Bioactivity of 2-Amino-9- (4-Oxoazetidin-2-yl) Nonanoic Acid from the Root Exudates of *Cleome viscosa* L

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Abstract

$C_{12}H_{22}N_2O_3$ [2-amino-9-(4-oxoazetidin-2-yl) nonanoic acid or 2-amino-9-β-lactum nonanoic acid(LNA), mol.wt. 242] has been isolated and purified from the root exudates of Cleome viscosa. Aqueous solution of this pure compound has been tested on bacteria (Escherichia coli, Pseudomonas aeruginosa and Staphylococcus aureus) and fungi (Aspergillus fumigatus, Aspergillus niger and Aspergillus tamarii). At a dosage of 500ppm 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. In A. tamarii, however, a dosage of 500ppm exerted the minimal stimulatory effect while maximal stimulation occurred at 1000ppm and above whereas in A. niger maximal stimulation was observed at 500ppm but at 2000ppm there was no effect. In rice, it caused complete inhibition of germination from 1000ppm to 125ppm concentration while in the case of mustard seeds, complete inhibition was noticed only at 1000ppm concentration. At 500ppm, 17.22% and 66.01% inhibition on shoot and root length respectively was detected. In case of gram seeds, MFCV exerted complete inhibition of germination up to 500ppm concentration. At 250ppm, it showed 100% inhibition in shoot length and 50.41% inhibition in root length.

Keywords: *Cleome viscosa,* Allelochemical, Antimicrobial activity, Lactam nonanoic acid (LNA)

Introduction

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

The current millennium demands that agricultural practices should be bio-intensive. To safeguard the environment is a major concern at present 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, undesirable effects on non-target biota etc. 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 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 which spell hazard (Khanh *et al.,* 2005). We now report our findings on a common weed, which can be a good source of such allelochemicals. *Cleome viscosa* L. (Capparidaceae) is a weed of woodland, grassland, fallow land, fields, roadsides and wasteland, often occurring on sandy soils, but sometimes on calcareous and rocky soils. It is found both under seasonal dry and humid conditions, from sea level up to 1000 m altitude. Common names are tickweed, wild mustard, spiderplant (En). In Bengali it is called Hurhuria (Bengali), The seeds have no dormancy and germinate readily after shedding. Plants start flowering 3–4 weeks after germination and the life cycle 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 and 2003; Rastogi *et al.,* 2003; Gupta and Dixit, 2009; Tiwari *et al.,* 2004; Clementine et al, 2008; Siriarcharungroj *et al.,* 2008). These findings were focused on the medicinal properties of crude extracts of *Cleome viscosa,* but chemical aspects have not been studied. We have therefore been interested in studying allelochemicals in root exudates of *Cleome viscosa,* which according to our field studies, is the first invader in wastelands.

Material and Methods

Collection of root exudates: Root exudates (RE) were collected after growing the plants of *Cleome viscosa* in root exudates trapping systems made with 110 mm diameter Buchner funnel and conical flasks of 500ml capacity. 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 put at the lower portion of the funnel for holding the soil in funnels. Funnels were kept on conical flasks painted black

containing distilled water. Five to six germinated seeds of *Cleome viscosa* from which root exudates has 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 attaining the age of 20-25 days, plant roots penetrated the soil of the Buchner funnels and emerged into the conical flasks containing distilled water. Root exudates 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. RE collection procedure were continued for 3-4 months.

Isolation and characterization of active compounds from root exudates: The entire collected RE of *Cleome viscosa was* slowly evaporated under hot air and then extracted with different solvents (Fig.1). Finally the compounds were purified by column chromatography and thin layer chromatography. Maximum amount of allelochemicals was recovered from the methanol fraction so we put emphasis on Methanol Fraction of *Cleome viscosa* (henceforth referred to as MFCV).

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

Spectral analysis: 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 *Cleome viscosa*. MS Analysis for determination of molecular weight of the compound was done with Mass Spectrometer (Micromass Q-TOF Micro^{TM}) in its positive ion mode.

