




Antioxidant activity, phenolic and flavonoid content of *Lawsonia inermis* and *Haplophyllum vermiculare*

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ABSTRACT

Introduction: Continuous exposure of oxidants to the skin may disrupt the antioxidant balance and leads to inflammatory skin diseases (ISD). The aim of the present study was to compare the antioxidant activity, phenolic and flavonoid content of two traditionally used plants in ISD, *Lawsonia inermis* and *Haplophyllum vermiculare*.

Methods: The hydroethanolic extract of the plants was prepared by maceration. Phenolic and flavonoid content of the extracts was measured respectively with Folin-Ciocateu and aluminum chloride methods. The monovalent reducing power and radical scavenging activity were also evaluated respectively by ferric reducing antioxidant power and 2,2-diphenyl-1-picryl-hydrazyl methods.

Results: The reducing power of *Lawsonia inermis* ($862.89 \pm 32.23 \mu\text{molFe}^{2+}/\text{g}$) was significantly higher than *Haplophyllum vermiculare* extract ($765.52 \pm 29.39 \mu\text{molFe}^{2+}/\text{g}$). The radical scavenging activity of *Lawsonia inermis* extract at a concentration of $1000 \mu\text{g}/\text{ml}$ ($\%65.72 \pm 0.77$) was also significantly higher than *Haplophyllum vermiculare* ($\%36.34 \pm 2.52$). The higher antioxidant activity of *Lawsonia inermis* is probably due to its higher phenolic ($96.76 \pm 3.34 \mu\text{g GAE}/\text{mg}$) and flavonoid content ($197.69 \pm 5.76 \mu\text{g QE}/\text{mg}$).

Conclusion: Henna leaves had higher antioxidant activity, phenolic and flavonoid content compared to aerial parts of *Haplophyllum vermiculare*, and may be more effective in improving oxidative stress, prevention and treatment of ISD.

Keywords:

Lawsonia inermis

Haplophyllum vermiculare

Antioxidative activity

Skin disease

Inflammation

Introduction

Skin is one of the innate immunity mechanisms that prevents the entry of germs and harmful substances into the body. Skin exposes to a variety of chemical and physical stimuli, such as reactive oxygen species (ROS) produced by immune cells or enzymatic activities and

environmental stimuli (UV light and pollutants) (Shah and Sinha 2013). Prolonged exposure to oxygen and other damaging agents make the skin particularly susceptible to oxidative stress (Kalkan et al., 2014). Oxidative stress is a condition in which ROS and other oxidants overcome the antioxidant system and disrupt

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cellular and tissue homeostasis. Changes in homeostasis lead to the development of various inflammatory skin diseases (ISD) such as psoriasis, contact dermatitis, atopic dermatitis and urticaria (Sies, 2015). Therefore, improving the antioxidant system seems to be very effective in treatment of ISD.

Lawsonia inermis, popularly known as henna, is a perennial tree of the Lythraceae family that grows in arid and warm areas. In traditional Iranian medicine, this herb is widely used on the skin because of its pleasant smell and color. The orange color of henna is derived from a compound called lawsone (2-hydroxy-1,4-naphthoquinone), which makes up about 2% of its leaf (Mastanaiah et al., 2011). The phytochemical analysis of *Lawsonia inermis* has indicated the presence of more than a hundred bioactive molecules like phenolic acids, flavonoids, coumarins, triterpenoids, naphthalene derivatives, organic acids, quinoids and xanthenes (Al-Snafi 2019).

Haplophyllum vermiculare is another herb that is especially used in rural areas of Fars province, Iran to reduce skin inflammation. The most common use of *Haplophyllum vermiculare* is skin inflammation following snake and scorpion stings. *Haplophyllum vermiculare* is a shrubby plant of the Rutaceae family and usually its aerial parts are cut off and put on the skin as a poultice in ISD. The phytochemical analysis of *Lawsonia inermis* has indicated the presence of alkaloids, lignans, coumarins, flavanoids, essential oil and volatile oil in the aerial parts of *Haplophyllum vermiculare* (Al-Snafi 2018). This study was performed for the first time to compare the antioxidant power, phenolic and flavonoid content of *Lawsonia inermis* leaves and aerial parts of *Haplophyllum vermiculare*, in order to evaluate their capacity to improvement of oxidative stress and eventually prevention and treatment of ISD.

