Synopsis of the Ph.D. thesis

on

Role of Apoptosis Inducing Factor in cell survival and mitochondrial dynamics in *Dictyostelium discoideum*

To be submitted to

The Maharaja Sayajirao University of Baroda

The Department of Biochemistry,
The Maharaja Sayajirao University of Baroda.

For the degree of

Doctor of Philosophy in Biochemistry

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Introduction

The mitochondrion, a membrane bound cell organelle, is often described as "the cellular powerhouse". It plays a crucial role in energy production, metabolism, apoptosis, cellular proliferation and other essential cellular processes (Green, 1998). It acts as a reservoir of suicidal proteins involved in mitochondrial mediated apoptosis. Upon stimulation of apoptosis, outer mitochondrial membrane gets disrupted followed by loss of mitochondrial membrane potential ($\Delta\psi m$) that leads to the release of two vital pro-apoptotic proteins, from inter-membrane space into cytosol viz., cytochrome c and Apoptosis Inducing Factor (AIF). The apoptogenic proteins, cytochrome c recruits downstream caspase cascade to execute cell death, whereas AIF takes part in caspase independent signaling pathway (Joza *et al*., 2005).

AIF is a phylogenetically conserved 67 kDa flavoprotein which is confined to the inter-membrane space of mitochondria. It has a mitochondrial localization sequence (MLS) at the N-terminus, two nuclear localization sequences (NLS), and NADH- as well as FAD-binding domains (Sevrioukova, 2011). AIF homologs are conserved throughout the eukaryotic kingdom namely insects, nematodes, fungi, plants and mammalian systems (Cande *et al*., 2002). It displays functional but not structural similarity with bacterial ferredoxin reductases (Mate *et al*., 2002).

I) AIF in cell death

AIF has a vital role in the induction of apoptosis. This apoptogenic activity is independent of its oxidoreductase function (Loeffler *et al*., 2001). Upon apoptotic insult, it translocates from mitochondria to the nucleus where it interacts with DNA directly to disrupt chromatin structure by recruiting nucleases or proteases and causes chromatinolysis. AIF has Poly(ADP-ribose) (PAR) binding site and its PARylation triggers AIF release from mitochondria to nucleus (Wang *et al*., 2011). The mitochondrio-nuclear translocation of AIF and large scale DNA fragmentation are the hallmark features of caspase independent cell death in mammalian cells (Cande *et al*., 2002). Several reports indicate that reduced cellular AIF levels make the cells highly susceptible to oxidative stress mediated cell death characterized by

Mitochondrial Membrane Potential (MMP) loss, translocation of AIF to the nucleus, Phosphatidyl Serine (PS) exposure etc. (Schulthess *et al*., 2009).

Beyond its role in cell death, AIF is proposed to have a cell protective role, maintaining mitochondrial homeostasis. It is also involved in cellular energy metabolism, possibly by stabilizing the Electron Transport Chain (ETC) complexes. Although AIF is well known to be involved in cell death, its cell survival function still remains unclear.

II) AIF in cell survival

1. Growth and development

AIF deficiency was found to be associated with growth arrest in fruit flies, which experienced defective complex I function and reduced ATP levels (Joza *et al*., 2005). T cell development is also regulated by AIF in Harlequin (Hq) mice (Banerjee *et al*., 2012). Reduced AIF expression manifested compromised OXPHOS (oxidative phosphorylation) in retina and brain, correlating with reduced expression of complex I subunits at the transcriptional and translational level, signifying AIF's role in optimum functioning of OXPHOS (Vahsen *et al*., 2004). AIF mutant mice have been shown to turn their energy metabolism towards glucose utilization through glycolysis in order to comply with cellular energy demands but not via oxidative phosphorylation due to impaired mitochondrial function (Joza *et al*., 2005). Local redox activity of AIF is required to sustain complex I activity, however, it is unknown whether AIF is necessary for an optimal assembly of mitochondrial ETC complexes and therefore in its biogenesis, or it participates in the maintenance of all ETC complexes. Thus, AIF may be crucial for maintaining the OXPHOS function in mitochondria, even though the underlying mechanism of its stabilizing function remains enigmatic.

