

## *In vitro* evaluation of anti-Alzheimer effects of dry ginger (*Zingiber officinale* Roscoe) extract

Maya Mathew<sup>1</sup> & Sarada Subramanian<sup>1\*</sup>

Department of Neurochemistry, National Institute of Mental Health and Neurosciences (NIMHANS),  
Hosur Road, Bangalore 560 029, India

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As the disease modifying therapies against Alzheimer's disease (AD) continue to exist as a major challenge of this century, the search for newer drug leads with lesser side effects is on the rise. A large number of plant extracts and phytochemicals are being actively pursued for their anti-Alzheimer effects. In the present study, the antioxidant activity, cholinesterase inhibition, anti-amyloidogenic potential and neuroprotective properties of methanolic extract of dry ginger (GE) have been evaluated. The extract contained  $18 \pm 0.6$  mg/g gallic acid equivalents of total phenolic content and  $4.18 \pm 0.69$  mg quercetin equivalents/g of dry material. GE expressed high antioxidant activity with an  $IC_{50}$  value of  $70 \pm 0.304$   $\mu$ g/mL in DPPH assay and  $845.4 \pm 56.62$   $\mu$ M Fe(II) equivalents/g dry weight in FRAP assay respectively. In Ellman's assay for the cholinesterase inhibitory activity, GE had an  $IC_{50}$  value of  $41 \pm 1.2$   $\mu$ g/mL and  $52 \pm 2$   $\mu$ g/mL for inhibition of acetyl- and butyrylcholinesterase respectively. Also, GE increased the cell survival against amyloid  $\beta$  ( $A\beta$ ) induced toxicity in primary adult rat hippocampal cell culture. Aggregation experiments with the thioflavin T binding studies showed that GE effectively prevented the formation of  $A\beta$  oligomers and dissociated the preformed oligomers. These findings suggest that methanolic GE influences multiple therapeutic molecular targets of AD and can be considered as an effective nontoxic nutraceutical supplement for AD.

**Keywords:**  $A\beta$  oligomers,  $A\beta$  toxicity, Anti-cholinesterase, Antioxidant, Ginger extract

Alzheimer's disease (AD) is an age-associated, irreversible, progressive neurodegenerative disease characterized by severe memory loss, unusual behaviour, personality changes, and a decline in cognitive function. It is the most common cause of dementia in the elderly and is regarded as the pandemic of the 21<sup>st</sup> century, imposing enormous social and economic burdens on patients and their families. Also the aging global population has led to a steep increase in the number of individuals with AD which is anticipated to be tripled by year 2050<sup>1</sup>.

Several biochemical and pathological mechanisms contribute to AD. They include: (i) imbalance in the rate of production of amyloid  $\beta$  ( $A\beta$ ) versus its clearance resulting in accumulation of  $A\beta$  in the form of senile plaques (amyloid hypothesis)<sup>2</sup>. These plaques can induce neuroinflammatory processes and trigger the production of reactive oxygen species (ROS)<sup>3</sup> and (ii) cholinergic deficits resulting due to

enhanced activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), contributing to the cognitive impairments seen in AD (cholinergic hypothesis)<sup>4</sup>. Based on these hypotheses, several anti-amyloid<sup>5</sup> and cholinesterase inhibitors<sup>6</sup> are being investigated for their disease modifying strategies. In the recent years, herbal therapy for AD is gaining momentum<sup>7</sup>. Several Ayurvedic medicinal plants and their constituent phytochemicals have shown promising pharmacological activities for treatment of AD<sup>8-10</sup>.

Ginger (*Zingiber officinale* Roscoe, Zingiberaceae) rhizome is consumed worldwide as a spice and flavouring agent over 2000 years and is attributed to have many medicinal properties. Of particular importance are the antioxidant<sup>11,12</sup> and anti-inflammatory properties<sup>13</sup> which are relevant for the treatment of AD. There are a few studies for validating the potential of ginger for neuroprotection against  $A\beta$  induced toxicity in cell culture studies<sup>14</sup> and reversal of behavioural dysfunction in rats<sup>15</sup>. These observations prompted us to identify the molecular targets of action of dry ginger contributing to its anti-Alzheimer effects. The results reveal that methanolic extract of dry ginger (GE) functions

<sup>1</sup> Both the authors contributed equally to this work

\* Correspondent author

Telephone: 91-80-2699 5165

Fax: 91-80-2656 4830

E-mail: sarada@nimhans.kar.nic.in; saradabs@yahoo.com

through multiple routes by exhibiting antioxidant property, inhibition of cholinesterase activity, prevention of A $\beta$  oligomerisation and conferring protection against A $\beta$  induced toxicity to primary adult rat hippocampal cells in culture.

