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ORIGINAL ARTICLE

Evaluation of Allelopathic Potential of *Rumex dentatus* Root Extract and Allelochemicals on *Cicer arietinum*

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The present study aimed to evaluate the allelopathic potential of root extract of *Rumex dentatus* L. and various allelochemicals on some physiological parameters in roots of *Cicer arietinum* L. The tested allelochemicals were benzoic, caffeic, cinnamic, ferulic, gallic, sinapic and vanillic acids. Seed germination of Cicer was inhibited by Rumex extract. Lipid peroxidation and hydrogen peroxide production increased gradually in response to extract concentration as well as allelochemicals treatment. The total soluble protein decreased whereas the total phenol increased under the various treatments. The activities of superoxide dismutase (SOD: 1.15.1.1) and catalase (CAT: 1.11.1.6) increased continuously with increasing extract concentration. However, peroxidase (POD: 1.11.1.7) increased sharply at 2% (w/v) followed by reduction at the higher concentrations and reached 1.1 U g-1 fresh weight at 10% (w/v) which was still higher than that of the control value. Ferulic acid was the most effective inducer for SOD activity followed by cinnamic acid. The POD activity increased remarkably particularly with cinnamic, benzoic and gallic acids. Sinapic, ferulic and coumaric acids enhanced CAT activity by 150.4%, 139.5% and 124.4%, respectively. The results reveal the possible use of R. dentatus as bioherbicide.

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The excessive and continuous use of synthetic herbicides have many negative impacts consequences on human health and environment and lead to increasing herbicidal resistance in many weed species (Yuan-quan *et al*., 2012). Scientists try to find biological eco-friendly solutions to minimize the perceived hazardous impacts from chemical herbicides and insecticides in agriculture production.

Allelopathy has a significant role in research involving sustainable agriculture, like biological weed and pest control. Allelopathic interactions are mediated by secondary metabolites, released through leaching, root exudation, volatilization and residue decomposition into the environment and affect growth and development in natural environments and agro-ecosystems (Cheema *et al*., 2013). These metabolites are known as allelochemicals which belong to a diverse range of chemical groups, and they have different sites and

modes of action. Allelochemicals may be distributed broadly among organs such as seeds, flowers, pollen, leaves, stems, and roots, or sometimes found in just one or two such locations (Zeng *et al*., 2008).

Phenolic compounds are one of the most diverse allelochemicals that interfere in several physiological processes associated with seed germination as well as plant growth and development (Weir *et al*., 2004). In particular, caffeic, ferulic, *p*-coumaric, hydroxybenzoic, sinapic, syringic, vainillic and phthalic acid have a phytotoxic effect on plant physiology (Lara-Nunez *et al*., 2009).

R. dentatus (Polygonaceae) is a widely spread weed in many countries including Egypt. It is distributed in many habitats including waste places, canal bank, orchards and cultivated fields. It has been used as a leafy vegetable in the Mediterranean diet (Hadjichambis *et al*., 2008). *R. dentatus* contains various bioactive phytochemicals including emodin, aloe-emodin, chrysophanol, physocin, parietin, nepodine (Choi *et al*., 2004), gallic, vanillic, isovanillic, cinnamic, *p*-hydroxycinnamic, syringic, benzoic, ferulic, succinic acids, anthraquinones and flavonoids (Zhang *et al*., 2012). Caffeic acid was reported in *R. aquatic* (Lee *et al*., 2011). Sinapic acid, benzoic acid, ferulic acid, coumaric acid and vanillic acid were recorded in *R. acetosa* (Kucekova *et al*., 2011).

These bioactive constituents characterized *R. dentatus* by various biologically activities such as antioxidant (Humeera *et al*., 2013), anti-inflammatory (Suleyman *et al*., 1999), antitumor, astringent and anti-dermatitis (Litvinenko and Muzychkina, 2003).

Many studies have reported the allelopathic activity of *Rumex* species such as *R. obtusifolius* (Zaller, 2006), *R. dentatus* (Anwar *et al*., 2013), *R. crispus* (Pilipavicius *et al*., 2012). On the other hand, many authors reported various allelopathic potential against *R. dentatus* (Anjum and Bajwa, 2007).

The mode of action of *R. dentatus* allelopathy on different plant physiological parameters is still not established. The major allelochemicals of plants are phenolic acids which exerts a biological stress (Lara-Nunez *et al*., 2009). Many studies have shown that phenolic compounds increase the generation of reactive oxygen species (ROS) and consequently produce oxidative stress (Cruz-Ortega *et al*., 2007). These ROS are toxic by-products of aerobic metabolism, key growth and development regulators or defense pathways (Mittler, 2002).

This study aimed to evaluate the allelopathic potentiality of *R. dentatus* root and different allelochemicals (reported in *Rumex*) on some physiological parameters of *C. arietinum* root. These parameters are lipid peroxidation, content of hydrogen peroxide, protein and phenol as well as the activities of antioxidant enzymes.

MATERIALS AND METHODS

Extract preparation

R. dentatus roots were washed several times with tap water, cut into small pieces of 5 cm in diameters. Roots were oven-dried at 60 °C for 48 h, and homogenized to fine powder using a grinder. About 250 g of the homogenized material was soaked in 1 L of distilled water and allowed to shake on orbital shaker for 24 h at room temperature (23-28 °C) for extraction. The extract was filtered using a Buchner funnel and Whatman No 1 filter paper. Various concentrations (2, 4, 6, 8 and 10% w/v) were prepared.