^{1}H-NMR and *^{13}CNMR* Analysis of purified MFCV compound were performed with the help of 600 MHz NMR Spectrometer (PROBHD 5mm DUEL 13C1, PULPROG Zg30, TD32768, SOLVENT MeOD) available at Chembiotek, Kolkata 700054. ^{1}HNMR spectra were detected on δ ppm (0-10) scale with end sweep at 0 ppm. ^{13}C -NMR spectra were also recorded on δ ppm (0-200) scale with end sweep at 0ppm. In both the cases samples were analyzed at ambient temperature, TMS as internal standard and $CDCl_3$ were used for dissolving the compound.

IR Analysis was undertaken to confirm the important functional group in the compound with the help of IR Spectrometer (JASCO-SP-Model No. 410 spectrophotometer). Solid-state spectrum was obtained by mixing the required quantity of sample in KBr plate.

Effects of MFCV at different concentrations on fungi: Inhibition zone test technique was adopted for testing the impact of extracted MFCV against three different fungal species viz., *Aspergillus tamarii, Aspergillus fumigatus, Aspergillus niger.* Few fungal spores of test fungi were transferred to PDA (Potato Dextrose Agar Media) slants and incubated for one week for colony growth. After one week, one loop full of fungal spore of each species was added separately to the sterile saline water and mixed well. 1ml of fungal spore suspension in water was then poured to sterile Petri dish containing molten PDA and allowed to solidify the plates. Four cups were cut at equidistant position and in these cups 0.5 ml solution of different concentration viz., 500ppm, 1000ppm, 1500ppm and 2000ppm of MFCV was added. Treated plates were incubated at 28±1°C for 24-48 hrs. After 48hr plates were taken out and observations were recorded for colony growth inhibition.

Effects of MFCV at different concentrations on bacteria: Inhibition zone test technique was employed for testing the effect of purified MFCV compound against three different bacterial strains i.e. *Pseudomonas sp*, *Staphylococcus aureus* and *Escherichia coli*. Initially the strains of test bacteria were transferred to NA (Nutrient Agar Media) slants and incubated for 24 hrs. 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 370C for two and half hr. 1ml broth was then added to sterile Petri dish containing molten NA medium and allowed the plates to solidify. After complete solidification four cups were cut at equidistant positions and in these cups 0.5 ml solution of different concentration viz., 500ppm, 1000ppm, 1500ppm and 2000ppm MFCV was added. Treated plates were incubated at 37±1°C for 24 hr. After this period observations on inhibition zone were recorded.

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

Results

Collection of root exudates: Nearly 35 liter of root exudates was collected through out the season

from 16 sets of root exudates trapping system. The entire collected root exudates were slowly evaporated to dryness under hot air.

Isolation and characterization of active allelochemical: The concentrated root exudates transferred to a 1000ml rotary flask and then extracted with hexane, ethyl acetate, acetone and methanol respectively (Fig. 1). Finally the compounds were purified by column chromatography and thin layer chromatography. In methanol fraction compound gathered in maximum amount. By repeatedly following the proposed scheme of fractionation we could extract the compound of interest, (methanol fraction of *Cleome viscosa,* henceforth referred to as MFCV) as a single pure compound (purity of >95%). At least eight repetitions of the procedure were necessary to achieve this goal.

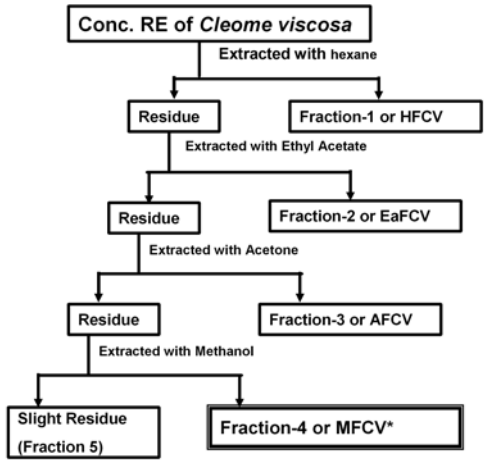

Fig. 1: Flow diagram of extraction of compounds from root exudates of *Cleome viscosa*

TLC analysis: After purification MFCV was run on TLC in the solvent system – Methanol : Acetone, 5 : 95. A single deep red spot with Rf value 0.63 was revealed after staining with methyl red. Purified MFCV is faint yellowish in color and oily at room temperature.

