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ELICITOR-INDUCED PHYTOCHEMICAL PROPERTIES AND TRANSCRIPTIONAL CHANGES OF GENES ASSOCIATED WITH 20-HYDROXYECDYSONE BIOSYNTHESIS IN *Asparagus officinalis*

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Abstract

Asparagus officinalis L. is predominantly known as a vegetable and medicinal herb, capable of producing a wide range of bioactive compounds known for their potent antioxidant and pharmaceutical properties. Here, we described the potential elicitors to enhance the phytochemical and phenolic compounds as antioxidant properties produced in *A. officinalis*. This was achieved by combining *in vitro* shoot cultures of *A. officinalis* with different concentrations of oxalic acid, salicylic acid (1, 2.5, and 5 mM), chitosan (1.4, 2.8, and 5.6 mM), and calcium chloride (27, 90, and 180 mM) foliar spraying. Total phenolic content and antioxidant capacity were significantly incremented by oxalic acid (2.5 and 5 mM) and chitosan (1.4 and 5.6 mM). Phytochemical screening showed the presence of saponin, terpenoid, and cardiac glycoside in all treatments. Compared with the control plants, *A. officinalis* strongly modified phytochemicals profiles after elicitation with 5 mM of oxalic acid. This study estimated the transcript changes of genes involved in 20-hydroxyecdysone (20E) biosynthesis which is the main bioactive compound that possesses several medicinal benefits in *A. officinalis* after being treated by 5 mM of oxalic acid. Oxalic acid increased the expression of the genes encoding key critical vital catalytic enzymes in an early precursor in the 20E biosynthetic pathway. Especially, squalene monooxygenase (*SQE*), cycloartenol synthase (*CAS*), Δ(24)-sterol reductase (*DHCR24*), sterol isomerase (*EBP*), Δ7-sterol-C5(6)-desaturase (*ERG3*), and 7-dehydrocholesterol reductase (*DHCR7*) exhibited the expression at 3.7, 5.7, 2.3, 2.1, 3, and was 5.5-fold higher than that of the control. These results suggested that elicitor-mediated metabolite farming using oxalic acid could be a valuable method for 20-hydroxyecdysone production in *A. officinalis*.

INTRODUCTION

A broad spectrum of plant-derived phytochemicals has specific biological functions to reduce the risk of diseases in mammals [1, 2]. The 20-hydroxyecdysone (20E), a polyhydroxylated steroid, has attracted widespread attention and interest due to the many potential therapeutic properties such as chronic disease prevention, lipid metabolism improvement, immunological response modulation, anabolic, anti-diabetic, anti-inflammatory, and

hepatoprotective with very low toxicity [3, 4]. Notably, the limited natural resources available and the relatively low content of 20E in the higher plants result in an excessive cost for 20E preparations.

Asparagus officinalis L. stands out among the very few crop plants that produces 20E as it is detected in the leaves, stems, and roots [4] and is well-known for being a rich source of many beneficial bioactive compounds such as saponins, flavonoids, dietary fiber, oligosaccharides, and antioxidants [5]. *A. officinalis* is an economically important crop in Thailand and worldwide because of its structural and sensory characteristic, therapeutic, and nutraceutical properties [6]. Additionally, in homeopathy, it has been used to treat heart pain, dyspnea due to hydrothorax, violent palpitation, deglutition in hydrophobia, and stone passage in urine [7]. However, there has been no reports concerning the production of *A. officinalis* in secondary metabolite farming for its health characteristics in consumers' purchasing decisions.

In addition, the *in vitro* culture of plants, such as *Vitex glabrata* [8]*, Achyranthes aspera* [9]*, A. bidentata* [10], and *Pfaffia glomerata* [11], have been reported to affect cell growth and modulate 20E accumulation. The cell and callus culture of *Ajuga turkestanica* produced 20E at a two-to-six-fold level increase [12]. The yield of 20E increased from 0.1 to 0.2 percent after treatment with the mutagen *N-nitroso-N-methylurea* at 8 mM [13]. With jasmonic acid at the concentration of 0.6 mM, 20E production in *A. bidentata* cell culture was stimulated to a 2.6-fold increment [10]. Therefore, the strategy of plant *in vitro* culture has potential for 20E, suggesting it as an alternative to produce plants rich in pharmaceutically active compounds.

Natural elicitors - i.e., chitosan, salicylic acid, etc. - have been proposed to be applied to enhance secondary metabolites production and nutritional quality of various economic crops [14, 15]. Chitosan is a well-known natural elicitor because of its eliciting and antibacterial activity [16]. Another natural elicitor is salicylic acid -a simple phenolic phytohormone with various roles in plant growth and development; it also has a role in preventing post-harvest damage to vegetables [17]. Besides chitosan and salicylic acid, calcium chloride is another elicitor that has a well-known role in physiology of plant tissue; it increases the stiffness of plant cell walls, thus delaying tissue ripening [18]. Application of calcium chloride retains cell turgor, membrane integrity, and tissue firmness, plus delays membrane lipid catabolism [19]. In addition to chitosan, salicylic acid, and calcium chloride, oxalic acid is one organic acid that is non-enzymatic antioxidant and helps in chelating free radicals and protecting plant from stresses could cause prolonging the shelf life of plant cells and improving growth characters [20]. These elicitors exert their effects by employing different mechanisms such as triggering the synthesis of phytochemicals and enhancing the production of antioxidant enzymes in plants [16, 21, 22].

We therefore investigated the effect of concentration and type of natural elicitors on phytochemical profiles, including phytochemical screening by colorimetric tests, phenolic content, and antioxidant capacities by spectrophotometric tests of *in vitro* cultivated *A. officinalis*. Additionally, the genes associated with the 20E biosynthetic pathway were also monitored to identify their changes in the expression after elicitation by qRT-PCR. The findings of this study can be used as a reference for further plant science research to increase the contents of 20E and other health-promoting substances.

MATERIALS AND METHODS

Plant Materials and Tissue Culture of *A. officinalis*

The nodal explants of *A. officinalis* were first surfaced sterilized by immersing them in running water for 15 min before being rinsed twice with distilled water and immersed in 3% bleach containing 1-2 drops of Tween-20 for 30 min. Again, the explants were rinsed five times with sterile distilled water and blotted on sterilized filter paper. The plants were grown on Murashige and Skoog (MS) medium (M5519, Sigma-Aldrich) including 1.5 mg/L 6-benzulaminopurine (BAP) under fluorescent light for 16 h photoperiod (40 μE m^{-2} S^{-1}) using cool daylight fluorescent incandescent tubes (40 W, Philips, Kolkata) at 25 ± 2°C. Plant cultures thus raised were transferred once every 3 weeks. Then, two-month-old *in vitro* regenerated *A. officinalis* were used as the plant material for elicitation.

