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EVALUATION OF ANTIFUNGAL ACTIVITY OF *CICER ARIETINUM* L.

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Abstract

The allelopathic potential of aerial parts of chickpea (*Cicer arietinum* L.) was investigated *in vitro* for their antifungal properties as natural alternatives of plant disease control. *Drechslera tetramera* (Mikinney) Subram. & Jain., and *Drechslera hawaiiensis* (M.B. Ellis) when tested against different concentrations of aqueous extracts of aerial parts of *C. arietinum* in liquid malt extract medium, the crude water extract showed most significant antifungal activity even at lower concentration of 5%. In case of extraction in Dichloromethane fraction, the inhibitory effect was found to be proportional with the applied concentration. *Cicer arietinum* was found to contain antimicrobial compound(s) for the control of plant pathogenic fungi.

Introduction

Allelopathy has been accepted widely as an important ecological phenomenon. Due to increased awareness about the risks involved in use of pesticides, much attention is being focused on the alternative methods of pathogen control. In the past two decades, a lot of work has been done on plant-derived compounds as environmentally safe alternatives to pesticides for plant disease control (Rice, 1984; Vyvyan, 2002). Extracts of many allelopathic plants are now known to exhibit antimicrobial activities. Different plant extracts have been evaluated for their antimicrobial properties by Mahmoud (1999), Digrak *et al.,* (1999), Bowers & Locke (2000), Eksteen *et al.,* (2001), Hol & Van-veen (2002), Magama *et al.,* (2003), Gulluce *et al.,* (2003), and Afolayan (2003). Pretorius *et al.,* (2002) tested crude extracts from 39 plant species for their antifungal potential against 7 economically important plant pathogenic fungi. The most significant mycelial growth inhibition was obtained with extracts from *Aristea ecklonii.* Petroleum ether and methanolic extracts of nine wild plant species were tested *in vitro* for their antimycotic activity against 8 phytopathogenic fungi and the petroleum ether extract of *Origanum syriacum* resulted in complete inhibition of mycelial growth of 6 out of 8 fungi tested (Abou-Jawadah *et al.,* 2002). Muhsin *et al.,* (2001) observed remarkable reduction in growth of 18 fungal species due to crude garlic bulb extract.

The chickpea (*Cicer arietinum* L.) is one of the most important human and domestic animal foods in South Asia and is thought to be the third most important pulse crop after dry beans, *Phaseolus vulgaris* L. and dry peas, *Pisum sativum* L. (Saxena, 1990). Chickpea secretes highly acidic exudates which have pH near to 1 (Rembold, 1981). These are mostly released through the trichomes located on all the plant, including pods. These exudates are reported to have a role as defence chemicals against soil pathogens (Pimbert, 1990; Li & Copeland, 2000) and are also involved in chickpea resistance to insect pests (Reed *et al*., 1987). Among these organic acids, malonic acid is the most abundant organic acid in nodules and roots, whereas malic acid is the major acid in leaves and stem (Lazzaro & Thomson, 1995; Li & Copeland, 2000).

Isoflavonoids are the major group of flavonoids and their presence is primarily restricted to the family *Leguminosae*. Among aromatic compounds, isoflavonoids are one of the major groups of antifungal compounds and a large proportion of these are formed as phytoalexins (Stevenson *et al*., 1998) but many also occur as preformed substances (Grayer & Harborne, 1994). The presence of isoflavonoids in chickpea was first reported by Bose & Siddiqui (1945). After that several constitutive flavonoid and isoflavonoid derivatives have been isolated from the roots, leaves and germinating seeds of *C. arietinum* (Bose & Siddiqui, 1945; Hösel & Barz 1970; Barz *et al.*, 1970; Wong, 1975).

The present study was designed to evaluate the antifungal potential of different extracts from aerial parts of *Cicer arietinum* L. against *Drechslera tetramera* (Mikinney) Subram. & Jain and *Drechslera hawaiiensis* (M. B. Ellis).

