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Investigating the use of pine needle extract as biological herbicide for weed control in bean crop

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Abstract

Aim : The study was undertaken to investigate the inhibitory effect of pine needle extract (*Pinus eldarica*) on the vegetative growth of weeds found in bean fields.

Methodology : Aqueous extracts [25, 50, 75 and 100% of pine needles were applied to common beans (*Phaseolus vulgaris*) and weeds (prosomillet and redroot pigweed). The effect extract on seedling growth, physiological and biochemical characteristics were subsequently investigated. The experiment was carried out completely a randomized design with three replicates. Phytochemical compounds of pine needle extracts were determined spectrophotometrically.

Results : Extracts of pine needles increased the Chl a, Chl b, total Chl and carotenoid contents of bean varieties in comparison to control; while the level of these pigments were considerably low in weeds. The lowest content of proline and phenolic compounds, as well as the highest peroxidation of biomembrane lipids was detected in redroot pigweed

treated with 100% needle extract. Although peroxidase and catalase activities dramatically increased in bean varieties proportional to the extract concentration applied. Auxin oxidase and nitrate reductase activities in both weeds significantly ($p < 0.05$) decreased due to treatment. The sensitivity of weeds to allelochemicals was higher as compared to bean varieties. Content of phenolics and proanthocyanidins were also high in pine needle extracts, which was correlated to the inhibitory effect on the weeds studied.

Interpretation : The results of this investigation point to a positive effect of the use of Eldar pine needle extracts as a natural herbicide in comparison to the chemical counter parts.

Key words: Allelopathic potential, Biological herbicide, *Pinus eldarica*, Prosomillet, Redroot pigweed

Pinus eldarica Medw.
Aqueous extraction of needles

Bean crop and its weeds in phytotron
Irrigation with needle extracts

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Introduction

Allelopathy is defined as interaction between plants and microorganisms in the form of stimulation or inhibition (Molisch, 1937). This natural phenomenon of production and release of secondary metabolites and interaction among living organisms has now become an applied subject of study in plant science. In addition, playing an important role against biological and non-biological stresses, secondary metabolites of plant species and allelopathy, prevent plants from being eaten, attract insects and animals, disintegrate pollen and seeds and play a role in plant nutrition (Taiz and Zeiger, 2006). Once released into the rhizosphere, these allelopathic compounds have a regulatory effect on the solubility, mobility, chelation and release of mineral elements (Jabran *et al.*, 2013). Release of allelochemicals in the environment can contribute to irrigation and rainfall (Aslam *et al.*, 2017), evaporation through stomata (Latif *et al.*, 2017), decomposition of plant residues (Sharma *et al.*, 2000) and exudation from root system (Dahiya *et al.*, 2017). Weeds pose a major threat to the natural environment and compete with crops for light, air, water, nutrients and space (Farooq *et al.*, 2013). They reduce the crop yield by 10-70% (Gidea *et al.*, 2016). Today, global efforts in modern agriculture are gearing towards reducing the use of chemicals by introducing new biological and ecological methods to control weeds. One of these methods is use of allelopathic compounds that play an effective role in controlling and eliminating weeds. Studies have shown that allelochemicals are more effective (Cheng and Cheng, 2015), more specific in function and less destructive as compared to chemical herbicides (Parasanta *et al.*, 2003).

Eldar pine or Afghan pine (*Pinus eldarica*) is an evergreen tree, indigenous to Iran, Afghanistan, Pakistan and Russia. Its rapid growth is remarkable (Michelozzi *et al.*, 2008). The allelopathic property of this plant prevents germination and growth of weed seedlings (Arouiee *et al.*, 2010). The most important secondary metabolites of pine needles are terpenoids, polyphenols and tannins (Lee, 2003), which have inhibitory effect on the neighboring plant species. Common bean (*Phaseolus vulgaris*) is one of the oldest leguminous crops grown throughout the world, including Iran. Bean seeds are important source of proteins in humans (Mensack *et al.*, 2010). However, the yield as well as quality, size and pod length of beans are severely affected by weeds (Amare and Mohammed, 2014). Keeping in view the above, the aims of this study was to investigate the effect of needle extract of Eldar pine on bean and common weeds during vegetative stage of seedling and further to assess the phenolic contents of pine needle extracts.