Elicitation Method

The four various elicitors, which were salicylic acid (SA), calcium chloride ($CaCl_2$), oxalic acid (OA) and chitosan (CHT) were dissolved in Milli-Q water and independent concentrations were prepared as shown in Table 1. The elicitors were applied as exogenous spraying on shoots with 10 mL of the test solution per treatment in the early morning every day, continuously for two weeks. The treatment with no spraying was used as a control.

The plants were collected and cut into pieces with a scissor, kept in liquid nitrogen, and then stored at -80ºC until the phytochemicals and gene expressions were measured.

Table 1. Types and concentrations of elicitors used in this study

Elicitors	Concentrations		
Salicylic acid	1 mM	2.5 mM	5 mM
Oxalic acid	1 mM	2.5 mM	5 mM
Chitosan	1.4 mM	2.8 mM	5.6 mM
Calcium chloride	27 mM	90 mM	180 mM

Phytochemical Screening

The phytochemical screening of whole stems and leaves tissues of *in vitro* cultivated *A. officinalis* was performed according to [23]. It was examined for alkaloids, anthraquinones, steroids, saponins, tannins, and glycosides. The qualitative results of the phytochemical screening are represented as + and - for the presence and absence of phytochemicals, respectively. The signs of +++, ++, and + are expressed the detection level of phytochemicals, which were classified as high, medium, and low, respectively.

Spectrophotometric Determination of Total Phenolic Content (TPC)

Total phenolic content (TPC) was evaluated using Folin-Ciocalteu's reagent [24]. Succinctly, 10 mg of plant tissues were extracted in 1 mL of 80% methanol at 4°C, then centrifuged at 10,000 RCF for 15 min. Subsequently, 1 mL of the extract was mixed with 0.2 mL of Folin's reagent (Sigma-Aldrich Chemie, GmBH, Stein-heim, Germany) and 1.6 mL of 5% Na_2CO_3 and then incubated for 20 min at room temperature for color development. The absorbance was then measured at 760 nm, using a spectrophotometer U-2900 (Hitachi High-Technologies Corporation). Results were calculated by comparing with the standard curve of gallic acid (standard equation: $y = 0.0029x + 0.1147$, $R^2 = 0.952$) and expressed as mg of gallic acid equivalents per g of fresh weight (mg GAE/g FW). Analyzes were done in three replicates.

Spectrophotometric Determination of DPPH Radical Scavenging Assay

Free radical scavenging ability of the plants was assessed by DPPH radical scavenging assay as methods of [25] and [26]. The ability of hydrogen atom donating was estimated by decolorization of solution of DPPH. It produces violet/purple color in methanol solution and fades to yellow color when antioxidants are present. For DPPH assay, the plants were extracted similarly to the method used for TPC determination. The 0.1 mM solution of DPPH in methanol was prepared, then 2.4 mL of this solution was mixed with 1.6 mL of methanol extract at various concentrations (20-150 µg/mL). The reaction was thoroughly vortexed and left in the dark for 30 min at room temperature. The absorbance was measured at 517 nm by using ascorbic acid as the reference. Percentage DPPH radical scavenging activity was calculated by the following equation:

$$\% \text{ DPPH radical scavenging activity} = \{(A_0 - A_1) / A_0\} \times 100$$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the extracts/standard. The percentage of inhibition was then plotted against concentration, and the IC_{50} was calculated. The experiment was set up with triplications for each concentration.

Gene Expression Analysis Using qRT-PCR

Total RNA was extracted from the frozen tissues and DNA eliminated by using the Plant Total RNA Extraction Kit (Geneaid, Taiwan). The total RNA quantity was checked by using a spectrophotometer at A260 wavelength, and the quality was checked by using gel electrophoresis. Then, reverse transcription polymerase chain reaction (RT-PCR) of samples were completed according to the manufacturer's instructions for the iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, CA, USA). The primers of the candidate and housekeeping *actin* genes (as an internal reference) were designed by using PrimerQuest Tool of Integrated DNA Technologies (http://sg.idtdna.com) and shown in Table 2.

Table 2. Gene accession number and primer sequences of the candidate genes involved in 20-hydroxyecdysone biosynthetic pathway and housekeeping gene used for qRT-PCR analysis in *A. officinalis* after elicitation by oxalic acid at the concentration of 5 mM

Gene	GenBank accession number	Forward sequence 5'-3'	Reward sequence 5'-3'	Product size (bp)
SQE	XM_020402269	GGCGGAAAGAACGAAGAAGG	TCCCTCCGAAGAGATACTGG	102
CAS	XM_020410366	CTTGAAGAGCGAGAGGCTGT	GCATCCGCATTAGCAAGTCT	94
DHCR24	XM_020405658	GCCTCAGTACTCCTGTGTCG	CCTATGGCGCCGTACTTCTT	100
EBP	XM_020389828	CAACTCTACGGGTGCTTGGT	TCGCGCCGATGTAATATGCC	100
ERG3	XM_020414024	CTCTCGTCCTCCTTCCCTTC	CCTCGACGAAATTGTGGAGG	104
DHCR7	XM_020408989	TTCAGCCGTCCTCACTTACAC	AGAACCATCGGCATGAACCA	100
Actin	LC385653	CCAAGGCAGAGTACGATGAA	CCACCTCAAGACAGCTAGATAC	102

SQE, squalene monooxygenase; CAS, cycloartenol synthase; DHCR24, Δ(24)-sterol reductase; EBP, sterol isomerase; ERG3, Δ7-sterol-C5(6)- desaturase; DHCR7, 7-dehydrocholesterol reductase.

The quantitative real-time PCR (qRT-PCR) was established in a BIORAD CFX96 Real-time PCR system (Bio-Rad Laboratories, CA, USA) with iQ™ SYBR Green Supermix (Bio-Rad Laboratories, CA, USA) under the following conditions: pre-denaturation at 95ºC for 3 min, 35 cycles of denaturation at 95ºC for 10s, annealing at 52ºC for 20 s, and extension at 72ºC for 10 min. The abundance of targeted gene transcripts was calculated relative to the control plants (with no treatment) according to the $2^{-\Delta\Delta CT}$ method [27]. Three independent replicates for each sample were evaluated, subsequently, the mean and standard error were analyzed.

