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Research article

The physiological and growth response of *Petunia hybrida*, *Tagetes erecta*, and *Calendula officinalis* to plant and human steroids

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Abstract: The application of plant hormones has been long considered in the production of the plants, but the use of a whole new set of plant growth regulators including brassinosteroids and human hormones including progesterone and estradiol has been an interesting field of study for plant researchers in recent decades. The present study aimed to explore the effect of plant and animal steroid growth regulators on growth and development of *Petunia*, *Tagetes* and *Calendula*. The first factor was devoted to steroid growth regulators including: No hormone, 1 mg L^{-1} progesterone, 1 mg L^{-1} estradiol, 1 mg L^{-1} 24-epibrassinolide, and 1 mg L^{-1} homobrassinolide. The second factor included three ornamental plant species including *Petunia hybrida*, *Tagetes erecta*, and *Calendula officinalis*. The recorded traits included morphological and biochemical compounds such as catalase, peroxidase, and superoxide dismutase. It was found that the highest leaf area, chlorophyll content, and peroxidase level were obtained from the plants treated with estradiol. The application of homobrassinolide had the highest effect of carotenoid and chlorophyll contents. Among the interactions, "24-epibrassinolide × *Petunia*" resulted in the highest leaf area, "estradiol × *Calendula*" resulted in highest superoxide dismutase activity, and "estradiol × *Petunia*" resulted in the highest peroxidase activity. We observed the desirable impact of steroids on the studied traits. Estradiol, homobrassinolide and 24-epibrassinolide improved some traits, but no specific effect was observed for the application of progesterone. According to the results, it is recommended to apply steroid growth regulators to improve the quality and yield of the plants.

Keywords: estradiol; brassinolide; progesterone; carotenoid; peroxidase

Highlights

- Estradiol increased chlorophyll content and peroxidase level.
- Homobrassinolide had the highest impact on the carotenoid level and chlorophyll content.
- No specific effect was observed for the application of progesterone on the studied traits.

1. Introduction

Petunia hybrida from the Solanaceae family has both annual and perennial cultivars and is originated from South America. In horticulture, most of its cultivars, even perennial cultivars, are used as annual plants [1]. *Tagetes erecta* from the Asteraceae family is an aromatic herb with feather-like leaves that is mostly grown in landscapes [2]. *Calendula officinalis* L. from the Asteraceae family is a beautiful and medicinal herb with a long flowering period [3].

Steroids play a major role as hormones both in plants and in animals. Plants produce a lot of steroids and sterols that have been introduced as hormones in animals [4,5]. Brassinosteroids (BRs) are steroidal hormones that regulate the growth and development of the plants. In plants, many steroids have been identified, but only the steroids commonly called brassinosteroids have been widespread in the plant kingdom and stimulate plant growth when applied exogenously. BRs have diverse physiological and morphological effects, including cell and stem elongation, root formation inhibition, leaf bending, proton pump activation, ethylene production, differentiation of vascular elements, and activation of stress responses. Brassinolide is the first plant steroid known for hormonal activity [6–8]. Brassinosteroids refer to steroid compounds that are cholestane derivatives. Various types of BRs have been identified in different plant species, including monocot and dicot angiosperms, gymnosperms, monilophyta, and mosses. They are capable of controlling developmental and physiological processes in plants. Some of these activities include cell division, cell elongation and photomorphogenesis [8,9]. In the mutant plants that lack brassinosteroids, growth variations are evident, including physiological complications such as dwarfness, male sterility, delayed flowering, and the loss of the apical dominance of the buds. Brassinosteroid transfer pathway in plants is among the well-studied and identified pathways [10,11]. Although it is said that BRs have the potential for hormonal activity, most of their effects are similar to those of other known hormones [12]. BRs can be used both in foliar spray and as seed immersion, but the foliar spray is highly dependent on the plant growth stage. Generally, young plants respond better to foliar spray than older plants [10].

