

Phytochemical Identification of *Clerodendrum phlomidis* Linn. by GC-MS Analysis and its Acetylcholinesterase Inhibitory Activity

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ABSTRACT

Background: Agnimantha is an important Ayurvedic medicinal plant mentioned in the text of Acharya Charak. Agni means fire and mantha means to rub together to produce fire in ancient times. **Objectives:** *Clerodendrum phlomides* Linn. (CP) leaf extracts examined for their phytochemical composition and antioxidant properties, as well as their ability to inhibit acetylcholinesterase (AChE). **Materials and Methods:** A coarse powder was ground from the leaves after being shade dried at room temperature. Alcoholic and hydroalcoholic extracts of leaf tissues were dried and used to identify phytochemicals by gas chromatography, *in vitro* radical scavenging assays, and AChE inhibition assays. **Results:** In the phytochemical screening procedures, fifteen constituents were identified in the alcoholic leaf extract of CP. AChE was inhibited by the extracts in a dose dependent manner as demonstrated by *in vitro* scavenging tests including FRAP assay (alcoholic 215.20 µg/ml and hydro alcoholic 288.70 µg/ml) and DPPH radical (alcoholic 167.70 µg/ml and hydro alcoholic 347.19 µg/ml). Inhibition of AChE was observed at 250 µg/ml of CP alcoholic and hydroalcoholic leaf extracts. **Conclusion:** We demonstrate that CP inhibits AChE activity and is capable of scavenging radicals *in vitro*, indicating that it could serve as an effective therapy for reversing the cholinergic deficit caused by Neurological diseases.

Key words: *Clerodendrum phlomides*, Antioxidants, Acetylcholinesterase, Phytochemical identification, GC-MS analysis, Oxidative stress.

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INTRODUCTION

Dysfunction and death of nerve cells are hallmarks of neurodegenerative disorders.^[1] The loss or dysfunction of certain types of neurons is central to neurological disorders such as Alzheimer's and Parkinson's.^[2] There are several causes of neuronal dysfunction, including synaptic loss, defects in long term potentiation (LTP), and disease-induced signal disruption.^[3] The loss of neurons is hallmark of neurodegenerative diseases.^[4] Researchers have extensively investigated neuroprotection as a treatment option for various neurological conditions, including strokes, neurodegenerative diseases, and trauma.^[5] In cerebral ischemia, the brain tissues are affected by necrosis and apoptosis. The leading cause of neurological disability in adults and the third leading cause of mortality.^[6] There are four major therapeutic targets in the process of ischemia-induced brain injury: inflammatory reaction, disruption of the blood-brainbarrier (BBB), oxidative stress, and neuronal apoptosis.^[7]

Current research focuses on the possibility of using antioxidants and neuroprotective properties to treat and prevent various neurodevelopmental disorders.^[8] "Rasayana" drugs modulate neuro-endocrine-immune systems and act as antioxidants in the human body.^[9] According to Ayurveda, Rasayana plants prevent aging, resurrect youth, increase

immunity, strengthen the brain, and prevent diseases. The properties of these ingredients support the idea that they will increase the body's resistance to harmful threats.^[10] In this study we attempt to identify potential Ayurvedic medicinal plant that inhibits AChE, a therapeutic target that has been implicated in several neurological disorders. Besides, antioxidant activity and major phytochemicals of the selected plants are also to be studied.^[11]

A lot of attention has been paid to *Clerodendrum phlomidis* (Verbenaceae family) because it contains many secondary metabolites and is used in Ayurvedic system of medicine. A shrub or tree growing up to 9 meters high, it can be found in India and Sri Lanka.^[12] There are various plant parts used for treating dyspepsia, digestive problems, colic, cholera, dysentery, post natal fever, and the measles during recovery. Its root and bark are bitter and used to treat debility and nervous disorders.^[13] The plant has traditionally been used to treat dysuria and retention of urine. An extracts of its leaves showed hepatoprotective properties.^[14] The aqueous extracts of the leaves have shown *in vivo* anthelmintic activity, and antidiabetic activity.^[15] The aim of this work is therefore to investigate and identify phytochemicals, antioxidant and AChE inhibitory activity.