Statistical Analysis

All experiments were performed in a randomized complete block design with three biological and technical replications. The Duncan tests SPSS statistical package (version 22; IBM Corp., NY, USA) was used to analyze the significant differences ($p \leq 0.05$) between means.

RESULTS AND DISCUSSION

Phytochemical Screening

Phytochemical screening of the *in vitro* cultivated *A. officinalis* tissues showed the presence of different secondary metabolites, which were saponins, terpenoids and glycosides according to the elicitors used (Table 3). Saponins were detected at a medium signal strength than that of control at certain concentrations of elicitors. While terpenoids and glycosides were detected in the elicitor treatments but not in the control (Table 3). These classes of phytochemicals act as important bioactive compounds of the *in vitro* cultivated *A. officinalis.* After the elicitor treatments, these secondary metabolites found in *A. officinalis* may interact with the signaling activity of the elicitors or function with the elicitors to control plant development and adapt to the environment. There are many studies employing these compounds, which they play some metabolic roles in controlling development of plant [28, 29]. In general, saponins and terpenoids are the class of secondary metabolites with basic functions in plant growth and development, and more specialized roles in stress tolerance [30, 31]. While glycosides play numerous roles in the early stages of plant development such as germination, bud formation, carbon transport, and possibly act as antioxidants [32]. Additionally, these phytochemical compounds detected are known to have pharmaceuticals and nutraceuticals properties. For example, saponins have been reported to be of great biological activities relevance to antiviral, anticancer, anti-inflammatory, insecticidal, and antifungal actions [33] due to their functions pertinent to steroids and cardiac glycosides [34]. Saponins from the shoots of *A. officinalis* exerted potential repressive activity on tumor, leukemia HL-60, metastasis of breast, colon, and pancreatic cancer cells [35]. Similarly, terpenoids are known to have antibacterial property [36]. Commonly benefits of glycosides are antioxidants and anti-inflammatory activities were also reported [37].

These phytochemical compounds were detected at a signal strength higher than that of the control when foliar sprayed by salicylic acid and oxalic acid (5 mM). The signals were greatest detected at oxalic acid treatment. Similarly, preharvest application of salicylic acid and oxalic acid showed the efficiency of oxalic acid in enhancing the accumulation of saponin in date plum [38]. Conversely, the plants grown without elicitor treatment only showed positive results for saponins (Table 3). This result contradicted the presence of alkaloids, terpenoids, glycosides, steroids, and proteins in naturally grown *A. officinalis* [39]. The occurrence of different phytochemicals between the *in vitro* and naturally grown *A. officinalis* may be due to the differences in the tissue, physiology of the plant part, and nutrient and endogenous hormone content [40]. Mashele et al. [41] reported that only naturally grown tissue of *A. laricinus* showed the presence of alkaloids, while *in vitro* tissues are devoid of alkaloids.

Therefore, foliar spraying of the natural elicitors can be used to support bioactive activities of *A. officinalis* and confirm the previous pharmacological properties *Asparagus* as the plant is predominantly assumed as medicinal plants [42, 43].

Total Phenolic Content

The TPC was determined by the Folin Ciocalteu method, as depicted in Figure 1. The TPC of the plants was relevant to the types and concentrations of elicitors. At all concentrations, oxalic acid and chitosan treatments showed significantly ($p \leq 0.05$) higher TPC compared to the control (Figure 1). Oxalic acid at the highest concentration (5 mM) presented the strongest effect, which was 9.67 mg GAE/g FW. This is agreement with the results of [14], whereby a 35% higher TPC content was found in asparagus spears sprayed with oxalic acid to delay the post-harvest damages during cold storage. The application of oxalic acid has been proposed to play a direct role in accumulation of phenolic content since it preserves the primary antioxidant compounds or free radical terminators found in the vegetative tissue [44].

Table 3. Preliminary screening of the natural elicitors, including salicylic acid (SA), calcium chloride ($CaCl_2$), oxalic acid (OA), and chitosan (CHT) on phytochemical compounds of the *in vitro* cultivated *A. officinalis*. Control (CT) is non-treated elicitor. The signs of +++, ++, and + are expressed levels of detecting phytochemicals according to high, medium, and low positive responses, respectively. The sign of – was obtained for a negative response

Metabolites	Test	CT	SA			OA			CHT			$CaCl_2$		
			1 mM	2.5 mM	5 mM	1 mM	2.5 mM	5 mM	1.4 mM	2.8 mM	5.6 mM	27 mM	90 mM	180 mM
Saponins	Foam test	+	+	+	++	++	++	++	++	+	+	+	+	+
Tannins	Ferric chloride test	-	-	-	-	-	-	-	-	-	-	-	-	-
Alkaloids	Dragendroff's test	-	-	-	-	-	-	-	-	-	-	-	-	-
Anthraquinones	Borntrager's Test	-	-	-	-	-	-	-	-	-	-	-	-	-
Terpenoids	Salkowski's test	-	+	+	++	++	++	+++	++	++	++	++	++	++
Steroids	Salkowski's test	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycoside	Keller-kiliani test	-	+	+	++	-	+	++	-	-	-	-	-	+

