Phytochemical Composition of *Nigella sativa* Extract as Potential Source for Inhibiting β-Amyloid Aggregation: Significance to Alzheimer's Disease

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Abstract

Objective: To monitor plant extract fractions and illuminate the components present in *Nigella sativa* leaves for anti-amyloidogenic activities for the treatment of Alzheimer's diseases.

Methods: This work has been carried out to study the action of *Nigella sativa* extracts toward inhibition of oxidative stress and amyloidosis. The anti-amyloidogenic assay by thioflavin-T fluorescence and transmission electron microscopy.

Results: The quantification of polyphenols was carried out for methanolic extract (CCMeOH) and elucidated by MS LAMPS database. For anti-amyloidogenic evaluations, among all the extracts, the CCMeOH was found to have the potential toward inhibiting the oligomers, fibrillation of Aβ42 with good defibrillation of amyloid cascading properties.

Conclusions: These results are also supported by the existence of polyphenols as the active ingredients. Multi-potent target drug therapy is a promising option in treating the Alzheimer's diseases. Methanolic extract of *Nigella sativa* shows potential activity against cholinergic enzymes, Aβ42 aggregation with antioxidant activity.

Key words: Alzheimer's disease, Acetylcholinesterase, Butyryl cholinesterase, β-Amyloid, *Nigella sativa*

1. INTRODUCTION

Alzheimer's disease (AD) is considered as the neurodegenerative disease that contributes to dementia-related brain disorders. The most probable hypothesis on AD is the amyloid hypothesis, inclusive of pathogenic hallmarks such as over expression of the protein, pathogenic mutation, aging, oxidative stress, impaired autophagy, proteasome impairment cumulative chairing in triggering of oligomerization of protein thus stimulating the neuroinflammatorycomplex and final staging for neurodegeneration. In the pathway leading to amyloidogenesis, the amyloid precursor protein is an integral membrane protein encoded by autosomal dominant familial AD gene. The amyloid precursor protein is cleaved by β-secretase and subsequent snipping by β-secretase releasing amyloid peptides into extracellular space. The cleaved proteinfragment amyloid beta is of 4 kDa, extracellular assembly of these monomers with intermediates composed into oligomers and protofibrils, which has a stellar role in the formation of insoluble senile plaques which disrupts the neuronal network. Subverting of neuronal cells ensues in the diminishing of the cholinergic network with higher activity of acetyl cholinesterase (AChE).[1] Neurotransmitter acetylcholine is hydrolyzed by the catalytic action of AChE, which belongs to serine hydrolases enzyme classification. The sister enzyme butyryl cholinesterase (BChE) is also well studied in drug inhibition that is said to have a similar role as that of AChE, which is present in glial cells. These two enzymes have been studied extensively as cholinergic neurons in the central nervous system of the brain and are studied for AD drug inhibition. Cholinergic hypothesis is the first ever hypothesis considered for therapeutic approaches for AD[2.] According to Alzheimer report, people living with dementia estimated to be 46.8 million worldwide and predicted to be doubled every 20 years, which will be increased to 74.7 million in 2030 and 131.5 million in 2050. Several generic drugs such as donepezil (Aricept), rivastigmine (Exelon), galantamine (Razadyne), memantine (Namenda) found to be symptomatic cure towards AD, where the first three are cholinergic drugs and latter acts as NMDA receptor antagonist. [3] In the past decade, researchers have come up with several drugs to treat AD that only slightly delay the inevitable symptomatic progression of the disease and they do not affect the main neuropathological hallmarks.

Therefore, there is an enormous medical need for new and more effective therapies and drugs, which are essentially free from deleterious side effects for the treatment of AD. Thus, this could add weight to the argument that the natural products are inherently better tolerated in the body than that of the synthetic chemicals and they have higher chances to be approved as new drugs for AD. *Nigella sativa* which belongs to Ranunculaceae family with finely divided linear leaves. It is an annual flowering plantgrowing up to 20-30 cm tall, which is widely distributed across India growing mainly in marshy and plain terrains, and the habitat of tropical and subtropical regions of Southeast Asia. It is one of the major herbal plants found in India locally called as black caraway. The Sanskrit name is Kalonji [4,5] and it is also called as karayal. Numerous traditional uses of *Nigella sativa* have been reported for antimicrobial, diuretic, antihypertensive,

anthelmintic, antidiabetic, anticancer and immunomodulatory, analgesic, analgesics and anti-inflammatory, spasmolytic, bronchodilator, renal protective gastroprotective, hepatoprotective and antioxidant properties.[6] Plant parts such as seed, flower and leaves are used to treat various disorders. Seeds are used to treat asthma, bronchitis, rheumatism and related inflammatory diseases. Fresh leaves juice is used to treat gas, colic, diarrhoea, dysentery, constipation, and haemorrhoids. [7,8]

