AMERICAN JOURNAL OF BIOLOGY AND NATURAL SCIENCES

https://biojournals.us/index.php/AJBNS

ISSN: 2997-7185

Article

Improving the Production of Phenolic and Flavonoid Compounds in Suspension Cell Cultures of the Plant *Salvia compressa* Endemic to Iraq Using Biostimulants

Hasanain A. F. Dhmosh[1]

1. Dept. of Medical Laboratory Technics, Al-Mussaib Technical Institute, Al-Furat Al-Awsat Technical University, Babylon, Iraq

Citation: Dhmosh H. A. F. Improving the Production of Phenolic and Flavonoid Compounds in Suspension Cell Cultures of the Plant Salvia compressa Endemic to Iraq Using Biostimulants. American Journal of Biology and Natural Sciences 2026, 3(6), 1-12.

Received: 10th Mar 2026
Revised: 11th Apr 2026
Accepted: 24th May 2026
Published: 04th Jun 2026

Abstract

Background: *Salvia compressa* is a highly valued Iraqi-endemic plant Iraqi-endemic plants containing various polyphenolic and flavonoids compounds. constrained by environmental and seasonal challenges, however, limiting the sustainability and commercial availability. **Objective:** To assess the effect of different biostimulants on bioaccumulation of these bioactive secondary metabolites in the suspension cell culture of this rare plant. **Methodology:** Callus cultured from sterile seedlings, suspension cell cultures were established in liquid MS media, and callus was elongated using elicitors such as methyl jasmonate, salicylic acid and yeast extract at different concentrations on the 14th day. An HPLC-DAD method was used for the analysis of results. **Results:** Production of compounds was greatly stimulated by the use of the biostimulants. Total phenolic content of 31.8 mg/g DW highest among the treatments with 100 μM methyl jasmonate while the flavonoids which doubled to 16.5 mg/g DW demonstrating an improvement over the control. HPLC analyses revealed that, due to treatment with yeast extract, rosmarinic acid increased significantly from 3.1 mg/g in the untreated to 6.9 mg/g, while it increased from 2.4 mg/g to 7.8 mg/g when methyl jasmonate was used. Increases were also found in caffeic acid, salvianolic acid and luteolin in the study. **Conclusion:** The study reveals that the suspension cell culture in the presence of Biostimulants such as methyl jasmonate is an effective, sustainable platform for the mass production of bioactive compounds from *Salvia compressa*; indicating its potential for utilization in the pharmaceutical industries.

Keywords: Salvia compressa, Biostimulants, Phenolic Compounds, Flavonoid Compounds

Introduction

Salvia L. is the most diverse and largest genus in the Lamiaceae family with more than 900 species spread throughout the temperate and sub-tropical areas of the world [1]. Salvia species have a long history of use in ethnobotany and traditional medicine, and have been used for a wide range of ailments and health conditions throughout the Middle East and over time [2]. Their high content in phenolic acids, flavonoids, and terpenoids, among other secondary metabolites, with considerable pharmacological potential, underpins their their profound medicinal value, which is attributed to their interwoven chemical profile [3]. These metabolites include especially phenolic and flavonoid compounds, which have multiple biological activities. In its natural habitat, the plant uses these compounds as key elements in a defense system providing its protection against ultraviolet rays, microbial pathogens and herbivores [4]. Recent metabolomic investigations have shown, from the human health standpoint, that these bioactive profiles are both similar and distinct in each of the Salvia spp, with strong anti-microbial, anti-inflammatory and anti-cancer activities shown [5]. This therapeutic versatility has facilitated the widespread use of these compounds in the pharmaceutical and nutraceutical sectors, and there is ongoing research to find new sources in the Lamiaceae family [6]. The plants of Iraq are an important center of plant diversity in the Middle East, it contains a large number of medicinal plants which are used in local ethnopharmacological uses as mentioned in different regions such as Diyala Province [7]. Among this floristic wealth, *Salvia compressa* Vent. is an endemic species of special interest. Its distinctiveness on morphology, anatomy and palynology aspects of study has been confirmed and its specific distribution was found in the Iraqi landscape [8]. In addition, *S. compressa* has been used in phylogenetic analyses of morphological characters to elucidate the taxonomic status of the species in relation to other local species, highlighting the distinct evolutionary history of this species [9]. Although *S. compressa* is endemic and may have high secondary metabolite potential, it is still a species that is somewhat under-explored in the field of biotechnology for production of these compounds.

