A preliminary investigation of anticholinesterase and antioxidant properties of various extracts of *Vernonia anthelmintica* in relation to the treatment of Alzheimer’s disease

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**ABSTRACT**
The complexity of the underlying pathogenesis of Alzheimer’s disease (AD) and substandard results of existing treatment, demands persistent research for the development of new therapeutics. As natural antioxidant has attracted considerable attention on this regards, the present study evaluated and validated antioxidant and anti-cholinesterase activity of *Vernonia anthelmintica* seeds. Different extracts were collected after sequential extraction of plant seeds by using pet ether, chloroform, ethyl acetate, methanol and water as solvents. Antioxidant activity of the extracts was tested by using superoxide, nitric oxide (NO) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assays, whereas, acetylcholine esterase (AChE) inhibition property was assessed by Ellman’s method. Results revealed that all the extracts produced some degree of both the activities in a concentration-dependent manner. While pet ether extract exhibited weakest, methanolic extract displayed potent radical scavenging and anti-cholinesterase properties in all the assays with IC₅₀ of 159.71 µg/ml against AChE inhibition and 98.51, 120.22 and 170.79 µg/ml against superoxide, NO and DPPH radical scavenging assays respectively. Presence of an array of secondary metabolites with modest flavonoid and phenol content in the methanolic extract is accountable for these desired activities. Collectively, reports from our experiments covey that *V. anthelmintica* possess significant AChE inhibitory and antioxidant property and thus can be further evaluated in search of potential disease-modifying therapeutic for management of neurodegenerative diseases like AD.

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**INTRODUCTION**
Management of slow progressive and most prevalent neurodegenerative disorder, Alzheimer’s disease (AD) become a worldwide challenge. Symptomology, including impairment in thinking, memory, behaviour and emotional functions are the pathological features associated with AD (Jazayeri et al., 2014; Foyet et al., 2019). Loss of neurons in the brain during the progression of the disease leads to the development of gaps in the temporal lobe and hippocampus which are responsible for stor-
ing and retrieving information and thus hampers the ability to remember, speaking, and making decisions (Kaufmann et al., 2016). Due to complexed aetiopathology of AD, several hypothesis were presented by scientists to explain the multifaceted disease mechanisms such as deposition and accumulation of extracellular amyloid plaques and hyperphosphorylated tau protein, mitochondrial dysfunction, oxidative and inflammatory stress, and cholinergic dysfunction (Lian et al., 2017).

Although several other treatment strategies are being tested, restoring cholinergic functions is still considered as a classical approach for the treatment of AD Acetylcholine (Ach) neurotransmitter is accountable for processing memory, deficiency of which in brain results in impaired learning and thinking skills. The decline in the cholinergic transmission is the first clinical observation in AD patients. Elevation in ACh levels is possible by inhibiting Acetylcholine esterase (AChE) enzyme, which breaks down ACh in the synaptic cleft (Uddin et al., 2020). AChE inhibitors act as a cognitive enhancer and provide some degree of symptomatic relief. However, side effects related to the marketed AChE inhibitors have encouraged the need for the development of other alternatives (Mehta et al., 2012). Presence of an array of secondary metabolites with a wide range of health beneficial properties in medicinal plants makes them a great source for the search of potential AChE inhibitors (Nwidu et al., 2017). Vernonia anthelmintica seeds are quite popular among the ethnic group for treating neurodegenerative disorders. It belongs to the family Asteraceae and commonly known as ‘kalijiri’ and ‘somraji’ in India (Nadkarni and Nadkarni, 1976). In addition, this plant and its parts have been used as a folk remedy for treating multiple ailments such for asthma, influenza, diarrhea, cardiovascular diseases, sinusitis, convulsion, leucoderma, psoriasis, paralysis, inflammatory swellings, fever, scabies, and an ulcer (Kritchikar and Basu, 1989; Acharya and Shrivastava, 2008). However, there is a lack of validation and scientific documentation on the use of this plant for the treatment of neurological disorders. The present study intended to investigate the antioxidant and AChE inhibition potential of V. anthelmintica seeds for the evolution of therapeutic agents for the management of neurodegenerative diseases.

MATERIALS AND METHODS

Plant material
Dried seeds of V. anthelmintica were gathered from the local market of The Nilgiris district, India and was authenticated and identified by Dr S. Rajan, Field botanist, Central Council for Research in Homeopathy, Department of AYUSH, The Nilgiris, Tamilnadu, India.

