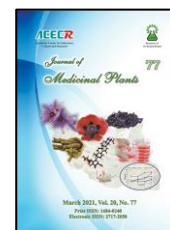




Institute of
Medicinal Plants

Journal of Medicinal Plants

Journal homepage: www.jmp.ir



Research Article

Improving biochemical traits, oleo-gum yield and compositions of asafoetida (*Ferula assa-foetida* L.) essential oil using 24-epibrassinolide in Kerman natural habitats (Iran)

Amir Saadatfar^{1,*}, Samira Hossein Jafari², Iraj Tavassolian³

¹ Department of Medicinal Plants, Research and Technology Institute of Plant Production (RTIPP), Shahid Bahonar University of Kerman, Kerman, Iran

² Faculty of Natural Resources and Desert Study, Yazd University, Yazd, Iran

³ Department of Rangeland and Watershed Management, Higher Education Complex of Shirvan, Shirvan, Iran

ARTICLE INFO

Keywords:

Secondary metabolites
Biochemical traits
Chemical composition
Ferula assa-foetida L.
Natural habitat

ABSTRACT

Background: Asafoetida (*Ferula assa-foetida* L.) is an endangered endemic plant which is widely used in food, cosmetics and pharmaceutical industries. It is necessary to enhance the quantity and quality of its secondary metabolites in order to meet requirements of the industries. **Objective:** This study investigated the impact of 24-epibrassinolide (EBL) on biochemical traits, essential oil yield and chemical compositions of *Ferula assa-foetida* in its natural habitat. **Methods:** We foliar sprayed the EBL at the concentrations of 0, 0.1, 0.5 and 1 μ M with three times during a week at fully matured leaves phase. One month after treatments application, leaf sampling and gum gathering of the plants was done and the mentioned parameters were measured. **Results:** ANOVA results indicated that 1 μ M EBL showed the best results for chlorophyll a, b, total chlorophyll, carotenoids, reduced sugar and protein contents (17.77, 13.08, 31.57, 7.50, 40.45 and 0.34 mg/g FW). The main components of the essential oil are α -pinene (8.82 %), β -pinene (11.57 %) and myrcene (1.12 %) that showed a significant increase at 1 μ M of EBL. Although, EBL at 0.5 μ M was proved to be the best treatment for leaf total phenol content (79.37 mg/g FW) and sulfurous compounds such as: (*E*)-1-propenyl sec-butyl disulfide (51.48 %), bis (1-methyl propyl) disulfide (0.9 %) and *n*-propyl sec-butyl disulfide (0.41 %). The highest amount of flavonoids (146.18; 162.56 mg/g FW) and essential oil yield (7.91 %; 8.16 %) were obtained at 0.5 and 1 μ M EBL concentrations, respectively compared to the control (6 %). **Conclusion:** Our results indicated the promising and positive effects of EBL, as an environmental friendly strategy, to improve oleo-gum quantity and quality.

Abbreviations: EBL, 24-Epibrassinolide; FW, Fresh Weight; PAL, Phenylalanine Ammonia Lyase; PVPP, Polyvinylpyrrolidone; PMSF, Phenylmethylsulfonyl Fluoride; EC, Electrical Conductivity; ANOVA, Analysis of Variance

* Corresponding author: saadatfar.amir@uk.ac.ir

doi: 10.29252/jmp.20.77.93

Received 9 December 2020; Received in revised form 1 February 2021; Accepted 3 February 2021

© 2020. Open access. This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (<https://creativecommons.org/licenses/by-nc/4.0/>)

1. Introduction

Medicinal and aromatic plants produce important secondary metabolites which are widely used in food, cosmetics and pharmaceutical industries [1, 2]. These compounds play an important role in plants survival under environmental conditions. It is vital to enhance the quality and quantity of plant biochemical traits in order to meet the market and industry requirements [3].

Asafoetida oleo-gum is obtained from different species of *Ferula* which is used in pharmaceutical, perfume, and other industries. *Ferula* is perennial plants genus belongs to Apiaceae family with 185 species [4]. Asafoetida (*Ferula assa-foetida* L.) is an endangered native herbaceous perennial plant that grows in arid rangelands of Iran, some parts of India and Afghanistan [5, 6]. Its fresh leaves are used by indigenous people in traditional foods [7]. The most important pharmacological uses of the oleo-gum obtained from this plant are for digestive and nervous disease treatments, anti-fungal, antioxidant activities and anti-tumor properties [8-10]. Asafoetida major components are resin (40-64 %), gum (25 %) and essential oil (10-17 %) [5]. Asafoetida essential oil has high commercial value and the main components consist of α -pinene, β -pinene, myrcene and sulfurous compounds [1, 11]. Finding methods to increase economical profit from this plant have significant role in sustainable development of rural communities. Plant growth regulators have been used to manipulate medicinal plants growth, essential oil compositions and yield under natural conditions, biotic or abiotic stresses [3, 12-15]. It is well documented that exogenous application of phytohormones such as brassinosteroides is helpful to improve the productivity of cereals, vegetables, oil seed and medicinal plants [3, 15].

Brassinosteroids are cost-effective and novel group of phytohormones which can function as growth regulators [3, 16, 17]. Among 70 types of identified brassinosteroids, brassinolide, 24-epibrassinolide (EBL) and 28-homobrassinolide are proved to have economic effects on plant growth, productivity, metabolism and high stability under field conditions [3]. They can protect plants from different biotic and abiotic stresses such as heat, drought, salt, heavy metals and fungi and virus diseases [18-22]. They are a type of steroid hormones which involved in a wide range of developmental, physiological and biochemical process [3, 23-26]. BRs application on different plants has been extensively studied recently. Exogenous application of BRs as an environmental friendly strategy reduced the phytotoxic effects of herbicides and insecticides [27, 28]. Application of 28-homobrassinolide improved *Satureja khuzestanica* essential oil yield and chemical compounds such as carvacrol and para-cymene [13]. Application of EBL (24-epibrassinolide) at 0.5 ppm significantly increased the essential oil yield and compositions of peppermint [29].

Although many studies have confirmed the potential of brassinosteroids to improve crops and several medicinal plants performance, there is no report about the effect of brassinosteroids on *Ferula assa-foetida* under natural conditions. According to the fact that *Ferula assa-foetida* is one of the most valuable and economically important plants which is widely used in different industries and in order to enhance its oleo-gum quality, the aim of this study was to investigate the effects of exogenous EBL application on biochemical traits, essential oil yield and chemical compositions of asafoetida oleo-gum under natural conditions.

2. Materials and methods

2.1. Plant materials, growth conditions and treatments

The present study was conducted in the natural habitat of asafoetida in Kerman province. The area is located at 30° 34' to 30° 39' N and 55° 00' to 55° 07' E with average annual precipitation of 176 mm. The average altitude is 2620 m above sea level and the average annual temperature is 14.8 °C. Soil texture was sandy loam. Foliar spray was performed at concentrations of 0, 0.1, 0.5 and 1 µM. For this purpose, several uniform asafoetida plants with the same ages (5-6 years old) were randomly chosen for each treatment. Their locations were determined by GPS and marked. All climatic and topographic (slope, aspect and height) conditions were relatively the same.

To make 24-epibrassinolide (EBL) solution, EBL powder (Sigma-Aldrich, Saint Louis, USA) was gently mixed with 96 % ethanol until completely dissolved, and then distilled water was added to make desirable concentrations. EBL treatment was applied by spraying aerial parts of the plants at fully matured leaves stage. This process replicated three times every other days in a week. One month after application of EBL, leaf samples were collected and the plants were prepared for gum gathering. Collected oleogums were used to extract the essential oil.

