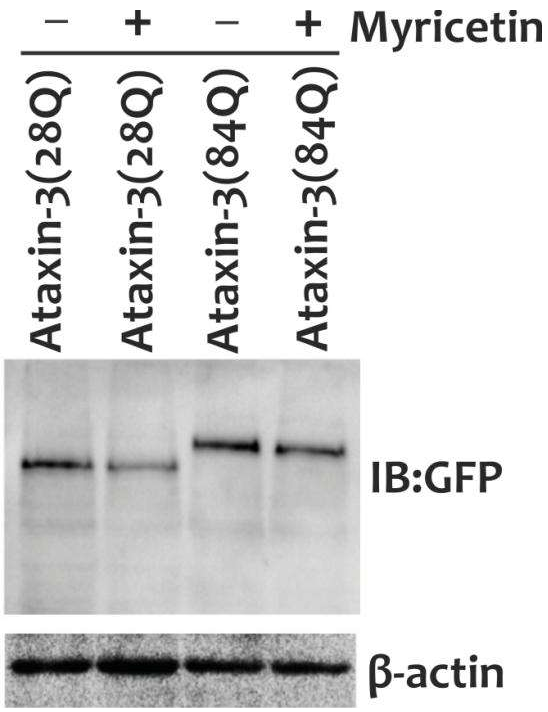


**Figure 4.29 :** *Myricetin treatment suppresses the expanded polyglutamin proteins:* Cells transfected with plasmid constructs of normal polyglutamine repeats containing EGFP-ataxin-3(28Q) and extended polyglutamine repeats containing EGFP-ataxin-3 (84Q) were subsequently treated with control or Myricetin and cell lysates were processed for western blot analysis by anti-GFP and β-actin antibodies.

Next, the direct effect of Myricetin on the number of aggregates of multiple misfolded proteins were observed. Therefore, as explained earlier, expanded-polyglutamine EGFP-HDQ74, mutant GFP-Δ9CAT and mutant SOD1 (GFP-SOD1-G37R) plasmids were exogenously expressed in cells in the presence or absence of Myricetin was checked. Effect of Myricetin was observed on various intracellular aggregates of different misfolded proteins. As shown in Figure 4.30, the total numbers of aggregates were reduced in the presence of Myricetin. Taken together, the above observations suggests that Myricetin treatment can increase endogenous levels of the chaperone Hsp70 and QC-E3 ubiquitin ligase E6-AP, which can significantly improve the survival of cells via enhanced degradation of misfolded proteins.