Germination bioassays

Seeds of *C. arietinum* were surface sterilized in

10% sodium hypochlorite for 10 min and then soaked in running tap water for 24 h. The seeds were then germinated between paper towels, moistened with distilled water (control) or different extract concentrations in sterilized plastic trays and were covered and incubated in dark at 25 °C for 72 h (El-Shora and Abo-Kassem, 2001).

Treatment with *Rumex* extract

The germinated seeds in a control treatment with well-grown *C. arietinum* root were then supported on plastic bowls containing 0.2 mM $CaCl_2$ solution and different concentrations of *Rumex* root extract and vigorously aerated for 5 day.

Enzymes assay

Root samples were prepared for enzyme analyses by homogenization of the fresh tissue material (5 g) with a mortar and pestle and a small amount of sand in a solution containing 50 mM potassium phosphate buffer (pH 7.0), 10% polyvinylpyrrolidone (PVP), 0.2 mM EDTA and 10 mL Triton X-100. After the homogenate was centrifuged at 12000 g for 20 min at 4 °C, the supernatant was used for immediate determination of enzyme activities.

Determination of superoxide dismutase (SOD) activity

Activity of SOD was assayed by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) (Becana *et al*., 1986). The reaction medium comprised 0.25 mL 50 mM Na–phosphate buffer (pH 7.8) with 0.1 mM Na_2-EDTA, 2.73 mL $O_2^{\cdot}$ generating solution and 20.45 mL extract. The $O_2^{\cdot}$ generating solution contained 2.2 mM riboflavin, 14.3 mM methionine, and 82.5 mM NBT. Glass cells containing the mixture were placed in a cylindrical bath lined with aluminum foil at 25°C and fitted with a 22 W fluorescent lamp. The reaction was initiated by turning the light on and the reduction of NBT was followed by reading the absorbance at 560 nm for 10 min. Blanks were run the same way but without illumination. One unit of SOD was defined as the amount of enzyme which produced a 50% inhibition of NBT reduction under the assay conditions.

Determination of catalase (CAT) activity

Catalase activity was measured by following the consumption of H_2O_2 at 240 nm. One mL reaction mixture contained 20 µl of the enzyme extract, 50 mM sodium phosphate buffer (pH 7.0) and 10 mM H_2O_2. The reaction was initiated by adding the protein extract. For each measurement, the blank corresponds to the absorbance of the mixture at zero time and the actual reading corresponds to the absorbance after 1 min. One unit of CAT activity was defined as 0.01 decrease in absorbance at 240 nm per mg protein per min (Aebi, 1984).

Determination of peroxidase (POD) activity

One mL reaction mixture contained 50 mM sodium phosphate buffer (pH 6.9), 3.2 mM guaiacol, 0.4 mM H_2O_2 and 20 µl of the enzyme extract. The reaction was initiated by adding the enzyme extract. The mixture was allowed to stand for 3 min and the absorbance was measured at 470 nm against a blank without the enzyme. One unit of POD activity was defined as 0.01 increases in the absorbance at 470 nm per mg protein per min (Chance and Maehly, 1955).

Lipid peroxidation analysis

Fresh plant leaves (0.2 g) were homogenized and extracted in 10 mL of 0.5% (w/v) thiobarbituric acid (TBA) made in 5% (w/v) trichloroacetic acid (TCA). The extract was heated at 95 °C for 15 min and then quickly cooled on ice (Zhang *et al*., 2008). After centrifuging at 5000 g for 10 min, the

absorbance of the supernatant was measured at 532 nm. Correction of nonspecific turbidity was made by subtracting the absorbance value taken at 600 nm. The MDA was calculated using an extinction coefficient of 155 mM cm^{-1}.

Determination of hydrogen peroxide (H_2O_2)

Fresh leaf tissue samples were ground in cold acetone (10% w/v) and centrifuged at 3000 g for 10 min. One milliliter of the supernatant was mixed with 0.1 mL titanium reagent (20% $TiCl_4$ in concentrated HCl) and 0.2 mL of 17 M ammonia solution and then centrifuged at 3000 g for 10 min. The precipitate was washed five times with acetone by resuspension and dissolving in 3 mL of 1 M H_2SO_4. The absorbance of the solution was measured at 410 nm against blanks, which had been prepared similarly but without plant tissue (Al-Aghabary *et al*., 2004).

Determination of total phenol

The amount of phenol in the root extract of *R. dentatus* was determined spectrophotometrically. An aliquot of the extract was mixed with 5 mL Folin-Ciocalteu reagent and 4 mL (75 g/L) of Na_2CO_3. The test tubes were vortexed for 15 s and allowed to stand for 30 min at 40 °C for color development. Absorbance was measured at 765 nm. Results obtained were expressed as mg g^{-1} of gallic acid equivalent (Sadasivam and Manickam, 2008).

Determination of protein content

The protein concentration was measured by the method of Lowery (Lowry *et al*., 1951).

Treatment with allelochemicals

Seedling of 5-days were incubated in 50 mL of half-strength Hoagland's solution containing 10 mL of benzoic, caffeic, cinnamic, coumaric, ferulic, gallic, sinapic and vanillic acids at 0.5 mM. Benzoic and cinnamic acids were dissolved in ethanol and added to the nutrient solution. Seedlings were incubated under a photosynthetic flux of 430 µmole m^{-2} s^{-1} and 28 °C. SOD, CAT, POD, MDA, H_2O_2, protein and total phenol were determined as previously mentioned.

