Brief communication

Lactam nonanic acid, a new substance from *Cleome viscosa* with allelopathic and antimicrobial properties

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Cleome viscosa L. (Capparidaceae) is well known for its medicinal properties. Lactam nonanoic acid (LNA) [2-amino-9-(4-oxoazetidin-2-yl)-nonanoic acid; $C_{12}H_{22}N_2O_3$, mol. wt. 242] has been isolated and purified from the root exudates of *Cleome viscosa*. The aqueous solution of this pure compound has been tested on bacteria (*Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus)* and fungi (*Aspergillus fumigatus, A. niger* and *A. tamarii*). At a dosage of 500 ppm and above, *P. aeruginosa* and *S. aureus* were totally inhibited while *E. coli* remained unaffected. On the other hand, growth of *A. niger* and *A. tamarii* was stimulated while there was no effect on *A. fumigatus.* This pure compound showed concentration-dependent inhibitory activity on rice, gram and mustard seeds.

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1. Introduction

Allelochemicals generally refer to the secondary metabolites released by intact living plants into their surrounding (Rice 1984; Dayan *et al.* 2000; Einhellig 2004). These metabolites exert inhibitory or stimulatory effects on other plants, fungi, bacteria, etc., in the surrounding environment, including the rhizosphere. Hence, they play a major role in agriculture and in the ecological network. These chemicals include flavonoids, tannins, alkaloids and aromatic acids, and have been observed to be active against weeds, pathogens and insects (Inderjit 1996; Duke *et al.* 2000). They also play an important role in protecting plants from certain pathogens.

The current trend demands that agricultural practices be bio-intensive. Safeguarding the environment is a major concern because continuous and injudicious use of synthetic products has led to problems such as diminishing resistance to pathogens, accumulation of residual toxic chemicals leading to contamination of food and environment, and undesirable effects on non-target biota. In this context the use of natural agrochemicals in order to develop environment-friendly, safe and compatible approaches has gained increasing attention. Natural agrochemicals reduce the input of synthetic chemicals and help to conserve natural fauna. These are effective and often quickly biodegradable, and hence they present no problems of toxic residue.

Identified strong bioactive allelochemicals are a source of biological herbicides and fungicides (Inderjit and Mukerji 2006). Such biopesticides can replace commercial pesticides that spell hazards (Khanh *et al.* 2005). We now report our findings on a common weed that is a potential source of such allelochemicals. *Cleome viscosa* L. (Capparidaceae) is a weed of woodland, grassland, fallow land, fields, roadsides and wasteland, often found growing on sandy soils but sometimes on calcareous and rocky soils. It is found in both seasonal dry and humid conditions, from mean sea level up to an altitude of

Keywords. Allelochemical; antimicrobial activity; *Cleome viscosa* L.; lactam nonanoic acid (LNA); root exudates

Abbreviations used: LNA, lactam nonanoic acid; RE, root exudates; MFCV, methanol fraction of *Cleome viscosa*; NA, nutrient agar; PDA, potato dextrose agar media; TLC, thin-layer chromatography

1000 m. The common names are tickweed, wild mustard and spider plant (English), and *hurhuria* (Bengali). The seeds have no dormancy and germinate readily after shedding. Plants start flowering 3–4 weeks after germination and the lifecycle is about 3 months. A number of pharmacological properties of this plant have been reported (Kirtikar and Basu 1935; Chatterjee and Pakrashi 1991; Singh and West 1991; Saxena *et al.* 2000; Devi *et al.* 2002, 2003a, b; Rastogi *et al.* 2003; Tiwari *et al.* 2004; Clementine *et al.* 2008; Sirrcharungroj *et al.* 2008; Gupta and Dixit 2009). These findings were focused on the medicinal properties of crude extracts of *C. viscosa* and the chemical aspects were not studied. We have, therefore, been interested in studying allelochemicals in root exudates (RE) of *C. viscosa,* which, according to our field studies, is the first invader in wastelands.

In this article, we report isolation and identification of lactam nonanoic acid from RE of *C. viscosa* and show that it exerts allelopathic effects on rice, mustard and gram and antimicrobial activity against the fungal species *Aspergilus niger* and *A. tamarii* and the bacterial species *Pseudomonas* sp. and *Staphylococcus aureus.*

2. Materials and methods

2.1 *Collection of root exudates*

RE were collected after the plants of *C. viscosa* were grown in RE-trapping systems made with a 110-mm-diameter Buchner funnel and conical flasks of 500 ml capacity. The central part (sieve portion) of the funnels was removed and the funnels were filled with soil collected from the field. Initially a small piece of muslin cloth was placed at the lower portion of the funnel to hold the soil in. The funnels were kept on conical flasks that were painted black and contained distilled water. Five to six germinated seeds of *C. viscosa* from which RE were to be obtained were sown in each funnel. After thinning, one to four plants, depending upon the growth or size of the plants, were allowed to grow. After the plants attained the age of 20–25 days, plant roots penetrated the soil of the Buchner funnels and emerged into the conical flasks containing distilled water. RE were collected from the conical flasks regularly at an interval of 5–7 days and the conical flasks were filled immediately with fresh distilled water. This RE were collection procedure continued for 3–4 months.