Material and methods

Collection and extraction of the plants

The leaves of *Lawsonia inermis* and aerial parts of *Haplophyllum vermiculare* were collected from Darab city, Fars province, Iran. The collected Seaweed species were confirmed by experts of Fasa Medicinal Plants Research Center. The voucher numbers of FM-PRC-100-20 and FM-PRC-100-24 Was assigned for *Lawsonia inermis* and *Haplophyllum vermiculare* respectively in the herbarium of Fasa Medicinal Plants

Research Center, Fasa University of Medical Sciences, Iran. Extraction was done by maceration method. The powders of each plants (100g) was immersed in ethanol (70:30 v/v) and kept at room temperature and darkness for one week with continuous stirring. The Solid and insoluble particles were removed by passing the extracts on a filter paper. The excess solvent was also evaporated at 50°C (Movaghari Pour et al., 2018). The concentrated extract was incubated at 50°C for 24h to dry (Hoseinzadeh 2019; Seyedalipour et al., 2016).

Measurement of phenolic content of herbal extracts

The phenolic content of *Lawsonia inermis* and *Haplophyllum vermiculare* extract was measured using the Folin–Ciocalteu (FC) method (Hoseini et al., 2019). Accordingly, 500µl of the FC reagent (10% v/v) was added to 100µl of each extract (1mg/ml) and incubated for 5min at room temperature and darkness. Then 400µl of sodium carbonate (7.5% w/v) was added to the sample and the resulting solution was kept at room temperature and darkness for 60min. Finally, absorbance of the samples was measured at 765nm by Synergy HTX multi-mode reader (Amoussa et al., 2015). Gallic acid was also used as standard and the phenol content of the extracts was reported in micrograms gallic acid equivalent (GAE) per miligram of dry weight (µg GAE/mg) (Yari et al., 2016). All measurements were done at least in duplicate.

Measurement of flavonoid content of herbal extracts

Aluminum chloride method was used to measure the flavonoid content of the extracts. Accordingly, 50µl of the aluminum chloride (10% w/v) and 50µl of the sodium nitrite (10% w/v) were added to 200µl of each extract (1mg/ml). The solution was incubated 6min at room temperature and darkness, then 700µl of sodium hydroxide (4% w/v) was added and the total volume of the solution was 1ml. After complete stirring, the resulting solution was again incubated at room temperature and darkness for 15min and the absorbance of the solution was read at 510nm using a Synergy HTX multi-mode reader. Quercetin was also used as standard and the flavonoid content of the extracts was reported in micrograms quercetin equivalent (QE) per miligram of dry weight (µg QE/mg) (Al-Sowayan and Mousa 2014; Ayyobi et al., 2017). All measurements were done at least in duplicate.

Evaluation of antioxidant activity

In the present study, ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods were used to compare the antioxidant power of *Lawsonia inermis* and *Haplophyllum vermiculare*. The FRAP assay was used to investigate the potency of monovalent antioxidants that capable of reducing Fe^{3+} to Fe^{2+} ions. Whereas in the DPPH method, the total radical scavenging activity is determined with reducing the stable nitrogen radical (DPPH radical) (Legault et al., 2011; Valverde Malaver et al., 2015).

Evaluation of the monovalent reducing power

As mentioned, the FRAP assay measures the antioxidant capacity of the extracts by reducing the Fe^{3+} ions (present in the Fe-TPTZ complex) to Fe^{2+} ions, so it's called monovalent reduction (Gallego et al., 2013). Working solution of FRAP assay is not stable and is recommended to be prepared just before testing. Therefore, the following solutions (A,B,C) are mixed at a ratio of 1–1–10, respectively. A: 10mM TPTZ solution in 40mM hydrochloric acid; B: $FeCl_3$ solution (20mM) and C: acetate buffer (300mM, pH = 3.6).

In the following, 1.5ml of the prepared FRAP working solution was poured into a test tube and 50ml of herbal extracts (1mg/ml) were added and mixed. After 10min incubation at 37°C, the absorbance of the colored solution was read at 593nm. Serial dilution of $FeSO_4$ solution (1mM) was used as standard and the antioxidant power of the extract was reported in $\mu mol Fe^{2+}/g$ (Wang et al., 2016). All measurements were done at least in duplicate.