Each and every part of the *Nigella sativa*plant is claimed to possess some therapeutic properties. However, the black seed is the most widely used part all over the world in various systems of medicine. Pharmacological investigations showed the medicinal properties of *Nigella sativa* such as diuretic, antihypertensive, antidiabetic, anticancer and immunomodulatory, analgesic, antimicrobial, anthelmintic, analgesics and anti-inflammatory, spasmolytic, bronchodilator, gastroprotective, hepatoprotective, renal protective and antioxidant properties. [9] Recent studies indicated that the plants belonging to the genus Nigella are rich in both fixed and essential oils, proteins, alkaloids, and saponin.[10] These scientific studies essentially shed insights on the effective usage of *Nigella sativa* leaves in exploring their potential properties towards treating AD.

Several contemporary pharmaceuticals that are being used in conventional medicine have natural plant origins, while they have been derived directly or indirectly from plants. The ethnopharmacological approach provided several paths to identify potential new drugs from plant sources. This information may also be useful as a starting point for the discovery of new drug molecules for the treatment of AD and cognitive disorders. Considering the importance of plant sources and continuation of our work towards the treatment of AD [11,12] herein, we wish to report the application of *Nigella sativa* leaves extract as an anti-AD agent. The present study focused on the screening of plant extracts to evaluate their AChE and BChE inhibitory activities, antioxidant assay and amyloidogenic inhibition supported by thioflavin-T (ThT) assay and morphological properties using transmission electron microscopy (TEM) technique.

2. MATERIALS AND METHODS

2.1. Samples and reagents

Amyloid beta 42 was acquired from EZ biolabs, butyrylcholine iodide from TCI, carbon coated copper grids from Grid tech. Besides, methanol, aluminum chloride, potassium acetate, sodium carbonate, ascorbic acid Tris (hydroxymethyl) aminomethane (Tris buffer), and dimethylsulfoxide (DMSO) were purchased from Merck, USA; Folin and Ciocalteu's reagent from Thomas Baker Limited; Galanthamine from Medchem; Express LLC and horse serum BChE from MyBioSource. 2 , 2 - Diphenyl- 1 - picrylhydrazyl (DPPH), gallic acid, protocateccheuic acid, catechin, chlorogenic acid, epicatechin, caffeic acid, vanillin, p-coumaric acid, sinapic acid, rutin hydrate, myrcitin, cinamic acid, quercetin, kaempferol, Electric eel acetylcholinesterase (Type-VI-S,

EC 3.1.1.7AChE) 500 U, 5,5- dithiobis (2-nitrobenzoic acid) (DTNB), ThT-T, 1,1,1,3,3,3- hexafluro-2-propanol (HFIP), uranyl acetate, acetylcholine iodide were purchased. All chemicals and reagents used in the experiment were of analytical grade.

2.2. Collection of plant material

The fresh leaves of *Nigella sativa* were collected in June to July (2019) from the talakona region of Andhra Pradesh, India. The plant material was identified by Botanist Dr. B. Ravi Prasad Rao, Professor, Sri Krishnadevaraya University, Ananthapuramu, India. Voucher specimen was deposited at Balaji College of Pharmacy, Rudrampeta, Ananthapuramu and the authentication number is 6246.

2.3. Preparation of plant extracts

Fresh leaves were collected and washed thoroughly to remove dust and left for drying under shade for over a week at room temperature in moist free condition. The dried leaves were subjected to mechanical shear to obtain a coarse powder. About 40 g of coarse powder was subjected to extraction by different solvent systems such as n-hexane, petroleum ether, and methanol with a volume of 200 mL by soxhlet extractor for a period of 72 h and solvent was removed by rotary evaporation. Aqueous extraction was carried out by steam boiling until water condensed to one-third of the initial volume and, allowed to cool and lyophilized to obtain a dried extract. The total extracts were stored at 4 ^{0} c for further use.

