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Full Length Research Paper

Autotoxicity of chard and its allelopathic potentiality on germination and some metabolic activities associated with growth of wheat seedlings

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In this study, the allelopathic effect of aqueous whole mature chard plant extract (*Beta vulgaris* L. var. Cicla) on wheat (*Triticum vulgare* L. var. Sides 1) and an associated weed (chard) was investigated. Plants used were sampled in 2006, and then plant extracts were obtained after they were ground and processed with distilled water. Twenty five of wheat grains and the same number of chard seeds of uniform size and weight were placed in a mixture on sterile filter paper in 15 cm Petri-dishes. Treated Petri-dishes were each supplied with 20 ml extract of 0.25,1, 4, 8, and 12% (w/v) while untreated control was supplied with 20 ml of distilled water. After 10 days the germination percentage, vigour value, seedling growth criteria and some physiological processes were counted. The aqueous extract retarded the germination of chard more effectively than that of wheat and the effect was concentration dependent. The lowest concentration stimulated the germination of both wheat and chard; on the other hand, the germination was retarded under the application of concentrations above 1%. However, 1% concentration had a positive effect on wheat and negative on chard. HPLC analyses of the water-soluble extract of whole chard plant residue revealed the presence of eight phenolic aglycones that show the abundant of chichimec acid, (+) camphor, hydroxybenzoic, *p*-coumaric and vanillic acids as well as trace amounts of coumarin and protocatechuic acids. This extract may be used as a bioherbicide to control the germination and growth of itself (autotoxicity).

Key words: Allelopathy; *Beta vulgaris* var. Cicla, weed control, *Triticum vulgar* L. sides, HPLC, bioherbicides, autotoxicity.

INTRODUCTION

Weeds are unwanted, undesirable and non economic plants that compete with crops for water, nutrients, and sunlight. Additionally, some weeds interfere with crop plants through allelochemicals which inhibit crop growth and development (Qasem and Foy, 2001; Bhowmik and Inderjit, 2003; Romero-Romero et al., 2005; Batish et al., 2007). El-Khatib et al. (2003), found that soil associated with *Chenopodium murale* and soil amended with its leaves and roots suppressed the growth of *Melilotus indicus*. However its roots and their exudates exert allelopathic effect on wheat by releasing water soluble phenolic acids as putative allelochemicals in soil (Batish et al., 2007). Weeds also may directly reduce profits by hindering harvest operations, lowering crop quality, and weeds left uncontrolled may harbor insects and diseases and produce seed or rootstocks which infest the field and affect future crops. In spite of using modern methods of weed management, weeds are still responsible for the decline in crop yield. Losses caused by weeds can be as high as 24% of the yield compared with 16.4 and 11.2% for disease and pests, respectively (Oerke and Steiner, 1996).

The overuse of herbicides has provoked increasing incidence of resistance to herbicides in weeds (Valverde

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Abbreviation: (W+C), Wheat germinated with chard; (C+W), chard germinated with wheat.

et al., 2000; Lemerle et al., 2001), disappearance of some susceptible weeds, which affect weed biodiversity (Itoh, 2004). Moreover, herbicides cause environmental pollution, unsafe agricultural products and human health concerns (Kohli et al., 1998; Xuan et al., 2004 a, b; Khanh et al., 2005). Due to the importance of sustainability, modern agriculture searches for new compounds which are environmentally friendly and challenges to reduce environmental damages and healthy hazards due to chemical inputs, minimizes soil erosion, and yet maintains a high level of production (Einhellig, 1995).

Allelopathy is a natural and an environment-friendly technique which may prove to be a unique tool for weed control, increase crop yields, decrease our reliance on both synthetic pesticides, and improve the ecological environment (Hess and Duke, 2000; Lovelace et al., 2001; Minorsky, 2002). Many recent researches suggested that allelopathy may provide alternatives to synthetic herbicides for weed control (Romeo and Weidenhamer, 1999; Khanh et al., 2005, 2006).

Many allelopathic studies on weeds found that the extracts of some weeds may be used as herbicides to control the germination and growth of other weeds (Pandey, 1994; Hari et al., 2002; Kadioiglu and Yanar, 2004). Moreover, some weed residue possesses allelopathic potential to increase the growth and yield of some crop (Dzyubenko and Petrenko, 1971; Bhowmik and Doll, 1982; Chivinge, 1985; Hagin, 1989; Uygur and skendero, 1995).

