agriculture

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# Exogenous Application of *Aloe vera* Leaf Extract Improves Silybin Content in *Silybum marianum* L. by Up-Regulating *Chalcone Synthase* Gene

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**Abstract:** Biotic elicitors such as *Aloe vera* extract (ALE) have been shown to stimulate growth and modify the bioactive composition of various plant species. ALE has a unique mixture of nutrients that support plant production and growth. In this study, the bio-stimulative effects of ALE foliar spray on plant production and growth, silybin levels, and *chalcone synthase* gene expression in *Silybum marianum* were examined. The findings indicated that foliar spray of all the ALE concentrations under study increased plant growth and yield. Additionally, by raising the silybin level of the plant extract, ALE increased the therapeutic value of *S. marianum*. Further, the activation of the *chalcone synthase* gene by ALE was analyzed by gene expression research. *S. marianum*'s growth and production were improved by the application of 60 mL/L ALE, while the silybin level and the *chalcone synthase* gene expression levels were improved by the application of 40 mL/L ALE. In addition, methanolic fruit extract that contained a higher silybin content also demonstrated a higher anti-microbial activity against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*.

**Keywords:** bio-elicitors; silymarin; high-performance liquid chromatography; quantitative reverse-transcribed PCR; disc diffusion assay; anti-microbial activity



**Citation:** Alkuwayti, M.A.; Aldayel, M.F.; Yap, Y.-K.; El Sherif, F. Exogenous Application of *Aloe vera* Leaf Extract Improves Silybin Content in *Silybum marianum* L. by Up-Regulating *Chalcone Synthase* Gene. *Agriculture* **2022**, *12*, 1649. https://doi.org/10.3390/agriculture12101649

Academic Editor: Fabio Sciubba

Received: 30 August 2022
Accepted: 4 October 2022
Published: 9 October 2022



## 1. Introduction

There are currently challenges related to crop production due to erosion of agri-resources, climate change, pollution, and non-sustainable farming practices. The use of chemical fertilizers and pesticides to increase crop growth and yield has only further aggravated the crop production problem, as this has caused ground water nitrate pollution, soil nutrient imbalance, and short- and/or long-term health issues for the whole biosphere. In view of this, the development of bio-stimulants has drawn considerable interest in recent years [1–3]. Any substance or micro-organism that is applied to plants with the intention of improving their nutrient utilization efficiency, abiotic stress tolerance, and/or crop quality attributes and production is referred to as a plant bio-stimulant. Plant extracts are amongst the several categories of bio-stimulants, including *Aloe vera* leaf extract [1–3], as it contains growth-stimulating substances, is considered to be environmentally friendly, and is an organic fertilizer source that includes a variety of bioactive components. A perennial plant with large leaves, *Aloe vera* has parenchyma cells that are covered in a clear mucin-like jelly, known as *Aloe vera* gel. *Aloe vera* gel is rich in minerals (e.g., calcium, iron, magnesium, potassium, phosphorus, and zinc), enzymes (e.g., amylase, catalase, lipase, oxidase, and superoxide dismutase), amino acids (e.g., alanine, glycine, leucine, and proline), vitamins (e.g., B-complex, C, β-carotene, α-tocopherol), and phytohormones (e.g., gibberellin, indoleacetic acetic, and abscisic acid). Furthermore, the application of water-diluted, cold-pressed *Aloe vera* gel as bio-stimulant has been reportedly used on several plant species [4–7]. The Middle East and the Mediterranean region are home to the annual or biennial medicinal plant known as milk thistle, or *Silybum marianum*

(L.) Gaertn. Bioactive compounds from the seeds and fruits of *S. marianum* are used as herbal medicines or supplementary for the prevention of various illness [8,9]. Silymarin, a combination of stereoisomeric flavonolignans discovered in *S. marianum* fruits, is the plant's most prominent secondary metabolite and is used to treat a variety of liver illnesses [10,11]. Natural silybin (a key element in silymarin) is a combination of silybin A and silybin B and is responsible for the majority of silymarin's hepatoprotective properties [10,12–14]. Silybin is present in various amounts in the *S. marianum* plant and has been shown to have great potential in the treatment of Alzheimer's disease [15] and cancer [16]. Indeed, the majority of the hepatoprotective effects of silymarin are due to silybin [17].

The chalcone synthase enzyme (CHS) family supports plants, fungi, and bacteria to synthesize a variety of secondary metabolites [18,19]. It is an allosteric enzyme that is essential for the synthesis of flavonolignans in *S. marianum* and other plants [17,20].

