Potential Cholinesterase Enzyme Inhibition and Antioxidant Bioassays of *Saxifraga flagillaris*; an approach to cure Alzheimer's disease

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Abstract

Production of surplus free radicals leads to impaired neurotransmission which results in Alzheimer Disease (AD). Inhibition of Acetyl and Butyryl-cholinesterase enzymes is considered to be the prominent healing process for AD coupled with scavenging the free radicals in the living system. Medicinal plants possess specific compounds which showed inhibition of the enzymes upon screening and are antioxidants. In current study, *Saxifraga flagillaris* n-hexane, dichloromethane and ethyl acetate fractions were used to assess the antioxidant and anti-cholinesterase activity. Antioxidant activity was carried out by ABTS and DPPH assays. The ethyl acetate fraction of *Saxifraga flagillaris* showed maximum antioxidant activity with IC_{50} of 11.54 for ABTS and 18.44 for DPPH assays. Acetyl and Butyryl-cholinesterase enzymes were inhibited in a dose dependent way as various concentrations of the extract were used (1000- 62.5 µg/mL). Acetylcholinesterase was inhibited with IC_{50} of 16.06 by ethyl acetate fraction of *Saxifraga flagillaris*. Similarly, Butyryl-cholinesterase was inhibited by *Saxifraga flagillaris* ethyl acetate fraction with IC_{50} of 16.94. These results suggest that the ethyl acetate extract of the plant possess metabolites which are potent antioxidants and also have high cholinesterase enzymes inhibition potentials. It is concluded that the plant extracts are best candidates for isolation of novel compounds to treat Alzheimer Disease.

Key Words; Antioxidants, Anticholinesterase; Alzheimer disease, Medicinal plants, phytochemicals

1. Introduction

Reactive oxygen species (ROS) can affect the human body both internally and outside. The basic biomolecules, such as enzymes, proteins, RNA, and DNA, might be damaged if the human body fails to eliminate these chemicals. When ROS production is out of control, it negatively impacts the immune system of humans, which leads to the onset of chronic illnesses in the body's major organs, including the heart, kidneys, lungs, spinal cord, and brain. Cancer, diabetes, and neurological disorders like Alzheimer's and Parkinson's disease are examples of such illnesses [1]. These ROS specifically impaired the activity of nerve cells of brain which are exceptionally more sensitive to undue ROS. Due to susceptibility of these nerve cells to oxidative stress suggested the instigation and development of neuro-degenerative diseases specially Alzheimer Disease [2-4].

Disease related to lifestyle has close association with oxidative stress that lead to production of ROS. Oxidative stress is a conditions where process of oxidation surpasses the anti-oxidant structures of the living organism bringing damage, with a result of imbalances in them [5]. Various studies of pathophysiology have proved that oxidative stress is responsible for damage in cells leading to tissue injury *in vivo* [6]. Without antioxidants, these ROS cause a number of major difficulties in the body, therefore their scavenging function is helpful in managing these chronic disorders, such as Alzheimer's disease [7]. Various agents like pollution, unhygienic food condition, and environmental factors are the chief causes of ROS [8]. Alzheimer's disease is one of the important nervous syndrome[9]. Amyloid bits deposition these are unsolvable proteins, are said to be responsible for causing the Alzheimer's disease [10]. Anomalies of mitochondria and RNA, DNA are said to be associated with Alzheimer's disease (nerve problem) [11] which causes the deficiency of neuro-transmitters like somatostatin [12], serotonin [13], noradrenalin[14] and especially of acetylcholine [15]. This deficiency collapse the synaptic function of meiosis. The synapsis activity was initiated by the neurotransmitter acetylcholine, and this function is terminated by the enzymes alpha and beta cholinesterase, respectively [16]. Resistant to insulin and diabetes T-II are additional causes of Alzheimer Disease. Unevenness of free radicals within the body tend to develop diseases in them which may become chronic with the passage of time [17, 18].

