

Production and Extraction of Phytotoxins from *Colletotrichum dematium* FGCC# 20 Effective Against *Parthenium hysterophorus* L.

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ABSTRACT

The aim of this work was to study the herbicidal potential of Cell free culture filtrate of *Colletotrichum dematium* FGCC#20 against *Parthenium* by employing different bioassays i.e. shoot-cut, seedling, detached leaf and seed germination. On solvent extraction of the Cell free culture filtrate, Ethyl acetate extracted fraction showed the presence of phytotoxic moiety.

Key words: *Parthenium hysterophorus*; *Colletotrichum dematium*; bioassay; Cell Free Culture Filtrate (CFCF); solvent extraction; hours post treatment (hpt)

INTRODUCTION

Parthenium hysterophorus L., popularly known as carrot weed or congress grass, native of West Indies, Central and North America was possibly introduced in India along with food grain imported under PL 480 scheme (Rao, 1956). Being an aggressive annual weed of disturbed sites, within the last three decades, it has established itself in the country and during the last few years; it has spread in almost all the states of the country. It is somewhat an unattractive member of Asteraceae. It is included in world's worst weeds. Different parts of the plant including pollen of the weed exert allelopathic effect on agricultural crops. Two sesquiterpene lactones such as parthenin and coronopilin exert allelopathic effect on agricultural crops (Deshpande et al., 2005). It also adversely affects human and animal health (Narsimhan et al., 1977).

Colletotrichum spp. are well known fungal pathogens, which cause severe diseases in a variety of plants. Many *Colletotrichum* species cause plant diseases commonly known as anthracnose on stems, leaves and fruits of a range of economically important plants, such as cereal crops, grasses, legumes, fruits and vegetables, and species of perennials. Strains of this pathogen are important model systems in bioherbicide studies and are expected to have the ability to meet some of critical agronomic needs once constraints are overcome.

It is well-documented that species produces phytotoxic metabolites which induce the symptoms similar to those of the pathogens themselves. Some of the metabolites have been used to select for resistance and have been shown to play a significant role in pathogenesis. *C. dematium* f. sp. *epilobii*, a specific and indigenous pathogen of fireweed *Epilobium angustifolium* L.

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subsp. *angustifolium* (family Onagraceae) has been investigated on biological weed control (Garcia-Pajon and Collado, 2003). It produces large necrotic lesions on the leaves and stem of the infected plant. Two Δ C-6 and C-8 methylflavonols were isolated from ethyl acetate extracts of liquid culture filtrates of the fungus. These demonstrated a high degree of antimicrobial and phytotoxic activity (Gohbara et al., 1978). These avoid the environmental problems as encountered by the use of chemicals.

Phytotoxins have not received extensive testing for their use as synergists of biocontrol agents. Thus, there is a need to study the weed pathogens, phytotoxins produced by them and their integration with biocontrol agents for a holistic approach for an integrated weed management. Looking into these challenges and world over emphasis towards developing 'environment friendly' technologies to avoid the hazards of chemicals, non-biodegradable pesticides and pollutants, the present approach could be a promising technology.

MATERIAL AND METHODS

Strain

Strain of *Colletotrichum dematium* FGCC # 20 was obtained from Fungal Germplasm Culture Collection (FGCC), Mycological Research Laboratory, Department of Biological Sciences, R.D.V.V. Jabalpur (M.P.) India. The culture was maintained on Potato Dextrose Agar (PDA); pH-6.0 medium at $4 \pm 1^\circ\text{C}$; light: $2.5 \times 10^4 \text{ erg/cm}^2/\text{s}$ in a refrigerator for further studies.

Morphological study

Slide culture and Camera Lucida (Line Drawings) were used to study the morphological characteristics of fungal strain and identification was done with the help of standard literature.

Preparation of Cell free culture filtrate (CFCF)

Fermentation

Richard's medium (Agarwal and Hasija, 1986) containing KNO_3 -10 g, KH_2PO_4 -5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -2.8g, Sucrose-35g, FeCl_3 -Trace, distilled water-1000ml; pH-3.84 \pm 1 was used for the fermentation. Ten mycelial bits (2.5mm) separated from seven days old culture of the fungus grown on PDA medium at $26 \pm 2^\circ\text{C}$ were transferred to 1000ml Erlenmeyer flasks containing 500 ml

medium. Inoculated flasks were incubated at $26 \pm 2^\circ\text{C}$; light intensity $3.5 \times 10^4 \text{ erg/cm}^2/\text{s}$ in B.O.D. incubator (Remi, India) for 7, 14, 21 and 28 days.

