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RESEARCH ARTICLE

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Antioxidant activity of *Trifolium resupinatum* L. exposed to different extracts from leaves, flowers and shoots of *Prangos ferulacea*

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Abstract

Prangos ferulacea is a member of Apiaceae family, is a popular fodder for livestock production and an important species in rangeland restoration. The objectives of the research are the comparative phytotoxic activity of aqueous and hydroalcoholic extracts obtained from different organs (flower, shoot and leaf) of *P. ferulacea* on antioxidant response of *Trifolium resupinatum* was investigated in a laboratory bioassay. Antioxidant enzyme activities including catalase (CAT), peroxidase (POD), ascorbic peroxidase (APX), and superoxide dismutase (SOD), in line with content of malondialdehyde (MDA) and hydrogen peroxide were measured. Results indicated that hydroalcoholic extract of *P. ferulaceae* flower possess the highest total phenolic content as well as highest phytotoxic effect on *T. resupinatum*. The highest antioxidant enzyme activity belonged to hydroalcoholic treatment. The treated *T. resupinatum* seedlings experienced lipid peroxidation at high extract concentrations (12% of hydroalcoholic and 100% of aqueous extract) as evidenced by increased concentration of MDA. In response to this, the activities of SOD, CAT, POD and APX increased at lower extract concentrations but significantly dropped as concentrations increased. According to results of this study, rehabilitation of *T. resupinatum* sites through the use of *P. ferulaceae* will probably not be successful.

Additional keywords: superoxide dismutase; peroxidase; lipid peroxidation; hydrogen peroxide.

Abbreviations used: APX (ascorbic peroxidase); CAT (catalase); MDA (malondialdehyde); NBT (nitroblue tetrazolium); POD (peroxidase); ROS (reactive oxygen species); SOD (superoxide dismutase).

Authors' contributions: HS designed the experiment, administered the experiment and wrote the manuscript. MB assembled input data, and analysed output data. Both authors read and approved the final manuscript.

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Introduction

Antioxidant enzyme activities in receiving plants can be affected by allelopathic compounds (Kaur *et al.*, 2012). It is known that one of early plant responses under this condition, is an oxidative burst characterized by the production of large amounts of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in the target plant tissues (Batish *et al.*, 2006; Kaur *et al.*, 2012). Numerous studies have supported the idea that increasing in ROS as a second messenger for the expression of defense genes (Orozco-Cadenas *et al.*, 2001; Gill and Toteja *et al.*, 2010). To avoid cellular damage by ROS, detoxifying antioxidants and scavenging enzymes such as ascorbate peroxidase (APX), peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) are produced in cellular compartments (Sadeghi & Robati, 2015). Of late, ROS-generation resulting in oxidative damage has been considered as one of the modes of action of allelochemicals in causing growth inhibition (Cruz-Ortega *et al.*, 2002; Romero-Romero *et al.*, 2005; Kaur *et al.*, 2012).

Prangos ferulacea is a member of Apiaceae family and one of the major plants to supply winter fodder of livestock. Due to high nutritional value of *P. ferulacea* most of Iranian dairy farmers prefer it rather than alfalfa for feeding animals (Eilami, 2008). Coskun *et al.* (2004) also indicated that *P. ferulacea* can be used as an energetic fodder. In recent decades, Excessive grazing and irrational use of grasslands has caused a reduction in plant cover, followed by a decrease in

plant diversity and density (Ibañez *et al.*, 2007). In Iran, about 45% of livestock productions are dependent on the current rangelands (Iranian Nomadic Organization, 1992). Due to high grazing pressures as well as repeated drought, the possibility of grassland natural recovery has significantly declined. Dairy farmers in many parts of Iran, such as Fars province rangelands, attempted to restore rangelands through seeding and planting forage plants such as *P. ferulaceae* in order to attain more sustainable ecosystems with a higher forage production (Azarfard, 2008; Eilami, 2008). Such interventions into rangeland ecosystems can affect various components of ecosystems such as plant, animal and microbial communities (Palmer *et al.*, 1997). Without considering the phenomenon of allelopathy, even using most compatible species for improving rangelands can have devastating effects on rangeland ecosystem. Hydroalcoholic and aqueous extracts are two common extraction methods in phytotoxicity bioassays (Cruz-Ortega *et al.*, 2002; Salam & Noghuch, 2010). Razavi (2012) reported that the essential oil of *P. ferulaceae* exhibited significant phytotoxic effect on lettuce. Moreover, it has been observed that usually no plant could be grown around *P. ferulacea*, probably due to the chemical effects of its compounds on other plants.