Preparation of seed extracts
The air-dried seeds were crushed into a coarse powder. Pulverised seeds were then extracted using pet either by soaking in a conical flask and was kept on a mechanical shaker for 24 hours. The marc was then dried and subjected to chloroform for the next 24 hours, followed by ethyl acetate, methanol and water-based on the increasing polarity of solvents. Extracts were then filtered using Whatman filter paper and concentrated under reduced pressure and the residue to be tested was lyophilized and protected from light.

Determination of the Total Phenol Content (TPC)
Based on the method discussed by (Singleton and Rossi, 1965), total phenol content in the various extracts of V. anthelmintica was determined. 10% Folin-Ciocalteau’s reagent (2.5 ml), 2% sodium carbonate solution (2 ml) and various extracts of V. anthelmintica (1 mg/ml) (0.5 ml) were mixed together and incubated at 45 °C for 15 minutes. Absorbance was read at 765 nm. Quantification of TPC in extracts was calculated based on standard curve prepared using gallic acid as reference at different concentrations (1, 0.5, 0.25, 0.125, 0.063, and 0.031 mg/ml).

Determination of Total Flavonoid Contents (TFC)
Aluminium chloride (AlCl₃) colorimetric method was used for determination of flavonoid content, where crude extract (1mg/ml) of plant seeds was mixed with methanol (3 ml), 10% AlCl₃ (0.2 ml), 1M potassium acetate (0.2 ml) and distilled water (5.6 ml). Absorbance was taken at 420 nm. Based on standard curve prepared using different concentrations (1, 0.5, 0.25, 0.125, 0.063, and 0.031 mg/ml) of gallic acid as a reference, quantification of the TFC was performed (Köksal and Gülçin, 2008).

Evaluation of antioxidant property
Superoxide scavenging activity
By using the method described by (Misra and Fridovich, 1972), superoxide generation activity of all the extracts were assessed. A mixture of 0.052 M sodium pyrophosphate buffer (1.2 ml), 186 µM phenazonium methosulphate (0.1 ml), 300 µM nitroblue tetrazolium (0.3 ml), and different concentrations (50, 100, 150, 200, and 250 µg/ml) of various extracts was added to 780 µM NADH (0.2 ml) and incubated for 90 seconds at 30°C followed by
### Table 1: The total phenolics and flavonoids content in the extracts of *V. anthelmintica*.

<table>
<thead>
<tr>
<th>Assays</th>
<th>Pet ether</th>
<th>Ethyl acetate</th>
<th>Chloroform</th>
<th>Methanol</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>% yield</td>
<td>8.77</td>
<td>9.58</td>
<td>5.66</td>
<td>19.31</td>
<td>12.12</td>
</tr>
<tr>
<td>Total phenolics (mg GAE/g)</td>
<td>31.68±0.71</td>
<td>24.89±0.22</td>
<td>12.93±0.08</td>
<td>9.59±0.03</td>
<td>16.27±0.26</td>
</tr>
<tr>
<td>Total flavonoids (mg GAE/g)</td>
<td>17.69±0.46</td>
<td>16.54±0.05</td>
<td>13.69±0.02</td>
<td>4.05±0.25</td>
<td>8.83±0.33</td>
</tr>
</tbody>
</table>

### Table 2: AChE inhibition activity with different solvents of *V. anthelmintica*.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>50 μg/ml</th>
<th>100 μg/ml</th>
<th>150 μg/ml</th>
<th>200 μg/ml</th>
<th>250 μg/ml</th>
<th>IC50 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pet ether</td>
<td>8.36±0.08</td>
<td>13.96±0.07</td>
<td>29.58±0.09</td>
<td>37.14±0.78</td>
<td>50.08±0.04</td>
<td>254.04</td>
</tr>
<tr>
<td>Chloroform</td>
<td>10.12±0.07</td>
<td>17.22±0.11</td>
<td>32.97±0.1</td>
<td>40.34±0.03</td>
<td>64.65±0.98</td>
<td>214.58</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>13.54±0.13</td>
<td>18.31±0.35</td>
<td>38.93±0.13</td>
<td>47.50±2.17</td>
<td>69.56±0.01</td>
<td>193.99</td>
</tr>
<tr>
<td>Methanol</td>
<td>23.64±1.56</td>
<td>31.02±0.54</td>
<td>40.36±0.72</td>
<td>65.06±0.84</td>
<td>76.37±0.40</td>
<td>159.71</td>
</tr>
<tr>
<td>Water</td>
<td>12.25±2.59</td>
<td>17.51±2.65</td>
<td>37.83±1.35</td>
<td>44.28±1.15</td>
<td>67.08±0.48</td>
<td>164.39</td>
</tr>
<tr>
<td>Galantamine</td>
<td>41.25±1.11</td>
<td>60.81±1.07</td>
<td>71.55±0.19</td>
<td>79.33±0.1</td>
<td>82.95±0.004</td>
<td>65.74</td>
</tr>
</tbody>
</table>