Autotoxicity is an intraspecific allelopathy, occurs when a plant species releases chemical substances that inhibit or delay germination and growth of the same plant species (Putnam, 1985; Singh et al., 1999). It has been reported in weed, for example, pokeweed (Edwards et al., 1988). Some researches indicated that some chenopdiaceae species produce allelopathic compounds; in some cases these compounds inhibit seed germination of the chenopods themselves. Also extracts of both *Avena sterilis* and *chenopodium maculatum* inhibit seed germination of themselves (Jefferson and Pennacchi 2003; Kadioiglu and Yanar, 2004). In order to minimize the negative impacts of varietal autotoxicity, careful selection of suitable crop varieties is necessary in a continuous cropping system (Oueslati et al., 2005; Wu et al., 2007).

Chard (*Beta vulgaris* L. var. Cicla) (Family: Chenopodiaceae) is one of the most harmful weeds in Egypt with an important effect on *Tritium vulgaris L.*

Wheat (*Trticium vulgare* L. var. sides) is an important crop in Egypt and yields a large amount of wheat straw residue. Utilizing the residue of chard (*B. vulgaris* L. var. Cicla) as a management tool for weed control is an alternative method for increasing crop productivity, and for reducing the dependence on synthetic herbicides. The objective of this study was to evaluate allelopathic potential of whole chard plant extract on seed germination of both wheat and chard and the ability of it to be a bioherbicide for chard control.

MATERIAL AND METHODS

Sampling of plant material

Whole mature chard plant was collected from different cultivated fields around Beni Seuf governorate during fruit development stage in 2006. Whole weed was dried for 4 weeks under room temperature. The dried weed was ground and stoked in plastic sacs in dark condition at room temperature until use. Grains of wheat were kindly obtained from the Crop Department, Sides Research Center, Ministry of Agriculture, Beni Suef, Egypt. Seeds of chard were kindly obtained from Weed Control Research Section, Field Crop Reasech Institute, Giza, Egypt.

Preparation of aqueous extracts

To obtain aqueous extract, 120 g of ground material was suspended in 1 L distilled water, shaking the suspension for 24 h on a shaker (200 rpm) at room temperature. The extract was centrifuged at 4000 g then filtrated through Buchner funnel. The filtrate was brought up to original volume with distilled water. Test solutions of water extract were prepared by diluting the original extract to obtain the different concentrations 0.25,1, 4, 8, 12% (w/v).

Germination test

Healthy plant seeds and grains were surface sterilized with 0.1% mercuric chloride for 5 min, washed with distilled water then sterile grains and seeds were dried on laboratory benches for 24 h. Twenty five of wheat grains and the same number of chard seeds of uniform size and weight were placed in a mixture on two sterile filter papers what man No. 1 in 15 cm Petri-dishes. Petri-dishes were treated each with 20 ml extract of 0.25,1, 4, 8, 12% (w/v) test solutions, while the control was treated with 20 ml of distilled water. The Petri-dishes were incubated in a dark germinating chamber for 10 days at 25/12^{o}C ± 2 and 97% relative humidity, each treatment was replicated five times in a completely randomized experiment design. After 10 days, the germination percentage and speed were calculated. The vigour value (V) has been chosen for measure the germination speed, it can be calculated by using the following formula (Bradbeer, 1988):

V= (a/1+b/2+c/3+d/4+…….+ x/n) X 100/S.

Where a, b, c… respectively, represent the number of seed which germinated after 1, 2, 3 days of imbibition, x is the number after n days and S is total number of germinated seeds.

Growth measurement of wheat and chard seedlings were determined after ten days of germination. The length of radicle and plumule of seedlings were measured. The fresh weight of seedling was recorded, and then oven dried at 60ºC to a constant weight to determine the dry weight. The water content of seedling was calculated on the dry weight basis. Other fresh seedlings were kept at -10ºC for enzyme assay, nucleic acids, and crude proteins determination. The dry seedlings were ground to fine powder and kept in sterilized dark glass vial for biochemical analysis.

Phenolic analyses

Phenolic extraction from dried tissues according to the method outlined by Jindal and Singh (1975). Phenolic content determined by Folin-Ciocalteau phenol reaction (AOAC, 1990).

