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Nigella Sativa : A Potential Inhibitor for Insulin Fibril Formation

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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) fluroscence 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.

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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 neurodegenartive 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

(Ahmad *et al.*, 2009; Ahmad, 2010). The most studied amyloidogenic protein is insulin. It is a globular protein which are formed by the β 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.

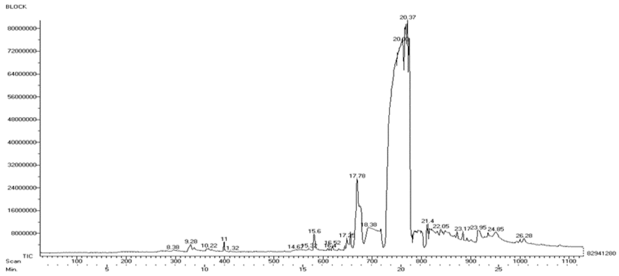

Figure 2: Ethyl E-11-octaadecenoate showed the highest peak

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 comparised 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

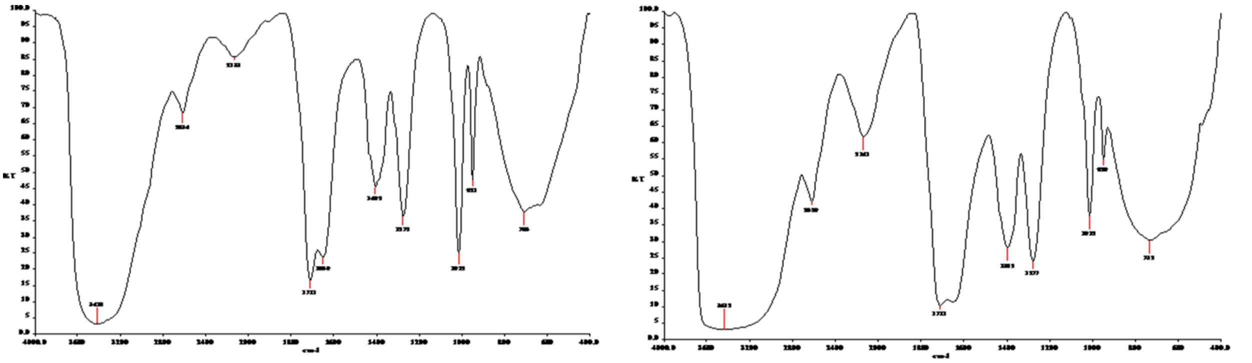

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Figure 1: (A) Insulin fibril (B) 50μg of an extract with insulin fibril

Sample **Name** **Inj Vol**	**SAMPLE-3** **5**	**Position** **InjPosition**	**P1-C6**	**Instrument Name** **SampleType**	**Instrument 1** **Sample**	**User Name** **IRM Calibration Status**	**Success**
Data Filename	SAMPLE-3.d	ACQ Method	30mins _+ESI _1003 2014	Comment		Acquired Time	2/25/2019 9:21:23 PM