Each value represents average of three analysis (mean±SD)

### Table 3: Superoxidescavenging assay with different solvents of *V. anthelmintica*.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>50 μg/ml</th>
<th>100 μg/ml</th>
<th>150 μg/ml</th>
<th>200 μg/ml</th>
<th>250 μg/ml</th>
<th>IC50 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pet ether</td>
<td>13.45±0.13</td>
<td>18.41±0.44</td>
<td>24.38±0.25</td>
<td>37.78±0.65</td>
<td>47.22±0.71</td>
<td>275.17</td>
</tr>
<tr>
<td>Chloroform</td>
<td>26.25±0.32</td>
<td>31.04±0.11</td>
<td>38.1±0.12</td>
<td>46.43±0.19</td>
<td>51.41±0.26</td>
<td>236.43</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>31.32±0.17</td>
<td>44.13±0.6</td>
<td>50.47±0.29</td>
<td>58.51±0.25</td>
<td>64.69±0.12</td>
<td>151.12</td>
</tr>
<tr>
<td>Methanol</td>
<td>40.43±0.3</td>
<td>51.54±1.66</td>
<td>58.75±0.32</td>
<td>68.77±0.33</td>
<td>76.06±0.31</td>
<td>98.51</td>
</tr>
<tr>
<td>Water</td>
<td>39.17±0.27</td>
<td>45.82±0.65</td>
<td>54.78±0.47</td>
<td>63.09±0.55</td>
<td>68.97±0.67</td>
<td>121.63</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>57.99±0.29</td>
<td>60.66±0.60</td>
<td>67.34±0.16</td>
<td>75.35±0.16</td>
<td>87.15±0.52</td>
<td>15.10</td>
</tr>
</tbody>
</table>

Each value represents average of three analysis (mean±SD)

### Table 4: NO scavenging assay with different solvents of *V. anthelmintica*.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>50 μg/ml</th>
<th>100 μg/ml</th>
<th>150 μg/ml</th>
<th>200 μg/ml</th>
<th>250 μg/ml</th>
<th>IC50 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pet ether</td>
<td>21.67±0.16</td>
<td>28.24±0.2</td>
<td>35.86±0.21</td>
<td>45.16±0.25</td>
<td>54.76±0.11</td>
<td>227.39</td>
</tr>
<tr>
<td>Chloroform</td>
<td>26.16±0.27</td>
<td>33.35±0.16</td>
<td>40.30±0.08</td>
<td>48.40±0.18</td>
<td>57.88±0.15</td>
<td>205.92</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>27.14±0.13</td>
<td>35.63±0.2</td>
<td>43.52±0.18</td>
<td>53.09±0.12</td>
<td>62.41±0.19</td>
<td>182.06</td>
</tr>
<tr>
<td>Methanol</td>
<td>37.05±0.2</td>
<td>45.14±0.23</td>
<td>56.29±0.05</td>
<td>66.69±0.22</td>
<td>72.15±0.55</td>
<td>120.22</td>
</tr>
<tr>
<td>Water</td>
<td>31.54±0.13</td>
<td>40.1±0.18</td>
<td>52.07±0.5</td>
<td>57.77±0.16</td>
<td>65.67±0.24</td>
<td>153.28</td>
</tr>
<tr>
<td>BHT</td>
<td>61.06±0.21</td>
<td>67.04±0.05</td>
<td>73.43±0.58</td>
<td>79.22±0.21</td>
<td>87.66±0.26</td>
<td>31.1</td>
</tr>
</tbody>
</table>

Each value represents average of three analysis (mean±SD)
addition of glacial acetic acid (0.1 ml) to stop the reaction. The absorbance of the organic layer was read at 560 nm after addition of n-butanol (4 ml). Ascorbic acid was used as a reference standard for the assay.

\[
\text{% Superoxide scavenging activity} = \left\{ \frac{(A_0 - A_1)}{A_0} \right\} \times 100
\]

The percentage inhibition of activity was calculated using the above formula where, \( A_0 \) is the absorbance of the control and \( A_1 \) is the absorbance of sample and all the tests were performed in triplicates.