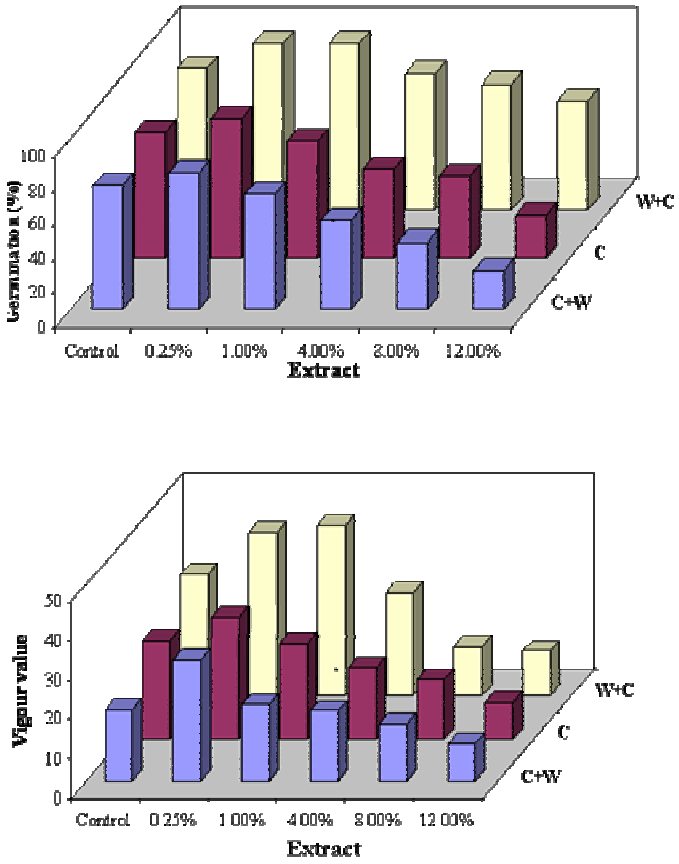

Figure 1a. Allelopathic effect of aqueous extract of whole chard plant on the germination percentage and the vigour value of 10-days-old seedlings.

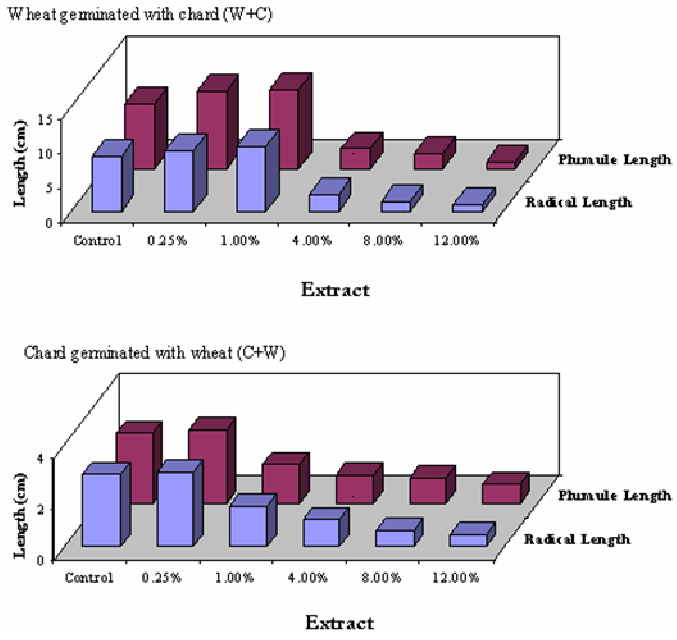

Figure 1b. Allelopathic effect of aqueous extract of whole chard plant on the growth criteria of 10-days-old seedlings.

Amylase analyses

The extraction was carried out by the method cited by Monerri et al. (1986), and assay methods adopted by Bilderback (1971).

Nucleic acids analyses

Extraction of nucleic acids by Mohamed and El-Sayed (1982), DNA and RNA were determined by Clark and Switzer (1977) and calculation of nucleic acids content according to the equations outlined by Schneider (1945).

Protein analyses

Extraction of soluble proteins by 0.3 M cold phosphate buffer (pH 6.8 - 7) and determination according to Bradford method (1976).

Statistical analysis

The experimental design was completely randomized with three replications. The results of bioassay experiments were analyzed with one-way analysis of variance and the mean values were separated at $P < 0.01$ and $P < 0.05$. The statistical analysis was done using the SPSS® / PC computer software package version 11.1., 2001.