It is necessary to find alternative inhibitors of these enzymes to treat Alzheimer's disease[19]. Many ailments, from simple to complex, are first treated with plants [20, 21]. Alzheimer's disease

is treated using a variety of medicinal herbs. It has been stated that using wild plants, vegetables, and spices helps treat Alzheimer's disease [22, 23]. There are numerous medications on the market to treat the illness, but they can have very serious side effects on the body [24]. Natural plant resources offer numerous healing properties and less negative impacts on humans than manmade medications [25-27]. According to studies on curcuma longa, the plant contains curcumin, a significant anti-inflammatory and antioxidant substance that can treat Alzheimer's disease [28, 29], *Bacopa monneiri* [30]. Ginkgolides, which is found in *Ginkgo biloba* and is neuroprotective, aids in the recovery of Alzheimer's patients[31]. *Acorus calamus* contained alpha and beta-asarone which inhibit the enzyme acetyl cholinesterase [32], *Withenia somnifera* contained chemical constituents which inhibit the enzyme acetyl cholinesterase [33, 34]. It is considered that using antioxidants will not only reduce the number of ROS but can also prevent long lasting diseases including Alzheimer's disease. Currently many antioxidants are available in the market like gallic acid esters, butyl hydroxyl toluene and anisole, hydroquinone. Their used is limited because these have tendency to replace the natural antioxidant of medicinal plants [35, 36].

The medicinal plant in the present study was selected due to their ethnobotanical significance. *Saxifraga flagillaris* is used in hair dandruff [39]. As far as, there is no information about the *Saxifraga flagillaris*'s anticholinesterase activity, hence he current study was preliminary focused on the anticholinesterase and antioxidant activities.

2. Materials and Methods

2.1 Plant sample

The part of the plant which are used locally for treatment of diseases were collected. These were thoroughly washed, shade air dried, rhizome were cut and oven dried.

2.2 Extraction Procedure

Dried part of plant was powdered using electric grinder. The powdered material was extracted with 70 % ethanol and then fractionated with organic solvents based on solvent polarity. The filtrate was concentrated using rotary evaporator. The extracts were kept at 4^{o}C for further use in activity [40, 41].

2.3 Antioxidant assays

Antioxidant activities of the extracts were carried out through DPPH and ABTS free radicals scavenging assays.

2.3.1. DPPH Bioassay

The technique of Brand-Williams, et al., [42] for scavenging free radicals through DPPH was used with few changes. 24mg DPPH was taken in 100ml of methanol, placed in dark area for 30 minutes at normal temperature to make its solution. The 1mg of plant extracts were dissolved in 1 ml of methanol and then different concentrations were made like, 1000, 500, 250, 125 and 62.5µg/ml respectively.

Plant extracts and DPPH solution were taken in a proportion of 1:1 and incubation was done for 30 minutes (23°C). Lastly, absorbance was obtained (517 nm) by spectrophotometer (thermo electron corporation USA). Ascorbic acid taken as standard.

The activity was measured in percent's with the given procedure;

Scavenging % = C.A. - S. A. / C.A. x 100

i.e.

C.A. = Control Absorbance; S.A.= Sample Absorbance

2.3.2. ABTS Assay

Antioxidant potency of selected medicinal plants fractions were performed through ABTS assay[43]. 7 mM of ABTS and 2.45 mM of potassium per-sulfate solutions were arranged and shake them well. At normal temperature, the mixture was kept in dark for 10- 12 hours for producing free radicals. Dilutions of the ABTS solution was made using Phosphate buffer (0.01 M), pH was 7.4, before any next step.

The absorbance of ABTS solution was attuned to 0.7 at 745 nm by mixing the methanol (50%). Scavenging potency of the plant extracts was examined by mixing 300µl of plant extract with ABTS solution of 3.0ml in a cuvette. The absorbance was taken by double beam spectrophotometer for 6 min. Ascorbic acid taken as standard. The assay was triplicated.

