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Research Article

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In vitro screening for Acetylcholinesterase Inhibition and Antioxidant activity of selected Medicinal Plants

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ABSTRACT
In the present study, four plant extracts (*Allium sativum* L., *Desmodium gangeticum* L., *Eclipta alba* L., and *Piper longum* L.) were considered and checked for their acetylcholinesterase inhibitory activity which is the main true enzyme which hydrolyses acetylcholine in the body. The dried coarse powder of plants was extracted with methanol by cold extraction method. The resultant was assessed for acetylcholinesterase (AChE) inhibitory activity by Ellman's method with few modifications. The antioxidant activity was determined by DPPH (1, 1-diphenyl-2-picrylhydrazyl) and FRAP (Ferrous reducing Antioxidant power) assays. Quantitative phytochemical (phenolic contents) analysis of endogenous substances was performed by standard spectrophotometric methods. Plant extract significantly inhibited AChE activity. Additionally, the plant extracts exhibited strong radical scavenging activity against DPPH and reduced the Ferric ion (FRAP) significantly when compared to that of standards. Plant extracts were found to be rich in phenolic (gallic acid equivalent/g of dry extract) content. Furthermore, a positive correlation was observed between the total phenolics and antioxidants as well as the anticholinesterase potential.
Keywords: Alzheimer's disease (AD), Acetylcholine (ACh), Acetylcholinesterase (AChE), DPPH, Antioxidant activity, FRAP, Ascorbic acid, Free radical.

INTRODUCTION

Inhibition of Cholinesterases, mainly Acetylcholinesterase (AChE) and therefore prevention of acetylcholine degradation in synapses of the cholinergic system is one of the most accepted palliative therapy opportunities for Alzheimer's disease (AD) today (Birks, 2006). Since the introduction of the first cholinesterase inhibitor in 1997, most clinicians would consider the cholinergic drugs, donepezil, rivastigmine (Birks *et al*., 2009), and galantamine (Prvulovic *et al*., 2010), to be the first-line pharmacotherapy for mild and moderate AD. The most that these drugs could achieve is to modify the manifestations of AD. Due to a lack of selectivity of cholinesterase inhibitor drugs on the market, AD patients suffer from side effects like nausea or vomiting.

The enzyme acetylcholinesterase (AChE) catalyses the hydrolysis of the ester bond of acetylcholine (ACh) to terminate the impulse-transmitted action of ACh through cholinergic synapses (Stryer, 1995). Although the basic reason for Alzheimer's disease (AD) is not clear so far, AD is firmly associated with impairment in cholinergic transmission. Several AChE inhibitors have been considered candidates for the symptomatic treatment of AD as the most useful relieving strategy (Howes *et al*., 2003).

Plants have formed the basis of the traditional medicine system that has been the way of life for thousands of years. Mostly, herbs and spices contain polyphenols which are the most powerful natural antioxidants and are highly valued for their antioxidant, anti-ageing antimicrobial effects. Antioxidants are widely used as ingredients in dietary supplements and are exploited to maintain health and prevent oxidative stress-mediated diseases. Antioxidant compounds like phenolic acids, polyphenols and flavonoids inhibit the mechanism that leads to degenerative diseases (Hamid *et al*., 2010).

The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases (Prakash *et al*., 2007).

The alkaloid piperine from the spice family Piperaceae has been reported to possess poly-pharmacological activities including anti-depressant and cognitive-enhancing effects. It has been suggested that its

neurocognitive benefits may be via its activity on the cholinergic system, particularly on the enzyme acetylcholinesterase (AChE), a pharmacological target for neurodegenerative diseases such as Alzheimer's disease (AD). Piperine, as seen in the historic remedies, is a vital compound that exerts antipyretic and anti-inflammatory properties for medicinal uses. Other biological effects that piperine possesses are; analgesic (Gupta *et al*., 2000), antidepressant (Lee *et al*., 2005), cognitive enhancing (Wattanathorn *et al*., 2008), cytoprotective and anti-oxidant (Selvendrian *et al*., 2003). The antioxidant properties in piperine have also been linked to improvements in cognitive function.