2.2. Biochemical parameters

2.2.1. Determination of chlorophyll and carotenoids contents

Chlorophyll a, b, total and carotenoid content were determined according to Lichtenthaler method [30]. Fresh leaves were homogenized in 3 ml of 70 % acetone. Then centrifuged at 12000 × g for 15 min. Absorbance was observed at 662, 645 and 470 nm to estimate chlorophyll a, b and carotenoid contents using a spectrophotometer (Cary 50, Germany),

respectively. 70 % acetone was taken as blank. Total chlorophyll content was determined by adding the contents of chlorophyll a and b.

2.2.2. Total phenol contents

Total phenol content was determined according to Swain and Hillis method [31]. Leaves powder (0.5 g) was extracted in 80 % methanol and centrifuged at 10000 × g for 10 min at 4 °C. Total phenol content in the supernatant was determined using Folin-Ciocalteu reagent. Its absorbance was measured at 725 nm against a blank control. The amount of total phenol content was calculated according to the standard graph of Gallic acid at different concentrations.

2.2.3. Total flavonoid determination

The total flavonoid content was determined using a modified spectrometrically method. In brief, 1 g of fresh leaves was weighed and grounded with 10 ml of 95 % methanol and then was filtered with Whatman filter. An aliquot of 1 ml of solution was mixed with 0.6 ml of 5 % sodium nitrate and vortexed well and kept in room temperature for 6 min. 0.4 ml of 10 % aluminum chloride was added and placed for further 6 min. 0.5 ml NaOH 1 M was added. The samples absorbance was measured at 510 nm with UV-Vis spectrophotometer (Cary 50, Germany) versus blank control. Four replications were used for each treatment.

2.2.4. Electrolyte leakage measurement

Electrolyte leakage was determined using electrical conductivity meter. Samples were washed by deionized water and dried by filter paper. Fresh leaves (1 g) were soaked in deionized water (20 ml) and incubated at 25 °C. Electrical conductivity (EC₁) of the solution was determined after 24 h. Samples were autoclaved (120 °C) for 20 min to kill the tissues completely

and release all electrolytes. After cooling the samples at 25 °C, final electrical conductivity (EC_2) was recorded. Then, the electrolyte leakage was determined by the following formula: Electrolyte leakage = $EC_1/EC_2 \times 100$.

2.2.5. Reduced sugar content determination

Reducing sugar content (mg/g FW) was estimated according to Nelson method [32]. Absorbance was measured at 620 nm using spectrophotometer.

2.2.6. Determination of phenylalanine ammonia lyase (PAL Enzyme) activity

Leaf powder (300 mg) was pulverized in cold mortar containing 50 mM Tris-HCl (pH = 8.8) and 15 mM beta-mercaptoethanol. The mixture was centrifuged at $5000 \times g$ for 30 min and the supernatant solution was used for PAL assays. In this method, PAL catalyzes and converts phenylalanine to cinamic acid. An aliquot of 1 ml of extraction buffer, 0.5 ml phenylalanine (10 mM) and 1 ml enzyme extraction were mixed and maintained at 37 °C for 60 min. The reaction was terminated by increasing 500 μ l of trichloroacetic acid (10 %). Cinamic acid concentration was estimated at 290 nm absorbance using spectrometer (UV-VIS, Cary 50, Germany). One unit of PAL activity was defined as 1 μ mol of produced cinamic acid in a minute.

2.2.7. Protein content

Leaf powder (0.5 g) was ground in 50 mM phosphate buffer (pH = 7) containing 1 mM EDTA, 2 % (w/v) polyvinylpolypyrrolidone (PVPP) and 2 mM phenylmethylsulfonyl fluoride (PMSF). The extract was centrifuged at $13000 \times g$ for 20 min. Protein content was estimated according to Lowry [33]. To calculate the amount of protein in the samples, a graph

(absorbance versus concentration) for standard solutions of protein was depicted and used.

2.3. Essential oil preparation

The essential oil of asafoetida oleo-gum was extracted by hydro-distillation method for 4 h, using Clevenger apparatus. The essential oil was dried using sodium sulfate and stored at 4 °C until analyzed. The yield of essential oil was calculated based on dry oleo-gum weight.

2.4. Identification of essential oil components

The essential oil was analyzed using model 6890 gas chromatography coupled with an Agilent model 5973-N mass spectrometry which equipped with an HP-5MS capillary column and phenyl methyl siloxane phase (30 m $\times$ 0.25 mm i.d. $\times$ film thickness 0.25 μ m). The ionization energy was 70 eV. Temperature was programmed from 60 to 246 °C with a rate of 3 °C/min. Injector and detector were maintained at 250 °C. Samples (1 μ l) were injected with a split ratio of 1:50. Helium was used as a carrier gas with a flow rate of 1.5 ml/min. Retention indices were determined using retention times of *n*-alkanes (C₈-C₂₅) which were injected after the essential oils under the same chromatographic conditions. The components of the essential oil were identified by comparing different parameters such as retention times, Kovats retention index and their mass spectra with those of a computer library, authentic compounds, Adams library and data published in the literature [34].

2.5. Statistical analysis

The experiments were arranged in completely randomized design and sampling was done randomly with five replications for each parameter. The collected data was analyzed by ANOVA test. Mean values were compared using

Duncan's multiple range test at $P < 0.05$. All analyses were conducted by SPSS and EXCEL software.

3. Results

The results showed that EBL treatment had significant effects on some biochemical parameters in *Ferula assa-foetida* leaves (Table 1).

EBL at concentration of 1 μM , significantly showed the maximum level of Chlorophyll a (17.77 mg/g FW) and b (13.08 mg/g FW), and total (31.57 mg/g FW) and carotenoids content (7.50 mg/g FW). While the minimum amounts of these parameters were observed in control (3.65, 2.55, 6.20 and 2.44 mg/g FW, respectively) and at 0.1 μM concentration (5.2, 3.39, 8.59 and 2.57 mg/g FW). There was no significant difference between these treatments in terms of chlorophyll and carotenoid contents (Fig. 1. a, b, c, d).

The results indicated that EBL treatment had a significantly increased the total phenol content in asafoetida leaves. The highest amount of total phenol was observed at 0.5 μM concentration (79.37 mg/g FW). EBL at 0.1 μM concentration (73.33 mg/g FW) was in the second level. There was no significant difference between 1 μM EBL concentration with control. The control treatment had the least amount of total phenol (63.91 mg/g FW) (Fig. 1.e).

EBL application markedly increased flavonoid levels compared to the control. There was no significant difference among different concentrations of EBL in terms of flavonoid

content. The amounts of flavonoids were 64.25, 151.26, 146.18 and 162.56 mg/g FW in control, 0.1, 0.5 and 1 μM treatments, respectively (Fig1.f). EBL treatment reduced the electrolyte leakage in asafoetida leaves, and the control treatment had the highest electrolyte leakage (77.87 %). The lowest electrolyte leakage was observed at 1 μM concentration (34.44 %) (Fig. 1.g).

Reduced sugar content increased significantly concomitant with EBL concentrations. The highest amount of reduced sugar was obtained from the plants treated with 1 μM EBL (40.45 mg/g FW). Control had the least reduced sugar content (13.05 mg/g FW, Fig. 1.h).