2.2 *Isolation and characterization of active compounds from root exudates*

The collected RE of *C. viscosa* were slowly evaporated under hot air and then extracted with different solvents (figure 1). Finally, the compounds were purified by column chromatography and thin-layer chromatography (TLC). The maximum amount of allelochemicals was recovered from the methanol fraction; so, we placed emphasis on the methanol fraction of *C. viscosa* (henceforth referred to as MFCV).

2.2.1 *Thin-layer chromatography:* TLC plates (20× 20 cm) were used for this study. Cellulose powder of TLC grade was used as a coating material and the plates were coated uniformly with 0.5-mm-thick layer of cellulose powder. A solvent mixture in the ratio of 95:5 of acetone:methanol was taken as the mobile phase (Stahl 1969). The plates were loaded with 20 µl solution (500 ppm of the MFCV compound) and developed up to a height of 18 cm in a glass chamber pre-saturated with the solvent system. The TLC plates were then taken out and dried under a stream of hot air. Finally, the compounds were detected by staining with methyl red reagent (Lederer and Lederer 1957) or under UV light (365 nm).

2.2.2 *Spectral analysis:* A Helios Gamma UV Spectrophotometer (Model No. NC: 9423 UVG 1002E) was used for recording the λ_{max} of the extracted and purified MFCV compound of *C. viscosa*. Mass spectrometry analysis for determination of the molecular weight of the compounds was performed with Mass Spectrometer (Micromass Q-TOF Micro™) in its positive ion mode.

^{1}HNMR analysis of purified MFCV was performed with the help of 600 MHz NMR Spectrometer (PROBHD 5 mm

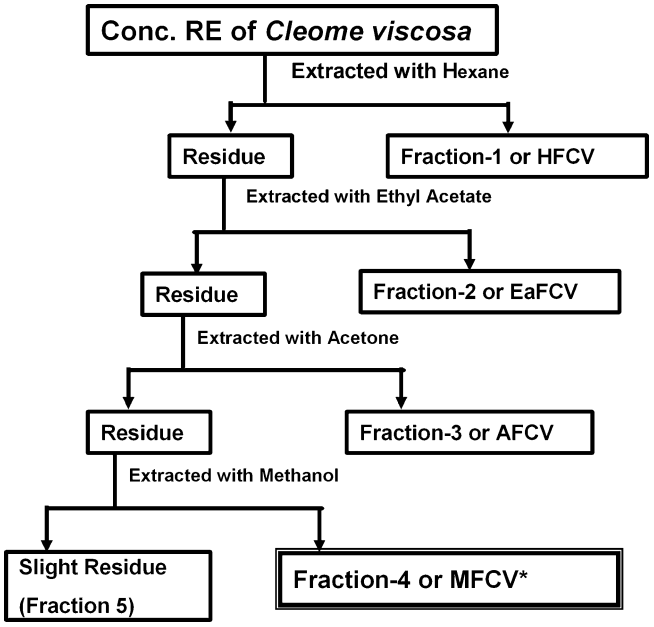

Figure 1. Flow diagram of extraction of compounds from root extracts of *Cleome viscosa* L.

DUEL 13C1, PULPROG Zg3D, TD32768, SOLVENT $CDCl_3$) available at Chembiotek, Kolkata. ^{1}HNMR spectra were detected on δ ppm (0–10) scale with end sweep at 0 ppm. ^{13}CNMR analysis was also performed with the help of 600 MHz NMR Spectrometer (PROBHD 5 mm DUEL 13C1, PULPROG zgpg, TD65536, SOLVENT $CDCl_3$) available at Chembiotek, Kolkata. ^{13}CNMR spectra of purified MFCV were detected on δ ppm (0–200) scale with end sweep at 0 ppm. In both the cases, the samples were analysed at the ambient temperature and $CDCl_3$ was used as the solvent for dissolving the compound.

FTIR analysis was conducted to confirm the important functional group in the extracted and purified MFCV samples of *C. viscosa* with the help of FTIR Spectrometer (Model No. QC/FTIR/006 available at Chembiotek, Kolkata).

2.3 *Effects of MFCV at different concentrations on fungi*

The inhibition zone test technique was adopted for testing the impact of extracted MFCV on three different fungal species viz. *A. tamarii, A. fumigatus* and *A. niger.* A few fungal spores of test fungi were transferred to potato dextrose agar media (PDA) slants and incubated for 1 week for colony growth to take place. After 1 week, one loop full of fungal spores of each species was added separately to the sterile saline water and mixed well. About 1 ml of fungal spore suspension in water was then poured into a sterile Petri dish containing molten PDA and PDA within the Petri plates were allowed to solidify. Four cups were cut at equidistant positions on four sides of the Petri dish and in these cups 0.5 ml solution of different concentrations viz. 500, 1000, 1500 and 2000 ppm of MFCV were added. The treated plates were incubated at 28±1°C for 24–48 h. After 48 h, the plates were taken out and observations were recorded for colony growth inhibition.