Desmodium gangeticum (L.) commonly known as Salparni, belongs to the family Papilionaceae. It is widely distributed mainly in the Himalayan territory at elevations up to 5,000 feet. It is also distributed in China, Philippine and tropical Africa (Sagar *et al*., 2010). Traditionally, the plant has been used as an antipyretic, diuretic, astringent, anthelmintic, laxative, and in the treatment of dementia (Ma *et al*., 2011). The plant has been reported to exhibit anti-inflammatory, antibacterial, antidiabetic, hepatoprotective, antiulcer, locomotor and wound-healing activities. *D. gangeticum* has been reported to contain alkaloids, flavonoids, steroids and terpenoids (Bhattacharjee *et al*., 2013). The aqueous extract of *Desmodium gangeticum* has been shown to reverse scopolamine-induced amnesia by decreasing whole-brain acetylcholinesterase activity (Joshi and Parle, 2006).

Garlic (*Allium sativum* L.) is one of the World's oldest medicines and has been employed not only for flavouring but also as a medical herb for its diverse biological activities, including anti-carcinogenic, antiatherosclerotic, antithrombotic, antimicrobial, anti-inflammatory and antioxidant effects (Augusti, 1996, Wargovich *et al*., 1996, Hunter *et al*., 2005, Brace, 2002, Leelarungrayub *et al*., 2006). The antioxidant activity of Allium spp. has been attributed mainly to a variety of sulphur-containing compounds and their precursors (Yin *et al*., 2002, Singh *et al*., 2004). Scientific evidence shows that allicin, diallyl disulphide and diallyl trisulphide appeared to be the main antioxidative compounds (Kim *et al*., 1997, Rabinkov *et al*., 1998). In addition, the antioxidant activity is also related to other bioactive compounds: dietary fibres, microelements (especially Se) and polyphenols (Lanzotti, 2006, Gorinstein *et al*., 2005).

Eclipta alba (L.) Hassk (synonym *Eclipta prostrata*) is an annual herbaceous plant, erect or prostrate, belonging to the Asteraceae family. It is also known as Bhringaraj in Ayurveda which has been generally utilized for a very long time as a part of the conventional prescription for ailments especially related to the liver and hair. There are four main varieties of the herb *Eclipta alba* based on the colour of their blossom, that is, red, yellow, white, and blue. The white and yellow ones assume an essential part in traditional medicine, but it is the white species (*Eclipta alba*) that is most commonly harvested for its therapeutic advantages as it grows wildly in moist places, as a weed, and it can be easily propagated. The extracts from the leaves and flowers of this medicinal herb can be applied in numerous ways, both topically and internally, to soothe many ailments. *Eclipta alba* shows versatile pharmacological effects that include hair growth, antimicrobial, antioxidant, anti-inflammatory, analgesic, antinociceptive, antileprotic, antihaemorrhagic, antimyotoxic, antiviral, antihepatotoxic, diuretic, hypotensive, hypocholesterolemic, hypotensive, immunomodulatory, nootropic, ovicidal, and spasmogenic activity (Sawant *et al*., 2004, Thakur and Mengi 2005, Pandey *et al*., 1997).

MATERIALS AND METHODS

Preparation of various extracts: All the above-mentioned plant materials were dried in shade and powdered in a grinder. The plant material was exhaustively extracted successively using methanol. The solvents from crude extracts were recovered under reduced pressure using rotary vacuum evaporator. Various extracts were screened for detection of Acetylcholinesterase and antioxidant activity. Extracts were also analysed for total Phenolic contents.

Acetylcholinesterase (AChE) inhibition assay: AChE Assay

AChE inhibiting activity was measured by the spectrophotometric method developed by Lopez *et al*., 2002 inspired by Ellman *et al*., 1961. The enzyme activity was determined by observing the increase of a yellow colour produced from thiocholine (resulting from acetylthiocholine hydrolysis by an enzyme) when it reacts with DNTB (5, 5'-dithiobis-2-nitrobenzoic acid) ion. This can be detected at 405 nm (Rhee *et al*., 2001). Ten per cent methanol in buffer was used as negative control (enzyme activity without extract), Tris-HCl buffer 50 mM, pH 8, 0.1% BSA as enzyme blank and Galanthamine as the reference standard. The substrate ATCI (Acethylthiocholine Iodide) 15 mM was prepared in water and enzyme (0.22 U/mL) in Tris-HCl buffer 50 mM, pH 8, 0.1% BSA. The kinetic reaction was followed for 3 min. The percentage of enzyme inhibition (I %) of the enzymatic reaction was determined by the following equation:

$I\% = (E - S) / E \times 100$

where E: The substrate hydrolysis kinetics by enzyme without test compound

S: The substrate hydrolysis kinetics by enzyme with the test compound.

