

Antibacterial and antioxidant activities of methanol extract and fractions of *Clerodendrum viscosum* Vent. leaves

Goutam Ghosh^{1*}, Suprava Sahoo², Debajyoti Das¹, Debasmita Dubey³ and Rabindra N Padhy³

¹School of Pharmaceutical Sciences, ²Centre of Biotechnology, ³IMS & Sum Hospital, Siksha 'O' Anusandhan University, Khandagiri Square, Bhubaneswar-751030, Odisha, India

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The present study was undertaken to evaluate antimicrobial and antioxidant activities of methanol extract as well as n-hexane, chloroform, ethyl acetate and aqueous fractions of *Clerodendrum viscosum* Vent. leaves. The antimicrobial activity, total phenolics and flavonoids were determined by agar-well diffusion, Folin-Ciocalteu and aluminum chloride colorimetric methods, respectively. The antioxidant potential was evaluated by using various *in vitro* methods such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), nitric oxide and hydrogen peroxide radical scavenging assays. The maximum inhibition zones for *S. aureus*, *B. subtilis*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* were 27, 25, 28, 23 and 22 mm for ethyl acetate fraction, respectively, whereas n-hexane fraction of methanol extract showed the weakest antimicrobial activity with inhibitory effect against *B. subtilis* and *K. pneumoniae*. The total phenolic content (TPC) in ethyl acetate fraction (87.28 mg GAE/g) was highest, followed by methanol extract (76.75 mg GAE/g), aqueous fraction (61.36 mg GAE/g) and chloroform fraction (28.46 mg GAE/g), respectively. The total flavonoid content (TFC) was in order of ethyl acetate fraction (77.48 mg quercetin equivalent/ g) > methanol extract (64.56 mg quercetin equivalent/ g) > aqueous fraction (49.66 mg quercetin equivalent/g) > chloroform fraction (32.85 mg quercetin equivalent/ g). The ethyl acetate fraction of methanol extract of *C. viscosum* leaves showed moderate free radical scavenging activity as evidenced by IC₅₀ values in DPPH (64.51 µg/mL), ABTS (28.32 µg/mL), nitric oxide (80.02 µg/mL) and H₂O₂ (77.45 µg/mL) scavenging assays. The results of present study showed that *C. viscosum* leaves possess high phenolic, flavonoid contents, potential antimicrobial and antioxidant activity and could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of ageing and age associated oxidative stress related degenerative diseases.

Keywords: *Clerodendrum viscosum*, Antimicrobial, Antioxidant activity, Total phenolic content.

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Introduction

The World Health Organization estimated that about 80 % of people in developing countries rely on traditional medicines based largely on species of medicinal plants for the treatment of various ailments¹. There has been an increasing interest in the study of traditional medicines and their popularity is increasing day by day in different parts of the world, since last few decades. The secondary metabolites from medicinal plants play an important role in the development of potent therapeutic agents, because of their less toxicity and economical viability². Now-a-days, many plants have been claimed to have promising antimicrobial and antioxidant activities. The emergence of multiple strains of antibiotic resistant

microorganism is now being the root cause to search potential antimicrobial compounds from plant source³. Moreover, the free radicals are highly reactive molecular fragments that contain one or more unpaired electrons in its outer orbit and are capable of independent existence, highly unstable and strongly react with biomolecules, leading to the structural modification and cell or tissue damage. They play an important role in oxidative stress related to the pathogenesis of various important diseases such as cardiovascular diseases, Parkinson's disease, Alzheimer's disease, cancerogenesis, neuro-degenerative, nephrotoxicity, diabetes and the ageing⁴. Antioxidants act as free radical scavengers and are thus found to play significant protective role against oxidative stress in a variety of diseases and phenolic compounds which are powerful antioxidants that can protect the human body from free radicals by acting as hydrogen donors and radical scavengers⁵. Over last few

*Correspondent author:

E-mail: goutamghoshsp@gmail.com

Phone: 09853126440

decades, a large number of synthetic antimicrobial and antioxidant agents have been used for the treatment of concerned diseases, however, are reported to be toxic to animals including human beings. Thus the time has come to find out new herbal drugs which can control antibiotic resistant microorganism and oxidative stress induced diseases satisfactorily. Hence, there is a need to search newer antimicrobial and antioxidant agents, preferably from the plants having pertinent ethnic claims, which is thought to be endowed with the required therapeutic efficacy and devoid of side effects.

