CHARACTERIZATION OF HERBICIDAL POTENTIAL OF TENUAZONIC ACID ISOLATED FROM *ALTERNARIA ALTERNATA* FOR THE MANAGEMENT OF *PARTHENIUM HYSTEROPHORUS*

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Abstract: Microbial secondary metabolites are potential herbicides with unique mode of action. Cell Free Culture Filtrate (CFCF) of *Alternaria alternata* was evaluated for its phytotoxicity against *Parthenium hysterophorus* . Shoot cut, seedling and detached leaf bioassays revealed the presence of a toxic metabolite in the CFCF. A significant reduction in chlorophyll and protein content were also noticed. Phytotoxic moiety was purified and characterized by using solvent partition, thin layer chromatography (TLC), FTIR and 1H NMR analysis. The acetone extract induced maximum phytotoxic damage at a concentration of 100 µg ml–1 and TLC purified fraction exhibited herbicidal potential. The toxic compound was identified as Tenuazonic acid on comparison with FTIR and 1 H NMR spectra. This is the first evidence of the herbicidal potential of Tenuazonic acid against *Parthenium hysterophorus* produced by submerged fermentation of *Alternaria alternata*.

Key words: *Alternaria alternata, Parthenium hysterophorus* , Mycoherbicide, Tenuazonic acid.

INTRODUCTION

Parthenium hysterophorus has gained global importance in the recent years as weed (Pandey et al., 1996). It is an obnoxious weed due to its fast multiplication, rapid dispersal, agricultural and health hazards. It causes dermatitis and other allergies to human beings. *Parthenium hysterophorus* is popularly known as Carrot weed, White head, Mutter kraut, abysinthe marron, gazargrass, and congress grass. With ever increasing population of P*arthenium* in both urban and rural localities, the associated problems are also growing phenomenally.

Biological control is the deliberated use of living organism to control a weed. During last two decades biological control has received considerable attention. These has been results of the intensive use of chemical herbicides coming under scrutiny due to an increasing number of resistant or tolerant weeds, effect of non-target organisms, contamination of soil, ground water and food etc. The characteristic of the microorganism that make them desirable candidates as biological control agents for pestiferous plant species have been extensively reviewed by Freeman (1977), Freeman et al., (1973) and Templeton et al., (1986). Exploitation of phytotoxic properties of microbial metabolites has attracted the attention of a large number of researchers involved in weed management programme (Fischer and Bellus, 1983 and Duke, 1986). Microbial secondary metabolites appear to be lucrative source of novel structures having unique mode of action which could be exploited as commercial herbicides (Kenfield et al., 1988 and Abbas and Duke 1995). Secondary metabolites having phytotoxic activity are identified as allelochemicals and represent myriad compounds such as hydrocarbons, triterpenoids, steroids etc.

In the present communication, we assess the mycoherbicidal potential of the secondary etabolites obtained from the fungus *Alternaria alternata* as eco-friendly herbicides for the management of the weed *Parthenium hysterophorus* . From the secondary 2 metabolites of the

fungus *Alternaria alternata* various phytotoxic effects have been reported during pathogenesis, including chlorosis or necrosis on leaves and other parts of the plant as well as inhibition of seed germination (Liakopoulou et al., 1997).

MATERIAL AND METHOD

Fungal strain

The strain of the test fungus *Alternaria alternata* (FGCC#101) was obtained from the Fungal ermplasm Collection Center (FGCC), Mycological Research Laboratory, Department of Biological Sciences, Rani Durgawati University, Jabalpur (M.P.) India. It was isolated earlier from the leaves of the target weed, *Parthenium hysterophorus* . This was maintained on potato dextrose agar medium (PDA: extract of 200 gm peeled potatoes; dextrose, 20 gm; agar, 20 gm and distilled water to make 1000 ml) at 4 ±1^{o}C in a refrigerator for further studies.