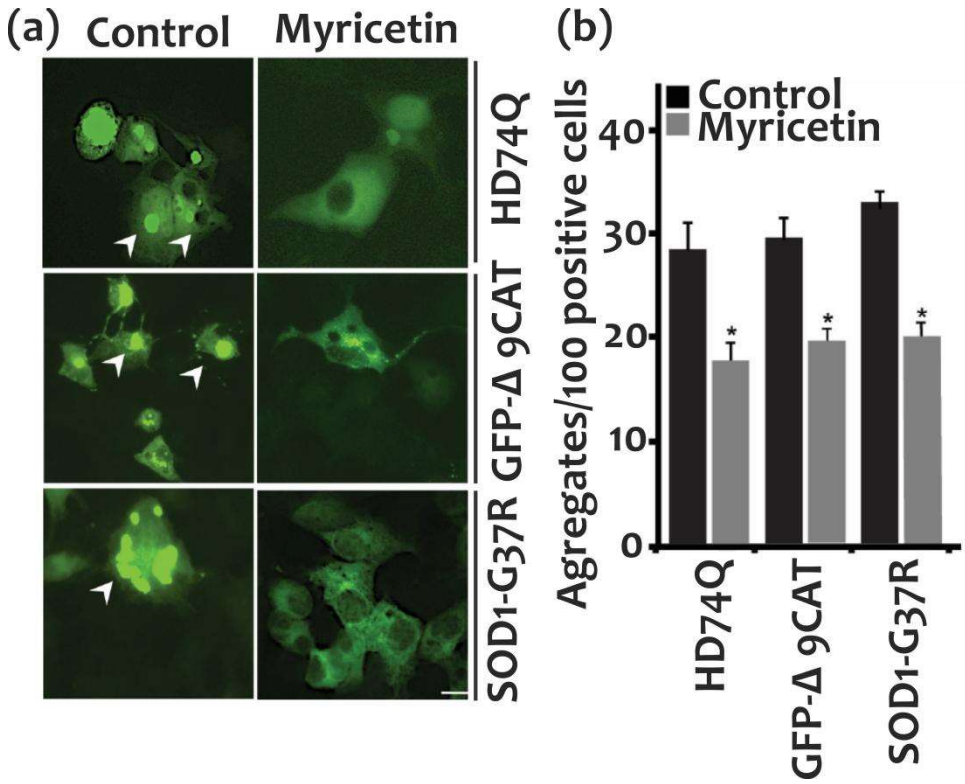


**Figure 4.30 :** *Myricetin treatment reduces the aggregation of neurodegenerative diseases associated abnormal proteins:* Cos-7 cells, after transfection with plasmid constructs of HD74Q, GFP-Δ9CAT, and GFP-SOD1-G37R, were given Myricetin and control DMSO treatment and numbers of aggregates were counted manually, under a fluorescence microscope. Quantification of a number of aggregates was performed in triplicates of three independent experiments, represented as mean ±SD with a value of *p<0.05 in comparison with control. Submitted as Joshi and Mishra et al., 2019.

## 4.2 DISCUSSION

The integrity of cellular fitness and health get often compromised during neurodegenerative diseases and ageing, which is linked with the accumulation of misfolded proteins. In this work, it has been shown that treatment of Myricetin increases the intracellular levels of few crucial proteins (HSF-1, Hsp70, and E6-AP) associated with PQC mechanism, which maintain cellular survival under proteotoxic insults, generated by aggregated proteins. How defects in cellular PQC machinery can cause the onset of such a diverse range of neurodegenerative diseases remains poorly understood. Recent research provides a diverse and emerging insight that insufficient removal of misfolded proteins, PQC dysfunctions, and loss of their clearance generate prominent pathogenic features of many neurodegenerative disorders [Chhangani and Mishra, 2013; Gestwicki and Garza, 2012; Kabashi and Durham, 2006]. To increase understanding for the various neurodegenerative diseases and their treatment methods it is important to identify molecules, which can improve cellular PQC functions and can also act as novel inhibitors of abnormal protein accumulation. Chaperone machinery is an integral component for protein folding and QC-E3 ubiquitin ligases result in the misfolded proteins degradation [Barral *et al.*, 2004; Chhangani *et al.*, 2013]. Therefore, it could be crucial to find out PQC mechanism-inducing compounds without having any toxicity and searched for them in Flavonoids. Earlier reports have suggested the medical importance of flavonoids in complex diseases like cancer, cardiovascular diseases, neurodegeneration and bacterial or viral infections, highlighting their significance and need of further research to obtain maximum therapeutic advantages [Havsteen, 2002; Spencer *et al.*, 2004].

To determine the potential of Myricetin against misfolded proteins aggregation, cells were treated with various expressing misfolded proteins. It has been observed that Myricetin reduces the aggregation of mutant GFP-Δ9CAT protein and also degrade the heat-denatured luciferase misfolded protein. In previous years, neuroprotective roles of Myricetin were well-explored because of its antioxidant properties, which helps in reduction of oxidative stress generated during multiple brain pathologies [Barzegar, 2016; Dajas *et al*., 2003]. Another study reported that Myricetin provides neuroprotection by reducing glutamate induced neuronal death and excitotoxicity by affecting three different pathways that are by inhibiting reactive oxygen species production, reducing $Ca^{2+}$ overload generated by phosphorylation of N-methyl D-aspartate receptor and finally by preventing caspase-3 activation [Shimmyo *et al.*, 2008].

Therefore, it was crucial to check the cytoprotective function of Myricetin against various stress conditions. Cells were treated with multiple stress inducing agents (autophagic dysfunction, proteasomal inhibition, and ER stress) in the presence or absence of Myricetin. Previous reports have indicated that QC-E3 Ubiquitin Ligases can recognize aberrant proteins via several molecular strategies based on the need of cells under stressed conditions [Chhangani *et al*., 2013; Houck *et al.*, 2012]. Earlier, it has also been observed that Myricetin can inhibit cytotoxicity and DNA damage generated by peroxynitrite; and thus provides neuroprotection in multiple neurodegenerative disorders [Chen *et al*., 2011; Peng and Kuo, 2003].