Clerodendrum viscosum Vent. belonging to family Verbenaceae is a flowering shrub, found in India, Myanmar, Thailand and Indonesia. Leaves in whorls, sessile, narrow lanceolate, sub-entire, glabrous, rather hard. Flowers are bluish-purple often white in pyramid shaped terminal panicles. The flowering and fruiting season is March to June. It is mostly used in traditional system of medicines; particularly the leaves and roots are widely used as antiseptic, anti-inflammatory, antipyretic, vermifuge, against leprosy and skin diseases⁶. Fumeric acid, caffeic acid esters, β -sitosterol, apigenin, acacetin and a new flavone glycoside, characterised as the methyl ester of acacetin-7-O-glucuronide were isolated from the flowers⁷. Scutellarin, hispidulin-7-O-glucuronide, poriferasterol and stigmasterol are the phyto-constituents of the aerial parts of *C. viscosum*^{8,9}. The methanol extract of *C. viscosum* has shown antioxidant and antimicrobial properties¹⁰. As no extensive antioxidant and antimicrobial study have been conducted earlier for the fractions of methanol extract, it was thought worthwhile to investigate the antibacterial, antioxidant activities, total phenolics and flavonoids content of various fractions of methanol extract of *C. viscosum* leaves.

Materials and Methods

Plant material

The leaves of *C. viscosum* were collected from Nayagarh district of Odisha in the month of January, 2012. The plant was authenticated by Dr. P. C. Panda, Senior Scientist, Taxonomy and Conservation Division, Regional Plant Resource Centre, Bhubaneswar. A voucher specimen (No. SPS/SOAU/2008/007) was deposited in the Department of Pharmacognosy, Siksha 'O' Anusandhan University, Bhubaneswar, Odisha.

Preparation of extract and fractions

The shade dried leaves were ground to coarse powder and extracted with methanol in a Soxhlet extractor for 24 h. The methanol extract was filtered

and then concentrated under reduced pressure at 40°C using vacuum rotary evaporator to yield a semisolid mass. The methanol extract was partitioned between n-hexane and water. The aqueous layer was further fractionated by using different polarity based solvents such as n-hexane, chloroform and ethyl acetate successively. Three solvent fractions (n-hexane, chloroform and ethyl acetate) were collected and concentrated with vacuum rotary evaporator. The yields of these fractions constituted 7.6, 6.6 and 8.5% of the methanol extract, respectively. The aqueous fraction was lyophilized and constituted 7.8% of methanol fraction. All the fractions and extracts obtained were investigated for antimicrobial and antioxidant activity using different standard methods.

Preliminary phytochemical screening

The methanol extract and its various fractions (n-hexane, chloroform, ethyl acetate and aqueous fractions) of *C. viscosum* were subjected to different chemical tests for the detection of phytoconstituents as per standard methods¹¹.

Test microorganisms

Microbial cultures of five different strains of both Gram positive and Gram negative bacteria were used for determination of antibacterial activity. All the stock cultures were collected from IMS and Sum Hospital, Bhubaneswar, Odisha, India. The bacterial strains were grown and maintained on Muller Hinton agar at 37°C.

Antibacterial activity test by agar-well diffusion method

Antibacterial activities of the methanol extract of *C. viscosum* and its various fractions such as n-hexane, chloroform, ethyl acetate and aqueous were evaluated by agar-well diffusion method¹². One strain from each bacterial species was used for monitoring antibacterial activities of plant extract and fractions. Bacterial lawn was prepared on 6mm thick agar layer and kept for 30 min in BOD incubator as described in the reported method. The wells (6 mm depth) were prepared with the help of sterilized cork borer and each well was based by 50 μ L molten MH agars. Further, wells were filled with 100 μ L aliquots of 30 mg/mL extracts and fractions of *C. viscosum*. Plates were incubated at 37°C for 18-24 h. Antibacterial activities were evaluated by measuring the diameter of zones of inhibition. Gentamicin 30 μ g/mL was used as reference controls, for all extract and fractions. Extracts causing the zone of inhibition of 20 mm or more were considered highly

active and that having a zone of inhibition less than 20 mm was considered moderately active.