Materials and Methods

Fresh and healthy plants of *C. arietinum* were washed thoroughly under running tap water, dried with blotting paper and were cut into small pieces. A 75% w/v stock solution of plant extract was obtained by soaking the crushed plant material in sterilized water for 48 hours at room temperature. It was then passed through muslin cloth and finally filtered through Whatman filter paper No. 1. The lower concentrations of 50, 25 and 5% were prepared by adding appropriate quantity of sterilized water in stock solution. The extract was stored at 4^oC in pre-sterilized flasks. To avoid contamination and prospective chemical alterations, the extract was ensured to be used within 3-4 days.

Basal medium for the growth of fungus was prepared by adding Malt extract (ME) 2% in water. Chloromycetin (250mg capsule in 100 ml of medium) was added to avoid bacterial contamination. ME (80ml) was distributed into 250ml flask. Plant extract (20ml) of each concentration was added separately to each flask in three replicates. Distilled water was added in place of extract in the control. Inoculum discs of 5mm diameter, obtained from 7-days old healthy growing fungal cultures of *Drechslera tetramera* and *Drechslera hawaiiensis* were transferred to these flasks aseptically and incubated at 25 ± 2^oC.

Plant extract prepared @ 75 gm in 100ml sterilized water was partitioned with100ml of Dicholoromethane for the second set of experiment. The solvent from the Dichloromethane and water fractions was removed using rotary evaporator (UTech-USA, RE-3000). The residue was re-dissolved in 100ml of sterilized water to get 75% stock solution. The further concentrations of 5, 25 and 50% were prepared by adding calculated amount of water by using the following formula:

$$N_1V_1= N_2V_2$$

For the assessment of fungal biomass yield, three harvests were designed at intervals of 5-days each. The mycelial biomass from triplicate samples for each treatment was collected on pre-weighed filter papers. Their dry weight yield was determined after 24 hours oven drying at 60^oC (Bajwa *et al.*, 2004). All the data was analysed by applying Duncan's Multiple Range (DMR) Test to compare the different treatments with one another statistically. The individual treatments were also compared with control for significant/insignificant difference by applying t-test.

Results

Effect of crude aqueous extract of *Cicer arietinum* on biomass production of *Drechslera tetramera*: *Drechslera tetramera* showed significant variation in dry biomass

when grown in different concentrations of *C. arietinum* (Fig. 1). The fungal biomass production exhibited reduction at lower extract concentrations of 5 and 25%, as compared to control at 5, 10 and 15 days incubation periods in crude aqueous extract. The inhibitory effect was found proportional with the incubation period and the percentage difference in dry biomass production from control was found highly significant at 15days incubation (Fig. 1). Insignificant reduction in first two harvests was observed when fungus was grown in 5% extract concentration, but at the 15 days the inhibitory effect was found to be highly significant. The higher concentrations increased the fungal biomass with time of incubation. The 50% extract concentration showed negative effect on fungal dry biomass production in first harvest, but after 10 and 15 days of incubation, increase in fungal growth recorded was not very significant (Fig. 2). The extract concentration of 75% markedly supported the mycelial yield and the increase in fungal biomass ranged from 6.6% after 5 days incubation to 15% after 15 days of incubation period (Fig. 1).

Effect of Dichloromethane and aqueous fractions of *Cicer arietinum* extract on biomass production of *Drechslera tetramera*: Dichloromethane (DCM) fraction showed the most promising antimycotic activity by reducing the fungal biomass up to 58% after 15 days of incubation (Fig. 1). Growth reduction increased as did the fraction concentration. There was insignificant increase in mycelial yield of 5% DCM fraction at 5 and 10 days of incubation period (Fig. 2), but in the final phase i.e., after 15 days of incubation the mycelial growth was found significantly depressed. At 25% DCM fraction the allelopathic stress was found to be increased with increase in time of incubation ranging from insignificant decrease in first harvest to highly significant in third. The higher regimes of 50 and 75% DCM fraction concentrations were found statistically highly significant with respect to the control at all incubation times.

No particular trend was observed in case of aqueous fraction, but generally lower concentrations of 5 and 25%, decreased the fungal dry biomass production whereas the trend was found reversed in case of higher concentrations (Fig. 1). The fungal biomass was found to be depressed after 5 days of incubation in 25 and 50% concentrations. At intermediate growth level of 10 days only 25% concentrated water fraction showed insignificant decrease in mycelial yield. The maximum dry biomass increment was observed at 75% after 10 days of incubation (Fig. 2).

