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Impact of natural plant growth-stimulating products on *RubisCO* gene expression, secondary metabolites, and antioxidant activities in *Calendula officinalis* plants

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تأثير المنتجات الطبيعية المحفزة لنمو النبات على التعبير الجيني لإنزيم RubisCO، المركبات الثانوية، والأنشطة المضادة للأكسدة في نباتات الأقحوان

ABSTRACT

Honey, pomegranate juice, and lemon juice are natural plant growth bio-stimulators with many bioactive substances that play essential roles in many plants' physio-biochemical processes, and development. The objectives of this research were to explore how foliar spraying with a 5% or 10% diluted pomegranate juice solution (DPJS), diluted lemon juice solution (DLJS), or diluted honey solution (DBHS), versus a control (distilled water; D-H_2O), positively influenced the leaf integrity, *RubisCO* gene expression, secondary metabolites, and antioxidant activities in *Calendula officinalis* plants. Foliar spraying with 5% or 10% individually from each DPJS, DLJS, or BHS significantly increased leaf SPAD index, relative water content (RWC), membrane stability index (MSI), total soluble (TS) sugar and K^+ contents, RubisCO content and activity, and *RubisCO* gene expression. Also, secondary metabolite and antioxidant capacity (total flavonoid, anthocyanin, proline, ascorbate, and glutathione contents) were significantly increased by foliar spraying of all the above-mentioned treatments. The second best treatment was 5%-DPJS or 10%-BHS, both of which were preceded by 5%-BHS as the best treatment, which the research results recommended for field application to obtain the best pharmaceutical contents from *Calendula officinalis*.

***Keywords*:** Natural plant growth biostimulators, Antioxidants, Phenolics, Flavonoids, Anthocyanins

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1. INTRODUCTION

Calendula officinalis L. (calendula or pot marigold) is one of the most salient members of the Asteraceae. Central, Eastern, and Southern Europe is the original home of calendula. It is a medicinal plant because it possesses many pharmaceutical properties, including antioxidant, anti-inflammatory, antibacterial, antitumor, and antiseptic properties (**Azizi *et al.*, 2021; Tavallali *et al.*, 2022; Ahmad *et al.*, 2023**). It contains flavonoids that reveal a strong antioxidant activity and play a role in human health by overcoming the oxidant-stimulated damage (**Rafiee *et al.*, 2015**). Because of the ability of calendula plants to flexibly adapt to various climates and soil types (**Basit *et al.*, 2018**), it is easy to expand its cultivation for more productivity with favorable ornamental and pharmaceutical properties that qualify calendula for use as home vase decoration and in the pharmaceutical industries.

At present, natural plant growth-stimulating products (NGSPs) are of interest to the agricultural sector due to their obvious benefits as nutrient and antioxidant solutions, their low prices and ease of application, and their exceptionally low harmful effects, minimizing the potential threats of toxic substances, especially for medicinal plants. They are a promising strategy for sustainable and clean farming under normal or stressful conditions (**Alzahrani and Rady, 2019; Semida *et al.*, 2019; Desoky *et al.*, 2021; Belal *et al.*, 2023; Rady *et al.*, 2023a, 2023b**). Research has focused extensively on NGSPs to ameliorate plant productivity with high quality through better nourishment which producers have to undertake practically in floriculture, including calendula. As novel NGSPs, diluted solutions made from pomegranate juice (PJS; **Kalefetoğlu Macar *et al.*, 2022; Yılmaz *et al.*, 2023**), lemon juice (LJS; **Rady *et al.*, 2023a**), and bee honey (BHS; **Semida *et al.*, 2019; Abou-Sreea *et al.*, 2021; Rady *et al.*, 2021, 2023b; Alghamdi *et al.*, 2023; Belal *et al.*, 2023; Tarfayah *et al.*, 2023**) possess several bioactive ingredients that stimulate plant growth and production in unstressed or stressful environments. The chief mechanisms targeted by NGSPs are allied closely to the nature of the growth-stimulating product. The chemical structure of the growth-stimulating product is multi-complex, and some compounds may act simultaneously, thus the exact mechanisms have not yet been identified and require further study (**Alzahrani and Rady, 2019; Semida *et al.*, 2019; Alghamdi *et al.*, 2023**). Pomegranate has outstanding nutritional quality and different antioxidants that promote immunity and overall health (**Cortez-Trejo *et al.*, 2022**). As an inexpensive material, pomegranate seeds are eco-friendly sources of antioxidants for extractions. They have antioxidant and nourishing advantages owing to their richness in phenolic acids, flavonoids, flavones, anthocyanins, coumarins, sterols, amino and fatty acids, nucleotides, minerals, vitamins, and polysaccharides (**Boroushaki *et al.*, 2016; Valero-Mendoza *et al.*, 2022**). Applying the PJS to plant leaves improves growth traits, productivity, and physio-biochemical attributes, overcomes reactive oxygen species (ROS), suppresses biomarkers of oxidative stress and genotoxicity, prevents stress-induced lipid peroxidation, and directly chelates transition metals. All these positive findings are attributed to increased antioxidant activities in plants grown in unstressed or stressful environments (**Boroushaki *et al.*, 2016;**

Kalefetoğlu Macar *et al.*, 2022; Yılmaz *et al.*, 2023).