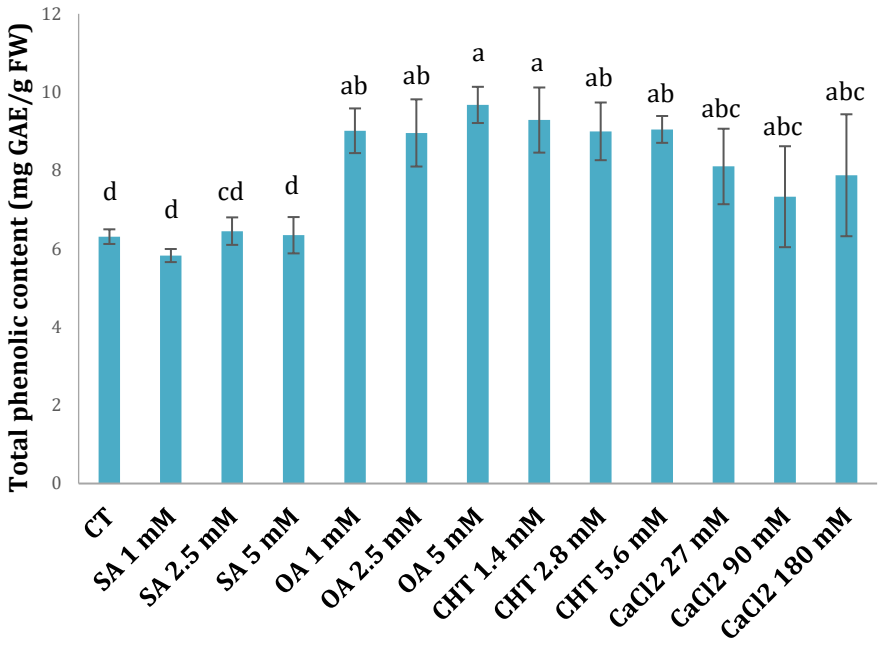

Figure 1. Determination of total phenolic content of *A. officinalis* under *in vitro* culture and foliar sprayed by various elicitors, including salicylic acid (SA), calcium chloride ($CaCl_2$), oxalic acid (OA), and chitosan (CHT). Data are represented as mean ± SE (n=3, $p \leq 0.05$). Level of significance among treatments as governed by ANOVA at $p \leq 0.05$ was represented as small letters.

The total amount of phenol content was also significantly increased by chitosan treatment. The total number of phenolics reached a maximal content of 9.28 mg GAE/g FW at the lowest concentration (1.4 mM) of chitosan (Figure 1). Exogenous spraying of chitosan increased phenolic content, hence destroy the hydrogen peroxide and free radicals. Additionally, the exogenous sprayings of 2.8 and 5.6 mM of chitosan also significantly increase the total phenolic content and revealed lower TPC than that of the 1.4 mM concentration (Figure 1). This is consistent with previous findings in soybean and tomato that showed chitosan at low concentrations could induce phenolic compounds, which enhanced the quality and shelf life [45, 46]. Ali et al. [47] also reported that the elicitor with a low concentration of chitosan enhanced the TPC in cell culture of *Artemisia absinthium*. These results indicated that the exogenous spraying of oxalic acid and chitosan under *in vitro* culture of *A. officinalis* can increase the accumulation of phenolic compounds, and thus the usefulness in the pharmaceutical and medical fields.

For salicylic acid treatment, although it has been reported to induce total phenols and antioxidants in natural-grown asparagus [48], but *in vitro* cultivated *A. officinalis* is no exception. In this study, the foliar sprayings of salicylic acid and calcium chloride had a slight effect on TPC (Figure 1). This result was consistent with that of Coste et al. [49] for *Hypericum hirsutum*, and of Cai et al. [50] for *Changium smyrnioides*. In both cases, salicylic acid was not affected on phenolic accumulation. Additionally, some natural elicitors have no function in the promoting production of phenolic compounds to prevent some common problems related with the presence of these compounds, such as browning. These properties have been detected in various tissues of vegetables and fruits, some of which are asparagus [51]. The elicitation by calcium chloride can delay browning without an improvement in the TPC accumulation [19, 51, 52]. Therefore, the dosage and type of the elicitors are major factors used for each specific case [53, 54].

DPPH Radical Scavenging Activity

The DPPH radical scavenging abilities of the cultivated *A. officinalis* tissues were compared with that of ascorbic acid as standard as shown in Figure 2. The performance of the various plant extracts and its concentrations is dependent on the ability to donate hydrogen to DPPH [55]. Figure 2A shows the free radical scavenging activity of the plants at the concentration of 0.1 mg/mL.

This study revealed that among the elicitor treatments, oxalic acid (2.5 and 5 mM), chitosan (1.4 and 5.6 mM), and calcium chloride (27, 90, and 180 mM) possessed slightly higher activity over the control with no significant difference detected in all the treatments (Figure 2A). On the contrary, for the free radical scavenging activity no increment occurred when the plants were foliar sprayed by salicylic acid.

