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IDENTIFICATION OF SMALL MOLECULE INHIBITORS AGAINST AMYLOID β (Aβ) OLIGOMERIZATION AND TOXICITY FROM NOOTROPIC AYURVEDIC HERBAL EXTRACTS

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ABSTRACT: Central pathogenic mechanism of Alzheimer's disease (AD) involves aggregation of amyloid β (Aβ) protein to form toxic oligomers. Identifying novel small molecules that inhibit the aggregation of monomeric Aβ to toxic oligomeric species can be an effective disease modifying strategy for AD. In the present study, plants being used in Ayurveda for treating dementia as well as to improve memory and cognitive function have been considered. These included *Nelumbo nucifera, Valeriana wallichii, Celastrus paniculatus*, and *Raulfia serpentina.* We tested the efficacy of these plant extracts and some of their active ingredients for their anti-amyloidogenic property by means of *in vitro* aggregation studies. The effect of each extract or phytocompound was tested on inhibition of aggregation as well as dissociation of preformed aggregates using thioflavin T fluorescence assay and transmission electron microscopy. Of the compounds screened, β-sitosterol and valerenic acid prevented the aggregation of Aβ while α-asarone and glycyrrhizin were effective in dissociating the preformed aggregates. More importantly, all the four phytocompounds have conferred protection against Aβ mediated toxicity in rat primary hippocampal cultures. These substances may hold therapeutic potential for AD treatment.

INTRODUCTION: Alzheimer's disease (AD), the most common cause of dementia in the elderly is an irreversible progressive neurodegenerative disorder. Although plenty of research is carried out on the mechanism of the disease, effective therapies are not available to halt or modify the disease progression. AD still exists as a pandemic in this century too.

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The number of AD patients is being expected to triple by 2050, with the increase in the aging population; leaving severe social and economic burden on patients and their families [1]. Current therapy for AD involves symptomatic palliative intervention using cholinesterase inhibitors and NMDA receptor antagonist which can retain the cognitive functions to some extent even though it cannot prevent the progression of the disease.

Amyloid β (Aβ) centric theory is best suited to explain the pathogenesis of AD, which states that imbalance between the production and clearance of Aβ leads to its progressive accumulation, triggering a cascade of events leading to synaptic dysfunction, microgliosis, neuronal loss, clinically

manifested with memory loss and impaired cognitive function [2]. Recent advances in AD research has pointed out that soluble Aβ oligomers and not fibrils are the major culprits in disease pathology causing synaptic dysfunction and neuronal cell loss [3, 4]. Identification of small molecule inhibitors of Aβ oligomerization can provide 'leads' for the drug development for AD [5, 6].

Hence it is appropriate to evaluate the existing CNS related therapies in traditional and alternative medicine for identification of novel therapeutic molecules [7, 8]. A large number of plant based compounds like curcumin [9], resveratrol [9], salvianolic acid [10], EGCG ((-)-epigallo-catechin-3-gallate) [11], ellagic acid [12] etc. have been found to be inhibiting Aβ aggregation and / or preventing the Aβ induced toxicity in cell culture studies. In order to select additional resources with therapeutic potential, we have earlier tested the methanolic extracts of 13 plants used in Ayurveda [13] for their anti-amyloidogenicity and selected *Glycyrrhiza glabra* for further studies. Four more ayurvedic plants have been screened in the current study owing to their CNS enhancing effect. They are *Nelumbo nucifera, Valeriana wallichii, Celastrus paniculatus*, and *Raulfia serpentina*.

A few of the phytochemicals from ayurvedic plants were previously studied elsewhere for their therapeutic potential against AD such as asiatic acid from *Centella asiatica* [14, 15], bacosides from *Bacopa monniera* [16] and withanolides from *Withania somnifera* [17-19]. It was our fond interest to test the active ingredients from the plants we have identified in our study on the aggregation of Aβ. Accordingly, we have included 4 phytocompounds - valerenic acid from *Valeriana wallichii* and glycyrrhizin from *Glycyrrhiza glabra*. In addition, α-asarone and β-sitosterol (**Figure 1**) have been included in the study.