Extraction of CFCF

CFCF was aseptically obtained by filtering the metabolized growth medium through pre-weighed Whatman filter paper No. 1. The supernatant was filtered through the filter paper $0.25 \mu\text{m}$ (Sartorius), under *in vacuo* conditions (Walker and Templeton, 1978).

Bioassays

Following bioassays were performed to assay the phytotoxicity of fungal CFCF against *Parthenium*.

Detached leaf bioassay

Parthenium leaves detached from the plant were surface sterilized with 0.2% NaOCl and were incubated in a sterilized moist filter paper in Petri plates using cotton and filter paper under continuous fluorescent light at $28 \pm 1^\circ\text{C}$. The phytotoxic effect due to the application of toxin was observed after 24, 48 and 72 hpt at room temperature (Sharma et al., 2004).

Shoot cut bioassay

Shoots (15 - 20 cm in length) were taken and tip of the shoots were sterilized by washing with tap water and with 0.1% NaOCl solution for 3 minutes and immediately washed in sterilized distilled water to remove any trace of the chemical. An inclined cut was made at the tip and the shoots were dipped in different dilutions of the filtrate. Different days old CFCF *viz.*, 7, 14, 21 and 28 days and different concentrations, i.e. 25, 50, 75 and 100% of CFCF were prepared using sterile Distilled water. The effect of dilutions of the toxic metabolites was observed on the shoots after 24, 48, and 72 hpt at room temperature.

Seedling Bioassay

The seedlings were raised in plastic pots containing soil, sand and peat in the ratio (1:1:1). The culture filtrates were sprayed to run off and incubated for different periods. Observation regarding the toxicity of the seedlings were made regularly (Abbas et al., 1995; Abbas and Boyette, 1992).

Seed germination bioassay

The toxicity of CFCF against *Parthenium* seeds was also tested by Seed Germination Bioassay.

Seeds were picked up randomly, dipped first in 0.01% NaOCl solution for 15 minutes, then dipped in toxin of different days old CFCF and different concentrations of CFCF and finally washed thoroughly with distilled water. Seeds were incubated in CFCF for overnight and placed in moist chamber. Plates were incubated at $26 \pm 2^{\circ}\text{C}$ in B.O.D. incubator. Seed germination percent was recorded after seven days. Seeds incubated in sterilized distilled water served as control (Singh and Pandey, 2001; Thapar et al., 2002).

The unmetabolized growth medium (Richard's) and distilled water were taken as control a and control b. The experiment was carried out in triplicate respectively.

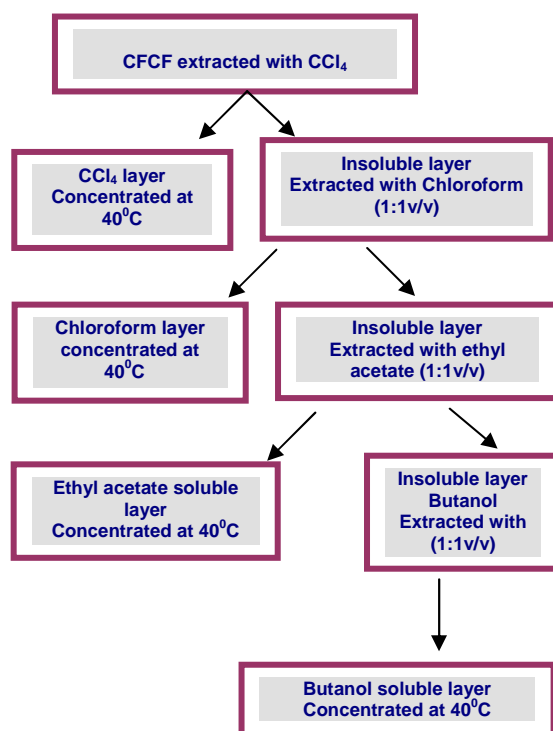
Thermal stability of phytotoxin

To ascertain the mode of extraction of the phytotoxic moiety, it was extremely essential to determine the thermal nature of phytotoxin (s). For this, 50 and 100% concentrations of CFCF of *C. dematium* was subjected to different temperature

treatment viz. 50, 100 and 121°C (autoclaved). Each treatment was carried out for 15 mins. The phytotoxic activity of each treatment was assessed using the shoot cut bioassay (Siddaramaiah et al., 1979) and detached leaf bioassay (Sharma et al., 2004). Each treatment was carried out in triplicate and CFCF at room temperature served as control and uninoculated medium served as second control.