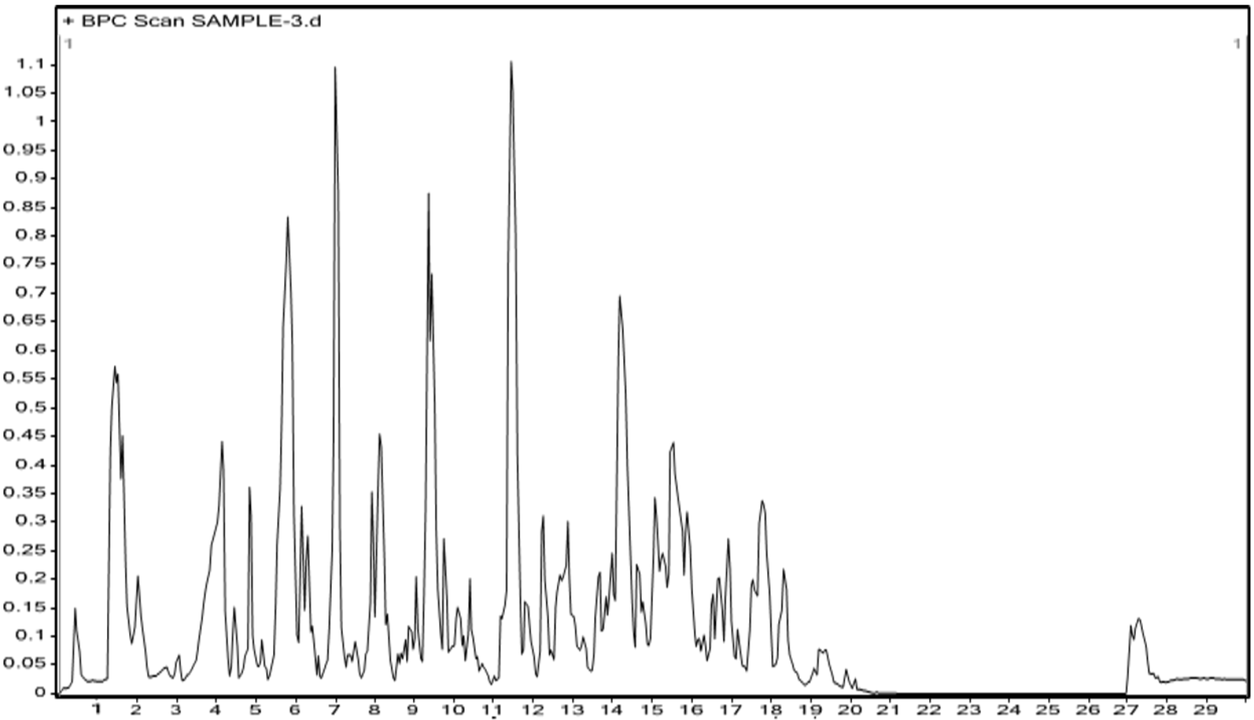

Figure 3: Positiveionization of *Nigella sativa* extract

Sample Name Inj Vol	**SAMPLE-1 5**	**Position InjPosition**	**P1-C4**	**Instrument Name SampleType**	**Instrument 1 Sample**	**User Name IRM Calibration Status**	**Success**
Data Filename	SAMPLE-1_-VE.d	ACQ Method	30mins_-ESI_10032014	Comment		Acquired Time	2/26/2019 8:37:33 PM

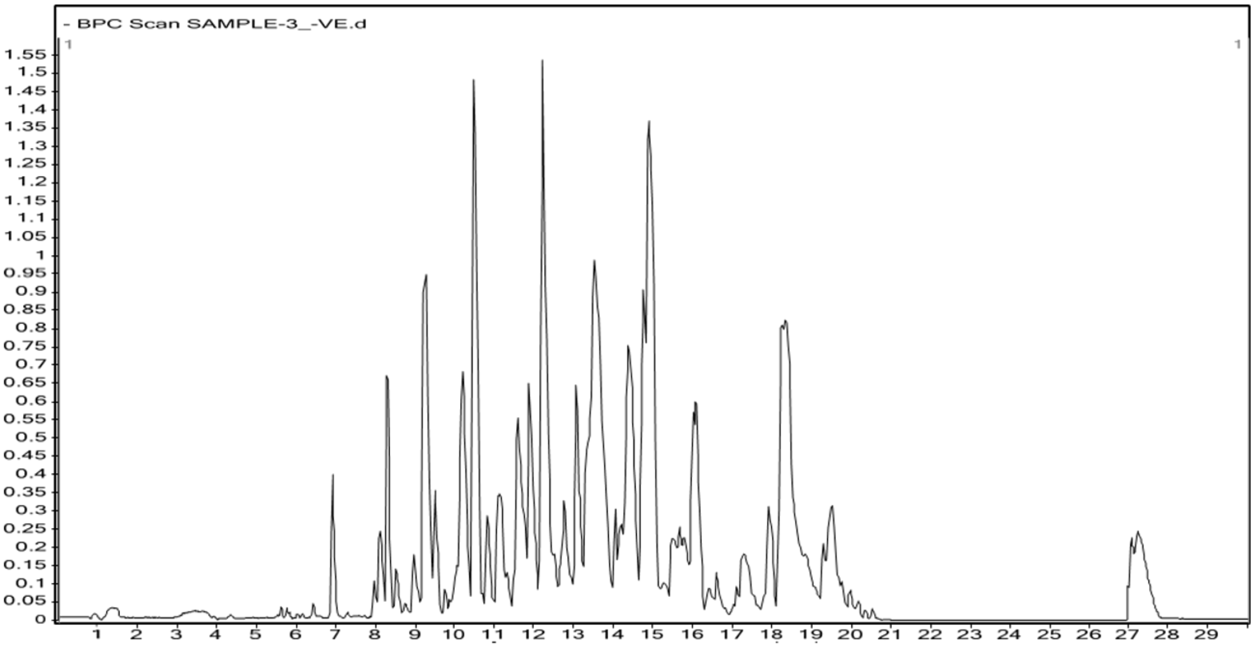

Figure 4: Negative ionization of *Nigella sativa* extract

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 spectrophotometry 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:

% scavenged (H_2O_2) = 1- Abs (std) / Abs (Cntrl) x 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) CO_2 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 invitro 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. Exitation 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 Zeta sizer 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 thet 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.