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MATERIALS AND METHODS

Chemicals

Sigma Chemical Co., St Louis, USA was the supplier of the following ingredients: Acetylcholinesterase (AChE), Galanthamine hydrobromide, Acetylthiocholine Iodide (ATCI), 1,1-diphenyl-2-picryl hydrazil (DPPH), 5,5'-dithio(bis)-2-nitrobenzoic acid (DTNB) and ascorbic acid. These analytical grade reagents were obtained from Himedia, Mumbai, India. They were trichloroacetic acid, Finol-Ciocalteu's phenol reagent, Ferric chloride (FeCl₃), Butylated Hydroxy Toluene (BHT), and potassium ferricyanide [K₃Fe(CN)₆].

Preparation of plant extract and plant collection

Clerodendrum phlomidis (CP) leaves from Regional Ayurveda Research Institute (RARI), Pune, Maharashtra, India were collected fresh and free from infection. An expert in Botany, Dr. Arun Manohar Gurav verified the authenticity of the plant specimen, which was deposited in a RARI herbarium with accession no. 2220 (Figure 1). After drying for 30 days at room temperature (28 ± 2°C), CP leaves (1000 gm) were ground in an electric blender into a coarse powder. In accordance with the Ayurvedic Pharmacopoeia of India (API, 2016), cold maceration was used to extract alcoholic and hydroalcoholic extracts (50:50). The powder of the plant material was macerated with a material to solvent ratio of 100 and 50:50 (w/v) for 24 hr with intermittent shaking followed by 18 hr in a static state. A further filtering was carried out on the extracts using Whatman filter paper No.1. A subsequent steps involves mixing the macerated material with 25 ml of solvent and allowed to stand for 15 min. The mixture is filtered, and the entire process is repeated. The filtrate, thus, obtained was evaporating into powder form. *In vitro* studies on AChE inhibitory activity and scavenging assays were carried out using alcoholic and hydroalcoholic extracts.

Qualitative phytochemical analysis

Phytochemical screenings of alcoholic and hydroalcoholic extracts were performed as per standard procedure.^[16-19] The presence of the

compounds is detected by color reactions as a qualitative method. These color reactions can only determine whether chemical groups are present in an extract, not their amounts.

Mass spectrometry analysis using gas chromatography (GC-MS)

Vellore Institute of Technology in Vellore, Tamil Nadu, India, conducted GC-MS analysis at SIF, the Sophisticated Instrumentation Facility (SIF) within the School of Advanced Sciences, Department of Chemistry. We used Elite-5MS (5% biphenyl and 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250 μm d_i) for the analysis. It was carried by helium at a flow rate of 1 ml/min, with an injector temperature of 260°C. GC-MS was injected with a 1 μL alcoholic extract at 60°C for 2 min, 300°C at a flow rate of 10°C per minute for 6 min, and 300°C for 12 min. A mass detector with the following parameters was used: 240°C for the transfer line, 240°C for the ion source, 70 eV for the electron impact, and 0.2 sec for the scan. There were fragments of 40 to 600 kDa in size. NIST library database (2008) was used to compare the GC-MS spectra of components.

Assay for radical scavenging with DPPH

Plant extracts were evaluated for their antioxidant activity by measuring 1-diphenyl-2-picrylhydrazil (DPPH) by using the Blois (1958),^[20] and Gomez-Alonso *et al.*, (2003).^[21] Hydrogen donating abilities of alcoholic and hydroalcoholic extracts were studied in the presence of DPPH stable radicals. We added samples and standards to 150 μl of 0.3 mM DPPH methanol solution at room temperature and allowed the mixture to react in the dark. In this experiment, the control and test samples were incubated for 30 min at 37°C. Methanol was used as a blank for calculating the absorbance at 517 nm over the 30 min incubation period. A reference material was used for the study: butylated hydroxy anisole (BHA).