Neurodegenerative disorders encompass a broad range of pathologies showing a common clinical feature of the presence of aggregates or inclusion bodies in neuronal cells that suggests the lack of PQC functions [Gestwicki and Garza, 2012; Nedelsky *et al*., 2008]. Although the functional role of molecular chaperone Hsp70 and E3 ubiquitin ligase E6-AP have been studied against misfolded proteins accumulation, but their precise enhanced cellular physiological roles based on the use of natural molecules like Myricetin are not well understood [Khan *et al*., 2018; Mishra *et al*., 2009]. To answer this question, various neurodegeneration associated cellular models were used in present study. It has been observed that administration of Myricetin significantly reduces the aggregations of mutant SOD1 (GFP-SOD1-G37R), mutant α-Synuclein (S87A) protein and expanded (EGFP-HDQ74; EGFP-Ataxin-3(84Q)) polyglutamine proteins. Overall, these results suggest the beneficiary potential of Myricetin to improve the survival of cells against the proteotoxic conditions mediated by the accumulation of misfolded proteins. Myricetin also inhibits the release of glutamate, a neurotransmitter that influences the various chronic brain diseases like epilepsy, stroke and few others [Chang *et al*., 2015b; Meldrum, 2000]. Further, studies on the role of Myricetin and other flavonoids in diseases like Alzheimer's, Parkinson's, Huntington's and multiple sclerosis revealed their higher therapeutic significance in protection from neurodegeneration [Dajas *et al*., 2003; Solanki *et al*., 2015].

In Parkinson's model Myricetin reduces the dopamine neuron degeneration, induced by 6-hydroxydopamine and 1-methyl-4-phenylpyridinium in substantia nigra-straitum [Ma *et al*., 2007; Zhang *et al*., 2011]. Recent investigation of Myricetin activity in HD and Spinocerebellar ataxia reported its interaction with RNA, specifically CAG motif, thus reduces the huntingtin protein translation and sequestration; also reduces the cytotoxicity in Huntington's and other polyQ disease models [Khan *et al*., 2018]. Moreover, the role of Myricetin was also observed in demyelinated mice model of multiple sclerosis as a potential molecule for alleviating motor defects [Zhang *et al*., 2016]. To find a possible cure against protein misfolding and aggregation problem is a challengeable task. The current study suggests that search of new modulators based on PQC mechanisms could provide new insights to design possible therapeutic strategies.

## 4.3 CONCLUDING REMARKS

Here, it has been demonstrated that use of Myricetin induces the endogenous levels of intracellular Hsp70 chaperone. This result strongly prompted to observe the direct effects of Myricetin on HSF-1 levels as in preliminarily experiments; it has been observed that the induction of Hsp70 by Myricetin is most likely mediated via activation of HSF-1. Upregulation of HSF-1 and Hsp70 could not significantly induce the clearance of misfolded proteins from the dense cellular pool, therefore an extended search was done for QC-E3 Ubiquitin Ligases, to identify or understand involvement of any E3 ubiquitin ligase as cytoprotective effect of Myricetin. It has been noticed in current observations that E6-AP E3 ubiquitin ligase was also induced after Myricetin treatment and this might explain the important role of Myricetin in the positive modulation of PQC system against misfolded proteins aggregation. But, how does Myricetin functionally perform the anti-aggregatory functions by the activation of HSF-1, Hsp70 and E6-AP levels are not clearly understood.

Present findings suggest that Myricetin retains the similar inductive effect under different concentrations and time intervals. These findings suggest that exposure of Myricetin protect cells from proteotoxic insults that could be due to the stabilization of QC E3 ubiquitin ligase E6-AP, which have been confirmed by cycloheximide chase experiment. Docking analysis also suggest the possible interaction of Myricetin with catalytic HECT domain of E6-AP. Taken together, current work suggests that Myricetin treatment can improve the molecular physiological functions of Hsp70 molecular chaperone and QC-E3 Ubiquitin ligase E6-AP linked to the PQC mechanism; which might provide a new therapeutic approach for neurodegenerative diseases.

...