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A report on antioxidant and antibacterial properties of *Callistemon viminalis* Leaf

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Abstract

Objectives: *Callistemon viminalis*, a common ornamental small tree, is traditionally used in folk medicine to treat various diseased conditions. This study was carried out to explore *in vitro* antioxidant and antibacterial activity of crude extracts of *C. viminalis* leaf using different solvent systems.

Methods: Maceration process was used for extraction with five different solvents and primarily qualitative and quantitative phytochemical analysis were carried out by standard methods. Antioxidant activity was explored by several *in vitro* assay models whereas disc diffusion method was used to evaluate antibacterial property.

Results: Qualitative assay detected the presence of different important phytoconstituents. Ethanol extract showed the highest content of phenolics (88.83±2.71 mg gallic acid/ g dry weight), flavonoids (65.48±2.74 mg of catechin/ g dry weight) and proanthocyanidins (17.39±0.64 mg of catechin/ g dry weight) except flavonol. The lower IC_{50} values of DPPH (32.80±1.71 µg/mL) and ABTS (46.20±1.33 µg/mL) assay were found by methanol extract than that of standards and other extracts. The highest superoxide and nitric oxide scavenging activity were found in ethanol (IC_{50}: 39.04±1.36 µg/mL) and chloroform extract (IC_{50}: 89.12±1.38 µg/mL), respectively. In disc diffusion assay, ethanol and petroleum ether extract showed highest activity against gram positive bacteria (inhibition zone: 22 mm against *S. aureus*) at 400 µg/ disc concentration, but the antibacterial activity of most extracts was relatively lower against gram negative bacteria.

Conclusion: The results of this investigation revealed potent antioxidant and antibacterial activity, therefore *C. viminalis* may have a promising therapeutic potential.

Keywords: antioxidant, antibacterial activity, *callistemon viminalis* leaf extract, phytochemicals

1. Introduction

Reactive oxygen species (ROS) such as superoxide anion radicals, hydroxyl radicals and hydrogen peroxide are formed as a byproduct during normal oxygen metabolism. But their excessive production leads to damage of proteins, DNA and lipids which is associated with the chronic degenerative diseases including cancer, coronary artery disease, hypertension and diabetes etc. [1, 2, 3]. An antioxidant helps organism to scavenge free radicals as well as to delay or prevent oxidative stress, thus prevents various degenerative diseases including cardiovascular diseases, cancers, neurodegenerative diseases, Alzheimer's disease and inflammatory diseases [1, 4]. Endogenous defense systems such as catalase, superoxide dismutase, peroxidase glutathione system etc. often fail to scavenge ROS. Synthetic anti-oxidants used in the food industry may be responsible for liver damage and carcinogenesis. Therefore exogenous anti-oxidants from natural sources are required [5, 6, 7]. Recently, plants have drawn attention for their different biological activities including antioxidant activity. The role of medicinal plants in disease prevention or control has been attributed to their constituents such as vitamins, terpenoids, phenolic acids, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids which are rich in anti-oxidant activity [8].

Different pathogenic microorganisms are responsible for a number of diseases of human being. Although modern science has developed many types of antibiotic against these microbes but generation of resistance is a global alarming issue. Therefore scientific research focuses on plant species to explore new and potential antimicrobial agents, nowadays [21, 37, 38].

Callistemon viminalis, commonly known as bottle brush, belongs to the family Myrtaceae which is a small tree or shrub with pendulous foliage. This ornamental plant is found in several areas with the exception of localities extremely cold and dry. It is also found along the streets and in the botanical gardens [9–11]. The leaves of *C. viminalis* are a tea substitute and have a delightfully refreshing flavour and fragrance. It has been used to prepare a hot drink locally referred to as "tea" for the treatment of various conditions like gastroenteritis, diarrhea and skin infections [12]. Various parts of *C. viminalis* used in traditional and folk medicine have been reported to have antihelminthic, antibacterial, antidiabetic, anti-inflammatory, analgesic, anticough and antibronchitis activities [13-15]. Previously, no studies have been carried out with different solvent extracts of *C. viminalis* leaves. Therefore, in this study we illuminated the antioxidant and antimicrobial properties of *C. viminalis* leaves with evaluating the phytochemical constituents using five different solvents namely ethanol, methanol, chloroform, n-hexane and petroleum ether.