The experimental design was carried out as a randomized complete block (RCB) with three replications. The inhibition percentage of germination was calculated. The data were subjected to ANOVA and the mean values were separated based on Least Significant Difference (LSD) at 0.05 probability level using COSTAT 6.3 program.

RESULTS

Allelopathic effect of *R. dentatus* extract on germination

The allelopathic activity of *R. dentatus* root aqueous extracts on germination inhibition of *C. arietinum* is shown in Fig. 1. After five days of treatment, all concentrations significantly inhibited the germination of *C. arietinum* (*P≤0.05*). The inhibition was concentration-dependent. It was observed that the higher concentration 10% (w/v) of root extract inhibited the germination by 34.9%.

Effect of *R. dentatus* extract on antioxidant enzymes

The activities of both SOD and CAT in *C. arietinum* L. roots significantly increased continuously (*P≤0.05*) when the seedlings of *C. arietinum* were subjected to various concentrations of *R. dentatus* root extract (Fig. 2). However, POD was significantly increased at 2% compared to the control followed by a continuous decrease in response to the higher concentrations. It was observed that the higher concentration 10% (w/v) of root extract enhanced the activity of SOD, POD and CAT by 143.7%, 37.5% and 191.0%, respectively.

Effect of *R. dentatus* extract on MDA and H_2O_2 contents

The results in Fig. 3 show that MDA and H_2O_2 contents in *C. arietinum* roots were significantly increased (*P≤0.05*) upon treatment with *R. dentatus* root extract and the increase was concentration-dependent. MDA increased by 279.0% while H_2O_2 increased by 191.0% at concentration 10% (w/v) respects to control.

Effect of *R. dentatus* extract on total phenol

Total phenol content increased significantly by subjecting the root of *C. arietinum* to the different concentrations of *R. dentatus* extract (*P≤0.05*) (Fig. 4). The total phenol content increased from 34.3% at the lower concentration (2% w/v) to 191.4% at higher concentration (10% w/v).

Effect of *R. dentatus* extract on protein content

The effect of *R. dentatus* root extracts on the protein content of *C. arietinum* roots was shown in Fig. (4). Protein content expressed significant decrease by increasing the extract concentration. The highest concentration (10% w/v) of the extract showed 70% decrease with respect to control.

Effect of allelochemicals on antioxidant enzymes

Among the tested acids, ferulic acid was the most inducer, which significantly increased SOD activity (*P≤0.05*), followed by cinnamic acid. The other remained acids expressed more or less comparable effects (Fig. 5). The POD activity in *C. arietinum* root was increased remarkably with cinnamic, benzoic and gallic acids. The rest of the tested acids expressed various percentages of increase ranged between 1.3 U g^{-1} fresh weight for coumaric and 2.3 U g^{-1} fresh weight caffeic acid (Fig. 5). CAT activity was enhanced by various acids used. The most effective acids were sinapic, ferulic and coumaric which enhanced the CAT activity by 150.4%, 139.5% and 124.4% respectively.

Effect of allelochemicals on MDA and H_2O_2 contents

The increase in MDA content was observed in *C. arietinum* root subjected to the various phenolic acids. Benzoic, sinapic, gallic and coumaric acids increased the content by 200%, 162.9%, 130.6% and 112.9%, respectively. The other remaining acids showed appreciable significance increase in MDA content (*P≤0.05*) ranged between 64.5% with cinnamic and 103.2% with vanillic acid (Fig. 6). All acids showed significant increase in H_2O_2 content in *C. arietinum* root (Fig. 6). Caffeic, cinnamic, sinapic and gallic acids increase the content by 371.1%, 279.9%, 279.7% and 232.6%, respectively.

Effect of allelochemicals on total phenol

All the allelochemicals significantly induced the total phenol content in *C. arietinum* root (Fig. 7). Caffiec, ferulic and benzoic were the most inducer. They induced phenol by 99.7%, 60.0 and 50.3%, respectively. The other remaining acids expressed moderate significance increase in total phenol content (*P≤0.05*) ranged between 2.0% with vanillic and 42.3% with cinnamic acid.

Effect of allelochemicals on protein content

All the tested allelochemicals significantly reduced total protein (*P≤0.05*) content in *C. arietinum* root (Fig. 7). Coumaric, vanillic and sinapic acids were the most potent inhibitors of protein where they reduce the content to 20.0%, 33.3% and 46.7% of the control. All other tested acids reduced the content with various rates.

DISCUSSION

The germination of *C. arietinum* seeds in the present study was inhibited by various concentrations of *R. dentatus* root extract. The inhibition is probably due to the presence of

phenols in the root of the *R. dentatus*. Phenols have been reported to interfere with the activities of respiratory enzymes in seed germination, thereby causing inhibitory effect on its germination (Lara-Nunez *et al*., 2009). This is may be due to the alteration of synthesis or activities of gibberellic acid (Olofsdotter, 2001), a hormone responsible for the stimulation of seed germination. It has been suggested by Saeid *et al*. (2010) that alteration in the synthesis or activities of gibberellic acid in the seed could be due to the presence of phenolic compounds.