Sex hormones of mammals, including estrogens, androgens, and progesterones, also belong to steroids, a group of compounds that have a stern-carbon skeleton. Various steroids of living organisms are distinguished according to the position and type of active groups (agents) attached to the stern. In mammals, steroid sex hormones play a key role in controlling reproductive and developmental processes; also, they are involved in the metabolism of minerals and proteins [7]. Extensive studies have been carried out with radioimmunoassay on the presence of mammalian steroids in plants (128 species from 50 plant families) [13]. Androsterone and progesterone have been found in over 80% of the studied species, androgens (testosterone and dihydrotestosterone) has been found in 70% of the species, and estrogen (estrogen and 17-beta-estradiol) has been observed in 50% of the species. The amount of steroid may significantly vary throughout plant development, depending on the species, variety, and plant organ [7]. Zhang et al. [8] used radioimmunoassay (RIA)

to measure the levels of total estrogens and 17-beta-estradiol in pollen and style of *Ginkgo biloba*, corn and *Brassica campestris*. The level of 17-beta-estradiol in pollens of these species was about 8–35 pg g^{-1} FW. It was 24–40 pg g^{-1} FW in the style of *Lilium davil*. They demonstrated the variations of its concentration over the flower development period. The 17-beta-estradiol and estrone were identified in the lipid fraction of organs and callus of *Solanum glaucophyllum* [14]. Also, 0.1 µg estrone increased the growth of pea seedlings by about 50% [15]. Similarly, 17-beta-estradiol and progesterone (0.25 µg per plant) improved the shoot growth of sunflower seedlings but inhibited their root growth, although root elongation was enhanced by 0.1 µg/plant progesterone. Testosterone at the rate of 0.1–0.25 µg/plant stimulated the formation of cotyledonary axillary bud formation [16]. Mammalian sex hormones have been reported to influence callus growth, epinasty formation, the increase in sugar and proteins content, reproductive growth and flowering, flower number, female/male flower ratio, pollination, and fertilization [7]. The similarity of the chemical structure of steroidal plant growth material (brassinosteroids) with the structure of the sex hormones of mammals has led researchers to investigate the effects of human sex hormones on different plants. The mechanism of sex hormones effects in plants is not yet elucidated and may involve elicitation [17,18] or hormone-like activity. In this sense, this experiment aimed to explore the effects of some sex hormones of mammals at various growth stages of the plants and to compare these effects with plant steroid, i.e. brassinolide.

2. Materials and methods

The study was carried out as a factorial experiment with two factors on the basis of a randomized complete block design with 15 treatments and three replications. The first factor was devoted to human and plant steroids at five levels of no hormone (a_1), 1 mg L^{-1} progesterone (a_2), 1 mg L^{-1} estradiol (a_3), 1 mg L^{-1} 24-epibrassinolide (a_4), and 1 mg L^{-1} homobrassinolide (a_5). The second factor included three plant species including *Petunia hybrida* (b_1), *Tagetes erecta* (b_2), and *Calendula officinalis* (b_3). Each experimental plot was composed of two pots, each one containing one single plant. In total, there were 90 experimental pots. The hormone was sprayed at three stages in 2-week intervals. The recorded traits included leaf length and width, leaf area, flower longevity, carotenoid level, chlorophyll *a*, *b* and total chlorophyll contents, and the enzymatic activities of catalase, peroxidase, and superoxide dismutase. To determine leaf area, the leaves of each plant were detached, their length (L) and widest width (W) were measured, and the leaf area (A) was calculated by the Eq 1 [19].

$$A = L \times W \times 0.75 \tag{1}$$

To measure carotenoid level, the treatments were sampled. Then, 0.5 g was weighed from the sample and was ground in a mortar containing 50 mL 80% acetone (80 mL acetone + 20 mL distilled water). Then, it was infiltrated, adjusted to 50 mL, and poured into cuvettes. The extracts were read at 645, 663, and 660 nm and were placed in the following equation, denoted by *A*, to determine carotenoid levels of the treatments [20] according to Eq 2.