PAL enzyme contents significantly decreased after EBR application (Fig. 1.i). The highest and the least amount of PAL enzyme was observed in control (0.19 U mg^{-1} protein) and 0.5 μM EBL concentration (0.07 U mg^{-1} protein), respectively.

The highest level of protein content was obtained at 1 μM concentration (0.34 mg/g FW), while no significant difference was observed among control, 0.1 and 0.5 μM EBL concentrations (Fig. 1.j).

EBL application caused a significant increase and improvement in essential oil contents at 1 μM (8.16 %) and 0.5 μM (7.91 %) concentrations. There was no significant difference between these concentrations. Control (6 %) and 0.1 μM EBL (6.10 %) treatments had the least amounts of essential oil (Fig. 2).

Table 1. Analysis of variance according to the experiment for measured biochemical parameters

Source	df	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Carotenoid	Phenol	Flavonoid	Electrolyte leakage	Reduced sugar	PAL Enzyme	Protein	Essential oil (%)
EBL	3	123.62**	68.65**	375.3**	16.71**	129.17**	6091.72**	1016.36**	10.57**	0.007**	0.0019*	4.07**
Error	16	1.25	1.06	26.34	0.27	8.79	319.91	26.69	1.02	0.0002	0.0003	0.02
CV%	-	11.87	15.88	13.17	12.25	4.15	13.64	8.78	2.48	12.45	6.48	1.94

*: ($P < 0.05$), **: ($P < 0.01$)

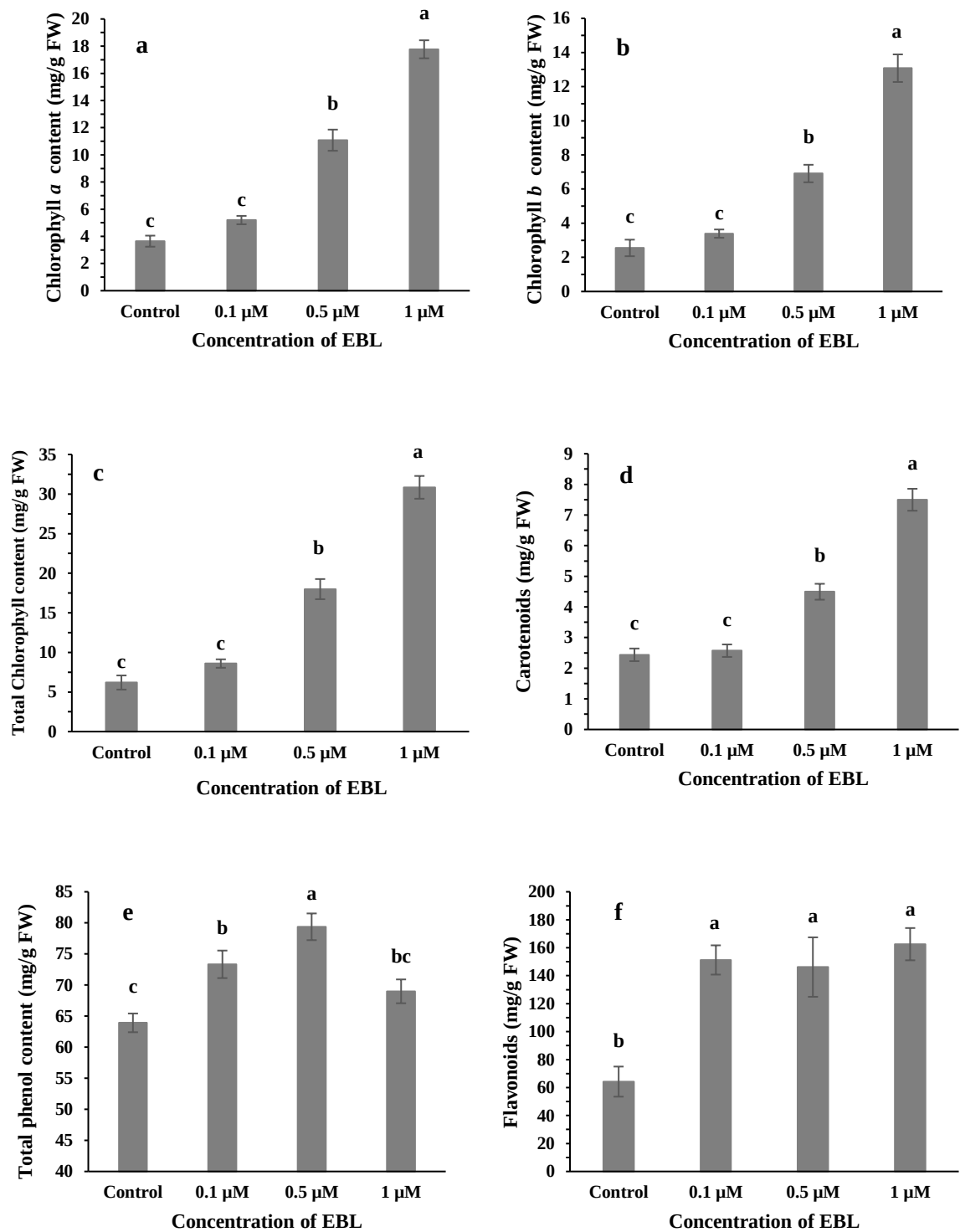


Fig. 1. Effects of EBL on Chlorophyll a (a), Chlorophyll b (b), Total Chlorophyll (c), Carotenoids (d), Total phenol content (e), Flavonoids (f), Electrolyte leakage (g), Reduced sugar content (h), PAL enzyme (i) and Protein (j) in *Ferula assa-foetida* leaves. Columns with different letters are significantly different.

