APPENDIX A

In Vitro Screening for Anti-Cholinesterase Activity of Methanolic extract of Saffron & Saussurea lappa Used for Cognitive Disorders
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A.1. Introduction:

Alzheimer’s disease (AD) as mentioned earlier in this thesis, is a multifactorial dementia characterized by cerebral accumulation of extracellular amyloid-β (Aβ) protein aggregates (senile plaques) and intraneuronal hyper phosphorylated twisted filaments of tau protein (neurofibrillary tangles) in brain areas associated with learning and memory (e.g., cortex, hippocampus, nucleus basalis of Meynhert), (Ballard, C et al., 2011) resulting, thus, in profound memory disturbances and irreversible impairment of cognitive function.

Abnormalities in cholinergic metabolism seen in AD are not viewed as the cause of the disorder, but cholinergic involvement is significant because it is universal, correlates with cognitive defects, and is one of the few pathophysiologic phenomena that can be addressed with currently approved treatment options, such as cholinesterase inhibitors (ChEIs) [e.g., tacrine (THA), rivastigmine, and galanthamine (GNT)]. Various clinical studies have been documented showcasing the beneficial effects of AChE inhibitors on cognition and behavior, suffer from short lives, show side effects which are caused due to stimulation of peripheral cholinergic system (Massoud, F et al., 2011) (Wallin, A et al., 2011) (Lockhart, I et al., 2009). However, to the best of our knowledge potent inhibitors of AChE belong to class of alkaloids like
galanthamine and physostigmine is due to positively charged quaternary ammonium group in the structure. Apart from its involvement in cholinergic synaptic transmission, AChE, is also known to accelerate the aggregation of Aβ during the early stages of AD, primarily via interactions through its peripheral anionic binding site. In this context, there is dire need of AChE inhibitors which can interact simultaneously to the catalytic as well as anionic binding site. Saffron derived from the stigmas of *Crocus sativus* is well known spice with reputed therapeutic uses. It is utilized as tonic, antidepressant, sedative and against dementia’s (Wallin, A et al., 2011) (Lockhart, I et al., 2009). Recently, two phase II clinical studies provide preliminary evidence of a possible therapeutic effect of saffron extract in the treatment of patients with mild to moderate AD (Akhondzadeh, S et al., 2007, 2010). In the present work, in vitro enzymatic kinetic studies have been undertaken as an attempt to explore the ability of saffron to act as potent inhibitor of AChE and to elucidate the possible mechanism of action. AChE from electric eel was used because (a) its oligomeric forms are structurally similar to those in AChE of vertebrates, nerve, and muscles (Sussman, JL et al., 1991) (Bon, S et al., 1979). In addition to that we used another plant extract of *Saussurea lappa* (SL) (K. Madhuri et al., 2012). SL has been traditionally known and well considered for its medicinal uses, such as anti-cancer, hepatoprotective etc. The SL plant was obtained from Kashmir (India) and was evaluated for its in vitro enzymatic kinetic studies to check its potency against AChE.
A.2. Materials and Methods:

A.2.1. Extraction

After the procurement of fresh Saffron and Saussurea lappa from Kashmir. One gram of dried stigmas of Kashmiri saffron (*Crocus sativus* L.) & *Saussurea lappa*, were frozen in a mortar using liquid nitrogen and ground into a fine powder. The powdered stigmas were extracted using 10 mL of 85% methanol, overnight at 4°C. The extract is then clarified by centrifugation and the supernatant was concentrated under vacuum. The resulting solid residual mass was weighed and dissolved in DMSO at a concentration of 100 mg/ml.

A.2.2. In vitro Acetylcholinesterase Inhibition assay:

Acetylcholinesterase (AChE) activity was measured following the Ellman’s method adapted to a microtiter plate assay. The assay involves the enzymatic hydroxylation of the acetylthiocholine (ATCI) substrate to form thiocholine which then reacts with 5, 5’-dithiobis [2-nitrobenzoic acid] (DTNB, Ellman’s reagent) to give yellow colored 2-nitrobenzoate-5-mercapto thiocholine and 5-thio-2-nitrobenzoate which can be read at 412 nm. AChE isolated from electric eel was used for the assay. The enzyme stock solution was stored at -80°C and diluted in a 0.1 % BSA in Tris-Cl buffer (pH 8) before use. Ellman’s reagent was dissolved in Tris-HCl buffer containing 0.1 M NaCl and 0.02 M MgCl₂ while ATCI was dissolved in deionized water. The saffron/lappa extracts in various concentrations (10, 5, 2.5, 1.25, 0.62, 0.31 mg/mL), Quercetin (230, 115, 57.5, 28.7, 14.3 μM) dissolved in Tris-HCl (pH 8.0) buffer with the final concentration of DMSO not exceeding 1%. To the 96 well plate added 100 μl of 3 mM DTNB, 20 μl of 0.26 U/ml of AChE, and 40 μl of buffer (50 mM Tris-
Cl pH 8.0), 20 µl of various concentrations of test samples were added to the wells. After brief mixing, the plate was incubated for 15 minutes at room temperature followed by reading the plate at 412 nm in Tecan multiple readers which served as a blank reading. To each well, 20 µl of 15 mM ATCI was added to initiate the enzymatic reaction and the hydrolyzed product of acetylthiocholine was followed by taking a reading at 412 nm at every 5 minutes for the next 15 minutes. The percentage inhibition of enzymatic activity was calculated using the following formula

\[
\% \text{ Inhibition} = \left[\frac{(E-S)}{E}\right] \times 100
\]

Where; E is the enzyme activity without test compound/extract and S is the enzyme activity in the presence of test compound/extract. Neostigmine bromide was taken as a positive control and assay was performed using triplicate wells for each drug concentration. IC50 is calculated from the plot of percent inhibition of AChE enzyme activity versus various concentrations of the test sample.
A.3. Results and Discussion:

Acetylcholinesterase (AChE) being the key enzyme in cholinergic nerves and whose inhibition to reverse the deficit of acetylcholine in cholinergic transmission has brought on therapeutic benefits in patients suffering from Alzheimer’s disease (AD). Also, such inhibitors were reported to prevent the formation of β-amyloid plaques and antioxidant properties. Many plant-derived natural products have been used for the treatment of CNS disorders in folk fore and Ayurvedic system of medicines. In the present, the methanolic extract of the stigmas of saffron, lappa and pure quercetin were evaluated for their anti-cholinesterase activity using Ellman’s colorimetric method in a 96-well microtiter plate. The percent inhibition of AChE activity at different concentrations of extracts and quercetin were given in Table A.1 and represented through bar graphs (Fig. A.1-3). Neostigmine bromide was used as a positive control which showed an IC$_{50}$ of 0.05 µg/mL (Fig. A.1:4) for AChE inhibitory activity. Among methanolic extracts, the saffron extract at 10 mg/mL completely inhibited the AChE activity whereas lappa extract at the same concentration showed only 23 % of AChE inhibition. On the other hand, quercetin also showed a dose-dependent inhibition of AChE activity with an IC$_{50}$ of 230 µM.
Table A.1. Statistical data representing the % AChE inhibition of various extracts.

<table>
<thead>
<tr>
<th>Drug (µM)</th>
<th>Saffron Extract</th>
<th>Lappa Extract</th>
<th>Drug (µM)</th>
<th>Quercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0000</td>
<td>110</td>
<td>23</td>
<td>230.0000</td>
<td>47</td>
</tr>
<tr>
<td>5.0000</td>
<td>37</td>
<td>10</td>
<td>115.0000</td>
<td>36</td>
</tr>
<tr>
<td>2.5000</td>
<td>8</td>
<td>3</td>
<td>57.5000</td>
<td>18</td>
</tr>
<tr>
<td>1.2500</td>
<td>-6</td>
<td>1</td>
<td>28.7500</td>
<td>10</td>
</tr>
<tr>
<td>0.6250</td>
<td>-6</td>
<td>-1</td>
<td>14.3750</td>
<td>8</td>
</tr>
</tbody>
</table>

Figure A.1. % AChE inhibition of (APA 1-4): quercetin, Lappa extract, saffron extract & Neostigmine bromide.
A.4. Conclusion:

Taken together, the present *in vitro* evaluation of the methanolic extracts of Saffron (stigma) and lappa showed promising AChE inhibition. Saffron is widely used as an energy drink could help in improving the cholinergic transmission and in long-term would benefit the person by preventing the occurrence of the symptoms of the AD. Also, the quercetin and crocins (saffron) being a well-known anti-oxidant has an additional advantage of preventing the formation of quinones of catecholamines which has a pathogenic role in the development of AD.
APPENDIX B