In addition to its hepatoprotective activity, silymarin, in particular, silybin A and B, has also displayed anti-microbial activity against a variety of bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*) and fungi (*Candida albicans*, *C. tropicalis*, and *C. krusei*) [21]. The anti-microbial activity of silymarin increases its medicinal value as natural plant products are becoming an alternative remedy for treating antibiotic- or drug-resistant microbes, which is an emerging public health threat [22].

The aim of this study was to investigate the use of different concentrations of ALE on the growth attributes and silybin (A + B) contents of *S. marianum*. To profile the expression patterns of the expression level of *chalcone synthase* 1(*CHS* 1), 2 (*CHS* 2), and 3 (*CHS* 3) genes in the plant petals, quantitative reverse-transcribed polymerase chain reaction (qRT-PCR) was used to reveal the molecular mechanisms underlying the responses to the ALE treatments. The anti-microbial property of *S. marianum* methanolic fruit extract was investigated to evaluate the biological activity related to changes in the silybin (A + B) content resulting from the ALE treatment.

## 2. Materials and Methods

### *2.1. Aloe vera Leaf Extract for Biotic Elicitors Preparation*

*Aloe vera* leaves were collected from a two-year-old plant growing at Al Sahab farm (25.2983, 49.6175) in Al Ahsa, Saudi Arabia. The *Aloe vera* leaf gel extract (ALE) was then extracted according to the method described in [5].

### *2.2. Analysis of the Aloe vera Leaf Sap (the Mucilaginous Jelly from Aloe vera Leaves)*

According to the methods described in [23,24], the phytohormone and macro- and micro-nutrient contents of the *Aloe vera* leaf extract were identified (Table 1)

**Table 1.** Chemical constituents of *Aloe vera* leaves.

| Chemical Constituents | Quantity | Unit |
|---|---|---|
| Nitrogen | 80.65 | mg/100 g |
| Phosphorus | 6.95 | mg/100 g |
| Potassium | 60.14 | mg/100 g |
| Iron | 0.229 | mg/100 g |
| Zinc | 0.028 | mg/100 g |
| Manganese | 0.0266 | mg/100 g |
| Calcium | 40.00 | mg/100 g |
| Copper | 0.0042 | mg/100 g |
| Magnesium | 14.44 | mg/100 g |
| Sodium | 51.12 | mg/100 g |
| GA3 | 16 | mg/100 g |
| IAA | 0.63 | mg/100 g |
| ABA | 3.06 | mg/100 g |
| Total carbohydrate | 10.1 | % |
| Glucose | 3.2 | g/100 g |
| Protein | 1.0 | mg/g |
| Sterol | 18.73 | mg/g |

### 2.3. Plant Sources and Growth Environments

The *S. marianum* plants were grown and harvested in the King Faisal University, Saudi Arabia, Agriculture and Veterinary Research and Training Centre. The experiment was conducted in the greenhouse of the King Faisal University Agriculture and Veterinary Research and Training Centre, King Faisal University (25.2713, 49.7077), where the average photoperiod was 14 h under 10,000 Lux light intensity (Phillips TLM 40 W/33RS) and the temperature was controlled between 32 and 36 °C. On 1 October 2019, seeds were planted in germination trays (with a depth of 1.0–2.0 cm) that contained a moist, 1:1 mixture of sand and peat moss.

After a month, the seedlings were transferred to plastic pots (37.5 cm in diameter and 27.5 cm in depth), each of which contained 25 Kg of a 1:1 moist sand and peat moss mixture. The experiment was conducted in a completely randomized design with 20 repetitions. During the vegetative growth stage of the *S. marianum*, the plants in the various treatment groups were sprayed with ALE solution at concentrations of 0 (control, distilled water), 20, 40, and 60 mL/L ALE (Table 1) every two months (December 1, February 1, and April 1). Around 8 a.m., ALE was sprayed onto the leaves. Thereafter, 50 mL of either distilled water (control) or an ALE solution (20, 40, or 60 mL/L) was sprayed across each plant. The pH of the irrigation water ranged from 6.5 to 7 throughout the experiment and was measured using a pH meter. Table 2 shows the mineral compositions and properties of the irrigation water.