2. Materials and Methods

2.1 Collection of plant material and authentication

For this study, mature *C. viminalis* leaves were collected from the Rajshahi University campus at the month of February, 2016 and authenticated by Department of Botany, University of Rajshahi.

2.2 Preparation of plant extracts

The leaves were first rinsed with water and air dried. After complete drying, these were grinded into a coarse powder by a grinding machine and stored in an airtight container for further use. Grinded plant material (20 g) was taken in a conical flask and extracted with five different organic solvents (200 mL) named ethanol, methanol, chloroform, n-hexane and petroleum ether in a mechanical shaker with temperature control (room temperature) at constant stirring rate at 200 rpm. It was left for 48 hours and solids were filtered using Whatman No. 1 filter. The extraction was repeated three times until complete extraction. Afterwards, the solvents were evaporated under reduced pressure at 40 ˚C using rotary evaporator and the residues were kept in small sterile glass vials under refrigerated conditions until used.

2.3 Qualitative analysis for phytochemicals

To determine the presence of different phytochemicals such as alkaloids, flavonoids, carbohydrates, triterpenoids, glycosides, saponins, resins and tannins standard qualitative tests were done by the methods previously described [16,17].

2.4 Determination of total phenolics content

The Folin-Ciocalteu method [18] was used for the estimation of total phenolic contents in each extract. In short, 2.25 ml of Folin–Ciocalteu reagent diluted (1:10) in distilled water was mixed with 300 µl of extract and kept for 5 minutes at room temperature. 2.25 ml of sodium carbonate (60 g/l) solution was then added to the mixture and absorbance was taken at 725 nm after 90 minutes incubation at room temperature. Gallic acid was used as standard and total phenolic content of each extract was calculated as gallic acid equivalents per gram of dry weight (mg GAE/g DW).

2.5 Determination of total flavonoids content

Total flavonoid contents were measured by the previously described method [18]. In short, 2.25 ml of distilled water was mixed with 0.5 ml extract in a test tube followed by addition of 0.15 ml of 5% $NaNO_2$ solution. The test tubes were kept at room temperature for 6 min. Then, 0.3 ml of a 10% $AlCl_3.6H_2O$ solution was added and allowed to stand for 5 min before the addition of 1.0 ml of 1 M NaOH. The mixture was then vortexed and absorbance was measured immediately at 510 nm. Catechin was used as standard and results were expressed as catechin equivalents per gram of dry extract (mg CAE/g DW).

2.6 Determination of total flavonols content

The content of total flavonols was estimated using the method previously described [19] where quercetin was used as standard. Briefly, 2.0 ml of extract/standard was mixed with 2.0 ml of 2% $AlCl_3$ ethanol and 3.0 ml (50 g/L) sodium acetate solutions and the mixture was allowed to stand for 2.5 hours at 20°C.The absorbance was taken at 440 nm. Extract/ standard were evaluated at a final concentration of 0.1 mg/mL. Total content of flavonols was expressed in terms of quercetin equivalent, mg of QUE/g of dry extract.

2.7 Determination of total proanthocyanidins

Total proanthocyanidins content was estimated based on the protocol reported by Sun *et al*. [20]. Shortly, 0.5 ml of extract/standard solution was mixed with 3 ml of 4% vanillin-methanol solution, followed by addition of 1.5 ml hydrochloric acid and the mixture was kept for 15 minutes at room temperature. Then the absorbance was recorded at 500 nm. Catechin was used as standard and content of total proanthocyanidins was expressed in terms of catechin equivalent, mg of CAE/g of dry extract.

2.8 Determination of antioxidant capacity

2.8.1 DPPH free radical scavenging assay

The DPPH free radical scavenging capacity was determined as previously described methods [21, 22] with a little modification. Briefly, 0.5 ml sample was mixed with 3.5 ml of 0.2 mM methanolic solution of DPPH free radical and the absorbance at 517 nm after incubation of 30 minutes at room temperature. Ascorbic acid and BHT was used as positive control. Radical scavenging activity was calculated by the following formula:

$$\% \text{ Scavenging Activity} = (A_{control} - A_{sample} / A_{control}) \times 100$$

Where, $A_{control}$ = Absorbance of control, A_{sample} = Absorbance of sample.