Production and extraction of Cell Free Culture Filtrate (CFCF)

The test strain was grown on PDA medium for 7 days at 25 ± 1^{o} C in the glass petriplates. 250 ml Erlenmeyer's flasks containing 100 ml of Richard's broth (KNO3, 10 g; MgSO4, 2.5 g; KH2PO4, 2.5 g; sucrose, 30 g and distilled water, 1000 ml) were seeded with 5 mm disc from 7 days old culture of *Alternaria alternata* (FGCC # 101) grown on PDA medium and was incubated at 25±1 ^{o}C in B.O.D incubator (Yorco, India) as stationary cultures and the CFCF was extracted after 7, 14, 21 and 28 days respectively.

Under aseptic conditions the metabolized growth medium was filtered through a pre-weighed Whatman filter paper No.1 and was centrifuged at 4000 rpm for 10 mins. The pellet was discarded and the supernatant was again passed through the Sartorius 0.45 µm, Minisart (Sartorius, Gottingen, Germany) under in vacuo conditions to obtain the final CFCF (Vikrant et al., 2006).

Screening of phytotoxicity of CFCF

Phytotoxicity of CFCF against *Parthenium hysterophorus* was tested in accordance with following methods.

Shoot cut Bioassay

Shoots of the weed were taken from healthy 4 weeks old plant and dipped in the CFCF obtained from 7, 14, 21, 28 days fermented broth in glass vials and these vials were capped with foil to make it air tight and placed in plant growth chamber (Yorco, India) at 26 ± 1 ^{o}C. The effect of different dilutions (25%, 50%, 75% and 100%) of different days old fermented broth on shoots was regularly observed after 24, 48 and 72 hours of treatment (Sharma et al., 1969 and Chiang et al., 1989). Phytotoxicity was determined by following the method of Abbas and Boyette (1992) on a rating scale of 0-5 (0, no symptoms; 1, slight chlorosis and necrosis; 3, marked chlorosis and necrosis; 4, marked chlorosis and high necrosis; 5, acute chlorosis and necrosis leading to death of shoots).

Seedling Bioassay

The seedlings were raised in pots containing soil, sand and peat (1:1:1). Different dilutions (25%, 0%, 75% and 100%) of the toxic metabolites obtained from 7, 14, 21, 28 days fermented broth were made and seedlings were sprayed to run off condition and were incubated for different periods. Observations regarding toxicity in the seedlings were made regularly on a 0-5 rating scale as described above Abbas et al., 1992).

Detached leaf Bioassay

Leaves detached from the plant were surface sterilized with 2% NaOCl. An area of approximately 3 mm2 on the upper leaf surface was gently scratched and 20 µl of the toxin was injected by using sterilized syringe. The leaves were than incubated on a sterilized moist chamber (Made in 9 cm petriplates using cotton and filter paper) under continuous 4fluorescent light at 26 ± 1^{o}C (Sharma et al., 2004). The effect was observed after 6, 12 and 24 hours at room temperature.

In all bioassays described above, sterilized unmetabolized growth medium was taken as control and the sterilized distilled water was taken as control over control (Sharma et al., 2004) and the experiments were carried out in triplicates.

Reduction in chlorophyll and protein content of *Parthenium hysterophorus* after all bioassays were screened to determine the mode of action of phytotoxin. The chlorophyll content was determined on a pre-weight basis by the method of Arnon (1949) and total protein content was determined by the method suggested by Lowry et al., (1951).

Thermal Stability of the phytotoxic moiety

To assess the mode of extraction of the phytotoxic moiety, it was extremely essential to ascertain the thermal nature of phytotoxin. The CFCF was subjected to different temperature treatment viz., 50^{o}C, 100 ^{o}C, 121^{o}C (autoclaved). Each treatment was carried out for 15 minutes. The phytotoxic activity of each treatment was assessed using shoot cut bioassay (Siddaramaiah et al., 1979). Each treatment was carried out in triplicates and untreated CFCF at room temperature taken as control.

Purification and characterization of the phytotoxic moiety

Extraction

The CFCF was obtained as described earlier and concentrated to ¼ folds of the original volume (Krishnamohan and Vidhyasekaran, 1989; Brain et al., 1945). This was further subjected to organic solvent extraction. Solvents used during the extraction procedure were Hexane, Petroleum ether, Carbon tetrachloride, Chloroform, Acetone and Ethyl acetate.