Effect of crude aqueous extract of *Cicer arietinum* on biomass production of *Drechslera hawaiiensis*: The results obtained from periodic biomass assays of *Drechslera hawaiiensis* in various concentrations of crude aqueous extract of *C. arietinum* showed significant antifungal activity in lower concentrations viz., 5-50% in comparison to control, in all the three growth phases (Fig. 3). A nominal depression was observed after 5 and 10 days of incubation by 5% extract concentration (Fig. 4). But at the final harvest i.e., after 15 days growth period, the mycelial yield was found to be significantly depressed as compared to the control (Fig. 4). The inhibitory effect was found to be decreased at 25 and 50% extract concentrations with increase in time of incubation. The maximum antimycotic activity was observed at early growth phase i.e., after 5 days of incubation. In contrast the higher concentration of 75% showed positive effects on dry biomass production of *D. hawaiiensis.* The increase in mycelial production ranged from insignificant at first harvest to highly significant after 15 days of incubation.

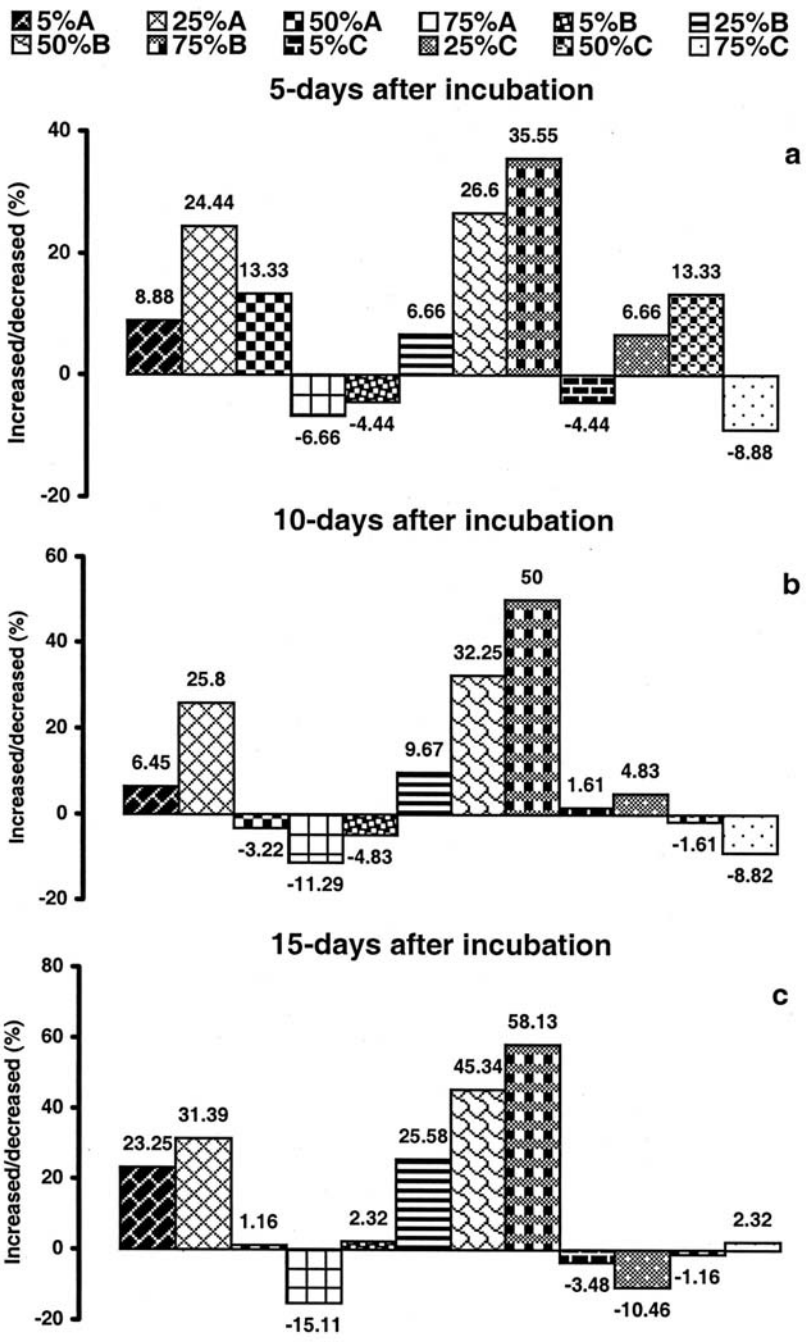

Fig. 1(a - c). Effect of extracts of *Cicer arietinum* on percentage losses in dry biomass production of *Drechslera tetramera* against control (extract free). Where A= Crude aqueous extract, B= Dicholoromethane fraction and C= Water fraction.

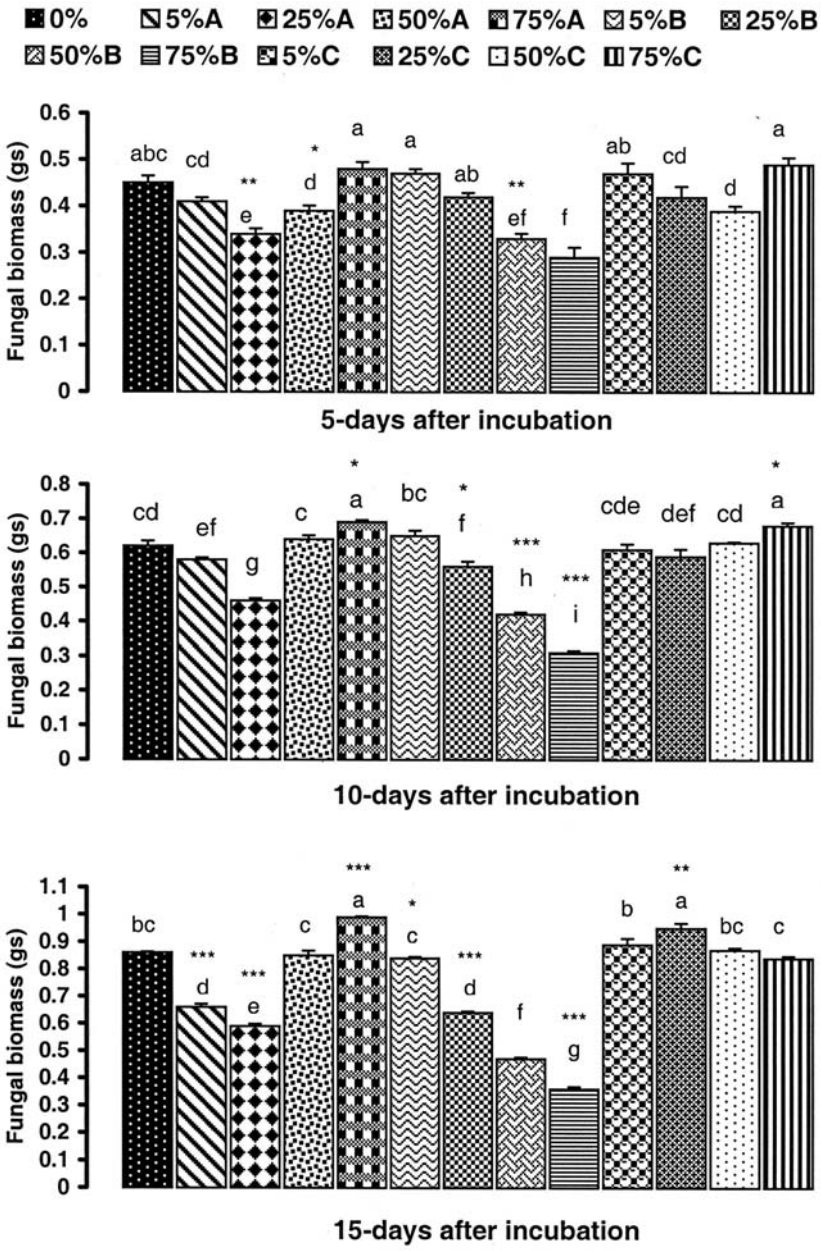

Fig. 2. Effect of different concentrations of extracts from *Cicer arietinum* on dry biomass production of *Drechslera tetramera* after 5, 10 and 15 days of incubation. Where A= Crude aqueous extract, B= Dicholoromethane fraction and C= Water fraction.
Vertical bars show standard errors of means of three replicates.
Values with different letters show significant difference (P = 0.05) as determined by DMR Test.
*, **, ***Show significant difference from control at 5, 1 and 0.1% level of significance respectively, as determined by t-test.

