Nigella Sativa: A Potential Inhibitor for Insulin Fibril Formation

Sandhya A, Gomathi Kanayiram*, Kiruthika L, Aafreen Afroz S
Department of Biotechnology, Dr MGR Educational and Research Institute, Maduravoyal, Chennai, Tamilnadu, India

**Article History:**
Received on: 03.08.2019
Revised on: 19.11.2019
Accepted on: 27.11.2019

**Keywords:**
Neurodegenerative diseases, Insulin fibril, Anti-aggregation, ThT fluorescence assay, SEM

**ABSTRACT**
The high order structure from proteins which are self-assembled are known as fibrils. They are collectively called as amyloid fibrils, which generally lead to neurodegenerative diseases like Alzheimer’s, Parkinson’s, Huntington’s, Type II diabetes. Insulin fibril aggregation is identified to be the major cause of neurodegenerative diseases. The effect of Nigella sativa extract is analyzed based on the fibril inhibition process. The formed fibrils is reduced with the concentration increase of Nigella sativa extract. Insulin fibril is found in Type II diabetes patients after repeated insulin injections subcutaneously. Insulin fibrils are formed in organisms or humans irrespective of their places like hips, shoulder, hands and abdomen. These are evident from the anti-aggregation assay. Thioflavin T (ThT) fluorescence and congo red (CR) assay confirms the inhibition of insulin fibril in the presence of Nigella sativa (NS) extract. Further, inhibition of fibril was confirmed by Scanning Electron Microscope (SEM), where no insulin fibrils was detected whose secondary conformational changes are studied using Fourier Transform Infrared spectroscopy (FT-IR). It is confirmed that insulin fibril inhibition depends on the various concentration of Nigella sativa. Based on the results obtained, it is demonstrated that Nigella sativa extract inhibits the fibril formation and it also provides a therapeutic strategy to prevent insulin fibril formation.

*Corresponding Author
Name: Gomathi Kanayiram
Phone: 9994876738
Email: gomes79@gmail.com

ISSN: 0975-7538
DOI: [https://doi.org/10.26452/ijrps.v11i1.1891](https://doi.org/10.26452/ijrps.v11i1.1891)

INTRODUCTION

The high order structure from proteins which are self-assembled are known as fibrils. These fibrils are insoluble in solvents like water. They are collectively called as amyloid fibrils, which generally lead to neurodegenerative diseases like Alzheimer’s, Parkinson’s, Huntington’s, Type II diabetes. Recently many studies showed the ability to form amyloid structures which is possessed by proteins and peptides, which is no way related to the disease (Souillac et al., 2002; Booth et al., 1997). Many kinds of research revealed the ability of amyloid fibril through the conformational change, which occurs from the native protein either by chemical denaturants, heating, pH change, mutations and various other factors (Ahmad et al., 2003; Uversky et al., 2001).

There many more amyloidogenic proteins/peptides which is been identified in recent researches like amyloid β peptide, a synuclein, insulin and islet amyloid polypeptide. Since several amyloidogenic proteins/peptides have been identified, the fibrils are the stable proteins (Li et al., 2002; Munishkina et al., 2008). They show their stability against the temperature, pressure (hydrolytic), denaturants, etc. Based on the current investigations on the fibrillation process through the investigations of fibril structures are influenced by the native proteins...
The most studied amyloidogenic protein is insulin. It is a globular protein which are formed by the \( \beta \) cells of the islets of langerhans, which contains two polypeptide chains in which A chain has 21 residues and B chain has 30 residues (Blundell et al., 1972).

In recent times many researches have been devoted on Insulin fibril (Störkel et al., 1983; Dische et al., 1988). Insulin fibril is found in Type II diabetes patients after repeated insulin injections subcutaneously (Swift et al., 2002; Sahoo et al., 2003). Insulin fibrils are formed in organisms or humans irrespective of their places like hips, shoulder, hands and abdomen (Dische et al., 1988; Swift et al., 2002). Upon repeated injections of insulin, the therapy effectiveness decreases, which makes the patients to loss the control of glucose levels in the blood (Albert et al., 2007; Yumlu et al., 2009).