Spectral analysis: UV Spectrophotometer Analysis showed a peak at 301 nm with absorption 2.202 at 500ppm. ESI-MS (Fig. 2) 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.

^{1}HNMR spectrum of purified compound of MFCV (Fig. 3) is constituted of fourteen peaks. Peak at δ 3.74 indicates the presence of terminal CH whereas the peak at δ 3.72 indicates internal CH. Peaks at δ 3.01 and δ 2.97 supports the presence of terminal and internal CH_2, respectively. Peak at δ 2.36 suggests the presence of terminal NH_2. Peaks from δ 2.04 - 1.53ppm indicate the presence of H.

^{13}CNMR spectra as shown in Fig. 4 are constituted of 12 peaks. Peak at δ 177.09ppm and δ 174.03ppm indicated the presence of terminal CO

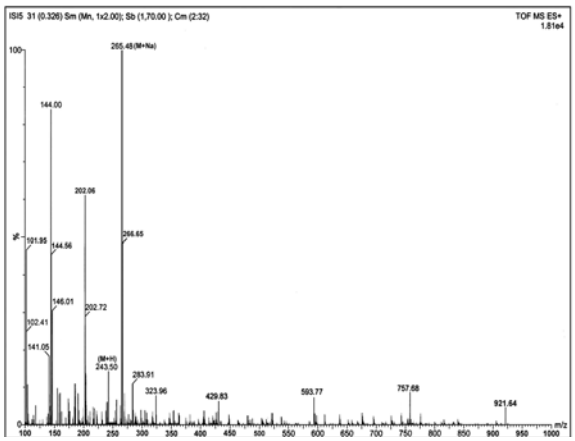

Fig. 2: Mass spectra of purified compound of *Cleome viscosa*

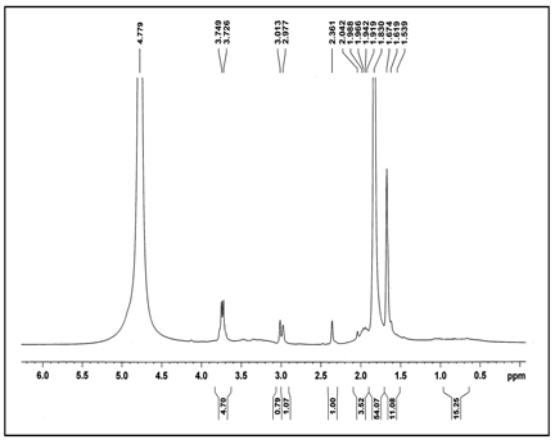

Fig. 3: ^{1}HNMR spectra of purified MFCV compound of *Cleome viscosa*

of the carboxyl group and internal CO group respectively. Peaks at δ 55.26ppm and δ 49.86ppm supported the presence of terminal and internal CH respectively. The peak at δ 49.57ppm - δ 48.14ppm is due to methylene (CH_2) carbons of substituted side chains. Hence the presence of alkane hydrocarbons is fully supported. The peak at δ 29.39ppm indicates a terminal NH_2 group attached to an alkyl chain.

Infrared Spectrum (Fig. 5) of purified MFCV showed broad absorption peak at 3369cm^{-1} indicaing N-H stretching vibration of alipathic primary amines. N-H bending (scissoring) vibration of primary amines observed at 1637 cm^{-1} (Silverstein and Webster, 1997). The peak at 1766 cm^{-1} indicates C=O stretching vibration of four numbered (ß) lactam ring. The most characteristic vibrational modes of alkenes are the out of plane bending vibrations at 833 cm^{-1}. The symmetrical stretching (v_sCO_2) was revealed at 1342 cm^{-1} and asymmetrical stretching ($v_{as}CO_2$), at 2397 cm^{-1}(Devi et al 1984).

