Biological activity and GC-MS determination of bioactive components of *Clerodendrum viscosum* (Vent.) from Siraha District, Nepal

Shivdev Yadav*, Santosh Kumar Yadav and Bimala Subba***

*Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

**Province Public Health Laboratory, Ministry of Health and Population, Janakpurdham, Nepal.

Abstract: *Clerodendrum viscosum* has a long history of medical use in Asian countries with a variety of bioactivities. Although the chemical constituents of plants are strongly influenced by their surroundings, little is known about the chemical composition and biological activity of *C. viscosum* that is growing in Nepal's Siraha District. Therefore, the aim of this study was to evaluate the total phenolic content, flavonoid content, and biological activity, GC-MS analysis of *C. viscosum* leaves that was collected from the Siraha District. The methanolic extract of *C. viscosum* leaf was prepared and the phytochemicals were assessed, its TPC and TFC were estimated, and antioxidant and α-amylase inhibitory activities were determined following standard methods. Hexane soluble fraction of methanol extract of *C. viscosum* was subjected for GC-MS analysis. The TPC of the extract was found to be 197.68±0.11 mg GAE/g and its TFC was 105.14±0.026 mg QE/g. The IC_{50} value for DPPH free radical was found to be 26.85 0.150 g/mL, while the IC_{50} value for α-amylase enzyme activity was found to be 121.48 0.424 g/mL. The presence of several bioactive components was revealed by the GC-MS chromatogram, with phytol being a major constituent.

Keywords: Antioxidant; *Clerodendrum viscosum;* Flavonoid content; Methanolic extract; Phenolic content.

Introduction

Over time, the utilization of medicinal plants has evolved and today plays a vital role in achieving positive health outcomes, and these plants have been found to be a good source of a wide range of compounds, including phenolics, nitrogen compounds, terpenoids, vitamins, and other metabolites, all of which have precious bioactivities such as antioxidant, anti-inflammatory, anticarcinogenic, antimutagenic, and antimicrobial properties[1]. Medicinal plants are considered to be a key repository of bioactive compounds that must be systematically analyzed and the natural products provide virtually endless opportunities for innovative medicine development due to their chemical diversity[2]. Chemists, biochemists, and pharmaceutics have all turned their attention to medicinal plants and their research is crucial in the identification and discovery of new treatment options that will probably be more effective and have fewer adverse effects than most modern drugs[3]. Plant extracts or bioactive components have been utilized in traditional therapies by around 80% of the world's population, and it is believed that over half of all modern clinical medications are derived from natural products[4,5].

Clerodendrum viscosum Vent. is a member of the Verbenaceae family and is found in Nepal, India, Myanmar, Thailand, and Indonesia as a flowering shrub[6,7]. The plant is a flowering shrub and is commonly found as a weed in crop fields, shallow areas, and along roads and railway tracks[7]. The genus is distinguished by its whole or toothed leaves, alternately oriented stems, terminally or axillarily cymose inflorescence, hypogynous and bisexual flowers, and ex-albuminous seeds[8]. The leaves are sessile, narrow lanceolate, sub-entire, glabrous, rather rigid and flowers in

Author for Correspondence: Bimala Subba, Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu, Nepal.
Email: bimalasubba@gmail.com; https://orcid.org/0000-0001-9211-5072

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in pyramid-shaped terminal panicles and are bluish-purple or white. March to June is the flowering and fruiting season[9]. The leaves contain a variety of components, including protein, free reducing sugar, clerodin a sterol, oleic, stearic, and lignoceric acids, glucuronide, tannin, and gallic acid. Several flavonoid compounds, such as apigenin, acacetin, quercetin, scutellarin, hispidulin-7-0-glucuronide, and cabruvin, as well as terpenoids like clerodin, acidic compounds like fumaric acid, stearic acid, and caffeic acid, anthraquinones, and alkaloids, are either localized to a specific part or found throughout the entire plant[7,10]. *C. viscosum* has a long history of use in Asian countries, particularly in medical applications in various indigenous systems of health care to cure a variety of human and animal ailments[7]. The leaves and roots are widely used as antiseptic, antioxidant, anti-inflammatory, antipyretic, anthelmintic, vermifuge, against leprosy, asthma, cancer, and certain skin diseases and the juice extracted from the leaves is laxative and cholagogue[9,10]. Phytochemical isolates and their investigation into biological models have supported documented traditional knowledge relating to diverse medicinal uses of *C. viscosum*[7].