Crystal Structure of HEWL
B.1. Introduction:

Hen egg white lysozyme (HEWL) is one of the most commonly used model proteins in various type of studies. HEWL is a monomeric globular enzyme that can hydrolyze the bacterial cell wall (Proctor, VA et al., 1998). Structurally, it is a helix-rich protein possessing a high degree of sequence similarity and structural homology to the human variant (Krebs, MR, et al., 2004), which is associated with the familial systemic amyloidosis (Pepys, MB, et al., 1993). HEWL is prone to aggregate in an acid environment at elevated temperature (Arnaudov, LN et al., 2005), in presence of chemical denaturants (Vernaglia, VA et al., 2004), using natural products to prevent fibril formation (Jayaraman J et al., 2014), or as a result of its non-enzymatic glycation (Adrover, et al., 2014). Therefore, HEWL has become an appealing model to study the protein misfolding and amyloid fibril formation mechanisms (Swaminathan, R et al., 2014).

HEWL thus become one of the models which have been used to study protein misfolding and amyloid fibril formation mechanisms. The procedure of preparing amyloids from HEWL is well known and has been described in detail. The simplest and most widely used method is to incubate a solution of HEWL (pH = 2) in elevated temperature.

B.2. Materials and methods:

Neutral HEWL, heat and mechanically (agitated), induced aggregated Lysozyme, and
Heat and mechanically treated (in presence of Gallic acid ) Lysozyme.

B.2.1. HEWL:

Lysozyme, purchased from Sigma (EC. No-235-747-3), was used.

B.3. Result and Discussion:

B.3.1. Crystallization:

The protein of HEWL (15mg/ml) was crystallized with the conditions viz., 0.2M sodium acetate trihydrate, 0.1M Tris hydrochloride & 30% w/v polyethylene glycol 4000.

B.4. Data collection:

The data was collected from the XRD1 beamline (which hosts macromolecular crystallography) available at ELETTRA, Trieste, Italy. The experimental setup consists in a Huber goniometer with ‘k’ geometry fully controllable from the remote.

B.5. Diffraction:

After the crystal was mounted onto the goniometer, the crystal got diffracted at a resolution of 1.80 Å. Structure of the lysozyme is solved by Molecular Replacement using (PDB ID: 1DPX) as template. Ribbon model of the HEWL protein is shown in Figure B.1, while as Table B.1, represents the Crystallographic data-collection statistics of HEWL. Though Heat treated and Gallic acid treated lysozymes yielded crystals unfortunately crystal didn’t diffract beyond 10 Å.

Though crystals could be obtained in other two prepared, no successful data collection is achieved.
**Figure B.1. Crystal Structure of HEWL**

*HEWL: Hen Egg White Lysozyme
(Values in parentheses correspond to the highest resolution shell)

\[ R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I(hkl)_i - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i \langle I(hkl)_i \rangle} \]

\[ R_{\text{model}} = \frac{\sum_{hkl} |F_o(hkl) - F_c(hkl)|}{\sum_{hkl} |F_o(hkl)|} \]

\( R_{\text{free}} \) was calculated using 5.0% of the total reflections.

Calculated with PROCHECK (Laskowski et al. 1993)
Table B.1. Crystallographic data-collection statistics of HEWL

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P 4₃ 2₁ 2</td>
</tr>
<tr>
<td>Cell constants a, b, c, α, β, γ</td>
<td>76.79 Å, 76.79 Å, 37.80 Å.</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>1.80 Å</td>
</tr>
<tr>
<td>% Data completeness (in resolution range)</td>
<td>99.8 (54.30-1.80)</td>
</tr>
<tr>
<td>&lt; I/σ(I) &gt;¹</td>
<td>4.02 (at 1.81Å)</td>
</tr>
<tr>
<td>R, R$_{free}$</td>
<td>0.179, 0.227</td>
</tr>
<tr>
<td>R$_{free}$ test set</td>
<td>558 reflections (5.11%)</td>
</tr>
<tr>
<td>Wilson B-factor (Å$^2$)</td>
<td>17.3</td>
</tr>
<tr>
<td>Anisotropy</td>
<td>0.000</td>
</tr>
<tr>
<td>Bulk solvent k$<em>{sol}$(e/Å$^3$), B$</em>{sol}$(Å$^2$)</td>
<td>0.33, 33.7</td>
</tr>
<tr>
<td>L-test for twinning$^2$</td>
<td>$&lt;</td>
</tr>
<tr>
<td>F$<em>{o}$-F$</em>{c}$ correlation</td>
<td>0.95</td>
</tr>
<tr>
<td>Total number of atoms</td>
<td>1130</td>
</tr>
<tr>
<td>Average B, all atoms (Å$^2$)</td>
<td>19.0</td>
</tr>
</tbody>
</table>