Effect of Dichloromethane and aqueous fractions of *Cicer arietinum* extract on biomass production of *Drechslera hawaiiensis*: Dichloromethane fraction was found superior in reducing the biomass production. The lower concentration of 5% slightly promoted the fungal growth after 5 and 15 days of growth. All the other concentrations i.e., 25-75% decreased the *in vitro* mycelial growth and this growth reduction was found proportional to the fraction concentration (Fig. 3). The maximum allelopathic stress was induced by 75% concentration causing a decline of 64% after 5 days of incubation period (Fig. 4). Statistically there was a decline in allelopathic stress with increase of incubation.

Generally the water fraction improved the biomass production. At initial growth stage the lower fraction concentrations (5-50%) depressed the mycelial yield up to 26%, while after 10 and 15 days of incubation period all the extract concentrations provided a considerably high boost in biomass productivity. At the final stage increased water fraction concentration promoted the biomass production ranging from insignificant increase at 5% up to highly significant at 75% (Fig. 4).

Discussion

Various plant extracts have been examined by different investigators for their antifungal activity with the objective of exploring environmentally safe alternatives of plant disease control. Significant effect of chickpea extracts was found on *D. hawaiiensis* and *D. tetramera* in reducing their mycelial growth. The extracts were found relatively more effective in decreasing the mycelial growth against *D. hawaiiensis* whereas *D. tetramera* exhibited greater resistance against allelopathic stress of *C. arietinum.* This difference in the susceptibility could be the cause of genetical difference in Physiological and morphological characteristics of different species (Shaukat *et al.,* 1983). Previous studies also support these results. Martinez *et al.,* (2000) reported variable effects of *Sargassum filipendula* extracts on *Aspergillus* species including *A. niger, A. flavus* and *A. parasiticus.*

Overall the general trends illustrated by both the tested species were found almost same. Greater inhibition of fungal growth was observed at lower concentrations of the crude water extract where as the higher concentrations supported the average mycelial growth rate per day. This may be because the optimal range of pH for growth of tested species lies in the acidic range. Since the exudates of *C. arietinum* contain several acidic compounds (Rembold, 1981), the enhancement in the dry biomass production at higher concentrations may be due to low pH level of medium. These results are also supported by the fact that some allelopathic substances have variable effects when applied in different concentrations, either inhibitory or stimulatory (Puruis *et al.,* 1985). The Dichloromethane fraction from crude aqueous extract showed stronger and broader spectrum of antimycotic activity. This activity was found proportional to the fraction concentration as the increase in concentrations decreases the biomass production significantly. Dichloromethane fraction (75%) showed maximum decrease in fungal growth which was 58% in *D. tetramera* and 64% in *D. hawaiiensis.* The water fraction after re-extraction of crude aqueous extract with dichloromethane was not found much effective against selected fungal species. The fraction in general promoted the mycelial growth and this positive effect increases with increase in time of incubation. *D. hawaiiensis* exhibited 45% increment in any biomass production at 75% water fraction after 15 days of incubation, whereas in case of *D. tetramera* it increases biomass up to 10% when grown at 25% fraction concentration.