Lemon juice solution (LJS) is rich in organic (citric, ascorbic, and malic) acids. The most predominant organic acids in LJS are citrate and ascorbate (**Nour *et al.*, 2010**). High concentrations of nutrient elements, vitamins, and sugars are explored in LJS (**Chanukya *et al.*, 2017; Rady *et al.*, 2023a**). Also, noticeable amounts of phenolic acids, flavonoids, and flavones are detected in LJS (**Klimek-szczykutowicz *et al.*, 2020**). Under normal or cadmium (Cd) stress, the use of LJS as a seed priming solution for *Phaseolus vulgaris* noticeably increases seed germination, growth traits, leaf integrity, leaf pigments, photosynthetic efficiency, activity of different antioxidants, and gene expressions, in addition to proline and soluble sugar contents as powerful osmoregulatory compounds (ORCs) (**Rady *et al.*, 2023a**).

As one of the highly effective NGSPs, foliar application of BHS to plants gives highly efficient performances in unstressed or stressful environments by reinforcing plant tolerance to adverse conditions (**Semida *et al.*, 2019; Abou-Sreea *et al.*, 2021; Alghamdi *et al.*, 2023; Rady *et al.*, 2021; Belal *et al.*, 2023; Tarfayah *et al.*, 2023**). The chief mechanisms that upregulate these affirmed findings are the bioactive ingredients of BHS, including antioxidants, ORCs, vitamins, nutrients, organic and inorganic acids, flavonoids, and phenolic acids. All bioactive ingredients of BHS can easily penetrate leaf tissue cells after foliar spraying to take advantage of their mode of action (**Rady *et al.*, 2021; Alghamdi *et al.*, 2023; Belal *et al.*, 2023**). As a consequence, the plant physio-biochemical profiles and different antioxidant compounds are favorably modified, water and nutrient absorption are improved, and the levels of both indicators of oxidative stress and stress damage are suppressed under normal or stressful conditions (**Semida *et al.*, 2019; Abou-Sreea *et al.*, 2021; Belal *et al.*, 2023; Tarfayah *et al.*, 2023**). Moreover, auto-oxidation is prevented and various oxidants (ROS) are neutralized in the presence of different enzymes and flavonoids of BHS (**Saxena *et al.*, 2010; Inés *et al.*, 2011; Abou-Sreea *et al.*, 2021; Belal *et al.*, 2023**). Foliar nourishment with DBHS shows higher plant biomass production, photosynthetic machinery, different antioxidant levels, ORCs, and nutrient contents, while effectively suppressing biomarkers of oxidative stress and indicators of stress-induced damages (**Abou-Sreea *et al.*, 2021; Rady *et al.*, 2021; Belal *et al.*, 2023; Tarfayah *et al.*, 2023**).

To date, PJS, LJS, and BHS have been applied in very few articles. Therefore, this work hypothesized that foliar spraying with PJS, LJS, or BHS would improve the pharmaceutical (secondary metabolites) properties of calendula plants. The objectives of this work were to explore the beneficial impacts of foliar spraying with PJS, LJS, or BHS (each at 5.0 or 10.0%) on SPAD index, RubisCO content and activity, *RubisCO* gene expression, leaf integrity, ORCs, antioxidant and secondary metabolites capacity in calendula plants. In addition, to test and identify the most cost-effective and eco-friendly NGSPs that can be economically applied as an alternative to synthetic chemicals.

2. MATERIALS AND METHODS

2.1. The site of cultivation and soil analysis

In 2022/2023, a plot of approximately 600 m^2 of soil with a clay loam texture on a private farm was selected for this study. The Abshwai district (29°19'39.2" N 30°39'16.9" E), Fayoum, Egypt, is the location of the farm. **Table 1** offers the main soil physicochemical characteristics (**Page *et al.* 1982; Klute 1986**).

Table 1. Physicochemical properties of the experimental soil

Soil layer (cm)	Particle size distribution: Silt (%)	Clay (%)	Sand (%)	Texture
0–30	40.2	37.3	22.5	Clay loamy

pH		7.80	N		604
ECe	(dS m^{-1})	2.11	P	(mg kg^{-1} soil)	80.1
CEC	(cmole kg^{-1})	15.0	K		588
$CaCO_3$	(%)	2.98	Fe		12.4
OM		1.02	Mn		7.69

OM= Organic matter, ECe= Electrical conductivity of soil paste, CEC= Cation exchange capacity, and $CaCO_3$= Calcium carbonate.

2.2. Transplanting, layout of experiments

The Egyptian Agricultural Research Center (Egy-ARC) was the provenance of the calendula (*Calendula officinalis* L., cv. Indian Prince) transplants at 45 days old. At a rate of one transplant $hill^{-1}$, the transplants were transplanted into the designated soil on September 25, 2022. The crop remained in the soil for more than 7 months (about 220 days). The experimental soil (about 600 m^2) was sectioned into 28 plots. Each plot was 17.5 m^2; 3.5 m (5 rows of 70 cm each) × 5 m. In each row, the hills were 25 cm apart. Planting density was about 55,000 plants per ha (100 plants per plot).