## Materials and Methods

**Materials**—Dimethylsulfoxide (DMSO), thioflavin T, 2,2'-diphenyl-1-picryl hydrazyl radical (DPPH), acetylthiocholine iodide (ATCI), AChE from electric eel (type VI-S lyophilized powder), butyrylthiocholine iodide (BTCl), BChE from equine serum, bovine serum albumin (BSA), 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), physostigmine, gallic acid, quercetin, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), PVDF membrane, anti-mouse secondary antibody conjugated with horse radish peroxidase, 3,3'-diaminobenzidine, formazan, 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  $\mu$ m pore size) was procured from Becton Dickinson (New Jersey, USA). The A $\beta$  used in the present study corresponding to human sequence was recombinantly expressed and purified as described earlier<sup>16</sup>. Oligomer specific A11 antibody was a kind gift from Dr. Charles Glabe (University of California at Irvine, CA, USA). Tissue culture ware was supplied by Nunc (Denmark). All other reagents used were of analytical grade and obtained locally.

**Preparation of methanolic ginger extract**—Rhizomes of ginger were procured from Arya vaidya sala, Kottakkal, Kerala and authenticated by Prof. Philomina A Vadakel, Department of Botany, Nirmala College, Muvattupuzha, Kerala. Rhizomes were desiccated in hot air oven and pulverized. Powdered ginger (5 g) was covered and tied in double layer muslin cloth and kept in the soxhlet extraction unit. Methanol (70 mL) was placed in the solvent round bottom flask and heated at 60 °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 °C. It was then solubilised in DMSO and 100 mg/mL stock was prepared and used for aggregation as well as cell culture studies. For measuring the total polyphenolic content (TPC), total flavonoid content

(TFC), antioxidant activities and cholinesterase inhibition assays, GE stock was prepared in methanol. The entire study was conducted using a single batch of GE to avoid batch-to-batch variation and maximise the product constancy.

**Estimation of total polyphenolic content**—TPC of the ginger extract was estimated using microplate assay method using Folin-Ciocalteu reagent<sup>17</sup>. 20  $\mu$ L each of the 10 mg/mL GE in methanol and the serially diluted gallic acid (1 to 50  $\mu$ g in methanol) were loaded on a 96 well microplate. Then 100  $\mu$ L of Folin-Ciocalteu reagent was added to each well and mixed well. After 5 min, 80  $\mu$ L of 7.5% sodium carbonate solution was added and mixed well. After incubating it in the dark for 2 h, absorbance was measured at 750 nm using microplate reader (Infinite M 200, Tecan). Gallic acid was used as standard to determine the total polyphenols.

**Estimation of total flavonoid content**—TFC was measured by colorimetric method<sup>18</sup>. To 0.1 mL of GE extract (10 mg/mL in methanol), 0.3 mL of methanol, 0.02 mL of 10% aluminium chloride, 0.02 mL of 1 M potassium acetate and 0.560 mL of distilled water were added. After 30 min, the absorbance was measured at 420 nm. Using quercetin as the standard, total flavonoid was represented as mg quercetin equivalents/g dry material.

### Determination of antioxidant activities—

(i) Scavenging effect on DPPH: Antioxidant potential of ginger was estimated using DPPH free radical scavenging assay in 96 micro-well flat plates<sup>19</sup>. Stock solutions of the extracts were prepared at 1mg/mL in methanol. Each well was filled with 200  $\mu$ L of extract in methanol starting from 1000  $\mu$ g/mL down to the lowest concentration of 10  $\mu$ g/mL. Then, 5  $\mu$ L of the DPPH solution (2.5 mg/mL in methanol) was added to each well. After keeping the plate in the dark for 30 min, the optical density of each well was read using microplate reader at 517 nm. Percentage inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = 1 - \text{OD (DPPH+Sample)} / \text{OD (DPPH)} \times 100$$

A graph of percentage inhibition of free radical activity was plotted against concentration of crude extract and 50% inhibition concentration (IC<sub>50</sub>) was obtained from the graph. The radical scavenging effect was examined and compared with gallic acid as positive control.