Then percentage DPPH radical scavenging activity was plotted against concentration, and from the graph IC_{50} was calculated.

2.8.2 ABTS radical scavenging activity

According to the method of Re *et al*. [23], the antioxidant capacity of different extracts of *C. viminalis* was determined in terms of the ABTS●+ radical scavenging assay.

ABTS●+ was generated by treating 7 mM ABTS stock solution with 2.45 mM potassium persulfate and the solution was kept in dark at room temperature for 12–16 hours before use, which is stable for 48 hours. The $ABTS^{\bullet+}$ solution was diluted with water to obtain an absorbance 0.70±0.02 at 734 nm. 3ml of ABTS●+ solution was added to 1 mL of test sample with various concentrations and mixed vigorously and the absorbance was taken at 734 nm after standing for 6 minute at room temperature. Ascorbic acid and BHT was used as standard compounds.

The ABTS●+ radical scavenging activity was expressed as

$$\% \text{ Scavenging Activity} = [(A_{control} - A_{sample}) / A_{control})] \times 100$$

Where, $A_{control}$ is the absorbance of the blank control (ABTS●+ solution without test sample) and A_{sample} is the absorbance of the test sample.

2.8.3 Scavenging activity of Superoxide

Superoxide scavenging activity of *C. viminalis* extracts was determined using the NBT (nitrobluetetrazolium reagent) method previously described by Sabu *et al*. [24] (with some modification) which is based on generation of superoxide radical by auto-oxidation of hydroxylamine hydrochloride in presence of NBT and gets reduced to nitrite. The color given by nitrite in presence of EDTA was measured at 560 nm.

Test solution of extracts (20–300µg/mL) was taken in a test tube, after that 1 mL of (5 mM) sodium carbonate, 0.4 mL of (0.24 mM) NBT and 0.2 mL of 0.1 mM EDTA solutions were added to the test tube and absorbance was taken at 560 nm immediately. About 0.4 mL (1 mM) of hydroxylamine hydrochloride was added to initiate the reaction and the reaction mixture was incubated at 25^0C for 15 min, and the reduction of NBT was estimated at 560 nm. Ascorbic acid and BHT were used as standards. The percentage of inhibition was calculated according to the following formula**:**

$$\% \text{ Scavenging Activity} = [(A_0 - A_1)/A_0] \times 100$$

Where, A_0 is the absorbance of the initial reading of sample/standard and A_1 is the absorbance of final reading.

2.8.4 Scavenging activity of nitric oxide

Nitric oxide scavenging activity of *C. viminalis* extracts was monitored by method previously described with some modification [25]. 2 mL of 10mM Sodium nitroprusside prepared by using 0.5mL phosphate buffer saline at pH 7.4, used for the spontaneous generation of nitric oxide, was mixed with 0.5 mL of extracts and incubated for two and half hours at room temperature. After incubation, 0.5 mL of Griess reagent [1.0 mL sulfanilic acid reagent (0.33% prepared in 20% glacial acetic acid)] was mixed and kept for 5 min at room temperature and 1 mL of naphthylethylenediamine dichloride (0.1% w/v)] was added to the mixture. Absorbance was measured at 546 nm after incubation of 30 min at room temperature. Ascorbic acid and BHT was used as positive control.

The NO radical-scavenging activity of the samples was expressed as

% Scavenging Activity = $[(A_o - A_1)/ A_o)] \times 100$

Where, A_o is the absorbance of the blank control (NO radical solution without test sample) and A_1 is the absorbance of the test sample.

2.9 Determination of antibacterial activity

Eight pathogenic bacterial strains were selected for the antibacterial activity test, four of which were gram positive i.e. *Staphylococcus aureus* (*S. aureus*), *Bacillus cereus* (*B. cereus*), *Bacillus subtilis* (*B. subtilis*) and *Streptococcus pyogenes* (*S. pyogenes*) and the remaining were gram negative i.e. *Escherichia coli* (*E. coli*), *Agrobacterium tumefaciens* (*A. tumefaciens*), *Shigella dysenteriae* (*S. dysenteriae*) and *Salmonella typhi* (*S. typhi*).