Although different compounds have been extracted from this plant, many of its constituents have yet to be completely studied for their pharmacological potential, necessitating more research to acquire a better knowledge of the phytochemicals against various diseases[7]. When assessing the quality of pharmaceutical materials, secondary metabolites are crucial markers. Environmental and developmental factors might have a special impact on a plant's secondary metabolites[11]. Therefore, this research was conducted to screen the phytochemicals, estimate total phenolic and total flavonoid content, determine antioxidant, α-amylase inhibitory activities of the methanol extract of *C. viscosum* leaves collected from Siraha District, Nepal and to analyze the bioactive phytochemical constituents in hexane soluble fraction of methanol extract (*C. viscosum* leaves) by GC-MS.

Materials and Methods

Chemicals and Reagents

The α-amylase, quercetin and acarbose were purchased from Sigma-Aldrich, Germany, and gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, Folin-Coicalteu reagent, and other chemicals and solvents were purchased from Fisher Scientific, India.

Collection and Identification of Plant Materials

The leaves of the C. *viscosum* Vent. were collected on the basis of an ethnobotanical approach from Siraha District of Madhesh Province, Nepal at the geographical coordinates 26°39'10"N 86°12'27"E. The collected plant materials were authenticated by Botanist, at the Botany Central Department of Tribhuvan University, Kathmandu, Nepal.

Preparation of Leaf Extract

The leaves collected were thoroughly washed with water, dried in the shade, and powdered. The chemical constituents of plant material were extracted with methanol using the cold percolation method. In summary, the powder of leaves was dipped in methanol for 72 hours at room temperature, with intermittent shaking. After that the solvent was filtered through the Whatman-1 filter paper and the filtrate was evaporated using a rotary evaporator under vacuum at 40 ˚C. To obtain a solid or semisolid residue, the concentrated filtrate was air-dried[12,13]. The dried extract was analyzed for the percentage yield using the given formula:

Percentage Yield = (Dry weight of extract / Dry weight of plant material) x 100%

Phytochemical Screening

The presence of phytochemicals in methanolic extract of *C. viscosum* leaves was determined using the standard technique. Alkaloids, flavonoids, glycosides, polyphenols, terpenoids, steroids, carbohydrates, saponins, and tannins were all screened for. As an analytical response to these tests, the color intensity or precipitate formation was used[13-16].

Estimation of Total Phenol Content (TPC)

To estimate the TPC of the leaf extract, the Folin-Ciocalteu's method was followed. For this, 200 µL of leaf extract (0.5 mg/mL) was mixed with 1000 µL of Folin-

Coicalteu's reagent (v/v; 1:10 diluted with distilled water). Then 800 µL of sodium carbonate aqueous solution (1.0 M) was added to the above mixture making a final volume of 2.0 mL. The mixture was left to stand at room temperature for 15 minutes and the absorbance was then measured with a spectrophotometer at a wavelength of 765 nm. The TPC of leaf extract was expressed in terms of milligrams of gallic acid equivalent per gram of dry weight of the extract (mg GAE/g) based on standard gallic acid calibration curve (10-100 µg/mL). To ensure the reproducibility of the experiment, each measurement was performed three times and the mean value and standard deviation were calculated[17, 18].

Estimation of Total Flavonoid Content (TFC)

The aluminum chloride ($AlCl_3$) colorimetric approach was used to estimate the TFC of the leaf extract, which is based on the development of a complex between $AlCl_3$ and flavonoid. In a short, 200 µL of leaf extract (0.5 mg/mL) was mixed up with 600 µL of ethanol and 50 µL of $AlCl_3$ (10%). Following that, 50 µL of potassium acetate (1.0 M) and 1100 µL of distilled water were added. The reaction mixture was left to stand for 30 minutes at room temperature and then using a spectrophotometer, the absorbance was measured at a wavelength of 415 nm. The TFC was estimated using quercetin (10-100 µg/mL) calibration curve, and the results were represented as milligrams of quercetin equivalent per gram dry weight of extract (mg QE/g). To ensure the reproducibility of the experiment, each measurement was performed three times and the mean value and standard deviation were calculated[19,20].