2.4. Determination of plant extract yield

Yield of plant subjected to subsequent solvent extraction was expressed as percentage yield that was calculated by

Yield (%) = (W2/W1)×100

Where, W2 is the dried extract weight and W1 weight of the original sample.

Determination of total polyphenols content and HPLC profiling, liquid chromatography mass spectrometry (LC-MS) and gas chromatography mass spectrometry (GC-MS)

2.5. Total phenolic quantification

Total phenol content of the plant extracts was determined using Folin-Ciocalteu reagent. Different concentration aliquots of the samples were taken and volume was made up to 750 µL using distilled water and 0.125 mL of Folin-Ciocalteu reagent was added. Then, the mixture was shaken well and allowed to stand for 6 min before adding 1.2 mL of 7% Na2CO3 and the final volume was adjusted to 3 mL and mixed thoroughly and incubated in dark for 90 min. The optical absorbance of the obtained extract was read at 760 nm. Gallic acid was considered as a standard to obtain the calibration curve. Average of three tests was considered and total phenolic content of all plant extracts was expressed as mg of gallic acid equivalents (GAE) per gram of the dried extract (mg GAE/g dry extract)[13].

2.6. Total flavonoid quantification

Total flavonoid content was measured by preparing different aliquots of the plant extracts. In this procedure, 0.1 mL 10% aluminum chloride and 0.1 mL potassium acetate (1 M) were added and final volume was made up to 3 mL by adding the distilled water. Then, the samples were incubated for 30 min at room temperature.

Absorbance was read at 415 nm and quercetin was considered as a standard to obtain the calibration curve. Triplicate of each tests was used and results were expressed in milligrams of quercetin equivalents (QE) per gram of dry extract (mg QE/g of dry extract) to quantify the total flavonoid content using the standard curve of quercetin [14].

2.7. HPLC fingerprinting of *Nigella sativa* methanolic extract (CCMeOH)

Phenolic acid profiling was carried on RPC18 column (150 mm× 4.5 mm) and detected with diode array detector (JASCO HPLC system; Easton, PA, USA) for the MeOH extract of Nigella sativa. The mobile phase used was 0.1% formic acid (solvent A) and methanol (solvent B). The solvent gradient elution program was used as per the reported method with minor modification [15]. The CCMeOH extract and all standards were dissolved in methanol to get concentrationof 1 mg/mL solution and filtered through 0.22 μm Merck syringe filters. A total of 20 μL of the sample and standards, such as gallic acid (1), protocatecheuic acid (2), catechin (3), chlorogenic acid (4), epicatechin (5), caffeic acid (6), vanillin (7), p-coumaric acid (8), sinapic acid (9), rutin hydrate (10), myricitin (11), cinamic acid (12), quercetin (13), and kaempferol (14) were injected into the column and the phenolic acids were detected at 270 nm. Triplicate injections were carried for each of standards and the sample. Retention time and UV-visible spectra of the area under peaks that were compared with those of the standards and samples were expressed as mean ± SEM.

2.7. TLC profiling of CCMeOH

Preliminary phytochemicals were further confirmed using TLC. Aluminum sheet with silica gel 60 F254 plate was used as stationary phase and solvent system n-hexane:ethyl acetate (4:2) used as a mobile phase for the development of chromatogram. Further, the CCMeOH extract was subjected to silica 60-120 mesh column chromatography with n-hexane:ethyl acetate (4:2) solvent system. All seventeen fractions were collected and out of which only sixteenth and seventeenth fractions were solvent dried and subjected to LC-MS along with whole CCMeOH extract [16].

2.8. Identification and quantification analysis of CCMeOH constituents by LC-MS

HPLC was performed using Waters Acquity liquid chromatography system with column Acquity UPLC BEH C18 1.7 μm×1.0 mm×50 mm and higher pressure limit upto 600 bar. Coupled with Waters Acquity Autosampler Electro Spray Ionization system. Quadru Pole Time of Flight (Waters, USA) fitted with the liquid chromatography system coupled on-line with an Electro Spray Ionization source and MassLynx software was used for data acquisition. Analytical conditions were: positive ion mass spectra and mass were recorded in the range m/z 100-800. Capillary temperature was kept at 250 and voltage 2.8 kV and Sheath gas flow rate was kept at 350 L/h. The resulting total ion chromatogram is a plot of time vs area showing the total response of each constituent on the basis of its molecular ion abundance. The mobile phase was composed of solvent A: 0.1% of formic acid in water, and solvent B: acetonitrile. The flow rate was 0.4 mL/min. The elution was performed starting at 10% of solvent which increased up to 95% solvent B after 4.5 min. The injection volume was 10 μL for the measurement in positive mode with total run time of 8 min. Column temperature was 50 ^{0}Cand sample temperature 20^{0}C. The library search was performed by using the database MS-LAMPS [17].