### Nitric oxide (NO) scavenging activity

NO scavenging property of all the extracts was evaluated using the method explained by (Ebrahimzadeh et al., 2009). Different concentrations (50, 100, 150, 200, and 250 \( \mu \text{g/ml} \)) of \( V. \text{anthelmintica} \) extracts (4 ml) was added to 25 mM sodium nitroprusside solution (1ml) and incubated at 37° C. After 3 hours, incubation solution (0.5ml) was mixed with Griess reagent (0.3 ml) and absorbance was measured at 570 nm. Butylated hydroxytoluene (BHT) was used as a reference standard for the assay.

\[
\text{% NO scavenging activity} = \left\{ \frac{(A_0 - A_1)}{A_0} \right\} \times 100
\]

The percentage inhibition of activity was calculated using the above formula where, \( A_0 \) is the absorbance of the control and \( A_1 \) is the absorbance of sample and all the tests were performed in triplicates.

### 1, 2-diphenyl-2-picryl hydrazyl (DPPH) scavenging activity

The effect of \( V. \text{anthelmintica} \) extracts on DPPH radical has been carried out, employing the method described earlier by (Koleva et al., 2002). Absorbance of mixture containing various concentrations (50, 100, 150, 200, and 250 \( \mu \text{g/ml} \)) of extracts (2 ml) with 0.01 M DPPH in methanol (0.5 ml) was analysed at 517 nm. Ascorbic acid was used as a reference standard for the assay.

\[
\text{% DPPH scavenging activity} = \left\{ \frac{(A_0 - A_1)}{A_0} \right\} \times 100
\]

The percentage inhibition of activity was calculated using the above formula where, \( A_0 \) is the absorbance of the control and \( A_1 \) is the absorbance of sample and all the tests were performed in triplicates.

### Table 5: DPPH scavenging assay with different solvents of \( V. \text{anthelmintica} \).

<table>
<thead>
<tr>
<th>Solvents</th>
<th>50 ( \mu \text{g/ml} )</th>
<th>100 ( \mu \text{g/ml} )</th>
<th>150 ( \mu \text{g/ml} )</th>
<th>200 ( \mu \text{g/ml} )</th>
<th>250 ( \mu \text{g/ml} )</th>
<th>IC50 ( \mu \text{g/ml} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pet ether</td>
<td>8.34±0.57</td>
<td>14.9±0.23</td>
<td>25.96±0.28</td>
<td>34.67±0.16</td>
<td>45.88±0.16</td>
<td>276.78</td>
</tr>
<tr>
<td>Chloroform</td>
<td>12.77±0.27</td>
<td>19.25±0.31</td>
<td>29.56±0.15</td>
<td>40.40±0.23</td>
<td>53.55±0.71</td>
<td>242.11</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>17.44±0.14</td>
<td>23.87±0.78</td>
<td>33.61±0.36</td>
<td>48.82±0.43</td>
<td>57.1±0.48</td>
<td>223.01</td>
</tr>
<tr>
<td>Methanol</td>
<td>24.55±0.88</td>
<td>32.07±0.11</td>
<td>44.76±0.48</td>
<td>54.64±0.16</td>
<td>70.30±0.78</td>
<td>170.79</td>
</tr>
<tr>
<td>Water</td>
<td>20.15±0.15</td>
<td>29.45±0.81</td>
<td>42.48±0.25</td>
<td>48.12±0.27</td>
<td>63.3±0.16</td>
<td>194.34</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>58.10±0.22</td>
<td>66.59±0.16</td>
<td>71.92±0.35</td>
<td>80.7±0.35</td>
<td>87.04±0.18</td>
<td>8.84</td>
</tr>
</tbody>
</table>

Each value represents average of three analysis (mean±SD)

### Statistical analysis

All experiments were done in triplicates, and results were analysed using GraphPad Prism 8.0.2 (263). All values were expressed as mean ± standard deviation (SD).

### RESULTS AND DISCUSSION

Complexity in understanding the etiopathogenesis and management of neuropsychiatric-related disorders provoked comprehensive investigation for the search of new and safe alternative which can act as multitargeted therapy (Mark and Cornelius, 2002). Due to the disparaging side effects of the currently available medications, ethnopharmacological studies by researchers have encouraged the systematic
screening of medicinal plants for the development of cost-effective novel drug treatment (Nair and Hunter, 2004; Rogers et al., 1998). *V. anthelmintica* is an integral part of traditional medicine for centuries and have been extensively used in Ayurveda and Siddha medicinal system for its end number of health profitable properties (Krithikar and Basu, 1989). On this context, the present study focused on the validation of antioxidant and AChE inhibition property of *V. anthelmintica* seeds.