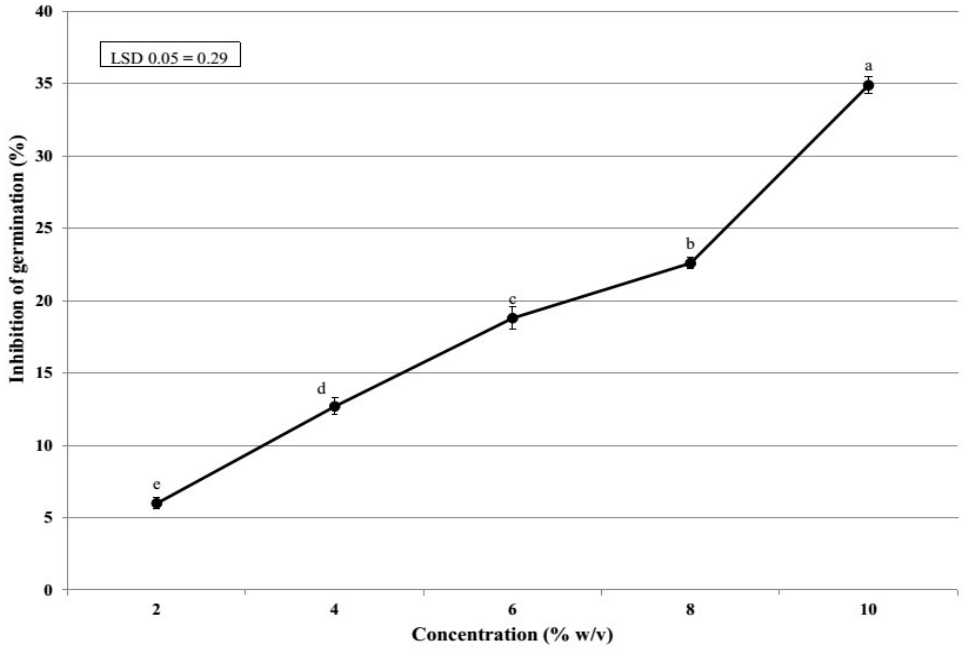

Figure 1 : Effect of different concentrations of *Rumex dentatus* root extract on germination of *Cicer arietinum*.

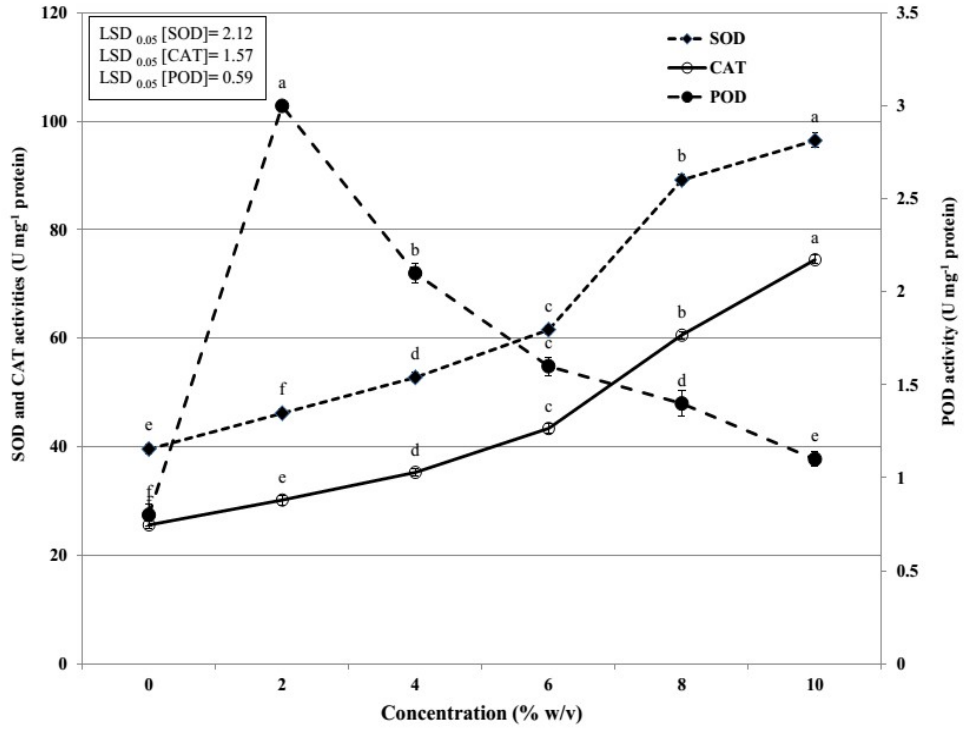

Figure 2 : Effect of different concentrations of *Rumex dentatus* root extract on the activities of antioxidant enzymes (SOD, POD and CAT) in *Cicer arietinum* root.