2.9. Identification and quantification analysis of CCMeOH constituents by GC-MS

GC-MS analysis of CCMeOH extract was performed on a Shimadzu (Tokyo, Japan) Make GCMS-TQ8030 with nonpolar Rxi 5Sil MS capillary column, full scan mode, injector mode-splitless, quadra polemass selective detector, injection temperature 250, GC-MS interface temperature was 250 , and the injection volume was 1 μL. Helium was employed as carrier gas, at a pressure of 57.5 kPa; flow rate was 1 mL/ min. Mass spectra were detected at 70 eV. Temperature programming was set as follows: column temperature was started from 60 (held for 2 min) and linearly increased by 5 ^{0}C /min to 200 (held for 2 min); after that it was increased by 3^{0}C/min to 220 (held for 1 min); further it was increased by 6 ^{0}C/min to 250 (held for 20 min). Total GC running time was found to be 64.67 min and the results were compared by using Wiley Spectral library search programme [18].

2.10. Antioxidant assay

The antioxidant activity was measured by the DPPH method [19]. The free DPPH in purple colour changes into yellow colour upon reaction with hydrogen donors; this is because of the reaction between hydrogen donors and DPPH free radical that leads to its reduction to corresponding hydrazine. Sample stock solution (10 mg/mL) was diluted with various concentrations with methanol and 1 mL of DPPH (0.04 mg/mL), and 3 mL of methanol were added into the samples, which were incubated for 30 min at room temperature. The absorbance was measured at 517 nm against the corresponding blank (methanol). All plant samples were dissolved in methanol. The IC50 values were calculated by linear regression of plot of standard curve where the abscissa represents the concentration of tested plant extracts and the ordinate the average percentage of antioxidant activity from three different tests. Ascorbic acid was used for standard curve and antioxidant activity was calculated by following equation.

Inhibition % = (Ac–As/Ac) ×100

Where, Ac is the absorbance of the control reaction (containing all reagents except the test sample), and As is the absorbance of the extracts/reference. Experiments were run in triplicate and the results were conveyed as average values with standard error of the mean (SEM).

2.11. Cholinesterase activity

Inhibition of AChE and BChE was determined by Ellman's method [20,21] with slight modifications [22,23]. To the reaction mixture 0.12 U AChE enzyme and plant extract 100 μL (1 mg/mL) was added along with 640 μL Tris-HCl buffer 50 mM pH 8 into the 1 mL quartz cuvette with path length of 1 mm. This reaction mixture was incubated at room temperature for 5 min. A total of 100

µL DTNB (7.5 mM) and 100 µL acetylcholinesterase (AChE) inhibitor (1 mM) were added and the reading was measured at 405 nm in spectrophotometer. The reaction was monitored for 104 s in the interval of 13 s. The velocities of the reactions were measured. Enzyme activity was calculated as the percentage of the velocities compared to that of the assay using buffer without any inhibitor. Inhibitory activity was calculated from 100 subtracted by the percentage of enzyme activity and every experiment was done in triplicate.

2.12. Anti-amyloidogenic activity

Preparation of amyloid beta 42 (Aβ42) peptide solution Aβ42 lyophilized powder formerly stored at -20 was dissolved in chilled HFIP and vortexed gently for 10 min. Then, the dissolved Aβ42 was allowed to form pellet at 25 and distributed in different vials to get final concentration of 1 µg/µL. The formed pellet of Aβ42 vials was defrosted at -20 for future use. The required working vial was thawed for 30 min by keeping at room temperature. The 50 µL of DMSO was added into the pellet, vortexed to mix thoroughly followed by adding 50 µL of sterile double distilled water to obtain final concentration of 1 µg/µL of 221.5 µM [24].