FRAP Assay

In 1986, Oyaizu^[22] measured the reductive power of alcoholic and hydroalcoholic extracts, as well as standard BHT. The 96-well plate contained 30 μl of extracts or BHT, phosphate buffer and potassium ferricyanide. Following 20 min of incubation at 50°C, the mixture was cooled to room temperature. To the mixture, trichloroacetic acid was added, followed by 120 μl distilled water and 25 μl of ferric chloride. The mixture was thoroughly mixed, then incubated for 30 min at room temperature. Microplate reader were used to measure absorbance at 700nm. As reference material, we used Butylated Hydroxy Toluene (BHT). Tests were done in triplicate, and the average of three measurements was used to plot the graph.

In-vitro AChE Inhibition assay

In-vitro assay for determination of Acetylcholinesterase (AChE) inhibition were standardized in 96-well microplate using methodology described previously by Mathew and Subramaniam, 2014^[23] and Ellman *et al.*, 1961^[24] To dissolve the drugs or standard concentrations in buffers of varying concentrations, in 96-well plates were filled with 100 μl of DTNB, 20 μl of AChE, and 40 μl of tris buffer, and 20 μl of drug/standard concentrations. A microplate reader measured the absorbance at 412 nm at the end of 15 min of gentle mixing on the plate. The effective concentration was determined by using the blank readings. ATCI was used to initiate the enzymatic reaction, and the absorption of the hydrolysis product was monitored every 30 sec over a period of 20 min. Triplicates of the experiments were performed using galanthamine hydrobromide as a standard control. Here are the results of the calculation:

The inhibition rate is calculated as (E S)/E × 100

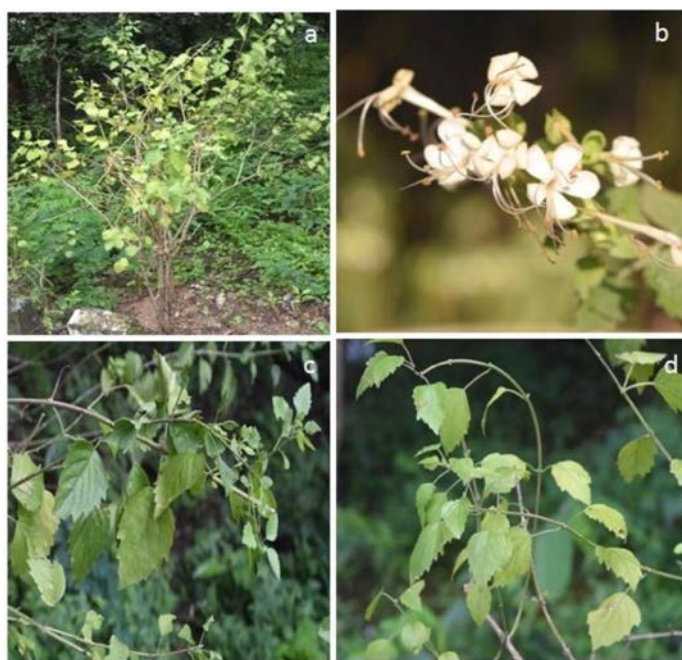


Figure 1: a) *Clerodendrum phlomidis* in its natural habitat, b) Flowering season
c) Mature leaves d) Young leaves

Figure 1: Photography images of *Clerodendrum phlomidis*.

The enzyme activity without extract/positive control is E, while the enzyme activity with extract is S. IC_{50} values are calculated by multiplying different concentrations of the positive control or/of each plant extract by their percent inhibition values.

RESULTS

Qualitative test

A phytochemical screening was performed on the hydroalcoholic and alcoholic leaf extracts of *Clerodendrum phlomidis* and the chemical constituents have been presented in the Table 2. In the hydroalcoholic extract, carbohydrates, reducing sugar, hexose sugar, cardiac glycosides, saponins, tannins and phenols were detected. In the alcoholic extract, reducing sugar, hexose sugars, cardiac glycosides and phenols were detected.