The percentage yield of extracts obtained after successive maceration extraction of *V. anthelmintica* seeds with various solvents ranged from approximately 8 to 19%. The highest yield was obtained after aqueous extraction (12.12%), proposing the highest solubility of polar compounds present in the seeds. Qualitative estimation of TPC and TFC have demonstrated the presence of the appreciable amount of phenolics and flavonoids ranging from 9.59 to 31.68 mg GAE/g of extract and 4.05 to 17.69 mg GAE/g of extract respectively. While aqueous extract exhibited the highest phenolic and flavonoid contents of about 31.68 and 17.69 mg GAE/g of extract, chloroform extract has shown minimal TPC and TFC among all the extracts with 9.59 and 4.05 mg GAE/g of extract respectively (Table 1).

Although several mechanistic approaches have been put forward which can explain underlying pathology of AD, loss of cholinergic synapses found to be the main culprit for the decline in the intellectual functions (Mehta et al., 2012), our study demonstrated that methanolic extract of *V. anthelmintica* seeds efficiently inhibited AChE activity with highest inhibition potential of 76% with IC$_{50}$ of 159.71 µg/ml. Ethyl acetate and aqueous extracts also displayed appreciable inhibition property with 69% and 67% respectively comparable to the standard galantamine. The order of AChE inhibitory activity of extracts was found to be methanol > water > ethyl acetate > chloroform > pet ether extract (Table 2).

Oxidative stress generated during AD progression exacerbates the condition by accelerating the neuronal damage (Feng and Wang, 2012). Further, it also facilitates the accumulation of plaque and neurofibrillary tangles along with neuroinflammation, which is considered to be other major hallmarks of AD by creating a neurotoxic environment (Alzoubi et al., 2013). Antioxidants play their protective role against oxidative stress-induced cell lipids and DNA damage by maintaining ion homeostasis (Serrano and Klann, 2004; Tuzcu and Baydas, 2006). Our study displayed that in addition to the notable AChE inhibitory activity, *V. anthelmintica* seed extracts exhibited potent antioxidant property against superoxide, NO and DPPH radicals in a concentration-dependent manner. With potent antioxidant activity, the methanolic extract showed 76% superoxide radical inhibition with IC$_{50}$ of 98.51 µg/ml at a concentration of 250 µg/ml when compared to standard ascorbic acid which displayed 87% inhibition with IC$_{50}$ of 15.10 µg/ml. However, the lowest activity was exhibited by pet ether with 47% inhibition at the highest concentration (Table 3).

Similarly, methanolic extracts also showed better NO (72% inhibition) and DPPH (70% inhibition) scavenging activity compared to all the extracts, followed by aqueous extract at 250 µg/ml concentration. IC$_{50}$ of methanolic for NO and DPPH scavenging assay was found to be 120.22 and 170.79 µg/ml respectively. Whereas, pet ether extract showed minimal antioxidant activity against NO (IC$_{50}$ of 227.39 µg/ml) and DPPH (IC$_{50}$ of 276.78 µg/ml) assay. The descending order of all the extracts for all radical scavenging activity was observed as: methanol > water > ethyl acetate > chloroform > pet ether (Tables 4 and 5).

Results from both radical scavenging and anti-AChE studies indicated that there is a correlation between antioxidant and AChE inhibition property of methanolic extract thus could be a promising candidate for the drug discovery against AD-related complications. Initial studies have shown the appreciable amount of phenols and flavonoids in the methanolic extract of the plant seeds which could be accountable for this desired property.

**CONCLUSIONS**

As several plant driven compounds are used for the management of AD, nature provides a valuable source for exploration of bioactive compounds with impressive antioxidant and acetylcholinesterase inhibition potential. Primary findings from the current study suggest that all the extract used in the study produced some degree of radical scavenging and anticholinesterase activity in a concentration-dependent manner.

The methanolic extract has shown the best results among other extracts tested. Synergistic interaction of the secondary metabolites could be responsible behind these activity. However, further investigation is needed in search of chemical composition and mechanism of action using *in vivo* and *in vitro* studies. Collectively our study demonstrates that *V. anthelmintica* could be a panacea in search of anti-AD therapy.
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Conflict of interest

None.

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