In Vitro Screening for Antioxidant and Anticholinesterase Effects of Uvaria littoralis Blume: A Nootropic Phytotherapeutic Remedy

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Abstract: Background: Oxidative stress is strongly linked in the development of numerous chronic and degenerative disorders. Medicinal plants with antioxidant and anticholinesterase activities exert a key role for the management of oxidative stress related disorders mainly Alzheimer's disease (AD). Therefore the purpose of this study was to assess antioxidant potentiality and anticholinesterase inhibitory activity of crude methanolic extract (CME), petroleum ether fraction (PEF), chloroform fraction (CLF), ethyl acetate fraction (EAF) and aqueous fraction (AQF) of Uvaria littoralis (U. littoralis) leaves.

Methods: The antioxidant compounds namely total flavonoids contents (TFCs) and total proanthocyanidins contents (TPACCs) were determined for quantities constituent's characterization. Antioxidant capacity of U. littoralis leaves were estimated by the iron reducing power (IRPA), 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and nitric oxide (NO) radical scavenging capacity. Anticholinesterase effects were estimated for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity.

Results: The EAF of U. littoralis leaves showed the highest TFCs as compared to CLF, CME, PEF and AQF. TPACCs were also found highest in EAF. The highest absorbance for IRPA was found in EAF (2.220 nm) with respect to CME and other fractions at the highest concentration. The EAF showed best DPPH and NO radical scavenging activity with IC₅₀ values of 31.63 and 55.47 µg/mL, respectively with regard to CME and remaining fractions. The PEF represents highest AChE inhibitory activity with IC₅₀ values of 35.19 µg/mL and CLF showed highest BChE inhibitory activity with IC₅₀ values of 32.49 µg/mL.

Conclusions: The findings of the current study demonstrate the presence of antioxidant phytochemicals, likewise, turns out antioxidant and anticholinesterase potentiality of U. littoralis leaves which could be a prestigious candidate for the treatment of neurodegenerative diseases especially AD.

Keywords: Oxidative stress, Antioxidant potentiality, Anticholinesterase activity, Uvaria littoralis, Alzheimer's disease.

INTRODUCTION

Oxidative stress (OS) is an imbalance between pro-oxidant/antioxidant homeostasis that block the biological system's ability to readily mask the reactive intermediate oxygen species or to repair the resulting damage [1]. Toxic effects are produced from any inconveniences in the normal redox state of cells through the production of peroxides and free radicals that damage cell components, including proteins, lipids and deoxyribonucleic acid (DNA). Production of reactive oxygen species (ROS) is a particularly a slaughters situation in a biological system which is created by the OS such species include free radicals and peroxides. Hydroxyl (OH⁻), superoxide (O²⁻) and nitric monoxide (NO⁻) radicals are the most common cellular free radicals. Likely hydrogen peroxide (H₂O₂) and peroxynitrite (ONOO⁻) are also referred dangerous [2-5]. There is sufficient scientific evidence that proves that oxidative stress is most common causative agents for neurodegenerative diseases, mainly Alzheimer's disease (AD) [6]. Cellular changes show that OS is an event that precedes the appearance of the pathological hallmark of the AD such as neurofibrillary tangles and senile plaques. The increased level of oxidative stress in the AD brain is reflected by increased protein and DNA oxidation, enhanced lipid peroxidation, decreased level of cytochrome c oxidase and advanced glycosylation end products [7, 8]. Ion imbalance and impaired metabolism caused by the weaken cell membrane which may produce from lipid peroxidation is also causative. Moreover oxidative stress can impair DNA methylation process which regulates gene expression. Amyloid-β (Aβ) proteins may play a major role in pathogenesis of AD and the trigger for OS is an active area of current research [9].
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Still AD is incurable threat, but Alzheimer's medications can temporarily slow the worsening of symptoms and improve condition of patients [10]. Acetylcholinesterase (AChE) inhibitors and N-methyl-D-aspartate glutamate receptor antagonists are the current standard therapy. Though these moderately improve quality of life, but it lacks a proper way of treatment [11]. Antioxidants are effective for breaking free radical chain reaction. Recently they emerged a great interest in the therapeutic potential of medicinal plants as antioxidants in reducing oxidative stress-induced tissue injury [12]. Ascorbic acid, carotenoids and phenolic compounds are more effective among the numerous naturally occurring antioxidants [13]. Although there is the availability of several synthetic antioxidants in a commercial manner, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), but they are quite unsafe and their toxicity becomes a higher degree of concern [14]. Therefore, strong restrictions have been put forward on their application and there lays a trend to substitute them with naturally occurring antioxidants [14].