Because of the importance of oxidative enzymes in stress responses, we mainly focused on the changes in the activities of enzymatic antioxidative defenses. *Trifolium respinatum* was chosen as a target plant since it is a valuable and dominant species in rangelands of Fars province. To the best of our knowledge at the time of carrying out this research, no comparative work has been done on the phytotoxicity effects of different extracting method in relation to inducing oxidative stress in receiving plants. This research aimed to evaluate the effect of different extraction methods from *P. ferulacea* different organs on antioxidant enzymes activity as well as ROS generation of *T. respinatum*.

Material and methods

Plant material

The scheme of the method followed appears in Fig. 1. The plant organs (flowers, shoots and leaves) of *P. ferulacea* were collected from cultivated plants in the Pooladkaf rangeland (30°14′ 32″ N and 51°59′ 38″ E; southern Iran, altitude: 2850 m asl) of Fars province. Voucher specimen (No. 12608) was deposited at the Herbarium of Fars Research Center for Agriculture, Shiraz, Iran. The plants were shade dried at room temperature for 14 days. The plant material was ground by using Endecott food processor and sifted through 0.5 mm mesh screen to remove large pieces of debris. *T. resupinatum* seeds were purchased from Pakan Bazr Co. (Isfahan, Iran). Damaged and under-sized seeds were discarded, and the assay seeds were selected for uniformity of size.

Preparation of extracts

To prepare the aqueous extract, shade dried samples of leaves, shoots and flowers of *P. ferulacea* were soaked at 100 g in 1000 mL distilled water for 48 h at room temperature followed by filtration through three layers of cheese cloth to remove any debris, and then centrifuged at 3000 rpm for 4 h as described by Correa *et al.* (2008). This stock extract was considered as 100% (w/v) and kept at 5°C until used. The stock solution was then two-fold serial diluted with sterile distilled water to give the final concentrations of 50%, 25%, 12.5%, 6.25%, 3.125% and 0 (control).

To prepare hydroalcoholic extract, dried and powdered plant material was submitted to extraction by maceration with ethanol–water (70:30) as a solvent at room temperature for 72 h. The ratio of plant material to solvent was 20:80 (w/v) and at the final extraction process the material was filtered and concentrated in rotary evaporator, thus obtaining the *P. ferulacea* dried extract. The resulting dried extracts were dissolved by distilled water to obtain stock solutions of 50 g/mL and stored at 4°C in the dark. Then the final concentrations of 0 (Control), 1.5, 3, 6 and 12% (v/v) were made from the stock solution (Tiffany *et al.*, 2004).

Germination bioassay

The seeds of *T. resupinatum* were surface-sterilized with 5% (w/v) sodium hypochlorite solution for 10 min and rinsed three times with distilled water to ensure viability. Twenty seeds were evenly placed in 9 cm Petri dishes lined with a double layer of Whatman No. 1 filter paper moistened with 4 mL of the respective different extract concentrations or distilled water as control. To prevent evaporation, Petri dishes were firmly sealed with parafilm and incubated for 7 days at 27 ± 2°C in a growth chamber (16 h light and 8 h dark). The plants were harvested 1 week after treatments for further analysis.

Activities of antioxidant enzymes

At the end of each treatment, antioxidant enzyme activity of *T. resupinatum* was measured. Fresh leaf tissues (500 mg) were titrated in 10 mL of 50 mM phosphate buffer pH 7.8, using an ice cooled sterilized pestle and mortar. The extract was centrifuged at 15,000 × g for 15 min at 4°C. The supernatant was

