Esterase’s properties in commonly used Indian spices for Alzheimer’s disease model

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Abstract

Esterase family of Hydrolases such as Acetylcholinesterase (AchE), Butyrylcholinesterase (BchE) and Carboxyl esterase (CE) have been estimated in Alzheimer’s disease (AD) model, normal brain of striatum, frontal cortex, hippocampus and in liver. In AD a loss of acetylcholine activity directly correlates with memory dysfunction due to the activation of acetylcholinesterase and butyrylcholinesterase enzymes. Therefore the effective treatment methods include restoration of cholinergic function and elevation of ACh level through inhibiting AChE and BChE. Hence to inhibit these enzymes four commonly used Indian kitchen spices viz., Cuminum cyminum, Elettaria carda momum, Cinnamomum verum, Syzygium aromaticum were selected because the extracts of these spices contain cholinesterase inhibitory activity in vitro. These spices were extracted with cold and hot aqueous solution and the anti-cholinesterase potential was measured in vitro. Elettaria cardamomum cold extract showed significant inhibition for AchE in all regions of brain for control and AD. Whereas Cinnamomum verum hot extract showed elevated activity for carboxyl esterase which is a neuroprotective factor. These findings suggest that dietary supplementation of cardamom and cinnamon in moderate amounts may aid in prevention delay in onset of AD.

Keywords: Acetylcholinesterase, Butyrlcholinesterase, Carboxyl esterase, Phytochemicals, Indian spices

Hydrolytic enzymes split different groups of biomolecules such as esters, peptides and glycosides. Hydrolytic enzymes break down protein, lipids, nucleic acids, carbohydrate and fat molecules into their simplest units. Some hydrolytic enzymes such as carboxylesterase, acetylcholinesterase and butyrylcholinesterase were stored in neurodegenerative diseases and brain tumors (Cataldo AM et al; 1996 and M Barbosa et al. 2001).

Carboxylesterases (CEs, EC 3.1.1.1) are members of the serine hydrolase super family and they are efficiently catalyze the hydrolysis of a variety of ester- and amide-containing chemicals as well as drugs (including prodrugs) to the respective free acids. They are involved in detoxification or metabolic activation of various drugs, environmental toxicants and carcinogens. CEs also catalyze the hydrolysis of endogenous compounds such as short- and long-chain acyl-glycerols, long-chain acyl-carnitine, and long-chain acyl-CoA esters (R.M. Wadkins et al. 2001; S. Benchari et.al. 2002).

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) acts on acetylcholine (ACh) to terminate its actions in the synaptic cleft by cleaving the neurotransmitter to choline and acetate (ting zhao et al., 2013) and thereby loss of acetylcholine (ACh) activity due to activation of these enzymes. This directly correlates with memory dysfunction and the loss of cognitive functions in AD patients was a continuous decline of the cholinergic neurotransmission in cortical and other regions of the human brain. In case of Alzheimer’s disease both enzymes are present in the brain which is detected in neurofibrillary tangles and neuritic plaques in cholinergic neurons. Cholinergic neurons are widely distributed throughout the mammalian central nervous system that exist as both projection neurons and inter neurons. There is evidence that central cholinergic systems may be important neural substrates for attention, an important component of the larger process termed cognition. A better understanding of the normal functions of central cholinergic systems will be a key step in exploring age related memory loss (peter et al., 2000).

In order to understand this concept earlier research studies showed that the causes and pathology of Alzheimer’s disease has been identified a number of biochemical changes that occur within the cells and throughout the brain, which contribute to improper cell functioning and cell death. This causes due to some biochemical aspects in which activation of enzymes such as Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) are involved. Increased activity of these hydrolases reported from postmortem AD brains. One of the most effective treatment strategies was suggested to restrain cholinergic function and elevate ACh level through inhibiting AChE and BChE. Among several theoretical options, the best approach has been the use of AChE inhibitors (AChEIs) which led to the introduction of tacrine as the first AChEI specifically approved for the
treatment of AD (Whitehouse 1993). Tacrine is a potent inhibitor (Ki 38 nM) of human acetylcholinesterase (hAChE), intum when the 2.4 A crystal structure of hCE1 in complex with tacrine, which is the first drug approved for treating Alzheimer’s disease.