Antibacterial activity of *C. viminalis* leaf extracts was monitored by employing agar disc diffusion method [26] against these strains. In short, different concentrations of plant extracts were obtained by dissolving in DMSO (final conc. of DMSO in each medium was 1%, so that it did not affect the bacterial growth). The bacterial suspension containing app. 5×10^5 CFU/mL was sprayed on the solid agar media plates and filter paper discs of 5mm diameter loaded with test extracts of different concentration were placed on the inoculated plates. These plates were kept at 4 ^{0}C for 6 hours in order to provide sufficient time to diffuse the test sample into the culture medium. The antibacterial activity was monitored by measuring the diameter of inhibition zone (in mm) after incubation at 37 ^{0}C for 24 hours. Standard discs of Azithromycin (15 µg/disc) was used as a positive control and negative control was prepared using respective solvent.

3. Results

3.1 Phytochemical screening of *C. viminalis* leaf extracts

Qualitative phytochemical analysis has revealed the presence of different phytochemicals including alkaloids, carbohydrates, flavonoids, glycosides, triterpenoids, resins, saponins, steroids and tannins in different extracts which are enlisted in table 1.

Table 1: The qualitative phytochemical investigation of five different extracts of *C. viminalis* leaf

Phytochemicals	Name of Test	Test sample				
		EECV	MECV	CECV	NECV	PECV
Alkaloids	Dragendorff's test	+	+	+	-	-
	Hager's test	-	+	+	-	-
	Wagner's test	+	+	+	-	-
	Mayer's test	+	+	-	-	-
Carbohydrates	Molisch' s test	++	++	+	-	++
	Benedict's test	+	+	-	-	+
	Fehling's test	++	++	-	-	-
	Anthrone test	+	++	-	-	++
Flavonoids	Shinoda's test	++	++	-	-	-
Glycosides	Molisch's test	++	++	+	-	++
Triterpenoids	Liebermann-Burchard's test	-	-	+	+	++
Resins		-	-	-	-	-
Saponins		-	-	+	+	-
Steroids	Liebermann-Burchard's test	-	-	+	+	++
	Salkowski reaction	-	-	+	+	++
Tannins		-	-	-	-	+

"++" indicates the presence of the relevant phytoconstituent at higher amount and "+" indicates smaller amount, whereas "–" indicates the absence.

Methanol and ethanol extracts of *C. viminalis* demonstrated the presence of alkaloids, carbohydrates, flavonoids and glycosides. Petroleum ether extract also indicated the presence of carbohydrates, glycosides, triterpenoids, steroids and tannins.

3.2 Total phenolics, flavonoids, flavonols and proanthocyanidins contents

Total phenolics, flavonoids, flavonols and proanthocyanidins contents of *C. viminalis* leaf extracts are shown in table 2, where relatively higher amount of phenolics, flavonoids and proanthocyanidin were found in ethanol extract of *C. viminalis* (88.83±2.71 mg GAE/g DW, 65.48±2.74 mg CAE/g DW and 17.39±0.64 mg CAE/g DW, respectively), but in methanol extract highest amount of flavonols (18.83±1.23 mg QUE/g DW) was found.

Table 2: Total Phenolics, Flavonoids, Flavonols and Proanthocyanidins contents of *C. viminalis* leaf

Name of extract	Phenolics (mg GAE/g DW)	Flavonoids (mg CAE/g DW)	Flavonols (mg QUE/g DW)	Proanthocyanidines (mg CAE/g DW)
MECV	78.79±1.92	46.41±2.23	18.83±1.23	15.03±0.69
EECV	88.83±2.71	65.48±2.74	16.05±1.28	17.39±0.64
CECV	33.63±1.57	23.63±1.20	6.03±0.52	10.91±0.47
NECV	35.39±0.94	13.32±0.70	14.42±0.70	11.79±0.52
PECV	25.52±1.32	31.22±0.74	11.74±1.32	7.25±0.51

Results are expressed as mean ± standard deviation. DW: Dry weight of extract.

3.3 Antioxidant capacity of *C. viminalis* leaf extracts

The antioxidant activity of *C. viminalis* leaf extracts was evaluated by four widely used different assays: DPPH, ABTS, Superoxide and Nitric Oxide (NO) scavenging assay and the IC_{50} values (the 50% maximal inhibitory concentration) were calculated (shown in Table 3). Extracts obtained by using different solvent system have shown antioxidant capacity at different levels.