**B.6. Conclusion:**

The protein HEWL was crystallized, with a space group of P 4₃ 2₁ 2, having a resolution of 1.80 Å. Structure is same as the previous reports.
APPENDIX C

Structural deformational analysis of amyloid mutants
C.1 Introduction:

Amyloids are residual aggregates of proteins, in neurodegenerative case they are a by-product of 36-42 amino acid long polypeptide known as Amyloid β (Aβ) and it’s residual clumping that leads to formation of fibular aggregations (Sinha, N et al., 2001) (Hamley et al., 2012) (Acerra, Net al., 2013). The aspect of interpreting the presence of neurodegenerative disease is by cognitive testing, brain imaging and genetic history. Effective understanding of neurodegenerative disease is difficult. Existing imaging technique such as MRI, CT and PET can only say the change in pathophysiological process and atrophy in volumetric change in brain (Petrella, J et al., 2003) (Vemuri, P et al., 2010) (Johnson, KA et al., 2012). The dysregulation in degradation process may be attributed to the formation of amyloid plaques. Protein-protein interactions work synergistically to execute specific cellular functionalities (Csizmok, V et al., 2016) (Keskin, O et al., 2016) (Song, JM et al., 2017).

Reports suggest the significance of interacting proteins partners and structural aspects of αβ and existing antibody. Understanding the inability of proteasomal degradation leading to amyloidogenesis is important. The current unavailability of anti-bodies to denature or hydrolyze αβ is an issue. Archetypal structural aspect of amyloids (αβ) formation leading to fibrillation with losing specificity is also been investigated through molecular modelling techniques. Mutations typically are held responsible for the disorder but how a mutations assortment induces profundity in specific formation is again a discussion. The residual correlated interactions of non-bonded Ca interactions can a bit
underlay the deformational pathway. Where the main identification of the work is to identify the residual perturbation and dynamics correlation with respect to elastic network model (ENM) and distance constraint model (DCM). The Hessian matrix within the ENM has been used to understand the low vibrational frequency mode of Cα atoms to understand the mean square fluctuation (MSF). This Appendix section of thesis, signifies to understand the perturbation in correlated motion of interatomic contacts. The mutation induced co-relational translations having low energy normal mode vibration with non-conformational variabilities leading to deformational space of the αβ is mainly studied.

C.2. Materials and Methods:

C.2.1. Reactome analysis of αβ:

ReactomeFIViz (Wu, G et al., 2008) uses the gene localization data and biochemical data to build protein-protein interaction (PPI) networks. The hit terms identifier of Amyloids related proteins were given to understand the putative chemical pathway relating to progression of amyloidogenicity. The hit terms used along with its functionality are amyloid αβ and APP. A detailed map regarding the protein pathology pathway was analyzed.

C.2.2. Solvent accessibility of the fibrils and antibodies:

To understand the solvent accessibility of the fibrils caver and hollow toolkits (Ho BK, et al., 2008) (Kozlikova, B et al., 2014) were used to analyze solvent accessible surface area to evaluate the possible space of mineralization.