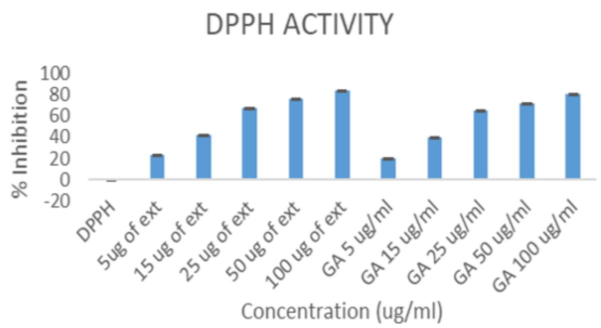

Figure 5: DPPH scavenging activity of *Nigella sativa* extract. Values are given as mean ± SD of triplicate experiments

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 corresponds to 86% of the entire GCMS chromatography.

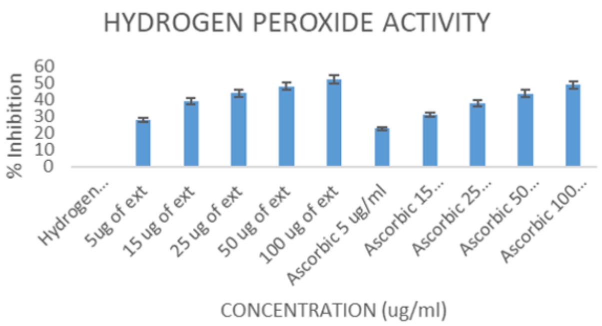

Figure 6: Hydrogen peroxide scavenging activity of *Nigella sativa.* Values are given as Mean ±SD of triplicate experiments

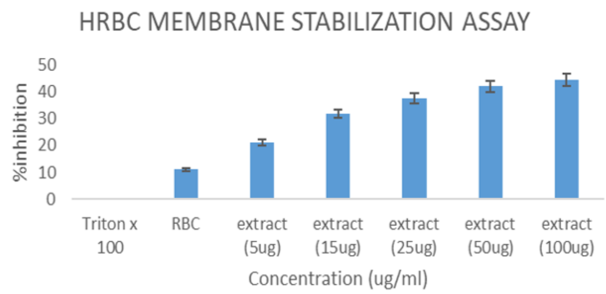

Figure 7: HRBC membrane stabilization assay

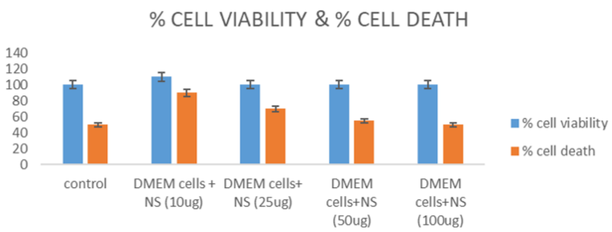

Figure 8: Cell viability and cell death using *Nigella sativa* extract

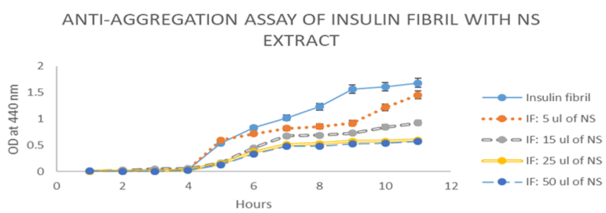

Figure 9: Anti-aggregation assay of insulin fibril with *Nigella sativa* extract

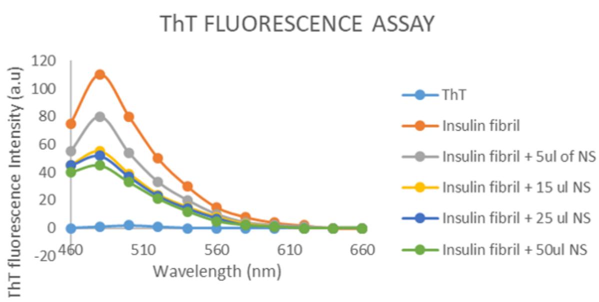

Figure 10: Thioflavin T assay of insulin fibril with *Nigella sativa* extract

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 HRLCMS 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, Octadecanedioic acid, flavonoids, steroids, alklaloids reveales 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* extract showed good activity as compared to positive control gallic acid (IC 50 value =2.46μg/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 95.14μg/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 deter