The clinical action of tacrine in alleviating symptoms of AD is thought to occur by inhibiting hAChE and prolonging the lifetime of acetylcholine neurotransmitter in human brain (Sompop Bencharat et al., 2003). There are also other kinds of AChE inhibitors, such as donepezil, galantamine and rivastigmine are available for the symptomatic treatment of patients with mild-to-moderate AD (noridayu et al. 2011). Since the limitations of the current drugs, it is necessary to look for new lead molecules from different sources such as natural product, which can be used to target these enzymes and helps in alleviating the symptoms of AD. A variety of plants have been reported to show Cholinesterase’s (ChEs) inhibitory activity which may be relevant for treatment of AD related to cholinergic deficit (kumar et al., 2012). The Indian spices and their active principles have been shown to inhibit aggregation of Aβ (curcumin, cinnamon, ginger), prevention of Aβ induced neurotoxicity in vitro (pepper) and improve memory in AD transgenic mice (combination of acupuncture and eugenol).

Therefore in present study, four commonly used Indian kitchen spices viz., Cuminum cyminum (cumin seeds), Elettaria cardamomum (Cardamom fruits), Cinnamomum verum (cinnamon bark), Syzygium aromaticum (Clove buds) have been selected and the extracts of these spices contain cholinesterase inhibitory activity in vitro when tested with commercially available AChE. The cold and hot aqueous extracts of these spices were prepared. Further, using the frontal cortex, striatum and hippocampus homogenates were prepared from aged non-transgenic AD model rats as the source of enzymes, the choline esterase activity of each spice extract have been measured. CE was also assayed in different regions of brain (Hippocampus, frontal cortex and striatum) and liver of control and non transgenic AD model rats.

It was observed that the anti-cholinesterase effect was highest with cardamom and cinnamon extracts. Marginal inhibition was noticed with clove and cumin. These findings suggest that dietary supplementation of cardamom and cinnamon in moderate amounts may aid in prevention delay in onset of AD.

**Materials and Methods**

**Materials**

Acetylcholinesterase (EC 3.1.1.7) from electric eel, butyrylcholinesterase (EC 3.1.1.8) from equine serum, acetylthiocholine iodide (ATChI), 5, 5’-dithio-bis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Aldrich, Bangalore. Butyrylthiocholine iodide (BTChI) was purchased from Paxmy Speciality Chemicals, Chennai. All other reagents used were of analytical graded and obtained locally. Frontal cortex, striatum, hippocampus and liver tissues used in the present study were provided by Central Animal Research Facility (CARF), NIMHANS, Bangalore. These tissues were earlier dissected from non-transgenic AD rat model and age-matched Sprague-Dawley rat, stored frozen at -80°C and made available for this study.

Therapeutic rat groups were treated daily with 37 mg of Lithium chloride (LiCl) per kilogram of body weight orally for 10 days at 24 hours interval time and then protein estimation and enzyme assays were carried out similar to that of the control group.

**Plant materials and Extraction**

All plant samples namely Eletetria cardamom fruit (Voucher no.001), Cinnamomum verum bark (Voucher no.002), Syzygium aromaticum flower bud (Voucher no.003), Cuminum cyminum fruit (Voucher no.004) were purchased from a local store in Bangalore, India. The plants were authenticated by a Botanist Dr M. D. Rajanna, professor, botanical garden, University of Agricultural Sciences, GKVK, Bangalore and voucher specimens of these samples are stored in a botanical garden & herbarium at University of Agricultural Sciences, GKVK campus, Bengaluru-560065.

Fresh samples of the plant materials were air dried at ambient room temperature and powdered in mixer. Cold extract was prepared by placing one gram of each sample in distilled water (1:10 w/v) for 24h. The clarified extract was obtained by filtering through Whatman filter paper No 3. Hot extract was prepared by boiling one gram of each sample in distilled water (1:10 w/v). The sample were boiled for 15-20 minutes and cooled. The samples were filtered using Whatman filter paper No 3. The filtered samples were collected and stored at -200°C until use.

**Phytochemical Screening**

Chemical tests were carried out using aqueous extract to identify various constituents using standard methods of Sofowara, Trease and evans and Harbone.

**Preparation of Tissue Homogenates**

18-month-old non-transgenic AD model rats induced by neonatal administration of monosodium glutamate were developed as described earlier (Madhavadas et al. 2013). These rats have been shown to exhibit AD-associated molecular changes viz., elevated amyloid β and AChE levels in their hippocampi with concomitant deterioration in their cognitive skills (Madhavadas et al. 2013). The hippocampi, other regions of brain and liver from such aged AD rats were dissected. The tissue (20 mg) was homogenized in high-ionic strength buffer [1 ml of 0.01 M Phosphate, pH 8.0 containing 1 M NaCl, 10% Triton X-100, 1 mM EDTA] followed by sonication (2 x 10 sec cycles) on ice. The soluble fraction serving as AChE source was separated by centrifugation at 10,000 rpm for 20 minutes at 4°C.