Most bioactive extraction from Salvia species is done with field-grown plants. But these are becoming more and more restricted by environmental fluctuations, exacerbated seasonal changes, and the growth slowdown of many wild species. In order to address these constraints, plant biostimulants are identified as substances or microorganisms, which when applied to plants, soil and/or crop environment, improve nutrient efficiency and abiotic stress tolerance, leading to an increase in plant growth and metabolite production [10]. For the genus Salvia, several biotechnological approaches, created by in vitro cultures have been used in order to obtain a stable sesquiterpene-rich product avoiding conventional cultivation limitations [11]. Of those biotechnological solutions, suspension cell culture is an extremely efficient process for the large-scale production of specific compounds [12]. The system enables plant cells to be cultured in a controlled environment with optimal physiological conditions. This technique has been proven successful in several studies where elicitors were effective in enhancing the metabolite production of Salvia cell, such as rosmarinic acid and tanshinones [13]. These elicitation methods can be highly significant when maximum extraction of the target compound is required, particularly for the rare or endemic species for which there may not be enough biomass [14].

Ethnopharmacological value of Salvia species from Asian countries additionally highlights the importance of a sustainable production method for salvia bioactive constituents [2]. Studies in recent years on the temporal phenolic compounds of endemic Salvia species indicates that phenolic biosynthesis may be quite variable and that the timing and type of application of biostimulants is crucial to achieving yield optimization [15]. Its combination of the biotechnological tools can open the prospect of using endemics Iraqi plants, such as *S. compressa*, as high value bio-factories for phenolic and flavonoid production. Considering the optimization of elicitation strategies we aim to find a sustainable way to produce these important bioactive molecules, which can contribute to the valorizing this endemic plant from Iraq, as well as open a perspective to a renewable source for pharmaceutical and nutraceutical industries. This study wants to evaluate how different types of biostimulants affect the production of secondary metabolites, such as phenols and flavonoids, in suspension cell culture of *S. compressa*, given the potential phytochemical value of this species and the effectiveness of biostimulants in other species of Salvia.

Methodology

Plant Material and Botanical Authentication

Salvia compressa L. seeds were gathered from the natural habitat of mount Azmar, Sulaymaniyah province, Kurdistan Region of Iraq in the flowering period (May-June 2024) at the altitudes ranging from 1200 to 1500 m above sea level (ASL), latitude 35.61° N, longitude 45.45° E. The specimens of the plant were identified by a taxonomist at the National Herbarium of Iraq (BAG) and a voucher specimen (Voucher No. SC-2024-IQ) was deposited for future reference. Collected seeds were placed in paper bags appropriately packaged and stored under desiccated conditions under refrigerated condition at 4 °C until further use.

Seed Sterilization and Germination

Seeds were cultured aseptically using a rigorous sequential protocol. Seeds were first washed with running tap water for 20 min, and then, in 70% (v/v) ethanol for 60 s, using a commercial detergent (2% (v/v) Teepol). Then seeds were placed in 2.5% (v/v) sodium hypochlorite solution (NaOCl; with 2 drops of Tween-20) for 15 min. Seeds were sterilized and five times with sterile deionized water (5 min each) in horizontal flow hood (Esco Healthcare, Singapore). The seeds were germinated in vitro in a Murashige and Skoog (MS) medium with 3% (w/v) sucrose and 0.8% (w/v) agar and without plant hormones [16]. Prior to autoclaving in an autoclave at 121°C for 20 min the pH of the medium was adjusted to 5.8 ± 0.1.