**Table 2.** Mineral compositions and properties of the irrigation water.

| Salinity Level (ppm) | Cations (meq L $^{-1}$) | | | | Anions (meq L $^{-1}$) | | | | Sodium Absorption Ratio |
|---|---|---|---|---|---|---|---|---|---|
| | $Ca^{2+}$ | $Mg^{2+}$ | $Na^{+}$ | $K^{+}$ | $CO_3^{2-}$ | $HCO_3^{-}$ | $SO_4^{2-}$ | $Cl^{-}$ | |
| 864 | 5.72 | 2.02 | 7.27 | 0.38 | 0.28 | 2.68 | 4.03 | 8.4 | 3.43 |

### 2.4. Vegetative Growth and Yield Components

Plants were harvested at 270 days post-sowing. Plant height (cm), number of branches/plant, and root and aerial part dry weight/plant (g) were measured for 10 randomly selected plants from each treatment group. All plants in the various treatment groups had their ripe fruits regularly collected and dried at room temperature. Both the quantity of capitula per plant and the dry weight (g) of the fruits per plant were noted.

### 2.5. Mineral Composition

Plant leaf samples were taken 270 days from 10 harvested plants and dried for 48 h at 60 °C. Quantification of nitrogen (N), phosphorus (P), and potassium (K) was performed as previously described [25].

### 2.6. Photosynthetic Pigment Determination

Four *S. marianum* plants that were 270 days old had the pigments (chlorophyll a (Chl-a), chlorophyll b (Chl-b), and carotenoid) extracted with 80% acetone from the third bottom fresh leaf [25].

### 2.7. HPLC Analysis of the Silybin (A + B) Content

Three biological replicates of each treatment group's air-dried fruit samples were analyzed by Waters 2690 Alliance HPLC system's (USA) with a Waters 996 photodiode array detector to measure the silybin (A + B) level [25]. Preparations of known concentrations of silybin (A + B) standards and *S. marianum* methanolic fruit extracts from various treatment groups were performed according to the method described in [25].

### *2.8. Total RNA Isolation, cDNA Preparation, and Gene Expression Analysis*

Total RNA was extracted from the frozen petals of four plants from each treatment group. For each cDNA synthesis reaction, 1 µg of total RNA was used. The conditions of cDNA synthesis and gene expression analysis were described in [25]. The primers used in the quantification of each *CHS* genes and the NADH dehydrogenase reference gene are listed in Table 3.

**Table 3.** Sequences of forward and reverse primers for real-time RT-PCR.

| Gene | Primer Sequences | Amplicon Length (bp) | GenBank Accession Number |
|---|---|---|---|
| *chalcone synthase* 1 (*CHS* 1) | CHS1F 5-TCTTGATTCCCTCGTTGGTC-3<br>CHS1R 5-TCTCAAACAACGGCCTCTCT-3 | 101 | JN182805.1 |
| *chalcone synthase* 2 (*CHS* 2) | CHS1F 5-AGGACATTGCGGAAAACAAC-3<br>CHS1R 5-AACGGCCTCTCTGTCTTCAA-3 | 184 | JN182806.1 |
| *chalcone synthase* 3 (*CHS* 3) | CHS1F 5-ACCCACCTCATCTTTTGCAC-3<br>CHS1R 5-CATCATGAGGCGTTTGATTG-3 | 105 | JN182807.1 |
| *NADH Dehydrogenase* (*NADH*) | ndhchs_L 5-TTCCGCATTTTGGAAATACC-3<br>ndhchs_R 5-CCCGTCTTGATTGAAAGGAA-3 | 134 | KC589999.1 |

### *2.9. Determination of S. marianum Methanolic Fruit Extract Anti-Microbial Activity by Disc Diffusion Assay*

#### 2.9.1. Preparation of *S. marianum* Test Solutions Using the *S. marianum* Methanolic Fruit Extract

The methanolic extract of *S. marianum* fruit was prepared as mentioned in Section 2.7 [25]. To prepare the *S. marianum* test solutions for the disc diffusion assay, methanolic extracts from *S. marianum* fruits of three individual plants from each of the control and ALE 40 treatments were separately dissolved in dimethyl sulfoxide (DMSO): water (*v:v* 1:1) to obtain solutions of 5 mg extract/mL. All six extracts (three from plants of the control group and three from plants treated with 40 mL/L ALE) were kept at 4 °C until use.