The plant, Uvaria littoralis (U. littoralis) Blume, belongs to the family Annonaceae is a woody climbing shrub [15]. Annonaceae comprises about 130 genera and 2,300 species [16]. Phytogeographically it is entirely tropical, with 39 genera being represented in tropical America. This plant family is recognized as being the center for isoquinoline alkaloids production in plants [16]. Among them several genera produce edible fruit. In folk medicines, their barks, leaves and roots of some plants of this family are used. Chemical compounds like flavonoids, alkaloids and acetogenins are extracted from the seeds and many other plants of this family. Flavonoids and alkaloids contained in the leaves and bark of several species of the family have shown insecticidal properties [17, 18].

There has been no study about antioxidant and anticholinesterase effects of the U. littoralis leaves. Therefore, the main objective of the present study was to evaluate the in vitro antioxidant and cholinesterase inhibitory activities of petroleum ether fraction (PEF), chloroform fraction (CLF), ethyl acetate fraction (EAF), aqueous fraction (AQF) and crude methanol extract (CME) of U. littoralis leaves.

MATERIALS AND METHODS

Chemicals and Drugs

Aluminium chloride (AlCl₃), ferrozine monosodium, DPPH (1, 1-diphenyl-2-picrylhydrazyl), Griess reagent, acetylthiocholine iodide, thiobarbituric acid and donepezil (Don) were purchased from Sigma-Aldrich, Japan. Butylated hydroxy toluene (BHT) was purchased from Merck, Germany. Gallic acid (GA) and quercetin were purchased from Wako pure chemicals Ltd., Japan. Unless otherwise specified, other chemicals were analytical grade and obtained from native sources.

Collection, Identification, Drying and Grinding of Plant Materials

The leaves of the plant, U. littoralis was collected during the month of October, 2016 from Comilla Hill track, Bangladesh in fresh condition and identified by an expert taxonomist. A voucher specimen was submitted to the National Herbarium Bangladesh (accession number: DACB-41884). In order to remove dirt, the leaves were cleaned with water, then the leaves were allowed to sun dried and then milled to generate powdered sample using a suitable grinder. The found milled powdered plant materials were reserved in an airtight glass conation awaiting extraction.

Extraction and Fractionation of Plant Materials

About 500 g dried leaf powder of U. littoralis was taken in an amber-colored glass bottle and soaked in 2 L methanol. The bottle was sealed for 10 days and shaken occasionally and stirred. The mixture was filtered by cotton and then by Whatman No.1 filter paper and finally filtrate was concentrated with a rotary evaporator at 52°C to find the crude methanol extract (CME, 45.39 g). About 20 g of CME was taken in a separating funnel and fractionated using modified Kupchan method [13]. After fractionation petroleum ether (PEF, 5.89 g), chloroform (CLF, 4.36 g), ethyl acetate (EAF, 2.86 g) and aqueous (AQF, 4.36 g) soluble fractions were obtained and used for the experiment purpose.

Estimation of Antioxidant Compounds

The antioxidant compounds were assayed for total flavonoid contents (TFCs) and total proanthocyanidin contents (TPACCs). A stock solution of 1 mg/mL was prepared for the CME and fractions just before the assay.

Total Flavonoid Contents

The method of Ordonez et al., was used for determining the TFCs [19]. A volume of 0.5 mL of 2%
AlCl₃ ethanol solution was added to 0.5 mL of plant extract/fractions solution. The mixture was incubated for 1 h at room temperature to complete the reaction. The absorbance was measured at 420 nm in contrast to a reagent blank. The TFCs were calculated by mg of catechin equivalent (QRE) per g of dried sample.

**Total Proanthocyanidin Contents**

The method described by Sun et al., was used to determine the content of TPACCs [20]. A volume of 0.5 mL of plant extract/fractions solution was mixed with 3 mL of 4% vanillin-methanol solution and 1.5 mL hydrochloric acid. After that the mixture was allowed to stand for 15 min at room temperature. The absorbance was measured at 500 nm in contrast to a reagent blank. The TPACCs were expressed in terms of mg of catechin equivalent (CTE) per g of dried sample.

**Estimation of Antioxidant Activity**

The antioxidant capacity was assayed for the iron reducing power activity (IRPA), DPPH radical scavenging and nitric oxide (NO) radical scavenging capacity. A stock solution of 1 mg/mL was prepared for the CME and fractions just before the assay.