Determination of Antioxidant Activity

The antioxidant activity of extracted leaf materials was determined by the scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals based on discoloration assay. This reagent functions as an antioxidant detector, with the purple color of the DPPH reagent changing to yellow or colorless depending on the electron transfer or hydrogen donor of a compound. Ascorbic acid was used as a standard and a mixture of methanol and DPPH was used as a control. A 0.4 mM DPPH solution in methanol was prepared and kept in a dark place. Different concentrations (20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL, 100 µg/mL) of methanolic extract and ascorbic acid were prepared. Then, 1.0 mL of each concentration of standard and extract solution was mixed with 1.0 mL of DPPH (0.4 mM) solution in methanol. The mixture of the reaction was incubated in dark at 37 ˚C for 30 minutes, and then the absorbance of was recorded at a wavelength of 517 nm using a UV-visible spectrophotometer[17,21,22]. The ability of sample extracts and the standard to scavenge the DPPH free radical was calculated by the following formula:

$$\textit{Percentage of Free radical scavenging activity} = (A_C\text{-}A_S/A_C) \times 100\%$$

Where A_C represents the control sample (DPPH + MeOH) absorbance and the A_S represents the sample extract or standard absorbance. Finally, DPPH scavenging activity was expressed as IC_{50} values, which established the sample concentration required to inhibit 50% of the DPPH. The lower the IC_{50} value, the higher the antioxidant efficiency.

In Vitro α-Amylase Inhibitory Assay

Iodine and starch interact to form a blue color complex. The α-amylase enzyme hydrolyzes starch into monosaccharides, and the intensity of the blue color decreases, indicating that starch is hydrolyzed by the enzyme. The intensity of the blue color will be greater if the extract contains compounds with α-amylase inhibiting action. The α-amylase inhibitory activity of methanolic extract of *C. viscosum* leaves was determined using a competitive inhibition-based assay. The substrate of starch was prepared by dissolving 200 mg of starch in 25 mL of NaOH (0.4 M) and heating for 5 minutes at 100 ˚C. The pH was fixed to 7.0 after cooling, and the final volume was increased to 100 mL by adding distilled water. Acarbose was used as a standard inhibitor of the α-amylase enzyme. A 400 µL starch solution was pre-incubated with 200 µL of acarbose or plant extract at various concentrations (40 µg/mL, 80 µg/mL, 160 µg/mL, 320 µg/mL, 640 µg/mL) for 5 minutes at 37 ˚C, followed by the addition of 200 µL of 50 µg/mL α-amylase (20 mM phosphate buffer with 6.7 mM NaCl, pH 6.9) and finally

incubated for 15 minutes at 37 ˚C. The reaction was terminated by adding 800 µL of HCl (0.1 M). After that, 1000 µL of iodine solution (2.5 mM) was added, and the absorbance was recorded with a spectrophotometer at a wavelength of 630 nm[21]. The test was performed in triplicate. The percentage inhibition at different concentrations was estimated by using the formula:

Percentage of Inhibition = [1-(A_2-A_1/A_4-A_3)] × 100%

Where, A_1 represents the reaction mixture absorbance containing plant extract, starch, and α-amylase, A_2 represents reaction mixture absorbance containing starch and plant extract, A_3 represents reaction mixture absorbance containing starch and α-amylase, and A_4 represents reaction mixture absorbance containing starch only. The concentration of plant sample required to inhibit the enzyme activity by 50% (IC_{50} value) was calculated from a curve of percentage inhibition versus concentration of the plant extract and compared with the standard acarbose, a potent α-amylase inhibitor.

Gas Chromatography – Mass Spectrometry

The qualitative analysis of hexane fraction obtained from methanol extract of *C. viscosum* was performed on a combined GC-MS instrument (GC-MS QP2010 ULTR SHIMADZU). An injector with a temperature setting of 230 ºC was used to inject a 2 µL aliquot of sample into the column. The GC program was initiated by a column temperature set at 40 °C for 5 min, increased to 250 ºC at a rate of 15 °C/min, held for 5 min. Helium was used as the carrier gas. The mass spectrometer was operated in EI mode. The chromatogram and spectrum of the peaks were visualized. The identification of components was based on comparison of their mass spectra with Wiley and NIST libraries[23,24].