Materials and Methods

Plant materials: Needles of *Pinus eldarica* were collected from Golestan Province, Iran and were subsequently sent for identification to the Herbarium of Ferdowsi, University of Mashhad. Freshly collected needles were oven dried at 40 °C for 48 hr and ground into a fine powder. Fifty gram of powder was

mixed with 500 ml of distilled water at room temperature for 24 hr. The extract was filtered to obtain a stock solution and further diluted to obtain different concentrations (25, 50, 75 and 100%). Seeds of common beans (*P. vulgaris* var. white and pinto) and prosomillet (*Panicum miliaceum*) and redroot pigweed (*Amaranthus retroflexus*) were collected from the Agricultural and Natural Resources Research Center of Golestan Province.

Similar shaped seeds were surface sterilized with 70% ethanol followed by immersion in 3% sodium hypochlorite and rinsing with sterile distilled water. Seeds were germinated in dark (25±2 °C) and the seedlings were transferred to 15 cm plastic pots containing garden soil. Ten ml each of pine needle extract solution was added to the pots for 21 days trial at 5 day interval, with ordinary water irrigation between each 5-day periods. The pots were kept under laboratory conditions (25±2°C, light intensity 250 µmol s⁻¹, 16 hr photoperiod) for 21 days. Seedlings irrigated with water served as control. The aerial parts of 3 week-old seedlings were harvested and stored at -20 °C for morphological and biochemical analyses.

Photosynthetic pigments (chlorophylls and carotenoids): Photosynthetic pigments were estimated spectrophotometrically using 100 mg fresh tissues extracted in 25 ml of 80% acetone to obtain chlorophyll-free homogen. After centrifugation at 3500 g for 10 min, the final volume of solutions were adjusted to 25 ml with 80% acetone. The absorption of the solution was read at 470, 646 and 663 nm and the pigment concentration was expressed in mg g⁻¹ f.w.t. (Lichtenthaler, 1987).

Relative Water Content (RWC): After determining the fresh weight, the leaves of a similar age were floated in distilled water at 20°C for 24 hr for rehydration. Following surface drying and measuring turgid weight, the leaves were oven-dried at 70 °C for 48 hr before being weighed again. The RWC of each sample was calculated by the formula: $RWC (\%) = 100 \times (FW - DW) / (TW - DW)$, where FW, DW and TW are leaf fresh, dry or turgid weight, respectively (Bian and Jiang, 2008).

Malondialdehyde (MDA) content : About 200 mg fresh leaf tissue was homogenized in 5 ml of 0.1% TCA and centrifuged at 10000 g for 5 min. To 1 ml aliquot of supernatant, 4 ml of 20% TCA containing 0.5% thiobarbituric acid was added and incubated at 95 °C for 30 min. There after, the reaction was stopped in an ice bath and centrifuged at 10000 g for 10 min. The absorbance of the supernatant was read at 532 nm and the value for non-specific absorption at 600 nm was subtracted. MDA content was calculated using extinction coefficient 155 mM cm⁻¹ by the formula $A = \epsilon bc$ described by Heath and Packer (1968).

Proline content: Total proline content was determined following the procedure of Bates *et al.* (1973). Leaf tissue (200 mg) was extracted in 5 ml of 3% sulfosalicylic acid and centrifuged at 4000 g for 10 min. Proline solution, ninhydrin and glacial acetic acid (1:1:1) were incubated at 100 °C for 1hr and the reaction was terminated in an ice bath. Chromophore was extracted in 4 ml of toluene and its absorbance was read at 520 nm.

Total phenolics: The concentration of phenolics was determined using Folin-Ciocalteu reagent (Singleton *et al.*, 1999). Briefly, the homogenized leaf tissue (100 mg) in 80% ethanol was centrifuged and mixed with distilled water, Folin-Ciocalteu and 25% Na_2CO_3 . After incubation at 25 °C for 20 min, the solution was centrifuged and absorbance was read at 725 nm. Total phenolic content was expressed as mg of gallic acid equivalent g^{-1} f.w.t.

Enzyme extraction: Leaf tissue was extracted in 100 mM potassium phosphate buffer containing 1 mM EDTA. The homogenate was centrifuged at 12000 g for 24 min and the obtained supernatant obtained was used for enzymatic analyses. Protein content was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Enzyme activities: Peroxidase (POX), polyphenol oxidase (PPO), catalase (CAT), auxin oxidase (IAA-OX) and nitrate reductase (NR) activities were estimated following the methods of Putter (1974), Raymond *et al.* (1993), Cakmak and Horst (1991), Rout (2006) and Steward *et al.* (1972), respectively.