Organic solvent extraction

Phytotoxins were extracted from the broth of 21 days old culture of *C. dematium* FGCC#20 grown as stationary culture ($28 \pm 2^{\circ}\text{C}$; 0.25 L batches) on Richard's broth. The CFCF was obtained as described earlier and concentrated to 1/50 folds of the original volume. Solvents used were carbon tetrachloride, chloroform, ethyl acetate and butanol. Concentrated CFCF was further subjected to organic solvent extraction scheme as shown below:



Assessment of the biological activity

All layers were subjected to *in vacuo* dessication at 40°C in a rotatory vacuum evaporator (Buchi, Germany) to remove the solvent and to obtain the

residues. Residues were named as Fraction A (carbon tetrachloride), Fraction B (chloroform), Fraction C (ethyl acetate), Fraction D (butanol). The test residues were prepared as stocks using

distilled water and were tested for their phytotoxic activity using detached leaf bioassay (Strobel, 1973; Karr et al., 1974).

RESULTS

Shoot cut bioassay

As evident from Figure 1, when shoots of test weed *Parthenium* were immersed in different days old CFCF of the test fungal strain, phytotoxic damage occurred which was observed after 12, 24

and 48hpt. The 21 days old fermented broth exhibited maximum phytotoxic damage to the *Parthenium* shoots after 48h, followed by 28, 14 and 7 days old CFCF. There existed a direct relationship between different days old CFCF and hpt of treatment with toxin. Phytotoxic effect was less after 12hpt and mild at 24hpt but was maximum at 48hpt. The shoots exhibited blackening of stem, drooping and curling of leaves, severe chlorosis and necrosis, finally leading to death of the toxin treated shoots.

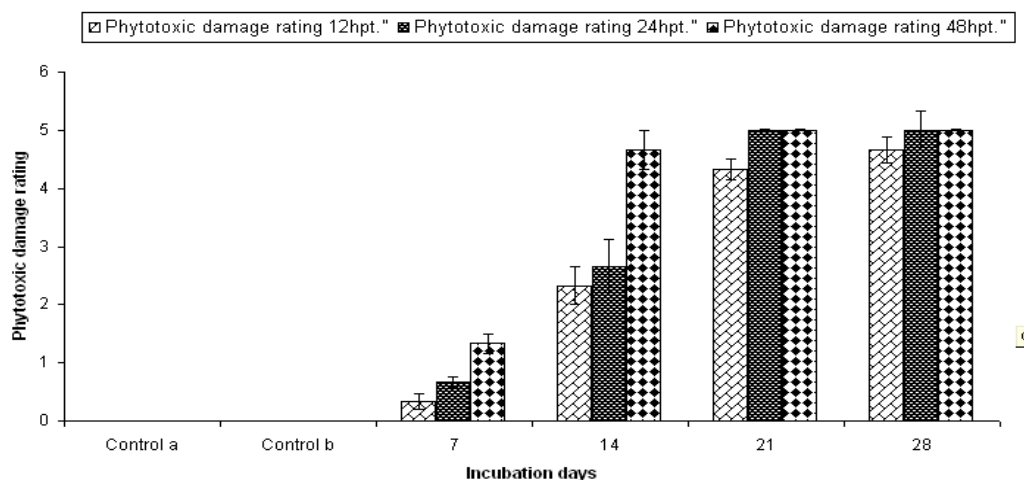


Figure 1 - Effect of different days old CFCF of *C. dematium* on *Parthenium* shoots.

Seedling bioassay

Phytotoxic damage rating was determined on *Parthenium* seedlings by spraying the CFCF of different incubation days of *C. dematium* FGCC# 20. When shoot cut bioassay was performed with different concentrations of 21 days old CFCF of *C. dematium* FGCC# 20, Maximum damage rating was observed after 48 hpt with 100% concentration of CFCF followed by 75 and 50%. Negligible effect was observed by 25% concentration. The results are documented in Fig 2.

