ABSTRACT: Amyloidogenesis is the process in which amyloid beta (Aβ) peptide aggregation results in plaque formation in central nervous system (CNS) are associated with many neurological diseases such as Alzheimer’s disease. The peptide aggregation initiated from peptide monomers results in formation of dimers, tetramers, fibrils, and protofibrils. The ability of allicin, a lipid-soluble volatile organosulfur biological compound, present in freshly crushed garlic (Allium sativum L.) to inhibit fibril formation by the Aβ peptide in vitro was investigated in the present study. Inhibition of fibrillogenesis was measured by a Thioflavin T (ThT) fluorescence assay and visualized by transmission electron microscopy (TEM). The molecular interaction between allicin and Aβ peptide was also demonstrated by in silico studies. The results show that allicin strongly inhibited Aβ fibrils by 97% at 300 µM, compared with control (Aβ only) (P<.001). These results were further validated by visual of fibril formation by transmission microscopy and molecular interaction of amyloid peptide with allicin by molecular docking. Aβ forms favourable hydrophobic interaction with Ile32, Met35, Val36, and Val39, and oxygen of allicin forms hydrogen bond with the amino acid residue Lys28. Allicin anti-amyloidogenic property suggests that this naturally occurring compound may have potential to ameliorate and prevent Alzheimer’s disease.

KEYWORDS: Alzheimer’s disease, allicin, amyloid β, fibrillogenesis, transmission electron microscope, Thioflavin T fluorescence assay, molecular docking

Introduction
The amyloid plaques deposited extracellularly in central nervous system (CNS) have been identified as one of the major pathological characteristics of Alzheimer’s disease (AD).1 The fundamental element of amyloid plaque revealed the presence of 1–40 and 1–42 amino acid sequences, termed the amyloid beta (Aβ) peptide. In brain, the disturbance between production and clearance of Aβ peptides formed by proteolytic cleavage of amyloid precursor protein (APP) directly correlated with the development of AD.2,3 The Aβ monomer is an unfolded, unstructured ~4 kD peptide, rich in hydrophobic residues. It has been proposed that the smallest stable form of the Aβ peptide exists as a dimer, trimer, or a tetramer. Furthermore, Aβ fibrillogenesis is a process where Aβ monomers are more susceptible to self-aggregate and form oligomers and fibrils. Amyloid fibrils contain characteristic crossed β-sheets which specifically bind to dyes such as Congo red and Thioflavin T (ThT) used in light microscopical staining and spectrophotometric/fluorescence techniques that are often used to analyse these fibrils.

The formation of well-ordered fibrillar aggregates consisting of Aβ plays a significant role in neurodegeneration and is considered one of the main pathogenic factors related to AD. Currently, there is no approved drug to target Aβ fibrillar assemblies. One possible strategy is the use of small molecules that efficiently and specifically inhibit the fibrillogenesis process. Previous studies have reported that some of the natural compounds such as polyphenols, curcumin, rosmarinic acid, tannic acid, catechin, and quercetin inhibit the formation of fibrillar assembly in vitro.4 Present study evaluated the anti-amyloidogenic potential of allicin measured by ThT assay validated by transmission electron microscopy (TEM) and molecular docking studies.

Materials and Methods
Chemicals/reagents
Allicin ((R, S)-diallyl disulfide–S-oxide) was purchased from Caymen chemical, India. Aβ peptide (Aβ1–42) was purchased from Abcam, India. Other chemicals such as cholesterol, uranyl acetate, glycine, sodium hydroxide, ThT were purchased from Sigma-Aldrich (India). All reagents were prepared in Milli-Q water (Millipore, India).

Thioflavin T fluorescence assay
ThT assay was used to quantify amyloid formation, in which the fluorescence intensity remarkably increases with the degree of β-sheet formation.5 ThT binds specifically to Aβ fibrils and this produces a shift in emission spectrum, the amount of β sheet formed being proportional to the fluorescent signal. Aβ was dissolved in Milli-Q water at a concentration of 1 μM. A mixture of Aβ with or without allicin solution (37.5–300 μM) was then incubated overnight at 37°C with constant rotation. After 24 h, 80 μL of ThT (10 μM) in glycine-NaOH buffer (pH 9.0) was added to each well with 20 μL of the Aβ with or without different concentrations of allicin in 96-well micro titration black
plate which was assayed in triplicates. Fluorescence intensities were recorded after 30 min of incubation at \(E_x\), 450 nm and \(E_m\), 490 nm using Molecular devices SpectraMax microplate reader equipped with Ascent software.\(^6\)

**Statistical analysis**

\(P\) value was calculated using Student’s \(t\) test by GraphPad prism software. The \(P\) value was considered statistically significant as it was less than .05 as compared with the controls.