C.2.3. Molecular Dynamics Simulation:

NMR derived amyloid αβ structure (PDB Id: 2LMN) was taken as input model. The models bond order geometry was optimized and hydrogen were fixed using Protein
preparation wizard Schrodinger (Madhavi Sastry et al., 2013). AMBER ff14SB force field was used to parameterize the receptors (Maier, JA et al., 2015). The molecular topology of the complex was generated in Antechamber Amber-tool (Wang, J et al., 2006). Vacuum minimisation was carried out to converge geometry to its lowest energy. Further the standard protocol was used to carry out Molecular simulations. Unrestrained minimisation was carried out on the total system. System initial temperature equilibration was maintained at 300 K using Berendsen thermostat condition (NTT=1). System temperature equilibration was carried for 500 ps steps, First solvent thermalization was processed followed by the Protein complex thermalization whereby keeping solvent fixed and gradually raising the temperature from 0 to 300 K by an increment of 50 K. Isobaric density equilibration was done by keeping a constant pressure of 1bar in Langevin thermostat condition (NTT =3)with system temperature kept at 300 K. Particle Mesh Ewald (PME) electrostatics was employed to approximate homogeneity in long-range Lennard-Jones electrostatic interactions with 10 Å. Bond geometry constraints were optimized using SHAKE algorithm and constraint geometry of rigid water molecule was improved using SETTLE algorithm. The 10 ns MD simulation was carried out in isothermal-isobaric ensemble (NPT) with periodic boundary conditions applied in Langevin thermostat condition with a frequency of collision every 2 ps with system temperature 300 k. The isothermal compressibility of water is set to $4.5 \times 10^{-5}$ bar. Random seed was generated and incremented at every consequent steps of restart to avoid artifacts. Analysis of the MD simulation were carried out using CPPTRAJ and PTRAJ. Image were processed either using chimera.

C.2.4. PCA analysis:

The CA analysis was calculated based on covariance matrix of the Cartesian coordinate deviants form the reference NMR structure, with the diagonalization of covariance
matrix. PCA tends to reduce the dimension of the data. All the calculation were chosen for Cα atoms to find maximum variance in implicit solvent system by stripping the solvent and ions from the trajectories. CPPTRAJ implemented in the amber was used for computing the PCA. The first three PCA with corresponding eigen values were utilized to understand the conformations (Roe DR et al., 2013)

C.2.5. DCCM analysis:

Dynamic cross correlation matrix (DCCM) is a matrix, where correlation is represented by the directionality with residual density represented with the direction of motion parallel of anti-parallel. The negative values suggest the residual motion along negative axis with respective to reference frame with anti-correlated motion or in opposite directions. The Residual interaction pattern also were analysed using Rinanalyser (Doncheva NT et al., 2011) and Bio3D, (Grant, BJ et al., 2006).

C.3. Result and Discussion.

C.3.1. Structural understanding of αβ:

The structural architecture of the αβ is random but upon proximity of other αβ peptides assembles into parallel beta shaped assembly. The fibril formation is totally based on residual symmetry of the αβ. The 1-42 sequence amyloid αβ is divided into 5 beta strands region with central beta as β3 which assemble to form parallel beta fibrils. The NMR structure 2LMN and 2LMP have 2 fold and three fold symmetric assembly of αβ. The αβ polypeptide being apart from the core β sheet region residues from 21-25 situated in loop region which are the area prone to 5 mutations namely Flemish, Italian, Dutch, Arctic and Lowa as shown in Figure C:1 while the Figure C:2 represents the structural architecture of the αβ with the mutational prone region.
Figure C:1 Schematic representation of mutational identities in amyloid αβ. Different color codes have been used to represent different type of amino acids.

Figure C:2 Archetypical representation of amyloid αβ with mutational zone represented in yellow spheres

C.3.2. Molecular dynamics analysis of mutants.

The αβ is a 42 amino acid long polypeptide chain, with 7 mutations in core β3 region. One of the main stabilizing factor for helical motif is hydrophobic patches. Once the αβ is cleaved by the secretase family of proteins the helicity of the protein is lost. The main contribution of hydrophobic stability in the APP is the bound lipophilic membrane and
the helical packing in intermediate trans-helical domains. Once the αβ is cleaved from APP the helical propensity is lost and becomes random coil but with hydrophobic nature it tends to aggregate. In solution upon binding the lowest energy conformation of αβ can be of random coil or beta sheet. Since the random coil is unstable in nature, αβ 1-42 assemble to form fibrils having intermediate parallel beta strands organization. The 7 variants αβ was taken for all atom molecular simulation for 10 ns. During the simulation we could perceive thermal factor of the mutants tends to be much higher than that of wild αβ. Mutants of αβ, i.e., Novel (APP 667), Flemish (APP 692), Dutch, Italian, Artic (APP 692) and Iowa (APP 693) with respect to wild αβ has clearly shown residual deviation along the mutations. The intermodal Ca occupancy during simulation tends to vary in case of mutants. The NMR derived structure generally accounts for the Ca movement calculated through the averaging of NMR space restraints. This also signifies that these sites are prone to calcification which can further induce fibril dynamism. That can be accounted for failure of degradation and evacuation through vacuole formation to plasma membrane through exocytosis. Ubiquitination of individual αβ is difficult because Ubiquitin is longer than that of αβ. The conformations of the αβ from wild to mutants suggests that wild αβ has conserved movement but the mutants have diverged sets of residual conformations. The resulting principal components projections involving orthogonal matrix having eigenvector representing the variance of Ca during simulated trajectory of wild αβ is shown in Figure C:3.