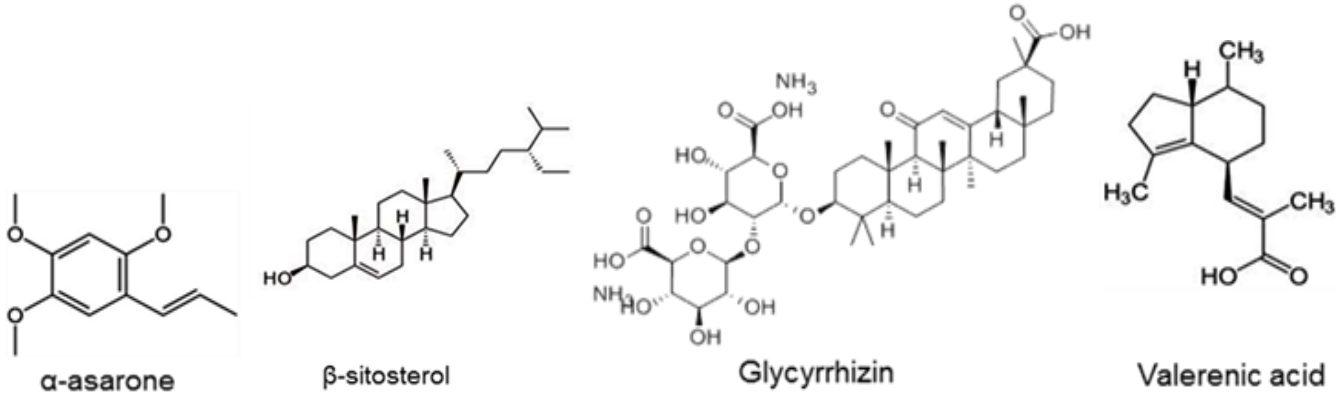


FIGURE 1: PHYTOCOMPOUNDS USED IN THE STUDY

MATERIALS AND METHODS:

Materials: Dimethylsulfoxide (DMSO), thioflavin T, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra zolium bromide (MTT), DME/F-12 powdered culture medium, insulin, transferrin, penicillin and streptomycin were purchased from Sigma Aldrich (Bangalore). Collagenase/dispase was obtained from Boehringer-Mannheim (Germany). Nitex membrane (102 μm pore size) was procured from Becton Dickinson (New Jersey, USA). The desiccated plant materials were obtained from Arya Vaidya Sala (Kottakkal). The Aß used in the present study corresponding to human sequence was recombinantly expressed and purified as described earlier [20]. Among the phytocompounds, valerenic acid and β-sitosterol were purchased from Chromadex, USA. Glycyrrhizin and α-asarone were obtained from Natural remedies, India. Tissue culture ware was supplied by Nunc (Denmark). All other reagents used were of analytical grade and obtained locally.

Preparation of methanolic extracts of the plant parts: Parts of the plants used for the study were dried in the shade and then pulverized. 5g each of powdered plant parts were covered and tied in double layer muslin cloth and kept in the Soxhlet extraction unit. 65ml of methanol was placed in the solvent round bottom flask and heated at 60^{o}C and the methanolic extract was collected in the upper chamber.

Filtered extract was concentrated to evaporate the methanol completely in vacuum centrifuge and the dried extract was stored in -20^{o}C.

Extracts were solubilised in DMSO and 100 µg/ µl stocks were prepared and used for aggregation studies.

Preparation and characterization of aggregates of Aβ: Native Aβ was dissolved in DMSO, followed by dilution with double distilled water and then with phosphate buffered saline to eventually prepare a 200 µM stock solution. This stock (200 µl) was incubated with 20 mM thioflavin T at 4°C for 24 h with intermittent shaking during the incubation to facilitate the formation of Aβ aggregates and was confirmed by measuring the fluorescence intensity (Excitation: 446 nm; Emission: 490 nm) in a fluorescence spectrophotometer (Shimadzu, RF 5301 PC).