Extract obtained using methanol as a solvent (MECV) indicated higher DPPH and ABTS radical scavenging activity with IC_{50} values of 32.80±1.71 µg/mL and 46.20±1.33 µg/mL, respectively which were comparatively higher than the reference compounds ascorbic acid (41.56±1.17 µg/mL in DPPH assay and 54.42±1.08 µg/mL in ABTS assay) and BHT (65.15±1.46 µg/mL in DPPH assay and 78.23±1.39 µg/mL in ABTS assay). During both of these above assays, the radical scavenging capacity of the extracts has followed the order: MECV > EECV > PECV > CECV > NECV. In superoxide radical scavenging assay, ethanol extract (EECV) exhibited highest antioxidant capacity, whereas chloroform extract (CECV) showed greater scavenging activity in nitric oxide scavenging assay. The order of scavenging activity among different extracts of *C. viminalis* leaf in superoxide scavenging assay is EECV > MECV > CECV > PECV > NECV and in nitric oxide scavenging assay is CECV > MECV > EECV > PECV > NECV. In all of the four assays, n-hexane extract exhibited a maximal IC_{50} value which indicates the lower antioxidant capacity among these five tested extracts.

Table 3: IC_{50} values of *C. viminalis* leaf *extracts* of different assays.

Extract Name	IC_{50} (µg/mL)			
	DPPH	ABTS	Superoxide	Nitric Oxide
MECV	32.80±1.71	46.20±1.33	65.55±1.69	96.5±0.99
EECV	37.08±1.07	52.19±1.45	39.04±1.36	100.3±2.35
CECV	72.44±2.01	105.20±1.86	44.13±1.52	89.12±1.38
NECV	135.3±1.85	169.5±2.14	201.30±2.68	169.91±2.41
PECV	59.23±1.46	89.13±1.37	61.38±1.83	121.6±1.98
Standard				
Ascorbic Acid	41.56±1.17	54.42±1.08	67.91±1.26	87.52±1.56
BHT	65.15±1.46	78.23±1.39	90.65±1.57	102.33±2.06

Results were expressed as mean ± standard deviation (n=3). BHT: Butylhydroxytoluene.

3.4 Antibacterial activity

The zones of inhibition measured in mm of five different solvent extracts of *C. viminalis* leaf at various concentrations against eight tested microorganisms were compared with that of standard antibiotic azithromycin and the result was represented in table 4. The extracts showed broad spectrum antibacterial activity against both of tested gram positive and gram negative bacteria. Among the extracts ethanol and petroleum ether extract of *C. viminalis* have demonstrated larger inhibitory zone against *Staphylococcus aureus* at a concentration of 400 µg/ disc (22 mm diameter of inhibition zone), whereas ethanol extract revealed higher inhibitory zone (19 mm) against *Streptococcus pyogenes* at the same concentration.

Table 4: Antibacterial Activity of different extracts of *C. viminalis* leaf extracts

Name of the Bacteria	Zone of inhibition(mm)															
	MECV			EECV			CECV			NECV			PECV			Az
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	
S. aureus	19	16	13	22	18	14	15	12	10	15	12	9	22	17	13	29
S. pyogenes	18	15	13	19	16	11	13	11	8	12	9	7	17	13	10	28
B. ceresus	18	14	12	18	13	11	13	10	8	12	8	N	18	15	11	27
B. subtilis	19	15	12	20	15	12	14	9	8	13	8	7	17	13	9	29
E. coli	16	14	11	17	14	12	14	11	9	11	N	N	16	12	11	28
S. typhi	13	9	N	13	8	N	8	N	N	9	N	N	12	7	N	26
S. dysenteriae	14	10	8	15	12	8	10	7	N	9	N	N	15	13	8	29
A. tumefaciens	16	13	11	17	14	11	13	11	8	14	10	7	17	13	9	31

Negative control excluded and disc diameter included; N: No inhibitory zone detected; A: 400 µg/ disc; B: 200 µg/ disc; C: 100 µg/ disc; Az: Azithromycin (15 µg/ disc)

