Anti-cholinesterase potential of *Cinnamomum tamala* (Buch.-Ham.)
T.Nees & Eberm. leaves

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Received 07.10.13, revised 25.11.13

*Cinnamomum tamala* (Buch.-Ham.) T.Nees & Eberm. (Lauraceae) leaves are well known as bay leaves which are popular for its aroma. A part from its extensive culinary uses, this spice has several uses in traditional practice for treatment of rheumatism, immunomodulation and also used as brain tonic. The cinnamon oil locally known as *Tejpat oil* obtained from the bay leaves used in alcoholic beverages and confectionaries. The present investigation was aimed to screen the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity of the extract of *C. tamala*. Standardization of the cinnamon oil of bay leaves was performed by HPLC method using linalool as biomarker. *In vitro* evaluation of anticholinesterase activity of bay leaves extract and oil was performed by TLC bio-autography and 96 well micro titer plate methods. HPLC analysis was confirmed the presence of linalool as an important phytoconstituents and its retention time was found to be 5.314 min. The outcome of the study demonstrated that the cinnamon oil obtained from leave of *C. tamala* possess maximum inhibition against AChE (IC\textsubscript{50}: 94.54 ± 0.774 µg/ml) and BChE (IC\textsubscript{50}: 135.56 ± 0.912 µg/ml) than the methanol extract. The result also explains that *C. tamala* is more sensitive to AChE enzyme than the BChE. Hence, *C. tamala* may be explored as anti-cholinesterase agent further for the better and safer management of Alzheimer’s diseases.

**Keywords:** *Cinnamomum tamala*, Acetylcholinesterase, Butyrylcholinesterase, Linalool, Spices

**IPC Int. Cl.** A61K 36/00, CO1, CO7, A23L 1/22, C12N, A61K 38/43, C11C 3/00, BO3B 3/02

*Cinnamomum tamala* (family Lauraceae) is also known as Indian Cassia. The leaves are commonly called as bay leaves. For centuries, it is used as home culinary spices for its rich aroma. In traditional healing practices, bay leaves are used as brain tonic, anthelminthic, diuretic and also used in treatment of liver and spleen inflammation. The cinnamon oil of the bay leaves is locally known as *Tejpat oil* is extensively used for flavoring foods and formulation of liquors and confections. This buff colored oil is used in wide range of diseases, viz. anti-flatulent, diuretic, hypoglycemic and analgesic in dental preparations. Reported GC–MS analysis of the oil showed the presence of 63 compounds in it, among them β-Caryophyllene, linalool and caryophyllene oxide are the main constituents. Apart from this, therapeutic phytochemicals compounds like, viz. α-pinene, camphene, myrcene, limonene, 1, 8-cineole, p-cymene, methyl eugenol and eugenol acetate are also reported in oil.

Level of acetylcholine in the brain decreases in the Alzheimer’s disease (AD); which is principally characterized by impaired memory and disturbed behavior thus, AChE and BChE inhibitors are being developed for the symptomatic treatment of AD. The results of previous studies at our laboratory indicated that essential oils obtained from spices possess significant AChE/BChE inhibitory activity. The current study was undertaken to evaluate its anticholinesterase potential of the standardize extract of *C. tamala* so as to validate the neuroprotective activity of this spices.

**Methodology**

**Collection and authentication of plant material**

The dried leaves (200 gm) of *C. tamala* was collected in the month of November 2011 from a commercial supplier of Kolkata, West Bengal, India and authenticated. A voucher specimen of the plant material (SNPS 1259) has been deposited in the herbarium of School of Natural Products Studies, Jadavpur University, India for further study.

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Chemical and reagents

5, 5-dithiobis[2-nitrobenzoic acid] (DTNB), Galantamine and Linalool was procured from Sigma (Poole, UK). Acetylcholinesterase enzyme (AChE) from bovine erythrocytes, butryrylcholinesterase (BChE) from equine serum, acetyl thiocholine iodide (ATCI), butyrylthiocholine iodide (BTCl), methanol and all other organic solvents (HPLC reagent grade) were purchased from Merck, India.