2. Regulation of ROS levels

Another observed phenomenon of AIF deficiency is altered ROS production as a consequence of complex I impairment. The oxido-reductase domain of AIF may be essential not only for stabilization of ETC complexes but also for an efficient antioxidant defense (Joza *et al*., 2005). Cerebellar granule cells from Hq mice showed more susceptibility to oxidative stress and increased

glutathione and catalase activity, suggesting that loss of AIF increases ROS production (Klein *et al*., 2002). On the contrary, reports from cancerous cell lines lacking AIF exhibited either increase or decrease in ROS levels (Apostolova *et al*., 2006). Thus, the exact mechanism of AIF in regulating ROS levels is yet to be unravelled.

3. Mitochondrial structure and mitochondrial DNA (mtDNA) content

Mitochondrial metabolism is tightly linked to the organelle structure and morphology. Apart from the role of AIF in maintaining assembly of mitochondrial ETC complexes, it is also implicated in regulating mitochondrial structure, mainly via the mitochondrial fission-fusion mechanism. Neuronal Mitochondria from forebrain-specific *AIF* null (tel. AIF^{Δ}) mice were found to be dilated and fragmented with aberrant cristae structure (Cheung *et al*., 2006). Additionally, Hq mice cerebella showed reduced levels of Mitofusion 1 (*Mfn1*), suggesting that alterations of mitochondrial fusion led to cerebellar degeneration (Chung *et al.,* 2011). OPA1, one of the mitochondrial fusion proteins was found to interact with AIF to maintain OXPHOS and mitochondrial morphology (Zanna *et al*., 2008). Deletion of an arginine residue (R201) of AIF leads to severe mitochondrial encephalomyopathy with almost depleted mtDNA content and fragmented mitochondrial network in affected patients (Ghezzi *et al*., 2010), suggesting its additional role in mtDNA maintenance. However, how AIF controls mitochondrial structure is yet to be understood.

Dictyostelium discoideum, a unicellular eukaryote, has been used as a model organism to study mitochondrial biogenesis and disease conditions (Annesley *et al*., 2009). As *Dictyostelium* lacks caspases, it provides a better system to explore non apoptotic function of AIF without any interference of caspases. Moreover, cellular functions of AIF can also be studied during the unicellular and multicellular phases of the life cycle of *D. discoideum*. Thus, the present study focuses to decipher the role of AIF in cell survival, by its antisense mediated down-regulation and to study growth, development and mitochondrial functions in constitutive *AIF* down-regulated *D. discoideum*

cells (*AIF* dR cells).

Hypothesis

Based on existing literature on the role of AIF in mitochondria, we hypothesize that AIF regulates mitochondrial structure and functions by maintaining the assembly of all of the complexes of ETC through protein-protein interactions, and hence might contribute to cell survival. Also, its oxidoreductase function may be involved in regulating ROS levels. As there are no known inhibitors of AIF and lethality due to *AIF* knockout, the present study would decipher AIF's role by down-regulating *AIF* using antisense technique in *D. discoideum.*

Significance of the study

The present study aims at addressing the importance of AIF in *D. discoideum* cell survival emphasising its role in growth, development, mitochondrial structure and functions. This study would be helpful to uncover the underlying mechanism behind mitochondrial diseases like mitochondrial encephalopathy wherein *AIF* levels get reduced, and this could be extrapolated to higher eukaryotes.

Proposed objectives

I. To study the effect of constitutive & prestalk specific down-regulation of Apoptosis Inducing Factor (AIF) on growth and development of *D. discoideum*.

II. To study the effect of constitutive *AIF* down-regulation on oxidative stress mediated cell death in *D. discoideum*.

III. To study the assembly and function of Electron Transport Chain (ETC) complexes in constitutive *AIF* down-regulated *D. discoideum* cells.

IV. To study the effect of constitutive *AIF* down-regulation on mitochondrial fission-fusion mechanism in *D. discoideum.*

Objective I: To study the effect of constitutive & prestalk specific down-regulation of Apoptosis Inducing Factor (AIF) on growth and development of *D. discoideum*.