$$\text{Carotenoid level} = 4.69(A_{660}) - 0.268(A_{645}) + 8.02(A_{663}) \tag{2}$$

In a similar procedure to measure chlorophyll contents of the treatments, 0.5 g of sample was weighed and ground in a mortar containing 50 mL 80% acetone (80 mL acetone + 20 mL distilled water). Then, the extract was infiltrated, adjusted to 50 mL, and poured into cuvettes. To determine chlorophyll content, it was read at 643 and 660 nm with a spectrophotometer. Chlorophyll *a* and *b* and total chlorophyll contents were estimated by the Eqs 3, 4 and 5, respectively [20].

$$\text{Chlorophyll a (mg/ml)} = 9.93(A_{660}) - 0.777(A_{643}) \tag{3}$$

$$\text{Chlorophyll b (mg/ml)} = 17.6(A_{643}) - 2.81(A_{660}) \tag{4}$$

$$\text{Total chlorophyll (mg/ml)} = 7.12(A_{660}) + 16.8(A_{643}) \tag{5}$$

To measure the enzymatic activity of peroxidase (POD), the extract was prepared as described above. Then, the variations of OD at 430 nm were read with a spectrophotometer once thirty seconds for two minutes [21]. The enzymatic activity of catalase (CAT) was measured through the following stages [22].

One gram of plant tissue that had been ground in 4 mL ethanol was added with (i) 0.01 mol phosphate buffer (pH = 7); (ii) 0.5 mL H_2O_2 0.2 mol; (iii) 2 mL acid reagent (dichromate/acetic acid mixture). Then, its absorption was read at 610 nm with a spectrophotometer. The enzymatic activity of superoxide dismutase (SOD) was measured by the Das et al. [23] procedure. Each unit of enzymatic activity was assumed to the quantity of SOD with the potential of 50% inhibition of nitrite formation [23]. Data were statistically analyzed with MSTATC Software Package, and the means were compared with the LSD test at 5% level.

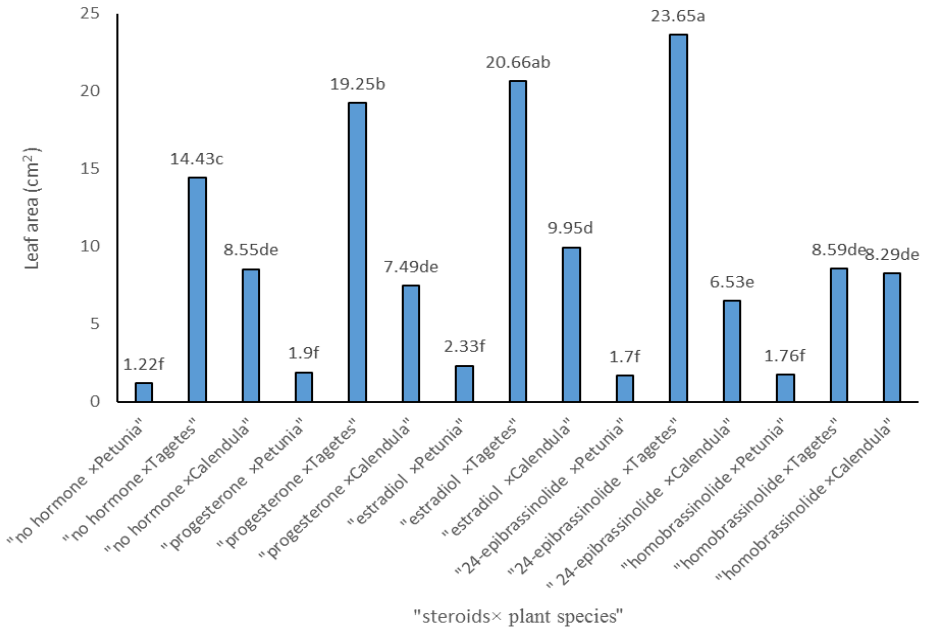

Figure 1. The interaction "steroids × plant species" for leaf area similar letter(s) imply the insignificant difference according to the LSD test.