Assessment of the Biological Activity

All the layers obtained in solvent extraction were subjected to in vacuo dessication at 40^{o}C to remove solvent and to obtain residues. Residues were named as obtained viz. Fraction 1 (Hexane), Fraction 2 (Petroleum ether), respectively. The test residues were prepared as stocks using 5 ml distilled water and were tested for their phytotoxic activity using detached leaf bioassay (Strobel, 1973; Karr et al., 1974 and Sugawara et al., 1985).

Purification of the phytotoxic Residue

Phytotoxic fraction was further clarified for characterizing the phytotoxic moiety. The analytical techniques employed were, Thin layer chromatography (TLC), FTIR (Fourier Transform Infrared spectroscopy) and 1H NMR (Nuclear magnetic resonance spectroscopy) analysis.

Thin layer chromatography (TLC)

Thin layer chromatography was performed on silica gel (0.25 mm) with Toluene: Ethyl acetate: Formic acid (6:3:1). The plates were allowed to run for about 75 minutes. Spots were visualized by spraying with 1% ninhydrin in acetone and Rf of each band was calculated. The plates were dried at 40^{0}C for 6 hours in an oven and scooped out the band, and then it was eluted with acetone and further tested for its phytotoxic activity against *Parthenium hysterophorus* by detached leaf bioassay. The samples were analyzed in triplicates.

FTIR and 1H NMR analysis

The dry TLC fraction eluted with Acetone was filtered through the Sartorius filter paper. The filtered sample was evaporated on a hotplate (Remi, India) at 40 oC till dryness. The dry powder was mixed with purified salt KBr. This powder mixture was then crushed properly in order to ensure that the pellet becomes translucent.

The FTIR analysis was carried out by using Shimadzu FTIR-8400, SCE model, and scanned against KBr blanks using DRS mode (DRS-8000), resolution-4 and the number of scans 20.

High-resolution ^{1}H nuclear magnetic resonance (NMR) spectra were obtained by using a Bruker AV-400 (Bruker 400 MHZ) spectrometer (Bruker Biospin, Switzerand).

RESULTS AND DISCUSSION

Effect of incubation days on phytotoxin production

It is evident from data presented in table 1 that CFCF obtained from fermented broth of *Alternaria alternata* had varied degree of toxicity against *Parthenium hysterophorus* . Maximum toxicity was recorded when shoots and seedlings were treated with CFCF obtained from 21 days old fermented broth. The initial symptoms observed within 6 hours of treatment on the affected shoots (Figure 1) and seedlings were prominent wilting and slight curling. At advanced stage rapid browning of stem, wilting of leaves, and veinal necrosis of leaves was observed. Leaves exhibited acute necrosis leading to the death of shoots and seedlings.

Significant reduction in photosynthetic pigment and protein contents was also recorded. Maximum reduction i.e., 85.9% was reported for total chlorophyll when shoots were treated with CFCF obtained from 21 days broth and the effect was less in chlorophyll b and chlorophyll a. 21 days old CFCF drastically reduced the protein content (82.7%) of shoots followed by 28 days old CFCF.

When biological content was determined for seedlings sprayed with CFCF, maximum reduction was observed in chlorophyll b content (84.9%) with 21 days old CFCF. Chlorophyll a and total chlorophyll showed less reduction. The protein content was markedly reduced to maximum 82.6% with 21 days old broth in the affected seedlings. Thus it is evident from the above discussion that the fungal pathogen produced maximum toxin at 21 days of incubation.

Effect of different concentrations of CFCF on Phytotoxicity

The phytotoxic damage was directly proportional to the concentration of CFCF. Data in table 2 shows the results of biological activities of CFCF of *Alternaria alternata* 7(FGCC#101) on *Parthenium hysterophorus* by utilizing different concentrations of CFCF. Effect of different dilutions of 21 days old broth was determined for their phytotoxicity against *Parthenium hysterophorus* by shoot cut and seedling bioassay. Maximum damage was recorded when shoots of the test weed were treated with 75% concentration followed by 100%, 50%, 25% concentration of CFCF.