2.13. Inhibition of oligomerization, fibrillation and defibrillation of Aβ42

Inhibition of Aβ42 oligomerization among all four extracts, each one (i.e. 50 µg/mL) was incubated with 25 µM of Aβ42 after dissolving in 0.4 mM DMSO with ratio of 1:2 in 50 mM Tris-HCl buffer (pH-7.4) incubated at 37 for 120 h[25]. Time course Aβ42 fibril formation inhibition by CCMeOH-kinetic study or time lapse study was carried out with 1:1 ratio concentrations of peptide and extracts. Aliquots were collected at different hours 0, 12, 24, 48, 72 and 120 h[26]. Formation of mature fibril, its inhibition by extract was performed by incubating CCMeOH with peptide in 1:1 (50 µM: 50 µg) ratio for 9 d at 37 and control was devoid of extract. There were four groups in ThT assay including CCAI9d (CC is CCMeOH, AI is along with incubation that refers to CCMeOH incubated with Aβ42 for 9 d), CCBI9d (CC is CCMeOH, BI is before incubation Aβ42 allowed to form fibrils for 120 h, then treated with extract and further incubated for a total of 9 d), ConAβ429d (control for fibrillization and defibrilization), and ConAβ42120h (Aβ42 incubated for 5 days i.e. 120 h). For defibrillation of Aβ42 fibrils by CCMeOH, defibrillation study was done by allowing the 50 µM peptide to form fibrils till 120 h and later 50 µg extract was added, mixed and incubated for 9 d. The control was devoid of extract so for defibrilization and fibrillization control was similar. Even 120 h data was measured to evaluate the augment in florescence intensity and configuration of mature fibrils [27]. For all experiment control Aβ42 was incubated without the extracts, and reaction mixture remained similar. Assays were carried out in triplicate.

2.14. ThTfluroscence assay

The formation of Aβ42 fibril and its inhibition by the extracts were observed by ThT dye. The binding efficacy of benzothiazole dye to the beta sheet structure of amyloid fibril enhances the fluorescence. The 25 µL (1 mM) of ThT in 50 mM glycine-NaOH (pH 9) was added to peptide solution and incubated for 30 min at 25. Later, using glycine buffer, final volume made up to 1 mL in 1 mm quartz cuvette to the incubated sample, fluorescence was measured at Ex450nm and Em482nm by adjusting the slit width to 5 nm and 10 nm, correspondingly, in spectro-flurophotometer [28, 29].

2.15. Transmission electron microscopy (TEM) study

The 10 µL of incubated aliquots were mounted on 400-mesh carbon coated copper grids, after 2-3 min excess was wicked off from edges of grid using ash less filter paper. Sample was then stained by 5 µL of 1% uranyl acetate (w/v) and excess wicked off. Uranyl acetate dissolved in sterile water was mixed carefully and filtered using 0.2 µm syringe filter to evade unsolubilized uranyl acetate. The sample was authorized to be dried and observed under microscope at120 kV Technai G2 BiotwinFei [30].

2.16. Statistic analysis

The results in spectroscopic studies were represented as triplicate mean±SEM of each sample and graph was plotted in Origin 8. One- way ANOVA (analysis of variance) followed by Dunnett's multiple comparison tests was carried out and P value less than 0.05 was regarded as statistically significant. Statistical analysis was carried out using GraphPad Prism 6.01.

3. RESULTS

3.1. Extraction yields, total phenolic and flavonoids content, and HPLC fingerprint profiling

Among all extracts, from non-polar to polar solvent, CCMeOH extract showed 6.423% w/w dry weight of total N. sativa extract. Total content of polyphenols was measured equivalent to mg of gallic acid per gram of dry extract. The phenolic content of CCMeOH was 182.25 mg GAE/g dry extract. Subsequently, the quantification of flavonoid content was expressed with respect to quercetin equivalents. The yield of flavanoid content was 441 mg QE/g of dry extract in CCMeOH which showed the presence of the highest amount of polyphenols in N. sativa followed by CCPE which contained 412 mg QE/g of dry extract (Table 1).

The presence of highest amount of total polyphenolic content in CCMeOH was further subjected to HPLC profiling. A total of 14 standards were compared as shown in Figure 1, in CCMeOH extract there existed sinapic acid (215.28 µg/mL), rutin hydrate (499.31 µg/mL), myricitin (317.49 µg/mL) and quercetin (119.20 µg/mL) as in Table 2 along with other polyphenols, which yielded less as compared to above mentioned contents.

3.2. Antioxidant activity

Maximum scavenging of synthetic DPPH radical activity was exhibited by CCMeOH with 93.01% at 400 µg/mL concentration. The highest activity of CCAq activity was 55.74% at 400 µg/mL. CCHex and CCPE extracts did not show any scavenging activity (Table 3).