RESULT

Germination

Effect of whole plant chard extract on germination and seed growth of wheat germinated with chard (W+C) and chard germinated with wheat (C+W) at the end of 10 days incubation period are shown in Figure 1a, b and c. Aqueous whole chard plant extract seemed to be of significant stimulatory effect on germination percentage and vigour value of treated W+C as compared with control at the concentrations of 1% by about 17.4 and 40.1%, respectively. While this concentration reduced the germination percentage of C+W by about 6.5%. The concentration of 0.25% seemed to have stimulatory effect on the germination percentage and the vigor value of all treated plants. On the other hand, chard extract at the concentrations of 8 and 12% caused significant and/or high significant inhibition for treated plants. Extract concentration of (1%) caused significant increases in radicle, plumule, fresh and dry weight of W+C by about 16.5, 21.5, 81.8 and 42.4%, respectively comparing with control, while at this concentration high significant inhibitions for radicle and plumule of C+W (43.8 and 44.3%, respectively) were recorded. The lowest level of chard extracts (0.25%) seemed to have stimulatory effect on all treated plants. The maximum stimulatory effect was observed in fresh weight of W+C (54.5%). The highest concentration applied (12%) produced high significant reduction in growth parameters, except for fresh weight of W+C. The highest reduction of radicle and plumule was 88.1 and 88.9% respectively, in case of W+C.

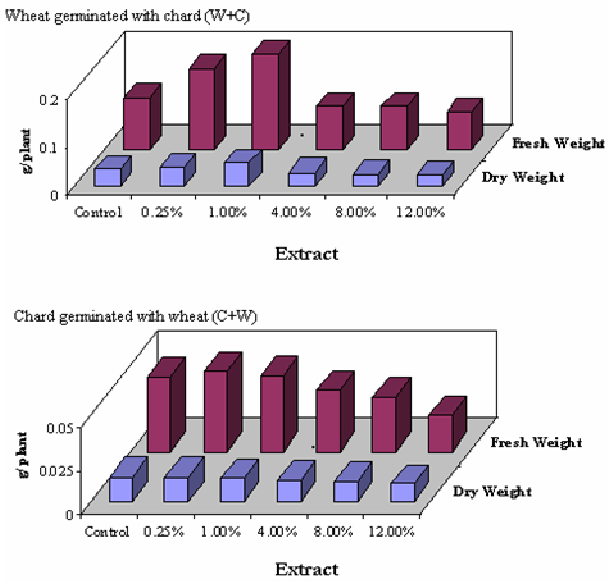

Figure 1c. Allelopathic effect of aqueous extract of whole chard plant on the growth criteria of 10-days-old seedlings.

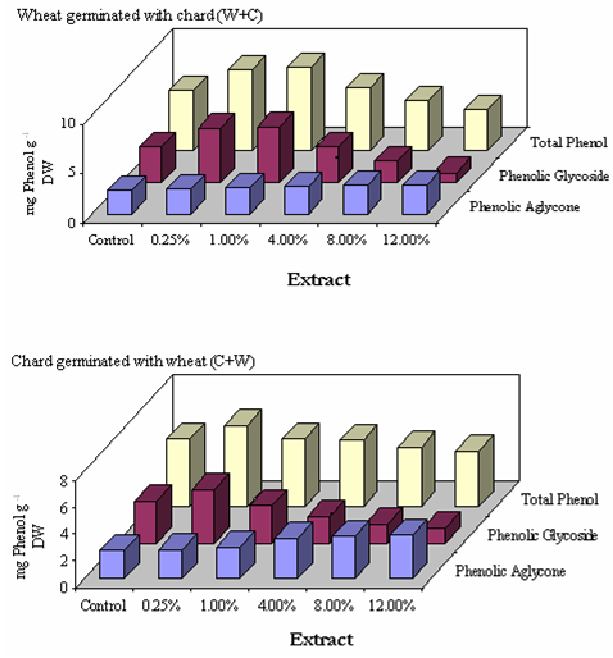

Figure 2. Allelopathic effect of aqueous extract of whole chard plant on the phenolic content of 10-days-old seedlings.