Chromatographic analyses of MFCV revealed that the compound is 2-amino-9-(4-oxoazetidin-2-yl) nonanoic acid or lactam nonanoic (LNA) acid (Fig. 6), however, complete confirmation is in progress to establish exact structure. In fact, there are no reports on the presence of lactam nonanoic acid (LNA) in *Cleome viscosa.*

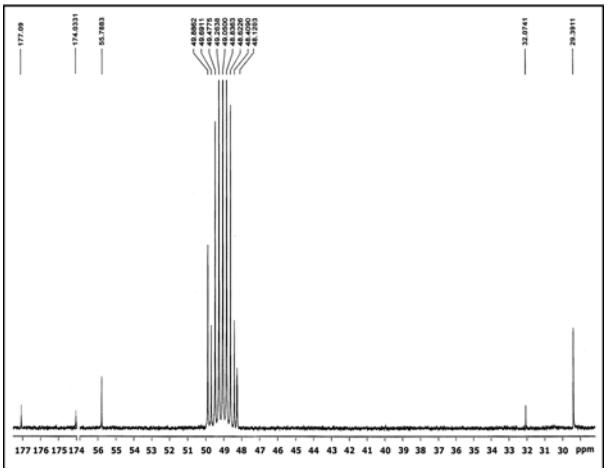

Fig. 4: ^{13}CNMR spectra of purified MFCV compound of *Cleome viscosa*

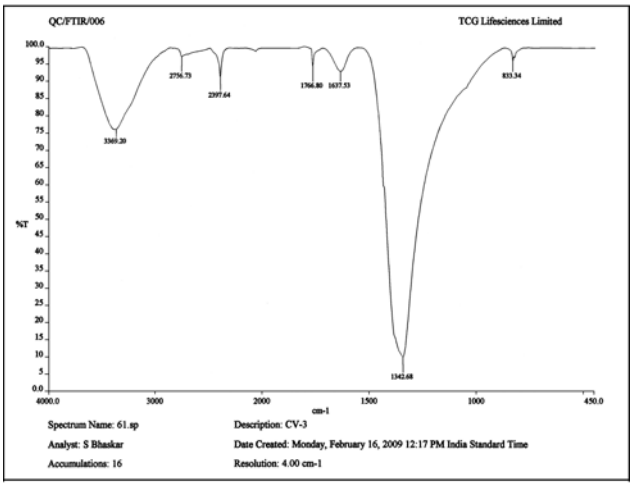

Fig. 5: IR spectra of purified MFCV compound of *Cleome viscosa*

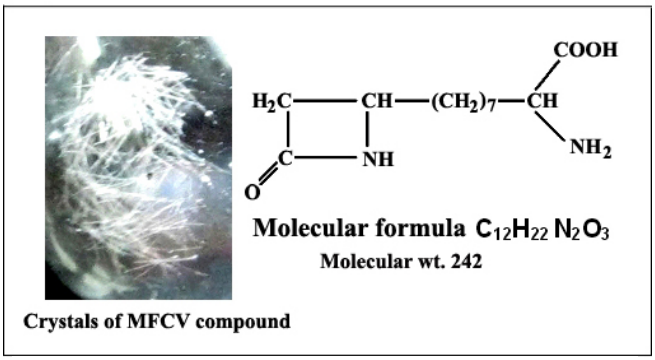

Fig. 6: Molecular structure of MFCV compound of *Cleome viscosa*

Effect of MFCV at different concentrations on the fungi: In the inhibition zone test, three fungal species viz. *Aspergillus niger, Aspergillus fumigatus* and *Aspergillus tamarii* showed differential effects at different concentrations of LNA. *Aspergillus niger* showed maximum stimulation at 500ppm. Stimulation decreased with increasing concentration and ceased at 2000ppm. At 2000ppm it did not show any effects. In case of A*spergillus tamarii,* the trend was reversed; maximum effect was detected at 1000, 1500, 2000ppm while at 500ppm the effect was minimal. In both Aspergillus *niger* and *Aspergillus tamarii,* the colour of the colony has changed due to the LNA of *Cleome viscosa.* Aspergillus *fumigatus* did not reveal any influence of LNA (Fig.7a, 7b).