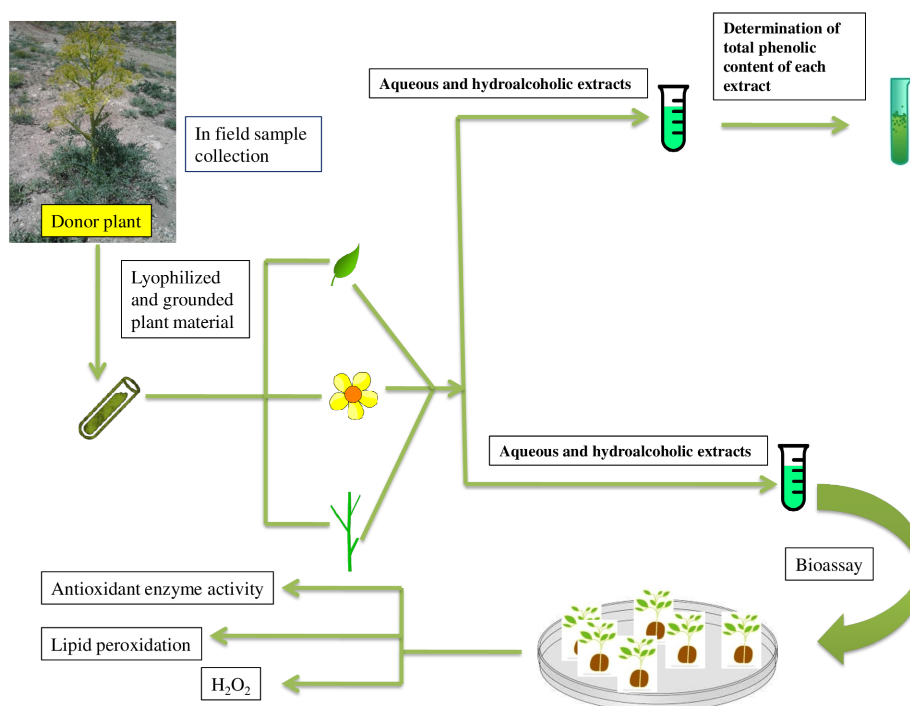

Figure 1. Method scheme.

separated in autoclaved Eppendorf tubes and used for the determination of the activities of the enzymes SOD, POD and CAT. Inhibition in photoreduction of nitroblue tetrazolium (NBT) was used to appraise the activity of SOD. The reaction mixture (500 µL phosphate buffer pH 7.8, 0.5 mL distilled H_2O, 100 µL methionine, 50 µL NBT and 50 µL sample extract) (1 mL/cuvette) was kept under light for 20 min. The optical density of the irradiated aliquot was read at 560 nm. Following Giannopolitis & Ries (1977), SOD enzyme activity per unit was based on the amount of enzyme that inhibited 50% of NBT photoreduction. The method of Chance & Maehly (1955) was used for the appraisal of CAT and POD activities. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (25 mM, pH 7.8), which contained 0.2 mM EDTA, 2% polyvinylpyrrolidone (PVP) and 2 mM ascorbate was used for APX (EC 1.11.1.11) extraction. Enzyme activity was determined according to the protocol of Zhu *et al.* (2004). The activities of all four enzymes were expressed as U/mg dw protein.

Membrane lipid peroxidation

Lipid peroxidation was determined by adapting the method described by Heath & Packer (1968). Enzyme extract (0.5 mL) was treated with 0.5% thiobarbituric acid (TBA) prepared in 20% trichloroacetic acid (TCA). The mixture was incubated in a water bath for 30 min, cooled immediately in ice chips and the absorbance was read at 532 nm. Lipid peroxidation was expressed in terms of MDA content.

Determination of hydrogen peroxide

For the estimation of H_2O_2, leaf tissue (40 mg) was extracted with 3 mL of 0.1% (w/v) TCA in an ice bath and centrifuged at 10,000 × g for 15 min (Singh & Prasad, 2014). The reaction mixture (2 mL) contained tissue extract (0.5 mL), 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI solution. The absorbance of the mixture was recorded at 390 nm. Concentration of H_2O_2 was calculated by using a standard curve prepared with graded solution of H_2O_2.

Determination of total phenolic content

Total phenolic content was estimated by the Folin–Ciocalteu colorimetric method, based on the procedure of Ahmed *et al.* (2011), using gallic acid as a standard phenolic compound. Briefly, 50 µL (four replicates) of the filtered extracts were mixed with 450 µL of distilled water and 2.5 mL of 0.2 N Folin–Ciocalteu reagent. After 5 min, 2 mL of saturated sodium carbonate (75 g/L) were added. The absorbance of the resulting blue-coloured solution was measured at 765 nm after incubation at 30°C for 1.5 h with intermittent shaking. Quantitative measurements were performed, based on a standard calibration curve of six points: 20, 100, 200, 300, 400, 500 mg/L of gallic acid in 80% methanol.