#### 2.9.2. Disc Diffusion Assay

The bacterial strains of *S. aureus*, *E. coli*, and methicillin-resistant *Staphylococcus aureus* (MRSA) were obtained from the College of Medicine, King Faisal University. The cultivation of all the bacterial strains was performed in nutrient broth (Sigma Aldrich, Cat. no. 7014) and the disc diffusion assay was performed according to the method described in [26]. The cultures were grown at 37°C at a speed of 200 rpm until the turbidity of the culture reached 0.3 at 600 nm. The cultures were inoculated into 10 mL of nutrient broth in a total volume of 100 µL of overnight bacterial culture. Subsequently, 5 mL of the homogenous bacterial culture of each of the bacterial strains was poured onto individual nutrient agar plates and each of the plates was gently swirled to ensure that the culture was spread evenly on the agar. Nine agar plates were inoculated with each bacterial strain. All inoculated plates were left unsealed in the bio-safety cabinet to allow the excess liquid to absorb into the agar before the test solution discs were placed onto the agar. A set of four discs of a 6 mm diameter, containing various test solutions, were placed onto the agar surface of each inoculated plate. Two of those discs contained the *S. marianum* test solutions from methanolic fruit extracts of the control and ALE treated plants, while the negative control disc contained the DMSO: water (*v:v* 1:1) solution. The positive control disc containing 10 µg of imipenem was purchased from Condalab, Madrid, Spain. The diameters of the incubation zone were then measured after each plate had been incubated at 37 °C overnight.

### 2.10. Statistical Analysis

ANOVA/MANOVA from Statistica's data analysis software system version 6 was used to analyze the data (StatSoft Inc.). The significance of differences between means was assessed using the Least Significant Test (L.S.D.) at $p = 0.05$ [27].

## 3. Results

### 3.1. The Analysis of ALE

The analysis of ALE, as shown in Table 1, revealed the presence of essential micro- and macro-nutrients (e.g., Mg, Zn, Cu, Mn, Ca, Fe, Na, Ca, K, nitrogen, phosphorus, and carbohydrates coupled with protein and K as osmoprotectants). An abundance of phytohormones, such as indole-3-acetic acid (IAA), gibberellins (GAs), and abscisic acid (ABA), were identified (Table 1). The phytohormones-rich composition of ALE suggested that this extract could be used as a plant bio-stimulant.

### 3.2. The Effects of ALE on the Growth Components and Yield of S. marianum

The height of plant, the number of leaves and branch, the dry weight of the root and aerial part under control, and ALE treatments are all presented in Table 4. The results indicate that the ALE foliar spray treatment increased all the above parameters compared with the control. The increase in the maximum plant height, the number of leaves, the branch number, and the dry weight of root and aerial part observed in the 60 mL/L ALE treatment group was significant compared to the control group (Table 4). Notably, the capitula number and fruit dry weight of *S. marianum* increased as the level of ALE increased (Table 5), with the maximum capitula number and fruit dry weight (13.5 n and 50.96 g, respectively) being observed in the 60 mg/L ALE treatment group, while a minimum (5.0 n and 16.65 g, respectively) was found in the control groups (Table 5).

**Table 4.** The impact of various ALE extract concentrations on the *S. marianum* L. plant's height (cm), number of leaves (n), branch number (n), and dry weight of root and aerial part (g/plant). The means and standard deviations were computed from measurements of 10 randomly selected plants.

| ALE (mL/L) | Plant Height (cm) | Number of Leaves (n) | Branch Number (n) | Root Dry Weight (g) | Aerial Part Dry Weight (g) |
|---|---|---|---|---|---|
| 0 | 50.75 $^{b*}$ ± 1.500 | 49.00 $^{b}$ ± 0.732 | 3.25 $^{b}$ ± 0.500 | 6.56 $^{b}$ ± 0.739 | 59.49 $^{b}$ ± 0.317 |
| 20 | 90.20 $^{a}$ ± 1.094 | 52.50 $^{b}$ ± 2.104 | 4.00 $^{ab}$ ± 0.200 | 6.27 $^{b}$ ± 0.213 | 68.762 $^{b}$ ± 0.983 |
| 40 | 91.40 $^{a}$ ± 2.915 | 66.80 $^{ab}$ ± 0.663 | 5.00 $^{a}$ ± 0.581 | 9.73 $^{b}$ ± 0.467 | 103.184 $^{a}$ ± 0.560 |
| 60 | 105.25 $^{a}$ ± 1.506 | 74.75 $^{a}$ ± 0.852 | 5.75 $^{a}$ ± 0.577 | 16.92 $^{a}$ ±0.666 | 111.67 $^{a}$ ± 0.692 |

* According to the L.S.D. test, means in a column that are distinguished by the same letter are not statistically different at the 0.05 level of probability.