Effects of MFCV at different concentrations on bacteria: In the inhibition zone test, *Escherichia coli*, *Pseudomonas* sp and *Staphylococcus aureus* revealed differential effect at different concentrations of purified LNA. *Pseudomonas* sp and *Staphylococcus aureus* were much more inhibitory to LNA at all concentrations. The colony color of *Pseudomonas* sp and *Staphylococcus aureus* has also changed due to this compound. No effect of LNA detected in *E. coli* (Fig. 8a, 8b).

Bioassay with MFCV compound of *Cleome viscosa:* - MFCV showed concentration dependent inhibitory activity on rice (var. Shamali), mustard and gram seeds (Fig. 9a and 9b). In case of rice, from 1000ppm to 125ppm concentration, it showed complete inhibition on germination. In case of gram seeds, MFCV exhibited complete inhibition on germination up to 500ppm concentration. At 250ppm, it showed 100% inhibition in shoot length and 50.41% inhibition in root length. In case of mustard seeds, only at 1000ppm MFCV exhibited complete inhibition. At 500ppm, it revealed 17.22% inhibition in shoot length and 66.01% inhibition in root length. The MFCV compound of *Cleome viscosa* showed maximum inhibitory activity on rice than gram and mustard.

Discussion

Lactam nonanoic acid (LNA) or 2-amino-9-(4-oxoazetidin-2-yl) nonanoic acid has been isolated and identified from the root exudates of *Cleome 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. But our experimental lactam nonanoic acid of *Cleome viscosa* was highly active on *Pseudomonas* sp (gram-negative) and *Staphylococcus aureus* (gram-positive) but had no effect on *Escherichia coli. Both S. aureus and P. aeruginosa* causes several infectious diseases on human beings. This weed, which easily grows in wasteland and other places, might in future be exploited as a source of bacteriostatic agents.

On the other hand, this β-lactam nonanoic acid (LNA) stimulates the growth of common soil borne fungi. *Aspergillus niger* showed maximum stimulation at 500ppm while at 2000ppm no effect was observed. In contrast, A*spergillus tamarii, was* maximally stimulated at 1000, 1500, 2000ppm but at 500ppm minimum the effect was minimal. This fact is of ecological interest. In nature, this weed may play an important role in ecological network. Kasten and Ralf (1997) showed that β-lactam antibiotics inhibit chloroplast division in a moss (*Physcomitrella patens)* but not in tomato

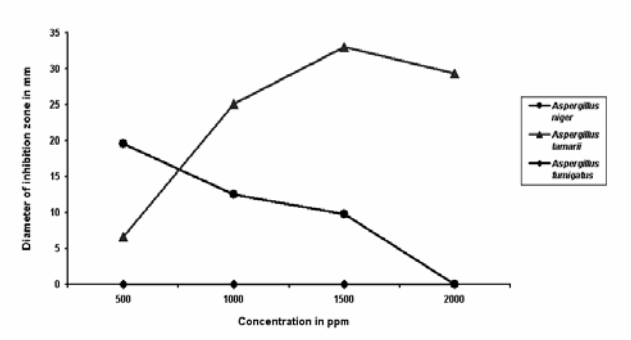

Fig. 7a: Effects of MFCV compound of *Cleome viscosa* at different concentration on *Aspergillus niger, Aspergillus tamarii* and *Aspergillus fumigatus,* Correlation is significant at 0.05 level (2-tailed)