Determination of radical scavenging activity

Total radical scavenging activity of the extracts is

determined by DPPH assay. The DPPH radicals has a purple color that turns to yellow after reduction by antioxidants in the herbal extracts. The intensity of colorlessness indicates the antioxidant power in different concentration (Carvalho et al., 2011; Wang et al., 2016). Accordingly, serial dilution of plant extracts (10, 50, 100, 200, 500 and 1000) was prepared using ethanol 70%. In the following 40 μ l of herbal extracts was added to 160 μ l of 0.3 mM DPPH and kept at room temperature and darkness for 30min (López et al., 2011). Finally, the optical absorption changes of the samples were determined at 517nm using Synergy HTX multi-mode reader. All measurements were done at least in duplicate. Ascorbic acid as a reference antioxidant was used for comparing the results. The antioxidant activity was calculated as percentage of inhibition relative to the control using the following equation:

$$\text{Antioxidant power} = \left[\frac{\text{optical absorption of control group} - \text{optical absorption of experimental group}}{\text{optical absorption of control group}} \right] \times 100$$

Statistic analysis

Data were expressed as the mean \pm SD and compared between experimental groups using t-test and one-way analysis of variance(ANOVA). The IC50 also calculated by Four Parametric Logistic Regression. Significant difference was determined at the level of $P < 0.05$. All analyses were performed using GraphPad Prism 8.0.2 (Chen et al., 2013).

Results

Phenolic and flavonoid content of the extracts

The phenolic content of the extracts was calculated by FC method based on the standard line equation of gallic acid ($y = 219.33x - 19.727$, $R^2 = 0.9971$). Accord-

TABLE 1: Antioxidant activity, phenolic and flavonoid content of *Lawsonia inermis* and *Haplophyllum vermiculare* extracts.

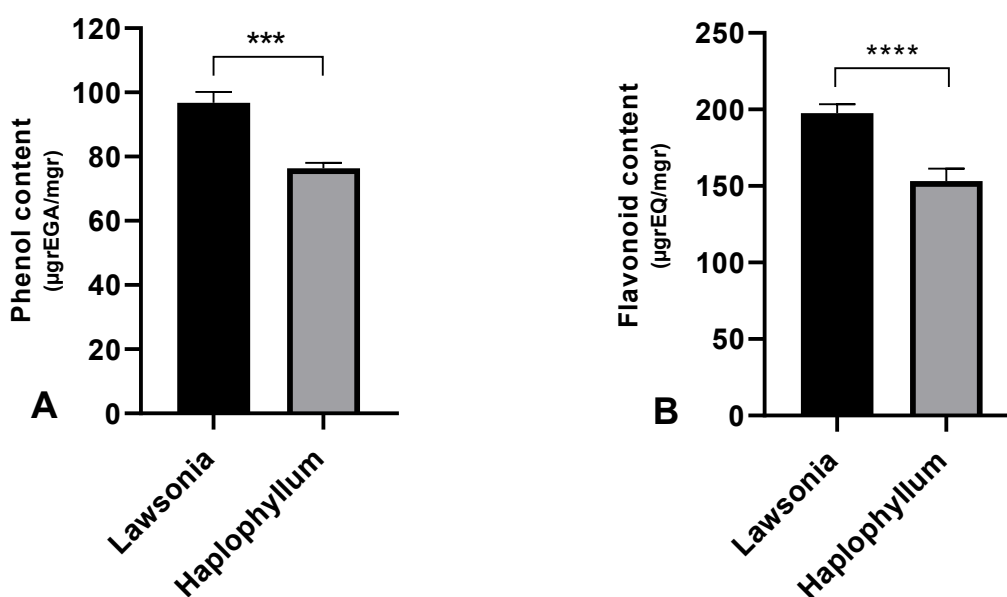
	Phenol content (μ g GAE/mg)	Flavonoid content (μ g QE/mg)	Antioxidant activity	
			FRAP assay (μ mol Fe^{2+} /g)	DPPH IC50 (μ g/ml)
<i>Lawsonia inermis</i>		197.69	862.8932.23	796.83
<i>Haplophyllum vermiculare</i>	76.33	153.20	765.52	1621
P-value	0.0008	<0.0001	0.0043	-

Data are expressed as mean \pm SD. Statistical difference between the groups was investigated by t-test and P-value <0.05 was considered as significant.

TABLE 2: Total radical scavenging activity (%) of different concentrations of *Lawsonia inermis* leaf, aerial parts of *Haplophyllum vermiculare* and ascorbic acid.