**Table 5.** The impact of various ALE extract concentrations on *S. marianum* L. number of capitula (n) and dry weight of fruits (g). The means and standard deviations were computed from measurements of 10 randomly selected plants.

| ALE (mL/L) | Capitula Number (n) | Fruits Dry Weight (g) |
|---|---|---|
| 0 | 5.0 $^{c*}$ ± 0.0816 | 16.65 $^{c}$ ± 0.387 |
| 20 | 8.2 $^{b}$ ± 0.295 | 19.80 $^{c}$ ± 0.425 |
| 40 | 8.4 $^{b}$ ± 0.164 | 31.13 $^{b}$ ± 0.237 |
| 60 | 13.5 $^{a}$ ± 0.462 | 50.96 $^{a}$ ± 0.149 |

* According to the L.S.D. test, means in a column that are distinguished by the same letter are not statistically different at the 0.05 level of probability.

### *3.3. Analysis of Chemical Contents*

#### 3.3.1. Photosynthetic Pigments and Mineral Contents

In comparison to the control plants, all of the ALE treatments increased the chlorophyll a and b and carotenoid contents in the leaves of *S. marianum* (Table 6), with the highest concentrations being found in the plants treated with 20, 40, and 60 mL/L of ALE (Table 6). Notably, the information in Table 7 demonstrates that, in comparison to the control plants, all ALE concentrations increased the nitrogen (N), phosphorus (P), and potassium (K) content in the leaves of *S. marianum*. Additionally, plants treated with 60 mL/L of ALE had a higher N content (Table 7), while plants treated with 20 and 40 mL/L of ALE produced the highest P+ and K+ percentages in the leaves, respectively (Table 7).

**Table 6.** The impact of various ALE concentrations on *S. marianum* L. chlorophyll a, b, and carotenoid (mg/100 g FW) pigment contents. The means and standard deviations were computed from measurements of 10 randomly selected plants.

| ALE (mL/L) | Chl a (mg/100 g F.W.) | Chl b (mg/100 g F.W.) | Carotenoids (mg/100 g F.W.) |
|---|---|---|---|
| 0 | 28.283 $^{c*}$ ± 0.544 | 6.426 $^{b}$ ± 0.146 | 34.883 $^{b}$ ±1.322 |
| 20 | 50.175 $^{a}$ ± 1.855 | 6.499 $^{b}$ ± 0.217 | 38.447 $^{b}$ ± 0.709 |
| 40 | 37.461 $^{b}$ ± 0.784 | 12.864 $^{a}$ ± 0.387 | 49.551 $^{a}$ ± 0.515 |
| 60 | 34.838 $^{b}$ ±0.187 | 9.548 $^{ab}$ ±0.386 | 51.006 $^{a}$ ±0.151 |

* According to the L.S.D. test, means in a column that are distinguished by the same letter are not statistically different at the 0.05 level of probability.

**Table 7.** The impact of various ALE concentrations on *S. marianum* L. nitrogen (N), phosphorus (P) and potassium (K) percentage. The means and standard deviations were computed from measurements of 10 randomly selected plants.

| ALE (mL/L) | N% | P% | K% |
|---|---|---|---|
| 0 | 2.015 $^{a*}$ ± 0.516 | 0.215 $^{b}$ ± 0.843 | 0.570 $^{b}$ ± 0.276 |
| 20 | 2.490 $^{a}$ ± 0.255 | 0.698 $^{a}$ ± 0.049 | 0.930 $^{ab}$ ± 0.042 |
| 40 | 1.725 $^{a}$ ± 0.262 | 0.368 $^{b}$ ± 0.092 | 1.145 $^{a}$ ± 0.014 |
| 60 | 2.180 $^{a}$ ± 0.283 | 0.280 $^{b}$ ± 0.021 | 0.720 $^{b}$ ± 0.020 |

* According to the L.S.D. test, means in a column that are distinguished by the same letter are not statistically different at the 0.05 level of probability.

#### 3.3.2. HPLC Analysis of Silybin (A + B)

The results of silybin (A + B) quantification and separation in the *S. marianum* fruit extracts by HPLC analysis are shown in Figure 1. Here, it can be seen that ALE application significantly increased the silybin (A + B) contained in the fruit of *S. marianum* compared to the control group (Figure 1), the latter having a silybin (A + B) content of 16.339 mg/100 g DW. This content significantly increased ($p < 0.05$) to 57.943, 69.591, and 43.021 mg/100 g DW in plants treated by ALE at 20, 40, and 60 mL/L ALE, respectively (Figure 1).