Figure C:3 Ca conformational movement of wild αβ shown as PCA analysis of first three Eigen vectors, where the function of PCA are plotted based on magnitude of first three Eigen vector.
The total residual fluctuations is captured by the corresponding eigenvalues. Most of the principle components representing Cα in wild αβ is found to be in lowest dimension having largest conformations concentrated. The Lowa mutant is also having the conformations concentrated in low energy occupancy. The APP 692 mutant has high dimensional conformations relating to the residues lying in the intermediate loop region of the connecting beta strands β3- β4 regions. Since the conformational freedom of the glutamic acid (APP 692) and aspartic acid (APP 693) is high we could expect calcium coordination to be possible which get occupied in residual voids in the loop region prone to mutations. Serine 26 and asparagine 27 could form co-ordination with calcium to increase the sturdiness of the fibril formations. The investigation has also shed the insights that the deformation energy and fluctuation occupancy factor is much minimal in the case of wild αβ without mutations. Dynamic cross correlation matrix also suggests the conserved interaction of wild αβ during the simulations. In almost all the cases the deformed residues are in the loop region were these segment are prone to mutation or the place where the existing mutation is present. The mutation actually contributes to imbalance in residual interactions. The physicochemical interaction between the amino acid regulates folding and kinetics of particular proteins. The wild tends to have intermodal contacting ensembles consistent during the simulation but the deformation of the αβ is evidential in the case of mutants. The radius of gyration of mutation suggest that Dutch variant where the glutamic acid is mutated to glutamine the residue becomes polar, the amine group tends to disintegrate the hydrogen bond pattern with neighboring residue. The Dutch mutant is having glutamic acid 22 replaced, the 20th phenylalanine residue conformation tends to be perturbed because of the induced amine polar sidechain due to induced dipole conformations. The amino group tends to form tends to disturb the phenylalanine conformation. In the wild case phenylalanine 19th and 20th of both chains
tends to have residual interactions stabilized by van der Waal non-bonded interactions. Phenylalanine 19, 20 which along with histidine 12, 13 stabilizes the central beta core strand β3 along with leucine 17 and valine 18. Conformational stress induced by the glutamic mutation actually hampers the stability which can be attributed to the high radius of gyration and root mean square deviation in comparison with wild αβ in Dutch mutant. Other mutations having increased radius of gyration are novel and Flemish mutants. Novel mutant is where the Histidine 8 is replaced with arginine though the mutation is in the loop region. In wild αβ Pi-Pi interactions stabilize the β3 loop region. Since non-bonded stacking interactions is absent there is sight deformation induced in loop β2 region. In the Flemish mutants, the alanine 21 in the loop region is replaced by glycine, which induces more flexibility in comparison to alanine. The essential dynamics analysis with respect to conformational deviation of Cα was evaluated to identify largest residual magnitude of Cα. The top three eigen values describing the residual motion was taken into consideration to understand the conformational fluctuations. In almost all the cases the second eigen value index around 67 has more number of Cα population concentrated along the origin without any fluctuation perturbations.