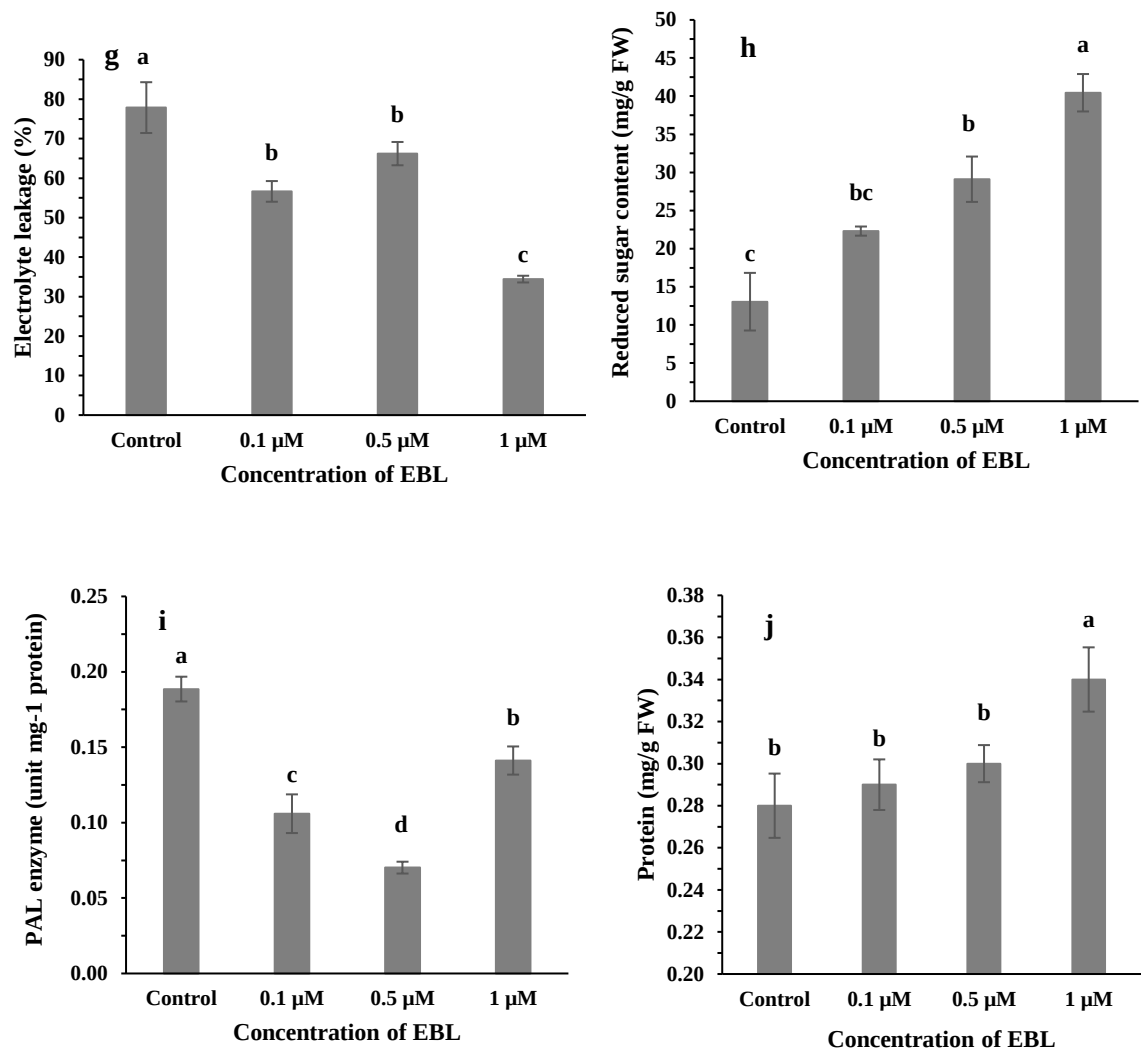


Fig. 1. Effects of EBL on Chlorophyll a (a), Chlorophyll b (b), Total Chlorophyll (c), Carotenoids (d), Total phenol content (e), Flavonoids (f), Electrolyte leakage (g), Reduced sugar content (h), PAL enzyme (i) and Protein (j) in *Ferula assa-foetida* leaves. Columns with different letters are significantly different. (Continued)

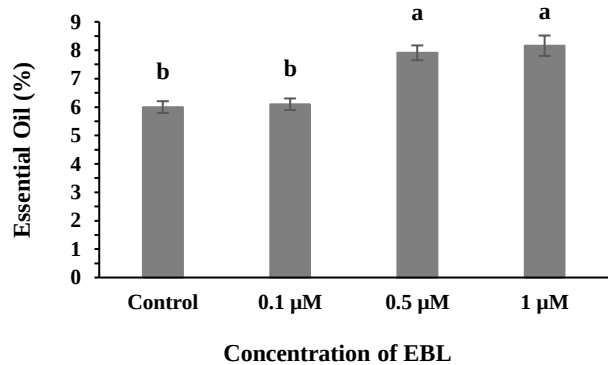


Fig. 2. Effects of EBL on essential oil content in *Ferula assa-foetida* gum

In response to EBL treatment, there was a progressive enhancement in the content and yield of the main components as compared to the control (Table 2). EBL treatment at 1 μM concentration proved the best and significantly elevated the contents of α -pinene, β -pinene and myrcene by 8.82 %, 11.57 % and 1.12 %, respectively compared to other treatments. Although, (*Z*)- β -ocimene (9.34 %), (*E*)- β -ocimene (8.49 %) compounds and sulfurous compounds, the most important compound in asafoetida, such as (*E*)-1-propenyl sec-butyl

disulfide (51.48 %), bis (1-methyl propyl) disulfide (0.9 %) and *n*-propyl sec-butyl disulfide (0.41 %) had the highest amount at 0.5 μM EBL concentration.

Other components with low percentages such as α -phellandrene (2.76 %), 2,3,4-trimethylthiophene (1.85 %), 2-ethyl-3,5-dimethylthiophene (0.41 %), *allo*-ocimene (0.27 %), α -guaiene (2.2 %), α -humulene (1.46 %), *cis*-cadinene-1,4-diene (0.46 %), γ -cadinene (0.84 %), and δ -cadinene (1.19 %) also showed significant increase at 0.5 μM EBL (Table 2).

Table 2. Effects of EBL on essential oil compounds (similar identified compounds in different concentrations) in *Ferula assa-foetida* gum.

	Chemical compounds	RT	KI	Concentration of EBL			
				Control	0.1 μM	0.5 μM	1 μM
1	α -Pinene	6.74	909	2.11 $\pm$ 0.08 ^c	3.22 $\pm$ 0.07 ^c	5.71 $\pm$ 0.12 ^b	8.82 $\pm$ 0.32 ^a
2	β -Pinene	8.08	953	5.87 $\pm$ 0.21 ^d	6.90 $\pm$ 0.14 ^c	10.54 $\pm$ 1.1 ^b	11.57 $\pm$ 0.61 ^a
3	Myrcene	8.45	966	0.84 $\pm$ 0.08 ^b	0.71 $\pm$ 0.02 ^b	0.96 $\pm$ 0.03 ^a	1.12 $\pm$ 0.03 ^a
4	α -Phellandrene	8.96	982	1.87 $\pm$ 0.02 ^c	2.05 $\pm$ 0.04 ^c	2.76 $\pm$ 0.06 ^a	2.36 $\pm$ 0.04 ^b
5	2,3,4-Trimethylthiophene	9.44	998	1.17 $\pm$ 0.02 ^c	1.71 $\pm$ 0.05 ^b	1.85 $\pm$ 0.1 ^a	1.74 $\pm$ 0.03 ^b
6	(<i>Z</i>)- β -Ocimene	10.14	1017	8.75 $\pm$ 0.18 ^b	7.64 $\pm$ 0.4 ^c	9.34 $\pm$ 0.26 ^a	8.65 $\pm$ 0.13 ^b
7	(<i>E</i>)- β -Ocimene	10.52	1027	5.75 $\pm$ 0.08 ^c	7.37 $\pm$ 0.08 ^b	8.49 $\pm$ 0.08 ^a	7.38 $\pm$ 0.08 ^b
8	2-Ethyl-3,5-dimethylthiophene	13.49	1105	0.18 $\pm$ 0.01 ^c	0.31 $\pm$ 0.02 ^b	0.41 $\pm$ 0.03 ^a	0.32 $\pm$ 0.02 ^b
9	<i>allo</i> -Ocimene	13.77	1112	0.28 $\pm$ 0.04 ^a	0.24 $\pm$ 0.03 ^b	0.27 $\pm$ 0.03 ^a	0.25 $\pm$ 0.05 ^b
10	<i>neo-allo</i> -Ocimene	14.36	1126	0.30 $\pm$ 0.03 ^a	0.26 $\pm$ 0.02 ^b	0.24 $\pm$ 0.03 ^b	0.25 $\pm$ 0.03 ^b
11	<i>n</i> -Propyl sec-butyl disulfide	15.16	1145	0.18 $\pm$ 0.02 ^c	0.27 $\pm$ 0.05 ^b	0.41 $\pm$ 0.08 ^a	0.32 $\pm$ 0.06 ^b
12	(<i>E</i>)-1-Propenyl sec-butyl disulfide	15.92	1164	50.28 $\pm$ 2.12 ^c	51.10 $\pm$ 3.11 ^b	51.48 $\pm$ 2.2 ^a	49.75 $\pm$ 2.14 ^d
13	Bis(1-methylpropyl) disulfide	17.20	1195	0.41 $\pm$ 0.03 ^c	0.79 $\pm$ 0.38 ^b	0.90 $\pm$ 0.06 ^a	0.49 $\pm$ 0.02 ^c
14	α -Guaiene	26.74	1424	1.89 $\pm$ 0.18 ^b	0.94 $\pm$ 0.08 ^c	2.20 $\pm$ 0.12 ^a	0.84 $\pm$ 0.03 ^d
15	α -Humulene	27.21	1436	0.60 $\pm$ 0.04 ^c	1.08 $\pm$ 0.03 ^b	1.46 $\pm$ 0.06 ^a	0.57 $\pm$ 0.02 ^c
16	<i>cis</i> -Cadinene-1,4-diene	28.82	1477	0.38 $\pm$ 0.04 ^b	0.21 $\pm$ 0.02 ^c	0.46 $\pm$ 0.05 ^a	0.22 $\pm$ 0.03 ^c
17	γ -Cadinene	29.62	1497	0.76 $\pm$ 0.07 ^b	0.30 $\pm$ 0.03 ^d	0.84 $\pm$ 0.08 ^a	0.26 $\pm$ 0.03 ^c
18	δ -Cadinene	29.94	1505	0.91 $\pm$ 0.07 ^b	0.32 $\pm$ 0.03 ^c	1.19 $\pm$ 0.13 ^a	0.25 $\pm$ 0.02 ^c