Biochemical analysis

The biochemical analysis of treated and non-treated seedlings were carried out after 10 days of exposure to allelochemicals of whole chard plant extract. Since chard seeds and wheat grains contain different food reserves, their metabolic changes during germination in response

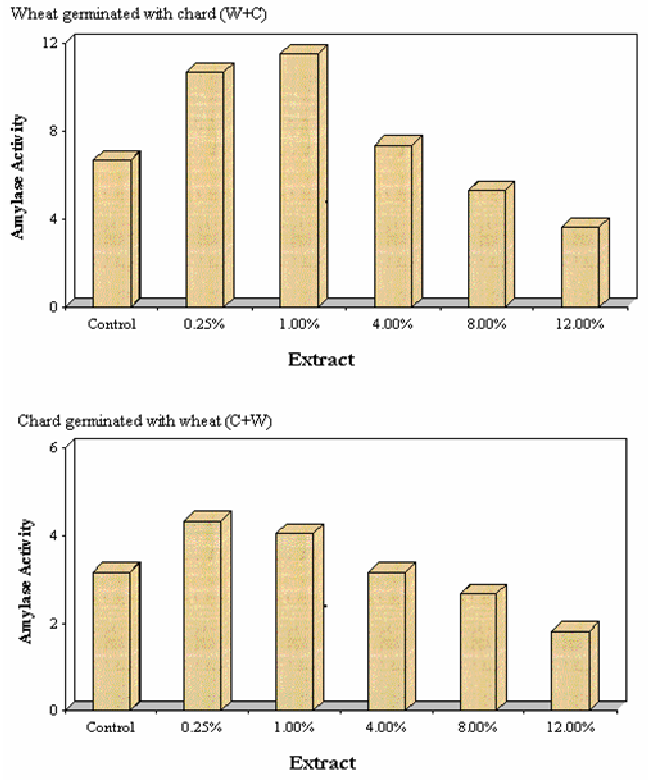

Figure 3. Allelopathic effect of aqueous extract of whole chard plant on the amylase Activity (Δ 620 g^{-1} fresh weight min^{-1}) of 10-days-old seedlings.

to the application of aqueous whole chard plant extract might provide a better understanding concerning the mode of action of these allelochemicals.

Phenolic content

The changes in the phenolic constituents of W+C and C+W were recorded in Figure 2. The increase in concentration of aqueous whole chard plant extract was accompanied by a gradual elevation in accumulation level of phenolic aglycone in shoots of W+C and C+W. The content of phenolic glycoside increased highly significantly (57.55%) in shoot of W+C at 1% but decreased in shoot of C+W. Phenolic glycoside detected in the control sample was more than the phenolic aglycone. This ratio increased in wheat shoot (W+C) at 0.25 and 1%, and then gradually decreased. While in case of C+W it increased at 0.25% and decreased gradually from 1 to 12%

Amylase activity

The activity of amylase enzymes are recorded in Figure 3. Allelochemical concentrations above 4% induced an

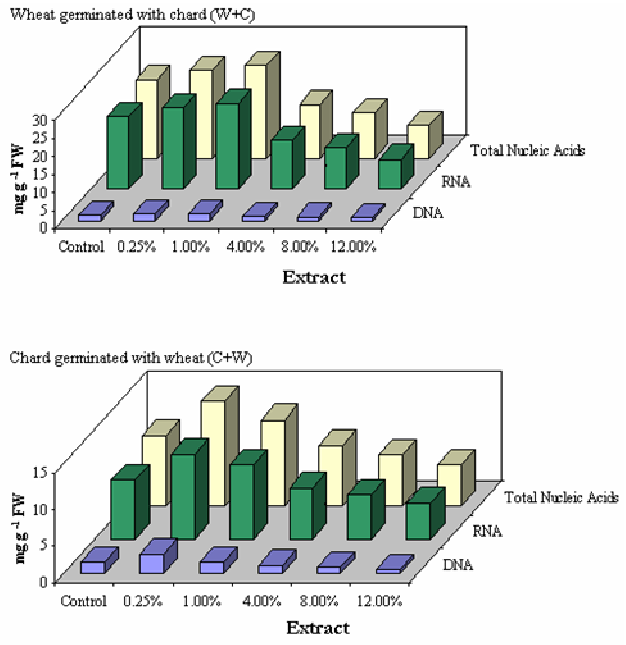

Figure 4. Allelopathic effect of aqueous whole chard plant extract on the nucleic acids content of 10-days-old chard seedlings.