The present study focuses on the kinetics of fibrillation as well as biophysical characterizations through the formation of insulin fibrils that binds to congo red and ThT characteristics. There are many natural and synthetic molecules or compounds which has very good antioxidant activity and properties which are related to neurodegenerative disorders (Shikama et al., 2010). *Nigella sativa* is found to exhibit the anti-amyloidogenic effect. Thus the compound is examined in-vitro for the effects at inhibition of fibril formed by insulin. The kinetic study for inhibiting the insulin fibril formation is done by UV-Vis spectroscopy, morphology through a scanned electron microscope (SEM). The fibril formed is finalized and assured with the confirmatory analysis using ThT (Thioflavin T assay) and Congo red assay (CR) using UV-Vis spectroscopy. Secondary structural changes was studied by Fourier Transform Infrared (FTIR) (Wild et al., 2004).

**Materials and Methods**

**Sample collection**

The *Nigella sativa* was purchased from the herbal store and authenticated by Dr. K. Gomathi, Associate professor, Department of Biotechnology. The seeds were washed and shade dried, finely powdered in a mixer grinder and stored using an airtight container for further analysis.

**Extract preparation**

300 ml of ethanol was mixed with the fine powdered *Nigella sativa* (100gms) in a brown bottle, which was kept in a shaker for 48 hrs. The ethanol extract was then filtered, and the process is repeated twice. The filtered extract was evaporated using vacuum pressure and used for subsequent analysis. The extract was directly used for antioxidant activity and other assays.

**Fourier transform infrared spectroscopy analysis**

Amide groups of the extracts were analyzed using FT-IR spectroscopy, of two spectrum, Perkin Elmer, the USA of room temperature ±24°C to 28°C of 340-5000 cm\(^{-1}\) range of the spectrum. Peaks of the amide group was analyzed using the IR spectroscopy correlation table (Souza et al., 2008).
Gas chromatography-mass spectrometry (GCMS) analysis

Chemical constituents of Nigella sativa were identified using GCMS, Agilent 5975C equipped with silica column with EI operating at 70eV. The injector temperature was set to 25°C and the temperature of the oven was set at 40°C for 1 min. The compounds of the extract was identified by mass spectra.

High-resolution liquid chromatography-mass spectrometer (HR-LCMS) analysis

HRLCMS of Nigella sativa were analyzed using Agilent 6200 series Liquid chromatography system at the sophisticated analytical instrumentation facility, IIT Mumbai. Hypersil gold 3micron (100 x 2.1 MM) was used. The solvent system comprised of 95% water (Solvent A): 5% acetonitrile (Solvent B) applying the gradient 0.01 - 20, 20 - 26, 26 - 30 with a flow rate of 0.2 ml/min with column temperature of 25°C. The injection volume was set to 5µL, with 30 mins run time. Sample ionization was achieved through the ESI interface with both positive and negative ionization mode (Kadam et al., 2018).

Antioxidant activity

2.2 Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Extracts containing various concentration was added to 100µl of 0.1 milli Molar DPPH radicals which is prepared in prior by dissolving it with ethanol. The DPPH without test sample serves as control (Gyamfi et al., 1999). The solution was placed in a dark place for half an hour. The absorbance was analyzed with UV Vis spectrophotometry. The radical scavenging activity was calculated for extracts using control.

Hydrogen peroxide scavenging activity

The modified method of Dehpour hydrogen peroxide is used to determine the scavenging activity of extracts. 40mM of hydrogen peroxide solution is prepared with phosphate buffer containing 7.4 pH. Various concentration of the extract was mixed with hydrogen peroxide solution containing in an Eppendorf tube. The concentration of the phosphate buffer and extracts was measured using UV-Vis spec-
Figure 4: Negative ionization of *Nigella sativa* extract
trophotometry at 560nm. Phosphate buffer without hydrogen peroxide serves as a blank (Ngonda, 2013). The percentage of hydrogen peroxide and extracts scavenging activity repeated in triplicates was calculated based on the formula given below:

\[
\% \text{ scavenged (H}_2\text{O}_2) = 1 - \frac{\text{Abs (std)}}{\text{Abs (Ctrl)}} \times 100
\]

**HRBC membrane stabilization assay**

2 ml of blood was drawn in a tube containing EDTA to prevent coagulation. Different concentrations of the extract was added to 100μL of blood. Triton X 100, along with RBC, serves as positive control and RBC alone serves as another control. The sample is incubated at 37°C, 30 mins. The sample is centrifuged at 5000 rpm for 15 mins and the supernatant is removed for analyzing the stabilization. The absorbance is measured using UV - Vis spectrometer at 517nm.