Influence of plant extracts on inhibition/ dissociation of Aβ oligomerization: To evaluate the influence of extracts on dissociation of Aβ oligomers, 200 µl of the above preformed Aβ aggregate was incubated with 200 µg of the extract or per tube for 24 h at 37°C followed by fluorescence measurement studies. To assess the effect of these extracts on formation of Aβ aggregates, soluble Aβ (20 µM) was co-incubated at 4° C along with 200 µg of the extract or the individual phytocompound (2 µM, in DMSO). After 24 h, an aliquot was taken for fluorescence measurements. The corresponding negative controls were obtained by using each of the extract alone incubated with thioflavin T in the absence of Aβ.

Transmission Electron Microscopy: 10 µl of each from the samples of aggregation as described above was mixed with 10 µl of 1% Phosphotungstic acid (PTA) and kept for 5 minutes. Then the mixture was placed on a collodion coated copper grid for 10 minutes. Excess solution was drained off by using Whatman filter paper by touching the edge of the grids. After air drying, the copper grids were scanned under Transmission Electron Microscope (Tecnai G2 Spirit Bio-twin – FEI Netherlands) at 80 KVA and related areas were captured using *Mega View-III* digital CCD camera at an original magnification of 68,000 X.

Primary cultures of adult rat Hippocampal cells: The experimental procedure for primary culture [21] was approved by the Institutional Animal Ethics Committee. Every effort was made to minimize the number of animals used and their suffering. 3-4 month old adult Sprague-Dawley female rats were decapitated; brains were dissected out to remove the hippocampi which were then kept in cold saline. Then it was transferred to DME/ F12 medium containing 10mM sodium bicarbonate, insulin (2.5 µg/ml), transferrin (50 µg/ml), 0.1% antibiotics (pencillin and strepto-mycin).

The tissue was minced aseptically, treated with 0.05% collagenase/ dispase containing 0.005% soybean trypsin inhibitor for 20 min at room temperature and washed twice by unit gravity. The cells were then triturated with a Pasteur pipette and filtered through Nitex membrane and washed extensively by centrifugation before plating.

The hippocampal cells were plated in DME/F-12 medium supplemented with the above listed substances at a density of 4 x 10^5 cells/ cm^2 and cultured at 37 ºC in a humidified atmosphere of 5% CO_2. After 24 h, the cells were incubated with oligomeric Aβ (10 µM) for further 24 h. The test wells were pretreated with phytocompounds (2 µM) in 2 µl of DMSO for 2 h prior to the addition of oligomeric Aβ. After incubation, the extent of cell viability was assessed by MTT assay.

Cell viability assay: Cell viability was measured by quantitative colorimetric assay with MTT method as described previously [22]. 20 µl/ well of 5mg/ml MTT solution was added and cells were incubated at 37°C for 4 h. Supernatants were then aspirated off and formazan crystals were dissolved with 200 µl of DMSO.

The absorbance of each well was determined at 570 nm using a microplate reader (Infinite 200, Tecan Group Ltd). The data expressed as mean + S.E.M. percentage cell viability measured in the absence of Aβ, considered as 100%.