3. Results

Analysis of variance of data showed that plant species influenced leaf size significantly ($p < 0.01$), but the effect of steroids and the interaction "steroids × species" was not significant on leaf size (length and width) (Table 1). The simple effect of steroids, species, and the interaction "steroids × species" were significant ($p < 0.01$) on leaf area (Table 1). The highest leaf area was related to the treatments with 1 mg L^{-1} estradiol (10.98 cm^2) and 1 mg L^{-1} 24-epibrassinolide (10.63 cm^2), and the lowest one (6.22 cm^2) was observed in plants treated with 1 mg L^{-1} homobrassinolide (Table 1). Also, *T. erecta* developed the highest leaf area of 17.32 cm^2, and *Petunia* grew the lowest leaf area of 1.78 cm^2.

According to the results of analysis of variance, the effect of steroids was significant ($p < 0.01$) on flower longevity. But, the effect of plant species and the interaction "steroids × plant species" did not influence this trait significantly (Table 1).

Plant carotenoid level was significantly affected by steroids ($p < 0.01$) and plant species ($p < 0.05$), but the interaction "steroids × species" was insignificant for this trait (Table 1). Means comparison for the effect of steroids on carotenoid level showed that the highest carotenoid level was 6.80 mg L^{-1} exhibited by the plants treated with 1 mg L^{-1} homobrassinolide and the lowest one was 4.85 mg L^{-1} observed in the plants treated with 1 mg L^{-1} 24-epibrassinolide.