At 75% concentration, initial symptoms of phytotoxicity were characterized by the appearance of necrotic patches within 6 hours of application with advancement in treatment time, severe necrosis was observed and at advanced stage, curling and wilting was recorded.

Drastic reduction in photosynthetic pigment and protein was also observed in shoots treated with phytotoxin at 75% concentration. A maximum reduction of 90.4% in chlorophyll a was recorded with 75% concentration followed by chlorophyll b and total chlorophyll and maximum reduction in protein content (78.5%) was observed when shoots were treated with 75% concentration of 21 days old CFCF. After treatment the seedling bioassay showed a maximum reduction in chlorophyll a (91.5%) and protein (76.9%).

Observations regarding phytotoxicity of fungal secondary metabolite obtained in the present investigation are in accordance with those recorded by many other workers working with biological activities of secondary metabolites from fungal extracts (Hoagland, 1990; Walker and Templeton 1978; Boyette and Abbas, 1995; Pandey et al., 1992; Mishra et al., 1996 and Saxena et al., 2000). Variation in biological activities of the phytotoxin in treated shoots and seedlings have also been recorded by several workers (Thapar et al., 2002; Joseph et al., 2002; Pandey et al., 2000 and Pandey et al., 2003).

Thermal Stability of Phytotoxin

As evident from the data (Table 3) that the phytotoxin was stable at 50 ^{o}C, 100^{o}C, and 121 ^{o}C. Thus it could be concluded that the phytotoxic moiety was thermo-tolerant and thermo-stable. Jose and Saura (1993), have reported similar observations during the production of Rhizopin from *Rhizopus nigricans*. Kurian et al., (1977) also reported the thermal stability and non-proteinaceous nature of the toxin produced by *Cristulariella pyramidalis.*

Purification and characterization of Phytotoxin

Solvent extraction and detection of phytotoxicity

The fractions so obtained from the solvent extraction were vacuum evaporated (Rotary evaporator, MAC, India) at 40-45 ^{o}C and their residues were tested using detached leaf bioassay to detect and isolate the phytotoxic moiety. 100 µg/ml concentrations of the residues were tested during the detached leaf bioassay. It was found that fraction 5 (Acetone extract) induced phytotoxic damage after 6 hours post treatment (hpt) during the detached leaf bioassay (Figure 2), this is depicted in Table 4. Other fractions did not induce phytotoxic symptoms. Robeson and Jalal (1991) extracted Tenuazonic acid from CFCF of *Alternaria alternata* by employing ethyl acetate. Similarly Yoshida et al., (2000) isolated toxic compounds from the CFCF of Colletotrichum dematium by fractionating it with an equivalent volume of n- hexane and ethyl acetate.

Thin layer chromatography

Phytotoxin fraction 5 was further clarified for characterizing the phytotoxic moiety. The analytical technique TLC was employed. A blue spot was visualized by spraying with 1 % ninhydrin in acetone (Figure 3). The Rf obtained was 0.5-0.6. Herbicidal potential of TLC purified fraction against the detached leaves of *Parthenium hysterophorus* is shown in Table 5 and figure 2. Similar results of TLC based toxin purification were observed and reported by Davis et al., (1977).

FTIR and 1H NMR Analysis of the phytotoxic band separated by TLC

The FTIR spectrum Figure 4) of the sample (Acetone extract) gave the following signals and assignments.

1. OH stretching: - 3500-3560 cm^{-1} (concentrating samples broadens the band and moves it to 3000 cm^{-1}).
2. Aliphatic C-H stretching: - 1380 cm^{-1} (weak), 1260 cm^{-1} (strong) and 2870, 2960 cm^{-1} (both strong to medium).
3. Presence of – C= O, Keto group, cyclic membered: - 1720 cm^{-1}.
4. Presence of primary amine: - doublet between 3400-3500 cm^{-1} and 1560-1640 cm^{-1} (strong).

