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Phytochemical screening, Antioxidant, Anti-Alzheimer and Anti-diabetic activities of *Centella asiatica*

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ABSTRACT

The present study was aimed at investigating the phytochemical bioactive compounds of the ethanolic extract of leaves of Centella asiatica (Family: Mackinlayaceae) and its antioxidative, AChE inhibitory and anti-diabetic activity which may ultimately lead to the finding of more effective agent for the treatment and management of Alzheimer Disease and Diabetes. For this purpose, phytochemical screening was done by variety of methods; Antioxidant activity was assessed by using the 1,1-diphenyl-2-picryl hydrazyl (DPPH) scavenging assay and measuring Super oxide radical scavenging activity; Anti-Alzheimer activity was assayed by quantifying acetyl cholinesterase (AChE) and butyrylcholinesterase (BChE) enzyme and Antidiabetic activity was investigated by measuring glucose in alloxan induced diabetic rat. There were presence of alkaloids, glycosides, steroids, flavonoids, tannins and reducing sugars; 50% scavenging (IC50) of DPPH and Superoxide were 40.4 and 109.57 µg/ml respectively; IC50 of AChE and BChE were 170 and 226 µg/ml respectively; Alloxan induced rat has 32.6%, 38.8% and 29.9% reduction of blood glucose level at 3rd hours after ingestion of 250mg/kg, 500mg/kg and 1000mg/kg of C. asiatica leaves extract respectively. C. asiatica was also tested for acute toxicity where no death or signs of toxicity even at the dose of 3000 mg/kg in rats was observed. The results of conducted study revealed that, the presence of the bioactive compounds, some of which possess an ideal structure for the scavenging of free radicals, could be responsible for the versatile medicinal properties of this plant including significant Antioxidant, Anti-Alzheimer and Antidiabetic activities.

Keywords: *Centella asiatica*, Phytochemical screening, Antimicrobial activities, Antioxidant activity, Anti-Alzheimer activity and Anti-diabetic activity.

INTRODUCTION

Centella Asiatic (synonym: Hydrocotyle asiatica L.), belonging to the family of Mackinlayaceae is native to most of the countries of Asia. Being herbaceous, it contains stems which are long, filiform and prostrate with long internodes containing roots, 1-5 leaves per node which are 50-350cm in radius, reniformed, deeply cordate, long petioled and oval or orbicular in shape, 3-6 small flowers which are purple to white-green in color and are arranged in umbels arising from the axils of the leaves [1]. It grows well in both tropical and sub-tropical countries. It is a popular herb that is either consumed fresh, or processed into tea or juice.

The plant has been claimed to exert various physiological effects and is traditionally used for various ailments including wound healing, bronchitis, asthma, diabetes, kidney troubles, urethritis, liver complaints, allergy, cancer, diuretic, and hypertension and to improve mental ability [2]. In ulcer, depression and venomous insufficiency *Centella Asiatic* is a potent drug [3, 4]. The plant is also found to improve the general behavior and mental ability of retarded children [5]. The anti-diabetic property of *C. asiatica* has been known to the ancient people of Bangladesh for centuries that are following Ayurvedic system of medicine. Although the plant is being long used in our country, a very few chemical and pharmacological study on the extract of this plant has been done in Bangladesh till now. The search for the phytochemical bioactive compounds from medicinal plant is always an alternative mean of finding new drugs. Phytochemical screening of *C. asiatica* will lead to the rationalization of the use of this plant in various diseases as mentioned and will also lead to the discovery of specific causative compound which have effective treatment roles in against specific diseases [6].

Alzheimer's disease (AD) is a progressive neurodegenerative disorder which has the characteristic features of memory impairment, cognitive dysfunction, behavior disturbances and deficits in activities of daily living [7]. Although the etiology of the disease is still not very clearly known there are two major hypotheses which inadequately explain the molecular mechanism of AD, namely: the cholinergic hypothesisand the amyloid cascade hypothesis [8]. The cognitive dysfunction in AD is supposed to be a result of degeneration of cholinergic neurons in the basal forebrain and associated loss of cholinergic neurotransmission in cerebral cortex and some other areas [9].