Fig 3 shows that 21 days old CFCF imparted

maximum damage to the seedlings after 72h, followed by 48 and 24hpt of spraying with 28, 14 and 7days.

The phytotoxic damage rating at different hpt with different concentrations of 21 days old metabolized broth on *Parthenium* seedlings is shown in Fig 4. Results indicated that 100% concentration of 21 days old CFCF caused the maximum phytotoxic damage after 72 hpt followed by 75%, 50% and 25% concentrations. Both the controls exhibited no effect after being sprayed on seedlings to run-off.

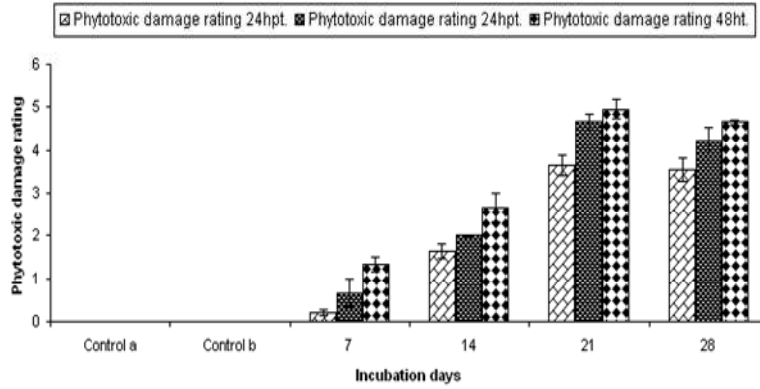


Figure 2 - Effect of different days old CFCF of *C. dematium* on Parthenium seedlings by Seedling Bioassay.

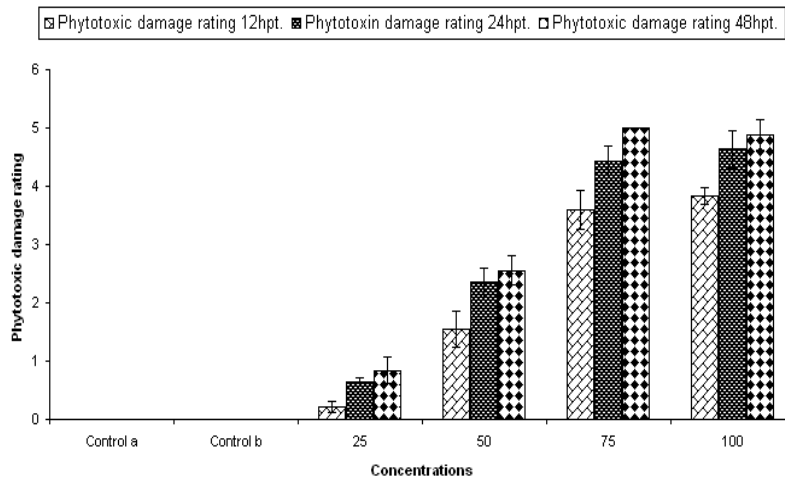


Figure 3 - Effect of different concentrations of 21 days old CFCF of *C. dematium* by Shoot cut Bioassay.

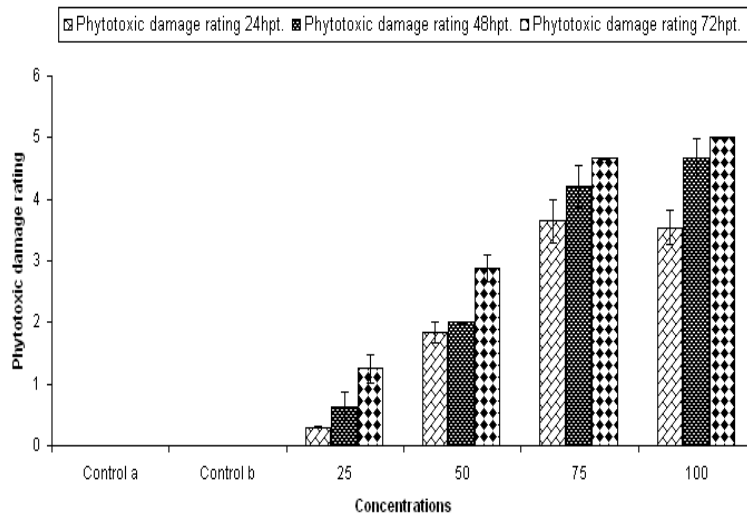


Figure 4 - Effect of different concentrations of 21 day old CFCF of *C. dematium* by Seedling Bioassay.