Cholinesterase assay: An assessment of cholinesterase inhibition was carried out in flat-bottom 96-well microtitre plates using the colorimetric method of Ellman et al. A typical run consisted of 5μL of electric eel AChE solution, at final assay concentrations of 0.03 U/mL; 200μL of 0.1 M phosphate buffer pH 7; 5μL of DTNB at a final concentration of 0.3mM prepared in 0.1 M phosphate buffer pH 7 with 0.12M of sodium bicarbonate; and 5μL of the test extract. The reactants were mixed and pre– incubated for15 minutes at 30°C. The reaction was initiated by adding 5μL of ATChI at a final concentration of 0.5mM. As a control the inhibitor solution was replaced with buffer. To monitor any non-enzymatic hydrolysis in the reaction mixture two blanks for each run were prepared. Change in absorbance at 405 nm was measured on a 96-well plate reader for a period of 6 min at 30°C. Similarly for butyrylcholinesterase assay same procedure was followed only difference being, AChE enzyme replaced by BuChE and ATChI replaced by BTChI (kumar et al. 2012).
Enzyme Activity of Carboxyl Esterase

Enzyme activity of the carboxyl esterase in tissue extracts were assayed by spectrophotometric methods by Gomari (1941) and later modified by Van Asperen (1962). α-naphthol is the product formed by the action of esterase enzyme on α-naphthyl acetate substrate. Enzyme reaction was initiated by adding 900 µl of 5 mM α-naphthyl acetate in Phosphate assay buffer (pH 7.0) to pre incubated 100 µl tissue extract and was incubated for 15 minutes at 27ºC. Subsequently, the reaction was stopped by the addition of 500 µl DBLS reagent and enzyme activity was measured at 600 nm. The rate of esterase activity was calculated by detecting the amount of α-naphthol formed.

Results and Discussions

Extraction and Phytochemical Screening of Indian spices the four commonly used Indian kitchen spices viz., Cuminum cyminum (cumin seeds), Elettaria cardamomum (Cardamom fruits), Cinnamomum verum (cinnamon bark), Syzygium aromaticum (Clove buds) were selected for anti-cholinesterase and CE activity. The cold and hot aqueous extracts of these spices were prepared. The various regions of brain and liver homogenates were prepared from 18 months aged normal and same age group of non-transgenic AD model rats as the source of enzymes. Then the anti-cholinesterase (Inhibition of AchE and BChE) and carboxylesterase (CE) activity was measured for each spice extract.

Phytochemical analyses were performed with these spice extracts. The results confirm the presence of constituents which are known to exhibit medicinal as well as physiological activities. The Phytochemical characteristics of the spice extracts investigated are summarized in Table 1.

Table 1: Phytochemical composition of spice extract

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>cardamom</th>
<th>Cinnamon</th>
<th>Clove</th>
<th>Cumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Tannin</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Saponin</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Flavanoids</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Triterpenoids</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Phenol</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

In cardamom, alkaloids were abundant in both hot and cold extracts where as flavanoids and triterpenoids were abundant in cold extracts and phenol in hot extracts. Cinnamon spices showed alkaloids which was similar to cardamom. Other phytochemicals such as tannin, flavanoids and triterpenoids were more abundant in hot cinnamom extract and flavanoids in cold extract. Clove and cumin showed more abundant tannin in cold and hot extracts.

However flavanoids and reducing sugars were abundant in both hot and cold extracts of clove. Apart from this, steroids were found to be more in hot extract and phenol in cold extract of clove. Hot and cold Cumin extracts were rich for saponin, steroids and reducing sugar. Phlobatannin and phenol were found more in hot extracts of cumin.

These phytochemical compounds are known to be biologically active and therefore aid the medicinal as well as physiological activities.

Anti-cholinesterase inhibition by Indian spices extract

In order to test the anti-cholinesterase activity, the influence of these four spice extracts on both AchE and BchE were evaluated *in vitro* using an inhibition assay with standard enzymes AchE (electric eel) (Fig. 1) and BchE (horse serum) (Fig. 2) and were summarized in Table 1.
maximum 90% of AchE inhibition was shown by *Eletteria cardamom* and 60.6% maximum BchE inhibition was obtained by *Cinnamomum verum*. An aqueous hot extract of *Cinnamomum verum* showed the maximum inhibition of 87.8% for AchE and 71% for BchE respectively.