**Iron Reducing Power Activity**

The method of Oyaizu was used to determine the iron reducing power activity [21]. A volume of 0.2 mL of plant extract/fractions solution was mixed with 0.5 mL of 200 mM/L trisodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min; then, 2.5 mL of 10% trichloroacetic acid (w/v) was added and the mixture was centrifuged at 650 rpm for 10 min. 0.5 mL supernatant solution was mixed with 0.5 mL deionized water and 0.1 mL of 0.1% ferric chloride and absorbance was measured at 700 nm in contrast to a reagent blank. In this test ascorbic acid (AA) was used as standard.

**DPPH Radical Scavenging Activity**

The method of Choi et al., was used for determination of DPPH radical scavenging activity [22]. A volume of 2 mL of methanol solution of plant extract/fractions or standard at various concentrations and 3 mL (0.02%) of methanol solution of DPPH was added then incubated at room temperature for 30 min in dark. The absorbance of the solution was measured at 517 nm in contrast to a reagent blank. In this test BHT was used as standard. The percentage inhibitions or scavenging of DPPH radical was calculated by using the following formula:

\[
\text{DPPH radical scavenging (\%) = } \left( \frac{A_o - A_i}{A_o} \right) \times 100
\]

where, \( A_o \) is the absorbance of the control and \( A_i \) is the absorbance of the sample/standard.

**Nitric Oxide Radical Scavenging Activity**

The method of Garrat was used to determine the nitric oxide radical scavenging activity [23]. A volume of 2 mL of 10 mM sodium nitroprusside in 0.5 mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of plant extract/fractions or standard at various concentrations and the mixture was incubated at 25°C for 2.5 h. An aliquot of 0.5 mL of the solution was added to 0.5 mL of Griess reagents [1.0 mL of sulfanilic acid reagent (0.33% prepared in 20% glacial acetic acid at room temperature for 5 min with 1.0 mL of naphthylenediamine chloride (0.1% w/v)]. The mixture was incubated at room temperature for 30 min. The absorbance was then measured at 540 nm in contrast to a reagent blank. In this test GA was used as standard. The percentage inhibitions or scavenging of NO radical was calculated by using the following formula:

\[
\text{NO radical scavenging (\%) = } \left( \frac{A_o - A_i}{A_o} \right) \times 100
\]

where, \( A_o \) is the absorbance of the control and \( A_i \) is the absorbance of the sample/standard.

**Estimation of Cholinesterase Inhibitory Activity**

The acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity were carried out by the use of bovine brain homogenate as acetylcholinesterase enzyme source and human blood as butyrylcholinesterase enzyme source. A stock solution of 1 mg/mL was prepared for the CME and fractions just before the assay.

**Acetylcholinesterase Inhibitory Activity**

The method of Ellman et al., with slight modification was used to determine the AChE inhibitory activity using acetylthiocholine iodide as a substrate [24]. Bovine brain was used as enzyme source and homogenized with 5 volumes of a homogenation buffer [50 mM Tris-HCl (pH 7.2), contained 1 M NaCl, 50 mM magnesium chloride and 1% Triton X-100] and centrifuged at 10,000 rpm for 15 min. The resulting supernatant was used as an enzyme source. All of the extraction steps were carried out at 4°C. Each plant
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Determination of TFCs

Quercetin standard curve ($y = 0.0075x + 0.1504, R^2 = 0.9887$) was used for the determination of TFCs. The TFCs of U. littoralis leaf extract and its fraction ranges from 61.58 (AQF) to 215.06 (EAF) mg QRE/g. The TFCs follow the following order: EAF > CLF > CME > PEF > AQF are specified in Figure 1.

Determination of TPACCs

Catechin standard curve ($y = 0.0019x + 0.2024, R^2 = 0.9723$) was used for determination of TPACCs. U. littoralis leaf extract and its fraction show a range of TPACCs from 22.03 (AQF) to 95.72 (EAF) mg CTE/g.
EAF shows highest content and follow the following order: EAF > CME > CLF > PEF > AQF (Figure 2).

**Determination of IRPA**

IRPA of the *U. littoralis* leaf extract and its fraction at different concentrations showed a dose dependent activity. The Figure 3, displayed the range of IRPA from 0.665 (CLF) to 2.220 (EAF) nm at 250 µg/mL. Results displayed the following order of antioxidant activity: AA > EAF > CME > AQF > PEF > CLF at the highest concentration.

**Determination of DPPH Radical Scavenging Activity**

The DPPH scavenging effects of *U. littoralis* leaf extract/fractions and standard are specified in Figure 4.