The trial was planned to arrange 7 treatments in a completely randomized block design (CRBD) and each treatment was distributed into 4 randomized plots. The 7 treatments were (1) control; treating calendula plants with distilled water, (2) treating plants with 5% pomegranate juice solution (PJS), (3) treating plants with 10% PJS, (4) treating plants with 5% lemon juice solution (LJS), (5) treating plants with 10% LJS, (6) treating plants with 5% bee honey solution (BHS), and (7) treating plants with 10% BHS. The honey used in the experiment is raw clover honey, the prevailing honey in Egypt. It was harvested from the college's apiary next to the experimental farm of the Faculty of Agriculture, Fayoum University (SE Fayoum; 29° 17'N; 30° 53'E), Egypt. Pomegranate and lemon fruits (Balady; Local varieties) were collected from an orchard in the Abshway area (29°19'39.2" N 30°39'16.9" E), Fayoum, Egypt. Three foliar sprays were performed six, eight, and ten weeks after transplanting (on the 6th of Nov., the 20th of Nov., and the 4th of Dec.). PJS, LJS, and BHS were sprayed in the early morning (before sunrise) at rates of 1.5, 2.0, and 2.8 L per plot for the 3 spray times, respectively. To achieve complete inflow into plant leaves, spray solutions were enriched with drops of Tween-20 solution. **Table 2** presents the chemical characteristics of pomegranate juice (PJ), lemon juice (LJ), and raw clover bee honey (BH).

Table 2. Chemical characteristics of the pomegranate juice (PJ), lemon juice (LJ), and raw clover bee honey (BH)

Components	Units	PJ	LJ	BH
Titratable acidity	(% as citric acid)	---	5.42±0.12	---
pH		3.78±0.25	3.10±0.22	4.28±0.21
TSS	(°Brix)	18.8±1.44	8.51±0.18	84.1±4.12
Total soluble sugars		20.1±1.48	1.21±0.03	80.9±3.82
Vitamin C	(%)	0.06±0.00	0.04±0.00	0.03±0.00
Total anthocyanins		0.12±0.00	0.02±0.00	0.06±0.00
Total phenols		0.26±0.01	0.14±0.01	0.13±0.00
Antioxidant activity	(mM Trolox eq.L^{-1})	18.2±0.88	17.5±0.78	19.2±0.92

TSS; Total soluble solids, and eq.; equivalent.

Before tilling the soil, according to Egy-ARC recommendations, a total amount of 980 kg of fully decomposed farmyard manure (fd-FYM) was added to the experimental soil (600 m^2; about 35 kg fd-FYM $plot^{-1}$). After plowing the soil, 28 kg of phosphate fertilizer (15% P_2O_5) and 14 kg of potassium fertilizer (50% K_2O) were added to the experimental soil (600 m^2). After a month of transplanting, 28 kg of urea (48% N) was added to the experimental soil. The plants were watered once every two weeks by surface irrigation. Other agricultural practices such as hoeing, weed control, and disease control were exactly followed. Twelve weeks after transplantation, plants from different treatments were sampled and the samples were immediately transferred to the relevant laboratories for all determinations.

2.3. Estimation of leaf integrity and osmoregulatory compounds

The leaf relative water content (RWC, %) and membrane stability (MSI, %) were measured by applying the protocols of **Osman and Rady (2014)**, **Rady (2011)**, and **Rady and Rehman (2016)**, respectively. The equations below were utilized:

$$RWC\ (\%) = \left[\frac{(fresh\ mass - dry\ mass)}{(turgid\ mass - dry\ mass)}\right] \times 100$$

$$MSI\ (\%) = \left[1 - (\frac{EC1}{EC2})\right] \times 100$$

Spectrophotometrically, the **Irigoyen *et al.* (1992)** procedure was practiced to evaluate the content (mg g^{-1} DW) of total soluble sugars. After centrifugation, newly prepared anthrone was added to the supernatant, and the mixture was boiled for 10 min. the OD was recorded at 625 nm. The **Bilal *et al.* (2017)** method was applied to evaluate K^+ content (mg g^{-1} DW) utilizing H_2O_2 and HNO_3 for sample suspension and digestion and K^+ content was quantified by ICP-MS (Optima 7900DV, Perkin-Elmer, Waltham, MA, USA). These analyses were performed using a Bausch and Lomb Spectronic 2000 Spec-trophotometer (Analytical systems divsions, Rochester, NewYork, USA).

2.4. Estimation of RubisCO content and activity, and *RubisCO* gene expression

The **Wishnick and Lane (1971)** method was utilized to evaluate the RubisCO content by spectrophotometry by applying $A_{280} \times 0.61$= mg mL^{-1}. Using spectrophotometry, the **Racker (1962)** method was utilized to evaluate RubisCO activity by monitoring NADH oxidation at 25 °C at 340 nm. One thousand mM Tris–HCl (pH 7.8) + NADH (6.0 mM) + 3-phosphoglyceraldehyde ($C_3H_7O_6P$) dehydrogenase (0.5%) + GSH

(100.0 mM) + 3-phosphoglycerate ($C_3H_7O_7P$) kinase (10 units per 20 µL) + ATP (200.0 mM) + α-glycerophosphate ($C_3H_9O_6P$) dehydrogenase-triose phosphate isomerase (0.05%) + RuBP (25.0 mM) + $MgCl_2$ (500.0 mM) + $KHCO_3$ (500.0 mM) + isolated solution of RubisCO in a final volume of 500.0 µL was the assay medium. The enzyme amount producing 1 µM of RuBP per minute is equal to 1 unit of enzyme. These determinations were performed using a Bausch and Lomb Spectronic 2000 Spec-trophotometer (Analytical systems divsions, Rochester, NewYork, USA).