3.3. Cholinesterase inhibition activity

Cholinesterase activity of both enzymes AChE and BChE ofCCMeOH found to be 51.19% and 63.86% respectively, and was compared with standard galantamine (96.68% and 83.68%) at 100 µg/mL. The other extracts n-hexane,

petroleum ether and aqueous extracts did not show the AChE and BChE inhibition activities.

3.4. Anti-amyloidogenic studies: ThT fluorescence and TEM analysis

Assessment of anti-amyloidogenic property was carried out by treating N. sativa extracts with Aβ42 to check Aβ42 oligomerization. The CCMeOH extract was treated with Aβ42 to analyse the inhibition activity kinetics of fibrillar structure, fibrillation, and defibrillation activity and control was taken without treating Aβ42 with N. sativaextract.

3.5. N. sativa extracts in inhibiting Aβ42 aggregation

All the four extracts (50 µg) were treated with Aβ42 (25 µM) at 37 for 120 h by ThT assay displayed promising inhibition activity as shown in Figure 2a among which, CCMeOH showed significant (P<0.0001) inhibition activity. Corresponding TEM electrogram of CCMeOH treated with Aβ42 was presented at 500 and 200 nm bar scale while control Aβ42 showed the aggregation of Aβ42 at 500 nm and 100 nm bar scale in Figure 2b. Comparing with control, electrogram showed a completely disorientation of the structural deformation and diminishing of secondary structure of Aβ42. The Aβ42 peptide aggregation kinetics study was conducted byfollowing series of stages - misfold, oligomers (dimmers, trimmers etc) in lag phase and protofibril formation at growth phase, later leading to amyloid fibrils representing sigmoid growth curve of insoluble fibrillar toxic species. As shown in Figure 3a ThT assay, the activity of CCMeOH in inhibiting Aβ42 aggregation process was significantly evident. CCMeOH was screened for time lapse studies, where a 25 µg/mL of the extract was incubated with 25 µM of Aβ42 with time intervals 0, 12, 24, 48, 72 and 120 h. As time increased, there was an increase in the fluorescence intensity in control Aβ42 as that of CCMeOH treated with Aβ42. This specifies a significant decrease in reduction of amyloid aggregation by CCMeOH. These results are also supported by electrogram in TEM analysis which was taken at 24, 48, 72 and 120 h in both controls (Aβ42) and CCMeOH with Aβ42 at 500 nm and 200 nm scale. In the Figure 3b, at 24 h (3b1A and 3b2A) of incubation there was globular structure formation but in CCMeOH treatment (3b3A and 3b4A) there was a drastic reduction in structural arrangement.

At 48 h control (3b1B and 3b2B), a structural network appeared between globular structures but in CCMeOH treatment (3b3B and 3b4B), there was an absence of these structure. Observing control (3b1C and 3b2C) and the CCMeOH treatment (3b3C and 3b4C) at 72 h, complete structural demodulation was found. At 120 h (3b1D and 3b2D), there was fibrilar structure and in CCMeOH treatment (3b3D b and 3b4D), a significant reduction of structural morphology was observed. This specifies the effective CCMeOH role in reduction of amyloid aggregation.

3.6. Inhibition of Aβ42 aggregation to form mature fibrils and defibrillation

Amyloid aggregation studies were carried out for 9 days (9 d) to see activity of CCMeOH on mature fibrils and study was performed at 50 µM:50 µg of Aβ42 and CCMeOH co-incubated at 37. The ThT assay was shown in Figure 4a. ConAβ429d in which Aβ42 was allowed to aggregate has shown increased fluorescence intensity compared to that of ConAβ42120h. We have compared 9d with 120h in order to know the increase in flurosence which specifies the growth of higher structural formation of Aβ42 aggregation. There is significant decrease in quenching of fluorescence and is evident in the decrease of fluorescence intensity. In TEM analysis, matured fibrillar structure formation was observed in control Aβ42 in Figure 4b (4b1A, 4b1B1 and 4b1B2). The CCMeOH treated with Aβ42 was shown in Figure 4b (4b2 A and B) both at 500 nm and 200 nm scale. A drastic decrease in structure, only in globular like structure, was observed which was evident from the electrogram. These studies give insights into the possible better activity of CCMeOH extract in inhibiting the amyloid aggregation. In Figure 4a, there was significant decrease in quenching of fluorescence as compared with control ConAβ429d. TEM electrogramsupported the result as in Figure 4c (4c2 A and B) there was a drastic change in structure of amyloid fibril. These observations essentially proved the effectiveness of the extract even in destabilizing the matured fibrils.