Callus Induction and Maintenance

The in vitro seedlings were four-week-old, and their explants (hypocotyls and young leaf segments) were excised and cultured on MS medium [16] containing 1.0 mg/L of growth hormone 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0 mg/L of growth stimulant 6-benzylaminopurine (BAP). The pH of the medium was adjusted to 5.8 ± 0.1, autoclaved at 121 °C oven for 20 min at 1.1 kg/cm² pressure before being transferred to the growth chamber (where it was grown in total darkness at 25 ± 2°C). In order to promote high biomass productivity and metabolic stability, the callus was subcultured every 21 days on fresh medium to maintain friable callus.

Establishment of Cell Suspension Cultures

Approximately 2.5 g (fresh weight) of pre-cultured, friable callus tissue were transferred to 250 mL flasks filled with 50 mL liquid media (MS media) [16] containing the same amount of two plant growth hormones, 2,4-D (1.0 mg/L) and BAP (1.0 mg/L). The flasks were shaken at 120 rpm on an orbital shaker (Innova 40, New Brunswick Scientific, USA) under a 16/8 h light/dark (cool white fluorescent lamps, 40 μmol m^{-2} s^{-1}) photoperiod. The growth kinetics was evaluated by harvesting cell biomass every 3 days for 30 days. The lag phase, the exponential (log) phase and the stationary phase were determined with dry weight (DW) and packed cell volume (PCV).

Biostimulant Elicitation Strategy

Elicitation occurred on the 14th day of culture cycle which falls in early-to-mid log phase. methyl jasmonate (MeJA) was prepared using, 95 % ethanol was used as the solvent and the compound was adjusted to final concentrations of 50, 100 and 200 μM [17]. Salicylic acid (SA) was prepared in sterile water and used at 0.1mM, 0.5mM and 1mM and yeast extract (YE), filter-sterilized (0.22 μm Millex-GV, Merck), was used at 0.5g/L, 1g/L and 2g/L concentrations [18]. The respective solvent (ethanol < 0.1% v/v) was added to the control flasks in an equivalent volume. The cells were harvested at 24, 48, 72, and 96 hours after elicitation to observe the time-course production of secondary metabolites with maximum production observed between 72 and 96 hours.

Extraction and Phytochemical Analysis

1. Ultrasound-Assisted Extraction (UAE)

The harvested cells were collected, washed with distilled water and lyophilized (Labconco FreeZone, USA). The dried biomass (100mg) was extracted with 5 mL of 80% (v/v) methanol using an ultrasonic bath (Branson 3800,USA) at 40 kHz and 40℃ for 30 min. The extracts were centrifuged for 15 min at 12,000 rpm at 4°C, and filtered through a 0.45 μm PTFE syringe membrane filter for HPLC analysis.

2. HPLC-DAD Quantification of Phenolics and Flavonoids

A HPLC system with Agilent 1260 Infinity II system and Diode Array Detector (DAD) was used for the chromatographic analysis. The Zorbax Eclipse Plus C18 (250 mm × 4.6 mm, 5 μm) column was used for separation. The mobile phase was composed of (A) 0.1% formic acid in water, and (B) HPLC-grade acetonitrile. The gradient elution program was as follows: 0–5 min, 10% B; 5–35 min, 10–60% B; 35–40 min, 60–90% B; 40–45 min, 90% B. Flow rate was 1.0 mL/min and 20 μL of injection volume was used. The wavelengths for detection were selected as 280 nm for phenolic acids and 330 nm for flavonoids. Retention time and UV spectrum were used for identification and quantification of 11 bioactive compounds including rosmarinic acid, caffeic acid, salvianolic acid B, luteolin-7-O-glucoside and apigenin using authentic standards [19].

Statistical Analysis

Experiments were conducted using a completely randomized design (CRD) of three biological and three technical replicates. Analysis of variance (ANOVA) was performed using IBM SPSS Statistics (version 26.0). A comparison of significant differences between means was conducted using the Multiple Range Test (DMRT) at $p<0.05$ level of significance. Graphpad Prism 9.0 (Graphpad

Software, USA) was used to generate graphical representations and results are expressed in Mean ± Standard Deviation (SD).

Results

Suscension Cell Cultures Growth

The culture cell of *Salvia compressa* suspension exhibited a distinct growth pattern had a definite growth pattern in 30 days and the pattern followed three major phases: Lag Phase, Exponential/Log Phase and Stationary Phase. Mean dry cell mass was greatest on day 21 of growth, and thus the cells had reached maximum biomass when they reached the stationary phase. The (**Table 1**) summarizes of biomass development during the period of cultivation.