The total phenolic content was expressed as gallic acid equivalents (GAE) in mg/g of dry material.

Statistical analysis

Each treatment had four replicates that were laid out as a three-way factorial experiment in completely randomized design. Analysis was performed with the statistical software SAS 9.1.3 (SAS Inst, 2004). Different extraction methods were the first factor and included aqueous and hydroalcoholic extract. The second factor was three different organs (leaves, shoots and flowers) of *P. ferulacea*, and the third factor was different concentrations of *P. ferulacea* aqueous and hydroalcoholic extracts which were applied on *T. resupinatum*. Significant differences of the means were determined by LSD test.

Results

Data analysis of variance showed that the effect of different extraction methods, organs and concentrations of *P. ferulacea* had significant effects on antioxidant enzyme activities, lipid peroxidation and hydrogen peroxide in *T. resupinatum* at 1% probability level (Table 1). The main effect of extraction method, different organs for extraction, and different concentrations were also significant. Furthermore, all two way and three way interaction effects were significant. These results indicated that different extraction methods, using different organs for extraction with different concentrations are dependent on each other.

Hydroalcoholic extracts caused a higher activity of APX, CAT and POD than aqueous extraction. In contrary, SOD activity was higher in aqueous extract treatments. Furthermore, higher content of lipid peroxidation and hydrogen peroxide were observed in the aqueous extracts.

For all antioxidant enzyme activities, control showed the minimum values in comparison to other treatments. APX activity was highest in the concentration of 6.25%. Concentration of 25% also showed highest activities for SOD and POD. There was no significant difference among 3.13, 6.25, 25%, and control related SOD activity. Content of MDA and hydrogen peroxide were highest in 100% while lowest in control.

In relation to APX activity, the highest value was obtained in 3%, while the lowest was observed in 12% and control. Related to other antioxidant enzymes including CAT, SOD, and POD, the lowest activity was obtained in the control. Furthermore, the highest activities were recorded for the 1.5% concentration. The concentration of 1.5% hydroalcoholic extracts showed lowest content of MDA and hydrogen peroxide, while the maximum content of these two traits were observed in the control and 12%, respectively. The result of total phenolic content obtained for *P. ferulacea* hydroalcoholic and aqueous extracts are given in Table 2. According to the result, generally, hydroalcoholic extract contained more phenolic content than the aqueous extract. The highest total phenolic content was determined in the hydroalcoholic extract of flower. Under both kinds of extract, the content of total phenolics in flowers was higher than in leaves, and in leaves was higher than in shoots (Table 2).

Three-way mean comparison for APX and CAT activity is presented in Fig. 2. Generally, there was more APX activity under hydroalcoholic extract than under the aqueous extract. The lowest activity

Table 1. Analysis of variance (ANOVA) for the effect of *Prangos ferulacea* extracts on antioxidant enzymes activity (ascorbic peroxidase, APX; catalase, CAT; superoxide dismutase, SOD; and peroxidase, POD), in line with content of malondialdehyde (MDA) and H_2O_2 of *Trifolium resupinatum*

Source	DF[1]	APX	CAT	SOD	POD	MDA	H_2O_2
Extraction method (A)	1	168634.46**	3.66*	91693.39**	599766.63**	9066.63**	96.63**
Organ (B)	2	14652.59**	218.61**	773.55**	194690.66**	4790.66**	90.66**
Concentration (C)	6	15351.13**	128.13**	37489.9**	75062.42**	562.42**	56.42**
A*B	2	23831.02**	135.01**	11004.31**	187115.51**	7715.51**	87.51**
A*C	4	28263.93**	163.73**	31561.28**	135345.61**	5645.61**	55.61**
B*C	12	8102.66**	70.36**	17474.38**	23068.06**	338.06**	68.06**
A*B*C	8	9772.01**	85.98**	15561.53**	32144.6**	154.6**	24.6**
Residual error	72	105.2	0.76	30.42	25.72	15.72	5.72
Coefficient of variation		14.25	10.6	5.53	3.45	13.23	7.34

[1]DF: degrees of freedom. [*]Significant at 5% probability levels, [**]Significant at 1% probability levels.