Detached leaf bioassay

Detached leaf bioassay was performed by treating *Parthenium* leaves with different day's old metabolized broth. Shown in Fig 5 clearly indicated that 21 and 28 days old CFCF brought maximum phytotoxic damage followed by 14 and 7 days old metabolized medium. In general effect was less after 12 hpt and gradually enhanced till 48 hpt.

Results on the treatment of detached leaves with increasing concentrations of 21 days old fermented medium is shown in Fig 6. Maximum damage was observed after 48 hpt with 100% CFCF of *C. dematium* FGCC# 20. In general, toxin treated leaves showed slight chlorosis, which increased with incubation time, leading to severe chlorosis, necrosis and complete death of leaf.

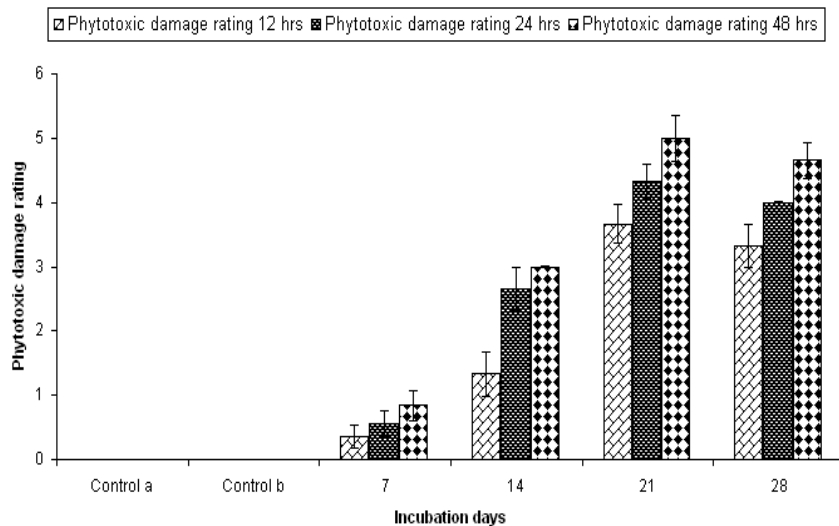


Figure 5 - Effect of different days old CFCF on Detached leaf Bioassay.

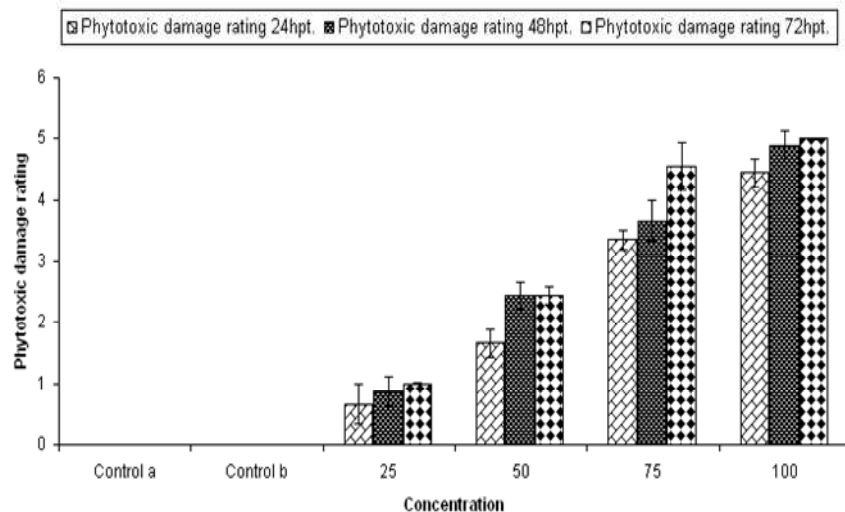


Figure 6 - Effect of different concentrations of 21 day old CFCF of *C. dematium* by Detached leaf Bioassay.

Seed germination bioassay

Results after the treatment of the seeds of *Parthenium* with toxins produced by *C. dematium* FGCC# 20 at different concentrations are

represented in Fig 7. In control (distilled water) negligible reduction occurred in germination and about 86.6% of the treated germinated seeds, followed by 25 and 50%. The percentage

germination observed with 75 and 100% was too negligible indicating nearly 90% inhibition of seed germination. Thus, the seeds imbibed the phytotoxin from *C. dematium* FGCC# 20 at higher concentration, hampering the germination.