Phytochemical analyses of pine needle extract

Total phenolic content: was estimated by the method of Swain and Hillis (1959) and the results were expressed as mg of gallic acid equivalent g^{-1} d.w.t.

Total flavonoid content: was assayed using a colorimetric assay as reported by Chang *et al.* (2002) and the results were expressed as mg quercetin equivalent g^{-1} d.w.t.

Total proanthocyanidin content: was determined by catechin assay (Sun *et al.*, 1998) and the results were expressed as mg catechin equivalent g^{-1} d.w.t.

Phenolic acid compounds: were analyzed following the method of Matkowski *et al.* (2008) and the results were expressed as mg caffeic acid equivalent g^{-1} d.w.t.

Flavonols: was determined by the method originally used by Mazza *et al.* (1999) and the results were expressed as mg of rutin equivalent g^{-1} d.w.t.

O-di phenol compounds: The method of Carrasco-Pancorbo *et al.* (2005), the content of DPC was measured and the results were expressed as mg of gallic acid equivalents g^{-1} d.w.t.

Statistical analyses : All the experiments were performed in three replicates using a full factorial design. The mean values were compared using Duncan's multiple range test at $P \leq 0.05$ (ANOVA, SPSS program-version 16).

Results and Discussion

Based on the analysis of variance, significant variations were observed for all the physiological and biochemical traits of

the plant interaction between plant species and pine needle extracts ($P < 0.05$) (Data not shown). Similarly, different concentrations of aqueous extracts increased the Chl a, Chl b and total Chl contents in the leaves of white and pinto beans as compared to control (Fig. 1 a, b, c). In redroot pigweed, the minimum Chl a, Chl b and total Chl contents was observed in with 25% needle extract. The pattern of changes in prosomillet pigments was more varied. The Chl b content of prosomillet was 2.6 times and 2.5 times more than control (Fig. 1 b). On treatment with the inhibitory effect of allelochemicals on Chl a, Chl b and total Chl contents in the leaves of sensitive plants has been reported (Gniazdowska and Borated, 2005; Oyerinde *et al.*, 2009). Chi-Ming *et al.* (2002) stated that phenolic compounds can decrease the biosynthesis of Chl a and Chl b and thereby reduce the photosynthetic capacity of plants. They reported that the lower level of chlorophylls in affected plants may be due to stimulation of chlorophyllase and Mg-dechelataase activities, also due to reduced chlorophyll biosynthetic pathway by allelochemicals such as phenolics, flavonoids, alkaloids, terpenoids, etc. (Yang *et al.*, 2004).

Different concentration of pine needle extracts increased carotenoid content of bean varieties in a concentration dependent manner. In addition, the maximum carotenoid content (2.66 mg g^{-1} f.w.t.) was found in pinto bean following treatment with 100% extract. With the increasing concentration of extracts above 50% the effect of extracts on total carotenoid concentration in prosomillet and redroot pigweed showed decreasing trend. (Fig. 1d). All carotenoids, including beta-carotene and xanthophylls, are potent antioxidants. They protect photosynthetic complexes against oxidative damage due to excessive production of reactive oxygen species (Howlitt and Pogson, 2006). Therefore, the increase in carotenoids level in bean varieties can be regarded as one of the defensive systems against pine allelochemicals.

As shown in Fig. 2, the leaves of bean varieties and weeds showed a significant decreasing trend in RWC ($P \leq 0.05$). However, redroot pigweed was more susceptible to needle extract application at each level, as the lowest percentage of RWC (57.22%) was recorded in 100% treatment (Fig. 2A). RWC is widely used to describe plant water status at a given time (Kramer, 1969). The metabolism of plant is affected, either directly or indirectly by water content of the plant both at cellular and at whole plant level, thus it is considered as a key factor which controls plant growth and development (Tanentzap *et al.*, 2015). Moderate reduction in leaf RWC of bean varieties can be regarded as a balance between growth process and coping with negative effects of allelochemicals. However, one of the allelochemical -occurring disorders is disturbance in root absorption of water and minerals, followed by severe disruption of water balance and RWC (Singh *et al.*, 2009); this was observed in redroot pigweed.