The enzyme acetyl cholinesterase (AChE) and butyrylcholinesterase (BChE) play an important role in the cholinergic deficit through enhanced degradation of the neurotransmitter acetylcholine (ACh). So, in most cases to improve neurotransmission and to alleviate cholinergic deficit, the focus is mainly on acetylcholinesterase (AChE) and butylcholinesterase (BChE) [10]. According to the amyloid cascade hypothesis, AD is associated with the accumulation of β-amyloid (Aβ) fibrils and senile plaque in the brain parenchyma. Several studies have reported that increased oxidative stress has a potential role in the inflammatory processes that eventually lead to the lipid peroxidation and formation of Aβ [11]. Many studies have shown that increased level of free radicals and reactive oxygen species induced the degeneration of neurons [12, 13 & 14]. Antioxidants can scavenge free radicals and ROS and can also attenuate inflammation pathways. Hence, antioxidants may be useful in the protection from neurodegeneration in AD. Although in some previous study [15]. the effect of C. asiatica extract on the Aβ levels in hippocampus of AD animal model was observed no study has yet been done on its cholinesterase (ChEs) inhibitory effect.

Diabetes mellitus is a metabolic disease characterized by high blood glucose levels due to absolute or relative deficiency of circulatory insulin levels [16]. It has an sdverse effect on carbohydrate, lipid and protein metabolism resulting in chronic hyperglycemia and abnormality of lipid profile. These lead to series of secondary complications including polyurea, polyphasia, ketosis, retinopathy as well as cardiovascular disorder [17]. The disease is a major degenerative ailment in the world today, affecting al least 15 million people [18]. Currentlly available therapy for diabetes includes insulin and various oral hypoglycemic agents such as sulfonylureas, metformin, glucosidasae inhibitors, troglitazone, etc. But these are reported to produce serious adverse side effects such as liver problems, lactic acidosis and diarrhea [19]. In addition they are not suitable for use during pregnancy. More over increased oxidative stress and generation of excessive free radicals in diabetic patients are thought to be the etiology of chronic diabetic complications [20]. Increased reactive oxygen species and oxidative stress are observed in type-1 and type-2 diabetes mellitus in some studies [21]. Besides, in diabetes glucose autoxidation, polyol pathway and non-enzymatic glycation of proteins lead to irregular formation of free radical [22]. This increased free radical generation along with declined antioxidant defense system may damage enzymes, cellular organelles, lipid peroxidation and further diabetic abnormalities [23]. So, if any medicinal plant can work as a potential antioxidant together with having anti-diabetic property then it could prevent or reduce diabetic complication more effectively

than the conventionally used anti-diabetic drug. Although many anti-diabetic drug has already available which have been commercially used by diabetic people but none of these have the dual property of reducing blood glucose level and scavenging free radicals. Moreover the side effects and cost of the drugs are not affordable for all.

Therefore, the present study was designed to investigate the compounds of the ethanolic extract of *C. asiatica* and its antioxidative, AChE inhibitory and anti-diabetic activity which may lead to the finding of more effective agent for the treatment and management of AD and Diabetes and related other disorders.

MATERIALS AND METHODS

2.1 Collection and Identification of plant materials

Fresh leaves of *C. asiatica* were collected from Savar, Dhaka, Bangladesh in July, 2011 and the plant samples were identified and authenticated by Bangladesh National Herbarium with accession number DACB 33537.

2.2 Preparation of plant extract

The leaves were washed with distilled water without squeezing to remove debris and dust particles, dried at room temperature and pulverized into a coarse material of about 1 mm in diameter. Pulverized powdered leaves (1.2 kg) of the plant were macerated with 6.0 and 5.0 L of ethanol, respectively at room temperature for 15 days with occasional shaking. The ethanolic extracts of leaves were collected, filtered by cotton plug followed by whatman filter paper (no. 1) and evaporated to dryness (45°C) under reduced pressure by rotary evaporator. The obtained crude extract was stored in a refrigerator at 4°C until time of use. The percentage yield of the extract was calculated using the formula below:

$$\% \text{ yield} = (\text{weight of the extract} / \text{weight of plant material}) \times 100$$

2.3 Photochemical Screening

For preliminary phytochemical analysis the freshly prepared crude ethanolic extracts of leaves were tested for the presence or absence of phytoconstituents such as reducing sugar, tannins, flavonoids, saponins, gums, steroids and alkaloids by using standard phytochemical procedures [24].