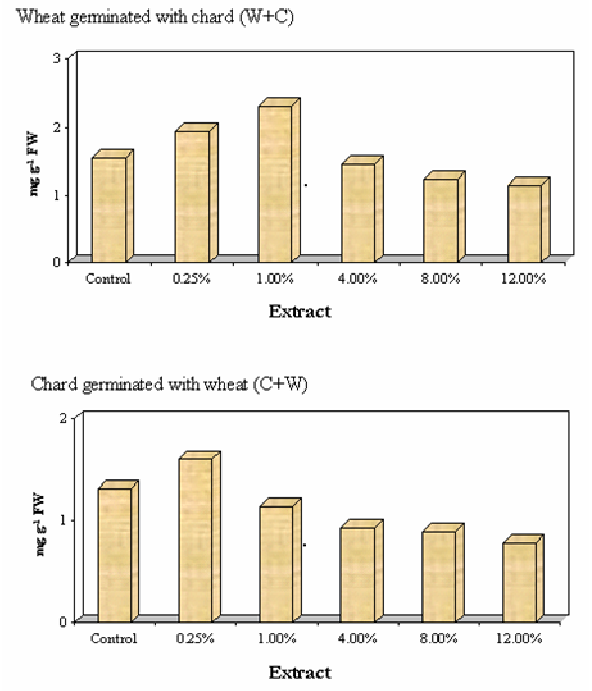

Figure 5. Allelopathic effect of aqueous whole chard plant extract on the nucleic acids content of 10-days-old chard seedlings.

inhibitory effect on the amylase activity of W+C and C+W. The lowest applied concentration 0.25% induced high stimulation in amylase activity of W+C by about (59.6%). At concentration of 1%, the amylase activity was stimulated by about 71.9 % in W+C.

Protein content

The changes in the protein content (Figure 4) of samples seedlings in response to allelochemicals exhibited a general pattern of increased accumulation at low concentrations and inhibitory effects at higher concentrations. The application of allelochemicals at low concentrations (0.25 and 1%) induced stimulatory effects on the accumulation of protein content of W+C. On the other hand, C+W at concentration 1% showed non-significant reduction in protein compounds as compared with respective controls. The 12% induced markedly higher levels of inhibition of protein content which accounted for 25.3 and 40.1% for applied allelochemicals for W+C and C+W, respectively. The results indicated that W+C at 4 and 8% recorded high stimulation (46.5%) and non-significant stimulation (12.1%), respectively; in the opposite direction, C+W recorded a high significant reduction in protein content (29.5 and 31.8%) for 4 and 8%, respectively.

Nucleic acids content

Nucleic acid contents in treated plant seedlings are illustrated in Figure 5. Data clearly revealed that the total nucleic acids contents markedly increased upon treatment with allelochemicals at 0.25 and 1% in all treated seedlings (change plant to seedlings). The maximum value obtained at 1% which was significant (18.5%) and highly significant (21.3%) in W+C and C+W, respectively. It then declined greatly with increases in allelochemicals application as compared with respective controls. Application of allelochemicals at 1% elevated the level of DNA and RNA but the DNA of C+W decreased by 1.4%. The highest applied concentration of allelochemicals produced the maximum reduction in the nucleic acids, DNA and RNA which reached (57.6 and 58.2%) and (58.2 and 38.4%) in W+C and C+W, respectively. It is interesting to mention that the inhibitory effect exerted by application of higher concentration on DNA was more than RNA as compared with respective controls.

Identification of phenolic composition in whole chard plant by HPLC

The analysis of plant phenolics using HPLC was based on the comparison of the retention time of a mixture of standard phenolics with those in plant samples. Each compound was quantified by peak area measurement relative to the standard peak area. Table 1 shows the retention times of a mixture of twenty five standards phenolic detected at 254 nm. Figure 6 shows the chromatogram obtained from HPLC analysis. Eight phenolic