(ii) Ferric reducing antioxidant power (FRAP) assay: This assay was conducted by the modified

method reported by Benzie and Strain<sup>20</sup>. The working FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ and 20 mM FeCl<sub>3</sub>.6H<sub>2</sub>O in a 10:1:1 ratio and heated to 37 °C prior to use. GE extract (0.15 mL) was incubated with 2.85 mL of FRAP reagent in dark. After 30 min, the absorbance was measured at 593 nm. Standard curve was constructed with 200–1000 µM FeSO<sub>4</sub>. Results were expressed as µM Fe(II) equivalents/g dry material.

*Determination of cholinesterase inhibitory activity using microplate assay based on Ellman's method*—AChE activity was measured using a 96-well microplate assay based on Ellman's method<sup>21</sup>. The enzyme hydrolyses the substrate acetylthiocholine resulting in the product thiocholine which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate which can be detected at 412 nm. Tris-HCl (50 mM, pH 8.0) was used as a buffer throughout the experiment unless otherwise stated. AChE used in the assay was from electric eel (type VI-S lyophilized powder, 518 U/mg solid, 844 U/mg protein). The enzyme stock solution (518 U/mL) was kept at –80 °C. The further enzyme-dilution was done with 0.1% BSA in buffer. DTNB was dissolved in the buffer containing 0.1 M NaCl and 0.02 M MgCl<sub>2</sub>. ATCI was dissolved in deionized water. In the 96-well plates, 100 µL of 3 mM DTNB, 20 µL of 0.26 U/mL of AChE, and 40 µL of buffer (50 mM Tris pH 8.0), 20 µL of ginger extract containing various concentrations (0-500 µg/ml) diluted in buffer were added to the wells. After mixing, the plate was incubated for 15 min at 25 °C and then the absorbance was measured at 412 nm in microplate reader and the readings were used as blank. The enzymatic reaction was initiated by the addition of 20 µL of 15 mM ATCI and the hydrolysis of acetylthiocholine was monitored by reading the absorbance every 5 min for 20 min. Physostigmine was used as positive control. All the reactions were performed in triplicate. The percentage inhibition was calculated by the following formula:

$$\text{Inhibition (\%)} = (E - S) / E \times 100$$

where E is the activity of the enzyme without ginger extract and S is the activity of enzyme with the extract. IC<sub>50</sub> value could be calculated from the % inhibition value of different concentrations of the ginger extract.

BTCI was used as a substrate to assay BChE enzyme, while all the other reagents and conditions were the same as described above for AChE assay.

*Preparation and characterization of aggregates of Aβ*—Recombinant 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).

*Primary cultures of adult rat hippocampal cells*—The experimental procedure for primary culture<sup>22</sup> was approved by the Institutional Animal Ethics Committee (Approval number: AEC/50/241(B)/N.C. dt. 24 January 2013). Every effort was made to minimize the number of animals used and their suffering. Adult (3-4 month old) 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 10 mM sodium bicarbonate, insulin (2.5 µg/mL), transferrin (50 µg/mL), and 0.1% antibiotics (penicillin and streptomycin). 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  $4 \times 10^5$  cells/cm<sup>2</sup> and cultured at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. After 24 h, the cells were incubated with oligomeric Aβ (10 µM) for further 24 h. The test wells were pretreated with GE (final concentrations of 0.02, 0.1 and 0.2 mg 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 (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method as described previously<sup>23</sup>. MTT solution (20 µL/well from 5 mg/mL stock) 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. The data were expressed as mean  $\pm$  SE. Percentage cell viability measured in the absence of A $\beta$  was considered as 100%.

**Dot blot assay for A $\beta$  oligomers**—A $\beta$  samples (2  $\mu$ g) were spotted on PVDF membrane, allowed to air dry and the unoccupied area was blocked for 1 h in 3% BSA in PBS. Next, the membranes were incubated for 1 h with the oligomer specific A11 antibody (1  $\mu$ g/mL)<sup>24</sup>, in 0.3% BSA in PBS solution for 2 h at RT. The antigen-antibody complex was visualized by using anti-mouse secondary antibody conjugated with horse radish peroxidase followed by 3,3'-diaminobenzidine as the chromogen.