Determination of Antioxidant activity: DPPH Assay

Free radical scavenging activity of different extracts was tested against a methanolic solution of 1, 1 diphenyl 2-picryl hydrazyl (DPPH). Antioxidants react with DPPH and convert it to 1-1-diphenyl -2-picryl hydrazine. The degree of discoloration indicates the scavenging potential of the antioxidant extract. The change in the absorbance produced at 517nm has been used as a measure of antioxidant activity. The samples of different extracts were prepared in various concentrations viz.

100, 150, 200, 250 µg/ml in methanol. 1 ml sample of the above concentrations was mixed with an equal volume of 0.1mM methanolic solution of DPPH (0.39mg in 10 ml methanol). An equal amount of methanol and DPPH was added and used as a control. Ascorbic acid solutions of various concentrations viz. 100, 150, 200, 250 µg/ml in distilled water were used as standard. After incubation for 30 minutes in dark, absorbance was recorded at 517 nm. The experiment was performed in triplicates. Percentage scavenging was calculated by using the following formula:

Scavenging effect (%) = $(A_0-A_1/A_0) \times 100$

A_0 is the absorbance of the control reaction.

A_1 is the absorbance in presence of all the extract samples and reference

A graph was plotted with concentration (µg/ml) on X axis and % scavenging on the Y axis and IC^{50} values were calculated, which represents the concentration of the scavenging compound that caused 50% neutralization (Sreejayan and Rao 1996).

FRAP Assay

Determination of Reducing Power: Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. It describes how easily one substance can give electrons to another. A powerful reducing agent is keen to donate electrons. This method measures the ability of antioxidants to reduce ferric ion. Reducing power was investigated using the method developed by Yen (Yen and Duh 1993). The samples of different extracts were prepared in various concentrations viz. 200, 400, 600 and 800 µg/ml in distilled water. 1.25 mL of sample aliquots were mixed with 1.25 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 1.25 mL of 1% potassium ferricyanide ($K_3Fe(CN)_6$). The mixtures were incubated at 50 ºC for 20 minutes. The resulting solution was cooled rapidly, mixed with 1.25 mL of 10% trichloroacetic acid and centrifuged at 3,000 rpm for 10 min. The supernatant (2.5 mL) was taken out and immediately mixed with 2.5 mL of distilled water and 500 µL of 1.0 % ferric chloride ($FeCl_3$) was then added. After incubation for 10 min, the absorbance (abs) against blank was determined at 700 nm. All samples were assayed in triplicate. The ascorbic acid standard was utilized for comparison.

Determination of Total Phenolic Content:

Folin-Ciocalteu Total Phenolic Assay

This assay measures the change in colour as metal oxides are reduced by polyphenolic antioxidants such as gallic acid and catechin, resulting in a blue solution with maximal absorption at 765 nm. The standard curve is prepared using gallic acid, and results are reported as gallic acid equivalents. Total phenols were determined by Folin-Ciocalteu reagent. The Folin-Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate used for the colourimetric assay of phenolic and polyphenolic antioxidants. However, this reagent does not only measure total phenols and will react with any reducing substance. The reagent, therefore, measures the total reducing capacity of a sample, not just the level of phenolic compounds. A dilute sample of different extract (0.5 ml of 1:10 g/ml) or gallic acid (standard phenolic compound) was mixed with Folin-Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by spectrophotometric measurements at 765 nm. The standard curve was prepared using 50, 100, 150, 200, and 250 mg /L solutions of gallic acid in methanol: water (50:50, v/v). The total phenolic content was expressed as mg/g equivalents of gallic acid which is a common reference compound (Banerjee *et al*., 2008, Pourmorad *et al*., 2006).

RESULTS AND DISCUSSION

The inhibition might come from the presence of phenolic acids, flavonoids and other antioxidant compounds. Antioxidant compounds might be implicated in AChE inhibition. Recent studies bound Alzheimer's disease to an inflammatory process induced by reactive oxygenated substances. The oxidative stress intervenes, for a share, in the physiopathology of neuronal degeneration.