Columns with different letters are significantly different.

4. Discussion

Exogenous application of EBL substantially improved the biochemical parameters, essential oil yield and chemical compositions in asafoetida. EBL application resulted in enhancement of chlorophyll a, b and total, There are several reports of increasing chlorophyll levels after brassinosteroids application in plants such as *Chlorella vulgaris* [35], *Glycine max* [36], *Pelargonium graveolens* [37], *Coleus forskoblii* [15], *satureja khuzestanica* [13], *Jasminum sambac* [29], *Mentha piperita* [3].

Foliar spray of EBL on cucumber significantly enhanced photosynthesis components including net CO₂ assimilation, rubisco activity and other enzymes involve in photosynthesis substances production such as sucrose phosphate synthase, sucrose synthase, and acid invertase activities. The most effective concentration was 0.1 μM EBL which improved Calvin cycle capacity [38]. The effect of EBL at 2 μM on broccoli revealed that chlorophyll content was increased significantly, chloroplast ultrastructure was maintained much better

compared to the control. Also, using the EBR was preserved the integrity of grana thylakoids in the chloroplast. The expression of chlorophyll genes degradation such as *BoACS4* and *BoACO3* with 2 μM of EBL reduced markedly. It was showed that EBR at low concentration could protect chlorophyll [39].

Carotenoid content was also increased after EBL application. Carotenoids are pigment with bioactivities in photosynthesis. The result of a study showed that brassinosteroids application caused increasing chlorophyll and carotenoids content in *Mentha arvensis* [40]. It was announced that elevated photosynthesis is parallel with enhancement of chlorophyll and carotenoids content in leaves [40].

Phenolic compounds contain hydroxyl groups that destroy free radicals. These important secondary metabolites release hydrogen atoms from their hydroxyl groups, scavenge the radicals to produce stable phenoxyl radicals. Therefore, they have an important role in antioxidant activity [14]. Brassinosteroids application has negative effects on generating of free radicals [40, 41].

The results of this study showed that foliar application of EBL led to a significant enhancement in total phenol content. The highest level of total phenol was observed at 0.5 μM EBL concentration. Enhancement of the total phenol and flavonoid contents after brassinosteroids application was reported in several plants such as tomato [42], sweet basil [43] and *Tinospora cordifolia* [14].

EBL treatment decreased electrolyte leakage significantly in leaves in comparison with control. All EBL treatments improved the integrity of cell walls. In a research, it was found that EBL application reduced the electrolyte leakage in *Raphanus sativus* seedlings [24].

Asafoetida plants treated with 1 μM of EBL contained elevated levels of reduced sugar content compared to control and other treatments. In the present study, higher level of reduced sugar content might be attributed to the higher photosynthesis because of elevated chlorophyll level. The same results were reported in terms of increasing carbohydrates levels like reducing sugar after brassinosteroids application in *Coleus* [15].

An increase in protein content was observed after EBL application. Brassinosteroids plays a major role in DNA and protein synthesis [23]. It has been reported that brassinosteroids affect transcription and translation of proteins [44-46]. Similar results were observed by the researchers who demonstrated that brassinosteroids application has positive effect on protein activation [40].

It was showed that exogenous application of EBL improved biochemical traits of *Ferula assafoetida* and showed that treated plants were more robust compared to the control [47]. Totally, BRs induce cell division and elongation by acidification of the cell membrane through H^+ -ATPases and trigger other enzymes that improve assimilation of photosynthetic compounds in treated plants [48]. BRs can increase the most important enzymes that increase photosynthesis such as rubisco enzyme activity and the expression of related genes [49]. It was reported that BR increases the sensitivity of auxin receptors, which has the combined effect with BR and enhance the impact of EBL [50].

EBL application influenced not only essential oil content but also its chemical compositions at 0.5 and 1 μM concentrations. There was an increase in the essential oil yield and the main components such as α -pinene, β -pinene, myrcene and sulfurous compounds, depending on doses of different concentrations. EBL application at

maximum level of concentration (1 μm) increased the main components such as α -pinene, β -pinene and myrcene. Most of chemical compositions including sulfurous compounds increased in 0.5 μm concentration of EBL. α -Pinene, β -pinene and myrcene substances are widely used in synthesizing artificial and aromatic compounds, cosmetics, flavorings, disinfectants and pesticides. These compounds have anti-microbial, anti-inflammatory and antibiotics properties [51, 52]. Thus these are the most demanded compounds by food, pharmaceutical and cosmetic industries. Sulfurous compounds like *n*-propyl sec-butyl disulfide, (*E*)-1-propenyl sec-butyl disulfide and bis (1-methyl propyl) disulfide are the key ingredients which reveal the quality of asafoetida gum [7]. In this study, EBL increased these compounds significantly and as a result elevated its quality. In contrast with the results of this study, it was found a decrease in α -pinene, β -pinene and myrcene contents in the presence of EBL in peppermint essential oil. These differences can be due to the growth regulator concentrations, time of application and plant species as mentioned in a study [3].

Brassinosteroids can effect on growth and metabolism and might indirectly cause increasing in essential oil yield. The other reason for enhancing essential oil can be due to inherent genetic potentiality of asafoetida plant which is activated under the effect of EBL. It was also mentioned the same reason in several studies [3, 16, 30]. It was reported that application of EBL increased the essential oil content in lavender [53]. Differences in essential oil content and the amount of chemical compositions may also be due to the influence of EBL on enzymes of the biochemical pathways as reported in a research [3]. There are several reports on the effect of brassinosteroids on secondary metabolites content in different kinds of plants include an

increased shikonin content in *Onosma paniculatum* [54], artemisin levels in *Artemisia annua* [55], carvacrol and para-cymene contents in *Satureja khuzestanica* [13], menthol, menthone amount in *Mentha piperita* and *Mentha arvensis* essential oil [3, 40] and an increase in geraniol content and decrease in citronellol content in geranium [37].