Thermal stability

Data presented in Fig 8 showed that the phytotoxicity of fermented broth of *C. dematium* FGCC# 20 did not change and was stable at 50, 100 and 121°C.

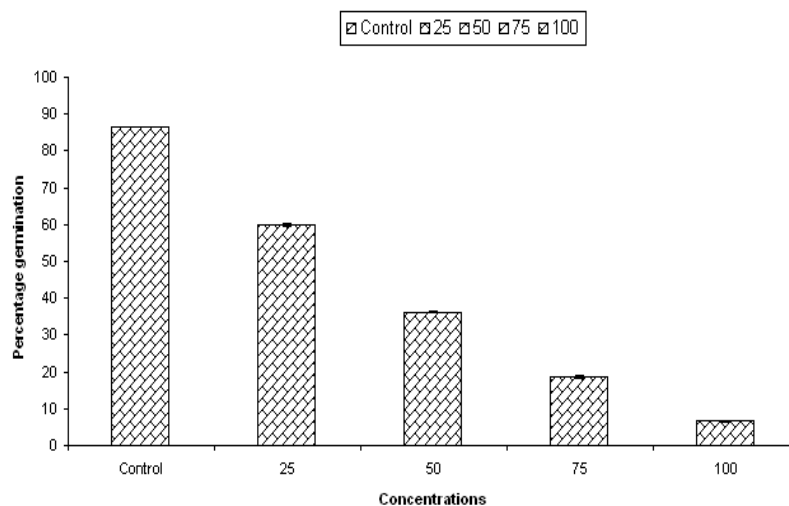


Figure 7 - Effect of different concentrations of 21 days old CFCF of *C. dematium* on Seed germination Bioassay.

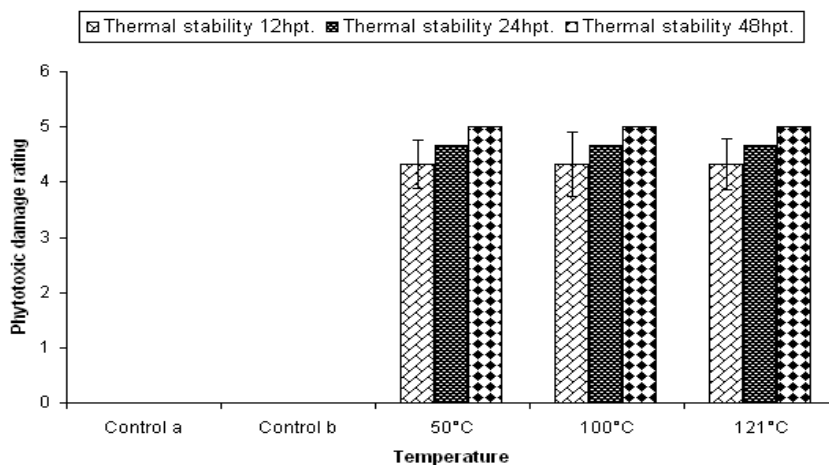


Figure 8 - Determination of thermostability of CFCF of *C. dematium* by Detached leaf Bioassay.

Isolation of phytotoxin(s)

Solvent extraction

As depicted in Fig 9 the following results are:

a. Fraction A (carbon tetrachloride fraction) was white in appearance. It produced least phytotoxic damage to *Parthenium* leaves even after 48 hpt.

b. Fraction B (chloroform fraction) was yellow in appearance. It also failed to produce significant phytotoxicity on *Parthenium* leaves at either 12, 24 or 48 hpt.

c. Fraction C (ethyl acetate fraction) was light brown in appearance and produced remarkable

chlorosis and necrosis on the leaves of test weed as early as 12 hpt. The Damage increased gradually and reached to its maximum after 48 hpt, ultimately leading to death of leaves after 48 hpt. d. Fraction D (butanol fraction) was pure white powder in appearance and produced phytotoxic effects on *Parthenium* leaves as severe chlorosis, necrosis after 48 hpt. The order of phytotoxicity of solvent extracted fractions was ethyl acetate >butanol > chloroform > carbon tetrachloride.

The phytotoxic moiety was present in ethyl acetate extracted fraction.