Cold maceration

Leaf of C. tamala was air dried in the shade and pulverized into coarsely powder in a mechanical grinder (Bajaj platini, India, PX 73 M, wattage: 550w, voltage: 230 v 50 Hz AC). The powdered plant material (190 gm) was extracted by cold maceration with (750 ml) methanol. The powder was soaked in methanol for 72 hrs with occasional stirring at room temperature. After 72 hrs, the same procedure was followed thrice for maximum extraction from the powder. All the extracted solvent then pooled together; filtered and dried under reduced pressure by rotary vacuum evaporator to yield a dark semisolid residue (76 gm). % yield was found to be (7.6) w/w. The extract was stored in the refrigerator and fresh solutions of the extracts were prepared for further study.

Phytochemical Screening

Phytochemical screening of the methanol extract of the leaves was performed to know the presence of the active chemical constituents such as sterol, carbohydrate, alkaloids, phenols, flavonoids, terpenoids, saponins and amino acid.

Preparation of cinnamon oil

Dried powdered leaves of C tamala were hydro-distilled in a Clevenger apparatus to obtain cinnamon oil. Briefly, leaves powder (100 gm) was soaked in water for 2 hrs and the oil was separated from the upper layer. The oil was then dried over anhydrous Na₂SO₄ and stored at 4°C. It was transparent, golden yellow coloured and with strong smell of spice. The yield of the oil was found to be (3.2%) v/w.

HPLC analysis of cinnamon oil of C. tamala

Cinnamon oil and linalool standard were prepared in methanol. The prepared sample was filtered through a whatman (Germany) NYL 0.45 µm pore size membrane filter. Further, Chromatographic analysis was performed in reverse phase high performance liquid chromatography (RP-HPLC) (Shimadzu Prominence, Kyoto, Japan) which equipped with Shimadzu LC-20 AD UFLC reciprocating pumps with variable Shimadzu SPD-M20A Prominence PDA detector. 20 µl of cinnamon oil and standard linalool were used for HPLC analysis at a concentration of 1 mg/ml. The prepared sample was injected into 20 µl capacity of Rheodyne loop injector. Standard linalool and cinnamon oil of C. tamala were analyzed on a Phenomenex-Luna (Torrance, CA, USA) C18 column (250×4.6 mm, 5 µ particle size) as stationary phase and methanol: water (80:20 v/v) as mobile phase in which 1 ml/min maintained the flow rate. During elution the temperature of the column was maintained at 55°C and reading was monitored at 210 nm. A calibration curve was prepared by using range of standards to quantify the percentage of linalool present in oil. The chromatographic peak corresponding to cinnamon oil was identified by comparing the retention time of a standard linalool.

TLC Bio-autography assay

TLC bio-autographic assay was used to detect the most bioactive constituents present in the extract by modified reported method. C. tamala methanol extract (10 mg/ml) was chromatographed over TLC plates (2.5 mm silica gel G, 60F254, Merck, Darmstadt, Germany). The plates were then developed with toluene-ethyl acetate 80:20 (v/v) as mobile phase. The dried TLC plates were subjected to AChE/ BChE enzyme inhibitory activities by spraying with Ellman’s reagent. Again, the plate was saturated by spraying DTNB/ATCI reagent for AChE inhibition at 3 U/ml was sprayed on it and waited for 5 min to appear a white spots within yellow background. This result was documented immediately for inhibition of the compounds. White spots appeared on the TLC plates was developed in the same way without the sample to determine the false-positive reaction and compared with true inhibition for further confirmation.