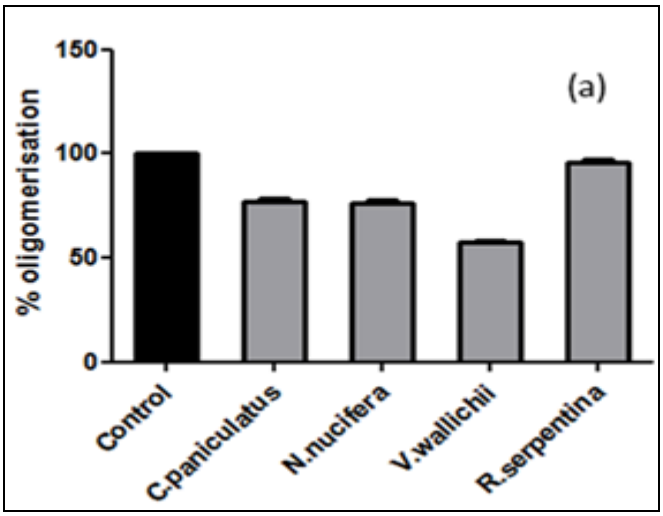
Statistical analysis: Statistical analysis of the data was performed using two way analysis of variance (ANOVA) by GraphPad Prism software. Data were expressed as mean ± standard error mean (SEM) for separate groups and differences were considered statistically significant, when p values were less than 0.05 ($p < 0.05$).

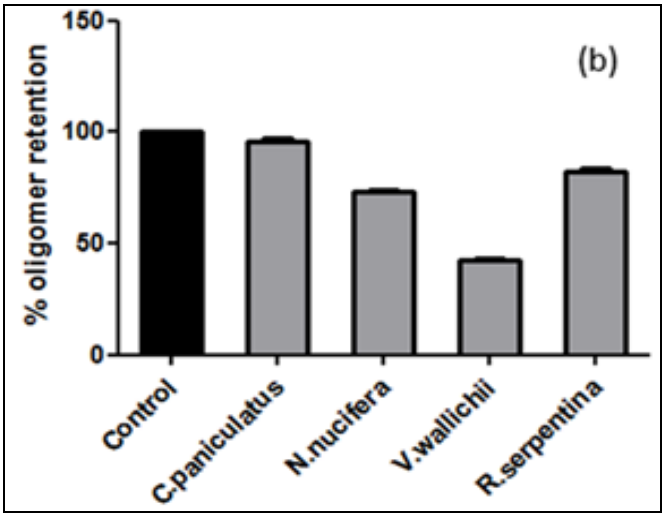
RESULTS AND DISCUSSION: In the past two decades, a large number of experimental studies have established a pathological role for Aβ in AD [2, 3]. However, recent debate has focused on whether Aβ fibrils or soluble oligomers are the major neurotoxic species which contribute to neurodegeneration and cognitive dysfunction. Considerable evidence has indicated that the early soluble oligomers are the primary neurotoxic species in AD brain compared to the fibrils [23, 24]. Preventing the formation of these oligomers is a promising therapeutic strategy against AD [25]. Screening of various phytocompounds and plant extracts from natural product libraries and traditional medicine have been carried out in search for identifying small molecule inhibitors which can prevent Aβ aggregation and toxicity [9-18]. Still, evidence for the capability of plant based products on Aβ oligomerization remains a challenge.

Plants have been used as a rich source of new bioactive compounds for drug discovery [26]. Phytochemical studies of the different parts of many of the plants have shown the presence of many valuable compounds as their secondary metabolites, such as lignans, flavonoids, tannins, polyphenols, triterpenes, sterols, and alkaloids, that show a wide spectrum of pharmacological activities, including anti-inflammatory, anti-amyloidogenic, cholinesterase inhibiting, lipid lowering, and antioxidant effects. Moreover use of the plants selected from Ayurveda dates back to 2000 B.C. and have proven to be less toxic with minimal side effects.

The current study was an attempt to evaluate the therapeutic potential of nootropic medicinal plants used in Ayurveda and some of their active phytocompounds against AD pathology.