There are a few studies regarding the effects of brassinosteroids application on the essential oil yield and the main components of medicinal and aromatic plants. A positive effect of EBL on asafoetida essential oil yield and its ingredients (like α -pinene, β -pinene, myrcene and sulfurous compounds) and even biochemical parameters of *Ferula assa-foetida* leaves has been reported for the first time under natural condition in this research. Further studies are needed to accurately ascertain the role of BRs in asafoetida plant.

5. Conclusion

According to the results, foliar application of EBL growth promoter at the appropriate dose can act efficiently in *Ferula assa-foetida*. Plants treated with 1 μm showed better results for chlorophylls, carotenoids, reduced sugar, protein contents and several main components like α -pinene, β -pinene and myrcene. EBL concentration at 0.5 μm proved to be the best treatment for leaf total phenol content and sulfurous compounds in asafoetida essential oil. EBL concentrations of 1 μm and 0.5 μm were the best treatments for flavonoids and essential oil contents. The results demonstrated the favorable and positive effect of EBL on leaf biochemical traits, essential oil yield and the main chemical composition of asafoetida gum which is very important economically. The results can be useful to apply in modern agriculture in order to enhance the quality of production.

Author contributions

The first author performed the experiment, collected data, analyzed and interpreted biochemical data. He is also the corresponding author. The second author participated in statistically analyzing and interpreting phytochemical data and prepared the manuscript. The third author designed and performed the experiment, collected the data and edited the manuscript.

References

1. Hossein Jafari S, Sepehry A, Soltanloo H and Karimian AA. Genetic differentiation between bitter and sweet asafetida plants using ISSR markers. *Mol. Biol. Rep.* 2018; 1-10. doi: 10.1007/s11033-018-4565-1.
2. Prins CL, Vieira IJC and Freitas SP. Growth regulators and essential oil production. *Braz. J. Plant Physiol.* 2010; 22: 91-102. doi: 10.1590/S1677-04202010000200003.
3. Coban O and Baydar NG. Brassinosteroid modifies growth and essential oil production in Peppermint (*Mentha piperita* L.). *J. Plant Growth Regul.* 2016; 1-7. doi: 10.1007/s00344-016-9614-1.
4. Eser N and Yoldas A. Identification of heat-resistant chemical components of *Ferula elaeochytris* root extracts by gas chromatography-mass spectrometry. *Trop. J. Pharm. Res.* 2019; 18: 55-60. doi: 10.4314/tjpr.v18i1.9.
5. Latifi E, Mohammadpour AA, Fathi B and Nourani H. Antidiabetic and antihyperlipidemic effects of ethanolic *Ferula assa-foetida* oleo-gum-resin extract in streptozotocin-induced diabetic wistar rats.

Acknowledgements

This research has been supported by Research and Technology Institute of Plant Production (RTIPP) (Shahid Bahonar University of Kerman, Kerman, Iran). The authors are grateful for the financial support for this research project (Project number: P/900/106).

Conflict of interest

The authors declare that they have no conflict of interest.

Biomed. Pharmacother. 2019; 110: 197-202. doi: 10.1016/j.biopha.2018.10.152.

6. Saeidy S, Nasirpour A, Djelveh Gh, Ursu AV, Marcati A, Gardarin Ch, Laroche C, Delattre C, Pierre G, Keramat J and Michaud Ph. Rheological and functional properties of asafetida gum. *Int. J. Biol. Macromol.* 2018; 118: 1168-1173. doi: 10.1016/j.ijbiomac.2018.06.177.

7. Pirmoradi MR. Morphological, Physiological, Phytochemical and Genetical evaluation of Asafoetida in Kerman Province. PhD Thesis, Horticulture Science, Faculty of Agriculture, Tarbiat Modares University, Iran; 2012: 78-98 [In Persian].

8. Angelini P, Pagiotti R, Venanzoni R and Granetti B. Antifungal and allelopathic effects of Asafoetida against *Trichoderma harzianum* and *Pleurotus* spp. *Allelopathy J.* 2009; 23: 357-368.

9. Dehpour A, Ebrahimzadeh N, Fazeland S and Mohammad NS. Antioxidant activity of the methanol extract of *Ferula assafoetida* and its essential oil composition. *Grasas Y Aceites* 2009; 60: 12-405. doi: 10.3989/gya.010109.

10. Kavosi G and Rowshan V. Chemical composition, antioxidant and antimicrobial activities of essential oil obtained from *Ferula assa-foetida* oleo-gum-resin: effect of collection time. *Food Chem.* 2013; 138: 2180-2187. doi: 10.1016/j.foodchem.2012.11.131.
11. Iranshahy M and Iranshahi M. Traditional uses, phytochemistry and pharmacology of asafetida (*Ferula assa-foetida* oleo-gum-resin)-A review. *J. Ethnopharmacol.* 2011; 134: 1-10. doi: 10.1016/j.jep.2010.11.067.
12. Dianat M, Saharkhiz J and Tavassolian I. Salicylic acid mitigates drought stress in *Lippia citriodora* L. Effects on biochemical traits and essential oil. *Biocatal. Agri. Biotechno.* 2016; 8: 286-293. doi: 10.1016/j.bcab.2016.10.010.
13. Eskandari M and Eskandari A. Effects of 28-homobrassinolide on growth, photosynthesis and essential oil content of *Satureja khuzestanica*. *Int. J. Plant Physiol. Biochem.* 2013; 5: 36-41. doi: 10.5897/IJPPB11.064.
14. Raghu K and Rao SSR. Effect of brassinosteroids on antioxidants content and radical scavenging activity of *Tinospora cordifolia* (Willd.) Miers ex Hook. F & Thoms. *J. Med. Plants Stud.* 2016; 4: 117-121.
15. Swamy KN and Rao SSR. Effect of brassinosteroids on the performance of *Coleus* (*Coleus forskoblii*). *J. Herbs, Spices and Med. Plants* 2011; 17: 12-20. doi: 10.1080/10496475.2011.556985.
16. Ding J, Wu JH, Liu JF, Yuan BF and Feng YQ. Improved methodology for assaying brassinosteroids in plant tissues using magnetic hydrophilic material for both extraction and derivatization. *Plant Methods* 2014; 10: 1-11.
17. Derevyanchuk M, Litvinovskaya R, Khripach V and Kravets V. Brassinosteroid-induced de novo protein synthesis in *Zea mays* under salinity and bioinformatics approach for identification of heat shock proteins. *Plant Growth Regul.* 2016; 78: 297-305. doi: 10.1007/s10725-015-0093-3.
18. Pociecha E, Dziurka M, Oklestkova J and Janeczko A. Brassinosteroids increase winter survival of winter rye (*Secale cereal* L.) by affecting photosynthetic capacity and carbohydrate metabolism during the cold acclimation process. *Plant Growth Regul.* 2016; 80: 127-135. doi: 10.1007/s10725-016-0149-z.
19. Rajewska I, Talarek M and Bajguz A. Brassinosteroids and response of plants to heavy metals action. *Front. Plant Sci.* 2016; 629: 1-5. doi: 10.3389/fpls.2016.00629.
20. Sharma P and Bharadwaj R. Effect of 24-epibrassinolide on seed germination, seedling growth and heavy metal uptake in *Brassica juncea* L. *Gen. Appl. Plant Physiol.* 2007; 33: 59-73.
21. Tao Y, Yu QX, Zhou YH, Shi K, Zhou J and Yu JQ. Application of 24-epibrassinolide decreases the susceptibility to cucumber mosaic virus in zucchini (*Cucurbita pepo* L.). *Sci. Hortic.* 2015; 195: 116-123. doi: 10.1016/j.scienta.2015.09.005
22. Clouse SD. A history of brassinosteroid research from 1970 through 2005: thirty-five years of phytochemistry, physiology, genes, and mutants. *J. Plant Growth Regul.* 2015; 34: 828-844. doi: 10.1007/s00344-015-9540-7.
23. Ramakrishna B and Rao SSR. 24-Epibrassinolide alleviated zinc-induced oxidative stress in radish (*Raphanus sativus* L.) seedlings by enhancing antioxidative system.