In vitro tests of methanolic extract of bulb of *Allium sativum* Linn. evaluated for its antioxidant property revealed DPPH, FRAP, AChE and total phenolic content effect. The antioxidant reacts with stable free radical, DPPH and converts it to 1, 1-diphenyl–2-picryl hydrazine. The ability to scavenge the stable free radical DPPH was measured by a decrease in the absorbance at 517 nm. A concentration-dependent assay was carried out with these extracts and the results are presented in Tables 1, 2 and 3. Methanolic extract of the sample showed the scavenging activity of DPPH that ranged from 16.28±0.26 to 34.48±0.36 and acetylcholinesterase activity was found in the range between 24.24±0.23 to 56.56±0.28. An assay of reducing power reveals the reductive capabilities of the bulb extracts compared to ascorbic acid. The reducing power of bulb extracts was very potent and the power of the extract was increased with increasing concentration. The absorbance values ranged from 0.292±0.22 to 1.124±0.62. The reducing power of the garlic extracts was a function of their concentration. The reducing power of the garlic extracts increased with their concentrations or on par with the results of Deore *et al* 2009. Phenolics are the widest-spread secondary metabolite in the plant kingdom. These diverse groups of compounds have received much attention as potential natural antioxidants in terms of their ability to act as both efficient radical scavengers and metal chelator. It has been reported that the antioxidant activity of phenol is mainly due to their redox properties, hydrogen donors and singlet oxygen quenchers (Rice-Evans *et al*. 1995). Therefore, in the present study, the total phenolic content present in the extract was estimated using the modified Folin- ciocalteu method. Polyphenols are used for the prevention and cure of various diseases which are mainly associated

with free radicals. The higher content of polyphenols may be attributed to the antioxidant potential of garlic.

Table 1. DPPH % scavenging of Plant extract

Conc (µg/ml)	% Scavenging DPPH (100µg/ml) MeOH extract				
	AS	DG	EA	PL	Ascorbic Acid
100	16.28± 0.26	34.48± 0.36	10.36± 0.22	14.40± 0.25	48.17± 0.43
150	22.24± 0.21	38.35± 0.56	12.36± 0.22	18.44± 0.28	66.08± 0.39
200	30.28± 0.22	40.28± 0.51	18.28± 0.28	37.95± 0.30	83.27± 0.56
250	34.48± 0.36	42.22± 0.67	24.86± 0.48	48.19± 0.34	91.26± 0.66

Each value represents the mean and standard deviation from three replicates

Table 2. AChE inhibitory activity of Plant extract

Conc. (µg/ml)	% Inhibition AChE (200 u/ml) AS	DG	EA	PL
100	24.24± 0.23	31.10± 0.52	18.52± 0.29	31.28± 0.24
150	31.26± 0.21	34.30± 0.42	24.25± 0.25	42.23± 0.28
200	42.28± 0.22	40.22± 0.20	37.26± 0.24	62.26± 0.20
250	56.56± 0.28	40.83± 0.05	46.25± 0.20	76.26± 0.24

Each value represents the mean and standard deviation from three replicates

Table 3. Reducing power ability: FRAP Assay

Conc (µg/ml)	Absorbance (700nm) MeOH extract				
	AS	DG	EA	PL	Ascorbic acid
200	0.292± 0.22	0.124± 0.12	0.202± 0.20	0.302± 0.20	0.362± 0.88
400	0.566± 0.42	0.222± 0.24	0.398± 0.18	0.596± 0.24	0.718± 0.32
600	0.824± 0.52	0.346± 0.18	0.582± 0.16	0.902± 0.26	1.086± 0.38
800	1.124± 0.62	0.420± 0.20	0.788± 0.18	1.224± 0.40	1.414± 0.42

All values in the table represent mean ± SD (n=3)

Table 4. Total phenolic content of four plants.

Plants	Conc. (µg/ml) vs Abs. (765nm)				Total phenolic content GAE mg/g
	100	150	200	250	
Allium sativum L.	0.226	0.386	0.418	0.522	15.23±0.20
Desmodium gangeticum L.	0.258	0.438	0.611	0.721	24.85±0.23
Eclipta alba L.	0.201	0.273	0.348	0.446	14.89±0.21
Piper longum L.	0.258	0.438	0.611	0.784	48.93±0.22
Mean Value ± Standard Deviation of three replicates					

So far as plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the selected plant extract. The content of total phenols in methanolic extracts was expressed in gallic acid equivalents (GAE). The phenols contain hydroxyls that are responsible for the radical scavenging effect mainly due to redox properties. The methanolic extracts of *Allium sativum* L. had phenol content of 15.23±0.20.mg GAE/g. Absorbance vs concentration graph was plotted for the Ferric ion reduction and the phenolic content.