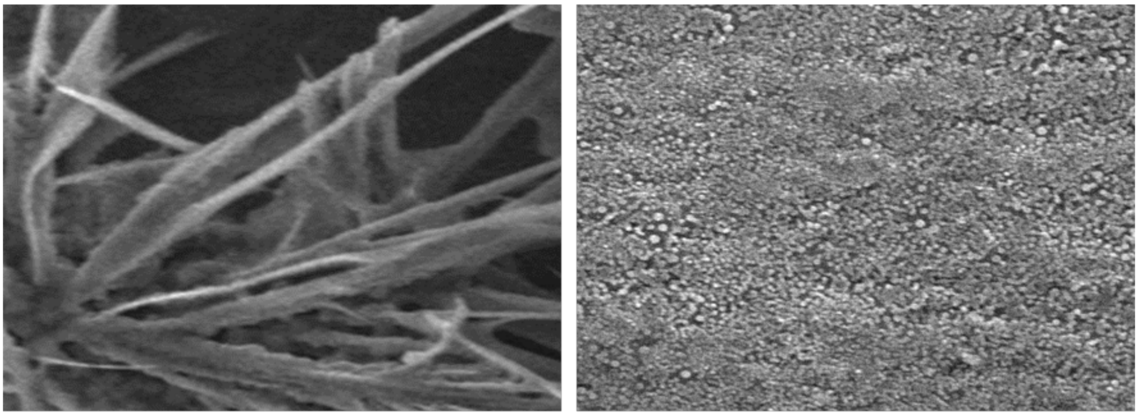

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Figure 11: (A) Insulin fibril (B) Insulin fibril with 50 ug of *Nigella sativa* extract

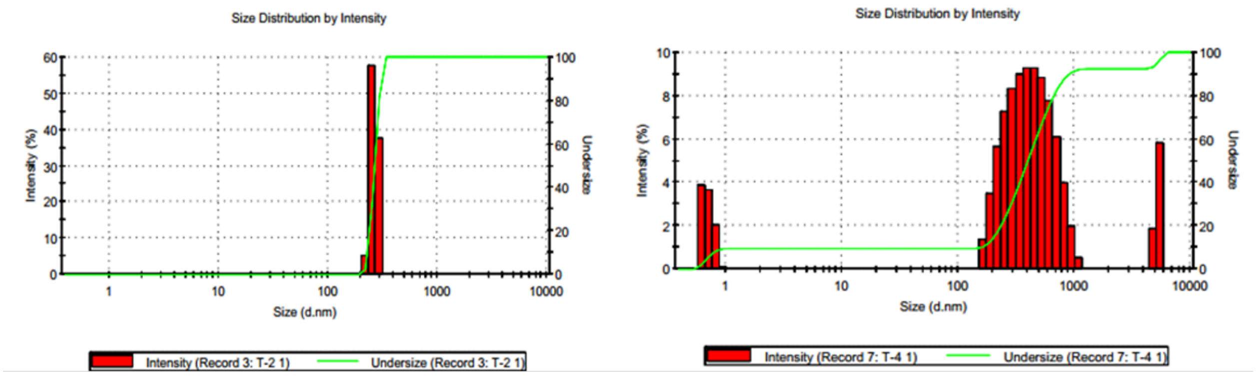

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Figure 12: (A) Insulin fibril (B) 50μg of *Nigella sativa* extract with insulin fibril

gent, destabilizes the RBC membrane and leakes 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 non-toxic 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 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 fibrillation 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

Table 1: GCMS results of *Nigella sativa* extract

Compounds	Rt	Area %
Tetra cosapentaene, 2,6,10,15,19,23 - hexamethyl	26.28	24.3
α-patchoulene	10.22	17.6
4-Isopropyl thiophenol	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, ethy ester	15.6	81.8
Flavone	16.4	7.4
Methyl 2,6,10 - trimethyldecanoiccanoate	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-carbohydrazide N 2-1-methylethenylideno	18.38	91.8

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 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 11 a and Figure 11 b 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 hydrodymaic radii of insulin in the presence and absence of extract is monitored by DLS. Few observations are recorded in previous reports. Figure 12 a and Figure 12 b 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_h 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.

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