Determinations of MIC

Minimum inhibitory concentration (MIC) of the plant extract and derived fractions was determined as described by the reported method¹³. Original stock solutions of plant extract (100 mg/mL) was prepared by completely dissolving 100 mg of plant extract in 500 μ L of dimethyl sulfoxide (DMSO) and adding 1 mL of peptone water sugar medium.

Each stock solution was diluted to obtain different concentrations with DMSO solution. Separate experiment was conducted for each solvent extract and fractions. An aliquot of 80 μ L of each dilution of a solvent extract/fraction was released to a well on a 96 well (12 x 8) micro-titer plate, along with an aliquot of 100 μ L MH broth (HiMedia), an aliquot of 20 μ L bacterial inocula (10^9 CFU/mL) and a 5 μ L-aliquote of 0.5 % of 2,3,5-triphenyl tetrazolium chloride (TTC). After pouring all the above to a well, the microplate was incubated at 37°C for 18 h. The development of pink colouration in a well indicated bacterial growth due to TTC and the absence of the colour was taken as inhibition of bacterial growth. First well of the micro plate was the control without any plant extract. The MIC value was noted at the well, where no colour was manifested.

Determination of total phenolic content

Total phenolic content (TPC) of *C. viscosum* was assessed using Folin-Ciocalteu reagent¹⁴ with little modifications. The stock solution (1mg/mL) of crude extract and each fraction was separately prepared in methanol. From each of the stock solution, 1.5 mL was taken in a 25 ml of volumetric flask and 10 mL of water and 1.5 mL of Folin-Ciocalteu's reagent were added to it. The mixture was kept for 5 min, and then 4 mL of 20% sodium carbonate solution was added and made up to 25 mL with doubled distilled water. Absorbance of the resulting solution was measured at 760 nm in a UV-VIS spectrophotometer after incubating the samples for 90 min. The total phenolic content was calculated as mg of Gallic acid equivalent by using equation obtained from the Gallic acid calibration curve. The determination of total phenolic compounds was carried out in triplicate and the results were averaged.

Determination of total flavonoid content

Total flavonoid content (TFC) was determined by using the aluminum chloride colorimetric method¹⁵. The extract and derived fractions of methanol extract

(1 mL) was added to distilled water (4 mL) in a flask. Then, 5% NaNO₂ (0.3 mL) was added. After 5 min, 10% AlCl₃ (0.3 mL) was added and after 6 min, 1 M NaOH (2 mL) was added. The mixture was diluted to 10 mL with distilled water. The absorbance of the solution was measured at 510 nm using a UV-VIS spectrophotometer. The results were calculated by extrapolating the absorbance of reaction mixture on calibration curve of Quercetin. All the determinations were performed in triplicate and TFC was expressed as mg Quercetin equivalent/ g of the extract.

DPPH radical scavenging assay

The DPPH radical scavenging assay was carried out as described by the reported method¹⁶ with some modifications. 1.5 mL of 0.1 mM DPPH solution was mixed with 1.5 mL of various concentrations (10 to 500 μ g/mL) of extract and fractions of methanol extract of *C. viscosum*. The mixtures were first kept in a dark place at room temperature and after 30 min the absorbance was measured using a spectrophotometer at 517nm. Methanol was used as control. The experiment was replicated in three independent assays. Ascorbic acid was used as positive controls. Inhibition of DPPH free radical in percentage was calculated by the formula:

$$\text{Inhibition (\%)} = [(A_{\text{control}} - A_{\text{test}}) / A_{\text{control}}] \times 100$$

Where A_{control} is the absorbance of the control (solution without extract) and A_{test} is the absorbance of samples (extract and ascorbic acid).