2.4 Evaluation of antioxidant activity

2.4.1 DPPH radical scavenging activity

The free radical scavenging activities of the ethanolic extracts of leaves of the plant on the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were estimated by the method of Brand-Williams [25].
Inhibitions were calculated by using the following equation:

$$\% \text{ inhibition} = [1 - (ABS_{sample} / ABS_{control})] \times 100 \quad \text{----- ----------(1)}$$

Where $ABS_{control}$ is the absorbance of the control reaction (containing all reagents except the test material) and ABS_{sample} is the absorbance of the sample material. Then percent inhibitions were plotted against respective concentrations. IC_{50} values were calculated as the concentration of each sample required to give 50% DPPH radical scavenging activity from the graph. Tert-butyl-1- hydroxytoluene (BHT) and Ascorbic acid were used as positive control. The experiment was performed thrice and the result was expressed as Mean±Standard Error of Mean (SEM) in every case.

2.4.2 Super oxide radical scavenging activity

The super oxide radical scavenging activity was assayed by the nitro blue tetrasolium reduction method which is based on the capacity of the sample to inhibit blue formazan formation by scavenging the superoxide radicals generated in Hydroxylamine-sodium carbonate-EDTA-NBT system. 1 mL of 50 mM sodium carbonate, 0.4 mL of 24 µM nitroblue tetrazolium and 0.2 mL of 0.1 mM EDTA were taken in a test tube. Then 100 µL of plant extract (conc. ranging from 1000 µg/mL to 7.513 µg/mL) was added to the test tube. Reaction was initiated by adding 0.4 mL of 1 mM hydroxylamine hydrochloride. The reaction mixture was incubated for 5 min at ambient temperature and then the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of formazan generated. The percentage inhibition of superoxide anion generation (I %) was calculated by formula (1). The experiment was performed thrice and the result was expressed as mean±SEM in every case.

2.5 Evaluation of ChEs enzyme activity

The inhibitory activities of were measured in vitro by Ellman method [26]. Then the product formation was calculated for each AchE and BchE concentration. The concentration of the extract that inhibited 50% of AchE activity [IC50] was estimated by method described by Alhomida et al. [27]. The method was performed by plotting % activity and % inhibition of AchE or BchE versus extract [inhibitor] concentration on the same graph. The concentration at the intersection of these two curves was the IC50 value.

2.6 Experimental animals

Adult Wister albino rats of both sexes weighing between 150-200 g were used for the experiments. The animals were procured from the animal resource branch of International Center for Diarrheal Disease and Research, Bangladesh (ICDDR, B). The animals were housed 5 per cage (polypropylene cage) in a temperature controlled room (25 $\pm 1^{0}$ C), with a light/ dark cycle of 12 h. For a week following their receipt, the animals were allowed free access to standard rat chow diet (ICDDR, B formulated) and fresh water *ad libitum* while they were acclimatizing to the environment. During the experiment all the rats had free access to standard rat chow and water at all times unless otherwise stated in the method section. At the start of the experiment, animals were randomly distributed so that body weight, initial total glucose and total cholesterol and other parameters were similar in all the experimental groups. The design and performance of research study involving rats have been approved by the institutional ethical review committee through the submission of a research protocol before the study.

2.7 Acute toxicity test

Acute toxicity was done by following the method of Lorke [28].

2.8 Anti-diabetic study

Alloxan monohydrate solution of 10 mg/ml was prepared in ice-cold citrate buffer (0.1M); pH of the ice was kept at 4.5 and was administered to the rats within 5 mins at a dose of 50 mg/kg bodyweight intraperitonially. The fasting blood sugar levels of each of the rats were checked every day with an autoanlyzer (Glucometer, Bioland G-423 S) glucose kit. After 8 days, animals with fasting blood sugar levels of 250 mg/dl and above were considered to be diabetic and were used for the study and assigned into five groups of five rats each. Group I served as the negative control and received tween 80 solution (solvent used to dissolve the extract) (10 ml/kg), group II–IV received the *C. asiatica* extract at the dose of 250, 500 and 1000 mg/kg respectively while group V served as the positive control and received the standard reference drug glibenclamide (2 mg/kg) all by gastric gavage. The blood glucose levels of the rats were measured at 0, 1, 2 and 3 h after administration of drug and extracts. Blood samples were collected by tail snip and the blood glucose measured with an autoanalyzer (Glucometer, Bioland G-423 S) glucose kit. At the end of the experiment percentage reduction of the glucose levels of the rats at the 3rd hour was calculated using the formula below:

$$\% \text{ reduction in glucose level} = \{(V_0 - V_t)/ V_0\} \times 100$$

Where $V_{0=}$ value at zero hour and V_t= value at subsequent hours.

2.8 Statistical analysis

Results were presented as mean±Standard Error of Mean (SEM) and the statistical analysis was done using one way analysis of variance (ANOVA). A p-value of $p < 0.05$ was considered to be statistically significant.

RESULTS

3.1 Plant extraction

The yield of the ethanolic leaf extract of *C. asiatica* was 6.87% (w/w) dry matter and was greenish in colour.

3.2 Phytochemical screening

Phytochemical screening of the leaf extracts of *C. asiatica* confirmed the presence of alkaloids, glycosides, steroids, flavonoids, tannins and reducing sugars (Table 1).

Table 1. Phytochemical screening of the leaf extracts of *C. asiatica*

Serial No.	Chemical Constituents	Test	Extract	Result
1.	Test for Reducing Sugar	Benedict's Test	ECA	+
		Fehling's Test	ECA	+
		Alpha Napthol Solution Test	ECA	+
2.	Test for Tannins	Ferric Chloride Test	ECA	+
		Potassium dichromate Test	ECA	+
3.	Test for Flavonoids	Hydrochloric Acid Test	ECA	+
4.	Test for Saponins	Foam Test	ECA	-
5.	Test for Gums	Molisch Test	ECA	-
6.	Test for Steroids	Libermann-Burchard Test	ECA	+
		Sulphuric acid Test	ECA	+
7.	Test for Alkaloids	Mayer's Test	ECA	+
		Wagner's Test	ECA	+
		Dragendroff's Test	ECA	+
		Hager's Test	ECA	+

+ Present, - Absent, ECA: Ethanolic Extract of leaves of C. asiatica.

3.3 In vitro antioxidant activity

The antioxidant activity of the ethanolic extract of *C. asiatica* was measured on the basis of its DPPH and superoxide radical scavenging activity. The concentration of the plant extract needed for 50% scavenging (IC_{50}) of DPPH and superoxide was found to be 40.4 and 109.57 µg/ml respectively. Two positive controls were used- Butyl hydroxyl toluene (BHT) and Ascorbic acid (AS) for which the IC_{50} values were found to be 16.34 and 5.0 µg/ml for BHT and 65.03 and 99.66 µg/ml for Ascorbic acid (AS) respectively. The results were shown in Table 2.

Table 2. In-vitro antioxidant activity of Ethanolic Extract of leaves (ECA) of *C. asiatica*

Extract/ Compound	Antioxidant activity (IC_{50} µg/mL)[a]	
	DPPH assay	Superoxide radical assay
ECA	40.4±3.42	109.57±0.65
BHT[b]	16.34±0.40	65.03±.67
AS[b]	5.0±0.004	99.66±1.82

[a]Values are expressed as mean±SEM. in three replicates. [b]positive control used. DPPH = 1, 1-diphenyl-2-picrylhydrazyl ECA= Ethanolic Extract of leaves of C. asiatica; BHT= Butyl hydroxyl toluene; AS= Ascorbic acid

3.4 In vitro ChEs enzyme activity

The ethanolic extract of *C. asiatica* leaves showed significant AChE and BChE inhibitory effects. The results are shown in Table 3.