**Influence of GE on inhibition/ dissociation of A $\beta$  oligomerisation**—To evaluate the influence of GE on dissociation of A $\beta$  oligomers, 200  $\mu$ L of the above preformed A $\beta$  aggregate was incubated with various concentrations (0.02, 0.1 and 0.2 mg in 2  $\mu$ L of DMSO) of the GE for 24 h at 37 °C followed by thioflavin T fluorescence assay. To assess the effect of GE on formation of A $\beta$  aggregates, 200  $\mu$ L of monomeric A $\beta$  (200  $\mu$ M) was co-incubated at 37 °C along with various concentrations (0.02, 0.1 and 0.2 mg in 2  $\mu$ L of DMSO) of the GE. After 24 h, an aliquot was taken for fluorescence measurements. The corresponding negative controls were obtained by using GE alone incubated with thioflavin T in the absence of A $\beta$ .

**Statistical analysis**—Statistical analysis of the data was performed using one way analysis of variance (ANOVA) by GraphPad Prism software. Data were expressed as mean  $\pm$  SE/SD for separate groups and differences were considered statistically significant, when *P* values were less than 0.05.

## Results

Extraction of dry ginger rhizomes in soxhlet extraction unit using methanol as the solvent gave a yield of 6.43% extract with respect to the starting material. Total polyphenolic content of methanolic GE prepared was found to be 18 $\pm$ 0.6 mg/ g gallic acid equivalents of the dry extract. Total flavonoid content was found to be 4.18 $\pm$ 0.69 mg quercetin equivalents/ g of dry material.

The methanolic GE manifested a significant effect in scavenging DPPH radical by virtue of its antioxidant activity with the IC<sub>50</sub> value of 70 $\pm$ 0.304  $\mu$ g/mL in DPPH assay whereas for gallic acid which served as the positive control, it was 1 $\pm$ 0.103  $\mu$ g/ mL. The reducing potential of GE estimated from its

ability to reduce TPTZ-Fe(III) complex to TPTZ-Fe(II) was 845.4 $\pm$ 56.62  $\mu$ M Fe(II) equivalents/ g dry weight.

The IC<sub>50</sub> value of GE for AChE inhibitory activity assayed using Ellman's method was found to be 41 $\pm$ 1.2  $\mu$ g/mL (Fig. 1) whereas for the physostigmine which was used as positive control, it was 0.075 $\pm$ 0.003  $\mu$ g/mL. Further, the GE extract inhibited BChE activity *in vitro* in a dose dependent manner with the IC<sub>50</sub> value 52 $\pm$ 2  $\mu$ g/mL. The extract brought about >80% inhibition of both the enzymes at concentrations of  $\geq$ 200  $\mu$ g/mL.

In primary adult rat hippocampal cell culture, when the oligomeric A $\beta$  was added, as anticipated, toxicity was observed in MTT assay resulting in drastic reduction of cell survival. However, when the GE was added prior to the addition of A $\beta$ , cell survival increased significantly in a dose dependent manner (Fig. 2).

To investigate the anti-amyloidogenic potential of GE, first oligomeric form of A $\beta$  and was prepared and the aggregation was confirmed by dot blot assay using oligomer specific A11 antibody. Freshly solubilised A $\beta$  existed as monomeric form as there was no binding to A11 (Fig. 3A), whereas, the oligomeric species which formed during the aggregation readily bound to the antibody (Fig. 3B). GE inhibited significant the formation of oligomers significantly at concentrations 0.1 and 0.2 mg when coincubated with A $\beta$ . Also, there was a dose dependent decrease in the thioflavin T fluorescence intensity when the GE was treated with preformed oligomers suggesting their dissociation (Fig. 4).

## Discussion

Although several research advances have been made in unravelling the neuropathology and

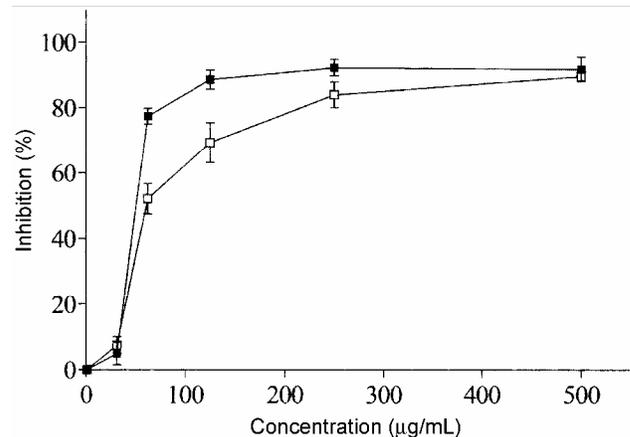


Fig. 1—Inhibition of acetyl cholinesterase activity (■) and butyryl cholinesterase activity (□) by various concentrations of ginger extract [Values are mean $\pm$ SD from 3 observations each].