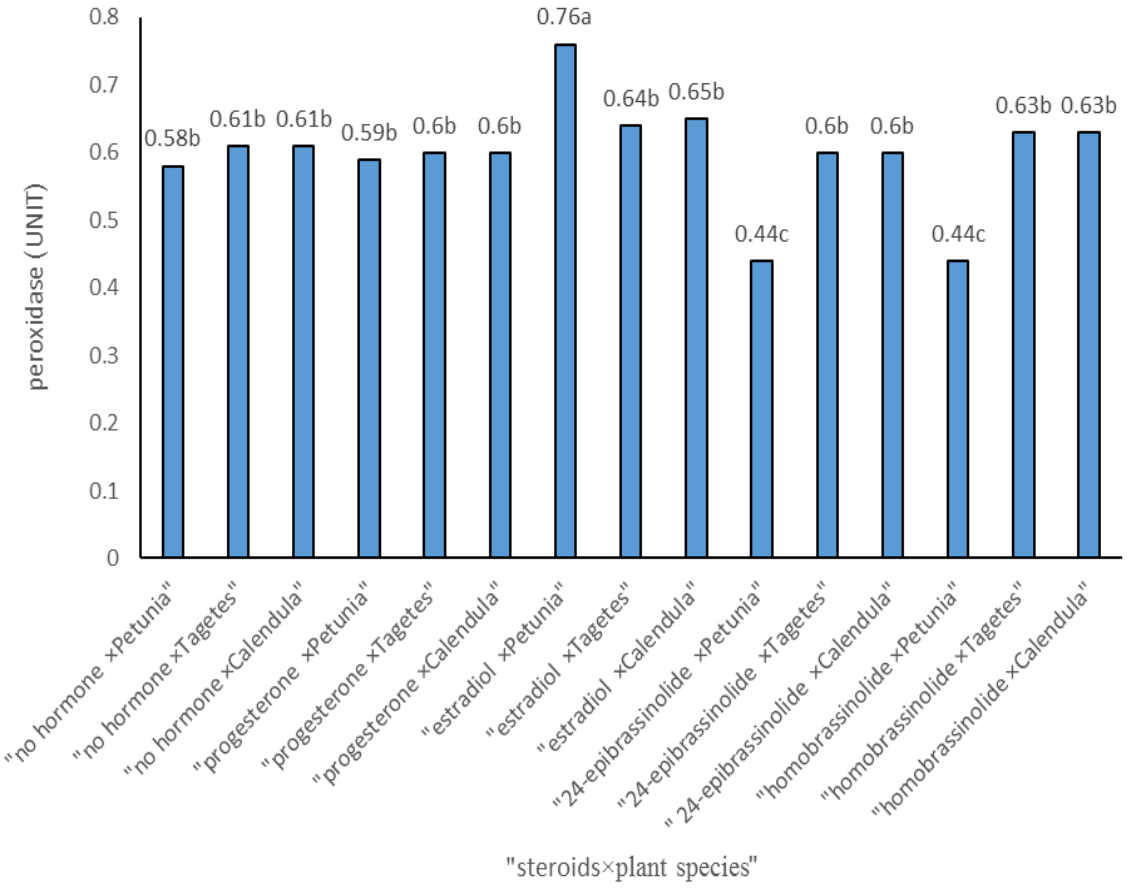

Figure 2. The interaction "steroids × plant species" for peroxidase activity.

Analysis of variance demonstrated that the simple effect of steroids on chlorophyll *a* was significant ($p < 0.01$), but the effect of plant species and "steroids × plant species" was insignificant on this trait (Table 1). According to means comparison for the data pertaining to the effect of steroids on chlorophyll *a* (Table 2), the highest chlorophyll *a* was related to plants treated with homobrassinolide, estradiol and control plants; and the lowest one was obtained by treatment with progesterone (Table 2). As the results of analysis of variance showed, chlorophyll *b* content was not changed significantly by the treatments of steroids, species, and their interaction (Table 1). It was also observed that steroids and plant species influenced total chlorophyll content significantly ($p < 0.01$ and $p < 0.05$, respectively), but "steroids × plant species" could not change this trait significantly (Table 1). Analysis of variance for data pertaining to the effect of different treatments on catalase (Table 1) indicated no significant differences in this enzyme. Peroxidase activity was significantly influenced by steroids and "steroids × plant species" ($p < 0.01$) and by plant species ($p < 0.05$) (Table 1). Means comparison for the effect of steroids on peroxidase (Table 2) showed the highest level of peroxidase in plants treated with estradiol and the lowest level in those treated with 24-epibrassinolide. Also, it showed that *T. erecta* and *C. officinalis* had higher peroxidase level than *P. hybrida* (Table 3). Means comparison for the interaction "steroids × species" for peroxidase level revealed that "estradiol × *P. hybrida*" was related to the highest and "24-epibrassinolide × *P. hybrida*" and "homobrassinolide × *P. hybrida*" were related the lowest level of peroxidase (Figure 2).

Analysis of variance showed that the treatments of steroids and plant species had no significant impact on superoxide dismutase. But, "steroids × plant species" was significant ($p < 0.01$) for this trait (Table 1). Means comparison revealed that "estradiol × *C. officinalis*" had the highest level and "no hormone × *P. hybrida*" had the lowest level of superoxide dismutase (Figure 3).