### *3.4. The Effect of ALE on CHS 1, 2, and 3 Gene Expression*

The impact of the three concentrations of ALE on the *CHS* 1, 2, and 3 gene expression was investigated using qRT-PCR (Table 8). The expressions of the *CHS* 1, 2, and 3 genes increased in all plants that were sprayed with ALE. The highest expression was detected in the treatment group with 40 mL/L ALE, followed by the 20 mL/L and 60 mL/L ALE groups (Table 8). This result is consistent with our morphological observation, wherein enhancement effects were observed in plant height, branch number, leaf number, the dry weight of the root, and the silybin content (69.591 g/100 g plant D.W.) in the 40 mL/L ALE treatment group.

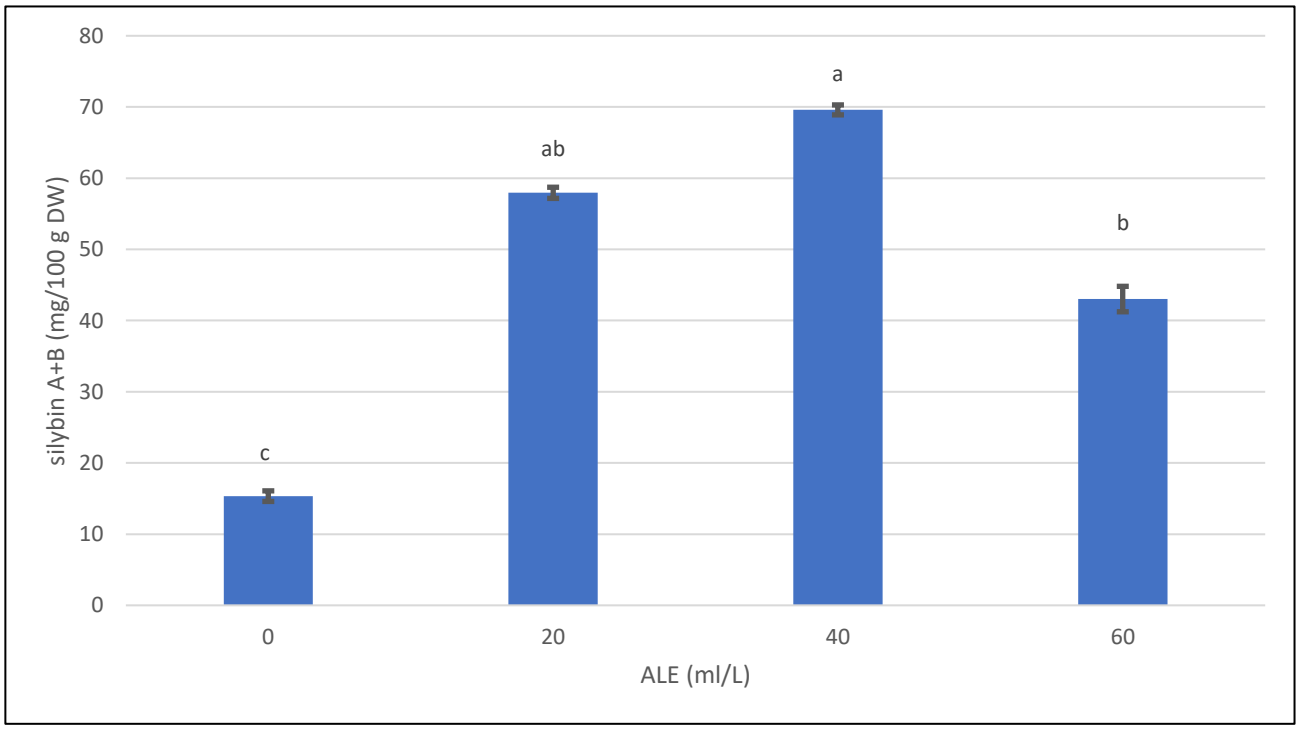


**Figure 1.** Impact of various ALE concentrations on *S. marianum* L. silybin (A + B) (mg/100 g DW) content. The error bars represent the standard deviations of the corresponding means. Means that are significantly different at 0.05 level of probability according to L.S.D. test are indicated with different letters above the corresponding bars.