**Cell viability and cell death**

The isolated lymphocyte cells are cultured in humidified 5% (v/v) CO₂ at room temperature in dulbecco's modified eagle (DMEM) medium, which contains 10% FBS and a hundred U/ml penicillin. 5 x 10^⁴ cell/ml of cells is placed in 96 well plate. Insulin fibril formed in prior with and without *Nigella sativa* was diluted with freshly prepared medium and added to the wells containing final concentration 2μmol/L. The same volume of medium is added to the control well. The plates were incubated at 37°C for 48 hrs. Cell viability was determined by using MTT toxicity assay. The MTT (5 mg/ml) was added to each well and incubated at 37°C for 3 hrs. The medium was removed and DMSO was added to each well. The plates were mixed well and the absorbance was measured at 490nm using a microplate reader.

**Fibril formation**

Fibril was prepared in vitro with Human recombinant insulin (10 mg/ml) of various concentrations at pH 2 in 20% acetic acid and 100 mM NaCl, that was agitated at 65 °C for 5 hours without stirring (Jayamani and Shanmugam, 2014).

**Anti-aggregation of insulin fibril using UV Vis spectroscopy**

The fibrillation kinetics of insulin fibril and *Nigella sativa* extract was observed using UV - Vis 1240 spectrophotometry (Shimadzu, Japan), whose absorbance was measured at 600nm. The anti-
aggregation effects of insulin fibril in the presence of *Nigella sativa* of various concentrations were monitored from 0 - 12 hrs (Ismail *et al.*, 2013).

**Thioflavin T assay**

The fibrillation and non-fibrillation inhibition was studied using the ThT assay. 1mM of ThT was made using glycine and NaOH of pH 8.5. The solution is taken and added to the extracts of different concentrations and mixed for 15 seconds. The measurements of ThT assay was carried out using a microtitre plate. Excitation wavelength at 450nm and emission wavelength at 480nm were recorded at time 0, 24, 48, 72 and 96 hrs. ThT without the aliquots of extract serves as a control. Phosphate buffer saline serves as a blank (Jayamani and Shanmugam, 2014).

**Scanning electron microscopy (SEM)**

SEM images of insulin and various concentrations of insulin, along with extracts, were captured by diluting the samples with buffer. 10 ml solution is added to a 1cm glass slide and made it to get dry in room temperature. Morphology of the insulin and insulin along with extracts were determined using the SEM imaging mode, under atmospheric conditions whose scan frequency is 0.5 Hz (Takai *et al.*, 2014).

**Dynamic light scattering (DLS)**

The particle size and its distribution in the presence, as well as the absence of insulin, is analyzed by photon Correlation spectroscopy (PCS) in a Zetasizer III (Malvern Instruments, Malvern, UK). Each samples were analyzed in batches to give a value as average and S.D for the particle with its diameter and by considering the refraction index and viscosity of dispersion (Nie *et al.*, 2016; Gong *et al.*, 2015). In-vitro release studies were performed using a 10mm reduced volume plastic cell at a temperature of about 37±5°C (Banerjee *et al.*, 2013; Sneideris *et al.*, 2015).

**RESULTS AND DISCUSSION**

**Fourier transform infrared spectroscopy analysis**

The most used technique to confirm the beta-sheet structure at the time of fibril formation is studied using the FT-IR technique. The insulin fibril’s structure and its behaviour is monitored by noticing the changes, especially in shape as well as in the frequency of the amide I and II bands. Amide I band is found to be more sensitive in the manner of formation changes that takes place when compared with the amide II band. Figure 1. shows the description of C=O FT-IR spectroscopy of insulin in the absence and presence of *Nigella sativa* extract of various concentrations. The Fourier Transform Infrared Spectroscopy analysis of insulin in the heated form and non-heated form is used as controls for the techniques. After heating, insulin without *Nigella sativa* is used to study the FT-IR spectrum, which also displays a change in the C=O band range from 1654 cm⁻¹ to 1634 cm⁻¹, which is a major feature of the beta structure, Figure 1. a. Based on the result, it is confirmed that the insulin amyloid fibrils is formed in the absence of extract. Figure 1. b, showed a change in the C=O band is inhibited with increasing concentrations of extract at 50 ug. It is found that the range at low wavenumber, attributed to ethanol extract, is more visible at higher concentrations of ethanol extract.