Identification of Phytochemicals by GC-MS

As shown in Figure 2 and Table 1, the plant extracts from *Clerodendrum phlomidis* were screened for phytochemical constituents. Gas chromatography shows the concentrations of compounds that elute based on their retention times. In the ethanolic leaf extract, the heights of the peaks correspond to the relative concentration of various constituents. As the eluted compounds are analyzed by mass spectrometry at different times, the structure and nature of the compound can be determined. These compounds mass spectra have been derived from GC-MS analysis based upon NIST library databases that identify them. A list of the compounds analyzed by GC-MS is presented in Table 1.

Table 1: Phytochemicals identified in the ethanol leaf extract of *Clerodendrum phlomidis* by GC-MS analysis.

S.No	RT (Min)	Name of the compound	Molecular formula	Molecular weight	Peak area %
1	17.53	3,7,11,15-Tetramethyl-2-hexadecan-1-ol	C ₂₀ H ₄₀ O	296	7.65
2	17.84	1-Octadecyne	C ₁₈ H ₃₄	250	3.19
3	18.07	1-Octadecyne	C ₁₈ H ₃₄	250	9.26
4	19.13	Tetraacetyl-d-xylopic nitrile	C ₁₄ H ₁₇ O ₉ N	343	1.91
5	19.84	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256	15.94
6	20.36	Nonadecanoic acid	C ₁₉ H ₃₈ O ₂	298	5.71
7	21.21	3-Decyn-2-ol	C ₁₀ H ₁₈ O	154	5.62
8	21.39	Z,Z-10,12-Hexadecadien-1-ol acetate	C ₁₈ H ₃₂ O ₂	280	12.94
9	21.84	2-Pentadecyn-1-ol	C ₁₅ H ₂₈ O	224	6.72
10	22.33	19,19-Dimethyl-eicosa-8,11-dienoic acid	C ₂₂ H ₄₀ O ₂	336	11.91
11	23.51	Silane, [[[3(beta.)-gorgost-5-en-3-yl]oxy]trimethyl-	C ₃₃ H ₅₈ OSi	498	6.55
12	24.79	Squalene	C ₃₀ H ₅₀	410	8.33
13	25.78	Silane, [[[3(beta.)-gorgost-5-en-3-yl]oxy]trimethyl-	C ₃₃ H ₅₈ OSi	498	1.74
14	26.91	Pseudoarsasapogenin-5,20-dien	C ₂₇ H ₄₂ O ₃	414	1.12
15	27.40	(2S,3S)-(-)-3-Propyloxiranemethanol	C ₆ H ₁₂ O ₂	116	1.33

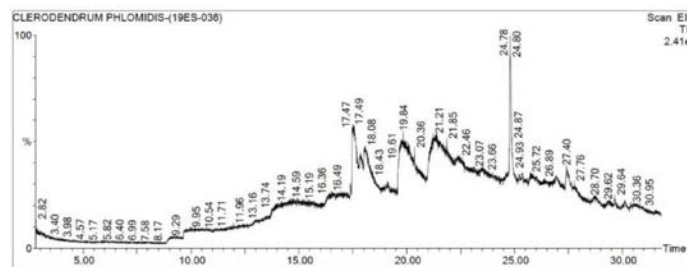


Figure 2: GC-MS analysis of alcoholic leaf extract of *Clerodendrum phlomidis*.

Assay for radical scavenging *in vitro*

In Table 3 and 4, antioxidant ability was determined by using DPPH and Ferric reducing agents. As compared with standard Butylated Hydroxy Toluene (148.70 µg/ml), the alcoholic and hydroalcoholic extracts of CP significantly decreased DPPH activity. At 250 µg/ml, CP exerts maximum scavenging effects on DPPH radicals and FRAP assays. Because the optimum dose of CP was calculated to be 200 µg/ml, increasing the CP concentration beyond the optimum dose did not result in significant increases in scavenging.

AchE inhibitory activity

In Table 5, we show the changes in acetylcholinesterase inhibitory activity. At five different concentrations of CP extracts, acetylcholinesterase inhibition was significantly reduced, and the IC_{50} concentration was determined to be 250.40 µg/ml for the alcoholic extract and 267.89 µg/ml for the hydroalcoholic extract. The alcoholic and hydro alcoholic extracts of CP show significant inhibition when compared with standard galanthamine hydrobromide. IC_{50} concentration of standard galanthamine was at 12.82 µg/ml.