The ^{1}H NMR spectrum (Figure 5) (Acetone) gave the following signals and assignments: 0.9 ppm (d + t, overlapping) of 2 x CH3, 1.3 ppm (m) of CH2, 2.0 ppm (m) of CH2, 2.5 ppm (s) of COCH3, 3.7 ppm (d) of ═CO-CH-N, 6.8 ppm (broads) of NH, and 9.3 ppm (broads) of OH.

The herbicidal compound was identified as tenuazonic acid (Figure 6) upon comparison with the IR spectra reported by Davis et al. (1977) and comparison of the above properties with those reported in the literature (Kaczka et al., 1964; Meronuck et al., 1972; Mikami et al., 1971; Steyn et al., 1976 and Stickings et al., 1959).

In conclusion, it is apparent from the present investigation that the phytotoxin (Tenuazonic acid) elaborated from *Alternaria alternata* (FGCC#101) are novel and exhibited potential to be exploited as natural herbicides for the target weed *Parthenium hysterophorus*. The present investigation provides us a break through for replacement of synthetic chemicals without incurring huge economic losses.

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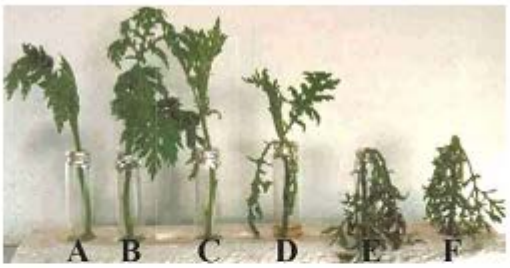
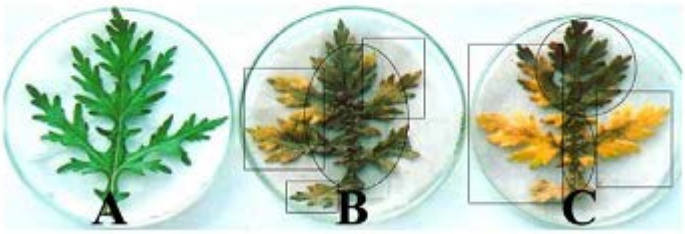


Fig 1



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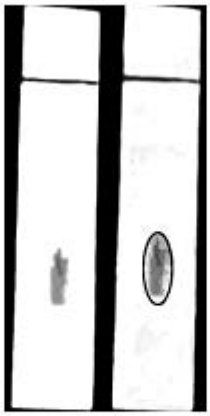