The results obtained with aggregation studies in the presence of various plant extracts as assessed by using thioflavin T binding fluorescence assay revealed that methanolic extract from *Valeriana Wallichii* was considerably effective in dissociating the preformed aggregates (**Figures 2a and 2b**). These results prompted us to further evaluate the influence of valerenic acid, the active ingredient from *Valeriana Wallichii* and glycyrrhizin, the main constituent from *Glycyrrhiza glabra*, the crude extract of which was shown to be effective in interfering with Aβ aggregation [13] in thioflavin T binding assays. In addition, we have included α-asarone and β-sitosterol in this study. α-asarone, purified from the rhizomes of *Acorus calamus* has traditionally been used for hundreds of years because of its beneficial effects on learning performance and an anti-aging effect. Recent studies indicated that α-asarone reduced the toxicity of $A\beta_{(25-35)}$ induced neuronal cell death and improved the spatial memory in rats [27]. β-sitosterol is one of several phytosterols, widely distributed in the plant kingdom. This compound has recently been shown to prevent high cholesterol induced platelet Aβ release. In addition, β-sitosterol inhibited the activity of β- and γ-secretases, the enzymes involved in Aβ generation [28]. The results obtained with these four phytocompounds showed that, valerenic acid from *Valeriana Wallichii* and β-sitosterol completely inhibited the formation of Aβ aggregates (**Figure 2c**) whereas glycyrrhizin from *Glycyrrhiza glabra* and α-asarone completely dissociated the preformed aggregates (**Figure 2d**).





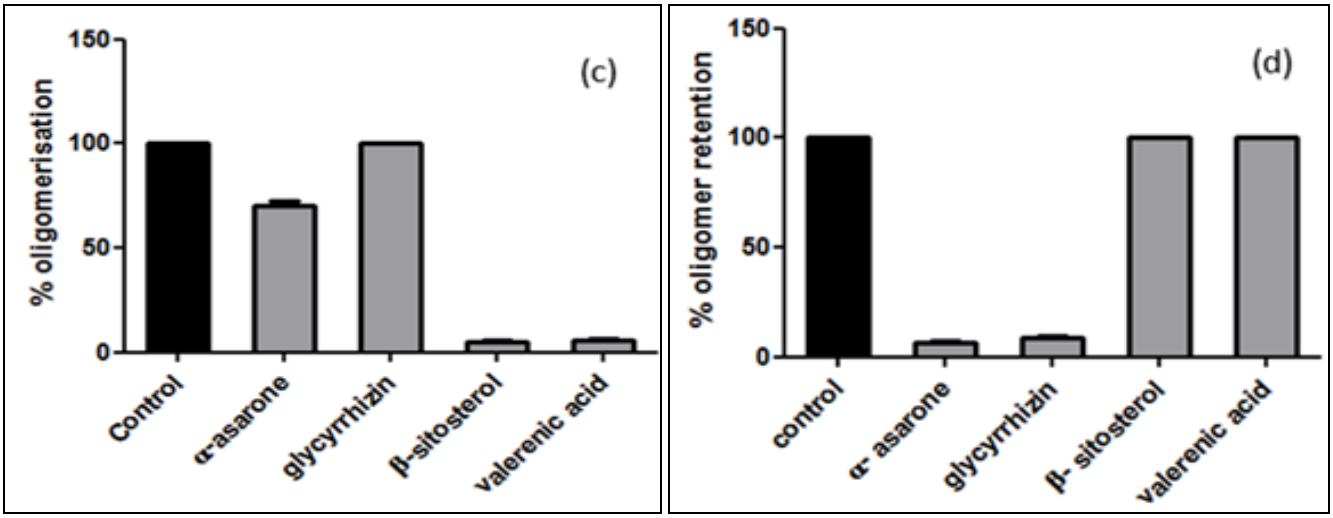


FIGURE 2: INFLUENCE OF PLANT EXTRACTS AND INDIVIDUAL PHYTOCOMPOUNDS ON AB OLIGOMERIZATION. Shown are the effect of plant components on formation of Aβ oligomers (a, c) or dissociation of preformed aggregates of Aβ (b, d). Data represented as mean ± S.E.M. (n = 3). *** P< 0.0001 versus control (Dunnett's test).