There are four isoforms of AIF in *Dictyostelium* namely AIFA (AIF), AIFB, AIFC and AIFD. Since there are no known inhibitors of AIF and *AIF* knockout is lethal, down-regulation of *AIFA* or *AIF* (henceforth *AIF* will be mentioned instead of *AIFA*) was carried out by using antisense strategy to establish constitutive and prestalk specific *AIF* antisense models. The constitutive pTX-*AIF* clone was previously made in the lab (Kadam *et al*., 2017) which was further used for all the downstream experiments. The prestalk specific pEcmB-*AIF* clone was made as follows: 5′ region of *AIF* gene (520 bp) was PCR amplified and was directionally cloned into pEcmB vector under EcmB (inducible) promoter. The rationale behind prestalk specific *AIF* antisense was to understand the role AIF in developmental cell death of *D. discoideum* as it is expressed only during slug stage of development. Both the clones (pTX-*AIF* and pEcmB-*AIF*) were transformed into *D. discoideum* cells and maintained under G418 selection pressure to generate the respective *AIF* down-regulated (*AIF* dR) cells.

AIF down-regulation was confirmed by monitoring *AIF* transcript levels by Real Time-PCR. *AIF* transcript levels displayed ~70% reduction in constitutive (p=0.0149, p=0.0195) and ~73% reduction in prestalk specific *AIF* dR cells (p=0.0109, p=0.0137) compared to control and the respective vector control cells (pTX-control & pEcmB-control). Also, no significant difference was observed in expression of other *AIF* isoforms namely *AIFB, AIFC and AIFD*, indicating no other isoforms could compensate the function of *AIFA* or *AIF*. Constitutive *AIF* down-regulated (dR) cells exhibited slower growth rate compared to control (p=0.0016) as well as pTX-control cells (p=0.0184). However, prestalk specific *AIF* dR cells displayed no significant change in growth as compared to control (p=0.9524) and pEcmB-control (p=0.6279) cells. The doubling time of constitutive *AIF* dR cells and prestalk specific *AIF* dR cells were 25.71±2.2 hrs and 14.37±0.75 hrs respectively whereas control cells, pTX-control and pEcmB-control cells exhibited doubling time of 14.24±0.77 hrs, 16.42±2.22 hrs and 14.95±0.80 respectively.

This slow growth could be explained by cell-cycle analysis using propidium iodide staining. Cell cycle analysis revealed significant decrease in the number of constitutive *AIF* dR cells in G0-G1 phase with a simultaneous increase in the number of cells in S phase at 48 hrs of growth compared to control cells. Additionally, constitutive *AIF* dR cells showed a stressed phenotype which was confirmed by studying the cellular morphology of constitutive *AIF* dR cells by Transmission Electron Microscope (TEM). Vacuole like structures were observed in constitutive *AIF* dR compared to control cells which indicate intrinsic stress in constitutive *AIF* dR cells (Kadam *et al*., 2017).

As *AIF* down-regulation led to significant effects on the unicellular stage of *D. discoideum,* we further studied its effect on developmental phase of the slime mold. Under starvation condition, constitutive and prestalk specific *AIF* down-regulation led to delayed morphogenesis as compared to control cells. Constitutive *AIF* down-regulation displayed a delay in initial stages of development i.e. tipped aggregate to migrating slug transition. Mature fruiting bodies were formed only at 48 hrs in constitutive *AIF* dR cells which were smaller as compared to fruiting bodies of control cells at 24 hrs. Nevertheless, prestalk specific *AIF* down-regulated cells exhibited delayed development during migrating slug (36-38 hrs) and fruiting body formation (48 hrs). This could lead to the alterations in differentiation pre-spore and pre-stalk markers. Real time PCR analysis showed increase in *EcmB* (pre-spore marker) (p=0.0441) and decrease in *D19* (pre-stalk marker) (p=0.0094) transcript levels in constitutive *AIF* dR cells compared to control cells and hence delay in developmental morphogenesis of *AIF* dR cells. Thus, we have shown that AIF is responsible for growth and development of *D. discoideum* (Kadam *et al*., 2017).

Objective II: To study the effect of constitutive *AIF* down-regulation on oxidative stress mediated cell death in *D. discoideum*

In addition to its cell survival function, AIF is known to be a pro-apoptotic factor. Reduced *AIF* levels make the cells highly susceptible to oxidative stress mediated cell death (Schulthess *et al*., 2009). Hence, to understand the effect of *AIF* down-regulation on cell fate during oxidative stress, various cell death