**Cholinesterase activity in Hippocampal region**

The activity of Cholinesterase’s were evaluated in vitro of homogenates prepared from hippocampal region dissected from control and non transgenic AD rats made available from laboratory using Ellman method and were summarized in Fig. 4 and Fig. 5.

![Figure 3: Acetylcholinesterase activity in hippocampus region.](image)

Tissue homogenates were prepared either using phosphate buffer, pH 8.0 (control P and test P) or high-ionic strength buffer (control H and test H).

The anticholinesterases showed increased activity at high ionic strength buffer (0.01M phosphate (pH 8) containing 1M Nacl, 10% triton X-100, 1mM EDTA) homogenates. Therefore the high ionic strength buffer was selected for homogenization to check the activity of these enzymes. The activity of both Acetylcholinesterase and Butyrylcholinesterase were found to be high in test homogenates when compared with their control.

![Figure 4: Butyrylcholinesterase activity in hippocampus region.](image)

Percentage inhibition of cholinesterase enzymes and activation of carboxyl esterase

Indian spices such as Cuminum cyminum (cumin seeds), Elettaria cardamomum (Cardamom fruits), Cinnamomum verum (cinnamon bark), Syzygium aromaticum (Clove buds) were selected for anti-cholinesterase and CE activity. The cold and hot aqueous extracts of these spices were prepared and used for the assay.

The inhibitory activities of the Indian spice extracts for cholinesterase were evaluated *in vitro* using the various regions of brain such as hippocampus, frontal cortex, striatum and liver homogenates as source of these 3 hydrolases. Percentage inhibition of cholinesterase enzymes and activation of carboxyl esterase are shown in Figure 5-12 and Table 3.

![Figure 5: Percentage inhibition of Choline esterase (acetylcholinesterase and butyrylcholinesterase) in striatum region](image)

![Figure 6: Percentage activation of carboxyl esterase in striatum region](image)

The present data has revealed that all of the extracts possessed potent AchE and BchE inhibitory activity at 100μg/ml concentration. Among the four spices screened, maximum AchE inhibition was shown by aqueous cold extract of Eletteria cardamom in all brain regions (control and test).
however striatum region showed maximum of 92.7% in control and 79.1% in AD model. Even the same cardamom extract showed maximum inhibition of 89.2% in control and 72.5% in AD model. Whereas aqueous cold extract of Cinnamomum verum showed inhibition by 21.7% in control and aqueous hot extract of Eletteria cardamom in AD model by 52.2% respectively in liver (Table 3).

Aqueous cold extract of Cinnamomum verum showed maximum BchE enzyme inhibition in all regions. Whereas aqueous cold extract of Cinnamomum verum showed maximum CE activity in all regions except in test liver sample (Table 3).
Tacrine is a potent inhibitor (Ki_38 nM) of human acetylcholinesterase. The reason for this result is known from earlier reports where 2.4 Ao crystal structure of hCE1 in complex with tacrine, which is the first time which is complimentary result for AD treatment. All of these Indian spices which showed complete % inhibition for AchE, also showed more activation for CE enzyme for the current study showed the importance of Indian spices towards physiological activities. The phytochemical compounds are known to be biologically active and therefore involved in medicinal as well as physiological activities. The phytochemicals like alkaloids and flavanoids play a role in the treatment of AD. Percentage inhibition of Cholinesterase and Activation of Carboxyl esterase with Phytochemicals of Indian spices are mentioned in Table 4.

Table 4: Percentage inhibition of Cholinesterase and Activation of Carboxyl esterase with Phytochemicals of Indian spices