96 well microtiter plate method

AChE and BChE inhibitory activity of the extract, cinnamon oil, linalool and standard galantamine was monitored spectrophotometrically in Bio Rad 96-well microtiter plate reader (680 XR, USA). Substrates
of ATCI/BTCI, reaction mixture of DTNB, bovine erythrocyte AChE and equine serum BChE were used as source of enzyme to measure the cholinesterase activity. The reaction was initiated with hydrolysis of the substrate ATCI/BTCI to form a resultant product thiocholine which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate which was yellow in color, catalyzed by AChE/BChE at a wavelength of 405 nm. 96-wells plates contained 125µl of 3mM DTNB, 25µl of 15mM ATCI (BTCI when measured the BChE inhibition), 50 µl of buffer and 25µl of sample was dissolved in phosphate buffer and the absorbance was read at 405 nm every 13 s for 65 s. After, 25 µl of 0.22 U/ml of AChE or BChE was added in each well and the absorbance was measured at every 13 s for 104 s. The experiments were done in triplicate. The % inhibition of the enzyme activity was calculated by comparing the rates of reaction of samples relative to blank sample (ethanol in phosphate buffer, pH = 8). Inhibition curves were obtained for each compound by plotting between absorbance and time. The linear regression parameters were determined for each curve and the IC₅₀ values were extrapolated.

**Statistical analysis**

Data were expressed as Mean ± SEM. IC₅₀ value was calculated from sigmoidal dose response curve (variable slope) obtained by plotting the percentage of inhibition versus the concentrations. All data analysis were performed by using Graph Pad Prism (4.0) and Excel data sheet (Microsoft office 2007).

**Results**

The preliminary phytochemical screening of the *C. tamala* methanol extract of leaves showed the presence of sterol, alkaloid, phenolic compound, flavonoid, tannin, terpene and saponin.

**HPLC analysis of cinnamon oil of *C. tamala***

HPLC profile of standard linalool and cinnamon oil was developed in RP-HPLC. Retention time (Rₜ) of linalool about 5.3 min and the correlation coefficient of 0.99 showed a good linearity in concentrations ranging from 0.5 to 25 µg/ml. The percentage of linalool present in cinnamon oil was calculated from the experimental peak areas by interpolation to standard calibration curves. The maximum concentration of linalool content was found to be 0.0328% in cinnamon oil. 210 nm detection wavelengths were used to quantify linalool in the samples. The HPLC chromatogram of standard linalool and cinnamon oil has been shown in (Figs. 1a & b). Thus, the result indicates presence of linalool as a biomarker compound in cinnamon oil. So, the present method could be used to quantify the linalool in cinnamon oil.

**TLC bio-autography assay**

Standardized methanol extracts of *C. tamala* (10 mg/ml) was spotted in TLC plates. Inhibiting spots of *C. tamala* extract on the TLC plate was developed with mobile phase of toluene-ethyl acetate 80:20 (v/v). Cholinesterase (ChE) inhibitory activity was determined by spraying with substrate solution of ATCI/BTCI, reactive agent DTNB and AChE/BChE enzyme concentration. After spraying, a white spot with yellow background was visualized in TLC plate. The most prominent spot was observed at the Rᵣ of 0.68, which is the Rᵣ value of *C. tamala* methanol extract, and it was not the corresponding false positive spot. From this it may be assumed that bay leaves have potential cholinesterase (ChE) inhibitory activity.

**96 well microtiter plate method**

The enzyme inhibitory activity was measured in a 96-well micro plate using a micro plate reader (Benchmark Micro plate Reader, Bio-Rad) at 405 nm with acetylthiocholine (1 mM) as substrate and
Ellman's reagent as DTNB. The IC₅₀ values of methanol extract, cinnamon oil, linalool and galantamine (standard) has been shown in Table 1. Cinnamon oil obtained from the C. tamala leaf showed better AChE/BChE activity than methanol extract. Linalool was found to be major biomarker of the cinnamon oil which showed strong inhibition of AChE with (IC₅₀: 55.10 ± 0.191 µg/ml). The methanol extract and its cinnamon oil showed AChE inhibitory activity with IC₅₀ value (108.43 ± 0.331 µg/ml) and (94.54 ± 0.774 µg/ml). In general, methanol extract, cinnamon oil, linalool and were found to be more potent on AChE than BChE. Cinnamon oil (IC₅₀: 135.56 ± 0.912 µg/ml) and biomarker linalool (IC₅₀: 92.46 ± 0.487 µg/ml) were found to be the most potent inhibitors of BChE. Methanol extract (IC₅₀: 144.97 ± 1.021 µg/ml) was least active on BChE. Reference compound galantamine were found to be most potent inhibitor on BChE (IC₅₀: 34.78 ± 0.364 µg/ml) then AChE (IC₅₀: 29.15 ± 0.075 µg/ml).