Concentration ($\mu\text{g/ml}$)	<i>Lawsonia inermis</i>		<i>Haplophyllum vermiculare</i>		P-value	Ascorbic acid	
	Mean	IC50	Mean	IC50		Mean	IC50
10	1.62		1.28		0.7056	1.83 ± 15.79	
50	5.49		2.29		0.3654	4.12 ± 63.12	
100	8.72	671.6	4.97	1621	0.0199	4.68 ± 84.10	30.99
200	14.03		8.25		0.03 ± 92.42		
500	36.50		21.03		0.0001	0.05 ± 92.62	
1000	65.72		36.32.52		0.0001>	0.40 ± 93.09	

Data are expressed as mean \pm SD. The P-value column indicate statistical differences between *Lawsonia inermis* and *Haplophyllum vermiculare* by t-test analysis. The P-value <0.05 was considered as significant.

**FIGURE 1.** Phenolic (A) and flavonoid (B) content of hydro-ethanolic extract of *Lawsonia inermis* leaf and aerial parts of *Haplophyllum vermiculare*. Data are expressed as mean \pm SD. Statistical difference between the groups was investigated by t-test and P-value <0.05 was considered significant. The bullet of (***) means $P < 0.001$ and the bullet of (****) means $P < 0.0001$.

ingly, the phenolic content of hydro-ethanolic extract of henna leaf extract was $96.76 \pm 3.34 \mu\text{g GAE/mg}$, which was significantly ($P = 0.0008$, Fig. 1) higher than the phenolic content of hydro-ethanolic extract of *Haplophyllum vermiculare* ($76.33 \pm 1.68 \mu\text{g GAE/mg}$). The flavonoid content of the extracts was also calculated by the aluminum chloride method based on the standard line equation of quercetin ($y = 415.98x - 7916.2$, $R^2 = 0.9869$). Accordingly, the flavonoid content of hydroalcoholic extract of henna leaf extract was $197.69 \pm 5.76 \mu\text{g QE/mg}$ which was significantly ($P < 0.0001$, Fig. 1) higher than the flavonoid content of *Haplophyllum vermiculare* ($153.20 \pm 8.16 \mu\text{g QE/mg}$).

Monovalent reducing power

The reducing power of the extracts was calculated using the FRAP assay based on the FeSO_4 standard line equation ($y = 2298.3x - 387.19$, $R^2 = 0.9973$). According to Table 1 and Figure 2, the antioxidant activity of hydroethanolic extract of *Lawsonia inermis* leaf was $862.89 \pm 32.23 \mu\text{mol Fe}^{2+}/\text{g}$, significantly ($P = 0.0043$) higher than *Haplophyllum vermiculare* extract ($765.52 \pm 29.39 \mu\text{mol Fe}^{2+}/\text{g}$).

Total radical scavenging activity

The radical scavenging activity of the extracts was determined by DPPH assay. The highest radical scavenging activity of the extracts was related to ascorbic acid ($\text{IC}_{50} = 30.99$), *Lawsonia inermis* extract ($\text{IC}_{50} = 671.6$) and *Haplophyllum vermiculare* extract ($\text{IC}_{50} = 1621$),

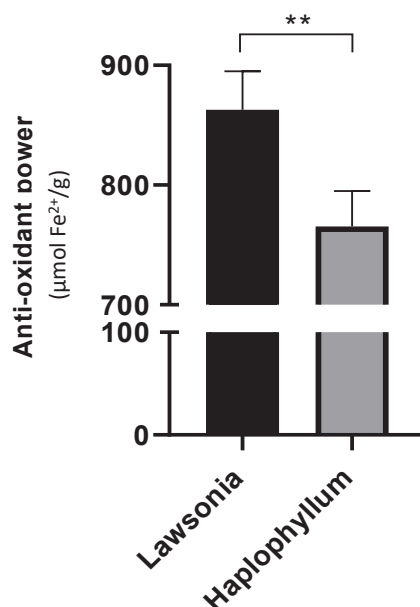


FIGURE 2. Monovalent reducing power of hydro-ethanolic extract of *Lawsonia inermis* leaf and aerial parts of *Haplophyllum vermiculare*. Data are expressed as mean±SD. Statistical difference between the groups was investigated by t-test and P-value <0.05 was considered significant. The bullet of (**) means P<0.01 in comparison of groups.