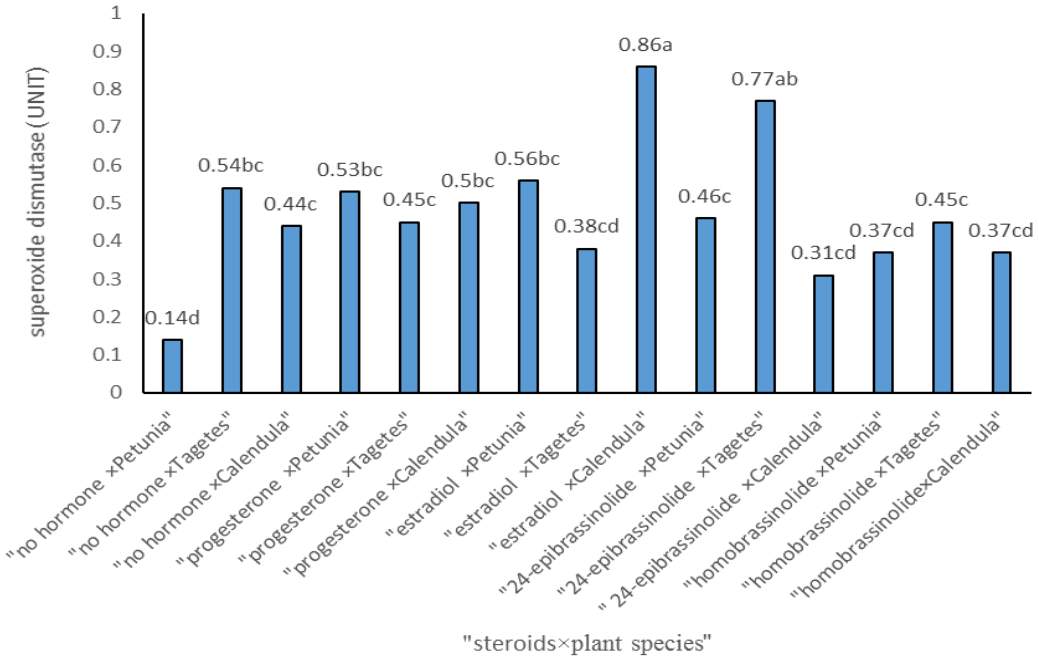

Figure 3. The interaction "steroids × plant species" for superoxide dismutase activity.

Table 1. Analysis of variance for the effect of experimental factors on the recorded traits.

Source of Variables	df	Means of squares										
		Leaf length	Leaf width	Leaf area	Flower longevity	Carotenoid	Chlorophyll *a*	Chlorophyll *b*	Total chlorophyll	Catalase	Peroxidase	Superoxide dismutase
Replication	2	0.01^{ns}	0.56^{ns}	4.77^{ns}	0.56^{ns}	0.48^{ns}	1.21^{ns}	1.17^{ns}	0.60^{ns}	0.46^{*}	0.03^{**}	0.14^{*}
Steroids (A)	4	0.19^{ns}	0.14^{ns}	34.75^{**}	82.78^{**}	5.46^{**}	7.96^{**}	0.78^{ns}	4.94^{**}	0.26^{ns}	0.03^{**}	0.08^{ns}
Species (B)	2	98.84^{**}	15.45^{**}	914.77^{**}	5.49^{ns}	3.14^{*}	1.56^{ns}	0.67^{ns}	4.21^{*}	0.05^{ns}	0.01^{*}	0.05^{ns}
A × B	8	0.47^{ns}	0.39^{ns}	37.63^{**}	29.54^{ns}	0.79^{ns}	0.81^{ns}	0.12^{ns}	0.97^{ns}	0.02^{ns}	0.02^{**}	0.11^{**}
Error	28	1.10	0.27	3.84	14.08	0.81	1.07	1.10	0.92	0.09	0.00	0.03
C.V.	-	22.53	23.94	21.56	33.44	14.48	18.93	39.17	11.80	28.01	10.27	37.17

**: significance at $p < 0.01$; *: significance at $p < 0.05$; ns: non-significance.

Table 2. Means comparison for the effect of experimental hormones on the recorded traits.

Treatment	Leaf area (cm^2)	Flower longevity (day)	Carotenoid (mg L^{-1})	Chlorophyll *a* (mg L^{-1})	Total chlorophyll (mg L^{-1})	Peroxidase enzyme (UNIT)*
No hormone	8.07 bc	7.67 c	6.59 a	5.75 a	8.66 a	0.60 b
1 mg L^{-1} progestrone	9.55 ab	11.11 bc	6.55 a	4.52 b	7.23 b	0.59 b
1 mg L^{-1} estradiol	10.98 a	9.11 c	6.44 a	6.37 a	8.72 a	0.68 a
1 mg L^{-1} 24-epibrassinolide	10.63 a	15.33 a	4.85 b	4.65 b	7.52 b	0.55 b
1 mg L^{-1} homobrassinolide	6.22 c	12.89 ab	6.80 a	6.53 a	8.93 a	0.57 b

Similar letter(s) in each column imply the insignificant difference according to the LSD test. *: peroxidase and catalase enzyme are expressed in µmol H_2O_2 consumption per minute per mg protein.