parameters were studied. Under oxidative stress, *AIF* downregulation led to early loss of mitochondrial polarization compared to control cells (Joza *et al*., 2008). In line with these reports, constitutive *AIF* dR cells showed lower MMP even under normal conditions, indicating compromised mitochondrial membrane potential. This was further validated by increased free cytosolic Ca^{2+} levels in constitutive *AIF* dR cells (Kadam *et al*., 2017). Our studies showed early AIF translocation during oxidative stress induced cell death in constitutive *AIF* dR cells. Both Annexin V and PI staining were positive in response to 0.03 mM and 0.05 mM H_2O_2 as early as 3h, indicative of simultaneous Phosphatidylserine (PS) externalization and Propidium Iodide (PI) staining. Annexin V staining was detected even in normal conditions in constitutive *AIF* dR cells suggesting that these cells are intrinsically stressed (Kadam *et al*., 2017). Mitochondrio-nuclear translocation of AIF and exposure of PS on outer leaflet of cell membrane are the characteristic features of caspase independent cell death (Cande *et al*., 2002).

Cerebellar granule cells of Harlequin mutant mice demonstrated oxidative stress, suggesting that AIF may be playing a role in ROS homeostasis (Klein *et al*., 2002). Hence, to study the role of AIF in ROS homeostasis and also to understand the vacuolated phenotype, ROS levels were monitored. We found significantly higher ROS levels in constitutive *AIF* dR cells as compared to control cells ($p<0.0001$). The presence of ROS was further monitored by Electron Paramagnetic Resonance (EPR) spectroscopy using POBN as a spin trap. Constitutive *AIF* dR cells exhibited triplet of POBN adducts which were absent in control cells, showing presence of hydroxyl radicals ($OH^{\cdot}$) in constitutive *AIF* dR cells. Elevation in ROS levels may result in oxidative damage to DNA and proteins. γ-H2AX foci formation was observed in constitutive *AIF* dR cells as compared to control cells indicating DNA damage. Protein carbonylation (PC) is also one of the oxidative stress markers; hence PC content was estimated in constitutive *AIF* dR cells. The results showed that PC content was significantly higher ($p=0.0004$) in constitutive *AIF* dR cells as compared to control cells. We have also shown supplementation of glutathione (GSH) could restore the growth and development of constitutive *AIF* dR cells as GSH is known to maintain the redox status of the cell. Constitutive *AIF* dR

cells in presence of 10 mM GSH showed rescue in growth and developmental delay as compared to untreated constitutive *AIF* dR cells suggesting that AIF may be acting as a ROS regulator (Kadam *et al*., 2017). Together, these results show that *AIF* dR cells are susceptible to oxidative stress as compared to control *D. discoideum* cells.

Objective III: To study the assembly and function of Electron Transport Chain (ETC) complexes in constitutive *AIF* down-regulated *D. discoideum* cells

AIFM1 (human AIF1) mutations have been reported with mitochondrial disorders, signifying the physiological importance of AIF in mitochondria (Kroemer *et al*., 2010). With this line of thought, we have explored *AIF* dR mediated mitochondrial impairment in *D. discoideum*. We showed that *AIF* downregulation induces ETC dysfunction with reduced respiration rate and impaired Oxidative Phosphorylation (OXPHOS). Constitutive *AIF* dR cells exhibited reduced activities of ETC complexes I (CI) (p=0.0243), III (CIII) (p=0.0211) and IV (CIV) (p=0.0127) and the resultant concomitant elevation in ROS levels, which were confirmed by EPR spectroscopy. CI/CII Oxygen Consumption Rate (OCR) ratio is also one of the parameters to confirm the impaired complex I function. CI/CII OCR ratio revealed that constitutive *AIF* dR cells exhibited ~50% reduction in CI dependent substrate oxidation relative to CII dependent substrate oxidation compared to control cells. We also found delay in development of *AIF* dR cells compared to control cells, signifying the role of AIF in multicellular development of *D. discoideum* (Kadam *et al*., 2017). Hence, OCR was also studied at major developmental stages of *D. discoideum* i.e. aggregate, slug and fruiting bodies under starvation condition. Interestingly, compromised OXPHOS was observed during *D. discoideum* development. We have also shown supplementation of GSH could restore the activities of the ETC complexes in constitutive *AIF* dR cells. Constitutive *AIF* dR cells in presence of 10 mM GSH showed restoration of complexes CI (p=0.0272) and CIII (p=0.0079) activities of constitutive *AIF* dR cells as compared to untreated constitutive *AIF* dR cells suggesting that AIF may be implicated in maintaining the redox status of the cell.