2.4 *Effects of MFCV at different concentrations on bacteria*

The inhibition zone test technique was employed for testing the effect of purified MFCV compound on three different bacterial strains i.e. *Pseudomonas* sp., *Staphylococcus aureus* and *Escherichia coli*. Initially the strains of test bacteria were transferred to nutrient agar (NA) media slants

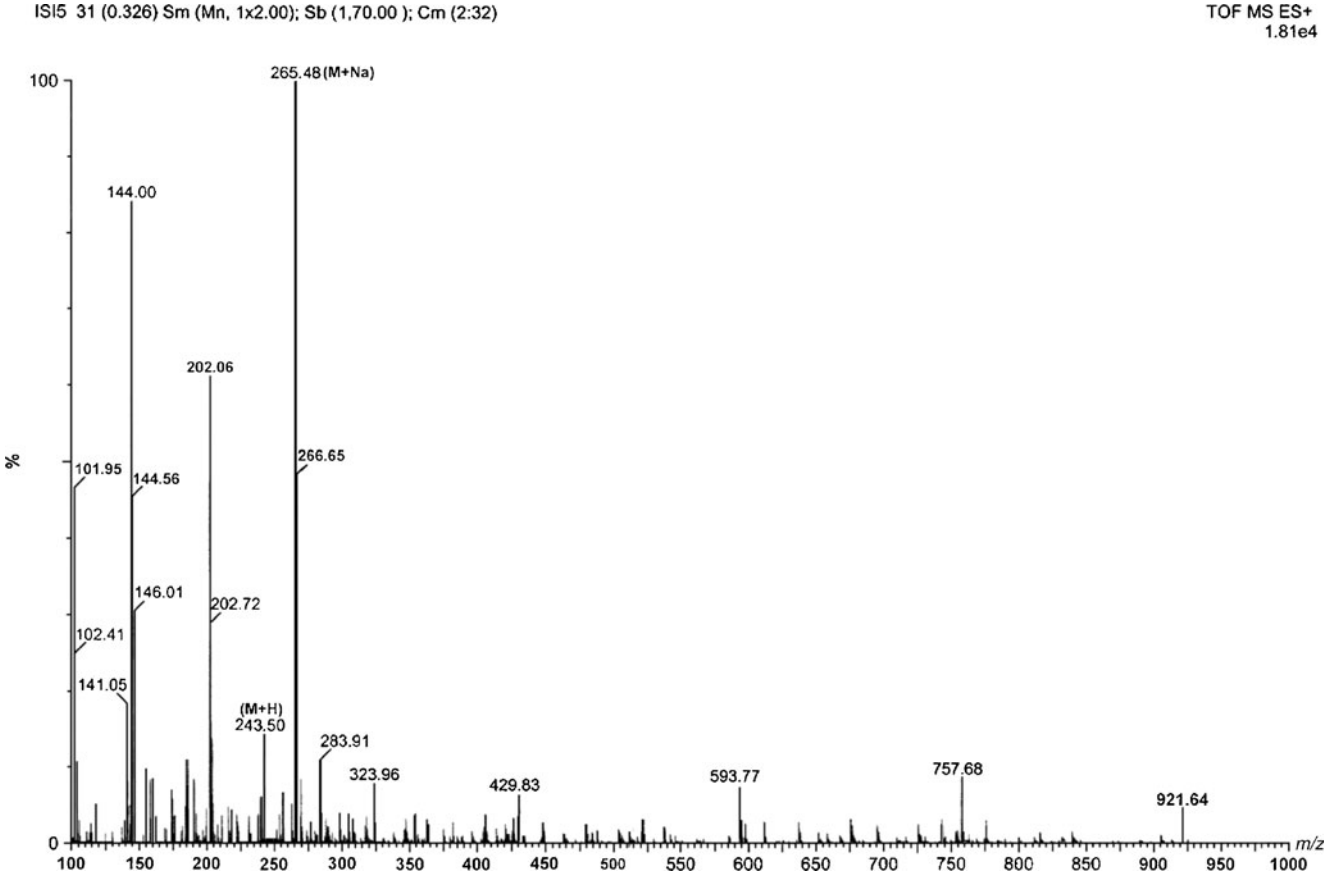

Figure 2. Mass spectra of purified compound of *Cleome viscosa* L.

and incubated for 24 h. The next day, one loop full solution from the slant was added to the nutrient broth and mixed well. The prepared nutrient broth was incubated at 37°C for 2.5 h. About 1 ml of the broth was then added to sterile Petri dish containing molten NA medium and the plates were allowed to solidify. After complete solidification, four cups were cut at equidistant positions and in these cups 0.5 ml solution of different concentrations viz. 500, 1000, 1500 and 2000 ppm MFCV were added. The treated plates were incubated at 37±1°C for 24 h. After this period, observations on the inhibition zone were recorded.