Data Analysis

The data generated during the study were entered and analyzed using Microsoft Excel. Wherever possible, the experiments were carried out in triplicate, and data were presented in mean ± standard deviation.

Results

Phytochemical Screening, Total Phenolic, and Total Flavonoid Contents

The yield of methanolic extract of *C. viscosum* leaves was found to be 22.89%. The qualitative tests for phytochemical screening showed that the methanolic extract of *C. viscosum* leaf contained a variety of components that includes alkaloids, flavonoids, polyphenols, terpenoids, steroids, carbohydrates, saponins, and tannins but no glycosides. TPC and TFC were expressed as mg GAE/g and mg QE/g of extract, respectively, using gallic acid and quercetin calibration curves. The extract's TPC was found to be 197.68±0.11 mg GAE/g, which was higher than its TFC of 105.14±0.026 mg QE/g.

Antioxidant Activity

The DPPH radical scavenging activity of *C. viscosum* leaf extract is shown as a dose response curve in (Figure 2) in comparison to normal ascorbic acid. At concentrations of 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL, and 100 µg/mL, the percentage of free radical scavenging activities of the methanolic extract of *C. viscosum* leaf was found to be 66.59%, 76.67%, 82.85%, 85.83%, and 92.33%, and that of ascorbic acid standard was found to be 73.76%, 80.75%, 84.85%, 89.70%, and 96.63%, respectively. The concentration of *C. viscosum* leaf extract necessary to inhibit 50% of the DPPH free radical (IC_{50} value) was found to be 26.85 ±0.150 µg/mL, while that of standard ascorbic acid was observed to be 22.56 ±1.770 µg/mL.

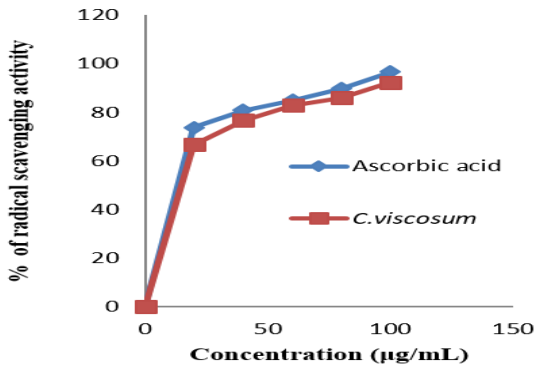


Figure 1: DPPH scavenging activities of ascorbic acid and the methanol extracts of *C. viscosum* leaves at different concentrations α-Amylase Inhibitory Activity.

At concentrations of 40 µg/mL, 80 µg/mL, 160 µg/mL, 320 µg/mL, and 640 µg/mL, the percentage of α-amylase inhibition activities of the methanolic extract of *C. viscosum* leaf was found to be 56.1%, 63.09%, 67.76%, 73.01%, and 78.47%, and that of acarbose standard was found to be 58.48%, 71.65%, 76.4%, 81.63%, and 87.4%, respectively (Figure 2). The concentration of the plant sample required to inhibit the enzyme activity by 50% (IC_{50} value) was found to be 121.48 ±0.424 µg/mL, while that of standard acarbose was observed to be 59.36 ±0.382 µg/mL.

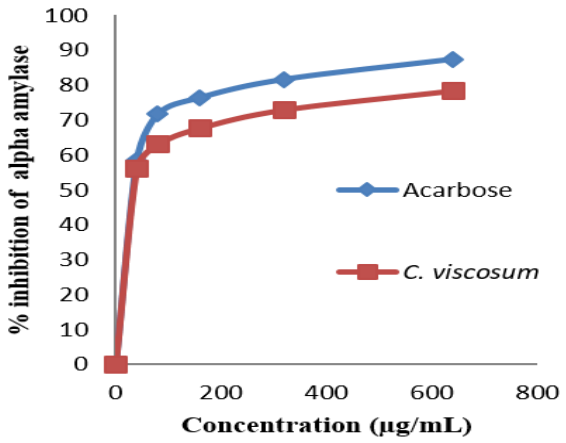


Figure 2: α-amylase inhibition activities of Acarbose and the methanol extracts of *C. viscosum* at different concentrations.