Scavenging activity was measured in percent's by following formula;

Scavenging %age = C.A. - S. A. / C.A. x 100

Where; C. A. =Control Absorbance; S. A. = Sample Absorbance

2.4 Anticholinesterase assays

Acetylcholinesterase (AchE) from electric eel and butyrylcholinesterase (BChE) from equine serum were taken to check the inhibition of enzymes potency for *Saxifraga flagillaris* n-hexane, Dichloromethane, ethyl acetate and aqueous fractions with using Ellman's assay [44, 45]. The bioassay is focused on the hydrolysis of acetylcholine iodide or butyrylthiocholine iodide by the particular enzymes, to form 5-thio-2nitrobenzoate anion couple with complexation with DTNB to produce a yellow color compound to be check with spectrophotometer.

2.4.1 Preparation of Solution

The extracts were dissolved in phosphate buffer (0.1M) with different concentrations (1000-62.5µg/m=L). For preparing 0.1M and 8.0±0.1pH phosphate buffer solution, K_2HPO_4 (17.4g/L) and KH_2PO_4 (13.6g/L) were prepared and mixed with 94% and 6% ratio individually. Potassium hydroxide was taken as to calibrate the pH. The AChE (518 U/mg) and BChE (7-16 U/mg) were dissolved in newly prepared buffer with 8.0 pH till to get a final concentration of 0.03U/mL and 0.01 U/mL respectively. DTNB solution of (0.0002273M) and ATChI and BTChI (0.0005M) were made in distilled water and put in Eppendorf tubes in fridge. Galathamine is use as standard.

2.4.2 Spectroscopic Analysis

A total of 5µl enzyme solution was added to a cuvette and 205 µl of sample solution was added to it with 5µl of DTNB reagent. The mixture was kept for 15 min. at 30^{o}C in water bath and 5µl of the substrate solution was added. Absorbance was measured at 412nm. The experiment was repeated three times and activity measured as enzyme inhibition with the given formula; (V = ΔAbs /Δt) as follow;

V ¼ ΔAbs=Δt

% enzyme activity ¼ V=Vmax _ 100

% enzyme inhibition ¼ 100-% enzyme activity

2.5 IC_{50} Values

Excel program was used to get the IC_{50} values of the extracts. It is the concentration at which 50% inhibition was observed.

2.6 Statistical analysis

The experiments were conducted in biological triplicates. The data was properly analyzed by ANOVA using SPSS software 21.0 package. The effect were considered significant if the $p > 0.05$.

3. Results

3.1 Antioxidant Activity

Antioxidant activity of selected medicinal plants in the present study was determined by ABTS and DPPH assays with IC_{50}. Ascorbic acid was used as standard drug for the activity (**Table.1**).

Table1. Ascorbic acid as Standard

Samples	**Conc. (µg/mL)**	**% ABTS inhibition (mean ±SEM)**	**ABTS IC50 (µg/mL)**	**% DPPH inhibition (mean ±SEM)**	**DPPH IC50 (µg/mL)**
Ascorbic acid	1000	87.68±0.35	15.70	89.23±0.30	13.324
	500	81.73±0.53		81.47±0.37	
	250	77.52±0.21		75.04±0.26	
	125	71.65±0.34		71.46±0.41	
	62.5	64.67±0.28		67.82±0.37	

3.2. ABTS and DPPH free Radical scavenging assay of *Saxifraga flagillaris*

Antioxidant activity of *Saxifraga flagillaris* showed (Table 2) that the ABTS and DPPH free radical scavenging assay of *Saxifraga flagillaris* were carried out using different concentration of hexane, DCM and ethyl acetate fractions. The ethyl acetate fraction was the most potent fraction with percent inhibition of 91.99±0.585% (ABTS assay) and 89.20±0.40% (DPPH assay) at 1000 µg/mL with IC_{50} of 11.54 and 18.44 respectively. While hexane and DCM fractions were least effective with ABTS assay IC_{50} of 2078.04 and 1992.03 respectively and with DPPH assay IC_{50}

of 1900.47 and 741.97 respectively. Overall the activity in the experiment was concentration dependent. IC_{50} of the plants are mentioned in (Table.2).