2.5 *Effects of MFCV on germination and subsequent growth of rice, mustard and gram*

The allelopathic potentials of the purified MFCV compound on the germination and seedling growth of rice, mustard and gram were determined by laboratory bioassay experiments. The experiments were laid out in replicated Petri plates (90 mm diameter) containing a layer of filter papers. About 30 mg of MFCV was dissolved in 30 ml of distilled water. This constituted the stock solution of 1000 ppm, from which further dilutions of 500, 250, 125, 62.5, 31.25, 15.62 to 7.81 ppm were made. Nine sets of experiments were performed, including the control. In the control set, 15 ml of distilled water was added instead of the treated solution. The seeds were surface-sterilized with 0.1% mercuric chloride solution, washed with distilled water and placed on filter papers in a Petri dish. After 4 days, shoot lengths and root lengths in the control and treated sets were measured (Mandal 2001).

3. Results

3.1 *Collection of root exudates*

Nearly 35 l of RE was collected through the season from 16 sets of RE-trapping systems. The entire collected RE were slowly evaporated to dryness under hot air.

3.2 *Isolation and characterization of active allelochemical*

The concentrated RE were transferred to a 1000 ml rotary flask and then extracted with hexane, ethyl acetate, acetone and methanol separately. Finally, the compounds were purified by

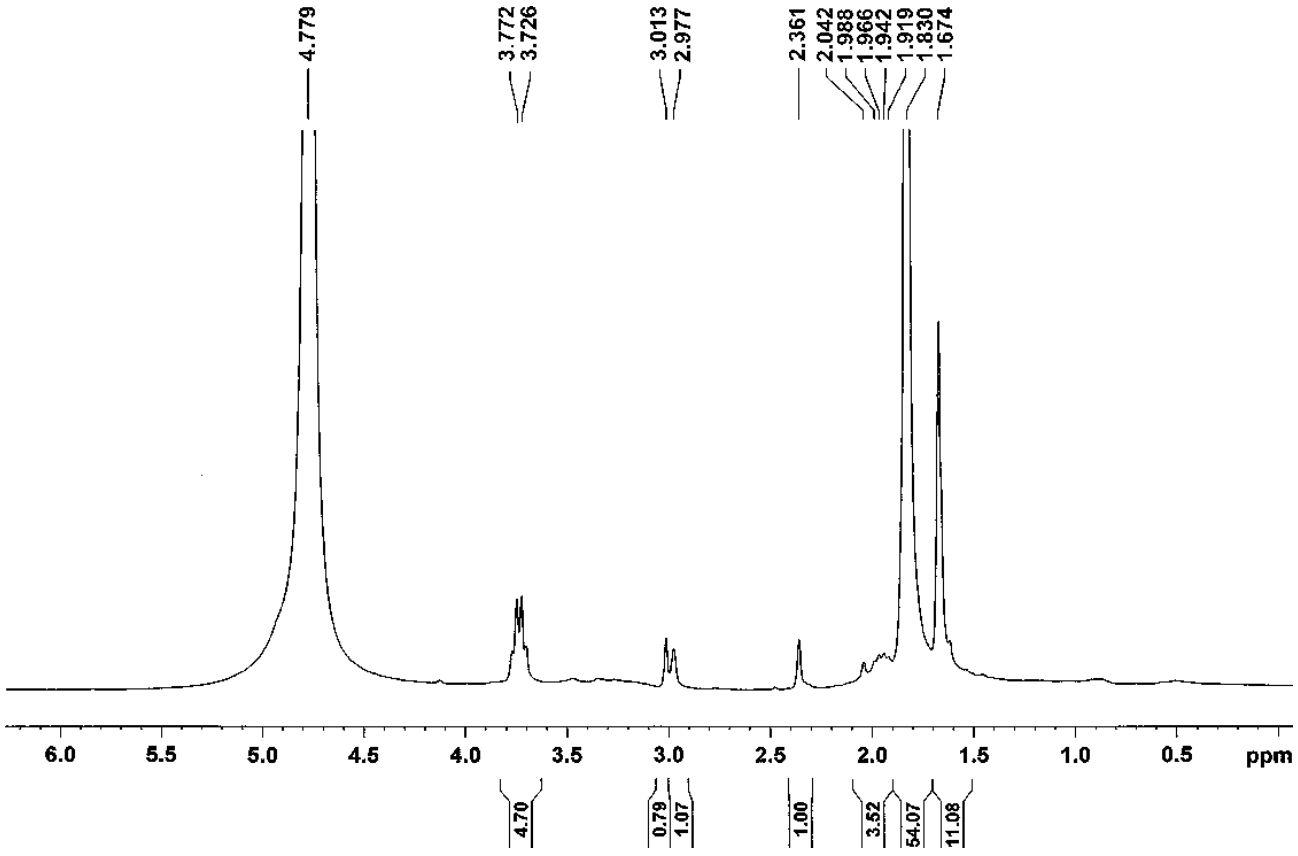

Figure 3. [1]HNMR spectra of the purified compound of MFCV of *Cleome viscosa* L.

column chromatography and TLC. In the methanol fraction, the compound gathered in maximum amount. By repeatedly following the proposed scheme of fractionation, we could extract the compound of interest, MFCV, as a single pure compound (purity >95%). At least eight repetitions of the procedure were necessary to achieve this.