The antioxidant activity of each sample was expressed in terms of IC₅₀, which is defined as the concentration of the test material necessary to cause a 50% reduction in initial DPPH concentration.

ABTS radical scavenging assay

The ABTS radical scavenging assay was determined according to the reported method¹⁷ with little modifications. The stock solutions of 7 mM ABTS and 2.4 mM potassium persulfate were prepared separately. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The resulting solution was diluted by mixing 1 mL of freshly prepared ABTS solution to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. The extract and individual fraction of methanol extract (1 mL) was allowed to react with 2.5 mL of the ABTS solution and the absorbance was taken at 734 nm after

7 min using the spectrophotometer. The ABTS scavenging capacity of the extract and fractions was compared with that of butylated hydroxytoluene (BHT) and percentage inhibition calculated as:

$$\text{ABTS radical scavenging activity (\%)} = [(A_{\text{control}} - A_{\text{test}}) / A_{\text{control}}] \times 100$$

Where A_{control} is the absorbance of ABTS radical with methanol; A_{test} is the absorbance of ABTS radical with sample extract/standard.

Nitric oxide (NO) scavenging activity

Nitric oxide scavenging activity was measured by the standard spectrophotometric method¹⁸ with little modification. Sodium nitroprusside (5 mMol) in phosphate buffered saline was mixed with a control without the test sample, but with an equivalent amount of methanol. Test samples at different concentrations were dissolved in methanol and incubated at 25° C for 30 min. After 30 min, 1.5 mL of the incubated solution was removed and diluted with 1.5 mL of Griess reagent (1% Sulphanilamide, 2% Phosphoric acid, and 0.1% Naphthyl ethylenediamine dihydrochloride). The absorbance of the chromophores formed during the subsequent coupling with Naphthyl ethylenediamine dihydrochloride was measured at 546 nm. All the tests were performed in triplicate and the graph was plotted with the mean values. The percentage of inhibition was measured by the following formula:

$$\text{NO radical scavenging activity (\%)} = [(A_{\text{control}} - A_{\text{test}}) / A_{\text{control}}] \times 100$$

Where A_{control} is the absorbance of the control (without extract) and A_{test} is the absorbance in the presence of the extract/standard.

Hydrogen peroxide scavenging activity

The ability of the extract and various fractions of methanol extract of plant to scavenge hydrogen peroxide was determined by the standard method¹⁹, with suitable

modifications. A solution of H₂O₂ (40 mM) was prepared in PBS (pH 7.4). The extract and each fraction of *C. viscosum* (4 mL), prepared in distilled water at various concentration were mixed with 0.6 mL of 4 mM H₂O₂ solution prepared in phosphate buffer saline and were incubated for 10 min. The absorbance of the solution was taken at 230 nm against a blank solution containing only plant extract in PBS. Ascorbic acid was used as positive control. The hydrogen peroxide radical scavenging activity of the extract and fractions was calculated using the following equation:

$$\text{H}_2\text{O}_2 \text{ radical scavenging activity} = [(A_{\text{control}} - A_{\text{test}}) / A_{\text{control}}] \times 100$$

Where A_{control} is the absorbance of H₂O₂ radical with methanol; A_{test} is the absorbance of H₂O₂ radical with sample extract or standard.

Results

Phytochemical screening

Preliminary phytochemical screening of methanol extract and the n-hexane, chloroform, ethyl acetate and aqueous fractions of the methanol extract of *C. viscosum* was carried out by standard qualitative chemical tests. The methanol extract contains flavanoids, polyphenolics, glycosides, steroids and triterpenoids. The chloroform, ethyl acetate, methanol and aqueous fractions reveal the presence of flavonoids, polyphenolics and glycosides, whereas n-hexane fraction contains only steroids and triterpenoids.