Table 3. Means comparison for the effect of plant species on the recorded traits.

Treatment	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Carotenoid ($mg\ L^{-1}$)	Total chlorophyll ($mg\ L^{-1}$)	Peroxidase enzyme (UNIT)*
Petunia hybrida	1.83 c	1.20 c	1.78 c	5.64 b	7.55 b	0.54 b
Tagetes erecta	6.84 a	3.22 a	17.32 a	6.68 a	8.45 a	0.61 a
Calendula officinalis	5.32 b	2.04 b	8.16 b	6.43 a	8.63 a	0.62 a

Similar letter(s) in each column imply the insignificant difference according to the LSD test. *: peroxidase and catalase enzyme are expressed in µmol H_2O_2 consumption per minute per mg protein.

4. Discussion

According to means comparison, the largest leaves were observed in *Tagetes erecta* and the lowest leaf length and width were related to *Petunia hybrida*. This shows that leaf size, which is an inheritable trait, is not determined by steroid growth regulators. Means comparison for the interaction "steroids × species" for leaf area revealed that the highest and lowest leaf area (23.65 and 1.22 cm^2) were obtained from "1 $mg\ L^{-1}$ 24-epibrassinolide × *T. erecta*" and "no hormone × *Petunia*", respectively (Figure 1). Leaf area is a key determinant of plant growth and production, so its estimation constitutes a major component of crop growth models [24]. Cristofori et al. [25] reported that the best model for estimating the leaf area of hazelnuts was a linear model calculated by multiplying leaf length by its width. We calculated leaf area by leaf length and width, too. The increase in leaf area due the application of brassinolide is related to the plant's light absorption capability and the resulting improvement of photosynthesis efficiency. This displays the favorable effect of brassinolide on cell division, and consequently, on leaf size, leaf anatomy, and the number of stomata [26]. The highest leaf area index of plants treated with brassinolide may be associated with the activity of plant meristem tissues and the increase in the number and size of the cells, which in turn enhances photosynthesizing area [27].

Means comparison showed that 24-epibrassinolide entailed the longest flower longevity of 15.33 days, and control plants (no hormone application) showed the shortest flower longevity of 7.67 days. Epibrassinolide strikingly doubled the flower longevity. The treatment of *Gazania splendens* L. with brassinosteroids reduced the flowering time and yielded the longest flower longevity [28]. In a study on the effect of benzyl adenine and epibrassinolide on the growth and development of *C. officinalis* L., Shabani Soltanmoradi [29] reported the longest flower longevity for the treatment of "no benzyl adenine × 5 $mg\ L^{-1}$ epibrassinolide".

Also, means comparison for the effect of plant species on the carotenoid level revealed that *T. erecta* and *C. officinalis* had the highest carotenoid levels of 6.86 and 6.43 $mg\ L^{-1}$, respectively, and *Petunia* had the lowest one of 5.64 $mg\ L^{-1}$. Carotenoids play a key role in the life of humans and animals as the precursor of vitamin A and retinoids. Furthermore, these compounds are known as an antioxidant, immune booster, mutation inhibitor, pigment precursor in mammals, and

nonphotochemical fluorescence quenchers [30,31]. Although the leaves of most plants contain similar carotenoid compounds, the carotenoids of their petals have different compounds depending on plant species [32]. Marigold petals contain a great deal of lycopene, a derivative of carotene [33]. The level of steroids may significantly change as the plant develops depending on the plant species, cultivar, and organ [7]. Brassinosteroids increase photosynthesis rate and photosynthesizing pigments production in plants [34]. Behnamnia et al. [35] reported that the application of brassinolide resulted in some physiological and biochemical variations in tomato seedlings including higher root volume, antioxidant content, carotenoids, and free proline content. Sardoei Kara et al. [36] found that seed priming and foliar application of 24-epibrassinolide for marigolds improved their growth traits and photosynthesizing pigments.