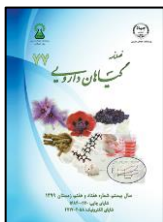
- Plant Growth Regul.* 2012; 68: 249-259. doi: 10.1007/s10725-012-9713-3.
24. Saini Sh, Sharma I and Pati PK. Versatile roles of brassinosteroid in plants in the context of its homoeostasis, signaling and crosstalks. *Front. Plant Sci.* 2015; 950: 1-17. doi: 10.3389/fpls.2015.00950.
25. Vriet C, Russinova E and Reuzeau C. Boosting crop yields with plant steroids. *Plant Cell* 2012; 24: 842-857. doi: 10.1105/tpc.111.094912.
26. Sasse JM. Physiological actions of brassinosteroids: an update. *J. Plant Growth Regul.* 2003; 22: 276-288. doi: 10.1007/s00344-003-0062-3.
27. Xia XJ, Huang YY, Wang L, Huang LF, Yu YL, Zhou YH and Yu JQ. Pesticides induced depression of photosynthesis was alleviated by 24-epibrassinolide pretreatment in *Cucumis sativus* L. *Pestic. Biochem. Physiol.* 2006; 86: 42-48. doi: 10.1016/j.pestbp.2006.01.005.
28. Akram A, Khan MA, Younis A and Ashfaq M. Exogenous application of 24-epibrassinolide on morph-physiological, Biochemical attributes and essential oil contents of *Jasminum sambac* L. *Pak. J. Agri. Sci.* 2014; 51:881-886.
29. Lichtenthaler HK, Bukhardt G, Kuhn G and Prenzel U. Light induced accumulation and stability of chlorophyll and chlorophyll-protein during chloroplast development in radish seedlings. *Z. Naturforsch.* 1981; 36: 421-430.
30. Swain T and Hillis WE. The phenolic constituents of *Prunus domestica* L. the quantitative analysis of phenolic constituents. *J. Sci. Food Agri.* 1959; 10: 63-68. doi: 10.1002/jsfa.2740100110.
31. Nelson NJ. Colorimetric analysis of sugars, In: Colowick S.P. and N.O. Kaplan (Eds), *Methods Enzymol* 3. Academic Press, New York; 1957: 85-86.
32. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with folin phenol reagent. *J. Biol. Chem.* 1951; 193: 265-275.
33. Adams RP. Identification of essential oil components by gas chromatography/mass spectrometry. Fourth ed., Illinois, USA: Allured Publishing Corporation; 2007: 809 p.
34. Bajguz A and Czerpak R. Physiological and biochemical role of brassinosteroids and their structure activity relationship in the green alga *Chlorella vulgaris* Beijerinck (Chlorophyceae). *Plant Growth Regul.* 1998; 17: 131-139. doi: 10.1007/PL00007026.
35. Cevahir G, Yentur S, Eryilmaz F and Yilmazer N. Influence of brassinosteroids on pigment content of *Glycine max* L. (soybean) grown in dark and light. *J. Appl. Biol. Sci.* 2008; 2: 23-28.
36. Swamy KN and Rao SSR. Effect of 24-epibrassinolide on growth, photosynthesis and essential oil content of *Pelargonium graveolens* (L.) Herit. *Russ. J. Plant Physiol.* 2009; 56: 616-620. doi: 10.1080/10496475.2011.556985.
37. Yu JQ, Huang LF, Hu WH, Zhou YH, Mao WH, Ye SF and Noguès S. A role for brassinosteroids in the regulation of photosynthesis in *Cucumis sativus*. *J. Exp. Bot.* 2004; 55: 1135-1143. doi: 10.1093/jxb/erh124.
38. Cai J, Luo F, Zhao Y, Zhou Q, Wei B, Zhou X and Ji S. 24-Epibrassinolide treatment regulates broccoli yellowing during shelf life. *Postharvest. Biol. Tech.* 2019; 154: 87-95. doi: 10.1016/j.postharvbio.2019.04.019.

39. Naeem M, Idrees M, Alam MM, Aftab T, Masroor M, Khan A and Moinuddin M. Brassinosteroid-mediated enrichment in yield attributes, active constituents and essential oil production in *Mentha arvensis* L., *Russ. Agri. Sci.* 2012; 38: 106-113. doi: 10.3103/S1068367412020176.
40. Naeem M, Idrees M, Aftab T, Alam MM, Masroor M, Khan A, Uddin M and Varshney L. Employing depolymerized sodium alginate, triacontanol and 28-homobrassinolide in enhancing physiological activities, production of essential oil and active components in *Mentha arvensis* L. *Ind. Crop. Prod.* 2014; 55: 272-279. doi: 10.1016/j.indcrop.2014.01.052.
41. Ahammed GJ, Zhou YH, Xia XJ, Mao WH, Shi K and Yu JQ. Brassinosteroid regulates secondary metabolism in tomato towards enhanced tolerance to phenanthrene. *Biol. Plantarum.* 2013; 57: 154-158. doi: 10.1007/s10535-012-0128-9.
42. Koca N and Karaman S. The effects of plant growth regulators and L-phenylalanine on phenolic compounds of sweet basil. *Food Chem.* 2014; 1-17. doi: 10.1016/j.foodchem.2014.06.065.
43. Bajguz A. Effect of brassinosteroids on nucleic acids and protein content in cultured cell of *Chlorella vulgaris*. *Plant Physiol. Biochem.* 2000; 3: 209-215. doi: 10.1016/S0981-9428(00)00733-6.
44. Fariduddin Q, Ahmed A and Hayat S. Response of *Vigna radiata* to foliar application of 28-homobrassinolide and kinetin. *J. Plant Biol.* 2004; 48: 465-468. doi: 10.1023/B:BIOP.0000041106.77930.d6.
45. Li P, Chen L, Zhou Y, Xia X, Shi K, Chen Zh and Yu J. Brassinosteroids-induced systematic stress tolerance was associated with increased transcripts of several defence-related genes in the Phloem in *Cucumis sativus*. *PLOS ONE* 2013; 8: 1-8. doi: 10.1371/journal.pone.0066582.
46. Evergetis E, Koulocheri SD and Haroutounian SA. Exploitation of Apiaceae family plants as valuable renewable source of essential oils containing crops for the production of fine chemicals: Part II. *Ind. Crop. Prod.* 2015; 64: 59-67. doi: 10.1016/j.indcrop.2014.10.069.
47. Mollaei S, Farahmand H and Tavassolian I. The effects of 24-epibrassinolide corm priming and foliar spray on morphological, biochemical and postharvest traits of sword lily. *Horticul. Environ. Biotech.* 2018; 59 (3): 325-333. doi: 10.1007/s13580-018-0033-z.
48. Khripach V, Zhabinskii V and Groot AD. Twenty years of brassinosteroids: steroidal plant hormones warrant better crops for the XXI century. *Ann. Bot.* 2000; 86: 441-447. doi: 10.1006/anbo.2000.1227.
49. Xia XJ, Huang LF, Zhou YH, Mao WH, Shi K, Wu JX, Asami T, Chen Z and Yu JQ. Brassinosteroids promote photosynthesis and growth by enhancing activation of Rubisco and expression of photosynthetic genes in *Cucumis sativus*. *Planta.* 2009; 230: 1185-1196. doi: 10.1007/s00425-009-1016.
50. Vert G, Walcher CL, Chory J, and Nemhauser JL. Integration of auxin and brassinosteroid pathways by Auxin Response Factor 2. *Proc. Natl. Acad. Sci. USA* 2008; 105: 9829-9834. doi: 10.1073/pnas.0803996105.
51. Hamid AA, Aiyelaagbe OO and Usman LA. Essential oils: its medicinal and pharmacological uses. *Int. J. Curr. Res.* 2011; 33: 86-98.