Antimicrobial activities of extract and fractions

The *in vitro* antimicrobial activities of methanol extract and various fractions of *C. viscosum* were evaluated against Gram positive (*S. aureus* and *B. subtilis*) and Gram negative bacteria (*E. coli*, *K. pneumoniae* and *P. aeruginosa*). The activity potentials were qualitatively assessed by the presence or absence of inhibition zones, zone diameters and MIC values. The results given in Table 1 showing that ethyl acetate fraction had greater potential as an

Table 1—Antimicrobial assay and MIC of methanol extract and fractions of *C. viscosum* against Gram +ve and Gram -ve bacterial strains (zone of inhibition in mm)

Strain	Methanol extract		n-Hexane		Chloroform		Ethyl acetate		Aqueous		Gentamicin (240µg/mL)	
	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC
<i>S. aureus</i>	24	3.12	-	-	21	3.12	27	3.12	22	6.12	30	240
<i>B. subtilis</i>	23	6.25	13	-	18	6.25	25	6.25	17	12.5	29	240
<i>E. coli</i>	25	6.25	-	-	15	6.25	28	6.25	23	6.25	33	120
<i>K. pneumoniae</i>	21	6.25	08	-	16	12.5	23	6.25	19	6.25	31	120
<i>P. aeruginosa</i>	22	6.25	-	-	19	3.12	22	3.12	20	6.25	29	120

Note: (-): absence of IZ/MIC; IZ: inhibition zone; MIC: minimum inhibitory concentration

antimicrobial agent against *S. aureus*, *B. subtilis*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* than crude extract and other fractions. The maximum inhibition zones produced by ethyl acetate fraction against *S. aureus*, *B. subtilis*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* were 27, 25, 28, 23 and 22 mm, respectively. Gentamycin was used as positive control since it is commonly used antibiotic against Gram positive and Gram negative pathogenic bacterial species.

On the other hand, n-hexane fraction of methanol extract showed the lowest antimicrobial activity with minimum inhibitory effect against *B. subtilis* and *K. pneumoniae* out of the 5 tested strains. The activity of the extract and fractions were presented in the following order: ethyl acetate fraction > methanol extract > aqueous fraction > chloroform fraction > n-hexane fraction. The most sensitive strains against all tested extracts were *S. aureus* and *B. subtilis*.

Total phenolic content (TPC) and total flavonoid content (TFC)

In order to determine the antioxidant compounds in methanol extract and fractions, the total phenolic and flavonoid contents of extract and of different fractions were determined by using modified Folin-Ciocalteu and aluminum chloride colorimetric method. TPC was calculated using the standard curve of Gallic acid (Standard curve equation: $Y = 0.004x + 0.063$, $R^2 = 0.998$) and TFC was calculated using the standard curve of Quercetin (standard curve equation: $Y = 0.013x + 0.487$, $R^2 = 0.886$). TPC in ethyl acetate fraction (87.28 mg GAE/g) was the highest, followed by methanol extract (76.75 mg GAE/g), aqueous fraction (61.36 mg GAE/g) and chloroform fraction (28.46 mg GAE/g), respectively. TFC was in order of ethyl acetate fraction (77.48 mg Quercetin equivalent/g) > methanol extract (64.56 mg Quercetin equivalent/g) > aqueous fraction (49.66 mg Quercetin equivalent/g) > Chloroform fraction (32.85 mg Quercetin equivalent/g).

DPPH radical scavenging activity

In the present study, the crude extract and all the fractions except n-hexane showed significant DPPH radical inhibiting activity at a concentration of 500 $\mu\text{g/mL}$. Figure 1 shows the dose response curve of DPPH radical scavenging activity of *C. viscosum* when compared with standard ascorbic acid. The IC_{50} values of methanol extract, n-hexane, chloroform, ethyl acetate, aqueous fractions and standard ascorbic acid were: 85.26, 160.5, 120.45, 65.25, 98.42 and

10.5 $\mu\text{g/mL}$, respectively. At 500 $\mu\text{g/mL}$, the percentage inhibition of the extract, n-hexane, chloroform, ethyl acetate and aqueous fractions of *C. viscosum* were 78.42, 33.25, 65.73, 86.24, 71.67, whereas at 500 $\mu\text{g/mL}$ the standard ascorbic acid shows 97.47 % inhibition (Fig. 1). The DPPH activity of *C. viscosum* was found to be increasing on dose dependent manner.