The inhibitory activity of AChE by *D. gangeticum* L. is presented above at a final concentration of 100-250 µg/mL. Percentage inhibition of *D. gangeticum* L. ranged from 31.60±0.52to 40.83±0.05. DPPH scavenging percentage was found in the range of 34.48±0.36 to 42.22±0.67. The amount of extract needed for 50% inhibition of DPPH free radical is known as the IC^{50} value of the extract. Lowering the IC^{50} value shows a better scavenging ability of the sample. The reducing power ability of Ferric ions in FRAP assay for DG was found to be effective as shown in Table 2. The absorbance values of the extract were in the range of 0.124±0.12 to 0.420±0.20. The standard Ascorbic acid reduced the Ferric ions significantly much higher in the range of 0.362±0.88 to 1.414±0.42 respectively. The total phenolic content in *D. gangeticum* L. was 24.85 ±0.23 mg GAE/g.

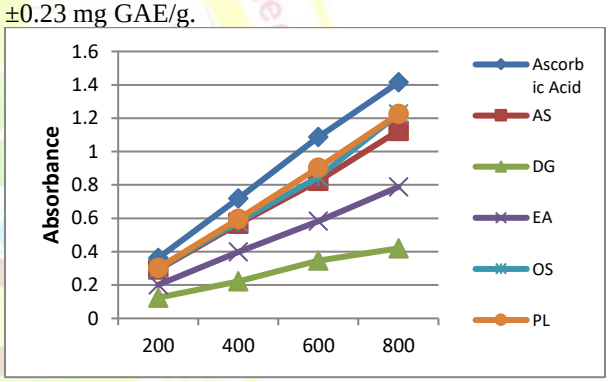

Fig. 1. FRAP Assay

The activity of *E. alba* L. was calculated as a range of 10.36±0.22 to 24.86±0.48 for DPPH whereas AChE inhibition activity was measured as a range of 18.52±0.29 to 46.25±0.20 (Table 2). The reducing power ability of Ferric ions in FRAP assay for EA was found to be effective as shown in Table 3. The absorbance values of the extract were in the range of 0.202±0.20 to 0.788±0.18. The total phenol content of *E. alba* L. was calculated as 14.89±0.21 mgGAE/g. which is the lowest among the studied medicinal plants. The highest DPPH scavenging percentage is of *Piper longum* L. and the lowest is *Eclipta alba* L. In respect of AChE inhibition activity, *P. Longum* L. showed the highest % whereas the lowest inhibition activity was of *D. gangeticum L.*

The methanolic extract of *Piper longum* L. showed a greater content of phenolics and augmented *in vitro* antioxidant activity and antiacetylcholinesterase activity. DPPH radical scavenging activity was found in the range

14.40±0.25 to 48.19±0.34. AChE inhibition percentage was between 31.28±0.24 to 76.26±0.24. Reducing power assays showed activity in the range of 0.302±0.20 to 1.224±0.40. Total phenols in *Piper longum* L. were found to be 48.93±0.22 mgGAE/g.

CONCLUSION

The overall acetylcholinesterase activity was found to be maximum in *Piper longum* L. followed by *Allium sativum* L., *Eclipta alba* L. and *Desmodium gangeticum* L, whereas DPPH radical scavenging and ferrous reducing power was maximum in *Piper longum* L. followed by *Desmodium gangeticum, Allium sativum* and lowest was found in *Eclipta alba*. (Table 1-3). Total phenol and flavonoids content were in the order, *Piper longum* followed by *Desmodium gangeticum, Allium sativum* and *Eclipta alba*.

The result of total Antioxidant activity and Total phenolic and flavonoids content showed that the plant extracts or the plants studied here can be seen as a potential source of new useful drugs. The present study reveals that the selected plants would exert several beneficial effects by virtue of their antioxidant activity and could be harnessed as drug formulation. The phytochemical characterization of the extracts, the identification of responsible bioactive compounds and quality standards are necessary for future study.

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