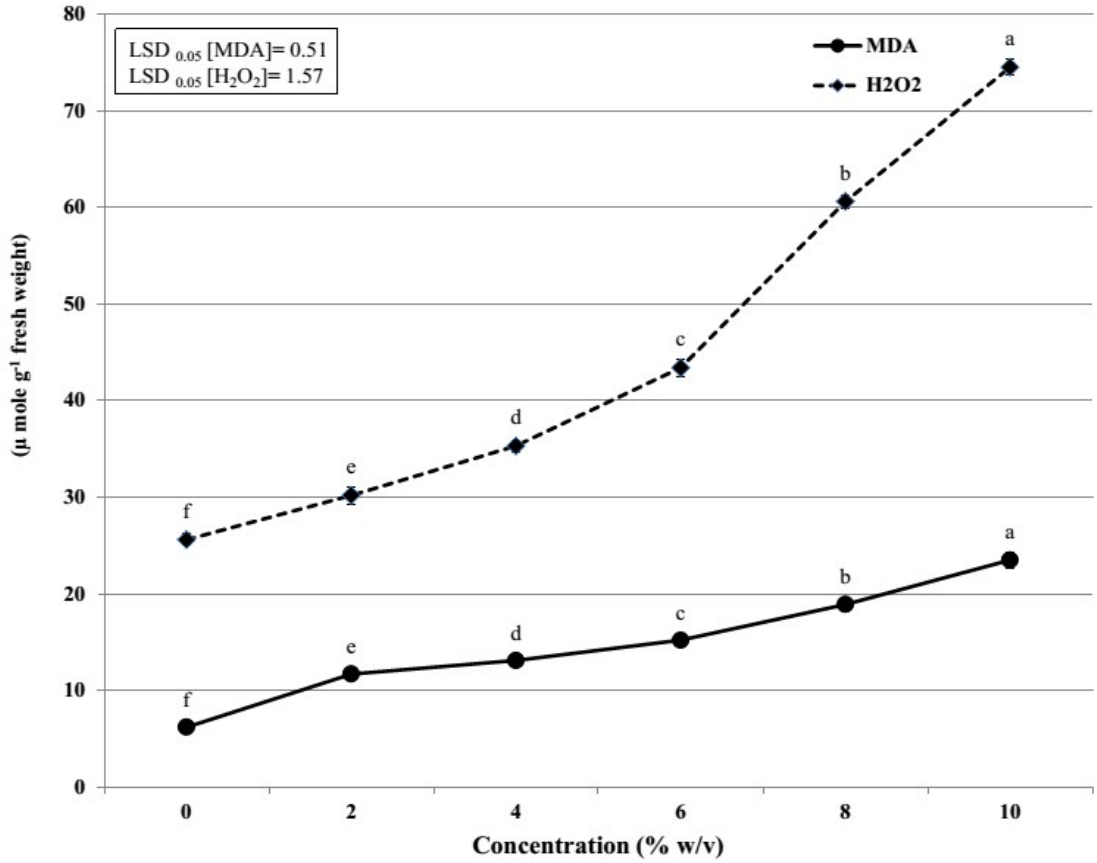

Figure 3 : Effect of different concentrations of *Rumex dentatus* root extract on lipid peroxidation and hydrogen peroxide contents in roots of *Cicer arietinum*.

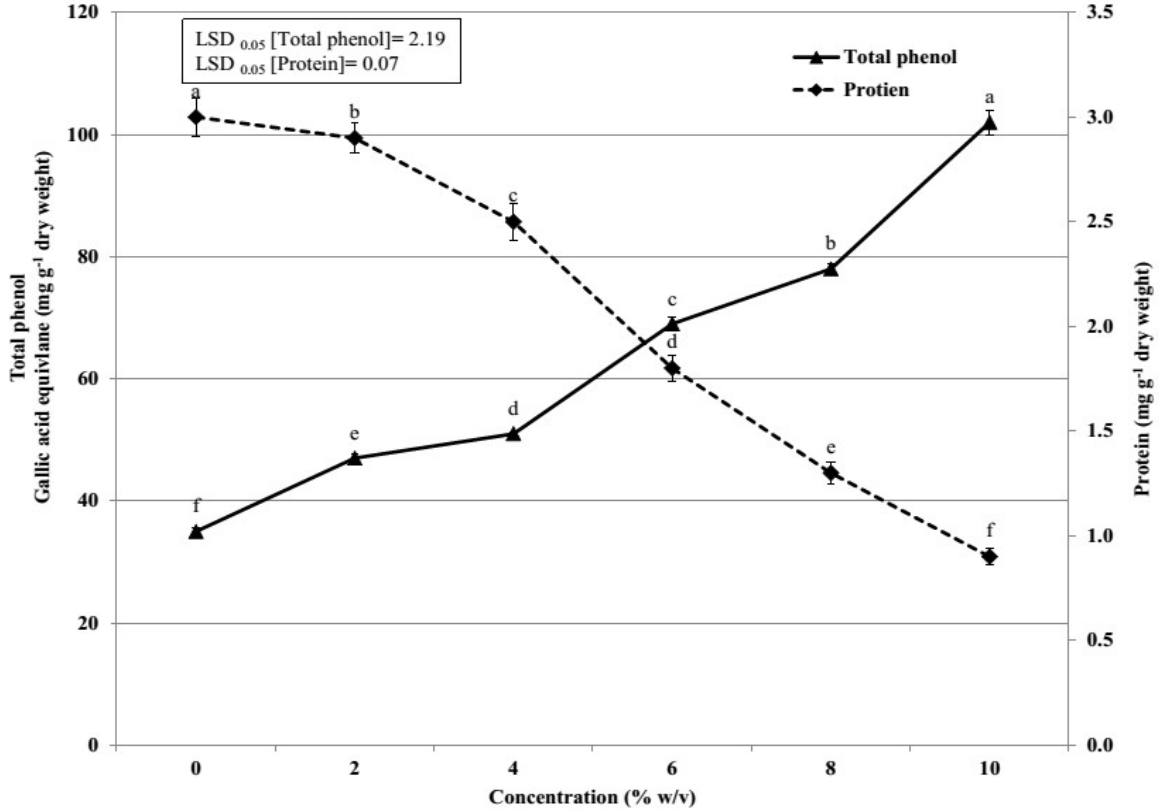

Figure 4 : Effect of different concentrations of *Rumex dentatus* root extract on protein and total phenol contents in roots of *Cicer arietinum*.