Table 2. Total phenolic contents of the hydroalcoholic and aqueous extracts of *P. ferulaceae* (mg GAE/g dw)

Organs	Hydroalcoholic extract	Aqueous extract
Leaf	25.63 b	19.7 b
Flower	37.45 a	28.41 a
shoot	8.91 c	5.63 c

Means in each column with different letters are significantly different using LSD ($p \leq 0.01$).

of APX was observed using the aqueous extraction of shoots, while the highest one was observed using the shoot hydroalcoholic extraction. Some of the applied treatments suppressed the germination of *T. resupinatum* completely such as: 100% of aqueous leaf extract, 50 and 100% of flower aqueous extract, as well as 6 and 12% of leaf extract in addition to 3, 6, and 12% of hydroalcoholic flower. Under aqueous extract treatments, the leaves showed higher activities than the other two organs (Fig. 2).

Totally CAT activity under hydroalcoholic extracts was higher than that of the aqueous extract. Under aqueous extracting method, the different concentrations of leaf organ obtained higher activities than that of other organs (Fig. 2). The activity of SOD under different concentrations of aqueous extract was relatively equal (Fig. 3). Under hydroalcoholic extraction treatments, shoot organ with 1.5% concentration showed highest SOD activity between all treatments, but generally leaves and flowers extracts had higher SOD activities than shoots (Fig. 3). POD activity was significantly higher in hydroalcoholic treatments compared with aqueous. Different organs (leaves, flowers and shoots) showed relatively similar activities in the case of POD (Fig. 3). Under hydroalcoholic extraction method, leaf organ showed higher activity of POD in general (Fig. 3). MDA and hydrogen peroxide (Fig. 4) contents were obviously lower in hydroalcoholic treatments compared to aqueous. Broadly speaking, under aqueous and hydroalcoholic extracts, leaf organ showed lower content of MDA and hydrogen peroxide.

Discussion

Results from our study clearly revealed that aqueous and hydroalcoholic extracts of *P. ferulacea* induced changes in antioxidant enzyme activity, lipid peroxidation and ROS generation in *T. resupinatum*. Recently, allelochemicals have been proposed to cause oxidative stress in target tissues and induce antioxidant mechanisms (Singh *et al.*, 2006; Sunmonu & Van Staden, 2014). In this study, *T. resupinatum* seeds exposed to aqueous extracts showed higher SOD activity resulting in high accumulation of H_2O_2 and MDA (Table 1). In the present study, elevation of MDA concentration in *T. resupinatum* seedlings treated with aqueous and hydroalcoholic extracts of *P. ferulaceae* is an indication of a stress signal which can stimulate the expression of stress-tolerance genes and gradually establish defense system (Sunmonu & Van Staden, 2014).

Degree of oxidative stress and antioxidant activity has been described to be closely associated with the resistance or susceptibility of a species to allelochemical stress (Sadeghi & Robati, 2015). According to the result, an initial increase in the activities of SOD, POD, APX and CAT in response to stress at lower concentrations of both hydroalcoholic and aqueous extracts was observed (Table 2). In aqueous treatments, this increasing trend was up to the concentration of 6.25% for APX and POD and 25% for CAT and SOD activity, while in the case of hydroalcoholic extract was up to 3% for APX and 6% for SOD, CAT and POD activity. This may be an induction of enzymes as a secondary defense system. Since at lower concentration the level of allelopathy stress was not very strong, the antioxidant defense system can protect the cell membrane and prevent excessive lipid peroxidation (MDA). This also accounted for the higher MDA content in higher concentrations (100% of aqueous and 12% of hydroalcoholic extracts) when compared with the control (Table 2). At higher concentration, the trend was reversed when MDA content increased dramatically indicating high concentration of ROS which was beyond the threshold of scavenging by the antioxidant enzymes. These findings are consistent with previous studies which reported up/down regulation in antioxidant enzyme activities under allelopathic stress (Sunmonu & Van Staden, 2014).