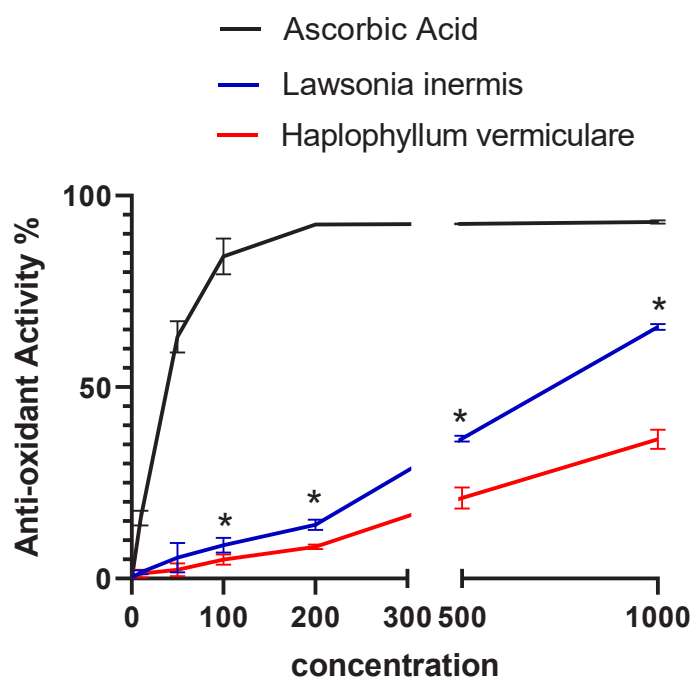


FIGURE 3. Total radical scavenging activity of hydro-ethanolic extract of *Lawsonia inermis* leaf, aerial parts of *Haplophyllum vermiculare* and Ascorbic Acid. Data are expressed as mean±SD and analyzed by t-test. The P-value <0.05 was also considered as significant. The bullet (*) Indicates a statistically significant difference between *Lawsonia inermis* and *Haplophyllum vermiculare* by t-test as P-value < 0.05.

respectively.

According to Table 2, the antioxidant activity of *Lawsonia inermis* extract at concentrations of 10 and 50µg/ml had no significant difference with *Haplophyllum vermiculare*. But at concentrations of 100, 200, 500 and 1000 there was a significant statistical difference between *Lawsonia inermis* and *Haplophyllum vermiculare*

extracts (Table 2). There was a direct relationship between the concentration of the extracts and their antioxidant activity (Karimi et al., 2010). The antioxidant activity of *Lawsonia inermis* and *Haplophyllum vermiculare* extracts at 1000µg/ml compared to 10µg/ml increased by 64.1% and 35.06%, respectively.

Discussion

Biological drugs have developed with increasing knowledge of the pathogenic mechanisms and inflammatory pathways in ISD. These drugs affect the specific and functional components of the immune system and have revolutionized in treatment of inflammatory diseases at first; however currently they are restricted. Therefore, the identification and production of alternative drugs has become critical (Goldenberg 2015). Numerous papers have shown the role of oxidative stress in the pathogenesis of ISD (Kalkan et al., 2014; Zhou et al., 2009). It is appeared that improving oxidative stress is a suitable therapeutic target in these diseases (Kaur et al., 2016).

The present study showed that the antioxidant activity, phenolic and flavonoid content of the *Lawsonia inermis* extract were significantly higher than *Haplophyllum vermiculare*. The higher antioxidant activity of the *Lawsonia inermis* extract was double confirmed by DPPH and FRAP assay, and indicated that *Lawsonia inermis* contains higher radical scavenging activity and reducing power. *Lawsonia inermis* extract on concentration of 1000µg/ml scavenged approximately 65% of the DPPH radicals and indicated more reducing power compared to *Haplophyllum vermiculare*. The high antioxidant potency of *Lawsonia inermis* leaves is probably due to its rich phenolic and flavonoid content (Mirzaei et al., 2010) and thus appears to be more effective in ISD prevention or treatment.

Multiple previous studies indicate high antioxidant activity of *Lawsonia inermis* (Al-Snafi 2019). For example Hasan et al. (2016) was reported the high DPPH radical scavenging activity of *Lawsonia inermis* by 79.16±0.98%. The antioxidant activity of petroleum ether fraction of *Lawsonia inermis* was comparable to that of ascorbic acid (78.07.3±1.2%). Hanachi et al. (2018) also showed that the aqueous extract of *Lawsonia inermis* flowers had high reducing power by FRAP assay. This high antioxidant power resulted in inhibition of gastric cancer cells (AGS line) and fibroblast cells proliferation. Rahmat et al. (2006) reported that essential oil of *Lawsonia inermis* showed high antioxidant effects by FTC and TBA assay that inhibited high proliferation of CaCo2, HepG2, MCF7 and MDA-MB-231 cancer cell lines. Therefore, *Lawsonia inermis* is likely to be effective in improving oxidative stress and suppressing proliferation of keratinocytes in ISD, especially

psoriasis.