We observed by means comparison of the impact of steroids on total chlorophyll that plants treated with homobrassinolide, estradiol, and control exhibited the highest total chlorophyll content, and those treated with progesterone had the lowest total chlorophyll content (Table 2). *T. erecta* and *C. officinalis* had the highest and *P. hybrida* had the lowest total chlorophyll (Table 3). Brassinosteroid can play a role in expressing genes specific to the synthesis of enzymes that affect chlorophyll generation [37]. A study on *Eriobotrya japonica* confirmed the desirable effect of epibrassinolide on chlorophyll content. This study expressed that *E. japonica* plants lost their growth parameters and chlorophyll content when exposed to salinity stress, but the application of epibrassinolide improved the plant growth by alleviating the harmful effects of salinity [38]. Swamy and Rao [39] reported that the growth increase in *Pelargonium graveolens* induced by 24-epibrassinolide application resulted in higher leaf photosynthesis rate, and ultimately, in higher biomass accumulation in shoots. The increase in photosynthesis rate was associated with higher chlorophyll contents of leaves induced by hormone application. Similarly, a study on *Gazania splendens* L. reported the effect of brassinolide application on the improvement of chlorophyll a, b, and a + b contents [28].

Given the fact that catalase plays a key role in plant resistance to stress by removing hydrogen peroxide, its boosted activity can imply plant's more resistance [40]. It has been reported that the application of 24-epibrassinolide increases the enzymatic activities of antioxidants (catalase, ascorbate peroxidase, and superoxide dismutase) in tomatoes significantly, whereas stomatal conductance, intercellular CO_2 concentration, and H_2O_2 level are lost [41]. But, this is not supported by our findings because, in our study, no hormone rates could influence catalase activity significantly.

These results imply that estradiol that is an animal hormone increases the level of peroxidase, the key antioxidant enzyme, in plants. In their ordinary activity cycle, plant peroxidases catalyze H_2O_2 recovery [42]. Peroxidase activity can be readily recognized in whole plant lifecycle from early germination stages to senescence by the control of cell elongation, defensive mechanisms, and several other functions. Peroxidases are involved in many cell processes, such as auxin metabolism, wood formation, lateral joints in the cell wall, responses to environmental stresses, and so on [43]. In our study, the treatment of 24-epibrassinolide had the least effect on this enzyme, which is not in agreement with these reports. However, this implies that the responses of the plants vary with hormone rate. A study on tomatoes reported that the application of 24-epibrassinolide mitigated water stress and enhanced the enzymatic activity of antioxidants, whereas it reduced H_2O_2 and malondialdehyde (MDA) levels; as well, it increased the concentration of abscisic acid [41]. The increased level of antioxidant property and the increased activities of superoxide dismutase, peroxidase, and catalase have been reported among the outcomes of brassinolide application [44].

In conclusion, we observed the desirable impact of steroids on the studied traits. Estradiol increased chlorophyll *a* content, total chlorophyll content, and peroxidase level, and homobrassinolide had the highest impact on the carotenoid level, chlorophyll *a* content, and total chlorophyll content. Also, the treatment of 24-epibrassinolide increased leaf area and flower longevity. In this study, no specific effect was observed for the application of progesterone on the studied traits. It was found that the highest leaf size, leaf area, carotenoid level, total chlorophyll content, and peroxidase level were related to *T. erecta*. Nonetheless, as regulators were applied, increases occurred in carotenoid level, total chlorophyll and peroxidase level in *C. officinalis*. According to the results, it is recommended to apply steroid growth regulators to improve the quality, pigments, active ingredients, and yield of the plants.

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Conflict of interest

All authors declare that they have no conflict of interests.

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