In the conditions used here for aggregation of Aβ, as evident from electron micrographs, oligomeric species were formed (**Figure 3a**). Aggregates were typically observed as spherical oligomers joined together in ordered clusters. Next, we compared the status of aggregates in the presence of the phytocompounds (**Figures 3b and 3c**). The electron micrographs, while confirming the results obtained with thioflavin T binding, clearly demonstrated the specificity of valerenic acid and β-sitosterol in prevention of Aβ aggregation alone and that of glycyrrhizin and α-asarone in dissociating the preformed aggregates only.

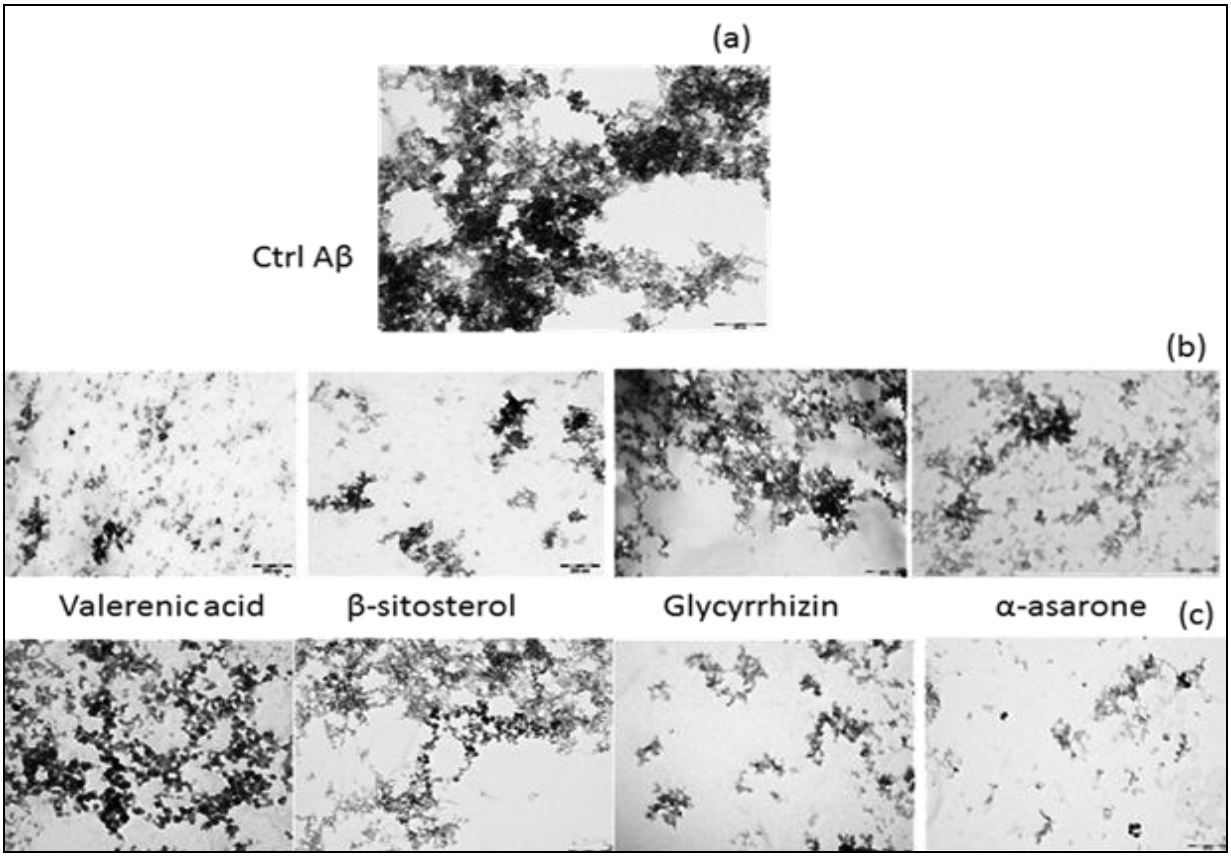


FIGURE 3: TRANSMISSION ELECTRON MICROGRAPHS OF Aβ. Panel (a) shows the image of Aβ oligomers (Ctrl Aβ). Panel (b) depicts aggregate morphology obtained upon coincubation of monomeric Aβ with phytocompounds for 24 h. Panel (c) shows the morphology obtained by treating the preformed aggregates of Aβ with phytocompounds for 24 h. Scale bar 200 nm.