![Figure 5: DPPH scavenging activity of *Nigella sativa* extract. Values are given as mean ± SD of triplicate experiments.](image)

![Figure 6: Hydrogen peroxide scavenging activity of *Nigella sativa*. Values are given as Mean ±SD of triplicate experiments.](image)

**Gas chromatography-mass spectrometry (GCMS) analysis**

The results of GCMS analysis for *Nigella sativa* helps to identify the number of compounds in it. These compounds are identified through mass spectrometry attached with Gas Chromatography. The various compounds present in the extract of *Nigella sativa* were found by the GCMS are shown in Table 1. The composition determined for the NS extract corre-
GCMS spectrum confirmed the presence of various components with different retention times, as illustrated in Figure 2. The mass spectrometer analyses the compounds eluted at different times to identify the nature and structure of the compounds. The large compounds are fragmented into smaller compounds giving rise to the appearance of peaks at different m/z ratios.

High-resolution liquid chromatography-mass spectrometer (HR-LCMS) analysis
HR-LCMS analysis of *Nigella sativa* extract showed 6 to 9 peaks showing various phytochemical constituents present in it. The HR-LCMS and mass spectra constituents are compared with the main library and all the compounds are identified. Identified compounds is Neuraminic acid, Didanosine, oxyphencyclimine, Repaglinidine, Etanidazole, Citrinin, Practolol, Recinnamine, Octadecanedioc acid, flavonoids, steroids, alkalkaloids reveals the presence in plant extracts. Figure 3 and Figure 4.

Antioxidant assay
The *Nigella sativa* extracts antioxidant activity was studied using 2,2 Diphenyl-1-picrylhydrazyl and hydrogen peroxide scavenging activity.

2,2 Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity
DPPH is known as a free radical which is more stable and it estimates free radicals. The effect on the antioxidants using DPPH is due to the ability of donating hydrogen molecules. As it is a radical which is free and takes up an H molecule to form a dimagentic compound which is more stable. The DPPH radicals reductions capability is determined by decreasing the optical density at 517 nm. The DPPH activity of the *Nigella sativa* is illustrated in Figure 5. The ethanol extract of *Nigella sativa* showed good activity as compared to positive control gallic acid (IC 50 value =2.46 µg/ml). Based on these results, the *Nigella sativa* extract showed the noticeable effect of antioxidant activity.

Hydrogen peroxide scavenging activity
Hydrogen peroxide scavenging activity in extracts is analyzed widely by measuring decrement hydrogen peroxide at 230 nm. The hydrogen peroxide assay of *Nigella sativa* is found to be dose-dependent, which is compared with the standard drug ascorbic acid scavenged hydrogen peroxide radical with IC 50 value 95.14 µg/ml. Figure 6.

HRBC membrane stabilization assay
Prevention of the hemolysis using *Nigella sativa* of the extract is determined using the HRBC membrane stabilization assay. Triton X 100, which is a detergent, destabilizes the RBC membrane and leaks hemoglobin. Different concentrations of the extract were analyzed, and the results suggests that *Nigella sativa* extract prevents the leaking of hemoglobin by stabilizing the RBC membrane and also protects the integrity of RBC. It is further proved that it is nontoxic in nature, and it can be used for further biological studies Figure 7.

Cell viability and cell death
The lymphocytes cells were isolated and the cells were treated with *Nigella sativa* extract, which
Figure 11: (A) Insulin fibril (B) Insulin fibril with 50 μg of Nigella sativa extract

Figure 12: (A) Insulin fibril (B) 50μg of Nigella sativa extract with insulin fibril.

showed changes resulting in cell death and viability. The assay demonstrated the statistical decrease in cell death resulting in an increase with the concentration of the NS. 100 μg NS extract showed 50% of cell death when compared to control; the extract showed a little effect on cell proliferation. Figure 8.