Table 2. ABTS and DPPH IC_{50} (µg/ml) of selected medicinal plants

Samples	ABTS IC_{50} (µg/mL)	DPPH IC_{50} (µg/mL)
n- hexane	2078.04	1900.47
DCM	1992.03	741.97
E.A	11.54	18.44
Ascorbic acid	15.70	13.324

3.4 Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzyme inhibition assay

Inhibiting the enzymes Acetylcholinesterase and ButyrylCholinestarse to break into acetylcholine and butyrylcholine which are said to be effective targets in the cure of many neural complaints specially Alzheimer's disease, senescent dementia and m. gravis. Medicinal Plants are conventionally used to improve cerebral activity and bring ease to illnesses related through Alzheimer's disease. The table showed the percent inhibition of with IC_{50} values of the selected medicinal plants. All the extracts exhibited a good inhibition percentages of the enzymes AcetylCholinestrase and ButyrylCholinestarse which occur in dose-dependent manner. Galantamine is used as standard drug (Table.3). At high concentrations the plant extracts have good antiAcetylCholinestrase and antiButyrylCholinestarse activity.

Table 3. Percent inhibition of AChE and BChE by Galantamine standard drug

Standard	Conc. (µg/mL)	ACHE% inhibition (mean ±SEM)	AChE IC50 (µg/mL)	BChE% inhibition (mean ±SEM)	BChE IC50 (µg/mL)
	1000	93.20±0.12		96.82±0.17	

Galantamine	500	86.82±0.25	23.89	87.93±0.13	27.18
	250	78.67±0.18		79.27±0.09	
	125	71.56±0.30		73.07±0.14	
	62.5	66.40±0.32		67.51±0.29	

The n-hexane and dichloromethane fractions of *Saxifraga flagillaris* produce less effective results as compared to its ethyl acetate fraction, these at 1000 (µg/mL) concentration n-hexane and dichloromethane fractions inhibit the acetylcholine by 33.82±0.09 and 38.70±1.76 percent respectively and butyrylcholine by 29.66±0.33 and 27.30±0.80% respectively.

The ethyl acetate fraction showed (Figure 8) the most effective inhibition of 98.06±1.07 percent acetylcholine and 95.87±0.64 butyrylcholine at 1000 (µg/ml) with IC50 value of 16.06 for acetylcholine and 16.94 for butyrylcholine. IC_{50} values of n-hexane and dichloromethane was 1675.26 and 1377.72 for acetylcholine and 2134.23 and 1893.97 for butyrylcholine which is very high as compared to standard Galantamine. Low IC_{50} value means effectiveness of the sample (Table. 4).

Table 4. AChE and BChE IC_{50} (µg/ml) of fraction of *Saxifraga flagillaris*

Samples	AChE IC50 (µg/mL)	BChE IC50 (µg/mL)
n-hexane	1675.26	2134.23
DCM	1377.72	1893.97
E.A	16.06	16.94
Galantamine	23.89	27.18