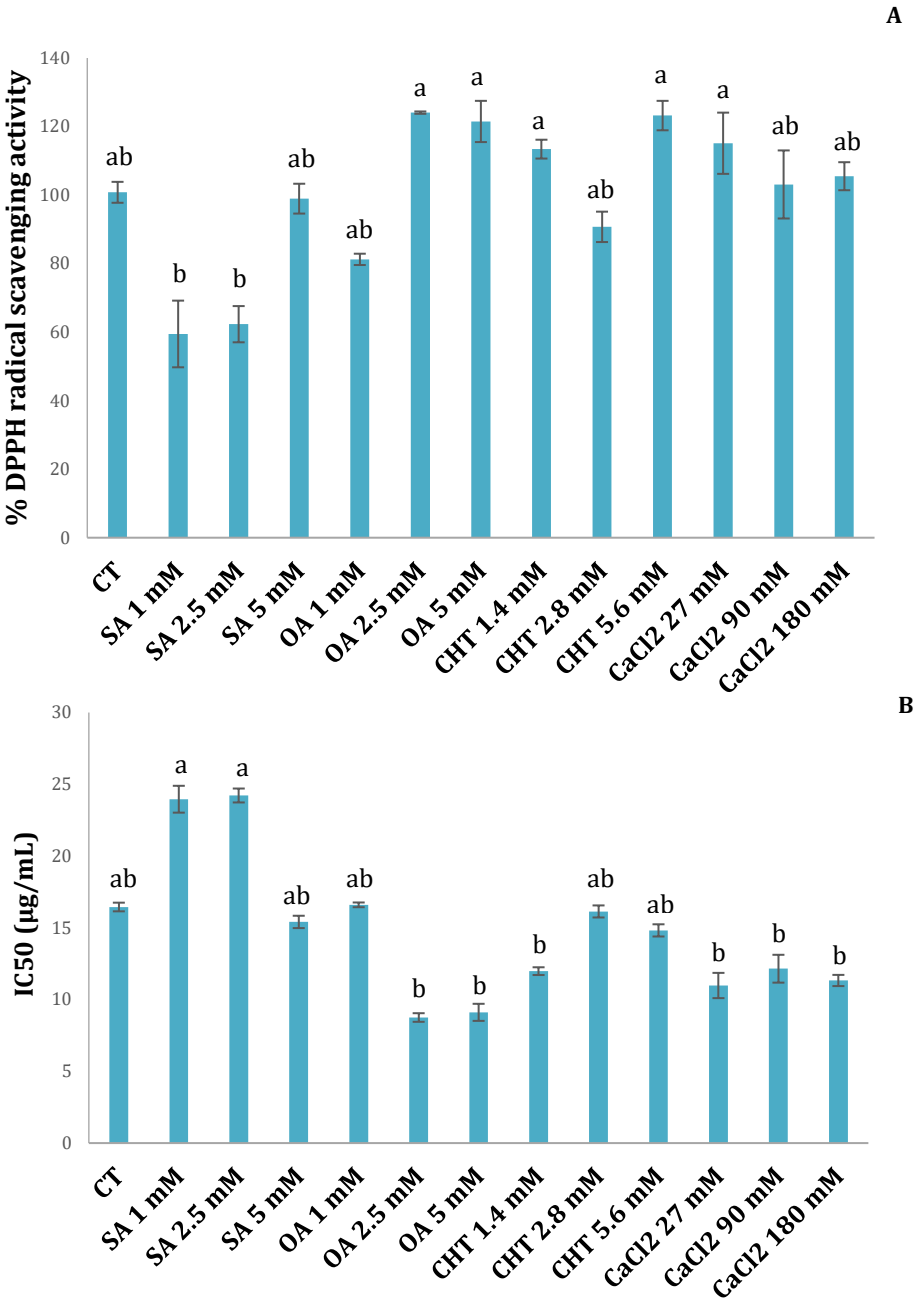

Figure 2. Determination of DPPH radical scavenging (A) and IC_{50} (B) of *A. officinalis* under *in vitro* and foliar sprayed by various natural elicitors, including salicylic acid (SA), calcium chloride ($CaCl_2$), oxalic acid (OA), and chitosan (CHT). Data are expressed as mean $\pm$ SE for all tests. Level of significance among treatments as governed by ANOVA at $p \leq 0.05$ was represented as small letters.

The same volume of the extracts against DPPH free radicals of each treatment was taken into consideration and represented as the IC_{50} value (Figure 2B). IC_{50} of the treatments of oxalic acid at the concentrations of 2.5 and 5 mM were 1.8-fold greater than that of the control plants (Figure 2B). This shows the enhanced capacity of electron transfer or hydrogen donating ability of antioxidant enzymes found in the plants treated with oxalic acid. In an interesting coincidence, the general profile of the antioxidant activity of *A. officinalis* (Figure 2) was very similar to that of the induced total amount of phenolic compounds (Figure 1) in the same treatments. Sun et al. [56] also reported that antioxidant capacity in many plant materials is connected primarily with their phenolic compound, indicating a significant positive correlation between total phenolic contents and antioxidant capacity. The results of analyses conducted in this study indicated the exogenous spraying of oxalic acid at 2.5 and 5 mM elicited total phenolic content

and the scavenging capacity in relation to the DPPH radical. Oxalic acid has been shown to increase the levels of antioxidant compounds and the antioxidant activity that improve fresh green-cut asparagus quality, through the reduction of harmful radicals [14]. Taking the results together, it can be concluded that the treatment of oxalic acid, especially at 5 mM might have greater responsibility for phytochemical and phenolic compounds and the radical scavenging activity of *A. officinalis* than other natural elicitors used in the study.

Effect of Oxalic Acid Application on the Genes Involved in 20-Hydroxyecdysone Biosynthesis

To understand the effect of oxalic acid elicitation on the 20E biosynthesis in *A. officinalis* culture, the expression level of key genes (*SQE*, *CAS*, *DHCR24*, *EBP*, *ERG3*, and *DHCR7*) from sterol biosynthesis was evaluated by using qRT-PCR. The fold changes of genes compared to the control are shown in Figure 3. We found that the foliar spraying of oxalic acid onto the plants was significantly influenced the expression of all these genes. The *CAS* and *DHCR7* gene transcripts were strongly increased up to 5.7- and 5.54-fold compared to that of control, respectively (Figure 3). The CAS and DHCR7 are generally regarded as the enzymes responsible for the synthesis of lanosterol and cholesterol, which are the precursor for 20E biosynthesis [57].

As the expressions of all examined genes involved in 20E biosynthesis were upregulated by oxalic acid at 5 mM (Figure 3), this implies that the biosynthesis of 20E is associated with the expression level of these six key genes and might be enhanced by oxalic acid treatment. This agrees with previous findings, in which reported that due to the expression of genes the high concentrations of phytochemicals increased after elicitation with oxalic acid in green lettuce [58] and wheat [59]. Effect of oxalic acid on *A. officinalis* response under *in vitro* culture had an implication at the genetic level, which led to the changes in relative expression of these genes. This elicitation could be related to the activation of the plant defense mechanisms, where secondary metabolite biosynthesis plays an important role in the plant defense [60]. This is supported by the function of 20E on the defense against herbivore insects [60] and mechanical damage [61], as well as the response to plant growth regulator application such as methyl jasmonate [62]. Related findings have been well-reported that exogenous spraying of oxalic acid improves gene expression related to plant protection such as phytic acid in mung bean [63] and various crop plants [20, 64, 65].