Anti-aggregation of insulin fibril using UV Vis spectroscopy

Insulin fibril formation was probed using the anti-aggregation assay, where the turbidity measurements is measured using UV-Vis Spectroscopy. The insulin fibril formation with or without Nigella sativa extract was studied by monitoring the optical density at 440nm as a function of time. The insulin fibrillation follows the nucleation - elongation mechanism. In the presence of insulin fibril (1:5), the insulin fibril formation is reduced by a decrease in elongation. Whereas 1:10 Nigella sativa extract shows an additional increase in inhibition when compared to 1:5. The Nigella sativa extract at higher concentrations showed 90% of inhibition of insulin fibrillation. Figure 9. Further, the results suggests that insulin fibril inhibited by Nigella sativa extract at different concentrations has no significant changes at a lag time, although fibril formation is decreased with an increase in time.

Thioflavin T assay

The insulin fibril formation and its inhibition is confirmed by ThT assay, as it is more sensitive to formed fibrils, and thus it is used to study and find the fibril formation. In Thioflavin T (ThT) assay, the fluorescence intensity changes is measured through insulin fibrils binding with ThT. ThT fluorescence increases suddenly when it binds to the aggregates of the beta-sheet. The fluorescence intensity will not change even when the ThT interacts with the monomers. Figure 10 exhibits the emission of ThT in the presence of various concentrations of the Nigella Sativa extract along with Insulin fibril as well as in the absence of Nigella sativa extract. Figure 10, showed that ThT has been revamped for insulin fibril in the absence of Nigella sativa extract, whereas the amount of fluorescence in ThT is found to be decreased as the concentration of Nigella sativa extract is increased at different molar ratios. 1:5 Molar ratio, decrease in fluorescence intensity when
Table 1: GCMS results of *Nigella sativa* extract

| Compounds                                                                 | Rt     | Area % |
|---------------------------------------------------------------------------|--------|--------|
| Tetracosapentaene, 2,6,10,15,19,23 - hexamethyl                           | 26.28  | 24.3   |
| α-patchoulene                                                             | 10.22  | 17.6   |
| 1.4-                                                                      | 11.32  | 4.9    |
| t-butyl catechol                                                           | 14.67  | 19.1   |
| 1-oxaspiro (2.5) octane 2,4,4 - trimethyl-8-methylene                     | 15.32  | 15.3   |
| Tetradecanoic acid, ethyl ester                                           | 15.6   | 81.8   |
| Flavone                                                                   | 16.4   | 7.4    |
| Methyl 2,6,10 - trimethyldecanoicanoate                                    | 16.52  | 34     |
| Hexadecanoic acid, ethyl ester                                           | 17.78  | 100    |
| Butanoic acid, 2-acetylamino-4-4-acetyloxy phenyl-4-oxo-methyl ester      | 20.03  | 100    |
| Ethyl E-11-octadecenoate                                                  | 20.37  | 100    |
| 10,13-Eicosadienoic acid, methyl ester                                    | 21.4   | 47.9   |
| 4-hexyl-1-7-methoxy carbonyl heptyl bicyclo (4.4.0) deca-2,5,7-triene     | 22.05  | 70     |
| Tetracosanoic acid                                                        | 23.17  | 32.5   |
| Propylene glycol monoleate                                                | 24.85  | 30.9   |
| (+)- longifolene                                                           | 11     | 22.7   |
| Thymoquinone                                                              | 9.28   | 27.8   |
| Ethyl 9- hexadecanoate                                                    | 17.25  | 34.2   |
| Acetin                                                                    | 8.38   | 14.5   |
| Coumarine-3-carboxyhydrazide N 2-1-methyleneylideno                       | 18.38  | 91.8   |

Compared to insulin in the absence of NS. Whereas 1:50 showed reduced changes in fluorescence intensity, which is observed by comparing it with control (ThT). This further confirms that NS has the greater ability to inhibit insulin fibril, which is formed in the absence of NS extract.

**Scanning electron microscopy (SEM)**

SEM is used to study and analyze the morphologic structure of fibrils formed by insulin and also provides *Nigella sativa* extracts effect on the formation of fibrils. SEM is often used to study the structural morphology of proteins and peptides or any other compounds as similar to TEM. Figure 11a and Figure 11b shows SEM images of insulin without *Nigella sativa* and with *Nigella sativa*, which is analyzed after incubating it for 12 hours at 65°C. Insulin without *Nigella sativa*, showed fibrils, whereas insulin with *Nigella sativa* at 1: 50 ratio, showed no fibrils. Thus it confirms that *Nigella sativa* inhibits insulin fibril, which is formed.

