Supporting Information to:

*In vitro* Acetylcholinesterase Inhibitory Activity of the Essential Oil from *Acorus calamus* and its Main Constituents

Pulok Kumar Mukherjee\(^1\),\(^2\)
Venkatesan Kumar\(^1\)
Mainak Mal\(^1\)
Peter J. Houghton\(^2\)

**Affiliation:** \(^1\) School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India
\(^2\) Department of Pharmacy, Pharmacognosy Research Laboratories, Franklin-Wilkins Building, King’s College London, London, UK

**Correspondence:** Dr. Pulok K. Mukherjee
School of Natural Product Studies
Department of Pharmaceutical Technology
Jadavpur University
Kolkata – 700032
India
Phone: + 91-33-2429-8313
Fax: + 91-33-24146046
E-mail: pknatprod@yahoo.co.in
Materials and Methods

Extraction of *A. calamus* rhizomes
Freshly collected plant materials were dried in shade, powdered (500 g), extracted with ethanol (70%) after standing for 48 h at room temperature, and the hydroalcoholic extract was drained off. This process of extraction at ambient temperature was repeated four times. The combined hydroalcoholic extract was filtered through filter paper and evaporated to dryness under reduced pressure in a Rotavapor at 45 °C. The extract was then freeze-dried and was further used for screening purpose. The percentage yield of the extract was 23.98 % (w/w).

Extraction of essential oil from *A. calamus* rhizomes
The essential oil was obtained by steam distillation of freshly dried and pulverized rhizomes of AC for 5 h (600 g). The apparatus was an all glass apparatus with Teflon stopcocks and sleeves. The essential oil is a pale yellow viscous liquid having a pleasant woody and spicy odour, which is bitter to the taste. The essential oil obtained was dried over anhydrous sodium. The percentage yield of the essential oil was 0.95% (v/w).

Chemicals
Acetylcholinesterase (AChE) enzyme from bovine erythrocytes, acetylthiocholine iodide (ATCI), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), β-asarone (70%), α-asarone (98%) and physostigmine were purchased from Sigma-Aldrich (Sigma-Aldrich; Poole, UK). Methanol and all other organic solvents (analytical reagent grade) were purchased from Merck (Poole, UK). AChE and DTNB stock solutions were prepared using phosphate buffer (pH 8), and the ATCI stock solution was prepared with glass distilled water.

GC/MS analysis of the essential oil
The identification and quantification of major constituents in the essential oil was carried out using GC/MS analysis (Fig. 1S). Essential oil, β-asarone and α-asarone samples were dissolved in methanol (2 μL). GC/MS analysis was performed on a Jeol AX505W instrument. The operating conditions were as follows: column fused silica capillary column, TC-wax, 60m × 0.25 mm, film thickness 0.25 μm; column temperature: 40 – 300 °C increasing at 5 °C/min to 150 °C,
then 15 °C/min to 300 °C, ending at 300 °C for 10 min; injector: 180 °C; carrier gas: helium at a flow rate of 2 mL/min; column head pressure: 180 kPa; injection volume: 2 μL; ionization energy: 70 eV; ion source temperature: 300 °C. Chemical components were identified by comparing their retention times and mass spectra with those of authentic samples.

Fig. 1S GC/MS total ion chromatogram of the essential oil from *Acorus calamus* L.
**Enzyme assay**

AChE activity was measured using a 96-well microplate reader [2], [3] based on the Ellman’s method [1]. The enzyme hydrolyzes the substrate acetylthiocholine resulting in the product thiocholine which reacts with Ellman’s reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptotthiocholine and 5-thio-2-nitrobenzoate which can be detected at 405 nm. In the 96-well plates, 125 μL of mM DTNB, 25 μL of 15 mM ATCI, 50μL of buffer and 25 μL of sample dissolved in buffer containing not more than 10% methanol were added to the wells followed by addition of 25 μL of 0.28 U/mL AChE. The absorbance was measured at 405 nm every 13 s for 65 s. 25 μL of 0.22 U/mL of enzyme were then added, and the absorbance was again read every 13 s for 104 s at 25 ºC. The absorbance was read using a Perkin Elmer microplate reader (Wallace VICTOR² 1420 Multilebel Counter; Perkin Elmer) at 405 nm. Enzyme activity was calculated using the slope of the absorbance VS time plot and expressed as percentage compared to an assay using a buffer without any inhibitor. Any increase in absorbance due to the spontaneous hydrolysis of substrate was corrected by subtracting the rate of the reaction before adding the enzyme from the rate after adding the enzyme. Percentage of inhibition was calculated by comparing the rates for the sample to the blank (10% MeOH in buffer). A range of concentration was used so that the IC₅₀ value could be calculated. The inhibition rate (%) was calculated by the equation:

\[
\% \text{ Inhibition} = \frac{[(\text{Blank} – \text{Blank positive control}) – (\text{Experiment} – \text{Experiment control})]}{\text{Blank} – \text{Blank positive control}}.
\]

**References**