**Transmission electron microscopy**

Equal volumes of allicin solution (37.5-300 \(\mu\)M) were incubated with 11 \(\mu\)M of A\(\beta\) peptides overnight at 37°C with constant rotation. Immediately after incubation, 5 \(\mu\)L of the sample was used for the preparation of TEM slides. Specimens were studied using TEM (JEOL 2100F). Samples containing A\(\beta\) with and without allicin were loaded on continuous carbon support film formed on copper grids. It was then negatively stained with 2% w/v aqueous uranyl acetate by the single droplet procedure.\(^7\) The Copper grids were routinely treated by glow discharge for 60 s, which renders the carbon surface hydrophilic, thus allowing satisfactory spreading of aqueous suspensions and then observed under TEM.\(^8\)

**In silico method**

Molecular docking studies were performed on nuclear magnetic resonance (NMR) structure of A\(\beta\)\(_{42}\) (PDB ID: 1IYT)\(^9\) using software Glide in Schrodinger suite. Receptor structure was preprocessed which includes protonation and optimization and then minimization with the root mean square deviation (RMSD) value of 0.30\(\AA\) using OPLS_2005 (Optimized Potentials for Liquid Simulations) force field using Protein Preparation Wizard in Schrodinger suite.\(^10\)

The structure of allicin was downloaded from PubChem (PubChem CID: 65036) and was prepared using LigPrep module in Schrodinger suite. The ionization states were generated using Epik, hydrogen atoms were added, charged groups were neutralized, and geometry was optimized.

Receptor grid generation module was used to a grid-enclosing residues are found to be important for aggregation, ie, 16–21 and 32–36. Extra precision docking was performed, keeping the ligand flexible, to recognize the possible binding post of ligand for A\(\beta\)\(_{42}\). GScore was used to rank the docked poses.\(^11\)

**Results**

**Effect of allicin on ThT-induced fluorescence on A\(\beta\) aggregation**

When A\(\beta\) (11 \(\mu\)M) was co-incubated with different concentrations of allicin (37.5-300 \(\mu\)M) for 24h at 37°C, the ThT fluorescence showed a concentration-dependent response, indicating that allicin suppressed the fibrillogenesis in a concentration-dependent manner, as shown in Figure 1. At low concentrations (37.5 and 75 \(\mu\)M), the effect was negligible, but amyloid formation was significantly reduced at 150 \(\mu\)M, with a marked reduction at 300 \(\mu\)M concentration. Allicin inhibited A\(\beta\) fibrils by 97% at 300 \(\mu\)M, compared with control (A\(\beta\) only) (\(P < .001\)) (Table 1).

**Effect of the allicin on A\(\beta\) fibril formation by TEM studies**

TEM was used to assess the inhibitory effect of allicin on A\(\beta\) fibril formation. A\(\beta\) peptide (11 \(\mu\)M) only (without allicin) incubated at 37°C for 24h formed extensive A\(\beta\) fibrils of considerable length which also formed large networks. These networks were thick, with fibrils tangled into pseudo-plaques (Figure 2A). With the increasing concentration of allicin (37.5-300 \(\mu\)M), the fibril formation was significantly reduced (Figure 2B to E). The morphology of the A\(\beta\) fibril networks progressively becomes less dense, less entangled, and increasingly sparse, indicating an inhibition of A\(\beta\) fibril formation.

![Figure 1. Concentration-dependent inhibition of A\(\beta\) aggregation by allicin (37.5 to 300 \(\mu\)M). B: Blank (No A\(\beta\)); C: Control (11 \(\mu\)M A\(\beta\)). Data are presented as mean ± SEM (n=3). **\(p < .01\), ***\(p < .001\) compared with control.](image)

| CONCENTRATION OF ALICIN (\(\mu\)M) | INHIBITION (%) OF A\(\beta\) AGGREGATION |
|-----------------------------------|----------------------------------------|
| 37.5                             | 4 ± 0.04                               |
| 75                               | 7 ± 0.03                               |
| 150                              | 44 ± 0.02**                            |
| 300                              | 97 ± 0.04***                           |

*Abbreviations: A\(\beta\), amyloid beta; ThT, Thioflavin T. Each value represents mean ± SEM (n=3). **\(p < .01\), ***\(p < .001\), compared with control.
Effect of allicin on Aβ fibril formation in the presence of cholesterol by TEM studies

Cholesterol is known to promote Aβ fibrillogensis, predominantly in the form of protofibrils, during overnight incubation. Aβ (11 μM) peptide co-incubated with cholesterol (520 μM) at 37°C for 24h promoted fibril formation (Figure 3A) (Control). Mature double helical fibrils were formed, which were often clustered around the cholesterol microcrystal. In the presence of allicin (300 μM), fibril formation was again much reduced (Figure 3B). In addition, the fibrils formed in the presence of allicin appeared much shorter in length and less well formed, compared with the control.