**Dynamic light scattering (DLS)**

The size and shape of the particles in the liquid phase is widely studied using DLS. The change in hydrodynamic radii of insulin in the presence and absence of extract is monitored by DLS. Few observations are recorded in previous reports. Figure 12a and Figure 12b represent the size of insulin in the presence and absence of extract where the results are graphed as the scattered intensity in the Y-axis and particle size in the X-axis. Insulin with NS showed the hydrodynamic radius value of 200-300 nm, while in the presence of NS extract, three inhabitant particles with R<sub>h</sub> 0.9nm, 200-1100 nm and 5000nm was observed. This results indicates, both peptides have an ability to interfere with the aggregation process and results in the formation of smaller size aggregates whereas the 50ug of the extract showed the aggregates formed are of smaller size comparatively. These results proves that NS extract inhibits aggregation of fibril and forms aggregates of smaller size.

**CONCLUSIONS**

The potentiality of *Nigella sativa* on fibril formation is studied and analyzed through various biophysical characterizations. SEM and anti-aggregation assay results exhibits that *Nigella sativa* inhibits the fibril
formation in vitro through the increase in the concentration of *Nigella sativa* extract. DLS and FTIR shows that there is a secondary structural changes which occurs at the time of the insulin fibril formation. The present results also demonstrates that *Nigella sativa* inhibits the fibril formation effectively at the in-vitro process. This work further shows that the *Nigella sativa* has a major role in inhibiting insulin fibril, and it also provides a therapeutic strategy to prevent formed insulin fibril.

ACKNOWLEDGEMENT

I thank Dr. K Gomathi, Associate professor, Department of Biotechnology, Dr MGR Educational and Research Institute, Chennai, for supporting my work and for guiding me. The author is thankful to Head of the department, Department of Biotechnology and Dr. MGR Educatiaonal and Research Institute, Chennai, for providing the workplace.

REFERENCES

Ahmad, A. 2010. DnaK/DnaJ/GrpE of the Hsp70 system have differing effects on α-synuclein fibrillation involved in Parkinson’s disease. International Journal of Biological Macromolecules, 46(2):275–279.

Ahmad, A., Millett, I. S., Doniach, S., Uversky, V. N., Fink, A. L. 2003. Partially Folded Intermediates in Insulin Fibrillation †. Biochemistry. 42:11404–11416.

Ahmad, A., Muzaffar, M., Ingram, V. M. 2009. Ca2+, within the physiological concentrations, selectively accelerates Aβ42 fibril formation and not Aβ40 in vitro. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1794(10):1537–1548.

Albert, S. G., Obadian, J., Parseghian, S. A., Hurley, Y., Mooradian, M., ., A. D. 2007. Severe insulin resistance associated with subcutaneous amyloid deposition. Diabetes Research and Clinical Practice, 75(3):374–376.

Banerjee, V., Kar, R. K., Datta, A., Parthasarathi, K., Chatterjee, S., Das, K. P., Bhunia, A. 2013. Use of a Small Peptide Fragment as an Inhibitor of Insulin Fibrillation Process: A Study by High- and Low-Resolution Spectroscopy. PLoS ONE, 8(8).

Blundell, T., Dodson, G., Hodgkin, D., Mercola, D. 1972. Insulin: The Structure in the Crystal and its Reflection in Chemistry and Biology. Advances in Protein Chemistry, 26:60143–60149.

Booth, D. R., Sunde, M., Bellotti, V., Robinson, C. V., Hutchinson, W. L., Fraser, P. E., Pepys, M. B. 1997. Instability, unfolding and aggregation of human lysozyme variants underlying amyloid fibrillogenesis. Nature, 385(6619):787–793.

Dische, F. E., Wernstedt, C., Westermark, G. T., Westermark, P., Pepys, M. B., Rennie, J. A., Watkins, P. J. 1988. Insulin as an amyloid-fibril protein at sites of repeated insulin injections in a diabetic patient. Diabetologia, 31(3):158–161.

Gong, H., He, Z., Peng, A., Zhang, X., Cheng, B., Sun, Y., Huang, K. 2015. Effects of several quinones on insulin aggregation. Scientific Reports, 4(1).

Gyamfi, M. A., Yonamine, M., Aniya, Y. 1999. Free-radical scavenging action of medicinal herbs from Ghana. General Pharmacology: The Vascular System. 32:238–247.