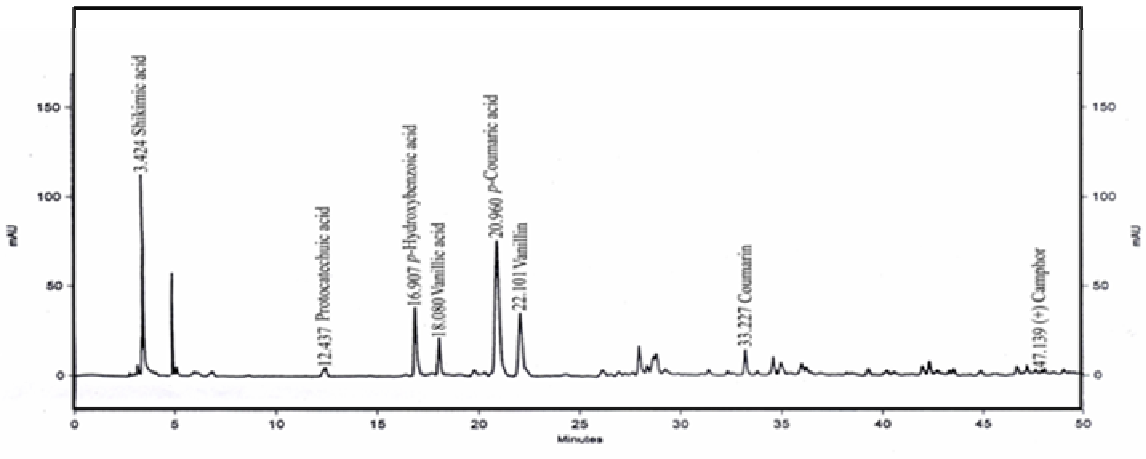

Figure 6. HPLC chromatogram of a whole chard plant phenolic aglycons. Wave leangth of the dector was fixed at 245 nm.

Table 1. The qualitative and quantitative analysis of phenolic aglycones of whole chard plant extract using HPLC.

Standard phenolic compounds	Retention time (min.)		Concentration (µg g^{-1} Dry weight)
	Standard	Sample	
Shikimic acid	3.424	3.424	1004.25
Gallic acid	8.160	-	
Pyrogallic acid	9.248	-	
Protocatechuic acid	12.736	12.437	5.063
Resorcinol	13.739	-	
Chlorogenic acid	16.331	-	
Caffeine	16.683	-	
Catechol	16.725	-	
P-Hydroxybenzoic acid	16.832	16.907	37.188
Caffeic acid	18.016	-	
Vanillic acid	18.037	18.080	13.625
Syringic acid	18.379	-	
m-Hydroxybenzoic cid	18.752	-	
p-Coumaric acid	22.208	20.960	23.625
Vanillin	22.411	22.101	17.438
Sinapinic acid	22.352	-	
Scopoletin	24.437	-	
Ferulic acid	24.853	-	
o-Coumaric acid	29.333	-	
Salicylic acid	30.389	-	
Coumarin	32.704	33.227	3.186
Cinnamic acid	36.149	-	
Trans- Cinnamic acid	36.171	-	
Apigenin	37.984	-	
(+) Camphor	47.861	47.189	365.813

compounds identified in water extract of plant tissues. The total phenolic aglycones reached 1.47 mg per gram dry weight, found as water-soluble secondary products. Among the identified aglycones in whole chard plant extract, shikimic acid and (+) camphor were the major phenolics (68 and 24%), respectively while both coumarin and protocatechuic acids were recorded in small amounts (0.2 and 0.3%), respectively. The other compounds in-

cluded *p*–hydroxybenzoic, *p*- coumaric and vanillic acids and vanillin accounted by about 2.5, 1.6, 0.9 and 1.2%, executively.

DISCUSSION

Eight phenolic compounds were identified and quantified in the water extract of chard residue using HPLC analysis. Some of the phenolic acids similar to that identified in chard residue were reported to play an important role in allelopathic interactions, and their biological activities on growth of some crop plants and weeds were studied using different bioassay tests. Chung et al. (2002) demonstrated that *p*-hydroxybenzoic, *p*-coumaric acids were the most active compounds in rice hull extracts which have inhibitory effect on the growth of barnyardgrass seedlings. Allelopathic activity in field situations is thought to be often due to joint action of mixtures of allelochemicals rather than to one allelochemical that has synergetic inhibitory or stimulatory effect depending on their concentrations (Einhellig, 1995; Blum, 1996; Inderjit, 1996). This indicated that all extracts above 0.25% can effectively control the establishment of chard, whereas 1% had no harmful effect on wheat. This response is attributed to the type and amount of phenolic compounds present in the extract. Wheat was identified as allelopathic crop (Lodhi et al., 1987; Rao and Pandya, 1992; Wu et al., 2001; Bertholdsson, 2005), containing secondary metabolites of allelopathic potential which may play a role in chard control. It is widely accepted that the production of secondary metabolite, particularly phenolic compounds, can play direct role in self-defense and plant protection to cope with the stress created by external conditions (Bennett and Wallsgrove, 1994; Dixon and Paiva, 1995).