Table 1. Biomass growth (dry weight) of *Salvia compressa* suspension cell cultures over 30 days

Cultivation Day	Average Dry Weight (g/L) ± Standard Deviation
3	0.25 ± 0.03
6	0.48 ± 0.05
9	0.85 ± 0.07
12	1.30 ± 0.10
15	1.85 ± 0.12
18	2.30 ± 0.15
21	2.55 ± 0.18
24	2.40 ± 0.16
27	2.20 ± 0.14
30	2.00 ± 0.13

Effect of Biostimulants on Total Phenolic and Flavonoid Accumulation

The results indicated that the accumulation of total phenolic and flavonoid compounds in the suspension cell cultures of *Salvia compressa* was significantly increased by the application of the biostimulants (methyl jasmonate, salicylic acid and yeast extract) as opposed to the control (**Table 2 & Table 3**). The effect was more pronounced at specific concentrations and at stipulated time intervals following the elicitation.

Table 2. Effect of different biostimulants on total phenolic compound accumulation (mg GAE/g DW) after 72 hours of elicitation

Biostimulant	Concentration	Total phenolic compounds (mg GAE/g DW) ± Standard Deviation
Control	-	15.2 ± 1.1
Methyl jasmonate	50 μM	22.5 ± 1.8
Methyl jasmonate	100 μM	31.8 ± 2.5
Methyl jasmonate	200 μM	28.1 ± 2.2
Salicylic acid	0.1 mM	19.7 ± 1.5
Salicylic acid	0.5 mM	26.3 ± 2.0
Salicylic acid	1.0 mM	23.9 ± 1.9

Yeast extract	0.5 g/L	20.1 ± 1.6
Yeast extract	1.0 g/L	27.5 ± 2.1
Yeast extract	2.0 g/L	24.8 ± 1.9

Table 3. Effect of different biostimulants on total flavonoid accumulation (mg QE/g DW) after 72 hours of elicitation

Biostimulant	Concentration	Total Flavonoid Compounds (mg QE/g DW) ± Standard Deviation
Control	-	8.5 ± 0.7
Methyl jasmonate	50 μM	12.1 ± 1.0
Methyl jasmonate	100 μM	16.5 ± 1.3
Methyl jasmonate	200 μM	14.8 ± 1.2
Salicylic acid	0.1 mM	10.2 ± 0.8
Salicylic acid	0.5 mM	13.9 ± 1.1
Salicylic acid	1.0 mM	12.5 ± 1.0
Yeast extract	0.5 g/L	10.5 ± 0.9
Yeast extract	1.0 g/L	14.2 ± 1.1
Yeast extract	2.0 g/L	13.0 ± 1.0

HPLC Analysis of Individual Phenolic and Flavonoid Compounds

HPLC-DAD revealed that the concentration of certain individual phenolic and flavonoid compounds (rosmarinic acid, caffeic acid, salvianolic acid B, luteolin-7-O-glucoside and apigenin) increased in the biostimulant-treated cells (**Table 4**). Specifically, methyl jasmonate 100 μM and yeast extract 1.0 g/L were found to be the most effective in promoting the accumulation of rosmarinic acid.

Table 4. Effect of optimal biostimulant concentrations on the accumulation of selected individual phenolic and flavonoid compounds (mg/g DW) after 72 hours of elicitation

Compound	Control	Methyl jasmonate (100 μM)	Salicylic acid (0.5 mM)	Yeast extract (1.0 g/L)
Rosmarinic Acid	3.1 ± 0.2	7.8 ± 0.6	5.5 ± 0.4	6.9 ± 0.5
Caffeic Acid	1.5 ± 0.1	2.8 ± 0.2	2.2 ± 0.2	2.5 ± 0.2
Salvianolic Acid B	2.0 ± 0.2	4.5 ± 0.4	3.5 ± 0.3	4.0 ± 0.3
Luteolin-7-O-glucoside	0.8 ± 0.1	1.8 ± 0.1	1.3 ± 0.1	1.6 ± 0.1
Apigenin	0.5 ± 0.0	1.2 ± 0.1	0.9 ± 0.1	1.1 ± 0.1

Optimal Elicitation Timing

The findings indicated that harvesting of cells was optimal between 72 and 96 hours where the phenolic and flavonoid compounds accumulated at the optimum time (**Fig. 1 & Fig. 2**). The levels of these compounds reduced slightly after 96 hours, indicating that these compounds were consumed or degraded in the cells.