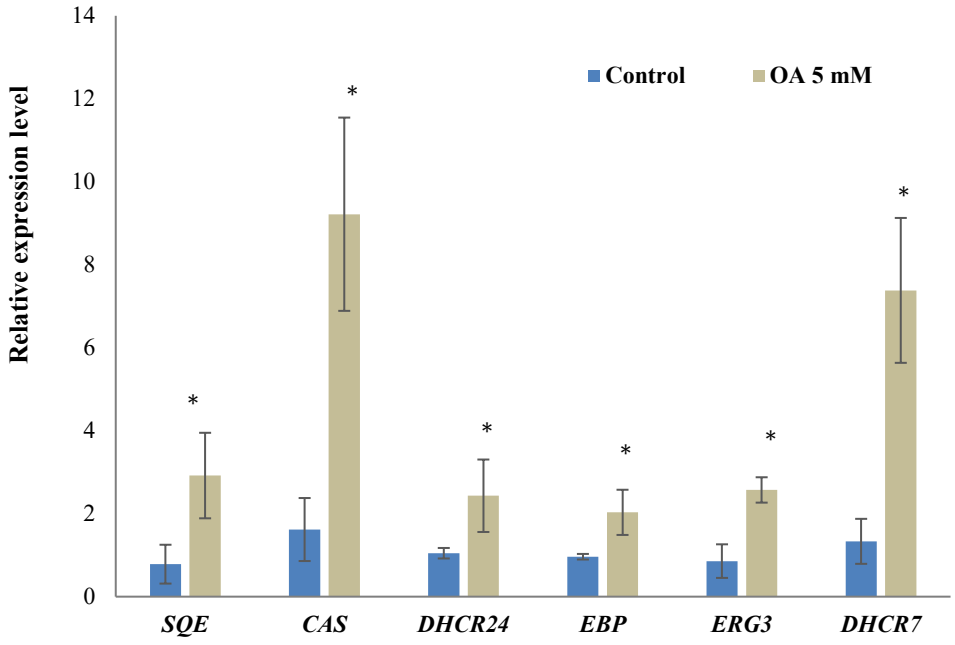

Figure 3. Effect of oxalic acid application on the expression levels of the genes involved in 20-hydroxyecdysone biosynthesis in cultivated *A. officinalis* under *in vitro*. Vertical bars represent standard error between different replicates of the same treatment, whereas asterisk indicates statistical significance.

CONCLUSION

Foliar application of natural elicitors in the culture of *A. officinalis* significantly increased phytochemicals in a dose-dependent manner of each elicitor. Especially, oxalic acid elicitation at 5 mM is a workable practice for increasing the production of phenolic compounds and antioxidant activity in *A. officinalis*, which may improve this plant value as a nutraceutical supplement. Furthermore, the six key genes associated with 20-hydroxyecdysone biosynthesis noticeably determined the effect of oxalic acid on 20-hydroxyecdysone elicitation were all upregulated. The increase in gene transcripts could be related to the increment of phytochemicals, which may lead to an enhanced 20-hydroxyecdysone biosynthesis. Consequently, it was possible to observe the spraying of oxalic acid to serve as an effective strategy for triggering the phytochemicals, antioxidant activities and medicinally important 20-hydroxyecdysone in *A. officinalis* during *in vitro* culture. Further investigations are needed to better understand the time course activated by oxalic acid elicitation, which results in the search for more beneficial phytopharmacological compounds increment in the plants.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

REFERENCES

1. González-Vallinas, M., González-Castejón, M., Rodríguez-Casado, A. and Ramírez de Molina, A. (2013) Dietary phytochemicals in cancer prevention and therapy: A complementary approach with promising perspectives. *Nutr. Rev.* **71**(9), 585-99.
2. Williams, D.J., Edwards, D, Hamernig, I, Jian, L, James, AP, Johnson, SK, et al. (2013) Vegetables containing phytochemicals with potential anti-obesity properties: A review. *Int. Food Res. J.* **52**(1), 323-33.
3. Dinan, L., Dioh, W., Veillet, S. and Lafont, R. (2021) 20-Hydroxyecdysone, from Plant extracts to clinical use: Therapeutic potential for the treatment of neuromuscular, cardio-metabolic and respiratory diseases. *Biomedicines*. **9**(5), 492.
4. Dinan, L., Savchenko, T. and Whiting, P. (2001) Phytoecdysteroids in the genus Asparagus (Asparagaceae). *Phytochemistry*. **56**(6), 569-76.
5. Lee, E.J., Yoo, K.S. and Patil, B.S. (2010) Development of a rapid HPLC-UV method for simultaneous quantification of protodioscin and rutin in white and green asparagus spears. *J. Food Sci.*. **75**(9), C703-C9.
6. Tandon, M., Shukla, Y.N. and Thakur, R.S. (1990) Steroid glycosides from *Asparagus adscendens*. *Phytochemistry*. **29**(9), 2957-9.
7. Iqbal, M., Bibi, Y., Iqbal Raja, N., Ejaz, M., Hussain, M., Yasmeen, F., et al. (2017) Review on therapeutic and pharmaceutically important medicinal plant *Asparagus officinalis* L. *Plant Physiol. Biochem.* **05**(01).
8. Thanonkeo, S., Chamnipa, N. and Thanonkeo, P. (2011) Induced accumulation of 20-hydroxyecdysone in cell suspension cultures of *Vitex glabrata* R.Br. *Afr. j. biotechnol.*. **10**, 10612-7.
9. John, R., Shajitha, P.P., Devassy, A. and Mathew, L. (2018) Effect of elicitation and precursor feeding on accumulation of 20-hydroxyecdysone in *Achyranthes aspera* Linn. cell suspension cultures. *Physiol. Mol. Biol. Plants*. **24**(2), 275-84.
10. Wang, Q., Zheng, L., Sima, Y., Yuan, H. and Wang, J. (2013) Methyl jasmonate stimulates 20-hydroxyecdysone production in cell suspension cultures of *Achyranthes bidentata*. *Plant Omics*. **6**, 116-20.
11. Fortini, E.A., Batista, D.S., de Castro, K.M., Silva, T.D., Felipe, S.H.S. and Correia, L.N.F., et al. (2020) Photoperiod modulates growth and pigments and 20-hydroxyecdysone accumulation in Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen] grown *in vitro*. *Plant Cell Tissue Organ Cult*. **142**(3), 595-611.
12. Lev, S., Zakirova, R., Saatov, Z., Gorovits, M. and Abubakirov, N. (1990) Ecdysteroids from tissue and cell cultures of *Ajuga turkestanica*. *Chem. Nat. Compd*. **1**, 51-2.
13. Zakirova, R. and Malikova, M.K. (2000) Effect of *N*-nitroso-*N*-methylurea on the biosynthetic activity of *Ajuga turkestanica* callus tissue. *Chem. Nat. Compd*. **36**(4), 384-6.
14. Barberis, A., Cefola, M., Pace, B., Azara, E., Spissu, Y., Serra, P.A., et al. (2019) Postharvest application of oxalic acid to preserve overall appearance and nutritional quality of fresh-cut green and purple asparagus during cold storage: A combined electrochemical and mass-spectrometry analysis approach. *Postharvest Biol. Technol.* **148**, 158-67.
15. Ahmed, Z.F.R., Alblooshi, S.S.N.A., Kaur, N., Maqsood, S. and Schmeda-Hirschmann, G. (2021) Synergistic effect of preharvest spray application of natural elicitors on storage life and bioactive compounds of date palm (*Phoenix dactylifera* L., cv. Khesab). *Horticulturae*. **7**(6), 145.
16. Romanazzi, G., Feliziani, E. and Sivakumar, D. (2018) Chitosan, a biopolymer with triple action on postharvest decay of fruit and vegetables: Eliciting, antimicrobial and film-forming properties. *Front. Microbiol*. **9**(2745).
17. Gomes, E.P., Vanz Borges, C., Monteiro, G.C., Filiol Belin, M.A., Minatel, I.O., Pimentel Junior, A., et al. (2021) Preharvest salicylic acid treatments improve phenolic compounds and biogenic amines in 'Niagara Rosada' table grape. *Postharvest Biol. Technol*. **176**, 111505.
18. Irfan, P.K., Vanjakshi, V., Prakash, M.N.K., Ravi, R. and Kudachikar, V.B. (2013) Calcium chloride extends the keeping quality of fig fruit (*Ficus carica* L.) during storage and shelf-life. *Postharvest Biol. Technol*. **82**, 70-5.
19. Mola, S., Uthairatanakij, A., Srilaong, V., Aiamla-or, S. and Jitareerat, P. (2016) Impacts of sodium chlorite combined with calcium chloride, and calcium ascorbate on microbial population, browning, and quality of fresh-cut rose apple. *Agric. Nat. Resour.* **50**(5), 331-7.
20. Malenčić, D., Vasić, D., Popović, M. and Dević, D. (2004) Antioxidant systems in sunflower as affected by oxalic acid. *Biol. Plant*. **48**(2), 243-7.
21. Ruiz-García, Y. and Gómez-Plaza, E. (2013) Elicitors: A tool for improving fruit phenolic content. *Agriculture*. **3**(1), 33-52.
22. Baenas, N., García-Viguera, C. and Moreno, D.A. (2014) Elicitation: A tool for enriching the bioactive composition of foods. *Molecules*. **19**(9), 13541-63.