GC-MS Analysis

The GC–MS chromatogram of hexane soluble fraction of methanol extract of *C. viscosum* leaves showed different peaks which indicated the presence of different bioactive/phytochemical compounds. The GC chromatogram of hexane soluble fraction of methanol extract of *C. viscosum* leaves is depicted in Figure 3. Based on the percentage of peak area of hexane fraction, phytol was observed as a major group of active compounds, the mass spectrum is shown in Figure 4., which is similar to reported mass fragmentation pattern of Phytol[25]. Table 1 listed the major phytocomponents obtained through GC-MS study of *C. viscosum*.

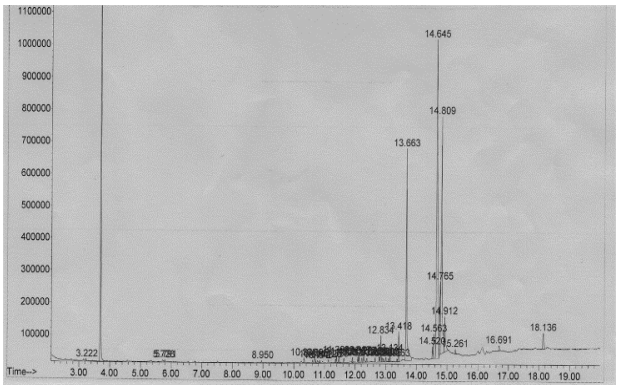


Figure 3: GC chromatogram of hexane soluble fraction of methanol extract of *C. viscosum* leaves.

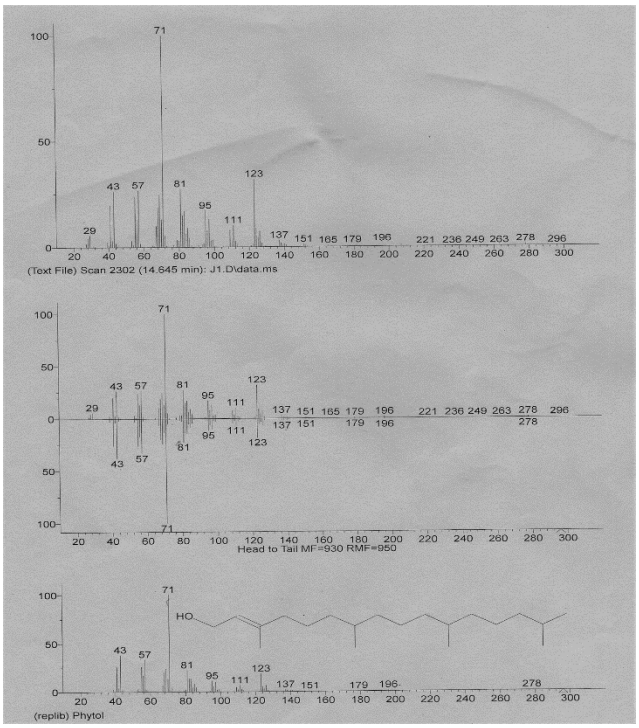
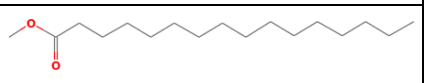
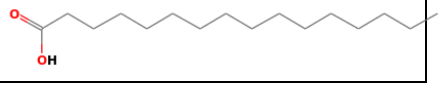
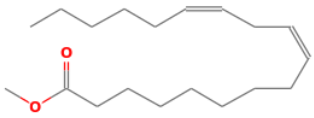
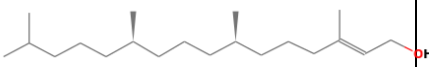
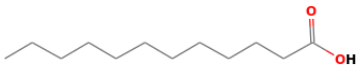
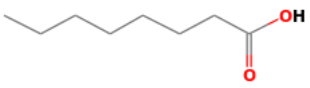
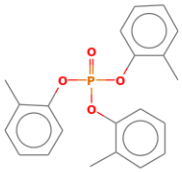
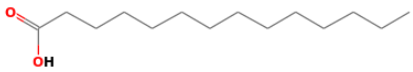
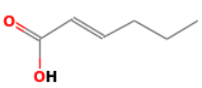
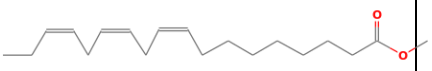


Figure 4: Mass spectrum of phytol.