In general, the highest activity value of each enzyme in *T. resupinatum* belonged to hydroalcoholic treatments (Figs. 2 and 3). We speculated that hydroalcoholic extract induced a stronger oxidative stress in *T. resupinatum* plant as evidenced by more treatments with no emergence (3, 6, and 12% of flower extract as well as 6 and 12% of leaf extract) (Figs. 2 and 3). Thus, we decided to measure the total phenolic content of each extract. As expected, total phenolic content of hydroalcoholic extract was significantly higher than aqueous (Table 2). In agreement of our findings, Ahmed *et al.* (2011) and Tawaha *et al.* (2007) also reported the higher phenolic content in hydroalcoholic extracts than the aqueous extract. Studies have also implicated phenolic content of plant extracts in allelopathic activity (Razavi, 2012; Kaur *et al.*, 2012; Zhang *et al.*, 2012). However, the phytochemical studies indicate the presence of other compounds in plant extracts such as saponins, tannins and nitrogen groups (Razavi, 2012); we just measured total phenolic content of each extract to have an index for comparing them with each other.

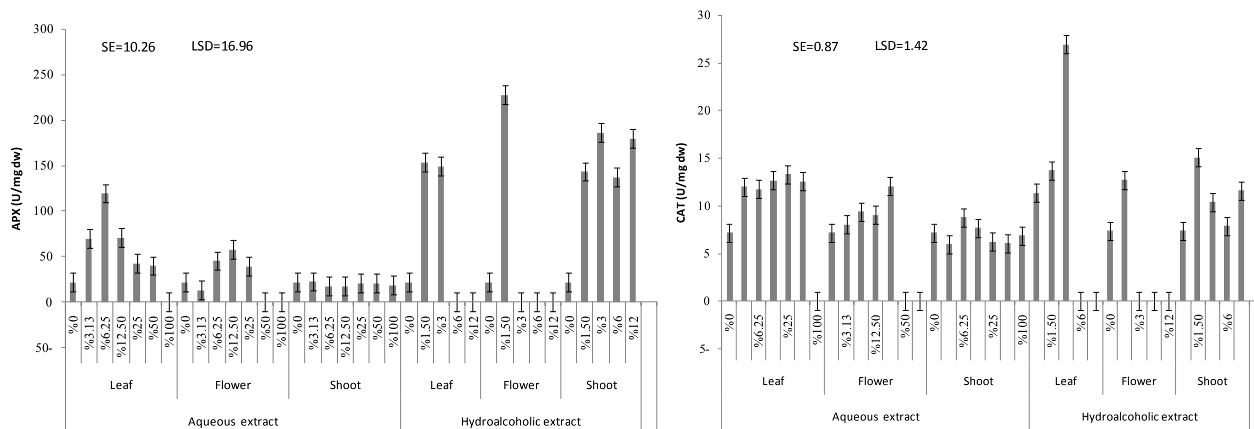

Figure 2. Mean comparison three way interaction of "extraction method × organ × concentration" for ascorbate peroxidase (APX) and catalase (CAT). Standard errors show that there were no significant differences between means with the same overlap ranges for each aqueous and hydroalcoholic extracts.

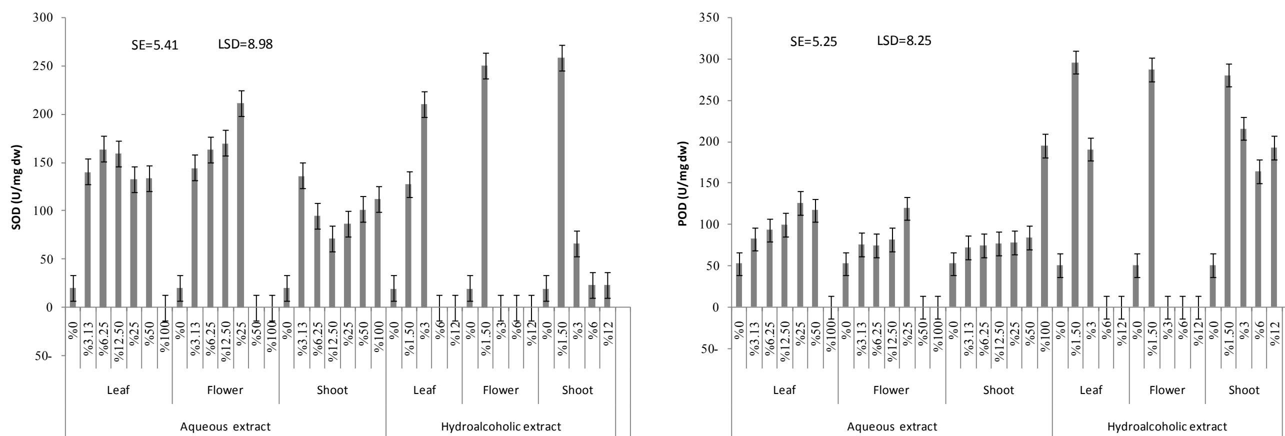

Figure 3. Mean comparison three way interaction of "extraction method × organ × concentration" for superoxide dismutase (SOD) and peroxidase (POD). Standard errors show that there were no significant differences between means with the same overlap ranges for each aqueous and hydroalcoholic extracts.