Alterations in ETC complexes assembly was further analyzed by Blue Native polyacrylamide gel electrophoresis (BN-PAGE). BN-PAGE revealed reduced abundance of the ETC complexes CI, CIII and CIV in constitutive *AIF* dR cells compared to control cells. In order to explore how AIF exerts its effect on the respiratory chain complexes, transcript levels of the subunits of the affected complexes were stimated by Real Time PCR. There was significant decrease in transcript levels of CI: *NAD1* (p=0.0062), *NAD6* (p=0.0013) and *NDUFS-3* (p=0.0308); CIII: *CYTB* (p=0.0051); CIV: *COX1/2* (p=0.0131); and CV: *ATP6* (p=0.0069) subunits in constitutive *AIF* dR cells compared to control cells. Compromised OXPHOS & reduced ETC complexes expression resulted in lower total NAD^+ levels (p=0.0405) as well as cellular (p=0.0166) & mitochondrial (p=0.0439) ATP pools in constitutive *AIF* dR cells compared to control cells. Thus, constitutive *AIF* dR cells exhibited glucose dependency for enhanced glycolysis, as constitutive *AIF* dR cells failed to grow in glucose free medium (GFM) compared to control cells (Kadam *et al*., 2017). Our results demonstrate the conserved role of AIF in the maintenance of functional ETC complexes in *D. discoideum.*

As the transcript levels of mitochondrial DNA (mtDNA) encoded subunits of the ETC complexes CI, CIII, CIV and CV were modulated in constitutive *AIF* dR cells, we strengthened this data by estimating mitochondrial DNA content. Mitochondrial DNA content was also found to be significantly lower in constitutive *AIF* dR cells as compared to control cells, implying AIF's role in mtDNA maintenance.

Objective IV: To study the effect of constitutive *AIF* down-regulation on mitochondrial fission-fusion mechanism of *D. discoideum*

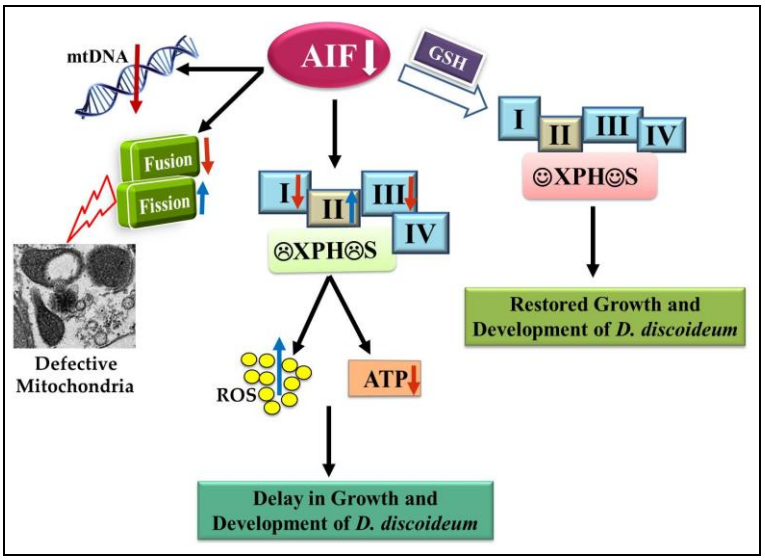
Knockdown of *AIF* led to fragmented mitochondria with altered cristae structure, suggesting AIF's role in maintaining mitochondrial structure possibly through its fission-fusion process (Milasta *et al*., 2016). Hence, we monitored the effect *AIF* down-regulation on mitochondrial fission-fusion mechanism. *CLUA*, a mitochondrial fusion gene, was found to be reduced significantly in constitutive *AIF* dR cells (p=0.020) compared to control cells. However, mitochondrial fission genes *DYMA* (p=0.0453), *DYMB* (p=0.0276),