With respect to Fig. 2 b, MDA content in white bean, pinto bean and prosomillet did not show any statistically significant difference as compared to control. In contrary, MDA content in redroot pigweed showed considerably increased by 82% and 88% at 50% and 100% treatment (Fig. 2 b). There is a direct

Fig. 1: Effects of different concentrations of pine needle extract on (a) chlorophyll a content (b); chlorophyll b content (c); total chlorophyll content and (d) carotenoid content of white bean, pinto bean, prosomillet and pigweed. For each column, different letters indicate significant differences ($P < 0.05$) according to Duncan's Multiple Range Test.

relationship between membrane destruction, membrane lipid peroxidation and intensity of allelochemical stress (Lara-Nunez *et al.*, 2006). Zunino and Zygallo (2004) reported that monoterpenoids increase peroxidation of membrane lipids. Lee, (2003) reported that monoterpenes are an important category of *P. eldarica* allelochemicals that may be due to the destruction of membrane system and subsequent increment of MDA content in weeds plants. However, the highest degradation of cell membrane in redroot pigweed may be due to weak radical scavenging potential of the plant. It was found a variation in the level of proline proportional to extract concentrations, when bean varieties were irrigated with needle aqueous extracts (Fig. 2 c). Despite high concentration of extract (100%) caused a significant increase in proline content of studied beans which was similar to earlier reports (Kalantar *et al.*, 2008). Adaptation of plants to both short and long-term abiotic stress condition is accomplished by accumulation of metabolites such as nitrogenous compounds (e.g. amino acids, and polyamines) (Razavizadeh *et al.*, 2014). When applied at high concentrations, allelochemical extracts create a drought-like situation due to disturbances in root water absorption, leading to water imbalance and reduced turgor

pressure in cells (Singh *et al.*, 2009). Accumulation of proline can be effective in maintaining osmotic balance and stabilizing the cell volume. It can also function as a source of nitrogen required for ATP production and as a non-enzymatic antioxidant under stress conditions (Rhodes, 2004), which was observed in beans treated with higher concentrations of needle extract. The other note worthy result was significant reduction in proline content in weeds after treatment with increasing concentration of needle extracts. The maximum decrease in proline content ($3.61 \mu\text{mol g}^{-1}$ f.w.t.) was found in redroot pigweed treated with 100% extract (Fig. 2 c). Hence, the lower proline content in weeds indicated that these species may not benefit much from the positive effects of increasing proline affected by pine needle extracts.

The results showed that the positive effect of 25% and 75% needle extract on total phenol content in bean varieties was remarkable (Fig. 2 d). Endogenous phenolic content of prosomillet and redroot pigweed decreased in a concentration-dependent manner, with redroot pigweed being more sensitive than prosomillet. The lowest level of phenolic content (0.40 mg g^{-1} f.w.t. and 0.43 mg g^{-1} f.w.t.) was found in redroot pigweed treated

Fig. 2 : Effects of different concentrations of aqueous extract of Eldar pine needle on RWC (a), MDA content (b), proline content (c) and total phenol content (d) of white bean, pinto bean, prosomillet and redroot pigweed. For each column, different letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

with 75% and 100% extract (Fig. 2 d). Phenolics as secondary metabolites are indirectly involved in the normal growth, development and reproduction of plant. Increases in the metabolism of flavonoids and phenolic compounds has been reported under different environmental stress conditions (Michalak, 2006). Due to high redox properties and antioxidant capacity of phenolic compounds (Bamdad *et al.*, 2006), they lead to increased oxygen reduction, scavenging of ROS and reduce their overproduction under stressful conditions (Sakihama and Yamasaki, 2002). Although some extract concentrations stimulated phenolic accumulation in bean varieties, pine extract in concentrations above 50% significantly reduced the total phenolics in redroot pigweed when compared with control. It is, thus, suggested that phenolic compounds may serve as part of defense mechanism only at high concentrations of pine needle extracts.

Evaluation of enzyme activities showed that the progressive application of needle extracts caused a statistically significant increase in POX and CAT activities in bean varieties, while a considerable decrease in both POX and CAT activity noted in both weeds. Notably, the lowest POX ($0.33 \text{ unit mg}^{-1} \text{ pro}$) and CAT ($0.078 \text{ unit mg}^{-1} \text{ pro}$) activities were recorded in redroot

pigweed treated with 100% extract (Fig. 3 a, c). Singh *et al.* (2009) reported a decrease in the activity of POX and CAT enzymes in maize leaves treated with tobacco extracts. Increased ROS generation and imbalanced redox states are induced by allelochemicals (Qian *et al.*, 2013). Therefore, regulation of intracellular concentration of ROS, especially hydrogen peroxide during oxidative condition, depends on the activation of antioxidant defense system such as POX and CAT (Caverzan *et al.*, 2016). The increased activities of POX and CAT in bean varieties suggest that these enzymes play an essential protective role against allelochemicals-induced oxidative stress, consequently enabling the crops to grow better. In the present study, PPO activity increased slightly in all test plants. However, a significant increase in their activity ($0.538 \text{ unit mg}^{-1} \text{ protein}$) was notable in redroot pigweed treated with 100% extract (Fig. 3 b).