ABTS radical scavenging activity

As per the Fig.2, results show that the scavenging activity values on ABTS of extract and the fractions (n-hexane, chloroform, ethyl acetate and aqueous) of *C. viscosum* decrease than that of BHT in the order of (BHT 98.25%) > ethyl acetate (89.76%) > methanol extract (87.52%) > aqueous (76.43%) > chloroform (67.48) > n-hexane fraction (26.37%).

Among all the extract and fractions, the ethyl acetate fraction exhibited highest scavenging effects against ABTS with an IC_{50} value of 74.25 $\mu\text{g/mL}$,

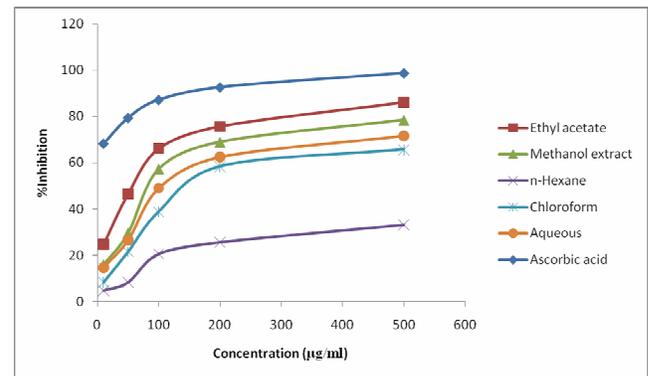


Fig. 1—DPPH radical scavenging activity of methanol extract/fractions of *C. viscosum* and standard drug, ascorbic acid. Values represent mean \pm SD of three replicates.

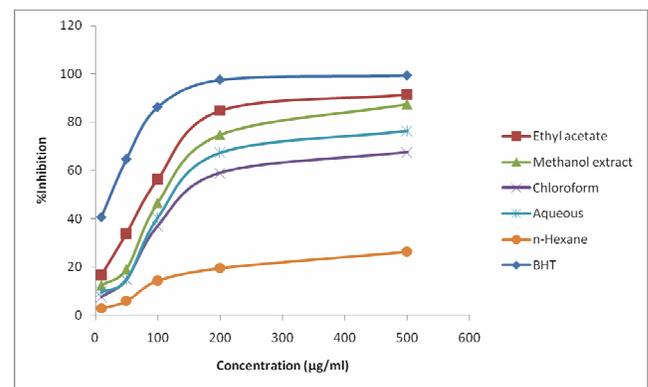


Fig. 2—ABTS scavenging activity of methanol extract/fractions of *C. viscosum* and standard drug, ascorbic acid. Values represent mean \pm SD of three replicates.

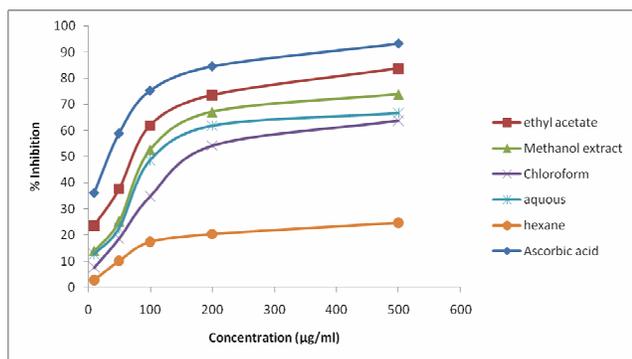


Fig. 3—NO radical scavenging activity of methanol extract/fractions of *C. viscosum* and standard drug, ascorbic acid. Values represent mean \pm SD of three replicates.

whereas IC_{50} value of standard BHT is 31.65 μ g/mL. The plant extract and derived fractions showed higher inhibitory activity in removing ABTS radicals from the reaction system at a concentration of 500 μ g/mL.

NO scavenging assay

The methanol extract and its derived fractions such as n-hexane, chloroform, ethyl acetate and aqueous fractions were found to be very much efficient in scavenging nitric oxide (Fig. 3) among which the ethyl acetate fraction displayed the highest activity. The IC_{50} values for methanol extract, n-hexane, chloroform, ethyl acetate and aqueous fractions were: 92.25, 175.5, 108.25, 80.65 and 98.35 μ g/mL, respectively. Ascorbic acid was used as a reference compound which has an IC_{50} value of 36.45 μ g/mL.