As it mentioned, *Haplophyllum vermiculare* probably is less effective in ISD prevention and treatment compared to *Lawsonia inermis*. The present study for the first time investigated the scavenging activity, reducing power and phenolic and flavonoid content of *Haplophyllum vermiculare*. *Haplophyllum vermiculare* on concentration of 1000µg/ml scavenged about 36% of DPPH radicals. The reducing power of *Haplophyllum vermiculare* was also lower than *Lawsonia inermis* by 12.71 % (consistent with phenolic and flavonoid content). Therefore, this plant is likely to be effective in inhibiting hypersensitivity reactions following by snake and scorpion stings, rather than improving antioxidant system. Of course, numerous studies need to be done to reach a conclusion.

As the best of our knowledge, no study has been performed on the *Haplophyllum vermiculare*. But some study indicated antioxidant activity of another species of *Haplophyllum* genus. For example in the study of Eissa et al. (2014a) the high antioxidant and cytoprotective effects of *Haplophyllum tuberculatum* aerial parts was indicated by employing H₂O₂ as oxidant inductor and the human astrocytoma U373-MG cell line as the cell model. The essential oils of *Haplophyllum tuberculatum* also showed inhibited ROS production and high scavenging activity (Eissa et al., 2014b).

Multiple studies have confirmed the efficacy of herbal antioxidants in the treatment of ISD. For example in a study, Antiga et al. (2015) showed that an antioxidant component of cinnamon (curcumin) can be effective as an complementary therapy in the treatment of psoriasis. In this study, oral administration of curcumin and topical administration of steroids compared with steroids alone had better effects on the psoriasis area severity index (PASI) reducing (Antiga et al., 2015; Hatcher et al., 2008). Mustard seed was another plant with high antioxidant activity that has been studied. In an animal study, oral administration of mustard seed has been shown to decrease the plasma malondialdehyde levels, infiltration and recruitment of T lymphocytes, dendritic cells and macrophages in the lesions as well as inflammatory cytokines. Oral administration of mustard seed also increased the activity of superoxide dismutase, catalase and glutathione peroxidase (Yang et al., 2013). The therapeutic effect of an antioxidant alga, *Dunaliella bardawil*, on psoriasis was also demonstrated in the study of

Greenberger et al. (2012). In that study, oral consumption of the the algae resulted in a significant reduction of PASI in patients with psoriasis compared with placebo group.

The use of antioxidant effects in the treatment of ISD is not limited to herbal compounds. Some chemicals with antioxidant activity have shown similar therapeutic effects. For example, dimethylfumarate (antioxidant drug) significantly reduced proliferation, epidermal thickness and keratinocyte differentiation and reduced PASI by 55%, as well as decreased inflammatory cell recruitment and infiltration in patients with psoriasis (Bovenschen et al., 2010). Utas et al. (2002) also reported that administration of propylthiouracil in patients with chronic psoriasis leads to a significant reduction of PASI by modifying the antioxidant system. In fact, propylthiouracil showed its effects by reducing malondialdehyde, inhibiting the production of ROS, increasing superoxide dismutase and glutathione peroxidase in plasma, erythrocytes and skin. Therefore, improving antioxidant activity is a suitable approach in ISD treatment and *Lawsonia inermis* probably is more effective in prevention and treatment of ISD compared to *Haplophyllum vermiculare*.

Conclusion

Development of oxidative stress and weakening of the antioxidant activity is one of the mechanisms involved in incidence and exacerbation of ISD. The native *Lawsonia inermis* leaf of Fars province compared to *Haplophyllum vermiculare* extract may be an appropriate option in the treatment of ISD (Lin and Huang 2016); therefore, it is suggested that the therapeutic effect of *Lawsonia inermis* leaves on different arms of the antioxidant system should be investigated in animal studies or future clinical trials. *Haplophyllum vermiculare* is also likely to be effective in improving hypersensitivity reactions and further studies are needed.

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Conflict of interest

The authors declare that they have no known com-

peting financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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