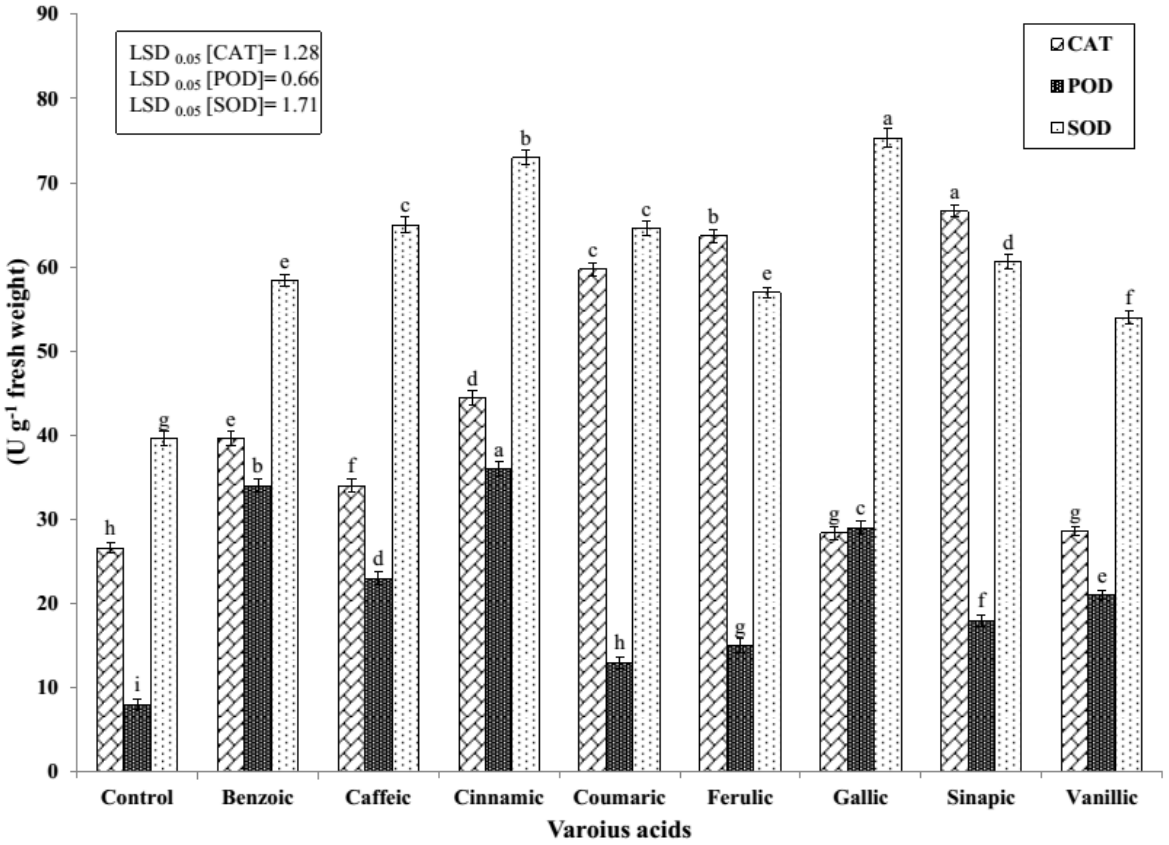

Figure 5 : Effect of various allelochemicals on the activities of antioxidant enzymes (SOD, POD and catalase) in *Cicer arietinum* roots.