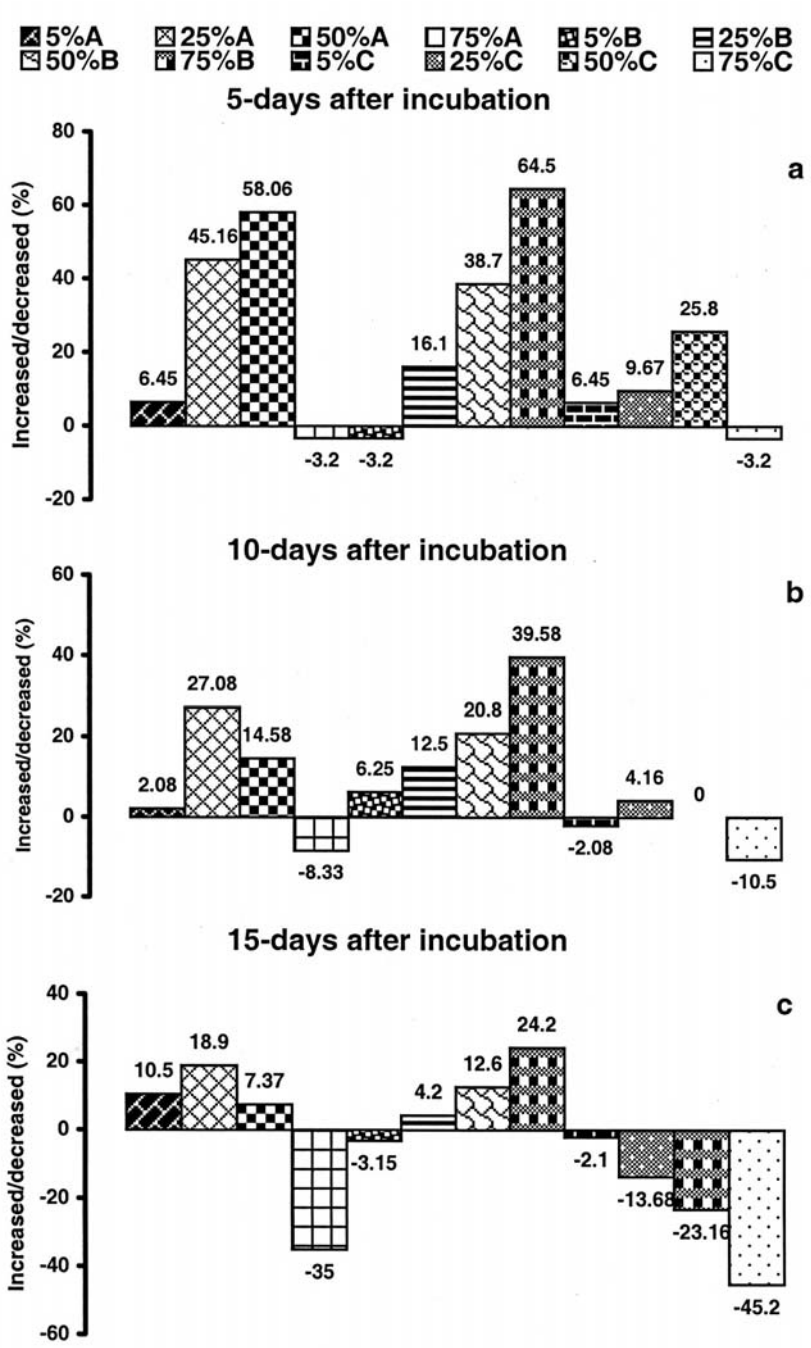

Fig. 3(a-c). Effect of extracts of *Cicer arietinum* on percentage losses in dry biomass production of *Drechslera hawaiiensis* against control (extract free). Where A= Crude aqueous extract, B= Dicholoromethane fraction and C= Water fraction.