In silico method

Molecular docking studies were done to know the mechanism of action of allicin with Aβ. It forms favourable hydrophobic interaction with Ile32, Met35, Val36, and Val39, and...
oxygen of allicin forms hydrogen bond with the amino acid residue Lys28 (Figure 4). As amino acid residue Met35 has been shown to mediate neurotoxicity and oxidative stress and amino acid residue Lys28 is involved in conformational changes in β-sheet formation, allicin is found to be effective in this case.

**Discussion**

The formation of neurotoxic oligomer and protofibrils are the main steps which lead to formation of plaques in CNS that results neurodegeneration in AD. It has been shown that insoluble Aβ oligomers and protofibrils results in cytotoxic effect compared with soluble peptides, when incubated with neuronal cells under in vitro condition. Furthermore, it has also been demonstrated earlier that Aβ induces formation of reactive oxygen species (ROS). In view of these studies, Aβ peptides are considered as potential therapeutic target that might have disease modifying effect in disease-like AD. The potential therapeutic strategy includes prevention of insoluble Aβ production, inhibition of oligomerization and fibril formation, clearance of insoluble Aβ peptides and hence prevention of neurotoxicity. In view of Aβ hypothesis, many scientists demonstrated anti-amyloidogenic potential of many phyto-compounds. One of the compounds namely multimeric quinacrine derivatives inhibits Aβ fibril formation. Another study demonstrated Aβ fibril formation in vivo was inhibited by carbazole derivatives. It was suggested that the compounds that bind to Aβ peptides might be useful against Aβ fibril formations. Based on these observations, inhibition of Aβ fibrillogenesis using allicin was investigated in this study. The morphological changes in the Aβ fibrils formed are reduced drastically in length and size when Aβ peptides were co-incubated with allicin. Cholesterol is a compound which accelerates fibril formation but in the presence of allicin, a significant reduction in fibril formation was observed in the present study that provides evidence of anti-amyloidogenic potential of allicin.

In *silico* studies shows oxygen atom present in the allicin molecule interact with important amino acids of the Aβ peptide responsible for crossed β-sheet formation and fibrils confirm this molecular interaction. The results presented in this study strongly support the claims of the beneficial effects of garlic extract on a number of neurological complications. Garlic extract contains a mixture of compounds in addition to allicin including several other water-soluble neuroactive sulphur containing compounds such as S-allyl-L-cysteine and S-allyl mercapto-L-cysteine, glutamyl-S-alkyl-L-cysteines, and S-alkyl-L-cysteinesulfoxides, including alliin S-(2-carboxypropyl) glutathione, γ-glutamyl-S-allyl-L-cysteine, γ-glutamyl-S-(trans-1-propenyl)-L-cysteine, and γ-glutamyl-S-allyl-mercaptopo-L-cysteine. On the basis of previous studies, garlic extract was shown to exhibit an anti-aging effect, improve learning and memory impairment and neurotrophic activities. In vivo studies on Alzheimer’s transgenic mice also showed a beneficial effect of garlic extract. Recently, garlic extract has been shown to exert an anti-amyloidogenic effect under in vitro conditions. Another in vitro study showed neuroprotective effect of garlic compounds by reducing apoptosis, apparently due to enhancement of endogenous anti-oxidant defences. It could be speculated that based on these studies, the effectiveness of garlic extract might be due to the presence of allicin and other related compounds. Recent studies have shown neuroprotective effect of allicin on ischaemia-reperfusion brain injury. Allicin also reduces neuronal death and ameliorates the spatial memory in Alzheimer’s rat model. However, further in vivo studies are required to confirm that allicin can be successfully used as an anti-amyloidogenic compound in the treatment of AD. However, the present in
vitro and in silico study demonstrated that allicin can be used as an anti-amyloidogenic compound that might have disease-modifying effect in AD.

In conclusion, allicin demonstrated significant anti-amyloidogenic potential by inhibiting Aβ fibril formation under in vitro condition, this study is giving a significant lead that allicin has a potential to be used as anti-aggregation compound that might have disease-modifying effects in AD. Furthermore, animal studies and pharmacokinetic studies are required to validate the anti-amyloidogenic potential in vivo models.

Author Contributions
SK designed and conceived the study. SK performed the experiments. HR helped in interpretation of results. All author contributed in writing of manuscript.

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