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**Figure 3:** IRPA of *U. littoralis* leaf extract and fractions. Values were reported as mean ± SD (n = 3). CME = Crude methanolic extract, PEF = Petroleum ether fraction, CLF = Chloroform fraction, EAF = Ethyl acetate fraction, AQF = Aqueous fraction, AA = Ascorbic acid.

**Figure 4:** DPPH radical scavenging assay of *U. littoralis* leaf extract and fractions. Values were reported as mean ± SD (n = 3). CME = Crude methanolic extract, PEF = Petroleum ether fraction, CLF = Chloroform fraction, EAF = Ethyl acetate fraction, AQF = Aqueous fraction, BHT = Butylated hydroxytoluene.
in which the scavenging percentages follow the following order: BHT > EAF > AQR > CME > PEF > CLF at 125 µg/mL. The established relationship between the percentage of DPPH radical scavenging and concentration was dose dependent. The IC\textsubscript{50} values of CME, PEF, CLF, EAF, AQR and BHT are depicted in Table 1.

### Table 1: IC\textsubscript{50} Values of *U. littoralis* Leaf Extract and Fractions for DPPH and NO Radical Scavenging Activity

<table>
<thead>
<tr>
<th>Sample/Standard</th>
<th>DPPH Radical (µg/mL)</th>
<th>NO Radical (µg/mL)</th>
</tr>
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<tbody>
<tr>
<td>CME</td>
<td>57.29±2.22</td>
<td>146.46±0.755</td>
</tr>
<tr>
<td>PEF</td>
<td>59.89±1.26</td>
<td>171.60±1.25</td>
</tr>
<tr>
<td>CLF</td>
<td>59.06±1.42</td>
<td>132.57±1.77</td>
</tr>
<tr>
<td>EAF</td>
<td>31.63±0.834</td>
<td>55.47±0.598</td>
</tr>
<tr>
<td>AQR</td>
<td>61.83±1.94</td>
<td>115.22±0.642</td>
</tr>
<tr>
<td>BHT</td>
<td>14.86±0.169</td>
<td>–</td>
</tr>
<tr>
<td>GA</td>
<td>–</td>
<td>27.80±0.162</td>
</tr>
</tbody>
</table>

Values were reported as mean ± SD (n = 3). CME = Crude methanolic extract, PEF = Petroleum ether fraction, CLF = Chloroform fraction, EAF = Ethyl acetate fraction, AQR = Aqueous fraction, BHT = Butylated hydroxytoluene, GA = Gallic acid. *P* < 0.01 significant difference from the standard.

Determination of NO Radical Scavenging Activity

Figure 5 displays the NO radical scavenging activity of *U. littoralis* leaf extract/fractions and standard in which the percentage of scavenging activity follows the following order: GA > EAF > AQR > CLF > CME > PEF at 125 µg/mL. The IC\textsubscript{50} values of CME, PEF, CLF, EAF, AQR and GA are shown in Table 1. Results expressed that the IC\textsubscript{50} value of EAF was statistically significant (*P* < 0.01) with respect to GA.

Determination of AChE Inhibitory Activity

Figure 6, revealed that the AChE inhibitory activity of *U. littoralis* extract/fractions and standard in the following order: Don > PEF > CLF > EAF > CME > AQR at 125 µg/mL. The IC\textsubscript{50} values of Don, PEF, CLF, EAF, CME and AQR were 16.793, 35.912, 38.513, 135.971, 137.712 and 153.68 µg/mL are expressed in Table 2.

Determination of BChE Inhibitory Activity

The BChE inhibitory activity of *U. littoralis* extract/fractions and standard were in the following order: Don > CLF > EAF > CME > PEF > AQR at 125 µg/mL are assumed in Figure 7. The IC\textsubscript{50} values of Don, CLF, EAF, CME, PEF and AQR were 16.71,
AD is a chronic neurogenarative disorder leading to death among the elderly people [25, 26]. Various factors have been identified for pathogenesis of AD, oxidative stress and impairment in the function of cholinergic activity has been found the major contributing factors [27, 28]. It is well established evidence that the compounds obtained from plant having antioxidants and anticholinesterase activity spatially AChE and BChE inhibitory activity would be effective for the management of AD [29, 30]. In this study antioxidant and anticholinesterase activities of the CME and its resulting fractions of *U. littoralis* leaves were tested.