3.2.1 *TLC analysis:* After purification, MFCV was run on TLC in the solvent system – methanol:acetone::5:95. A single deep red spot with R_f value 0.63 was revealed after staining with methyl red. Purified MFCV is faint yellowish in colour and oily at room temperature.

3.2.2 *Spectral analysis:* UV spectrophotometer analysis showed a peak at 301 nm with absorption of 2.202 at 500 ppm. ESI-MS of purified faint yellowish MFCV revealed positive-mode molecular ion peak $[M + H]^+$ and $[M + Na]^+$ at *m/z* 243 and 265, respectively, suggesting a molecular weight of 242 (figure 2).

^{1}HNMR spectra of the purified compound of MFCV (figure 3) consisted of 12 peaks. The peak at δ 3.77 ppm indicates the presence of terminal CH, whereas the peak at δ 3.72 ppm indicates internal CH. Peaks at δ 3.01 ppm and δ 2.97 ppm support the presence of terminal and internal CH_2, respectively. The peak at δ 2.36 ppm suggests the presence of terminal NH_2. Peaks from δ 2.04 to 1.67 ppm indicate the presence of CH_2 of substituted aliphatic side chains.

^{13}CNMR spectra (figure 4) consisted of 12 peaks. Peaks at δ 177.08 ppm and δ 173.87 ppm indicate the presence of terminal CO of the carboxyl group and internal CO group, respectively. Peaks at δ 55.04 ppm and δ 49.86 ppm support the presence of terminal and internal CH, respectively. The peaks from δ 49.57 to δ 31.92 ppm are due to methylene (CH_2) carbons of substituted side chains. Hence, the presence of alkane hydrocarbons is fully supported. The peak at δ 28.99 ppm indicates a terminal NH_2 group attached to an alkyl chain.

In IR spectra (figure 5), the peak at 3401 cm^{-1} suggested the presence of aliphatic primary amines (Silverstein and Webster 1997). The strong absorption peak at 2065 cm^{-1} indicated N–H stretching vibration of amine. A broad and strong absorption peak at 605 cm^{-1} indicated the N–H wagging of primary amines. The peak at 1049 cm^{-1} indicated the C–N stretching vitrations of primary aliphatic amines. A strong absorption peak at 1632 cm^{-1} and a weaker absorption peak at 1384 cm^{-1} confirmed, respectively, asymmetrical and symmetrical stretching of the carboxylate ion group. The broad strong absorption peak at 2395 cm^{-1} indicated the superimposed O–H stretching