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Spices extract</th>
<th>Control region</th>
<th>Test AD region</th>
<th>ST region</th>
<th>LV region</th>
</tr>
</thead>
<tbody>
<tr>
<td>%AChE Inhibition</td>
<td>1. Cardamom cold extract</td>
<td>89.2</td>
<td>72.5</td>
<td>37.7</td>
<td>79.1</td>
</tr>
<tr>
<td></td>
<td>Cardamom hot extract</td>
<td>73.6</td>
<td>49.1</td>
<td>71.1</td>
<td>36.9</td>
</tr>
<tr>
<td></td>
<td>Cinnamon cold extract</td>
<td>76.5</td>
<td>35.3</td>
<td>71.1</td>
<td>48.7</td>
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<tr>
<td></td>
<td>Cinnamon hot extract</td>
<td>68.0</td>
<td>39.4</td>
<td>64.4</td>
<td>44.8</td>
</tr>
<tr>
<td></td>
<td>Clove cold extract</td>
<td>51.8</td>
<td>27.1</td>
<td>6.0</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>Clove hot extract</td>
<td>44.5</td>
<td>25.9</td>
<td>0.0</td>
<td>1.8</td>
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<tr>
<td></td>
<td>Cumin cold extract</td>
<td>68.7</td>
<td>35.0</td>
<td>59.5</td>
<td>45.2</td>
</tr>
<tr>
<td></td>
<td>Cumin hot extract</td>
<td>64.2</td>
<td>26.1</td>
<td>50.0</td>
<td>35.9</td>
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<tr>
<td>%BChE Inhibition</td>
<td>1. Cardamom cold extract</td>
<td>0.47</td>
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<td>8.3</td>
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<td>1.6</td>
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<tr>
<td></td>
<td>Clove cold extract</td>
<td>0</td>
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<tr>
<td></td>
<td>Cumin cold extract</td>
<td>0</td>
<td>0.0</td>
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<tr>
<td></td>
<td>Cumin hot extract</td>
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<td>0.0</td>
<td>0.0</td>
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<tr>
<td>%CE Activation</td>
<td>1. Cardamom cold extract</td>
<td>46.3</td>
<td>12.9</td>
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<td></td>
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<td>46.3</td>
<td>0.56</td>
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The reason for this result is known from earlier reports where 2.4 Ao crystal structure of hCE1 in complex with tacrine, which is the first drug approved for treating Alzheimer's disease (Sompop Bencharit et al., 2003). Tacrine is a potent inhibitor (K_i 38 nM) of human acetylcholinesterase (hAcChE). Tacrine co-crystallized in the active site of human recombinant CES1 but was not found to inhibit the enzyme due to the large size of the active site. Hence tacrine did not significantly inhibit CES1 activity in the assay (Bencharit et al.2003). Therefore the current study showed the importance of Indian spices towards the activation of CE even though it belongs to a family of esterase especially with cold extract of Cinnamomum verum in control than in test AD and showed inhibitory activity for cholinesterases.

The lack of BChE inhibitory activity of the spice extracts with tissue homogenates may be due to the interference by some of the constituents present in these extracts. Further confirmation of this speculation is achievable by fractionation of the extracts prior to enzyme assays.

Percentage inhibition of Cholinesterase and Activation of Carboxyl esterase with Phytochemicals of Indian spices--novel

The phytochemical compounds are known to be biologically active and therefore involved in medicinal as well as physiological activities. The phytochemicals like alkaloids and flavanoids play a role in the treatment of AD. Percentage inhibition of Cholinesterase and Activation of Carboxyl esterase with Phytochemicals of Indian spices are mentioned in Table 4.

All of these Indian spices which showed complete % inhibition for AchE, also showed more activation for CE enzyme for the first time which is complimentary result for AD treatment. All these spices, showed maximum AchE inhibition in striatum region at the same time activated CE enzyme in hippocampus,
i.e., region of learning and memory. Among all the spices cardamom and cinnamon showed maximum anticholinesterase inhibition. This can be due to the presence of phytochemicals alkaloids and flavonoids.

The importance of Phytochemicals is discussed since earlier reports of natural alkaloids such as galantamine or alkaloid-related synthetic compounds (such as rivastigmine) are considered as beneficial for patients with mild-to-moderate AD. Many natural alkaloids have been studied as AChE-Is, but relatively few compounds have entered in therapeutic use. There are many different classes of compounds have been considered, namely indole derivatives (such as Physostigmine and related compounds), isoquinoline and related derivatives (such as galantamine and lycorine-type alkaloids), steroids, terpenoid alkaloids and many other derivatives that showed significant inhibitory effects on AChE (Stefano Dall’Acqua et al; 2013).

Epidemiological studies suggest that a lower rate of incidence of AD in Indian population correlating with higher consumption of traditional Indian spices in their daily diet. The possible beneficial effect seen with spices could be due to their cholinesterase inhibitory activity. This is shown with aqueous extracts of spices such as cardamom, cinnamon, cloves and cumin seeds in control and AD model from non-transgenic rats. The anti-cholinesterase effect was highest with cinnamon and cardamom extracts. The same spices showed activation for CE (hippocampus) which is neuroprotective factor. Marginal inhibition was noticed with clove and cumin. These findings suggest that dietary supplementation of cardamom and cinnamon in moderate amounts may aid in prevention and delay in onset of AD and Modulation of CE activity may help in the treatment of AD.

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References