Same inhibitory zone (18 mm) was found for the methanol, ethanol and petroleum ether extracts of *C. viminalis* against *Bacillus ceresus*, whereas ethanol extract was more effective (inhibition zone: 20mm) against *Bacillus subtilis* at 400 µg/ disc concentration. Moreover, extracts of this plant was found to be less active against gram negative bacteria compared to gram positive strains. At a concentration of 400 µg/ disc, both ethanol and petroleum ether extract showed similar inhibitory zone (17 mm) against *E. coli* and *Agrobacterium tumefaciens* followed by the other three extracts. Petroleum ether extract also exhibited higher inhibitory zone (16 mm) against *Shigella dysenteriae* at the above concentration. Most of the extracts showed a relatively lower antibacterial activity against *Salmonella typhi.*

4. Discussion

In the present investigation we used five different solvent extracts of *C. viminalis* to detect the presence of alkaloids, flavonoids, carbohydrates, triterpenoids, glycosides, saponins, resins and tannins as well as to evaluate the total phenolic content, flavonoid content, flavonol content, proanthocyanidin content and antioxidant activity. We also assessed the antibacterial activity of the extracts against some gram positive and gram negative bacteria. The results of our current investigation varied from extract to extract. Previously it has been reported that the differences in polyphenol contents and biological activities of plant extract depend on the type of solvent used [27, 28].

Numerous biological activities such as antioxidant capacity, antimicrobial activity, anti-inflammatory activity etc. of phenolic compounds and flavonoids have been demonstrated by a large number of studies. There is ample evidence that diets rich in polyphenolic compounds has a major contribution in the prevention of many oxidative stress associated diseases because of their antioxidant properties [29-31]. The present study also identified the *C. viminalis* as a rich source of polyphenolic compounds.

The DPPH assay has been widely used for measuring the free-radical scavenging activity of plant extracts where stable DPPH free-radical is reduced by antioxidants leading to the development of a yellowish colored compound [32]. The ABTS assay measures the total antioxidant potential of plant extracts and has also been used widely [33]. The present investigation found that methanol extract can decrease the concentration of DPPH and ABTS free radicals significantly compared to that of standard reference compounds, ascorbic acid and butylhydroxytoluene (BHT), suggesting that the extract possesses phytochemicals with potent antioxidant properties. Ethanol extract also exhibited potent antioxidant activity which was almost similar to that of methanol extract, whereas remaining extracts showed moderate activity.

Superoxide anion can generate reactive oxygen species which induce oxidative damage in lipids, proteins and DNA, and thereby contribute to the development of various pathological conditions [34]. In our present study, superoxide produced from dissolved oxygen by PMS-NADH coupling reaction decreases the yellow dye (NBT^{2+}) to produce the blue formazan. The ethanol extract demonstrated the highest superoxide scavenging activity, whereas n-hexane extract showed the lowest activity compared to that of standards. Antioxidant compounds have been reported to have the ability to inhibit the generation of superoxide in the *in vitro* reaction mixture.

Although nitric oxide is involved in many physiological processes and regulation of cell mediated toxicity, excessive NO can react with oxygen resulting in the generation of free radicals such as nitrite and peroxynitrite anions which have injurious effect in the cells. Therefore excessive production of NO in our body should be neutralized [34]. In our investigation it was found that *C. viminalis* leaf extract compete with oxygen to react with NO, thus inhibiting the production of the anions. The chloroform extract has exhibited the highest NO scavenging activity among five extracts.

Various studies conducted previously have reported that antibacterial activity exhibited by different plant extracts is due to the presence of different phytochemicals [35, 36]. In the present study each extract of *C. viminalis* leaf showed considerable broad-spectrum antibacterial activity against some pathogenic bacteria which suggests that it may be a promising source for the treatment of various infectious diseases. Among the five extracts under this study, ethanol, methanol and petroleum ether extracts showed potent antibacterial activity which suggests that active compounds having antibacterial potency may be extracted with ethanol, methanol and petroleum ether in higher amounts.

5. Conclusion

The present study concluded that *C. viminalis* leaf possesses strong antioxidant and antibacterial activity, and can be a promising source to discover drug for the treatment of various human diseases. It can also be suggested that different type of solvents has a significant influence on antioxidant and antimicrobial properties of the extracts under this investigation. Potent antioxidant and antibacterial activities of *C. viminalis* leaf, revealed in the present investigation may be important in the future research works to explore the underlying mechanism of its numerous pharmacological properties. Moreover, further investigation is required to identify and characterize the active principles present in the extract.

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