Statistics

Each experiment was performed atleast three times. The data are given in figures as Mean \pm SE and the bars denote \pm S.E. values. Data were analyzed by Analysis of Variance (ANOVA) Genstat, Hyderabad, India with a significant level of (P=0.05)

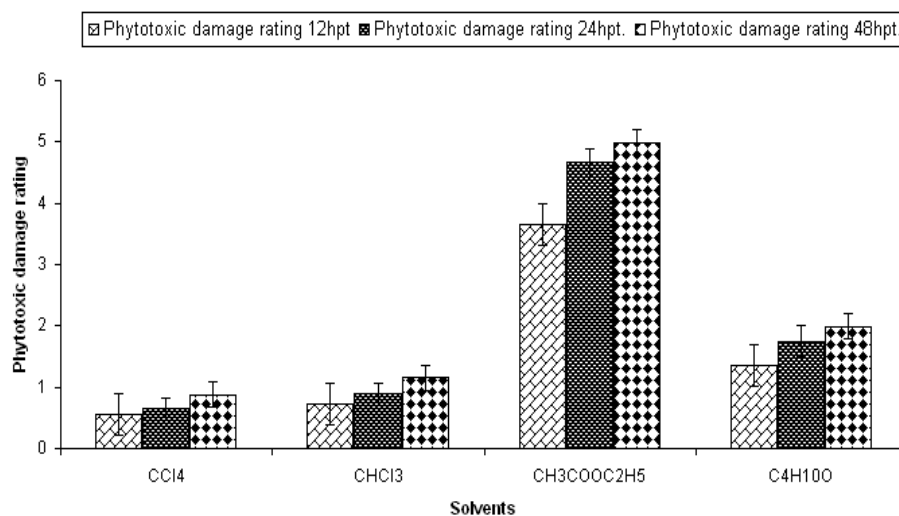


Figure 9 - Effect of different solvent extracted fractions of the CFCF of *C. dematium* on Detached eaves of *Parthenium*.

DISCUSSION

Variations in phytotoxicity with different days and different concentrations have been reported by earlier workers (Pandey et al., 2004, 2005; Quereshi et al., 2006; Quereshi and Pandey, 2007; Shukla and Pandey, 2006, Thapar et al., 2002). Observations regarding the phytotoxic damage rating on treatment with different days old metabolized broth and 21 days old CFCF of different concentrations on test weed seedlings have been reported by Joseph et al. (2002) which strengthen the present data. Similar results on detached leaf bioassay have been obtained by Sharma et al (2004). Thapar and Singh (2003) studied the effect of leachates of *Amaranthus viridis* on *Parthenium* weed. Jeyalakshmi et al. (1998) carried out a study indicating CFCF of *Trichoderma* sp. exerting inhibitory effect on seed

germination of *Parthenium*. Similarly observations regarding thermo-stability of phytotoxic moiety have also been made by Siddaramaiah et al. (1979). Kurian et al. (1977) also recorded thermostable and non-proteinous nature of toxin produced by *Cristularia pyramidalis*. Barbosa et al. (2002) reported similar results regarding thermostability of phytotoxin produced by *Bipolaris euphorbiae* effective against the weed *Euphorbia heterophylla*. Solvent extraction to recover the organic biological entities with novel activity has also ben considered as one of the most effective method by many other workers (Vikrant et al., 2006; Pandey et al., 2002) who have employed similar solvents for extracting and isolating the phytotoxic compounds from the CFCF of other fungi. In several earlier reports on the isolation of tenuazonic acid from aqueous media, the metabolite was extracted into an

organic solvent (Benzene, Chloroform or ethyl acetate) prior to purification. Tenuazonic acid was extracted from CFCF of *A. alternata* by employing ethyl acetate (Robeson and Jalal, 1991). Similarly, Yoshida et al. (2000) isolated toxic compounds from the CFCF of *C. dematium* by fractionating it with an equivalent volume of n-hexane and ethyl acetate.

The biological control of the weeds with microorganisms has provided an effective and eco-friendly management for many weed problems. Thus, the main aim of this paper was to highlight the herbicidal efficacy of the phytotoxins of *C. dematium* FGCC#20 as novel and lucrative source of potential herbicides for the management of weed, *Parthenium hysterophorus* and the presence of the phytotoxic compounds in ethyl acetate extracted fraction.

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