DISCUSSION

More than 4000 years ago, India's Ayurvedic system of medicine used various plants to treat a variety of neurological conditions and to improve memory and cognitive function.^[25] Using the Rasayana therapy, our bodies are protected against ageing due to their enhanced ability to fight pathogens. Herbs used in Rasayana treatments contain antioxidants that protect against free radical damage.^[26] CP extracts were examined to determine and compare their anticholinesterase and antioxidant activity. A phytochemical is a natural bioactive compound produced by plants that protects the plants from environmental stress or pathogen attack.^[27] Secondary metabolites enables plants to survive by protecting them against external threats. It is possible to classify phytochemicals' biosynthesis into several categories: phenolics, alkaloids, steroids, terpenes, and saponins, for example.^[28] Also, phytochemicals have the ability to inhibit mutagenesis, cancer, or infection.^[29] Anticholinesterase properties in plant-derived phytochemicals are promising candidates for use in the development of drugs.^[30] *In vitro* tests were conducted to identify phytochemicals, determine antioxidant potential, and to examine CP extracts' ability to inhibit acetylcholinesterase. CP demonstrated a concentration-dependent antioxidant effect. The leaf extracts of CP have been screened for phytochemical constituents and the chemical compounds have been identified through GC-MS and qualitative analysis. We found that 250 µg/ml concentration of CP was optimal for inhibiting cholinesterase activity *in vitro*. A number of compounds found in CP, such as squalene, octadecynl, and n-Hexadecanoic acid, provide free hydrogen and electrons to the hydroxyl radicals, stabilizing them and resulting in a relatively stable radical

Table 2: Qualitative estimation of *Clerodendrum phlomidis* leaves.

Sr.No	Phyto constituent tested	Test performed	<i>Clerodendrum phlomidis</i> leaves	
			Hydroalcoholic extract	Alcoholic extract
1	Carbohydrates	Molish's test	+	-
2	Reducing sugar	Fehling's test	+	+
		Benedict's test	+	+
3	Pentose sugar	Phloroglucinol Reagent test	-	-
		Tollen's Phloroglucinol Test	-	-
4	Hexose sugar	Cobalt chloride test	++	++
		Biuret test	-	-
5	Protein	Biuret test	-	-
6	Amino acid	Ninhydrin Test	-	-
		Test for cysteine	-	-
7	Steroids	Libermann-Burchard Test	-	-
		Salkowaski reaction	-	-
8	Glycoside	General test	-	-
9	Cardiac glycosides	Legal test	-	+
		Keller-Killiani Test	-	+
10	Anthroquinone glycoside	Borntrager's test	-	-
		Modified Borntrager's test	-	-
11	Saponins	Foam test	+	-
		Lead acetate solution test	+	-
12	Coumarin glycoside	Aromatic odour test	-	-
		Fluorescence test	-	-
13	Flavonoids	Shinoda test	-	-
		Lead acetate test	-	-
14	Alkaloids	Dragendorff's test	-	-
		Mayer's test	-	-
		Wagner's test	-	-
15	Tannins	Lead acetate solution test	+	-
		Gelatin solution test	+	-
16	Phenol	Neutral FeCl ₃ test	+	-
		Indophenol reaction	-	+
17	Starch	Iodine test	-	-

Table 3: Percentage inhibition of *Clerodendrum phlomides* on DPPH radical scavenging assay.