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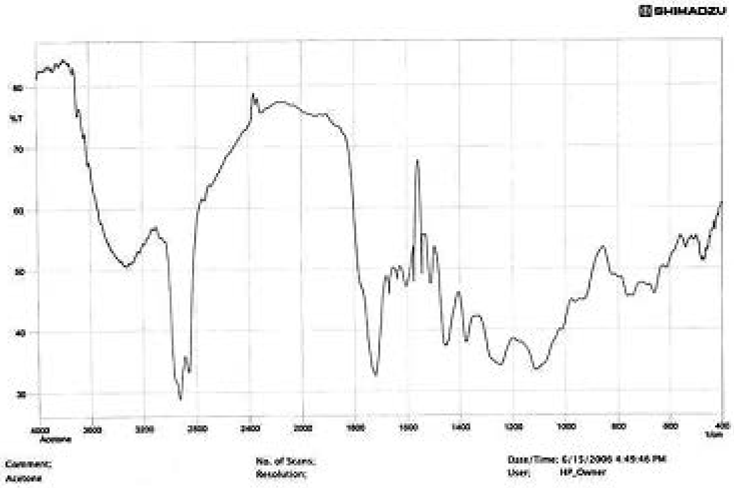
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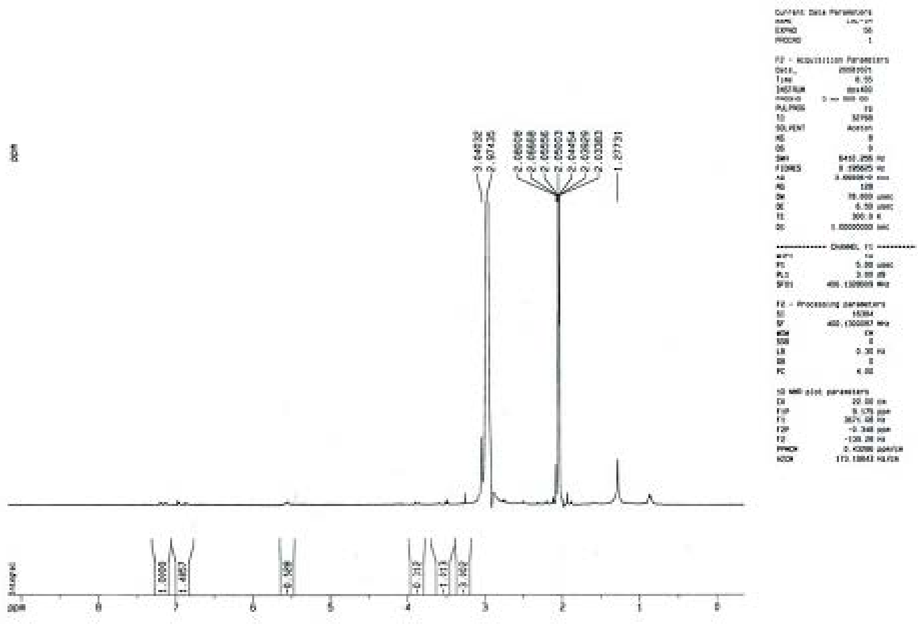

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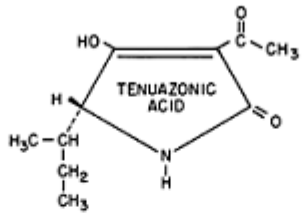

Fig 6

Figure legends

Fig. 1

Detection of phytotoxicity by shoot cut bioassay, A, Control a: Uninoculated Richard broth (no symptoms); B, Control b: Distilled water (no symptoms); C, 7 days old CFCF (slight curling); D, 14 days old CFCF (prominent wilting and slight curling); E, 21 days old CFCF (browning of stem, wilting of leaves, and veinal necrosis); F, 28 days old CFCF (browning of stem, wilting of leaves, and veinal necrosis).

Fig. 2

Detection of phytotoxicity by detached leaf bioassay; A, Control: Uninoculated Richard broth (no symptoms); B, Acetone extract; C, TLC purified fraction (Circle and oval shows area of necrosis and rectangle shows area of chlorosis and yellowing).

Fig. 3

TLC plate of the phytotoxic fraction, circle shows the blue spot (R_f: 0.5-0.6)

Fig. 4

FTIR spectrum of Tenuazonic acid.

Fig. 5

^{1}H NMR spectrum of Tenuazonic acid.

Fig. 6

Structure of Tenuazonic acid (Davis et al. 1997).

Tables

Table 1

Reduction in biological content in a Shoot cut and Seedling Bioassay of *Parthenium hysterophorus* by treating with CFCF of *Alternaria alternata* (FGCC#101) of different incubation days

Incubation days	Percentage Reduction in Biological Contents (Mean ± S.E.M)							
	Shoot cut Bioassay				Seedling Bioassay			
	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Protein	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Protein
7 day	10.4 ± 0.7	28.5 ± 0.44	30.3 ± 0.26	38.9± 0.36	18.2 ± 0.75	36.7 ± 0.29	20.4 ± 0.60	35.6 ± 0.40
14 day	39.5 ± 0.95	42.4 ± 0.26	48.8 ± 0.26	50.4 ± 0.36	52.7± 0.46	45.3 ± 0.11	48.3 ± 0.44	42.9 ± 0.43
21 day	74.5 ± 0.86	65.6 ± 0.26	85.9 ± 0.65	82.7 ± 0.46	74.2 ± 0.82	84.9 ± 0.08	70.5 ± 0.26	82.6 ± 0.10
28 day	64.9 ± 0.53	60.7 ± 0.4	72.5 ± 0.62	70.4 ± 0.72	65.6 ± 0.26	70.2 ± 0.02	60.5 ± 0.17	75.4 ± 0.66
Control a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Control b	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Control a: uninoculated Richard broth; Control b: distilled water; Incubation temperature: 26±1 °C; Medium: Richard broth