Next, the effect of these phytocompounds on Aβ induced primary rat hippocampal cell toxicity was evaluated. In order to compare the general metabolic state of the cells upon treatment with oligomeric Aβ either in the presence or absence of the individual phytocompounds, MTT reduction was assayed. Preliminary investigations using trypan blue exclusion assay revealed that half maximal effective concentrations of these phytocompounds to inhibit Aβ induced cell death were in the range of 0.8 – 2 μM (Data not shown). Accordingly, in the MTT assay, these phytocompounds were tested at 2 μM concentrations. **Figure 4** shows that incubation with Aβ alone (10 μM) resulted in a significant decline in the ability of cells to reduce MTT. This was reversed to a considerable extent by the phytocompounds tested in the present study.

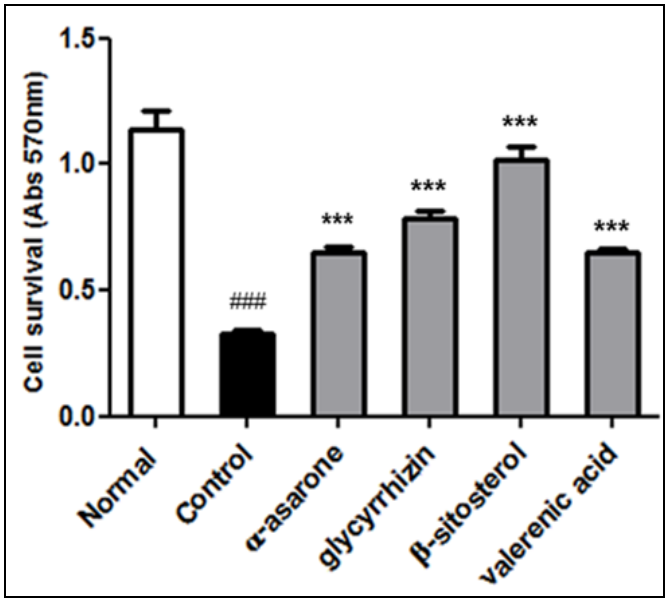


FIGURE 4: THE PROTECTIVE EFFECT OF VARIOUS PHYTOCOMPOUNDS ON OLIGOMERIC Aβ-INDUCED TOXICITY IN RAT HIPPOCAMPAL CELLS MEASURED BY THE MTT ASSAY. Results are expressed as absorbance at 570 nm. Shown are the values obtained with untreated cells (Normal), cells treated with Aβ alone (Control), or pretreatment with individual phytocompounds followed by incubation with Aβ. Data represented as mean ± S.E.M. (n = 3). *** P< 0.0001 versus Aβ alone control (Dunnett's test) and ### P< 0.0001 versus untreated (Student's t-test).

In summary, the current findings offer direct evidence on the influence of the extracts from *Valeriana Wallichii* and the phytocompounds α-asarone, β-sitosterol, glycyrrhizin and valerenic acid on Aβ oligomer formation/ dissociation.

More importantly, the results suggest that valerenic acid and β-sitosterol may further be tested for prevention of AD whereas glycyrrhizin and α-asarone can be evaluated for their efficacy in treatment of AD.

However, since this study provides only results obtained from *in vitro* studies, further studies must be carried out in animals, to assess the activity and also the bioavailability of the isolated compounds or extracts, which is especially critical for their action in the central nervous system.

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