Acetylcholinesterase and NADH oxidase inhibitory activity of some medicinal plants.

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Acetyl-cholinesterase (EC 3.1.1.7) comprise a family of enzymes which include serine hydrolases. Acetyl-cholinesterase (AChE) catalyzes the hydrolysis of the neurotransmitter acetylcholine and termination of the nerve impulse in cholinergic synapses. AChE inhibitory activities were determined as given in Materials and Methods. Results show that the following plants have more than 50% inhibition of AChEase enzyme activity at 250 µg plant extract. *Onosma bracteatum* 61.12±4.28, *Orchis mascula* 59.80±2.2, *Cymbopogon jawarancusa* 70.6. AChE is used in the treatment of Alzheimer's disease and other related diseases. NADH oxidase (EC 1.6.99.3) catalyzes the two-electron reduction of oxygen to peroxide or the four-electron reduction of oxygen to water. 50% inhibition of NADH oxidase inhibitory activity was recorded at 250 µg plant extract of *Alhagi Maurorum* 60.48±1.40 with *Myristica fragrans* 52.69±2.99, *Nepeta hindostana* 57.53±3.43, *Pinus gerardiana* 70.43±7.0. Other plant extracts showed <50% inhibitory activities against the two enzymes.

Key words: Medicinal plants, acetyl cholinesterase, NADH oxidase.

INTRODUCTION

Acetylcholinesterase (AChE) comprises a family of enzymes which include serine hydrolases. They share about 55% of amino acid sequence identity, and have similar catalytic properties. The different specificities for substrates and inhibitors are due to the difference in amino acid residues of the active sites of AChE and BChE. The enzyme system is responsible for the termination acetylcholine at cholinergic synapses (Cygler et al., 1993). The major function of AChE is to catalyze the hydrolysis of the neurotransmitter acetylcholine and termination of the nerve impulse in cholinergic synapses (Quinn, 1987). Cholinesterase inhibitors are considered to be an important and ongoing strategy to introduce new drug candidates for the treatment of Alzheimer's disease and other related diseases (Bertaccini et al., 1982).

NADH oxidase (EC 1.6.99.3) catalyzes the two-electron reduction of oxygen to peroxide or the four-electron reduction of oxygen to water. NADH oxidase share the ability to reduce oxygen, their physiological role is to reduce oxygen and to catalyse different reactions (Serve et al., 2003). NADH oxidases are becoming a potential target for therapeutic interventions in vascular disease (Rajagopalan et al., 1996).

Plants secondary metabolites have been used as inhibitors of various classes of enzymes. Several thousand plant extracts have been screened against AChE from different parts of the world (Ingkaninan et al., 2003; Orhan et al., 2004; Ferreira et al., 2006; Adsersen et al., 2006; Mukherjee et al., 2007; Vinutha et al., 2007). Recently, Shahwar et al., (2010) and Lee et al., (2011) have demonstrated the AChE inhibition activities from various plants. These plants, therefore, have potential therapeutical importance. Literature survey has revealed hardly a report on the NOX inhibition studies of plant extracts. The present studies were carried out to screen methanolic extracts of 50 plants of various families against AChE and NOX. The presented work is the first in the series of several papers to come.
**MATERIAL AND METHODS**

**Chemicals**

Acetylthiocholine iodide, 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB) and nicotinamide adenine dinucleotide, reduced form (NADH+2) were purchased from Sigma Chemical Inc, USA. All other chemicals were of analytical grade from Merck/Fluka/Sigma.

**Preparation of plant extract**

Plants were dried under shade and ground in an electric grinder to prepare a fine powder. Ground powder (100 g) was soaked in 400 ml methanol for two weeks with occasional shaking. The soaked material was filtered through miracloth. This process was repeated 3 times with 200 ml methanol to extract maximum material and all contents were pooled. The solvent was evaporated at room temperature or under vacuum with rotary evaporator. The residue was collected in small glass bottles and stored at 4°C. Plant methanolic residue was weighed and dissolved in 100 mM Tris buffer pH 7.4 which dissolved maximum residue and this stock solution of 1 mg/ml was used for the determination of enzyme activities. This was done to avoid denaturation of enzymes activities. This was done to avoid denaturation of enzymes due to organic solvent during the assay and get reproducible data.

**Preparation of enzyme source**

Fresh chicken liver (1 g) was purchased from the local market, washed with 50 mM Tris-HCl buffer pH 7.4 and homogenized in 10 ml extraction buffer (50 mM Tris-HCl buffer pH 7.4, 1mM MgCl2, 1mM CaCl2, 0.32M sucrose) in a homogenizer for 15 sec each after 10 sec intervals (De Silva et al., 2006). Test tube was placed in ice bucket to avoid heating. Contents were filtered through 3 layers of miracloth and centrifuged at 15,000 rpm for 15 min at 4°C (Model 1710, Kubota, Japan). The supernatant was used as a source of enzyme. Enzyme source was made fresh everyday and used within 4 hours. Protein was determined by Bradford method and 40-60 µg protein (10 µl) was used per assay.

**Determination of AChE activity**

Enzyme activity was measured by the method of Ellman et al., (1961). Enzyme activity reaction mixture (200 µl) consisted of 160 µl of 50 mM Tris HCl buffer, pH 7.4, with/without plant extract followed by the addition of 10 µl enzyme (40-60 µg protein) from fresh chicken liver homogenate in 96-well plates. The contents were mixed and preincubated for 10 min at 25°C. Plates were pre-read at 412 nm using Synergy HT BioTek (USA) plate reader. The reaction was initiated by the addition of 10 µl of 1 mM DTNB and 3 mM substrate acetylthiocholine iodide. After 15 min incubation, absorbance was measured at 412 nm within 4-7 min. Control experiments were carried out to correct for non-enzymatic hydrolysis by adding enzyme after the addition of DTNB. Absorbance values were subtracted from the control and data presented as percent inhibition of enzyme activity. All experiments were carried out with their respective controls in triplicate.