C.3.3. Fluctuation and deformation analysis of αβ in comparison with wild αβ:

The N-terminal residue such as Asp1 and Ala 2 are having a residual fluctuation high in almost all the cases including wild αβ and αβ mutations. Four residue which are important and lying in core loops are HIS 6, ALA 21, GLU 22, and ASP 23. These important mutants leads to residual deformation and fluctuations of those residue. The wild αβ tends to very little deformation and almost insignificant fluctuation and deformity but the Dutch variant where glutamic acid 22 is replaced with asparagine is having highest deformity but we could see the C-terminal in β5 regions tends to fluctuate more as shown in the Figure C:4. The vector motion of wild and Dutch mutant is sown in Figure C:5.
The dynamics cross correlation of interatomic contacts shows considerable variations in interatomic contacts during the course of simulations. Overall the mutations induced imbalance in overall stability of the parallel beta assembly. Figure C:6 depicts the Interaction study of wild Cα with residual non-bonded contact approximated within 7 Å radius during simulated time.

Figure C:4 Deformational and fluctuation analysis of wild and Dutch mutation of αβ.
Figure C:5 Free energy analysis of Wild αβ with respect Cα Principle components. Where PCA1 and PCA2 represents first and second principle components of motion respectively. Total energy $E_{TOT}$, $E_{IM}$ total energy of Implicit solvent $E_{VanderWaal}$ represent of Vander Waal energy, $E_{surf}$ represents electrostatic surface energy, $E_{GB}$ represents generalized born solvation energy and $E_{el}$ represent electrostatic energy.

Figure C:6 Interaction study of wild Cα with residual non-bonded contact approximated within 7 Å radius during simulated time. (a) conserved dynamic cross correlating community where internal nodes is represented by red lines. (b) Correlating motion of Cα atoms grouped into communities during the simulated time (c) Cα residues with color representing the communities having correlated motion. (d) Residual interatomic correlation of reference frame (e) Dynamic cross correlation of inter-residual interactions.
3.3.4. Conclusion:

The induced stress thus on tau protein tends to disassociate from microtubule causing neurofibrillary tangles. The mutational effects of inducing deformation is evidentially seen with our results and also the mutational stress induces residual fluctuation in between β3-β4 loop regions. To comprehend the total understanding in short is the mutational stress induces deformation and residual fluctuation. Since the degree of randomness or entropy of the αβ is high calcium induced stability is required for fibril formations, which modulates the failure of αβ proteolysis. Addressing the essentiality of αβ formation is important to address the prospect of druggability of αβ related disorders. Our results clearly signify the mutation introduced deformation with respect to energy.
APPENDIX D

Antioxidant and Anticancer activities of Methanol extract of seeds of *Datura Stramonium*
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Antioxidant and Anticancer activities of Methanol extract of seeds of

*Datura Stramonium*

D.1. Introduction:

*Datura stramonium* is commonly known as Jimson weed or Datura, it belongs to the family of Solanaceae. Datura refers to species of shrubby herbaceous plants which produces large, white or purple trumpet-shaped flowers. It is 60-120 cm in height, branched and pubescent plant. Seeds are used as purgative, in a cough, fever and asthma. Seeds are used for smoking for its narcotic action (Khan J *et al*., 2013) The primary biologically active substances in *Datura stramonium* are the alkaloids - atropine and scopolamine. Atropine has been used in treating Parkinson’s disease, peptic ulcers, diarrhea and bronchial asthma (Ivancheva S *et al*., 2006). Recent Insights have found the role of anticancer potential of Nutraceuticals (Pitchaiah G *et al*., 2017). Patients receiving anthracycline-based neoadjuvant chemotherapy for breast cancer (Sambasivam G *et al*., 2017). Seeds and leaves of *D. stramonium* used to treat psychotic patients, insomnia and depression, relax the smooth muscles of the bronchial tube and asthmatic bronchial spasm. *D. stramonium* is a plant with both poisonous and medicinal properties. Studies to have demonstrated that it has great pharmacological potential with great value and usage in folklore. Seeds of Datura are used in the treatment of analgesic, anthelmintic and anti-inflammatory, intestinal pain, infestation, toothache, and anti-pyretic activity.
D.2. Materials and Methods:

D.2.1. Preparation of extract

After the procurement of seeds from the Jammu and Kashmir, India, lying at a latitude of 33° 72N and a longitude of 74° 53E. The seeds were crushed to the fine powder. The seed powder (100 g) were soaked in methanol and extracted by maceration method for 72 h. Then, the supernatant was filtered by filter paper. Soaking process was repeated once again in the same powder and the supernatant was filtered. All the supernatant was collected together and concentrated using rotary evaporator which yielded greenish-black colored sticky residue.