23. Iqbal, E., Salim, K.A. and Lim, L.B.L. (2015) Phytochemical screening, total phenolics and antioxidant activities of bark and leaf extracts of *Goniothalamus velutinus* (Airy Shaw) from Brunei Darussalam. *J. King Saud Univ. Sci*. **27**(3), 224-32.

24. Fukumoto, L.R. and Mazza, G. (2000) Assessing antioxidant and prooxidant activities of phenolic compounds. *J. Agric. Food Chem.* **48**(8), 3597-604.

25. Sharma, O.P. and Bhat, T.K. (2009) DPPH antioxidant assay revisited. *Food Chem.* **113**(4), 1202-5.

26. Elshafie, H.S., Sakr, S.H., Sadeek, S.A. and Camele, I. (2019) Biological investigations and spectroscopic studies of new moxifloxacin/glycine-metal complexes. *Chem. Biodivers*. **16**(3), e1800633.

27. Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. **25**(4), 402-8.

28. Erb, M. and Kliebenstein, D.J. (2020) Plant secondary metabolites as defenses, regulators, and primary metabolites: The blurred functional trichotomy. *Plant Physiol*. **184**(1), 39-52.

29. Weng, J.-K., Lynch, J.H., Matos, J.O. and Dudareva, N. (2021) Adaptive mechanisms of plant specialized metabolism connecting chemistry to function. *Nat. Chem. Biol*. **17**(10), 1037-45.

30. Shabani, L., Ehsanpour, A., Asghari, G. and Emami, J. (2009) Glycyrrhizin production by *in vitro* cultured *Glycyrrhiza glabra* elicited by methyl jasmonate and salicylic acid. *Russ. J. Plant Physiol*. **56**(5), 621-6.

31. Yendo, A.C., de Costa, F., Gosmann, G. and Fett-Neto, A.G. (2010) Production of plant bioactive triterpenoid saponins: Elicitation strategies and target genes to improve yields. *Mol. Biotechnol.* **46**(1), 94-104.

32. Gleadow, R.M. and Møller, B.L. (2014) Cyanogenic glycosides: Synthesis, physiology, and phenotypic plasticity. *Annu. Rev. Plant Biol*. **65**(1), 155-85.

33. Ashour, A.S., El Aziz, M.M.A. and Gomha Melad, A.S. (2019) A review on saponins from medicinal plants: Chemistry, isolation, and determination. *J. Nanomed. Res*. **7**(4), 282-8.

34. Adedapo, A., Mogbojuri, O. and Emikpe, B. (2009) Safety evaluations of the aqueous extract of the leaves of *Moringa oleifera* in rats. *J. Med. Plant Res*. **3**(8), 586-91.

35. Sakaguchi, Y., Ozaki, Y., Miyajima, I., Yamaguchi, M., Fukui, Y., Iwasa, K., et al. (2008) Major anthocyanins from purple asparagus (*Asparagus officinalis*). *Phytochemistry*. **69**(8), 1763-6.

36. Sathya Bama, S., Jayasurya Kingsley, S., Sankaranarayanan, S. and Bama, P. (2012) Antibacterial activity of different phytochemical extracts from the leaves of *T. procumbens* Linn.: Identification and mode of action of the terpenoid compound as antibacterial. *Int. J. Pharm. Pharm. Sci*. **4**(1), 557-64.

37. Kren, V. and Martínková, L. (2001) Glycosides in medicine: "The role of glycosidic residue in biological activity". *Curr. Med. Chem*. **8**(11), 1303-28.

38. García-Pastor, M.E., Giménez, M.J., Valverde, J.M., Guillén, F., Castillo, S., Martínez-Romero, D., et al. (2020) Preharvest application of oxalic acid improved pomegranate fruit yield, quality, and bioactive compounds at harvest in a concentration-dependent manner. *Agronomy*. **10**(10), 1522.

39. Begum, A., Rao, K., Dutt, R., Giri, K., Sindhu, K., fathima, U., et al. (2017) Phytochemical screening and thin layer chromatography of Indian *Asparagus officinalis* Linn. *Int J Approx Reason*. **5**, 1520-8.

40. Kumar, V., Singh, S., Bandopadhyay, R., Sharma, M. and Chandra, S. (2014) *In vitro* organogenesis secondary metabolite production and heavy metal analysis in *Swertia chirayita*. *Open Life Sci*. **9**(7), 686-98.

41. Mashele, S., Fuku, S. and Al-Azzawi, A.M. (2013) Phytochemistry and free radical scavenging activity of *Asparagus laricinus*.