Concentrations in (µg/ml)	Standard Butylated Hydroxy Toluene	<i>Clerodendrum phlomides</i> leaf	
		Alcoholic % Inhibition	Hydro alcoholic % Inhibition
50 (µg/ml)	16.25 ± 0.15	15.97 ± 0.25	13.96 ± 0.47
100 (µg/ml)	31.25 ± 1.41	22.8 ± 0.21	18.68 ± 0.13
150 (µg/ml)	55.20 ± 0.12	47.2 ± 0.52	22.17 ± 0.17
200 (µg/ml)	60.41 ± 0.21	82.07 ± 0.16	35.12 ± 0.30
250 (µg/ml)	87.50 ± 0.32	94.72 ± 0.05	56.19 ± 0.53
IC ₅₀ value	148.70 ± 14.28	161.70 ± 16.20	347.19 ± 30.12

Table 4: Ferric reducing antioxidant power (FRAP) assay on *Clerodendrum phlomides*.

Concentrations in (µg/mL)	Standard Butylated Hydroxy Toluene % O.D. value at 700 nm	C P leaf % O.D. value at 700 nm	
		Alcoholic	Hydro alcoholic
50 (µg/ mL)	0.15 ± 0.17	0.26 ± 0.00	0.16 ± 0.01
100 (µg/mL)	0.22 ± 0.02	0.34 ± 0.12	0.22 ± 0.02
150 (µg/mL)	0.39 ± 0.03	0.54 ± 0.04	0.37 ± 0.03
200 (µg/mL)	0.47 ± 0.04	0.68 ± 0.05	0.46 ± 0.04
250 (µg/mL)	0.62 ± 0.05	0.89 ± 0.07	0.62 ± 0.01
IC ₅₀ value	149.60 ± 14.22	215.20 ± 21.11	288.70 ± 28.10

Table 5: Acetylcholinesterase inhibitory activity on *Clerodendrum phlomides*.

Concentrations in (µg/mL)	<i>Clerodendrum phlomides</i> leaf		Concentration in (µg/mL)	% Inhibition of Galanthamine hydrobromide
	Alcoholic	Hydro alcoholic		
			5 (µg/ mL)	31.10 ± 0.01
50 (µg/ mL)	0.55 ± 0.01	0.57 ± 0.02	10 (µg/ mL)	41.95 ± 0.04
100 (µg/mL)	10.55 ± 0.03	2.93 ± 0.04	15 (µg/ mL)	51.72 ± 0.05
150 (µg/mL)	13.74 ± 0.02	3.83 ± 0.03	20 (µg/ mL)	59.89 ± 0.06
200 (µg/mL)	30.88 ± 0.03	5.64 ± 0.04	25 (µg/ mL)	74.81 ± 0.05
250 (µg/mL)	50.99 ± 0.01	11.03 ± 0.02	30 (µg/ mL)	82.62 ± 0.03
IC ₅₀ value	250.40 ± 25.01	267.89 ± 26.12	IC ₅₀ value	20.82 ± 2.02

A possible effect of the phenolic compounds found in the extract of CP could be the cause for inhibition of acetylcholinesterase. There is evidence that several plant species used in alternative medicine for the treatment of neurological disorders can inhibit AChE and act as antioxidants, thus validating their use in traditional medicine. There are other molecular targets related to their traditional use that may not inhibit AChE.

CONCLUSION

The results from *in vitro* experiments indicated that CP exhibited significant antioxidants properties. Furthermore, both alcoholic and hydroalcoholic extracts have anticholinesterase activity in a dose dependent manner.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AChE: Acetylcholinesterase; **GC-MS:** Gas chromatography mass spectrometry; **FRAP:** Ferric reducing antioxidant power; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **BHA:** Butylated hydroxyl anisole; **DTNB:** 5,5'-Dithiobis(2-nitrobenzoic acid); **ATCI:** Acetylthiocholine iodide; **IC₅₀:** Half-maximal inhibitory concentration; **NIST:** National Institute Standard and Techniques.

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GRAPHICAL ABSTRACT



SUMMARY

- *Clerodendrum phlomidis* is a widely growing medicinal plant belonging to the family of Verbenaceae
- The study is designed to evaluate the anticholinesterase activity
- The phytochemical screening studies have been carried out by qualitative and GC-MS analysis
- The alcoholic and hydroalcoholic extracts of *Clerodendrum phlomidis* exhibited a significant dose dependent inhibition of *in vitro* radical scavenging assays.

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