In order to further characterize the inhibition potential, the dose response relationships of the acetylcholinesterase and butyrylcholinesterase inhibition have been shown in (Figs. 2 & 3). A dose dependent AChE/BChE inhibition enzyme was observed with the extract, cinnamon oil, linalool and standard compound used. The linear plot showed that linalool has higher % inhibition value comparing to the standard galantamine (Figs. 2 & 3).

Discussion

AChE/BChE are the chief enzymes which involves in symptom generation and underlying progression of AD. In progression of AD, concentration of AChE is lost up in specific brain regions; whereas BChE levels chiefly the G1 form, rise with in hydrolyzing excess of Acetylcholine (ACh). Though, the specificity of BChE is very less towards hydrolyzing ACh but it is generally viewed as an analogue of AChE, thus it plays an important role in aggravating AD, especially at a very low concentration of AChE. BChE is an enzyme which catalyses the hydrolysis of ACh, a cholinergic neurotransmission regulates in AD progression. Thus, the BChE inhibition emerged to be another approach to intervene in the progression of AD. Literature study reveals that AChE/BChE inhibitors of plant origin are well utilized for the management of mild to moderate form of AD. In the present study BChE inhibitory activity was targeted along with the AChE inhibitory activity of bay leaf. % inhibition activity of the cinnamon oil, and its extracts showed significant increment in the enzyme inhibition compared to the standard compound linalool.

During phytochemical screening, it was observed that terpene is one of the phytoconstituents of the methanol extract. Lopez and Pascual-Villalobos have shown in Table 1- IC₅₀ values of the C. tamala extract, cinnamon oil, Linalool and galantamine against acetylcholinesterase and butyrylcholinesterase (values are expressed as Mean ± SEM, N = 6).

<table>
<thead>
<tr>
<th>Sample</th>
<th>AChE inhibition</th>
<th>BChE inhibition</th>
</tr>
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<tbody>
<tr>
<td>Methanol extract</td>
<td>108.43 ± 0.331</td>
<td>144.97 ± 1.021</td>
</tr>
<tr>
<td>Cinnamon oil</td>
<td>94.54 ± 0.774</td>
<td>135.56 ± 0.912</td>
</tr>
<tr>
<td>Linalool</td>
<td>55.10 ± 0.191</td>
<td>92.46 ± 0.487</td>
</tr>
<tr>
<td>Galantamine</td>
<td>29.15 ± 0.075</td>
<td>34.78 ± 0.364</td>
</tr>
</tbody>
</table>

Values are Mean ± SEM, N= 6 (N = Number of replicates)

Fig. 2-Dose response curve of C. tamala methanol extract, cinnamon oil, linalool and galantamine against acetylcholinesterase

Fig. 3-Dose response curve of C. tamala methanol extract, cinnamon oil, linalool and galantamine against butyrylcholinesterase
been reported that linalool, monoterpenoid compound showed a reversible competitive AChE inhibition\textsuperscript{22}. Linalool is a terpene alcohol compound and it is widely used in purification process of flavour and fragrance obtained from natural products\textsuperscript{23}. Linalool is an important phytomarker of bay leaves, as evidenced from the HPLC standardization and ChE inhibitory activity. These findings suggested that AChE/BChE inhibitory activity of the methanol extract and its cinnamon oil may be due to the monoterpenoid constituents.

The results of the present study also indicated that cinnamon oil of bay leaves was found to be more active in comparison methanol extract. This may be due the maximum content of linalool in the cinnamon oil of \textit{C. tamala}. Further finding suggest that the affinity of bay leaves was more towards the AChE than BChE. Plants therefore provide useful resource of therapeutic lead compounds\textsuperscript{24,25}. Bay leaves and its oil may be explored further as a potential lead in development of anticholinesterase inhibiting agents for the management of Alzheimer’s disease.