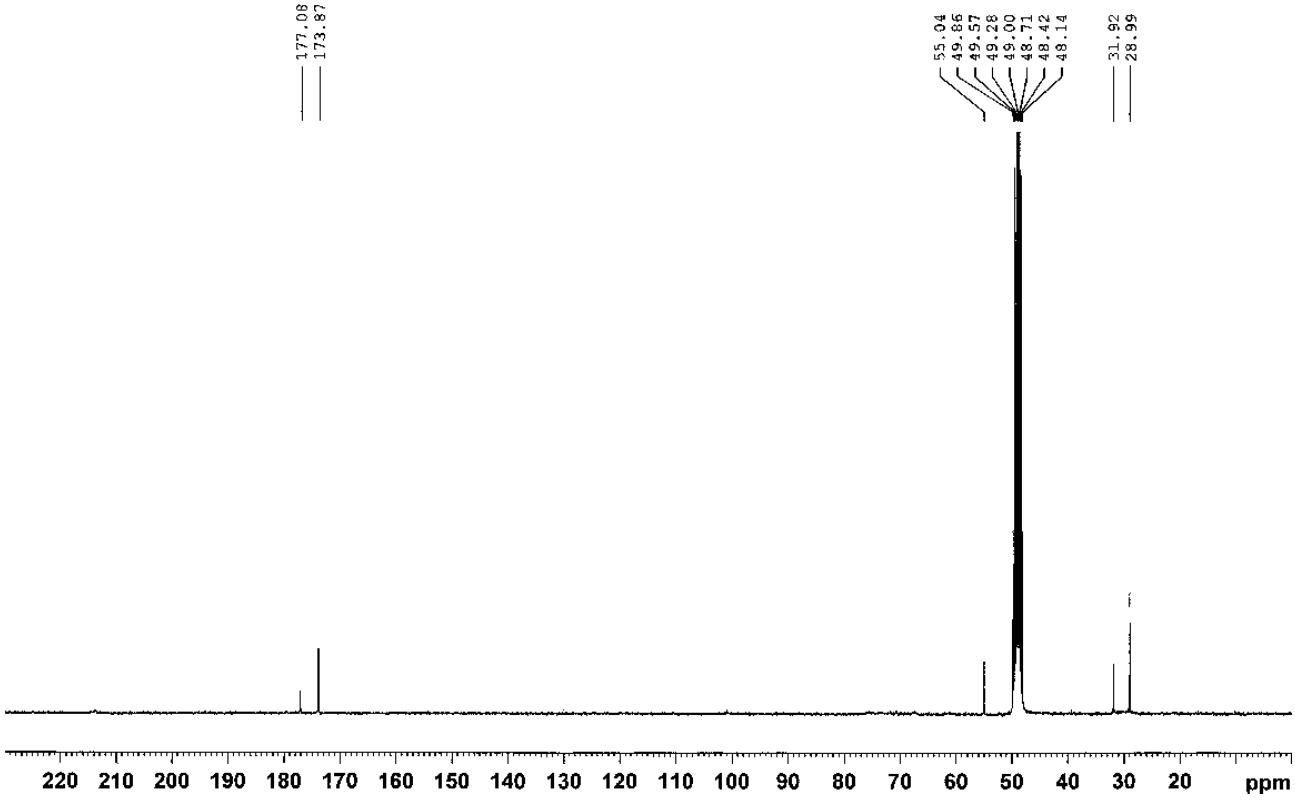

Figure 4. ^{13}CNMR spectra of the purified compound of MFCV of *Cleome viscosa* L.

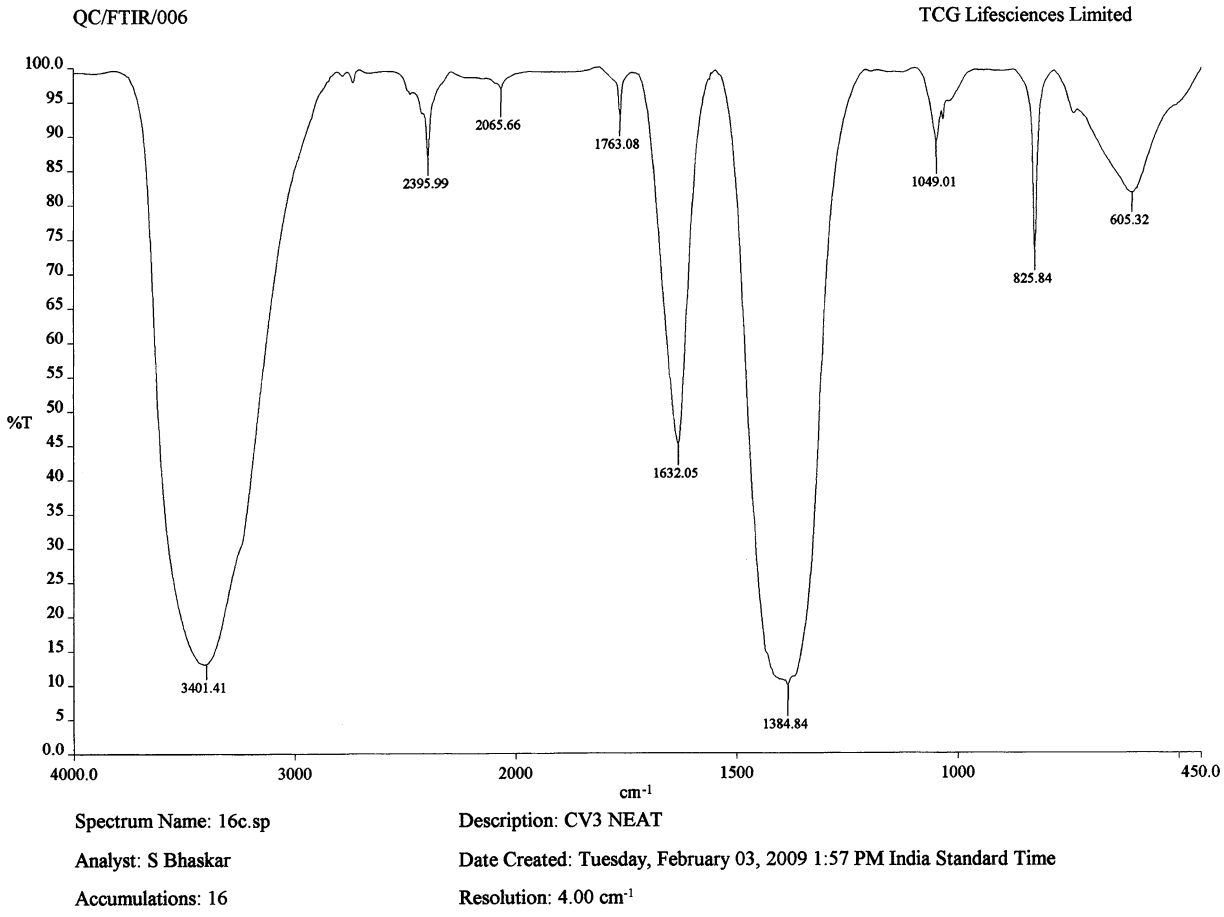

Figure 5. IR spectra of the purified compound of MFCV of *Cleome viscosa* L.

band of carboxylate ion. The peak at 1763 cm^{-1} indicated the C=O absorption of four-membered ring (β) lactam. The broad absorption band at 825 cm^{-1} indicated the N–H out-of-plane wagging in lactams.