**Table 8.** Fold differences of *CHS* 1, 2, and 3 expressions in various ALE treatment. For each treatment group, four biological replicates were used to calculate the means and standard deviations. PCR duplicates were run for every biological replicate.

| ALE (mL/L) | CHS1 | CHS2 | CHS3 |
|---|---|---|---|
| 0 | 1.000 $^{c*}$ ± 0.439 | 1.000 $^{c}$ ± 0.168 | 1.000 $^{c}$ ± 0.147 |
| 20 | 7.018 $^{a}$ ± 0.000 | 3.014 $^{b}$ ± 0.001 | 2.098 $^{b}$ ± 0.036 |
| 40 | 8.362 $^{a}$ ± 1.669 | 9.050 $^{a}$ ± 1.311 | 3.536 $^{a}$ ± 0.231 |
| 60 | 5.375 $^{b}$ ± 0201 | 1.389 $^{c}$ ± 0.035 | 1.211 $^{c}$ ± 0.017 |

* According to the L.S.D. test, means in a column that are distinguished by the same letter are not statistically different at the 0.05 level of probability.

### *3.5. The Effect of ALE on Anti-Microbial Activity of S. marianum Methanolic Fruit Extract*

The disc diffusion assays (Table 9) indicated that methanolic fruit extracts from the *S. marianum* plants displayed anti-microbial activity to various extents against *S. aureus*, *E. coli*, and MRSA. Among the three tested bacteria strains, extracts from plants not treated with ALE (control) were more effective against *S. aureus* and *E. coli*, while those from plants treated with 40 mL/L ALE were more effective against *S. aureus* and MRSA. The increase in silybin (A + B) content as observed in the methanolic fruit extract of plants treated with 40 mL/L ALE (Figure 1) increased the anti-microbial activity against *S. aureus* (almost comparable to that demonstrated by 10 µg of Imipenem) and MRSA, but not *E. coli*. Despite the higher silybin (A + B) content in the fruit extract from plants treated with 40 mL/L ALE, a decrease in anti-*E. coli* activity was observed in the corresponding fruit extract.

**Table 9.** Zone of inhibition (mm) exhibited by various test solutions on plates inoculated separately with *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and Methicillin-resistant *Staphylococcus aureus* (MRSA). The means and standard deviations were computed from the measurements of nine replicate plates. The test solution positive control was 10 µg Imipenem. Three *S. marianum* extract test solutions (5 mg/L) were prepared from methanolic fruit extracts from three individual plants of each of the control and ALE 40 mL/L treatments. No discs containing the test solution negative control (DMSO: water, v:v 1:1) produced an inhibition zone, in all nine replicate plates.

| Test Solution | Inhibition Zone (mm) | | |
|---|---|---|---|
| | ***S. aureus*** | ***E. coli*** | **MRSA** |
| *S. marianum* extract (control) | $12^{c*} \pm 1$ | $12^{b} \pm 2$ | $7^{c} \pm 2$ |
| *S. marianum* extract (ALE 40) | $17^{b} \pm 2$ | $5^{c} \pm 2$ | $14^{b} \pm 1$ |
| Imipenem 10 µg | $18^{a} \pm 3$ | $20^{a} \pm 2$ | $25^{a} \pm 2$ |

* According to the L.S.D. test, means in a column that are distinguished by the same letter are not statistically different at the 0.05 level of probability.

## 4. Discussion

The current study's objective was to investigate the impact of foliar application of various ALE concentrations as a natural elicitor on growth, yield, and phytochemical composition, and the *CHS* 1, 2, and 3 gene expressions of *S. marianum* growing in a greenhouse. The foliar application of the different concentrations of ALE increased plant growth and yield in *S. marianum* compared to the control treatment, with the highest difference being observed in plants treated with 60 mL/L ALE due to the presence of GA3, IAA, carbohydrate, and micro-elements, etc. in ALE (Table 1). Notably, IAA, which was found in ALE at the concentration (0.63 mg/100 g), plays an important role in cell elongation and the promotion of the stem growth, while gibberellins (16 mg/100 g) enhance plant height and several macro-elements found on the ALE analysis in Table 1 [28]. Similarly, ALE enhancement of plant growth and yield has been well documented in several plants [2,4–6], with ALE increasing the photosynthetic pigments and the potassium, phosphorus, and nitrogen contents in *S. marianum* leaves, compared with control plants.

Micro- and macro-elements, such as Cu, Fe, Mg, and K, are crucial for the formation of chlorophyll and are notably present in ALE (Table 1) [29]. Indeed, these results support other reports in this field [2,4,30].