Table 3. Anticholinesterase activity of the ethanolic extract of *C. asiatica* leaves

Sample	**AChE[a] IC_{50}(µg/ml)**	**BChE[b] IC_{50}(µg/ml)**
Ethanol extract	170.2±1.6*	226.4± 2.3*
Eserine	0.02±0.01*	0.04±0.03*

*IC_{50} values are mean±SEM (n=3); *p<0.05 when compared between the sample and the control; [a]AChE = acetylcholinesterase; [b]BChE= Butylcholinesterase*

3.5 Acute toxicity test

The acute toxicity in rats produced no death or signs of toxicity even at the highest dose of the extract (3000 mg/kg).

3.6 Anti-diabetic study

The result of the effect of *C. asiatica* on the fasting blood glucose levels of alloxan-induced diabetic rats is presented in Table 4. Effect of *C. asiatica* on the fasting blood glucose level of alloxan-induced diabetic rats.

Group	Treatment	Fasting blood glucose level (mg/ml)				% reduction at at the 3rd hour
		0 h	1 h	2 h	3 h	
I	Alloxan+ Tween80 (1%)	390.0±5.7*	395±6.2*	392.2±5.5*	393.7±5.5*	-
II	Alloxan+ ECA(250mg/kg)	335.6±5.9*	217±4.3*	265.2±4.9*	226.2±3.4*	32.6
III	Alloxan+ ECA (500mg/kg)	409.2±5.7*	377.6±4.2*	354.2±4.1*	250.4±6.5*	38.8
IV	Alloxan+ ECA (1000mg/kg)	340.2±5.0*	298.6±4.4*	268±5.0*	238.4±4.4*	29.9
V	Alloxan+glibenclamide(5mg/kg)	318.2±1.8*	168.8±1.4*	116.0±0.7*	102.6±1.3*	67.75

*ECA- ethanolic extract of leaves of C.asiatia; *p<0.05 when compared with the negative group.*

The result showed that there is no significant change in the blood glucose levels of rats in group I that received tween 80 solutions (negative control). The ethanolic leaf extract of *C. asiatica* in all the doses used including the reference drug caused a time dependent and significant ($p < 0.05$) reduction of the blood glucose levels of the alloxan-induced diabetic rats when compared to the negative control group with the extracts at the doses of 250, 500 and 1000 mg/kg. This decreased the blood glucose levels by 32.6%, 38.8% and 29.9% respectively at the 3rd hour while the reference drug (glibenclamide, 2 mg/kg) decreased the blood glucose levels by 67.75% at the 3rd hour.
Also the extract at the different test doses caused various degrees of reduction (29.9% - 38.8%) of the blood glucose levels of the test rats at 1st, 2nd and 3rd hours when compared to the negative control rats (Table 4). The highest activity of *C. asiatica* extract in this experiment was observed at the dose of 500 mg/kg while the reference drug glibenclamide (2 mg/kg) had a superior activity when compared with *C. asiatica* extract.

DISCUSSION

Phytochemical screening of the plant extract confirmed the presence of several bioactive compounds like alkaloids, flavonoids, tannins and steroids which could be responsible for the versatile medicinal properties of this plant.

The antioxidant activity exerted by the plant extract was statistically significant. It supports previous finding of the antioxidant activity of the ethanolic extract of *C. asiatica* is due to the presence of substances with free hydroxyls [29]. The presence of several phenolic constituents such as flavonoids, tannins etc. which possess an ideal structure for the scavenging of free radicals contributes to the anti-oxidant activity of the plant extract.

The present study demonstrated that the *C. asiatica* extract could exhibit significant ChEs inhibitory effect. By inhibiting the enzyme AChEs and BChEs *C. asiatica* might be able to increase the level of neurotransmitter acetylcholine and thus improve synaptic transmission in the AD brain. The anti-oxidant activity of the plant extract also implicated its neuroprotective effect in AD through the scavenging of reactive free radicals and ROS which otherwise play important role in the formation of neurofibrillary tangles and neurotic plaques [11]. A previous study [14]. had already shown a reduction in fibrillar amyloid plaques by C. asiatica extract in AD animal model. By acting through all these mechanisms C. asiatica could be a very good anti-AD agent. Therefore, further study should continue to isolate the causative chemical compounds and the precise and detailed underlying mechanism of action of these compounds.

The acute toxicity test of *C. asiatica* in rats produced no death or signs of toxicity even at the dose of 3000 mg/kg which shows that the extract was well tolerated and the test doses safe in the animals.