Ismail, N., Ismail, M., Shahid, I., Latifah, A. L. 2013. Anti-aggregation effects of thymoquinone against Alzheimers -amyloid in vitro. Journal of Medicinal Plants Research, 7(31):2280–2288.

Jayamani, J., Shanmugam, G. 2014. Gallic acid, one of the components in many plant tissues, is a potential inhibitor for insulin amyloid fibril formation. European Journal of Medicinal Chemistry, 85:352–358.

Kadam, D., Palamthodi, S., Lele, S. S. 2018. LC-ESI-Q-TOF-MS/MS profiling and antioxidant activity of phenolics from L. Sativum seedcake. Journal of Food Science and Technology, 55(3):1154–1163.

Li, J., Uversky, V. N., Fink, A. L. 2002. Conformational Behavior of Human α-Synuclein is Modulated by Familial Parkinson's Disease Point Mutations A30P and A53T. NeuroToxicology, 23(4-5):66–75.

Munishkina, L. A., Ahmad, A., Fink, A. L., Uversky, V. N. 2008. Guiding Protein Aggregation with Macromolecular Crowding †. Biochemistry, 47(34):8993–9006.

Ngonda, F. 2013. In-vitro Anti-oxidant Activity and Free Radical Scavenging Potential of roots of Malawian Trichodesma zeylanicum (burm. f.). Asian Journal of Biomedical and Pharmaceutical Sciences, 3(20):21–25.

Nie, R., Zhu, W., Peng, J., Ge, Z., Li, C. 2016. A-type dimeric epigallocatechin-3-gallate (EGCG) is a more potent inhibitor against the formation of insulin amyloid fibril than EGCG monomer. Biochimie, 125:204–212.

Sahoo, S., Reeves, W., Demay, R. M. 2003. Amyloid tumor: A clinical and cytomorphologic study. Diagnostic Cytopathology, 28(6):325–328.

Shikama, Y., Kitazawa, J., Yagihashi, N., Uehara, O., Murata, Y., Yajima, N., Yagihashi, S. 2010. Local-
ized Amyloidosis at the Site of Repeated Insulin Injection in a Diabetic Patient. Internal Medicine, 49(5):397–401.

Sneideris, T., Darguzis, D., Botyriute, A., Grigaliunas, M., Winter, R., Smirnovas, V. 2015. pH-Driven Polymorphism of Insulin Amyloid-Like Fibrils. 10.

Souillac, P. O., Uversky, V. N., Millett, I. S., Khurana, R., Doniach, S., Fink, A. L. 2002. Elucidation of the Molecular Mechanism during the Early Events in Immunoglobulin Light Chain Amyloid Fibrillation. Journal of Biological Chemistry, 277(15):12666–12679.

Souza, D., Devi, L., Shridhar, P. D., Naik, M. P., C. G. 2008. Use of Fourier Transform Infrared (FTIR) Spectroscopy to Study Cadmium-Induced Changes in Padina Tetrastromatica (Hauck). Analytical Chemistry Insights, 3:135–143.

Störlke, S., Schneider, H. M., Müntefering, H., Kashiwagi, S. 1983. Iatrogenic, insulin-dependent, local amyloidosis. Laboratory Investigation; a Journal of Technical Methods and Pathology, 46(1):108–111.

Swift, B., Hawkins, P. N., Richards, C., Gregory, R. 2002. Examination of insulin injection sites: an unexpected finding of localized amyloidosis. Diabetic Medicine, 19(10):881–882.

Takai, E., Ohashi, G., Ueki, R., Yamada, Y., Fujita, J.-I., Shiraki, K. 2014. Scanning electron microscope imaging of amyloid fibrils. American Journal of Biochemistry and Biotechnology, 10(1):31–39.

Uversky, Vn, Li, J., Fink, A. L., et al. 2001. Evidence for a partially folded intermediate in alpha-synuclein fibril formation. Journal of Biological Chemistry, 276(14):10737–10744.

Wild, S. H., Roglic, G., Green, A., Sicree, R., King, H. 2004. Global Prevalence of Diabetes: Estimates for the Year 2000 and Projections for 2030: Response to Rathman and Giani. Diabetes Care, 27(10):2569–2570.

Yumlu, S., Barany, R., Eriksson, M., Rocken, C. 2009. Localized insulin-derived amyloidosis in patients with diabetes mellitus: a case report. Human Pathology, 40:1655–1660.