**Traditional significance of study to the farmers/society/researchers and some constructive recommendations**

Leaves of \textit{C. tamala} are widely employed as spice. The essential oil obtained from it after distillation are very useful in traditional medicine and used for several ailments especially by people in Northen part of India. The plant species is also considered as a potential source of economic value to farmers by supplying the raw material to local industries. The dried leaves of the plant has been extensively used in traditional practice by Manipuri (India) farmer to protect the food crops against pest manifestation. This common practice has been observed in the farmers of Manipur, India. This is due to the fact that the leaves of the plant produces an odour that repelled the agriculture insects in paddy crops\textsuperscript{26}. Some traditional healers and rural farmers of many parts of India, also advise to take the mixture of three to four pieces of Tejpata (\textit{C. tamala}), 50 gm Ada (\textit{Zingiber officinale}) and Aswatha (\textit{Ficus religiosa}) leave extract along with water to reduce cough and cold in domestic animals\textsuperscript{27}. Over the years, traditional healer of Uttarakhand region (India) are prescribing mixtue of water and leaf powder (5 gm) of this plant, taken on daily basis to cure diabetes condition\textsuperscript{28}. Due to its aromatic character, it is kept in clothes and also chewed on mouth to keep away bad odour. Traditionally, in Punjab, leaves of the extract have been used in treatment of rheumatism, colic pain and diarrhoea\textsuperscript{29}.

The plant parts have been used traditionally for its different activities i.e carminative, anthelmintic, diuretic, colic, dyspepsia, and diarrhoea, which have also been verified scientifically as well. Eswaran \textit{et al}. (2010) studied the gastroprotective activity of an hydro-alcoholic (ethanol 50\%) extract of \textit{C. tamala} leaves in ethanol (EtOH), cold-restraint stress and pylorus ligation induced ulcers in rats at successive dose label of 50,100 and 200 mg/kg, p.o, twice daily for 5 days. Pretreatment with \textit{C. tamala} extract significantly decrease gastric ulcer in dose dependent manner and simultaneously shows antioxidant activity. The experimental study shows that the gastroprotective effect of \textit{C. tamala} leave extract in rats due to decrease level of H+K+ATPase activity, volume of gastric juice, acid output of the affected region and increase the level of catalytic enzyme in mucus membrane of gastric wall\textsuperscript{30}.

The leaves of the \textit{C. tamala} has been used in Indian ayurvedic medicine as nerve tonic to brain and also an ingredient of cyavana’s elixir, a rejuvenating liquid herbal preparation used for repairing of nervous tissue and associated disorder\textsuperscript{31}. A wide range of bioactive components from leaves and essential oil of \textit{C. tamala} extract have been isolated and most of them have shown useful biological activities belonging mainly polyphenolic, monoterpenes, sesquiterpene and flavonoid. Recent examination of several monoterpenoids compounds have revealed the potent acetylcholinesterase activity against pest insect, among them fenchone, γ-terpinene, geraniol and linalool have shown highest acetylcholinesterase inhibition activity\textsuperscript{22}. Our result also suggest that linalool, monoterpenoid compound, a prominent bioactive constituents of essential oils and extract of \textit{C. tamala} leave have shown strong cholinesterase inhibition activity by \textit{in vitro} study. Cholinesterase (acetylcholinesterase and butyrylcholinesterase) inhibitors are the current clinical strategy and USA FDA approved compounds used for treatment of Alzheimer’s disease\textsuperscript{7}. Several plant species have been proved to be effective in CNS and related disorder by variety of \textit{in vitro} and \textit{in vivo} study, and their mechanisms of actions have been well established through neurochemical approaches.
Conclusion
Leaves of *C. tamala* is an important culinary spice with active medicinal properties and aromatic character of cinnamon oil is extensively used as flavouring agent in confection industry. Linalool, an active monoterpene compound have been identified as medicinal important phytoconstituents for CNS activities. The outcome of this study further explores the cholinesterase inhibitory activity of *C. tamala* leave extract and its oil and provide supportive evidence of anticholinesterase agents for the treatment of Alzheimer's disease.

Acknowledgement
Thanks are also due to the AICTE for providing QIP research scholarship to the first author.

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