Among the fractions the highest TFCs was found in EAF with respect to remaining. TPACCs consist of flavan-3-ol units including catechin, epicatechin, catechin gallate and epicatechin gallate. Inhibition of Aβ aggregation is an important strategy for AD. Some flavonoids have been identified and active in the modulation of the pathological processes of AD [31]. Like flavonoid among the plant extract/fractions the highest TPACCs was found in EAF. A number of studies recommended the potent role of phytoconstituents and proanthocyanadins rich natural compounds for the controlling of neurological disorders including AD [32].
Antioxidant decreases free-radical-mediated damage in neuronal cells and help to inhibit dementia in mammalian brain cells [33]. Medicinal plant with reducing power donates an electron might be stabilized free radicals and inhibits the radical chain reactions [34]. Among CME and the four different fractions, EAF showed the highest IRPA with absorbance of 2.220 nm at highest concentration. Free radicals are linked to numerous diseases and there are strong reasons to believe that the free radical as a pathogenic factor for AD as stated earlier might be due to its toxicity and its role in various reactions such as Fenton’s, involving iron [35]. In this study for the DPPH scavenging activity, EAF showed the highest scavenging. The highest percentage of NO radical scavenging activity was also reported for EAF. The study suggested that metals play a foremost catalytic role in the formation of free radicals and attention has centered on the role of many metals in AD [6].

Only symptomatic treatment proven for AD is the use of cholinesterase (ChE) inhibitors [36]. In case of healthy brain, AChE dominates (i.e., 80%) and butryrylcholinesterase (BChE) is considered to show a minor role in controlling brain acetylcholine (ACh) levels [37]. In the AD brain, BChE activity rises, whereas AChE activity remains constant or declines [37, 38]. In fact, both enzymes are likely to have connection in controlling ACh levels and exert potent therapeutic targets to upgrade the cholinergic deficit [37]. Recent study showed that BChE may also have a role in the etiology and progression of AD by regulation of synaptic AChE levels [39, 40]. Therefore, specific AChE and BChE inhibitors development can improve clinical outcomes of AD patients [40]. In this study the highest AChE inhibitory activity was found in PEF and the highest BChE activity was found in CLF. Inhibition of AChE and BChE is suggested as the foremost therapeutic strategy against AD [41]. Several studies suggested that nootropic medicinal plants are effective for controlling Alzheimer’s pathogenesis by regulating ChE [3, 42].

CONCLUSION

The results acquired from the current study establish that EAF of U. littoralis leaves showed the highest antioxidant activity while PEF and CLF showed the highest anticholinesterase inhibitory activity with respect to remaining extract and fractions. Consequently U. littoralis leaves could be used as natural antioxidants, alternative anticholinesterase drugs and effective to treat various cognitive and neurodegenerative disorders, particularly AD. Nevertheless, further studies are needed to isolate and identify responsible nootropic compound(s).
ABBREVIATIONS

_Uvaria littoralis_ = _U. littoralis_

AD = Alzheimer's disease
OS = Oxidative stress
DNA = Deoxyribonucleic acid
ROS = Reactive oxygen species
OH\(^{-}\) = Hydroxyl radical
O\(^{2-}\) = Superoxide radical
NO\(^{-}\) = Nitric monoxide radical
NO = Nitric oxide
H\(_{2}\)O\(_{2}\) = Hydrogen peroxide
ONO\(^{•}\) = Peroxynitrite
Aβ = Amyloid-β
BHA = Butylated hydroxyanisole
BHT = Butylated hydroxytoluene
AlCl\(_{3}\) = Aluminium chloride
DPPH = 1, 1-diphenyl-2-picrylhydrazyl
Don = Donepezil
GA = Gallic acid
QRE = Quercetin equivalent
CTE = Catechin equivalent
CME = Crude methanolic extract
PEF = Petroleum ether fraction
CLF = Chloroform fraction
EAF = Ethyl acetate fraction
AQF = Aqueous fraction
AA = Ascorbic acid
TFCs = Total flavonoids contents
TPACCs = Total proanthocyanidins contents
IRPA = Iron reducing power

AChE = Acetylcholinesterase
BChE = Butyrylcholinesterase
ChE = Cholinesterase
ACh = Acetylcholine

AUTHORS’ CONTRIBUTIONS

This work was carried out in collaboration between all authors. Authors MSU and MA designed the study, wrote the protocol and managed the analyses of the study. Author AH provided the plant sample. Authors AR, MSU, MMM, and MAS performed the laboratory experiments and prepared the manuscript. Authors MMR, YA, MRR, MMAD and MJU reviewed the scientific content of the manuscript. All the authors read and approved the final manuscript.

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ETHICAL APPROVAL

The study protocol was approved by the ethics committee of the Department of Pharmacy, Southeast University, Dhaka, Bangladesh. The study was conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

The authors proclaim that they have no competing interests.

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