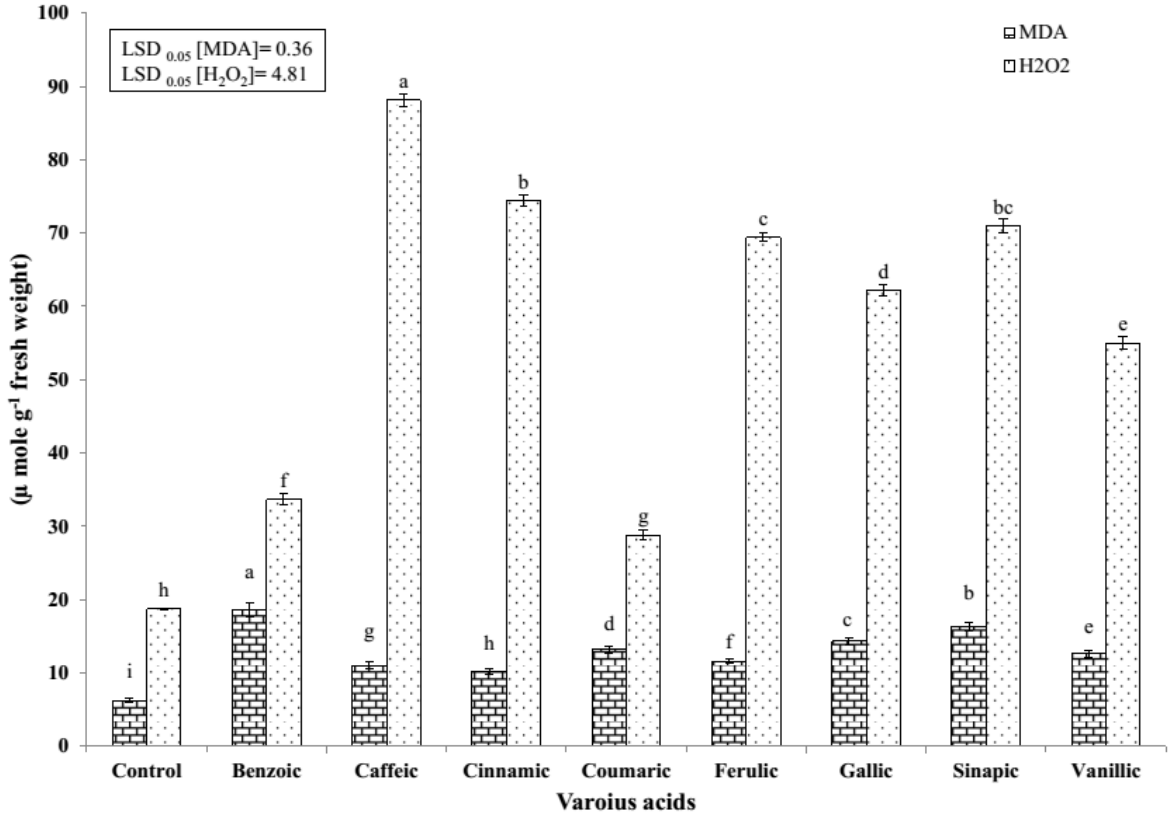

Figure 6 : Effect of various allelochemicals on hydrogen peroxide and lipid peroxidation contents in roots of *Cicer arietinum*.

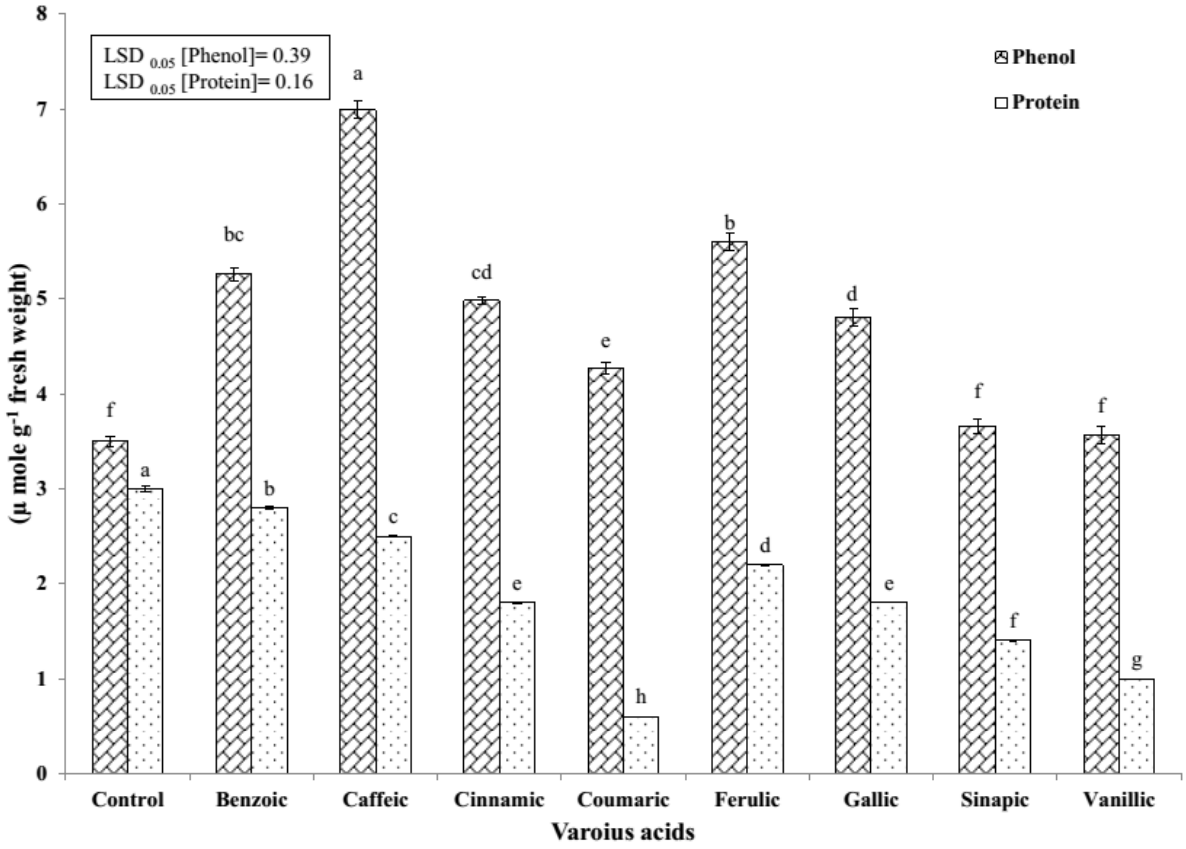

Figure 7 : Effect of various allelochemicals on protein and total phenol contents in roots of *Cicer arietinum*.

Superoxide dismutase is the key enzyme and first line of defense in the plant cell controlling the oxidative balance within the cell. The gradual increase in SOD activity in response to increasing root extract suggested an increased production of superoxide radicals in treated root. The induction of SOD in a concentration-dependent manner suggests that the oxidative stress condition led to an increased antioxidant capability of root cells. However, this increase may not match the production of ROS thus resulting in an increased peroxidation of cell membrane in root cells (Shalata and Tal, 1998).