3.7. TLC, LC-MS and GC-MS analysis of CCMeOH

The TLC of CCMeOH extract showed different bands (Figure 5), which shows the presence of various phytochemical presences. For further study of these phytochemials, we performed GC-MS and LC- MS. GC-MS analysis of CCMeOH extract was carried out. Peaks obtained were shown in chromatogram (Figure 6) and a total of 32 compounds were identified with their peak number, retention time (RT), area under peak (%) and height of the peak (%) as represented in the Table 4. Most of the identified compounds have medicinal properties. For LC-MS study, the column chromatography was carried out with 60-120 mesh silica. Total of seventeen fractions were collected, among which only sixteenth and seventeenth were brown compared to rest that was yellow or green in colour, and were eliminated and subjected to LC-MS along with whole CCMeOH extract. The whole extract of CCMeOH shown in the MS spectra (Figure 7Aa and 7Ab) demonstrated the various peaks represented the presence of phytochemicals that was considered with retention time 3.124 (7Aa) and 5.839 (7Ab) of LC chromatogram. The column fraction of CCMeOH was brown colour fraction as in Figure 7B and the MS spectra showed retention time 3.124 (7Ba) and 3.517 (7Bb) of LC chromatogram. Both the spectra mass was compared with help of MSLAMPS database. In Table 5, we could be able to identify thirteen polyketides and one prenol lipids as in Figure 7A showing the MS spectra of RT time (7Aa) 3.124 and (7Ab) 5.839 of LC chromatogram for CCMeOH whole extract. Both the spectra mass was also compared with help of MSLAMPS database. As in Table 6, we could be able to identify nine polyketides for CCMeOH column fraction from MS chromatogram 7Ba and 7Bb. Some of the mass peak in Figure 7B with RT 3.124 (7Ba) MS spectra of CCMeOH column fraction was as same as in

CCMeOH whole extract in Figure 7Aa with RT 3.124 (7Aa) which was excluded. Both the whole fraction and column fraction molecules compared with mass number represented indicate the presence of flavanoids like kaempferol, myricitin, catechin, quercetin, chrysosplenol etc. Spectral studies give an insight into the presence of active ingredients present in the extracts which could be isolated, and the studies in detail show the plausible mechanism that may play a vital role in inhibition of pathogenesis of AD.

4. DISCUSSION

N. sativa is a common climber with bipinnate, oblong, obtuse, and mucronate leaves. In ayurveda, it consumed as astringent with a bitter taste. Advances in studies on leaves of N. sativa have highlighted many activities as discussed above. Our version of current study gives an insight into the fractional study of leaves extracts from non-polar to polar solvent, which can guide for the better active fraction or active moieties for AD at in vivo condition. Plants produce various secondary metabolites such as alkaloids, terpenoids sterols, saponins, polyphenols, anthraquinones, glycosides etc. We have quantified the presence of phenolic and flavonoid contents among the four different extracts of N. sativa. The potential CCMeOH with the highest yield could explain better activity against the oxidative stress and the cholinergic inhibition. The HPLC fingerprinting also showed the presence of gallic acid, protocatechuic acid, catechin, chlorogenic acid, epicatechin, caffeic acid, vanillin, p-coumaric acid, sinapic acid, rutin hydrate, myricitin, cinnamic acid, quercetin, and kaempferol.