52. Youssef AA and Talaat IM. Physiological effect of Brassinosteroids and Kinetin on the growth and chemical constituents of lavender plant. *Ann. Agri. Sci. (Cairo)* 1998; 43: 261-272.
53. Yang YH, Zhang H and Cao RQ. Effect of brassinolide on growth and shikonin formation in cultured *Onosma paniculatum* cells. *J. Growth. Regul.* 1999; 18: 89-92. doi: 10.1007/PL00007054.
54. Wang JW, Kong FX and Tan RX. Improved artemisin accumulation in hairy root cultures of *Artemisia annua* by (22S, 23S)-

homobrassinolide. *Biotech. Let.* 2002; 24: 1573-1577. doi: 10.1023/A:1020377130657.

How to cite this article: Saadatfar A, Hossein Jafari S, Tavassolian I. Improving biochemical traits, oleo-gum yield and compositions of asafoetida (*Ferula assafoetida* L.) essential oil using 24-epibrassinolide in Kerman natural habitats (Iran) . ***Journal of Medicinal Plants*** 2021; 20(77): 93-107.
doi: 10.29252/jmp.20.77.93



مقاله تحقیقاتی

بهبود ویژگی‌های بیوشیمیایی، تولید اسانس و ترکیبات شیمیایی شیرابه آنقوزه (*Ferula assa-foetida* L.) با استفاده از ۲۴- اپی‌براسینولید در رویشگاه‌های طبیعی استان کرمان (ایران)

امیر سعادت فر^{۱*}، سمیرا حسین جعفری^۲، ایرج توسلیان^۳

^۱ گروه گیاهان دارویی، پژوهشکده فناوری تولیدات گیاهی، دانشگاه شهیدباهنر کرمان، کرمان، ایران

^۲ دانشکده منابع طبیعی و کوهپژوهی، دانشگاه یزد، یزد، ایران

^۳ گروه مرتع و آبخیزداری، مجتمع آموزش عالی شیروان، شیروان، ایران

اطلاعات مقاله	چکیده
گل‌واژگان: متابولیت‌های ثانویه خصوصیات بیوشیمیایی ترکیبات شیمیایی آنقوزه رویشگاه طبیعی	مقدمه: گیاه آنقوزه (<i>Ferula assa-foetida</i> L.) گیاهی بومی و در معرض انقراض بوده که به طور وسیعی در صنایع غذایی، آرایشی و دارویی مورد استفاده می‌باشد. به دلیل افزایش تقاضا در صنایع مختلف، بالا بردن کمیت و کیفیت متابولیت‌های ثانویه این گیاه ضرورت دارد. هدف: در این مطالعه تأثیر محلول‌پاشی ۲۴- اپی‌براسینولید (EBL) بر ویژگی‌های بیوشیمیایی، تولید اسانس و ترکیبات شیمیایی شیرابه آنقوزه در رویشگاه طبیعی آن مورد بررسی قرار گرفت. روش بررسی: محلول‌پاشی EBL بر اندام هوایی گیاه با غلظت‌های ۰، ۰/۱، ۰/۵ و ۱ میکرومولار با فاصله سه بار در طول یک هفته در زمان برگ‌دهی کامل انجام شد. پس از یک ماه، نمونه‌برداری از برگ‌ها و بهره‌برداری از شیرابه انجام و پارامترهای مورد نظر اندازه‌گیری گردید. نتایج: نتایج آنالیز واریانس حاکی از این بود که EBL در غلظت ۱ میکرومولار بهترین نتیجه را در مورد کلروفیل a، b، کلروفیل کل، کاروتنوئید، قندهای محلول و میزان پروتئین (به ترتیب ۱۷/۷۷، ۱۳/۰۸، ۳۱/۵۷، ۷/۵۰، ۴۰/۴۵ و ۰/۳۴ میلی‌گرم بر گرم وزن تر) نشان داد. مهم‌ترین ترکیبات اسانس شامل آلفا- پینن (۸/۸۲ درصد)، بتا- پینن (۱۱/۵۷ درصد) و میرسن (۱/۱۲ درصد) نیز در غلظت ۱ میکرومولار EBL افزایش معنی‌داری نشان دادند. اما غلظت ۰/۵ میکرومولار EBL بهترین تیمار برای محتوای فنل کل برگ (۷۹/۳۷ میلی‌گرم بر گرم وزن تر) و ترکیبات گوگردی مانند (E)-۱- پروپیل سکو- بوتیل دی سولفید (۵۱/۴۸ درصد)، بیس (۱- متیل پروپیل) دی سولفید (۰/۹ درصد) و n- پروپیل سکو- بوتیل دی سولفید (۰/۴۱ درصد) بود. بیشترین مقدار فلاونوئیدها (۱۴۶/۱۸ و ۱۶۲/۵۶ میلی‌گرم بر گرم وزن تر) و بازدهی اسانس (۷/۹۱ درصد و ۸/۱۶ درصد) به ترتیب در غلظت‌های ۰/۵ و ۱ میکرومولار در مقایسه با شاهد (۶ درصد) به دست آمد. نتیجه‌گیری: نتایج نشان‌دهنده اثرات امیدبخش و مثبت EBL، به عنوان یک استراتژی دوستدار محیط زیست، برای بهبود کمیت و کیفیت صمغ آنقوزه بود.

مخفف‌ها: EBL، ۲۴- اپی‌براسینولید؛ FW، وزن تر؛ PAL، فنیل آلانین آمونیا لیاز؛ PVPP، پلی‌وینیل پلی‌پیرولیدون؛ PMSF، فنیل متیل سولفونیل فلوراید؛ EC، هدایت الکتریکی؛ ANOVA، آنالیز واریانس

* نویسنده مسئول: saadatfar.amir@uk.ac.ir

تاریخ دریافت: ۱۹ آذر ۱۳۹۹؛ تاریخ دریافت اصلاحات: ۱۳ بهمن ۱۳۹۹؛ تاریخ پذیرش: ۱۵ بهمن ۱۳۹۹

doi: 10.29252/jmp.20.77.93

© 2020. Open access. This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License (<https://creativecommons.org/licenses/by-nc/4.0/>)