Table 1. Major phytochemicals identified in hexane soluble fraction of methanol extract of *C. viscosum* leaves.

Peak	RT (Min)	Peak Area (%)	Base m/z	Name of the compound	Molecular formula	Molecular weight	Molecular structure
1	13.417	1.07	74	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45	
2	13.663	11.87	60	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	

3	14.520	0.45	67.1	9, 12-octadecadienoic acid (Z,Z)-, methyl ester	$C_{19}H_{34}O_2$	294.47	
4	14.645	29.13	71	Phytol	$C_{20}H_{40}O$	296.53	
5	14.765	3.68	60	Dodecanoic acid	$C_{12}H_{24}O_2$	200.32	
6	14.912	2.64	28	Octanoic acid	$C_8H_{16}O_2$	144.21	
7	12.927	0.13	91	Phosphoric acid, tris (2-methylphenyl) ester	$C_{21}H_{21}O_4P$	368.36	
8	12.086	0.22	91	Benzene, (1-pentylheptyl)-	$C_{18}H_{30}$	246.43	
9	12.277	0.42	73.05	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.37	
10	5.726	0.17	73	2- Hexenoic acid, (E)-	$C_6H_{10}O_2$	114.14	
11	14.563	1.19	79.1	9, 12, 15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	$C_{19}H_{32}O_2$	292.46	

Discussion

Medicinal plants have been used in human health care since ancient times due to their numerous therapeutic characteristics. Humans continue to rely on medicinal plants to address their fundamental health care needs and the search for new medicines with better or enhanced therapeutic activities derived from medicinal plants has become increasingly valuable in enhancing health care [2,7]. Plants have long been a rich source of phytochemicals, as well as having a variety of biological activities. The existence of secondary metabolites such as phenolics, terpenoids, or alkaloids determines a plant's therapeutic potential [14]. In this study, phytochemical screening showed that the methanolic extract of *C. viscosum* leaf contained a variety of components that includes alkaloids, flavonoids, polyphenols, terpenoids, steroids, carbohydrates, saponins, and tannins. This result is supported by the study of Gosh et al [9] who also identified flavonoids, polyphenolics, steroids, and triterpenoids in the methanolic extract of *C. viscosum* leaves. According to Nandi et al [7], the alkaloids, saponins, sterols, carbohydrates, tannins, and terpenoids found in *C. viscosum* leaves have been shown to have biological effects as a therapeutic agent against various animal diseases.

In medicinal plants, phenolic and flavonoid compounds are abundant, and their bioactive phenolic and flavonoid content acts as antioxidants and free radical scavengers. Because phenolic compounds have redox characteristics, they can act as antioxidants and flavonoids are a type of secondary plant metabolite that is easily ingested by humans, and they are likely to have anti-inflammatory, anti-

allergic, and anti-cancer properties[17,20]. Because *C. viscosum* contains a high concentration of tannins and polyphenolic compounds, these components functions effectively as antioxidants[9]. In this study, the TPC of methanolic extract of *C. viscosum* was found to be 197.68±0.11 mg GAE/g and its TFC was 105.14±0.026 mg QE/g. In addition to our findings, Ghosh et al[9] found lower levels of TPC (76.75 mg GAE/g) and TFC (64.56 mg QE/g) in the methanolic extract of *C. viscosum*. Similarly, Swargiary et al[14] reported that the TPC of *C. viscosum* extract was 154.54±3.89 mg GAE/g and TFC was 22.57±1.35 mg QE/g. The results of our study revealed a higher level of TPC and TFC than the above two studies, which could be attributed to the effect of the extraction solvent on the content of phenolic and flavonoid compounds, as well as other factors such as environmental variation, temperature, harvesting time, climate, and storage conditions[2,13].