RT-qPCR was applied to quantify the transcript level of *RubisCO* as a selected target gene by reverse transcription. After isolation using the protocol of hot borate (**Wan and Wilkins, 1994**), total RNA was treated with DNase I, and then the first strand (cDNA) was synthesized. An ABI 7500 RT-qPCR System (Applied Biosystems, USA) was applied. An initial hot start was implemented at 95 °C for 10 min. Following this step, 40 cycles of 95, 58−60, and 72 °C for 30 seconds, 1 min, and 1 min, respectively, were implemented. With the use of CT value versus the level of the actin gene expression, the level of the expression was normalized. The specific primer pairs of the actin and *RubisCO* genes for the qPCR designed utilizing Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA) are as follows: TGGGTTTGCTGGAGATGAT (forward; 5'–3') and CAGTAGGAGAACTGGGTGC (reverse; 5'–3'), and GCAGGCTGAAACAGGTGAA (forward; 5'–3') and ACGGTGGATGTGAAGAAGTAGA (reverse; 5'–3'), respectively. The level of the gene relative expression was computed using the formula $2^{-\Delta\Delta Ct}$ (**Livak and Schmittgen, 2001**).

The **Krishnaveni *et al.* (1984)** method was applied to evaluate glucose content in a 100mg fresh leaf sample utilizing a reagent (glucose oxidase-peroxidase), from which 1 mL was added to deproteinized extract (0.25 mL). One mL HCl (3 N) was added to the reaction mixture to terminate the reaction. Spectrophotometrically, the OD was measured at 550 nm. The **Jones *et al.* (1977)** technique was practiced to evaluate sucrose content in a 1g fresh leaf sample. Two hundred mM buffer (imidazole, pH 7.6) + imidazole base (80.0 mM) + adenosine triphosphate (APT, 1000.0 µM) was the reaction mixture. Spectrophotometrically, the OD was written at 340 nm. Eighty-five µL of G-6-dehydrogenase (seventy units per mL) was added to the reaction mixture. The OD was re-read after well-mixing after 5 min. The **McCready *et al.* (1950)** method was applied to evaluate starch content through acid hydrolysis of leafy samples. Then, the extract received $HClO_4$ (55%), incubated for 30 min, and then received a reagent (cold anthrone, 20 mL). The mixture was heated at 90 °C and then cooled in ice. Spectrophotometrically, the OD was written at 635 nm. These analyses were performed using a Bausch and Lomb Spectronic 2000 Spectrophotometer (Analytical Systems divisions, Rochester, NewYork, USA).

2.5. Estimation of secondary metabolite and antioxidant compound contents (LMACs)

Methanol extracts were prepared from freeze-dried plant leaf samples. A 1.5g sample was macerated into 15 mL of 100% methanol (**Guedes *et al.*, 2022**). Three-time extraction was conducted and total phenolic compound content (TPCC, mg gallic acid equivalents g^{-1} DW) was evaluated by applying the **Mongkolsilp *et al.* (2004)**

method. Spectrophotometrically, total flavonoid content (TFC) was assessed by applying a modified method (**Matvieieva *et al.*, 2019**) utilizing a leaf sample that was homogenized in ethanol (70%). After centrifugation at 14000 r.p.m. for 10 min (Eppendorf Centrifuge 5415C). Supernatants were used for determining TFC. The OD was recorded at 510 nm and the TFC content (mg rutin equivalent g^{-1} WW) was computed by the calibration plot: C (rutin) = 1.7427D (R2 = 0.9936). The total content of anthocyanins (TCAs) in plant leaves was evaluated with a modified method (**Sukwattanasinit *et al.*, 2007; Lee *et al.*, 2008**). The TCAs content (mg cyanidin-3-glucoside equivalent g^{-1} FW) was evaluated from the OD taken by a nucleic acid/protein analyzer (Beckman Coulter, Inc., USA) at 536 and 700 nm in buffers (pH 1.0 and 4.5, respectively). The TCAs were computed as follows: TCAs (mg g^{-1} FW) = $(A_{536} - A_{700})$ pH 1.0 – $(A_{536} - A_{700})$ pH 4.5.

Spectrophotometrically, the content of free proline (µmol g^{-1} DW) was evaluated following the **Bates *et al.* (1973)** method. After conducting an extract centrifugation, the supernatant received a newly prepared acid-ninhydrin solution. After incubating the mixture at 90°C for 30 min, the reaction was ended in ice. A toluene solution was utilized for an additional extraction to obtain the toluene phase, which read at 520 nm. The detailed procedures of **Kampfenkel *et al.* (1995)** and **Griffth (1980)** were practiced to evaluate both contents (µmol g^{-1} FW) of ascorbate (AsA) and glutathione (GSH), respectively.

These analyses were performed using a Bausch and Lomb Spectronic 2000 Spectrophotometer (Analytical Systems divisions, Rochester, NewYork, USA).

2.6. Statistical analysis

The experimental treatments were arranged in a completely randomized design. Using one-way ANOVA, data were analyzed statistically, and then Duncan's Multiple Range Test was followed to assess significance among different means, at a 5% level of probability ($p \leq 0.05$).

3. RESULTS

Seven foliar spray treatments were applied to calendula (*Calendula officinalis* L.) plants to improve their pharmaceutical quality; secondary metabolites and antioxidant capacity. The treatments were 5 and 10% diluted pomegranate juice solution (5%-DPJS and 10%-DPJS), 5 and 10% diluted lemon juice solution (5%-DLJS and 10%-DLJS), and 5 and 10% diluted bee honey solution (5%-BHS and 10%-BHS), plus foliar spraying with distilled water (d.H_2O) as a control.