At 500 μ g/mL, the percentage inhibition of methanol extract, n-hexane, chloroform, ethyl acetate and aqueous fractions were: 73.85, 24.75, 63.76, 83.76 and 66.78%, respectively whereas for the standard ascorbic acid, the percentage inhibition was 93.28%. The ethyl acetate fraction showed maximum activity of 83.76% at 500 μ g/mL, whereas ascorbic acid at the same concentration exhibited 93.28% inhibition.

H₂O₂ radical scavenging activity

The H₂O₂ radical scavenging assay exhibits the ability of extract, all the fractions and standard Ascorbic acid to inhibit hydroxyl radical-mediated deoxyribose degradation in a Fe³⁺-EDTA-Ascorbic acid and H₂O₂ reaction mixture. The results are displayed in Fig. 4. The IC_{50} values of the methanol extract, n-hexane, chloroform, ethyl acetate, aqueous fractions and standard ascorbic acid were: 90.45, 165.5, 102.5, 82.55 and 98.25 μ g/mL, respectively. The IC_{50} values of extract and all the fractions were

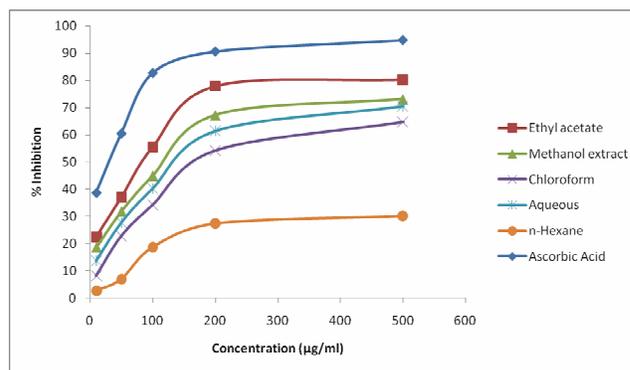


Fig. 4—H₂O₂ radical scavenging activity of methanol extract/fractions of *C. viscosum* and standard drug, ascorbic acid. Values represent mean \pm SD of three replicates.

less than that of the standard. At 500 μ g/mL conc, the percentage inhibition values for the above mentioned extract and n-hexane, chloroform, ethyl acetate, aqueous fractions of *C. viscosum* and the standard were: 73.18, 30.16, 64.77, 80.26, 70.56 and 94.88%, respectively. The ethyl acetate fraction of *C. viscosum* showed highest scavenging effects with IC_{50} value of 80 μ g/mL. As shown in Fig. 4, the extract and all the fractions demonstrated hydrogen peroxide decomposition activity on a concentration dependent manner.

Discussion

Plants provide sophisticated traditional medicinal systems that have been in existence for thousands of years and continue to serve mankind with newer remedies²⁰. The research work on natural antioxidants and natural antibacterial agents akin to synthetic drugs were continuously emerging into the scientific limelight and the exploitation of the various secondary metabolites of the plant was highlighted in recent time.

Amongst the Gram-positive and Gram-negative bacteria, Gram-positive bacterial strains were more susceptible to the plant extracts as compared to Gram negative bacteria. This is in agreement with previous reports that plant extracts are more active against Gram-positive bacteria than Gram-negative bacteria²¹.

It is widely reported that phenolic and flavonoid compounds may significantly contribute to overall antimicrobial activity²². The antibacterial activity of the plant extracts might be attributed to the presence of bioactive compounds such as tannins, phenolic compounds, polyphenols and flavonoids²³. Among these bioactive compounds, phenolics were the most important active compounds against bacteria²⁴. Thus

the results of antibacterial activities obtained in the present study for extract and fractions of *C. viscosum* were correlated to their total phenolic contents.

Medicinal plant and their bioactive phenolic and flavonoids content play important role in scavenging of free radicals. Phenolic compounds such as flavonoids and tannins found in the extracts are considered to be the major contributors to the antioxidant activity of medicinal plants²⁵. Since *C. viscosum* is rich in tannins and polyphenolic compounds, these compounds exhibited effectively as the antioxidant activity.