FSZA (p=0.0067) and *FSZB* (p=0.04), were found to be increased significantly in constitutive *AIF* dR cells compared to control cells. The relative transcript levels of mitochondrial fission-fusion genes were analyzed at different developmental stages also (aggregate, slug and fruiting bodies). *CLUA* transcript levels were observed to be significantly reduced in constitutive *AIF* dR cells compared to control cells during aggregate (p=0.0246) as well as slug stages (p=0.0291). *DYMA* (p=0.0284) and *FSZB* (p=0.0228) transcript levels were significantly increased at slug stage of constitutive *AIF* dR cells compared to control cells. *FSZA* transcript levels were observed to be significantly elevated in constitutive *AIF* dR cells compared to control cells during aggregate (p=0.0407) and slug (p=0.0471) stages whereas no significant difference was found in *DYMB* transcript levels of constitutive *AIF* dR cells compared to control cells. Overall, constitutive *AIF* dR cells exhibited increased mitochondrial fission and decreased mitochondrial fusion genes transcript levels as compared to control cells. Further, perturbations in mitochondrial morphology were visualized by TEM. Mitochondria of constitutive *AIF* dR cells were defective with aberrant cristae and dilations (mitochondrial hole). Interestingly, constitutive *AIF* dR cells exhibited more number of mitochondria compared to control cells (p=0.0191), corroborating increased mitochondrial fission and reduced mitochondrial fusion process. These results highlight the prime role of AIF in maintaining mitochondrial structure and morphology.

Conclusion

Although the role of AIF in *D. discoideum* cell death is well studied, its role in cell survival still remains elusive. Our *AIF* dR studies suggest the importance of AIF in growth and developmental morphogenesis of *D. discoideum*. Our results provide evidence for the protective function of AIF as a ROS regulator and its essential role in regulation of mitochondrial function and cell survival. Moreover, it might be involved in stabilization of assembly of ETC complexes and maintaining the mitochondrial morphology and mtDNA content through interaction with the proteins that are crucial for mitochondrial homeostasis. This study opens new

avenues to unravel AIF's role in mitochondrial integrity and function. One of the intriguing facets of the most common complex I mitochondriopathies is hypomorphic and/deletion AIF mutations, which causes 30% of mitochondrial deficiencies. The AIF deficient models could be instrumental for therapeutic approaches in complex I and AIF deficiencies thus making it possible to identify target metabolic and stress-response pathways. Thus the present study demonstrates that AIF is essential for cell survival, maintaining mitochondrial structure and function in *D. discoideum*.



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Award:

Council of Scientific & Industrial Research - Senior Research Fellowship (CSIR-SRF), 2018.

Publications:

1. **Kadam A**, Jubin T, Mir H, and Begum R. (2017) Potential role of Apoptosis Inducing Factor in evolutionarily significant eukaryote, *Dictyostelium discoideum* survival. *BBA-General Subjects*, 1861(1 Pt A):2942-2955.
2. Jubin T, **Kadam A**, and Begum R. (2019) Poly (ADP-ribose) polymerase (PARP-1) regulates developmental morphogenesis and chemotaxis in *Dictyostelium discoideum. Biol. Cell* 111:1-11.

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Manuscripts under preparation:

1. **Kadam A**, Jubin T, Mehta D, Begum R. Insights into the functional aspects of Apoptosis Inducing Factor in mitochondrial homeostasis maintenance.
2. **Kadam A**, Jubin T, Mehta D, Begum R. Apoptosis Inducing Factor: Pro-apoptotic or Anti-apoptotic?
3. **Kadam A**, Jubin T, Roychaudhary R, Mehta D, Begum R. Cross-talk between PARP-1 and mitochondria.

Posters presented & conferences attended during Ph.D.:

1. **Kadam A**, Jubin T, Mehta D, Begum R. Involvement of Apoptosis Inducing Factor (AIF) in *Dictyostelium discoideum* mitochondrial fission-fusion at International Conference on "Proteins, miRNA and

Exosomes In Health and Diseases" held at The M. S. University of Baroda, Vadodara, Gujarat, India on 11th - 13th December, 2018.

2. **Kadam A**, Jubin T, Begum R. "Potential role of Apoptosis Inducing Factor (AIF) in mitochondrial functions of *D. discoideum"* at National Symposium on "Omics…to Structural Basis of Diseases" held at The M. S. University of Baroda, Vadodara, Gujarat, India on 30th Sept. and 1st Oct. 2016.
3. **Kadam A**, Alex T, Mir H and Begum R. "The dual role of Apoptosis Inducing Factor in *D. discoideum* life and death", at Three day National Symposium on Emerging Trends in Biochemical Sciences (29-31st December, 2014), The M. S. University of Baroda, Vadodara-390002.

Workshop attended during Ph.D.:

1. Three day workshop on 'Advanced Microscopy and Imaging Techniques', held at Dr. Vikram Sarabhai Institute of Cell & Molecular Biology, Faculty of Science, The M. S. University of Baroda, Vadodara (1st -3rd December, 2015).

Date: 24.04.2019

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