3.1. SPAD, leaf integrity, and osmoregulatory compounds (ORCs)

Tables 3 and **4** display that foliar spraying 5% or 10% of DPJS, DLJS, and DBHS significantly increased SPAD index (relative chlorophyll content), relative water content (RWC), membrane stability index (MSI), and ORCs (total soluble sugars; TS sugar and K^+) contents relative to foliar spraying with d.H_2O. Relative to the d.H_2O treatment, the best treatment was 5%-BHS, increasing SPAD index by 36.2%, RWC by 21.3%, MSI by 25.3%, TS sugar content by 121.9%, and K^+ content by 18.2%. In addition, 5%-DPJS was the second-best treatment for SPAD index, RWC, and MSI, all of which increased by 34.6%, 20.2%, and 23.5%, while 10%-BHS was the second-best treatment for TS sugar and K^+ contents (which increased by 123.8 and 46.2%, respectively) relative to the d.H_2O treatment.

Table 3. Impact of foliar nourishment with diluted solutions of pomegranate juice (DPJS), lemon juice (DLJS), and bee honey (DHS) on leaf SPAD index and leaf integrity of *Calendula officinalis* plants

Treatments		SPAD index	RWC (%)	MSI (%)
NGSPs	Conc.			
Control (d.H_2O)		38.7±2.08^{d}	73.7±3.36^{c}	67.7±4.55^{d}
DPJS	5.0%	52.1±3.13^{ab}	88.6±3.28^{a}	83.6±5.60^{a}
	10.0%	47.8±2.93^{c}	79.7±2.51^{bc}	75.1±3.38^{bc}
DLJS	5.0%	50.0±3.10^{abc}	87.4±3.08^{a}	81.2±5.44^{a}
	10.0%	47.0±1.18^{c}	78.7±4.03^{c}	72.4±4.43^{cd}
DHS	5.0%	52.7±2.69^{a}	89.4±8.78^{a}	84.8±3.23^{a}
	10.0%	48.6±3.02^{bc}	85.9±3.69^{ab}	79.5±7.92^{ab}

In each column, means (± SE; standard error) followed by different lowercase letters are significantly different according to the Duncan Multiple Range Test ($P \leq 0.05$). NGSPs; natural growth-stimulating products, Conc.; concentration, and d.H_2O; distilled water.

Table 4. Impact of foliar nourishment with diluted solutions of pomegranate juice (DPJS), lemon juice (DLJS), and bee honey (DHS) on osmoprotectants and *RubisCO* activity of *Calendula officinalis* plants

Treatments		TS sugar content (mg g^{-1} DW)	K^+ (mg g^{-1} DW)	RubisCO content (mg mL^{-1})	RubisCO activity (U mL^{-1})	Relative expression of RubisCO
NGSPs	Conc.					
Control (d.H_2O)		16.0±0.31^{e}	21.4±0.05^{b}	0.31±0.01^{d}	0.68±0.02^{d}	1.00±0.02^{c}
DPJS	5.0%	22.2±0.57^{c}	24.6±0.04^{a}	0.37±0.01^{c}	0.82±0.03^{c}	1.55±0.03^{b}
	10.0%	29.1±0.66^{b}	24.9±0.03^{a}	0.37±0.01^{c}	0.79±0.03^{c}	1.60±0.04^{b}
DLJS	5.0%	17.4±0.41^{d}	25.2±0.04^{a}	0.40±0.02^{b}	0.88±0.03^{b}	1.60±0.04^{b}
	10.0%	17.9±0.46^{d}	21.5±0.04^{b}	0.42±0.02^{b}	0.89±0.04^{b}	1.55±0.04^{b}
DHS	5.0%	29.5±0.68^{b}	25.3±0.04^{a}	0.48±0.02^{a}	0.98±0.04^{a}	1.80±0.05^{a}
	10.0%	35.8±0.86^{a}	25.9±0.03^{a}	0.49±0.02^{a}	0.95±0.04^{a}	1.75±0.04^{a}

In each column, means (± SE; standard error) followed by different lowercase letters are significantly different according to the Duncan Multiple Range Test ($P \leq 0.05$). NGSPs; natural growth-stimulating products, Conc.; concentration, d.H_2O; distilled water, TS sugar; total soluble sugars, and K^+; potassium ion.

3.2. RubisCO content and activity, and *RubisCO* gene expression

Foliar spraying with 5% or 10% of DPJS, DLJS, and DBHS significantly increased RubisCO content and activity, and *RubisCO* gene expression compared to foliar spraying with d.H_2O (**Table 4**). Generally, the best treatment was 5%-BHS, outperforming the d.H_2O treatment by 54.8% for RubisCO content, 44.1% for RubisCO activity, and 80.0% for *RubisCO* gene expression. The second-best treatment, in general, was 10%-BHS, outperforming the d.H_2O treatment by 58.1% for RubisCO content, 39.7% for RubisCO activity, and 75.0% for *RubisCO* gene expression.