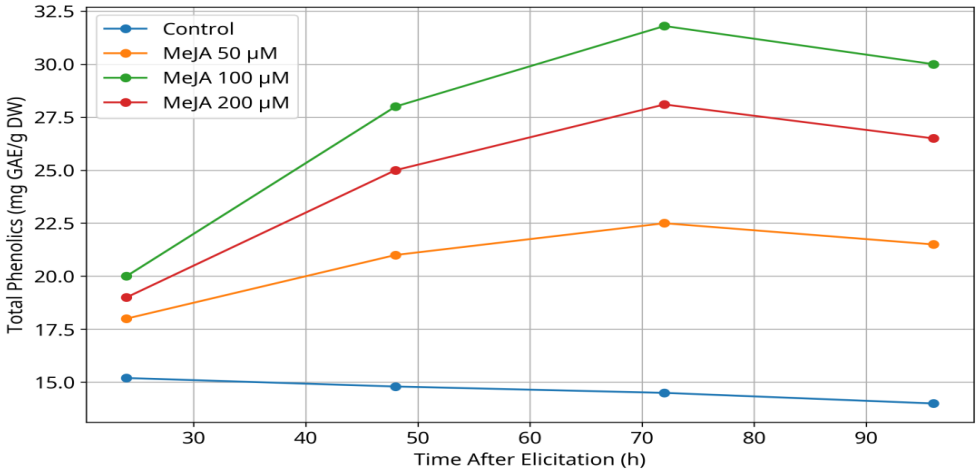

Fig. 1. Effect of different methyl jasmonate concentrations on total phenolic compound accumulation over time

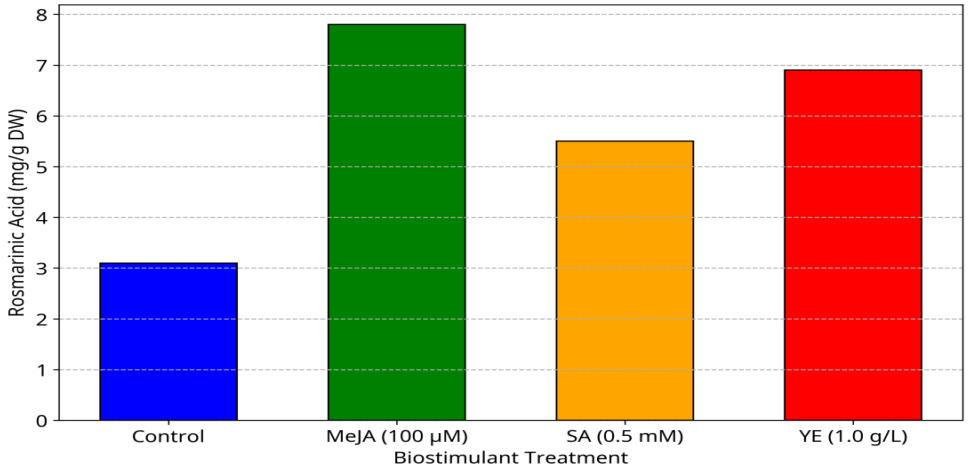

Fig. 2. Comparison of optimal biostimulant effects on rosmarinic acid accumulation

HPLC Chromatograms

(**Fig. 3 & Fig. 4**) shows the HPLC chromatograms of the control and methyl jasmonate-treated group and these results show that there is a significant increase in the phenolic and flavonoid compound peaks following elicitation.