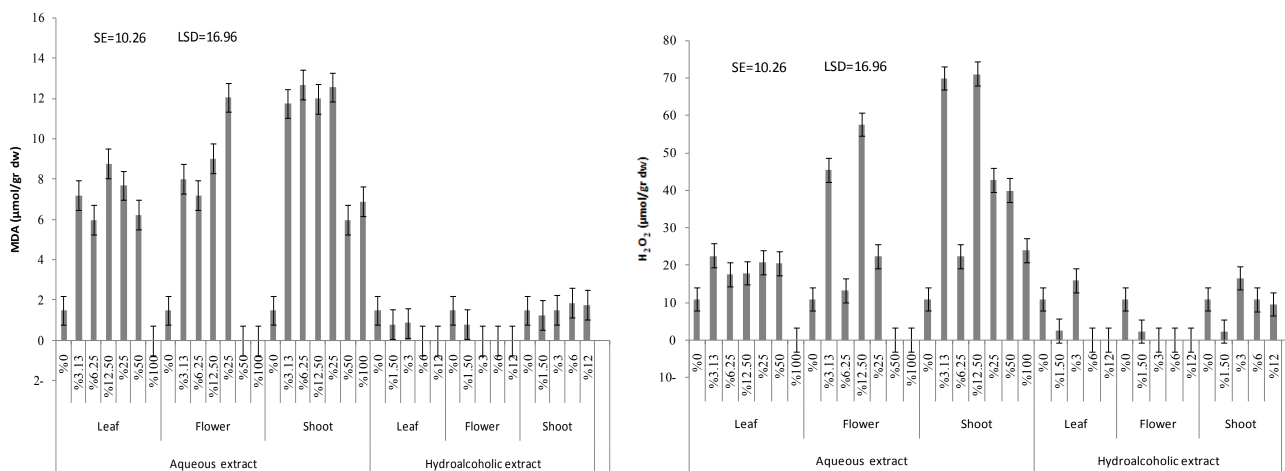

Figure 4. Mean comparison three way interaction of "extraction method × organ × concentration" for malondialdehyde (MDA) and hydrogen peroxide (H_2O_2). Standard errors show that there were no significant differences between means with the same overlap ranges for each aqueous and hydroalcoholic extracts.

In relation to the phytotoxicity effect of different organs, results of the present study showed that the phytotoxic effect of flower extracts (both hydroalcoholic and aqueous) was higher than of the two other organs (leaves and shoots) as evidenced by no emergence of *T. resupintum* (Figs. 2, 3 and 4). In Figs 2 to 4 values of zero showed no emergence in respected treatments. This observation can be due to the higher phenolic content of flower extracts (Table 2). Ahmed *et al.* (2011) also reported that phenolic content of hydroalcoholic and aqueous extract of *P. ferulacea* fruit was higher than of shoot and root.

In this research we have compared the phytotoxicity effect of two different extraction methods. According to the results, the phytotoxicity effect of hydroalcoholic extract was significantly higher than of aqueous extract, since in nature the allelopathic interaction between plants is closer to the aqueous method. Thus, we suggest using this method in primary evaluations of rangeland restoration.

This study was undertaken to compare the effect of different extraction methods from *P. ferulacea* different organs' on antioxidant enzymes activity as well as ROS generation of *T. respinatum*. Because of the importance of oxidative enzymes in stress responses, we mainly focused on the changes in the activities of enzymatic antioxidative defenses. Hydroalcoholic extract of *P. ferulaceae* induced a higher antioxidant enzyme activity in *T. resupinatum*. Among the three tested organs, flower's extract showed a higher phytotoxicity effect and contained a higher phenolic content as well. According to the result of the present study, rehabilitation of *T. resupinatum* sites by using *P. ferulaceae* will probably not be successful. A better understanding of the physiological basis of changes in allelopathic interaction could be used to select suitable plants for the restoration of destructive rangeland.

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