3.3. Antioxidant capacity

Except for the total phenolic compound content (TPCC) that was not affected, foliar spraying with 5% or 10% of DPJS, DLJS, and DBHS significantly increased secondary metabolites and antioxidant capacity (total contents of flavonoids; TFC, anthocyanins; TCAs, proline; Pro, ascorbate; AsA, and glutathione; GSH) compared to foliar spraying with d.H_2O (**Table 5**). The best treatment was 5%-BHS, outperforming the d.H_2O treatment by 103.6% for TFC, 77.3% for TCAs, 116.8% for Pro content, 159.8% for AsA content, and 156.3% for GSH content. In addition, 5%-DPJS was the second-best treatment for TFC and TCAs,

both of which increased by 71.4 and 72.7%, and 5%-DLJS was the second-best treatment for Pro, AsA, and GSH contents, all of which increased by 94.4%, 118.3%, and 104.2% relative to the d.H_2O treatment.

Table 5. Impact of foliar nourishment with diluted solutions of pomegranate juice (DPJS), lemon juice (DLJS), and bee honey (DHS) on the secondary metabolites and antioxidant capacity of *Calendula officinalis* plants

Treatments		TPCC (mg GAE g^{-1} DW)	TFC (mg RE g^{-1} WW)	TCAs (mg g^{-1} FW)	Pro content (µmol g^{-1} DW)	AsA content (µmol g^{-1} FW)	GSH content (µmol g^{-1} FW)
NGSPs	Conc.						
Control (d.H_2O)		3.63±0.12^{a}	0.28±0.01^{e}	0.22±0.01^{c}	78.4±2.2^{f}	2.24±0.05^{d}	0.96±0.02^{e}
D**P**JS	5.0%	3.64±0.14^{a}	0.48±0.03^{b}	0.38±0.02^{a}	119.2±3.3^{c}	4.04±0.09^{c}	1.74±0.04^{c}
	10.0%	3.58±0.11^{a}	0.30±0.01^{de}	0.30±0.01^{b}	91.3±2.8^{e}	4.01±0.08^{c}	1.38±0.03^{d}
D**L**JS	5.0%	3.64±0.12^{a}	0.54±0.03^{a}	0.39±0.02^{a}	152.4±3.8^{b}	4.89±0.11^{b}	1.96±0.04^{b}
	10.0%	3.57±0.12^{a}	0.32±0.02^{d}	0.29±0.01^{b}	104.4±3.0^{d}	4.03±0.09^{c}	1.40±0.03^{d}
D**H**S	5.0%	3.62±0.13^{a}	0.57±0.03^{a}	0.39±0.02^{a}	170.0±4.2^{a}	5.82±0.14^{a}	2.46±0.05^{a}
	10.0%	3.58±0.12^{a}	0.38±0.02^{c}	0.31±0.02^{b}	108.2±3.1^{d}	4.11±0.09^{c}	1.72±0.04^{c}

In each column, means (± SE; standard error) followed by different lowercase letters are significantly different according to the Duncan Multiple Range Test ($P \leq 0.05$). NGSPs; natural growth-stimulating products, Conc.; concentration, TPCC; total phenolic compounds content, TFC; Total flavonoid content, TCAs; total content of anthocyanins; Pro; free proline, AsA; ascorbate, GSH; glutathione, and d.H_2O; distilled water.

4. DISCUSSION

The use of natural plant growth-stimulating products (NGSPs) in agriculture to maximize plant production and quality has gained widespread recognition recently. Among these NGSPs, the diluted solutions made from pomegranate juice (PJS; **Kalefetoğlu Macar *et al.*, 2022; Yılmaz *et al.*, 2023**), lemon juice (LJS; **Rady *et al.*, 2023a**), and raw clover bee honey (DBHS; **Semida *et al.*, 2019; Abou-Sreea *et al.*, 2021; Rady *et al.*, 2021, 2023b; Alghamdi *et al.*, 2023; Belal *et al.*, 2023; Tarfayah *et al.*, 2023**). As shown in **Table 2**, PJS, LJS, and BHS possess several bioactive compounds (bio-ACs) that bio-stimulate plant growth, productivity, and quality. The main bio-ACs that PJS, LJS, and BHS are rich in are soluble sugars, vitamin C, anthocyanins, and phenols. However, BHS generally contains more bio-ACs than PJS and LJS and possesses the greatest antioxidant activity; 19.2 mM Trolox eq. L^{-1} compared to 18.2 and 17.5 mM Trolox eq. L^{-1} for PJS and LJS, respectively. This explains the superiority of BHS (especially at 5% concentration) over 5 and 10% PJS and 5 and 10% LJS in the results obtained in this study (**Tables 3−5**). In addition, the superiority of 5% BHS over 10% BHS may be because 10% BHS has more vital bio-ACs than the plant needs and may cause toxicity or impair some vital processes of plant cells (**Bartucca *et al.*, 2022; Alghamdi *et al.*, 2023; Belal *et al.*, 2023**).

Immediately after foliar spray treatment, the bio-ACs can penetrate the leaf surface cells through two paths. The leaf cuticle is the 1st path, and the ectodesmata can serve as an appointed path (**Schonherr and Riederer, 1989**). The second path is stomata, through which the bio-ACs can simply inflow into the leaf and move to meristematic and/or biosynthesizing cells to support cell metabolic processes and support plants in unstressed or stressful environments (**Rady *et al.*, 2021; Alghamdi *et al.*, 2023**). The bio-ACs present in NGSPs, especially 5% BHS, can protect plants against any adverse growing conditions and bio-stimulate plant

growth, physio-biochemical indices, yield, and quality (**Tarfayah *et al.*, 2023; Rady *et al.*, 2021; Abou-Sreea *et al.*, 2021; Alghamdi *et al.*, 2023; Belal *et al.*, 2023**). In addition, NGSPs, as an easy-to-prepare and inexpensive strategy, can be used as nutritious solutions due to their contents of vitamins and sugars (**Table 2**) that provide plants with these vital bio-ACs to maximize their performance. The chief mechanisms targeted by these bio-ACs are related closely to the nature of the growth-stimulating product. The chemical structure of a growth-stimulating product is complex and two or more bio-ACs can act simultaneously, so the exact mode of action of the growth-stimulating products has not yet been identified and requires further study (**Alzahrani and Rady, 2019; Semida *et al.*, 2019; Alghamdi *et al.*, 2023**).