Chromatographic analyses of MFCV revealed that the compound to be 2-amino-9-(4-oxoazetidin-2-yl)-nonanoic acid or LNA (figure 6). In fact, there are no reports on the presence of LNA in *C. viscosa.*

3.3 *Effect of MFCV at different concentrations on the fungi*

In the inhibition zone test, three fungal species viz. *A. niger, A. fumigatus* and *A. tamarii* showed differential effects at different concentrations of LNA. *A. niger* showed maximum stimulation at 500 ppm. Stimulation decreased with increasing concentration and ceased at 2000 ppm. In the case of *A. tamarii,* the trend was reversed – the maximum effect was detected at 1000, 1500 and 2000 ppm while at 500 ppm the effect was minimal. In both *A. niger* and *A. tamarii,* the colour of the colony has changed because of the LNA of *C. viscosa. Aspergillus fumigatus* did not reveal any influence of LNA (figure 7).

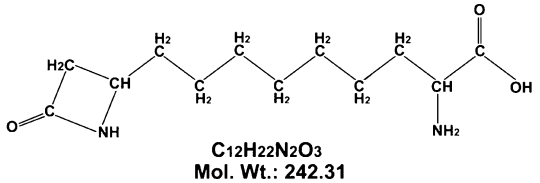

Figure 6. Molecular structure of 2-amino-9-(4-oxoazetidin-2-yl)-nonanoic acid of *Cleome viscosa* L.

3.4 *Effects of MFCV at different concentrations on bacteria*

In the inhibition zone test, *E. coli*, *Pseudomonas* sp. and *S. aureus* revealed differential effects at different concentrations of purified LNA. *Pseudomonas* sp. and *S. aureus* were much more inhibitory to LNA at all concentrations. The colony colour of *Pseudomonas* sp. and *S. aureus* had also changed because of this compound. In *E. coli*, no effect of LNA detected.

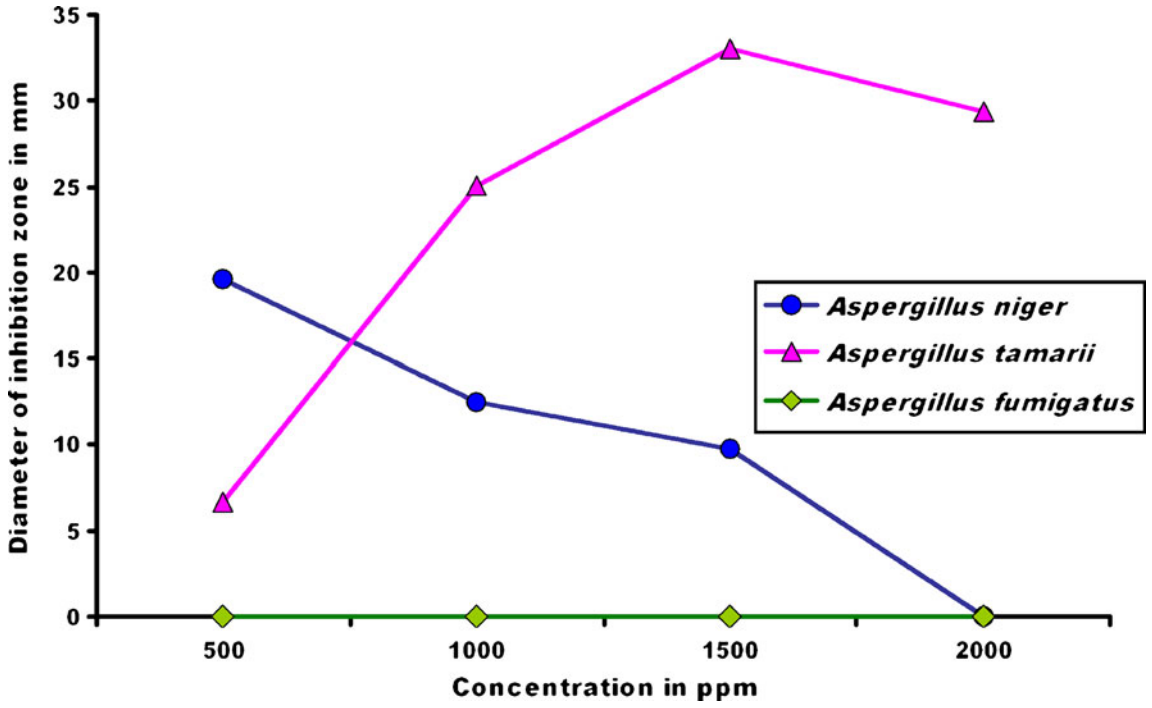

Figure 7. Effects of MFCV of *Cleome viscosa* L. at various concentrations on *Aspergillus niger, A. fumigatus* and *A. tamari*. Correlation is significant at the 0.05 level (two-tailed).