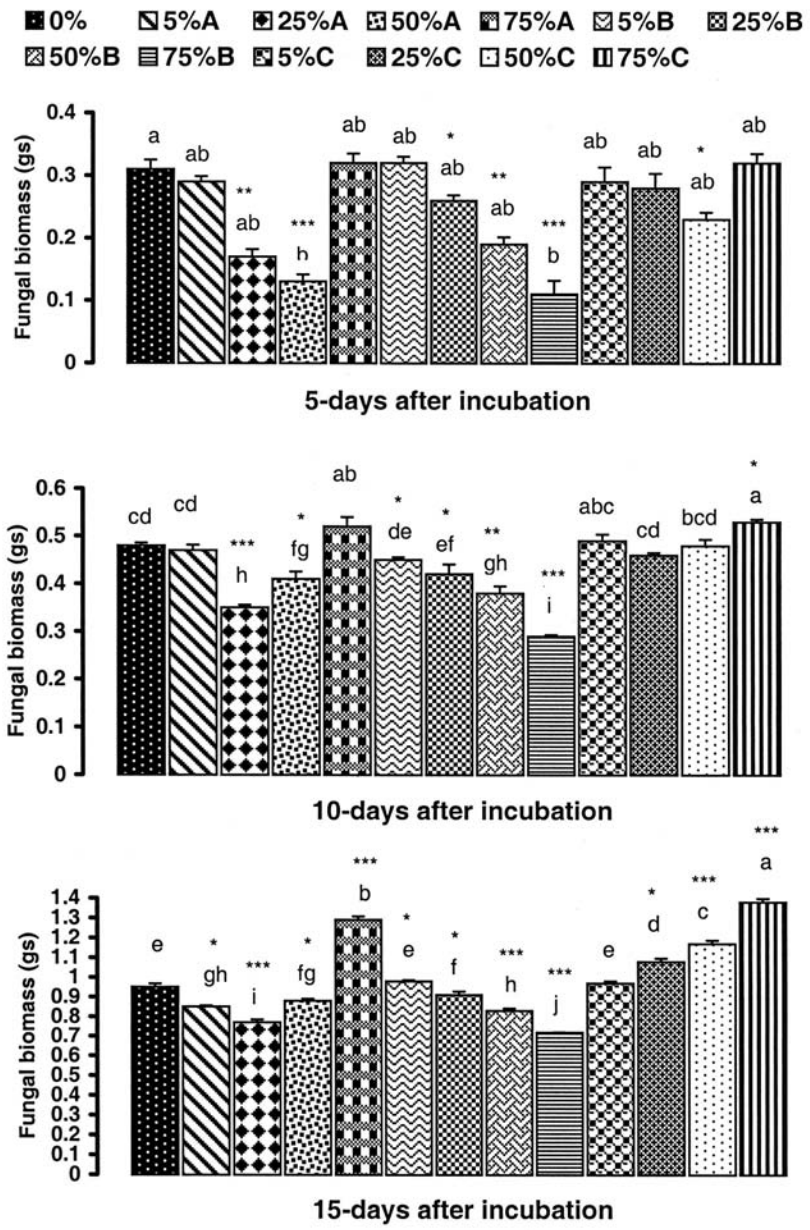

Fig. 4. Effect of different concentrations of extracts from *Cicer arietinum* on dry biomass production of *Drechslera hawaiiensis* after 5,10 and 15 days of incubation. Where A= Crude aqueous extract, B= Dicholoromethane fraction and C= Water fraction.
Vertical bars show standard errors of means of three replicates.
Values with different letters show significant difference (P = 0.05) as determined by DMR Test.
*, **, ***Show significant difference from control at 5, 1 and 0.1% level of significance respectively, as determined by t-test.

In case of crude aqueous extract the highest tested concentration i.e., 75% caused a persistent positive impact on growth of both fungal species. This increase in biomass production may be due to detoxifying ability of the fungi to allelochemicals or the ability of fungal species to exploit them as nutritional source (Sicker, 1998). Some allelochemicals are also known to enhance the growth at different concentrations (Mughal *et al.*, 1996).

The most pronounced allelopathic stress was observed at intermediate growth stage, whereas at initial and final stages both fungi showed species specific variations. In case of *D. tetramera* the maximum decrease was observed at the final harvest but in contrast *D. hawaiiensis* exhibited more retarded growth at initial growth stages. High cellular respiration and low rate of mitosis can be the possible causes for reduction in dry biomass production (Singh, 1999). In case of dichloromethane fraction the incubation period showed contrasting effects on both fungi. In case of *D. tetramera* more decrease in biomass production was observed with increase in time of incubation. Whereas *D. hawaiiensis* showed maximum reduction at initial growth stages and the percentage reduction in dry weight was compared to the control decreases with extended exposure to allelochemicals.

These results are clear indication for the possible use of crude aqueous extract from *C. arietinum* as well as their fractions to control fungal pathogens.

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