molecular mechanisms of AD, only a few treatment options exist currently. Various potential therapeutic or preventive compounds have been tested in clinical trials, but none of them showed any clear therapeutic benefit against the disease. There is a severe lack of effective therapies with respect to the predicted dramatic increase in AD cases in the coming decades which call for the demand on new drug candidates. In this regard, a large number of direct and indirect activities of traditionally used plants and its active constituents that influences various therapeutic targets of AD have been explored<sup>11,12</sup>.

Ginger (*Zingiber officinale* Roscoe) is extensively consumed as a spice in foods and beverages worldwide. It has several medicinal properties including carminative, anti-emetic, spasmolytic, peripheral circulatory stimulant, anti-inflammatory, anti-cancer, anti-hypertensive, anti-diabetic and antioxidant action due to its various active phytochemicals such as monoterpenes, oxygenated monoterpenes, sesquiterpenes, zingerone, polyphenols

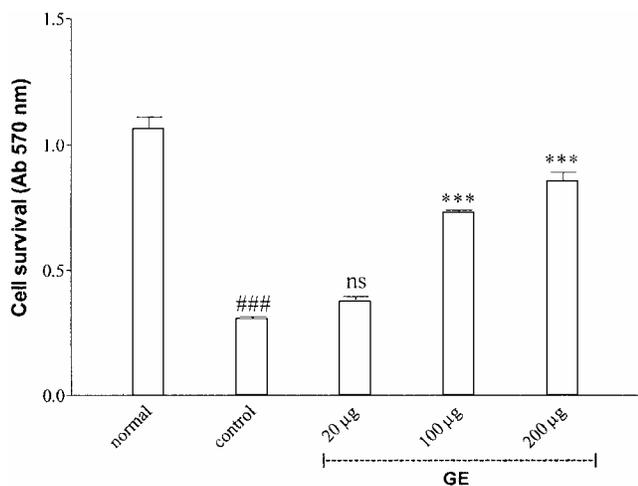


Fig. 2—The protective effect of GE on oligomeric A $\beta$ -induced toxicity in rat hippocampal cells measured by the MTT assay. Results are expressed as absorbance at 570 nm. [The values obtained with untreated cells (normal), cells treated with A $\beta$  alone (control), or pretreatment with GE followed by incubation with A $\beta$ . Values are mean $\pm$ SE from 3 observations each. *P* values : <0.0001; \* vs A $\beta$  alone control (Dunnett's test) and # vs untreated (Student's t-test)].

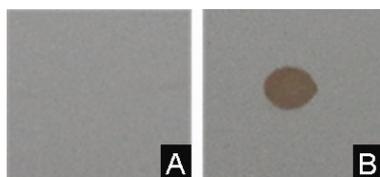


Fig. 3—Dot blot using oligomer specific A11 antibody with A $\beta$ . Blots obtained with monomeric A $\beta$  (A) and oligomeric A $\beta$  (B).

like paradols, gingerols and shogaols other than essential oils<sup>25-27</sup>. The pungency of ginger is due to gingerols, a group of homologous phenols which are the major active components responsible for ginger's antioxidant activity. 6-gingerol, 8-gingerol, 10-gingerol, 6-shogaol, paradol and methyl-6-isogingerol are the predominant gingerols present in the ginger rhizomes<sup>28</sup>. A few studies have tested the efficacy of GE against AD. In an *in vitro* study, Guo *et al.*<sup>29</sup> reported marginal blocking effect of aqueous extract of fresh ginger in binding of fluorescence tagged A $\beta$  to the plaques in AD brain sections. In another independent study, Oboh *et al.*<sup>30</sup> showed that aqueous extracts of red and white ginger contained AChE inhibitory activity with IC<sub>50</sub> values of 3.03 and 2.86 mg/mL respectively.