Although, Sasikumar *et al.* (2002) reported that shoot and root extracts of eucalyptus can increase PPO activity of *Phaseolus mungo*; Chum *et al.* (2012) reported that treatment with benzoic acid reduces the enzyme activity in *Brassica oleracea*. PPO is involved in the response to various types of non-biological stresses. Indeed, it can react with other antioxidant

Fig. 3 : Effects of different concentrations of aqueous extract of Eldar pine needle on the activity of POX (a), PPO (b), CAT (c), IAA-OX (d) and NR (e) of white bean, pinto bean, prosomillet and redroot pigweed. For each column, different letters indicate significant differences ($P<0.05$) according to Duncan's multiple range test.

enzymes to scavenge ROS more effectively. Never the less, over-oxidation and decomposition of phenolic compounds under severe stressful condition like presence of allelochemicals in soil is another function of PPO (Williams and Hogland, 2003). Hence, imbalance in PPO activities may cause less efficiency of different parts of antioxidant defense system.

Similar variations in enzymatic activities of IAA-OX and NR were recorded under the application of pine extracts on bean

varieties and on the weeds, where IAA-OX and NR enzymatic activities were negatively correlated with the extract concentrations applied. It is worth noting that higher concentrations (75% and 100% v/v) of aqueous extract, there were maximum reductions in IAA-OX (49.4% and 53.8%) and NR (26.1% and 37.2%) activities of prosomillet and redroot pigweed (Fig. 3 d, e). Mierziak *et al.* (2014) stated that most allelochemicals, such as phenolic acids and flavonoids can regulate the activity of IAA-oxidase, with different effects

Fig. 4 : Total phenolic content: gallic acid equivalents, Total flavonoid content: quercetin equivalents, Total proanthocyanidin content: catechin equivalent, Phenolic acid compounds: caffeic acid equivalents, Flavonol compounds: rutin equivalent and O-di phenol compounds: gallic acid equivalents. Data presented are mean value of three replicates $\pm$ SE.

depending on plant species and environmental conditions. NR is one of the key enzymes for nitrogen acquisition and nitrate assimilation. Both environmental and hormonal factors have been implicated to alter NR gene expression (Katalin *et al.*, 2000). It has also been reported that NR activity is affected by developmental stages of plant and stress conditions (Sangwan *et al.*, 2014). High reduction in NR activity in target weeds might interfere with the uptake and assimilation of nitrogen into amino acids and further into proteins and subsequent by hampering normal growth and development.

The content of total phenolics, total flavonoid content, total proanthocyanidin content, phenolic acid compounds, flavonol compounds and o-di phenol compounds of needle extract is shown in Fig. 4. The extracts contained high levels of phenolics (74.979 mg GAEg⁻¹ d.w.t.) and proanthocyanidins (39.479 mg CEg⁻¹ d.w.t.) which are known as the main allelochemicals (Fernandez *et al.*, 2018). Similarly, a number of other studies have documented high levels of phenolics in other pinus species, *P. halepensis* (Refifa *et al.*, 2016), *P. nigra* (Garcia-plazaola *et al.*, 2008) and *P. sylvestris* (Karolewski, 1990). A comparative study revealed that aqueous extract of green needles of pine significantly delayed the germination and affected the seedling growth of radish and wheat greater than mature needles, as extracts of green needles contain higher amount of phenolic contents as compared to mature needles (Refifa *et al.*, 2016). The inhibitory properties of pine needles on other plant species has already been explained due to richness in phenolic compounds (Fernandez *et al.*, 2018), which was also reported in our results.

In conclusion, the present study confirm that the allelopathic effects of pine needle extracts differ between crops and weeds. Plant defense mechanisms-consisting of enzymatic and non-enzymatic antioxidant processes-exhibit clear variations. Furthermore, deficiencies in mentioned defense capabilities in the weeds, in particular redroot pigweed, were observed. It is thus recommended to use the extracts of Eldar pine needles as a natural herbicide instead of the chemical versions.

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