Table 2

Reduction in biological content in a Shoot cut and Seedling Bioassay of *Parthenium hysterophorus* by treating with CFCF of *Alternaria alternata* (FGCC#101) of different concentrations

Concentration of CFCF %	Percentage Reduction in Biological Contents (Mean ±S.E.M)							
	Shoot cut Bioassay				Seedling Bioassay			
	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Protein	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Protein
7 day	24.5 ± 0.62	40.4± 0.17	36.6 ± 0.62	20.4 ± 0.3	38.4 ± 0.44	50.8 ± 0.26	42.4 ± 0.89	25.8 ± 0.36
14 day	64.8 ± 0.44	68.9 ± 0.2	57.5 ± 0.1	42.8 ± 0.82	65.1 ± 0.1	70.4 ± 0.3	59.4 ± 0.1	40.1 ± 0.87
21 day	90.4 ± 0.6	89.6 ± 0.26	71.3 ± 0.7	78.5 ± 0.46	91.5 ± 0.53	84.7 ± 0.36	64.5 ± 0.15	76.9 ± 0.2
28 day	78.5 ± 0.56	68.4 ± 0.17	66.4 ± 0.46	65.1 ± 0.26	80.4 ± 0.69	76.2 ± 0.26	68.1 ± 0.35	68.4 ± 0.26
Control a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Control b	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Control a: uninoculated Richard broth; Control b: distilled water; Incubation temperature: 26±1 °C; Medium: Richard broth

Table 3

Thermal nature of phytotoxin from *Alternaria alternata* (FGCC#101), phytotoxicity rating against the detached leaves of *Parthenium hysterophorus*

Temperature	Phytotoxic Damage
50°C	4.5 ± 0.5
100°C	4.5 ± 0.0
121°C	4.5 ± 0.5
Control a	4.5 ± 0.0
Control b	0 ± 0.00

Control a: CFCF in room temperature; Control b: uninoculated Richard broth; R.H.: 80%; Room temperature: 26±1 °C

Table 4

Herbicidal potential of phytotoxic solvent extract fractions of CFCF of *Alternaria alternata* (FGCC#101) against the detached leaves of *Parthenium hysterophorus*

Exposure time	Control	Hexane	Petroleum ether	Carbon tetrachloride	Chloroform	Acetone	Ethyl acetate
6 hours	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3 ± 0.5	0 ± 0
12 hours	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4 ± 0.5	0 ± 0
24 hours	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4.5 ± 0.0	0 ± 0

R.H..: 80%; Room temperature: 26±1 °C; Control: distilled water

Table 5

Herbicidal potential of TLC purified fraction of CFCF of *Alternaria alternata* (FGCC#101) against the detached leaves of *Parthenium hysterophorus*

Exposure time	Control	TLC fraction (Acetone extract)
6 hours	0 ± 0	1 ± 0.0
12 hours	0 ± 0	4 ± 0.5
24 hours	0 ± 0	4.5 ± 0.0

R.H..: 80%; Room temperature: 26±1 °C; Control: distilled water

Phytotoxic damage scoring for table 5, 6 and 7:-

0: 0 – 4% plant death (no effect)

1: 2 – 19% plant death (slight chlorosis & lower leaf drop)

2: 20 – 49% plant death (marked chlorosis and slight necrosis, drooping of entire twings)

3: 50 – 79% plant death (acute chlorosis and marked necrosis)

4: 80 – 94% plant death (high chlorosis and high necrosis)

5: 95 – 100% plant death (acute chlorosis and necrosis)