**Determination of NOX activity**

NOX activity was determined in total volume of 200 µl reaction mixture which consisted of 160 µl 50 mM Tris HCl buffer, pH 7.4 containing 1 mM EDTA Na2, with/without plant extract followed by the addition of 10 µl enzyme (40-60 µg protein) from fresh chicken liver homogenate. The contents were mixed and preincubated for 10 min at 25°C. The reaction was initiated by the addition of 10 µl of 3 mM NADH+2. After 45 min incubation at 25°C, absorbance was measured at 340 nm using Synergy HT BioTek (USA) 96-well plate reader. All experiments were carried out with their respective controls. Results are mean of three independent determinations.

**RESULTS**

**Standardization of AChE assay and plant inhibitory activity**

AChE assay was standardized with varying concentrations of enzyme and substrate to reach the final protocol. The effects of varying concentrations of the enzyme on the rate of reaction and varying concentrations of substrate on the rate of reaction were determined (Figure 1-2). After standardization of assay conditions as given, AChE inhibitory activity was determined using 250 µg plant extract as given in Table 1.

**Standardization of NOX assay and plant inhibitory activity**

NOX assay was standardized with varying concentrations of enzyme and substrate to reach the final protocol. The effects of varying concentrations of the enzyme on the rate of reaction and varying concentrations of substrate on the rate of reaction were determined as given in

![Figure 1. Effects of various concentrations of enzyme on the rate of reaction.](image-url)
Figure 2. Effects of varying concentrations of substrate on the rate of reaction.

Figure 3. Effect of concentration of the substrate on the rate of reaction. 1mM NADH reduced sodium salt were used as a substrate, 10µl enzyme was added per assay.

Table 1. AChE inhibition activity of 250 µg methanolic extracts of plants exhibiting >50% inhibition (n=3, mean±sem).

<table>
<thead>
<tr>
<th>Plant</th>
<th>Family</th>
<th>AChE inhibition (%)</th>
</tr>
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<tbody>
<tr>
<td>1. Onosma bracteatum</td>
<td>Boraginaceae</td>
<td>59.73±5.29</td>
</tr>
<tr>
<td>2. Orchis mascula</td>
<td>Orchidaceae</td>
<td>56.99±0.31</td>
</tr>
<tr>
<td>3. Cymbopogon jawarancusab</td>
<td>Poaceae</td>
<td>72.36±1.67</td>
</tr>
</tbody>
</table>

Table 2. NOX inhibition activity of 250 µg methanolic extracts of plants exhibiting >50% inhibition (n=3, mean±sem).

<table>
<thead>
<tr>
<th>Plant</th>
<th>Family</th>
<th>NOX inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alhage Maurorum (whole plant)</td>
<td>Fabaceae</td>
<td>60.48±1.40</td>
</tr>
<tr>
<td>Myristica fragrans (nuts)</td>
<td>Myristicaceae</td>
<td>52.69±2.91</td>
</tr>
<tr>
<td>Nepeta hindostana (seeds)</td>
<td>Lamiaceae</td>
<td>62.63±1.40</td>
</tr>
<tr>
<td>Pinus gerardiana (fruit)</td>
<td>Pinaceae</td>
<td>75.81±5.83</td>
</tr>
</tbody>
</table>

Figure 4. Effect of varying concentrations of enzyme on the rate of reaction. 1mM NADH reduced sodium salt was used as substrate, with varying concentrations of enzyme added per assay.

DISCUSSION

Enzymes are the primary targets for the development of new drugs because of the simplicity of enzyme based assays. The inhibitor interacts with the enzyme or enzyme-substrate complex with a decreasing in the rate of reaction. The enzyme inhibition assays have prompted us to carry out a primary screening of the methanolic extracts of plants commonly used in traditional medicine in Pakistan. Therefore these studies were conducted to investigate the presence of inhibitors of some therapeutically important enzymes. A stock solution of 1mg/ml was prepared in 100 mM Tris buffer pH 7.4 for all 50 plant extracts and used in the screening of enzyme inhibitors. Table 1 describes the AChE percent inhibition activity. Maximum AChE inhibitory activity was found in O. bracteatum (59.73±5.29%), O. mascula.
(56.99±0.31%), C. jawarancusa (72.36±1.67%) at 250 µg plant extract. More than 50% inhibition of NOX inhibitory activity at 250 µg plant extracts of A. maurorum (60.48±1.40%), M. fragrans (52.69±2.99%) C. sativum (0.27±1.50%), N. hindostana (62.63±1.40%) and P. gerardiana (75.81±5.83%). Activities of other plant extracts was <50% against the two enzymes (data not shown).

These inhibition studies should be taken cautiously since the enzyme source was a crude source and not purified. The need to assay these extracts for inhibition studies still remains with the purified enzymes. Further, the plants extracts exhibiting greater enzyme inhibitory potential should be subjected to the isolation and purification of active principles by standard chromatographic and spectroscopic methods. This paper is the first in the series of several papers.

Conclusions

In summary, five plant extracts possess moderate levels of inhibitory activity against NADH oxidase and all plant extracts possess more or less Anti-AChEase activity. Plant extracts should be further subjected to chromatographic separations and phytochemical analyses and the purified AChEase should be used to carry out more inhibition studies.

REFERENCES