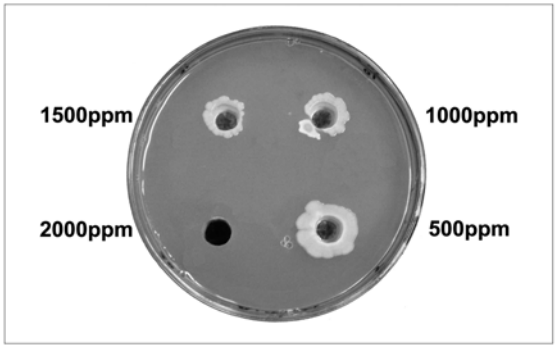

Fig. 7b: Effects of MFCV compound of *Cleome viscosa* at different concentration on the fungus, *Aspergillus niger.*

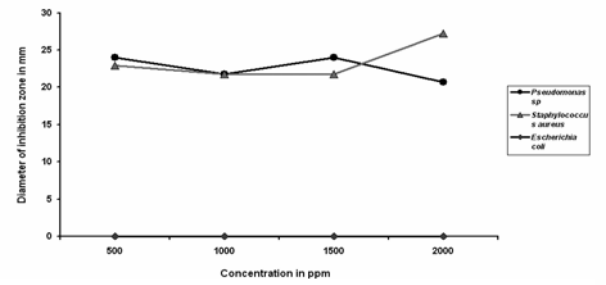

Fig. 8a: Effects of MFCV compound of *Cleome viscosa* at different concentration on *Pseudomonas* sp., *Staphylococcus* sp and *Escherichia coli* Correlation is significant at 0.05 level (2-tailed)

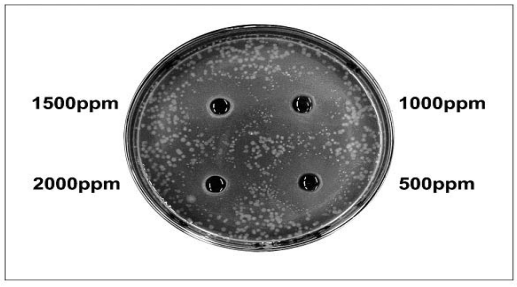

Fig. 8b: Effects of MFCV compound of *Cleome viscosa* at different concentration on the bacterium, *Pseudomonas* sp.

(*Lycopersicon esculentum*). We noted that in both *Aspergillus niger* and *Aspergillus tamarii,* the colour of the colony changed probably due to lactam nonanoic acid of *Cleome viscosa. Aspergillus fumigatus* did not exhibit any effect due to LNA. Therefore, lactam nonanoic acid (LNA) probably acts as allelopathic agents in *Cleome viscosa.* The secretion of lactam nonanoic acid may provide a competitive advantage for *Cleome viscosa* and its antibacterial activity on *Staphylococcus aureus* and *Pseudomonas aeruginosa* are responsible for medicinal property.

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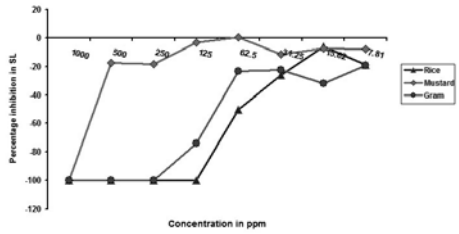

Fig. 9a: Effects of MFCV compound of *Cleome viscosa* on the shoot length (SL) growth of rice, mustard and wheat. Correlation is significant at 0.01 level (2-tailed)

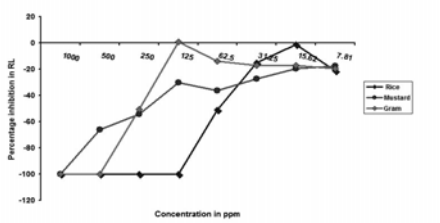

Fig. 9b: Effects of MFCV compound of *Cleome viscosa* on the root length (SL) growth of rice, mustard and wheat. Correlation is significant at 0.01 level (2-tailed)

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