The concentration of diverse secondary plant products is extensively influenced by growing conditions, with stressful situations having a significant effect on the metabolic pathways, leading to the build-up of associated natural products.

In the current study, various ALE concentrations significantly increased the silybin composition to various extents, significantly impacting *S. marianum* growth compared to the control group. Notably, 40 mL/L of ALE treatment gave the greatest increase in silybin composition (approximately five times that of the control treatment). Furthermore, the second and third highest enrichments in silybin composition were detected for the 20 and 60 mL/L ALE treatments (approximately four and three times that of the control treatment, respectively). Interestingly, the incorporation of ALE has also been reported to alter the composition of secondary metabolites [4,6].

In plants, fungi, and bacteria, chalcone synthase (CHS) is a crucial enzyme for the bio-synthesis of a wide range of compounds [17,18]. In *S. marianum*, the expression of the CHS 1, 2, and 3 genes has been linked to flavonoid bio-synthesis, including silybin composition [17]. Our results indicated that *CHS* 1, 2, and 3 expression was mildly enhanced in all the treatment groups that received ALE foliar spray, the highest expression being observed in the treatment group that received the 40 mL/L ALE foliar spray. Several research groups also observed *CHS* 1, *CHS* 2, and *CHS* 3 genes from *S. marianum* involved in the silymarin bio-synthetic pathway using different approaches [11,14].

Imipenem is an β-lactam group of antibiotics and is active against both Gram-positive and Gram-negative bacteria, even those that are resistant to certain antibiotics such as MRSA [31]. Our disc diffusion assay results were in accordance with this as the Imipenem

discs exhibited inhibition zones of 18, 20, and 25 mm against *S. aureus*, *E. coli*, and MRSA, respectively. The anti-microbial activity of ethanolic and methanolic extracts of *S. marianum* fruit against various bacteria species including *S. aureus* and *E. coli* have been previously reported [26,32]. In a study of methanolic extracts [32], the authors observed bigger inhibition zones in plates inoculated with *S. aureus* compared to those of *E. coli*. In contrast, in another study with ethanolic extract [28], the authors observed inhibition zones of similar sizes between plates inoculated with *S. aureus* and *E. coli*, which is consistent with the observation in the current study. The increase in silybin (A + B) content in the methanolic extracts from *S. marianum* treated with 40 mL/L ALE resulted in an increase in anti-microbial activity against *S. aureus* and MRSA, but not *E. coli*. Similar results were also observed by Rad et al. [30], whereby increases in the silybin content in the *S. marianum* ethanolic extract enhanced the anti-microbial activity against *E. coli* to a lesser extent compared to *S. aureus.*

## 5. Conclusions

In this study, the impacts of three different concentrations of *Aloe vera* leaf extract (20, 40, and 60 mL/L) on *S. marianum* growth, yield, silybin content, *CHS* 1, 2, and 3 gene expressions, and anti-microbial activity were investigated. The outcomes revealed that the foliar application of all the tested ALE concentrations improved plant growth and yield, and increased the medicinal value of *S. marianum* by enhancing the levels of bioactive components (silybin A and B) and the anti-microbial activity in the fruit extract. Furthermore, gene expression analysis demonstrated that ALE enhanced the synthesis of silybin (A + B) via the activation of *CHS* genes. Among the different concentrations of ALE tested, 60 mL/L of ALE was found to give the best enhancement effects in terms of growth and yield, while 40 mL/L of ALE resulted in strong enhancement effects on silybin content and *chalcone synthase* gene expressions. The increase in silybin (A + B) composition in the extract of plants treated with 40 mL/L ALE was also reflected in the increase in its anti-microbial activity against *S. aureus* and MRSA.

**Author Contributions:** All authors equally participated in this study. F.E.S. conceived and designed the study. F.E.S., M.F.A. and Y.-K.Y. conducted the research, curated, and analyzed the data. F.E.S. and M.A.A. prepared the first draft. M.F.A. and M.A.A. revised and edited the final manuscript. All authors have read and approved the final manuscript.

**Funding:** The authors extend their appreciations to the Deanship of Scientific Research, King Faisal University, for funding this research work through grant number Grant1367.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** Assistance from the greenhouse staff at the Agriculture and Veterinary Research and Training Centre, King Faisal University, was highly appreciated.

**Conflicts of Interest:** The authors declare no conflict of interest.

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