Chlorophyll content measured in this study through SPAD index in calendula leaves was markedly improved in all treatments; 5 and 10% concentrations of BHS, PJS, and LJS with a marked superiority of the 5% BHS treatment over the other treatments (**Table 3**). This positive finding may be because 5% BHS contains sufficient plant needs of vital bio-ACs (soluble sugars, vitamin C, anthocyanins, and phenols). All these vital bio-ACs confer the greatest antioxidant activity (19.2 mM Trolox eq. L^{-1}) as multiple biocatalysts with multiple mechanisms that support the photosynthesis process to improve net CO_2 assimilation and photosynthetic efficiency to benefit plant performances (**Rady *et al.*, 2021; Alghamdi *et al.*, 2023; Belal *et al.*, 2023**). The improvement in chlorophyll content (SPAD index) with 5% BHS treatment was linked to enhanced RubisCO activity and *RubisCO* gene expression, upregulated osmoregulatory compounds (ORCs; including soluble sugars, K^+, and proline), secondary metabolite, and antioxidants, maintained cell membrane integrity, and increased RWC (**Tables 3-5**), all of these multiple mechanisms were involved in maximizing MSI to reduce water consumption by calendula plants. These multiple mechanisms were conferred due to the presence of the vital bio-ACs in the BHS (**Table 2**) to contribute to maintaining the balance of cellular ions necessary for chlorophyll biosynthesis in calendula leaves to further trigger the photosynthesis process. The promotion in the chlorophyll content (SPAD index) with 5% BHS treatment was also linked to proline content (**Tables 3** and **5**). As discussed in **Merwad *et al.* (2018)** and **Rady *et al.* (2019)**, proline contributes to triggered efficiency of photosynthesis and generation of adenosine triphosphate (ATP). Like other ORCs (**Table 4**), proline accumulated more in calendula plants in the 5% BHS treatment compared to the other treatments. Therefore, the ORCs promoted with 5% BHS treatment positively reflected the photosynthesis and RWC of calendula plants. Treating plants with BHS leads to a notable enhancement in the chlorophyll content (SPAD index), whether under normal conditions of plant growth or stress (**Semida *et al.*, 2019; Rady *et al.*, 2021; Alghamdi *et al.*, 2023**).

Through the "Calvin cycle", CO_2 is assimilated in plant photosynthesis and is upregulated at the levels of enzymes and substrates. In the 1st step of the "Calvin cycle", RubisCO is catalyzed to fix CO_2, positively affecting the net rate of photosynthesis. Thus, in photosynthesis, RubisCO is a fundamental enzyme, which functions to limit the rate of CO_2 assimilation (**Han *et al.*, 2023**). In this report, the RubisCO content and activity, *RubisCO* gene expression, and sugars contents tested in calendula plants were

markedly improved in all treatments; 5 and 10% concentrations of BHS, PJS, and LJS with a marked superiority of the 5% BHS treatment over the other treatments (**Table 4**). The marked improvement in chlorophyll content (SPAD index) by 5% BHS treatment led to an improvement in metabolic processes, including photosynthesis, through upregulation of *RubisCO* gene expression. Foliar spraying with 5% BHS upregulated *RubisCO* genes. Like *RubisCO* gene expression, CO_2 fixation-related RuBisCO enzyme implicated in the "Calvin cycle" is up-regulated by all treatments with the superiority of 5% BHS treatment. *RuBisCO* stimulation at the gene level (**Table 4**) proposed that photosynthesis is improved through the upregulation of *RubisCO* genes and *RuBisCO* protein by plant growth regulators (**Li *et al.*, 2016**). For metabolic processes, especially photosynthesis, and gene expressions of the enzymes, including RuBisCO, involved to occur most completely, an optimal cellular water content (RWC) is required to help optimize these processes and stabilize cellular membranes. As shown in **Table 4**, the promoted RubisCO content and activity, and *RubisCO* gene expression with the 5% BHS led to enhanced soluble sugar contents. These outcomes agreed with **Iñiguez *et al.* (2021)** and **Han *et al.* (2023)**. Our outcomes highlight the prominence of the 5% BHS treatment on photosynthesis, as well as the RubisCO content and activity, *RubisCO* gene expression, and their reflection on glucose, sucrose, and starch contents in calendula plants.