The antidiabetic activity of *C. asiatica* was evaluated in alloxan-induced diabetic rats by testing its effect on fasting blood glucose level using autoanalyzer (Glucometer, Bioland G-423 S) glucose kit. The fasting blood sugar test is a carbohydrate metabolic test which measures plasma or blood glucose levels after a fast (usually 8–12 h). During fasting the body stimulates the release of the hormone glucagon, which in turn releases glucose into the blood through catabolic processes. Normally, the body produces and processes insulin to counteract the rise in glucose levels but in diabetes, this process does not occur and tested glucose levels normally remain high. Alloxan is one of the usual substances used for induction of diabetes mellitus apart from streptozotocin and has a destructive effect on the beta (β) cells of the pancreas as previously reported [30]. Pancreas is the primary organ involved in sensing the organism's dietary and energetic states via glucose concentration in the blood and in response to elevated blood glucose, insulin is secreted. However, alloxan causes diabetes through its ability to destroy the insulin-producing β-cells of the pancreas [31]. When there are not enough available beta-cells to supply sufficient insulin to meet the needs of the body, insulin-dependent diabetes results.

The result of the present study indicates that the ethanolic leaf extract of Centella asiatica at the test doses used and the reference drug glibenclamide (2 mg/kg) exhibited a time dependent significant ($p < 0.05$) reduction of the blood glucose levels of the alloxan-induced diabetic rats with different levels of percentage reduction at the 3rd hour when compared to the negative control rats. The different test doses of the extract (250, 500 and 1000 mg/kg) caused various degrees of reduction of the blood glucose levels of the test rats at 1st , 2nd and 3rd hours while the blood glucose levels of the negative control rats where increased.

It is well documented that antidiabetic drugs treat diabetes mellitus by lowering glucose levels in the blood. The result obtained from this preliminary study clearly shows that *C. asiatica* caused marked hypoglycemic activity in alloxan-induced diabetic rat model which indicates antidiabetic potentials of the extract. In this experiment, the dose of the extract of 500 mg/kg produced the highest antidiabetic effect and this may suggest that this dose may be the effective antidiabetic dose of the crude extract even though there might have some limitation due to the crude nature of the extract.

The exact mechanism by which the plant extract lowered the blood glucose level is not yet clear but [32]. attributed the anti-hyperglycemic effects of medicinal plants to be due to their ability to restore the function of pancreatic tissues by causing an increase in insulin output or a decrease in the intestinal absorption of glucose. *C. asiatica* may have worked through this mechanism or by stimulation of surviving beta cells to release more insulin just like glibenclamide. Medicinal plants and herbal extracts containingglycosides, flavonoids, tannins, etc. have been reported to demonstrate antidiabetic activities [33]. and our phytochemical stduy have shown that *C. asiatica* contains all the above phytoconstituents. It is therefore possible that the phytochemicals present in the plant may be responsible for the observed antidiabetic activity.

However, variations in the hypoglycemic activity of the different doses of extract may be tied to the possible prooxidative activity of the extract at the concentrations shown. It should be noted that every antioxidant/phytochemical is in fact a redox (reduction-oxidation) agent protecting against ROS generation and in some circumstances promoting free radical/ROS in others. Excessive antioxidant action can adversely affect some physiological processes. It is known that some of these phytochemicals in plants have balanced biochemistry i.e. having a redox mixture of being in oxidized form and partly in a reduced form depending on concentration [34]. This however, needs further investigation. It has been reported that plant phytochemicals not only depend on individual levels but also on the ratios of various components and their redox states [35]. It might be possible that due to having dual property of anti-diabetic and antioxidant activities this natural medicine can better manage the diabetes and diabetes associated complications which usually accompanied by increased oxidative stress. However, further study is required for the study of the effects on lipid profile, isolation and characterization of the anti-diabetic bioactive compound and the establishment of the exact mechanism(s) of action.

CONCLUSION

The specific compounds responsible for this antioxidant, anti-alzheimer and antidiabetic are needed to be found and further investigation by fractionation of the extracts and then analysis for active compounds responsible for these activities could be done in future. This might lead to the development of new drugs. On the otherhand, this study support the traditional use of the plants for the purposes mention above.

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