3.5 *Bioassay with MFCV compound of Cleome viscosa*

MFCV showed concentration-dependent inhibitory activity on rice (var. Shamali), mustard and gram seeds (figure 8). In rice, it caused complete inhibition of germination from 1000 to 125 ppm concentration, while in the case of gram seeds, MFCV exhibited complete inhibition on germination up to 500 ppm concentration. At 250 ppm, it showed 100% inhibition in shoot length and 50.41% inhibition in root length. In the case of mustard seeds, MFCV exhibited complete inhibition only at 1000 ppm. At 500 ppm, it revealed 17.22% inhibition in shoot length and 66.01% inhibition in root length. The MFCV of *C. viscosa* showed maximum inhibitory activity on rice when compared with gram and mustard.

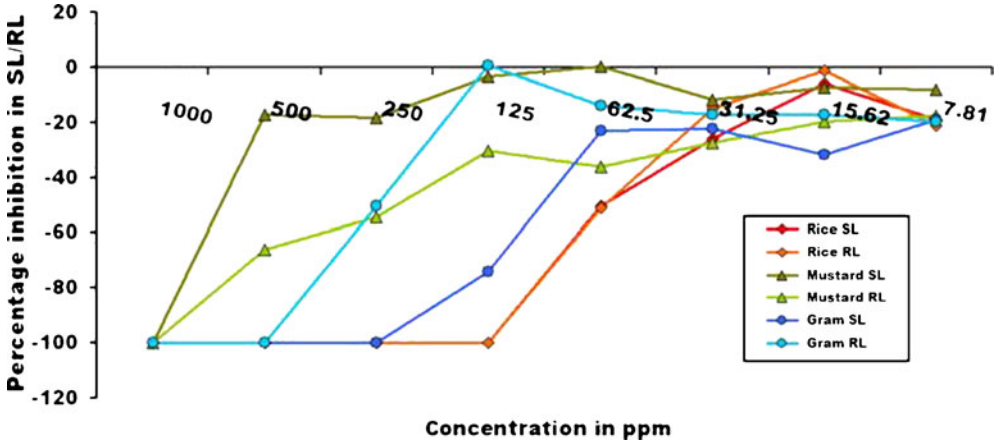

Figure 8. Effects of MFCV of *Cleome viscosa* L. at various concentrations on the shoot and root length growth of rice, mustard and gram. Correlation is significant at the 0.05 level (Pearson two-tailed).

4. Discussion

LNA, or 2-amino-9-(4-oxoazetidin-2-yl)-nonanoic acid, has been isolated and identified from the RE of *C. viscosa*.

β-lactam antibiotics are a broad class of antibiotics and are known to be mainly active only against Gram-positive bacteria, but recently broad-spectrum β-lactam antibiotics active against various Gram-negative organisms have also been developed. However, our experimental LNA of *C. viscosa* was highly active on *Pseudomonas* sp. (Gram-negative) and *S. aureus* (Gram-positive) but had no effect on *E. coli.* Both *S. aureus* and *P. aeruginosa* caused several infectious diseases in humans. This weed, which easily grows in wastelands and other places, might in future be exploited as a source of bacteriostatic agents.

On the other hand, β-LNA stimulates the growth of common soil-borne fungi. *A. niger* showed maximum stimulation at 500 ppm while no effect was observed at 2000 ppm. In contrast, *A. tamarii* was maximally stimulated at 1000, 1500 and 2000 ppm but at 500 ppm the effect was minimal. This fact is of ecological interest. In nature, this weed may play an important role in the ecological network.

Kasten and Ralf (1997) showed that β-lactam antibiotics inhibit chloroplast division in a moss (*Physcomitrella patens*) but not in tomato (*Lycopersicon esculentum*). We noted that in both *A. niger* and *A. tamarii,* the colour of the colony changed probably due to LNA of *C. viscosa. Aspergillus fumigatus* did not exhibit any effect due to LNA. This compound also showed concentration-dependent inhibitory activity on rice, wheat and gram seeds. This inhibitory activity was higher in rice than in gram or mustard. Therefore, LNA probably acts as allelopathic agents in *C. viscosa.* The secretion of LNA may provide a competitive advantage for *C. viscosa*, and its antibacterial activity on *S. aureus* and *P. aeruginosa* are responsible for its medicinal property. In the future, biologically active plant-derived chemicals can be expected to play an increasingly significant role in the commercial development of new products for regulating plant growth and for insect and weed control.

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