42. Fouché, G., Cragg, G., Pillay, P., Kolesnikova, N., Maharaj, V. and Senabe, J. (2008) *In vitro* anticancer screening of South African plants. *J. Ethnopharmacol*. **119**(3), 455-61.

43. Kubota, S., Konno, I. and Kanno, A. (2012) Molecular phylogeny of the genus Asparagus (Asparagaceae) explains interspecific crossability between the garden asparagus (*A. officinalis*) and other Asparagus species. *Theor. Appl. Genet.* **124**(2), 345-54.

44. Cefola, M. and Pace, B. (2015) Application of oxalic acid to preserve the overall quality of rocket and baby spinach leaves during storage. *J. Food Process. Preserv.* **39**(6), 2523-32.

45. Köhle, H., Young, D.H. and Kauss, H. (1994) Physiological changes in suspension-cultured soybean cells elicited by treatment with chitosan. *Plant Sci. Lett*. **33**(2), 221-30.

46. Pearce, G., Marchand, P.A., Griswold, J., Lewis, N.G. and Ryan, C.A. (1998) Accumulation of feruloyltyramine and p-coumaroyltyramine in tomato leaves in response to wounding. *Phytochemistry*. **47**(4), 659-64.

47. Ali, M., Abbasi, B.H. and Ali, G.S. (2015) Elicitation of antioxidant secondary metabolites with jasmonates and gibberellic acid in cell suspension cultures of *Artemisia absinthium* L. *Plant Cell Tissue Organ Cult.* **120**(3), 1099-106.

48. Wei, Y., Liu, Z., Su, Y., Liu, D. and Ye, X. (2011) Effect of salicylic acid treatment on postharvest quality, antioxidant activities, and free polyamines of asparagus. *J. Food Sci*. **76**(2), S126-32.

49. Coste, A., Vlase, L., Halmagyi, A., Deliu, C. and Coldea, G. (2011) Effects of plant growth regulators and elicitors on production of secondary metabolites in shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum*. *Plant Cell Tissue Organ Cult.* **106**(2), 279-88.

50. Cai, J., Ma, Y., Hu, P., Zhang, Y., Chen, J. and Li, X. (2017) Elicitation of furanocoumarins in *Changium smyrnioides* suspension cells. *Plant Cell Tissue Organ Cult*. **130**(1), 1-12.

51. Chen, Z., Zhu, C., Zhang, Y., Niu, D. and Du, J. (2010) Effects of aqueous chlorine dioxide treatment on enzymatic browning and shelf-life of fresh-cut asparagus lettuce (*Lactuca sativa* L.). *Postharvest Biol. Technol.* **58**(3), 232-8.

52. Silveira, A.C., Aguayo, E., Chisari, M. and Artés, F. (2011) Calcium salts and heat treatment for quality retention of fresh-cut 'Galia'melon. *Postharvest Biol. Technol.* **62**(1), 77-84.

53. Ishikawa, A., Kitamura, Y., Ozeki, Y. and Watanabe, M. (2007) Different responses of shoot and root cultures of *Glehnia littoralis* to yeast extract. *J. Nat. Med.* **61**(1), 30-7.

54. Li, Y., Kong, D., Fu, Y., Sussman, M.R. and Wu, H. (2020) The effect of developmental and environmental factors on secondary metabolites in medicinal plants. *Plant Physiol. Biochem*. **148**, 80-9.

55. Benmehdi, H., Behilil, A., Memmou, F. and Amrouche, A. (2017) Free radical scavenging activity, kinetic behaviour and phytochemical constituents of *Aristolochia clematitis* L. roots. *Arab. J. Chem*. **10**, S1402-S8.

56. Sun, T., Powers, J.R. and Tang, J. (2007) Evaluation of the antioxidant activity of asparagus, broccoli and their juices. *Food Chem*. **105**, 101-6.

57. Sripinyowanich, S., Kil, E.-J., Petchsri, S., Jo, Y., Choi, H., Cho, W.K., et al. (2021) *De novo* transcriptome assembly of two

Microsorum fern species identifies enzymes required for two upstream pathways of phytoecdysteroids. *Int. J Mol. Sci*. **22**(4), 2085.

58. Moreno-Escamilla, J.O., Alvarez-Parrilla, E., de la Rosa, L.A., Núñez-Gastélum, J.A., González-Aguilar, G.A. and Rodrigo-García, J. (2017) Effect of different elicitors and preharvest day application on the content of phytochemicals and antioxidant activity of butterhead lettuce (*Lactuca sativa* var. capitata) produced under hydroponic conditions. *J. Agric. Food. Chem*. **65**(26), 5244-54.

59. El-Shabrawi, H., Bakry ba, Ahmed, M. and Abou-El-Lail, M. (2015) Humic and oxalic acid stimulates grain yield and induces accumulation of plastidial carbohydrate metabolism enzymes in wheat grown under sandy soil conditions. *Science*. **6**, 175-85.

60. Hunter, M. (2001) Issues in agricultural and forest entomology: Out of sight, out of mind: The impacts of root-feeding insects in natural and managed systems. *Agric. For. Entomol*. **3**, 3-9.

61. Schmelz, E.A., Grebenok, R.J., Galbraith, D.W. and Bowers, W.S. (1998) Damage-induced accumulation of phytoecdysteroids in spinach: A rapid root response involving the octadecanoic acid pathway. *J. Chem. Ecol*. **24**(2), 339-60.

62. John, R., Shajitha, P.P., Devassy, A. and Mathew, L. (2018) Effect of elicitation and precursor feeding on accumulation of 20-hydroxyecdysone in *Achyranthes aspera* Linn. cell suspension cultures. *Physiol. Mol. Biol. Plants*. **24**(2), 275-84.

63. Jin, X., Yang, R., Yan, X., Zhou, Y., Wang, X. and Gu, Z. (2016) Malic acid and oxalic acid spraying enhances phytic acid degradation and total antioxidant capacity of mung bean sprouts. *Int. J. Food Sci*. **51**(2), 370-80.

64. Mucharromah, E. and Kuc, J. (1991) Oxalate and phosphates induce systemic resistance against diseases caused by fungi, bacteria and viruses in cucumber. *J. Crop Prot*. **10**(4), 265-70.

65. Zheng, G., Zhao, R. and Peng, X. (1999) Oxalate-induced resistance of muskmelon to WMV-2. *Sci. Bull*. **44**, 1794-7.