The ethyl acetate fraction of methanol extract of *C. viscosum* exhibited highest total phenolic content is in agreement with other reports²⁶. Phenolic compounds of plants are also very important because of their hydroxyl groups confer scavenging ability and possess a high antioxidant effect. The high concentration of polyphenolics in the ethyl acetate fraction may be due to purification and concentration of phenolics throughout the fractionation procedure and it is probably responsible for its high free radical scavenging activity.

Flavonoids are naturally occurring compounds in plants and are thought to have positive effects on human health. Flavonoids have shown a wide range of antibacterial, antiviral, anti-inflammatory, anticancer and anti-allergic activities²⁷. Flavonoids have been shown to be highly effective scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals implicated in several diseases²⁸. So comparing with these findings from the literature of plant products, our results suggested that flavonoids found in methanol extract and various fractions of *C. viscosum* may be the major contributors for the antioxidant activity.

DPPH is a relatively stable radical at room temperature and accepts an electron or hydrogen radical to become stable diamagnetic molecule. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH which reacts with suitable reducing agent²⁹. DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. The low IC₅₀ value of ethyl acetate fraction of methanol extract of the subjected plant is due to presence of high polyphenolic and flavonoid compounds³⁰.

Hydrogen peroxide occurs naturally at low concentration levels in the air, water, human body,

plants, microorganisms and food. H₂O₂ is rapidly decomposed into oxygen and water and this may produce hydroxyl radicals (-OH) that can initiate lipid peroxidation and cause DNA damage²⁸. Ethyl acetate fraction of *C. viscosum* efficiently scavenged hydrogen peroxide which may be attributed to the presence of phenolic groups that could donate electrons to hydrogen peroxide, thereby neutralizing it into water.

ABTS radical scavenging assay involves a method that generates a blue/green ABTS⁺ chromophore via the reaction of ABTS and potassium persulfate. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate. All the fractions of methanol extract showed strong ABTS scavenging activity and these observations are supported by other researchers³¹.

NO is a reactive free radical generated from SNP in aqueous solution at physiological pH and reacts with oxygen to form nitrite. Suppression of released NO may be partially attributed to direct NO scavenging, as the extracts of *C. viscosum* decreased the amount of nitrite generated from the decomposition of SNP *in vitro*. The mechanisms by which NO may inhibit lipid peroxidation are not entirely clear, however, one possible mechanism relates to the ability of NO to terminate propagation of lipid peroxidation reactions³².

Conclusion

In the present study, we conclude that the ethyl acetate fraction of methanol extract of *C. viscosum* has significant amount of total phenolics and flavonoids and has shown higher *in vitro* antioxidant and antimicrobial activity compared to the crude extract and other fractions. The highest scavenging activities of the ethyl acetate fraction may be due to its polyphenolic compounds, tannins and flavonoids, which were concentrated in the fraction during fractionation. The scavenging effect on DPPH represents the fraction's direct radical scavenging activity. However, in the hydroxyl radical and NO scavenging assay, hydroxyl radicals are generated by the Fenton reaction and the inhibition of deoxyribose degradation could be attributed to the inhibition of radicals. Since this investigation is a preliminary study, a detailed study of the antioxidant mechanisms of specific phenolic components is an absolute necessity. For further work on the profile and nature of chemical constituents of *C. viscosum* leaves will provide more information on the active principles responsible for their antioxidant properties. This may also lead to the

development of new natural antioxidants which can result in ways of combating the serious health related problems.

Despite the widespread use of *C. viscosum* as folk medicines, literature contains few reports on its antioxidant activity. In the present experiment, we conceded a systematic record on the relative free radical scavenging activity of the fractions of methanol extract. The finding of this study suggests that *C. viscosum* leaves could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of ageing and age associated oxidative stress related degenerative diseases. The present result confirms the free radical scavenging and antibacterial activity of the plant which can be accounted for the traditional uses of the plant in treating such type of diseases.

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