5. Discussion

The intake of medicinal plants has increased in the most recent age as a result of more severe side effects from synthetic medications. Alkaloids, terpenoids, phenols, flavonoids, and other secondary metabolites are among those found in medicinal plants which are used as effective antioxidants, ant aging agents, as memory restorative agent's etc [46]. These chemical components, such as flavonoids, are found in many fruits, vegetables, and cultivated and wild medicinal plants. They have a variety of biological uses, including as metal binders, free radical scavengers, and in the protection, suppression, and activation processes of numerous major bodily systems. It is hypothesized that flavonoids have a vital role in brain cells [47, 48]. Alzheimer's disease specifically causes memory loss and generally has an impact on daily life. There is currently no effective medicine available to treat the illness. Worldwide, scientists are constantly interested in screening medicinal plants for new and potentially beneficial chemicals to cure disease. Lack of acetylcholine results in a lack of cholinergic neurotransmitters, which are thought to play a role in short-term memory. Using a choline stars inhibitor helps to some extent prevent the condition [49]. It is reported that the plant with 50% inhibition is classified as potent plant, above 30% is moderate, below 30% is low and near to 5% is no inhibition [50]. Free radical production is thought to contribute to Alzheimer's disease. Antioxidant activity of the chosen medicinal plants was also tested in the current study. Various concentrations were used to assess the activity (1000 µg/mL -62.5 µg/mL). ABTS and DPPH assay have been mostly used to check the antioxidants potential of samples[51]. Similarly, the *Saxifraga flagillaris* fractions also have good scavenging activity and its ethyl acetate fraction scavenged the free radicals through ABTS and DPPH with IC_{50} of 11.54 and 18.44 respectively. While hexane and DCM fractions were least effective with ABTS assay IC_{50} of 2078.04 and 1992.03 respectively and with DPPH assay IC_{50} of 1900.47 and 741.97 respectively. Many findings indicate that ethyl acetate fraction produces positive outcomes when compared to other solvent extracts [52]. Oxide-nanoparticles of selenium, cerium, and melanin are utilized in addition to the use of green plants because they not only act as antioxidants but also help to restore mitochondrial activity, which is a factor in the excessive production of ROS. These powerful oxides may one day be used to treat disorders like anxiety and Alzheimer's that are brought on by stress [53-55]. Antioxidant disease treatment has been demonstrated to significantly improve cognitive performance and communication deficits in Alzheimer's disease patients [56]. According to theory, plants are the biggest source of antioxidants and effective cholinesterase enzyme inhibition for treating Alzheimer's disease [57-59].

In present study, the selected medicinal plant used to screen for treating Alzheimer Disease. All the plant samples showed moderate to strong inhibition of the enzyme. *Punica granatum* fruit extract also possess anticholinesterase activity with good IC_{50} value of 77±6.2µg/ml showed to improve the cognitive abilities. The plant possess punicalgins which are potent antiaging substsnce [60]. Juice of the plant reduce the amount of amyloid produced in body [61]. It assist the present findings. Begum et al [52] reported similar results that the ethyl acetate fraction of *A. augusta* produced good inhibition of the both enzymes while extracts of other solvents were not much effective like the ethyl acetate fraction.

The enzyme Acetyl cholinesterase and butyryl chcholinesterase is the drug target due to its function in acetylcholine hydrolysis. The enzyme inhibitor are the only way to get rid of the mental disease like Alzheimer. Similarly ethyl acetate fraction of *Saxifraga flagillaris* also gave low IC_{50} value which is considered to possess potent compounds to treat the disease Alzheimer. Herbal extracts either singly or in combination used as to cure Alzheimer Disease. These medicinal herbs have particular chemical components that prevent acetylcholine esterase from working. According to reports, nitrogenous chemicals, which are naturally occurring alkaloids, make up the majority of the acetylcholine esterase enzyme inhibitors. So these medicinal plants extracts might have strong alkaloids to inhibit the enzyme [62-64]. The efficient screening of medicinal plants for evolving new compounds to treat Alzheimer diseases, takes place from time to time. Rivastigmine and galantamine like plant derived compounds were used as cholinesterase inhibitors[65-67]. Some of the analyses on AChEIs have been focused on alkaloids. More than 35 alkaloids have been purified from plants so far with AChEI activity [68]. The plant *Saxifraga flagillaris* ethyl acetate fraction might have strong alkaloids that make inhibition of the enzyme acetylcholinesterase. While some reports suggest that coumrine also inhibit the acetyl cholinesterase [69, 70].

Conclusion

The current experiment concluded that antioxidants are extremely important in the treatment of chronic disorders like Alzheimer's disease, which is brought on by the body producing too many free radicals (ROS) as a result of compromised mitochondrial function. Also, it is advised that this medicinal plant be thoroughly examined for the treatment of Alzheimer's disease. Doing so will

enable the identification and isolation of novel components that will aid in the creation of new medications specifically designed to treat Alzheimer's disease.

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