Norwood et al.,[31] have deliberate the probable activity of CCMeOH for diverse other reactive oxygen species such as nitric oxide, hydroxyl, superoxide, singlet oxygen and hypochlorous acid with scavenging activity with IC50 values being 24.9 µg/mL, 0.44 µg/mL, , 33.72 µg/mL, 61.13 µg/mL and 170.51 µg/mL, respectively. The CCMeOH shows iron chelating activity of 279.85 µg/mL and oxidative activitites of CCMeOH pour light on combating the oxidative stress. Oxidative stress caused by Aβ42 is by intercalation of peptide aggregates into lipid bilayer thus resulting in oxidative alteration of proteins and lipid peroxidation. Also, the cell model studies have paved evidence of increase in levels of hydrogen peroxide and lipid peroxides upon treatment of Aβ to the cells [32-34]. AChE is a membrane bound enzyme that has a major role in cognition and the evidences show the role of AChE in amyloidosis, where AChE can colocalize with amyloid aggregates that are present in pre-amyloid diffuse deposits, mature senile plaques, and cerebral blood vessels [35]. Interaction of AChE that occurs between the growing Aβ fibrils formed complex thus triggeringthe assembly of Aβ peptides [36]. Suppressing the action of AChE is an essential for reducing the pathogenesis. To suppress AD pathology polyphenols have major role in combating the disease and show potential activities against AChE enzyme. Quercetin, rutin, quercetin 3-glucuronide, kaempferol, myricitin, epigallocatechein, epicatechein, catechin etc have shown the AChE and BChE inhibition activity [37]. Scientific studies have untangled the reason behind the process of amyloidosis. Beta-sheet rich senile plaques structures are the major hallmark in AD. For in vivo quantification of these amyloid beta fibrils ThT assay is used, where the fluorescent dye has higher affinity towards fibrillar beta-sheet rich structures inducing red shift in the excitation spectrum, thus increasing the quantum yield whichhelps the study condition in the formation of Aβ fibrillar structure and its inhibition at in vivo condition. The reason behind the formation of beta structure of peptides is due to interaction between planar aromatic rings of amino acids forming - interactions or -stacking which plays major role in supra molecular structure like senile plaques. Parallel displaced, T shaped, parallel staggered, or herringbone are the four higher order possible clusters formed by aromatic rings. Formation of secondary structure is crucial and the elucidation of structural morphology of peptides to senile plaques and its inhibition is an essential approach towards the therapeutic approach. Natural drug therapy has its wider application and positive merits over the synthetic therapeutic treatment. Plants are rich source of polyphenols and various metabolites, over the year's scientific evidences have supported the elucidation and provided proof for the active moieties that have been used to treat various diseases. In kinetic study of Aβ42 aggregation myricitin has more significant role in intercalating the conformational transition from monomer to oligomer than defibrillation of aggregation cascade[38]. Kaempferol- 3-O-rhamnoside has shown effective activity against neurotoxicity of SH-SY5Y induced by Aβ42 and also anti-amyloidogenic property by inhibiting fibrillogenesis and secondary structural transformation of the peptide [39]. Studies on major polyphenols like epicatechin, epigallocatechin, along with the 3-O-gallate esters of epicatechin gallate and epigallocatechin gallate which contained flavan-3-ol unit of catechins in the structure system showed key interaction between Aβ42, PrP106-126, and ataxin-3 oligomers inhibition and also presence of gallate has enhanced the activity [40]. Detailed studies give an insight towards the probable mechanism of action of structural based interaction in inhibiting the, protofibrils or mature fibrils and oligomers. Over the years, plants have gained attraction for finding the drug like candidature for treating the AD. With growing statistics of dementia, it gives the call towards searching or developing the proper therapeutic approach in need to treat the disease. Over the decade, there has been only treatment of mild and moderate condition of AD. Drugs accessible for symptomatic cure are not enough to fighting the diseases that is imposing on the society. Natural source could yield the better option for its nutraceutical property. Plants like N. sativa can be a better option to search the active moieties for multi-targeting drug approach as it exhibits aggregation inhibitory property in addition to cholinergic activity. Various studies show the presence of polyphenols, terpenoids and other metabolites which are potential source of medicinal value. Previous studies have shown whole aqueous extract of N. sativa leaves as anti-amyloidogenic potential. Accordingly, we have studied from non-polar to polar solvent extract in which the

CCMeOH extract has shown better antioxidant, anticholinergic and excellent anti-amyloidogenic property that gives insights to exploring the novel drug-like candidature for AD therapeutics. Thus, N. sativa could be a potential source of moieties that could possibly have aggregation inhibitory property in addition to the cholinergic activity for treating AD. All in all, the natural products that are rich in polyphenols, and possess multiple functions, might be preferable choices for the cure of AD. The further studies will be involved to know the better mechanistic approach of N. sativa extracts towards the treatment of AD.

5. CONCLUSION

These results are also supported by the presence of polyphenols as the active ingredients. Multi-potent target drug therapy is a promising option in treating the Alzheimer's diseases. Methanolic extract of N. Sativa shows potential activity against cholinergic enzymes, Aβ42 aggregation with antioxidant activity.

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