D.2.2. Phytochemical screening

The methanol extract of seeds of *D. stramonium* was subjected to preliminary phytochemical analysis using standard methods (Harbome JB et al., 1973). The methanol extract was screened for different classes of phytoconstituents such as alkaloids, steroids, terpenoids, phenolic compounds, flavonoids, saponins and glycosides using specific standard reagents (Raaman N et al., 2006). Various radical scavenging assays (Hydroxyl, Ferric, Nitric oxide) and reduction assays such as Phosphomolybdenum reduction assay were carried out to evaluate the antioxidant capacity of the extract.

D.2.3. Cytotoxicity activity

D.2.3.1. Cell line and culture

Human breast cancer MCF7 cell lines were obtained from National center for cell sciences Pune (NCCS). The cells were maintained in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 50 μg/mL CO₂ at 37°C. The Cytotoxicity of an extract of seeds of *D.*
stramonium on MCF7 cells was determined by the MTT assay according to the method
of (Mosmann T et al., 1983)

D.2.3.2. Reagents

RPMI-1640 was purchased from GIBCO/BRL Invitrogen (Caithersburg, MD). Fetal bovine serum (FBS) was purchased from Gibco laboratories. Trypsin, methyl thiazolyl diphenyl- tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from (Sisco research laboratory chemicals in Mumbai). All of other chemicals and reagents were obtained from Sigma Aldrich Mumbai.

D.3. Results and Discussion:

D.3.1. MTT assay

The methanol extract of seeds of D. Stramonium was subjected to preliminary phytochemical analysis using standard methods. The methanol extract was screened for different classes of phytoconstituents such as alkaloids, steroids, terpenoids, phenolic compounds, flavonoids, saponins and glycosides using specific standard reagents. Experiment on the cytotoxicity of methanol extract of seeds of D. stramonium on human breast adenocarcinoma MCF-7 cells showed increasing cytotoxicity with increasing concentrations of the extract and the viable cells detected by MTT assay. Results depicted in Figure D.1, summarize the cytotoxic effects of the extract and the concentration Vs % of cell viability on MCF-7 breast cancer cell lines respectively. The concentration-dependent cytotoxic effect on this cell line has too been vouched by the data presented in Table D.1. The IC50 of methanol extract of seeds of on cytotoxic activity of breast adenocarcinoma (MCF-7) cell line was 113.05 μg/mL concentration. The morphological representation of cytotoxicity of the extract on MCF7 cell line too have been provided in Figure D.1.
% Cell viability = A570 of treated cells / A570 of control cells × 100%

Figure D.1. Cytotoxic effects of the extract on MCF-7 breast cancer cell lines.

Table D.1. Cytotoxic activity of methanol extract of seeds of *D. stramonium* on MCF7 cell line

<table>
<thead>
<tr>
<th>Concentration µg/mL</th>
<th>Cell death %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 1.62</td>
<td>34.61</td>
</tr>
<tr>
<td>2 3.12</td>
<td>41.85</td>
</tr>
<tr>
<td>3 6.25</td>
<td>56.67</td>
</tr>
<tr>
<td>4 12.5</td>
<td>63.38</td>
</tr>
<tr>
<td>5 25.0</td>
<td>66.30</td>
</tr>
<tr>
<td>6 50.0</td>
<td>72.52</td>
</tr>
<tr>
<td>7 100.0</td>
<td>76.30</td>
</tr>
<tr>
<td>8 500.0</td>
<td>86.71</td>
</tr>
</tbody>
</table>
**D.4. Conclusion:**

The results of the present work indicated that the methanolic extract of seeds of *D. stramonium* is a potential source of natural antioxidants and significantly inhibit free radicals by dose-dependently. The difference in the antioxidant activity may be ascribed to their different group of phenolic and flavonoid compounds. The methanolic extract of *D. stramonium* showed higher phenolic content contributes to the higher antioxidant activity. Based on the results obtained, it can be concluded that the plant contains essential phytochemical constituents and possess active antioxidant property, moreover our studies do confirm that extracts exhibit a cytotoxic effect on the breast cancer cell line MCF-7. Further investigations based on the clinical trial and animal model will authenticate, the mechanism of action of constituents in disease prevention.