In the present study, dry ginger was evaluated for its multiple anti-Alzheimer effects. Methanolic extraction was preferred as it gave a better yield of various phytochemicals<sup>12</sup>. The antioxidant activity of any plant extract containing polyphenol and flavonoid components is due to their capacity to be donors of hydrogen atoms or electrons and to capture the free radicals. DPPH analysis is one of the tests used to prove the ability of the components of the plant extracts to act as donors of hydrogen atoms. In addition, FRAP method is another simple yet useful method which can provide antioxidant concentration based on the reduction of Fe<sup>3+</sup> to Fe<sup>2+</sup> by antioxidants in acidic medium<sup>20</sup>. The methanolic

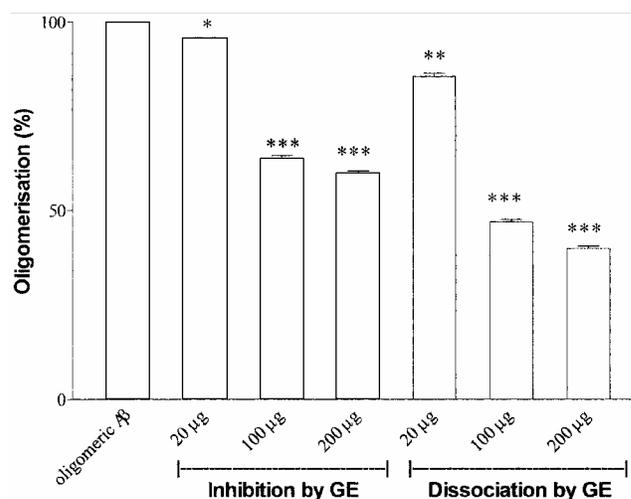


Fig. 4—Influence of methanolic GE on A $\beta$  oligomerisation. The effect of GE on formation of A $\beta$  oligomers or dissociation of preformed aggregates of A $\beta$ . [Values are as mean $\pm$ SE from 3 observations each. *P* values \* < 0.05, \*\* < 0.001 and \*\*\* < 0.0001 considered more significant versus control (Dunnett's test)].

GE manifested a significant effect in scavenging DPPH radical and ferric reducing ability by virtue of its antioxidant activity.

At present, AChE inhibitors are the first group of drugs approved by the FDA to treat mild to moderate AD. Many recent reports identifying new AChE inhibitors from plant sources<sup>6,31</sup> as the currently approved drug galantamine is derived from a plant based compound. The extent of anti-cholinesterase activity obtained with methanolic extraction in our study is >50 times more effective compared to the inhibitory activity of aqueous extracts<sup>30</sup> suggesting the preference for methanolic extraction of the dry ginger. This difference in the extent of cholinesterase inhibition could be due to the presence of certain specific secondary metabolites in methanolic extract or the synergistic effect of one or more components. Since AChE and BChE inhibition have been accepted as an effective model for managing AD<sup>32</sup>, the cholinesterases inhibition by GE could be of great importance as a possible therapeutic substance in management of AD.

In primary cell culture, GE conferred protection against A $\beta$  induced toxicity and improved the cell survival in a dose dependent manner. It is pertinent to mention at this juncture that GE per se was not toxic to the cells in culture and a concentration of up to 2 mg per  $4 \times 10^5$  cells was well tolerated by the cells in absence of A $\beta$  (unpublished data). Antioxidant activity is one of the mechanisms by which phytochemicals reduce the A $\beta$  induced toxicity in cell culture studies. Alternatively, these phytochemicals could intercalate the toxic A $\beta$  aggregates and dissociate those species into non-toxic monomers thus preventing the cell death<sup>33</sup>. Since preventing the formation of the toxic oligomers is an effective therapeutic strategy against AD, the anti-amyloidogenic potential of GE was also investigated and established.

In summary, methanolic extract of dry ginger showed therapeutic properties by acting on various molecular targets of AD. Ginger has no known acute toxicity at the usual doses consumed for dietary or medicinal purposes. In experimental trials, ginger at a dose as high as 2 g daily has been shown to be well tolerated by both experimental animals and humans with occasional reports of mild gastrointestinal complaints<sup>34</sup>. Hence, ginger can be regarded as an effective herbal supplement for AD therapy. Further studies with *in vivo* models of AD are necessary to confirm the therapeutic role of whole ginger extract.

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