Osmoregulatory compound contents (ORCs; including soluble sugars, K^+, and proline) and leaf integrity (RWC and MSI) tested in calendula plants were markedly improved in all treatments; 5 and 10% concentrations of BHS, PJS, and LJS with a marked superiority of the 5% BHS treatment over the other treatments (**Tables 4** and **5**). Frequently, RWC and MSI are harnessed to examine the water balance and membrane integrity of leaf cells and the gross volumetric water that can be held at full capacity. The induced increments in ORCs by 5% BHS treatment were in favor of the integrity of calendula leaves; increased RWC and MSI. These results agreed with the findings of **Semida and Rady (2014)** and **Desoky *et al.* (2021)**. ORCs (present in the tested NGSPs, especially 5% BHS), including soluble sugars, proline, and K^+ were associated with safeguarding MSI and RWC in an integrated status in calendula leaves. This positive state helped sustain optimal metabolic activities through ideal cellular osmosis (**Semida and Rady, 2014; Desoky *et al.*, 2021**) as a favorable mechanism for calendula productivity. This desirable finding could be because 5% BHS augmented the accumulations of ORCs (soluble sugars, K^+, and proline; **Tables 4** and **5**) to upregulate osmotic pressure to preserve cell membranes and turgor pressure for healthy plant productivity (**Semida and Rady, 2014; Abou-Sreea *et al.*, 2021; Rady *et al.*, 2021; Alghamdi *et al.*, 2023; Belal *et al.*, 2023**).

Secondary metabolites are found in medicinal plants in major contents, representing various chemical classes. They are biosynthesized via various biochemical pathways and accumulate in different plant organs by different biochemical and regulatory mechanisms to enable plants to acclimate to different growing environments. The phytochemical examination of calendula leaf extracts explored that they contain many secondary metabolites, including anthocyanins, flavonoids, and phenolics (**Ahmad *et al.*, 2023**). Secondary metabolites and antioxidant capacity (total

phenolic compound contents; TPCC, total flavonoid content; TFC, total anthocyanins content; TCAs, free proline; Pro, ascorbate; AsA, and glutathione; GSH) tested in calendula plants were markedly improved in all treatments; 5 and 10% concentrations of BHS, PJS, and LJS with a marked superiority of the 5% BHS treatment over the other treatments (**Table 5**). Phenols found in all plants, including flavonoids, have antioxidant effects and are secondary metabolites (**Yousefi *et al.*, 2019**). Phenolics, flavonoids, and anthocyanins have various plant survival-related physiological functions. They are naturally produced during plant growth and development for protection against any excess formation of oxidants under normal or stress conditions (**Verma and Shukla, 2015**). BHS as a biostimulant may have positive effects on the synthesis of secondary metabolites in both direct and indirect manners. **Mrid *et al.* (2021)** stated that the direct influence of the biostimulants is on the phenolic production, including flavonoids and the indirect manner is via the influence on plant performance. Treating plants with BHS increases total phenolics, whether under normal conditions of plant performance or stressful environments (**Abou-Sreea *et al.*, 2021; Rady *et al.*, 2021, 2023b; Tarfayah *et al.*, 2023**). Biostimulants may enhance enzyme activity related to triggering phenolic biosynthesis and improving inflorescence quality (**Danaee and Abdossi, 2018**), and, as an efficient biostimulant, BHS may play this role. Flavonoids were markedly increased by the application of 5% BHS in this study. An increase in flavonoids has been stimulated to contribute (auxiliary role) to the recovery of tissue cells from oxidants (**Velicka *et al.*, 2022**). As reported by **Mutha *et al.*, (2021)**, flavonoids comprise a class of the most isolated, identified, and diversified polyphenolic compounds. In addition, anthocyanins, glycosides of anthocyanidins, are prominent phenolics (**Coklar and Akbulut, 2017**). Therefore, the stimulated increase in TPCC by applying 5% BHS treatments should be followed by an increase in TFC and TCAs as shown in this study (**Table 5**). Like TPCC, TFC, and TCAs, the low molecular mass antioxidants (LMAs), including GSH, AsA, and Pro increased by applying 5% BHS treatment to provide further mechanisms to eliminate any excess oxidants formed in tissue cells to benefit calendula plant performance. This can be attributed to BHS's abundance of various antioxidants, including vitamin C (AsA), anthocyanins, and phenols, with high antioxidant activity; 19.2 mM Trolox eq. L^{-1} (**Table 2**). Treating plants with BHS notably reinforced different LMAs, whether under normal conditions of plant growth or stress (**Semida *et al.*, 2019; Abou-Sreea *et al.*, 2021; Alghamdi *et al.*, 2023; Belal *et al.*, 2023; Rady *et al.*, 2021, 2023b; Tarfayah *et al.*, 2023**).

Potassium ions; K^+ with sufficient soluble sugars and proline contents found in calendula leaves help plant cell retains sufficient water required for vital processes (**Seleiman *et al.*, 2020; Rehman *et al.*, 2021**) helping increase the medical (secondary metabolites) compounds in calendula plants.

5. CONCLUSIONS

The foliar spray strategy with natural plant growth-stimulating products (NGSPs), including pomegranate juice (PJS), lemon juice (LJS), and raw clover bee honey (BHS), tested in this study, resulted in significant improvements in *RubisCO* gene expression and secondary metabolite and antioxidant capacity with the observed

superiority of the 5% BHS treatment over the other treatments. *RubisCO* gene expression supported increased soluble sugars and secondary metabolite and antioxidant capacity, all of which improved by 5% BHS treatment. These findings suggest the use of a foliar spray of 5% BHS as a workable multiple biostimulant possessing numerous bioactive compounds to enhance the production of secondary metabolite and antioxidant capacity in calendula plants with highly marketable quality for a wide range of use as medical/pharmaceutical industries. However, more research is required to optimize BHS treatment for the above-mentioned purposes, especially for long-distance transportation.

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