Free radicals, such as superoxide, hydroxyl radicals, and nitric oxide, as well as other reactive species, like hydrogen peroxide, single oxygen, and hypochlorous acid, that are produced during normal metabolism or under adverse conditions, can damage biological systems and be a factor in a many disorders, including neurodegeneration, chronic inflammation, and immune system function. Antioxidant compounds are responsible for scavenging these free radicals and reactive species[26,27]. In this study, the leaf extracts of *C. viscosum* exhibited excellent dose-dependent DPPH radical scavenging activity, which increased with concentration. The percentage of free radical scavenging of both methanolic extract and ascorbic acid was found to be higher at 100 µg/mL concentrations and lower at 20 µg/mL concentrations. Dey et al[28] observed a greater percentage of free radical scavenging (75.79%) at a concentration of 100 µg/mL, although Hossain et al[10] reported only 13.02% inhibition by *C. viscosum* leaf extract. In our study, the IC_{50} value of *C. viscosum* leaf extract for DPPH free radical was found to be 26.85 ±0.150 µg/mL. In the study of Dey et al[29], the IC_{50} values of methanol extract of *C. viscosum* was 13.95 ± 0.44 µg/mL, 85.26 µg/mL in the study of Ghosh et al[9], and 8.93 µg/mL in the study of Hossain et al[10]. The differences in antioxidant activity of *C. viscosum* leaf extract in different investigations could be attributed to the presence of metabolites at varying levels that stabilize free radicals by donating hydrogen atoms[29]. Our findings in this quantitative DPPH free radical scavenging antioxidant assay revealed that methanolic *C. viscosum* leaf extracts have significant anti-oxidant properties, and thus this plant can be used in the development of medicines and the food industry for its anti-oxidative properties and health benefits.

Major digestive enzymes such as α-amylase and α-glucosidase are responsible for the breakdown of starch into oligosaccharides, disaccharides, and ultimately glucose, which can result in high blood glucose levels if not utilized for energy generation and results in diabetes. Inhibiting these two digestive enzymes using bioactive compounds derived from plants has been shown to be one of the most efficient strategies for managing hyperglycemia[19,30]. We investigated the α-amylase inhibitory activity of a methanolic extract of *C. viscosum* using acarbose as a standard α-amylase enzyme inhibitor. The methanolic extract of *C. viscosum* and standard acarbose were found to inhibit α-amylase enzymes in a concentration-dependent manner. The percentage of α-amylase inhibition of both methanolic extract and standard acarbose was found higher at 640 µg/mL and lower at 40 µg/mL concentrations. In this study, the IC_{50} value of methanolic extract of *C. viscosum* leaf for α-amylase enzyme activity was found to be 121.48 ±0.424 µg/mL and that of standard acarbose was 59.36 ±0.382 µg/mL. The leaf extract of *C. viscosum* inhibited α-amylase moderately as compared to the standard inhibitor acarbose as the IC_{50} value of standard acarbose was lower than that of plant extracts. Plant extracts, as opposed to single component acarbose, are well recognized to be mixtures of many components. The observed α-amylase inhibitory activity of the plant extract may be related to the presence of different phytoconstituents identified during phytochemical screening, as well as the plant extract's also showed antioxidant potential. Previous literatures have also shown that *C. viscosum* has anti-diabetic properties[10,28].

The GC-MS analysis is the preliminary step toward establishing the nature of active principles in medicinal plants and whether a particular plant species contains a specific compound. By using GC-MS, the distinct bioactive/phytochemical components of the methanolic extract of the leaf of *C. viscosum* were examined. The findings of GC-MS analysis demonstrated that the percentage of compounds viz., Hexadecanoic acid, methyl ester (1.07%), n-Hexadecanoic acid (11.87%), 9, 12-octadecadienoic acid (Z,Z)-, methyl ester (0.45%), Phytol (29.13%), Dodecanoic acid (3.68%), Octanoic acid (2.64%), Phosphoric acid, tris (2-methylphenyl) ester (0.13%), Benzene, (1-pentylheptyl)- (0.22%), Tetradecanoic acid (0.42%), 2- Hexenoic acid, (E)- (0.17%), and 9, 12, 15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- (1.19%) were found as the major bioactive compounds in the methanol extract of *C. viscosum*. Based on their retention times, the GC-MS spectrum profile revealed the presence of key phytochemical components, and the peak heights demonstrate the relative quantities of the phytochemicals present in the extracts. Phytol was identified as a substantial group of active compounds in the current study among the recognized phytochemicals in the analyzed sample. The biological properties of phytol and its derivatives, including antibacterial, antidiabetic, antioxidant, anti-inflammatory, and other properties, have been demonstrated from a pharma-medico perspective[31]. Phytol is found to be effective at different stages of the arthritis[25].

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