Our data demonstrate that the stimulatory and inhibitory effect of whole plant chard extract is a function of concentration and species dependent. This result agrees with the view of Rice (1984), that allelopathic compounds that were inhibitory at some concentration and were stimulatory to the same processes in very small concentrations. The response to the allelochemicals varied according to the sensitivity of test plants (Jadhav and Gaynar, 1992; Nsolomo et al., 1995) Although the negative effect of higher concentration of whole plant chard allelochemicals is often assumed that the response of seeds or seedlings to plant extracts is due entirely to allelopathy, the extract may also exert negative osmotic effects on the test species (Bell, 1974). Generally, possible mechanisms suggested for the reduction in seedling growth, are the inhibiting effect on the organelle membranes which produce adenosine triphosphat (ATP), resulting in lower ATP production due to the displacement of the mitochondrion and photosynthetic electron flow. During the initial germination stages and in the post germination stages, the mitochondrion oxidative phosphorilation is the main ATP synthesis agent (Morohashi and Suguimoto, 1988). The strong inhibitory effects of extract on roots might have been caused by the fact that roots were in direct contact with the extract and subsequently with inhibitory chemicals as described in earlier works with various crops and weeds (Bhowmik and Doll, 1984; Qasem, 1995).

Under the different treatment of whole chard residue extract at 1% caused the accumulation of phenolic aglycone in chard seedlings that plays a role in chard inhibition and this change may be attributed to absorption of water-soluble aglycone from external medium. On the other side wheat seedlings contained a high level of phenol glycoside that acts as defensive and protective compounds in plants (Kleiner et al., 1999).

Autotoxic behaviour has been reported in chard which agreed with Edwards et al. (1988) who indicated that pokeweed possesses autotoxic behavior. This autotoxic behaviour could be involved in germination and seedling development control (Alias et al., 2003). In connection with the allelopathic potential, four chenopod species were susceptible to allelopathy by extracts isolated from leaves of their own respective species (Jefferson and Pennacchio, 2003).

The adverse changes in amylase activity could be attributed to interference of allelochemicals with enzymes action (Einhellig, 1986; Abu El-Soud, 2001). The enhancement of amylase activity could accelerate starch hydrolysis and hence the utilization of soluble sugar during seed germination. On the other hand, the depression in amylase activity as the result of exposure to high level of allelochemicals could suggest the retardation of starch hydrolysis and hence delay seed germination.

This stimulation was obvious in wheat and chard seedlings treated with aqueous whole chard extract at (0.25 and 1%) and (0.25%), respectively. These findings are in agreement with that of Baziramakenga et al. (1997) who reported that the low concentrations of allelochemicals vanillic and ferulic acids stimulated the biosynthesis of nucleic acids by increasing the incorporation of ^{32}P into DNA in soybean seedlings. Inversely, allelochemicals treatments at higher concentration gradually reduced the contents of both DNA and RNA and this reduction was more or less similar in DNA as well as RNA, and concentration dependent. Additionally, Seigler (1996) demonstrated that allelopathic compounds interact with nucleic acid metabolism causing modification of DNA and RNA. The obvious reduction in the DNA and RNA levels by high levels of the applied allelochemicals coincides with its depressive effect on the activity of amylase enzyme. This agrees with the general knowledge of enzyme activity and the contents of nucleic acids (Finer et al., 1969). In this regard, Schuab et al. (2001) reported that treatment of *p*-hydroxybenzoic acid at 0.1 and 1 mM caused a decrease in radicle protein content of (Glycine max (L.) Merrill), and by increasing the applied concentration up to 10 mM, the protein content increased. This

could be attributed to inhibition of enzyme activity which degrades the seed reserve substances. It is important to mention that the pattern of changes in protein contents by allelochemicals treatment follow the same patterns of changes in nucleic acids fractions. Cameron and Julian (1980) showed that phenolic acids reduced incorporation of ^{14}C-tyrosine into protein in *Lactuca sativa* seedlings.

Conclusion

The present study showed that chard is one of autotoxic species. This autotoxic behaviour could be involved in the species' own population control. Immersing of wheat grains and chard seeds in the concentration (1%) of whole chard plant extract before sowing may play a good role in enhancing the germination of wheat and inhibiting that of chard.

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