Treatment of root with phenolic acids as allelopathic agents resulted in increasing the activities of SOD and POD, two important enzymes for detoxification of overproduced ROS. The marked increase in the antioxidant enzymes (SOD, POD and CAT) have been observed in other studies on the mode of action of allelochemicals such as phenol. Also, report of Niakan and Saberi (2009) revealed an increase in activities of antioxidant enzymes in target plants treated with phenolic compounds at low levels.

The increased activities of SOD and POD in soybean seedlings after treatment with benzoic and cinnamic acids reported by other author Baziramakenga *et al*. (1997). SOD and POD were stimulated by exposure of plant to environmental stress such as salinity, UV radiation, drought, heavy metals, temperature extremes, nutrient deficiency, air pollution, herbicides and pathogen attacks (Gill and Tuteja, 2010). SOD and POD activities were the most important protective enzymes in active oxygen eliminating system and there is a certain relation between the resistance to stress and the

change in plant protective system (Jinhu *et al*., 2012). At low doses, phenolic compounds such as caffiec, ferulic and vanillic acids have been reported to stimulate the antioxidant enzyme activity (Hegab and Ghareib, 2009). On the other hand, at high concentration, plant phenolic compounds suppress protein content and increase lipid peroxidation (Hegab and Ghareib, 2009). Generally, the present results illuminate that the effect of the allelopathy may directly induce the increase of antioxidant enzyme activities to eliminate ROS.

The present results indicate that the higher concentrations of *R. dentatus* root extract or allelochemicals resulted in an increase of MDA content. These results are in consistent with other studies (Ghareib *et al*., 2010) for tomato plants treated with high level of phenolic extract. Benzoic acid and cinnamic acid themselves damages cell membrane integrity by a decrease in SH groups (Baziramakenga *et al*., 1997). Both compounds induced lipid peroxidation that resulted from free radical formation in membrane. Oxidation or cross-linking of plasma membrane SH groups was suggested as this first mode of action. Hence, it seems likely that derivatives of cinnamic and benzoic acid cause structural changes in membrane that include alterations in a variety of membrane protein.

Allelochemicals can damage cell membrane through interaction with the membrane constituents (Garcia-Sanchez *et al*., 2012). The damage can also occur because of an impairment of some metabolic function necessary to the maintenance of membrane function (Rice, 1984). Foyer *et al*. (1994) stated that environmental stresses could produce ROS which brings about membrane peroxidation leading to membrane damage. The toxicity of many quinones and phenols can largely be attributed to the formation of semiquinone radicals that donate electrons to molecular oxygen by forming O_2^- (Weir *et al*., 2004) and H_2O_2 (Cruz-Ortega *et al*., 2007). Also, other study Kaushik *et al*. (2010) reported that catechin from *Centaurea stoebe* induced ROS mediated phytotoxicity in *Arabidopsis thaliana*.

Allelochemicals are known to be involved in many ways in production of active oxygen species (Imatomi *et al*., 2013). It is conceivable that a decrease of enzymic and non-enzymic free radical scavengers caused by stress (Salt *et al*., 1995) may also contribute to the shift in the balance of free radical metabolism towards H_2O_2 accumulation, and H_2O_2 and O_2^- may interact in the presence of certain metal ions or metal chelates to produce the highly reactive hydroxyl radical (OH^-). The increased H_2O_2 and OH^- production might be involved in the lipid peroxidation observed in *C. arietinum* root.

Total phenol content increased by subjecting the root of *C. arietinum* to the *R. dentatus* extract and allelochemicals. The increase in phenol content is attributed to the response of plant defense system against allelochemicals which cause reduction of harmful ROS (Garcia-Sanchez *et al*., 2012).

The reduction in total soluble protein at the highest concentration may be attributed to the effect of allelochemicals on DNA replication or transformation by intercalation with nucleic acids by ionic bonding with their negatively charged phosphate groups. The protein content was stimulated by lower concentration of *R. dentatus* root extract or allelochemicals. This stimulation correlated with stimulation in nucleic acid content. As *p*-coumaric acid increased incorporation of 3S S methionine into protein (Baziramakenga *et al*., 1997).

The protein content was decreased by the

highest application of allelochemicals and this may be due to the accumulation of phenolic glycine that interferes with the cytoplasmic ribosomes and production of RNA, which in turn inhibited protein synthesis (Hegab and Ghareib, 2010). In this respect, it has been reported that cinnamic acid derivatives depressed translation activity of polysomal mRNAase of been cells that reduced the protein synthesis (Bolwell *et al*., 1988). The increase in the antioxidant system of *C. arietinum* under treatment with *R. dentatus* root extract could be attributed to the ability of extract compounds to improve the scavenging system (Vaidyanathan *et al*., 2003).

In conclusion, since phenolic allelochemicals are broadly found throughout the plant kingdom, their concentration and differences in the sensitivity of receiving species are keys to their allelopathic action. At very low concentrations, they may be stimulatory, whereas higher concentrations inhibit functions in the receiving species. Derivatives of cinnamic acid and benzoic acid, coumarins and various phenolic compounds have multiple target sites whereby they alter the physiology of plants. Also, it may be concluded that allelochemicals first act by altering permeability and protein functions of the cell membrane. The present results suggest that *R. dentatus* root extract could be used as bioherbicide.

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