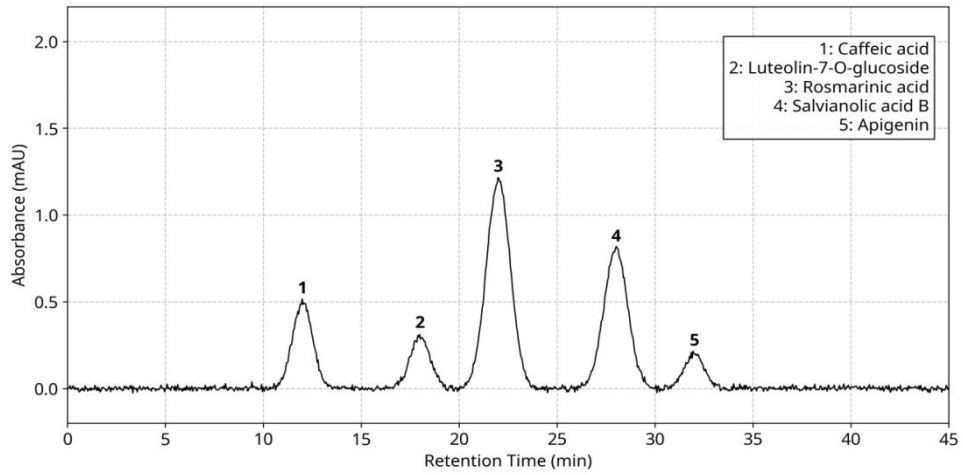

Fig. 3. HPLC chromatogram of *Salvia compressa* (control group)

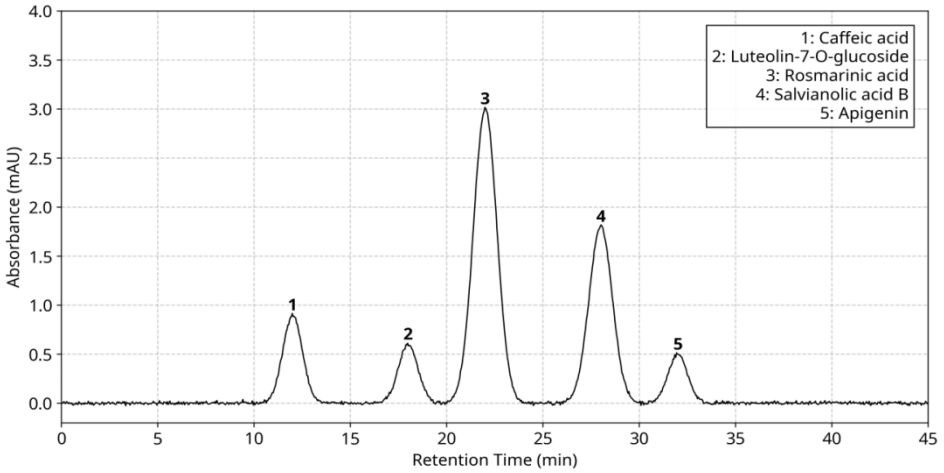

Fig. 4. HPLC chromatogram of *Salvia compressa* (MeJA 100 μM treated group)

Discussion

Cell suspension cultures are a powerful biotechnological resource to produce high-value secondary metabolites, and it essentially overcomes obstacles of environment and season limitations associated with conventional forms of agriculture. The endemic species Salvia compressa endemic to Iraq was used in this study to examine its biosynthetic capacity of phenolic and flavonoid compounds under the effect of different biostimulants. The findings opens new avenues for applying methyl jasmonate (MeJA), salicylic acid (SA) and yeast extract (YE) resulted in a considerable increase in the concentrations of the bioactive compounds, which provided new opportunities to apply the Iraqi botanical resources.

The significant increase in the levels of rosmarinic acid (RA) when exposed to 100 μM MeJA is consistent with the recent research trends highlighting the importance of jasmonates as important signal transduces in plant stress response. In a recent experiment on cell suspension cultures of Lavandula angustifolia, MeJA treatment was found to induce the expression of gene(s) encoding important enzymes of the RA biosynthetic pathway leading to a significant rise in productivity [20]. This genetic response is the reason behind our observations because MeJA triggers our phenylpropanoid pathway that produces phenolic acids in the family Lamiaceae. MeJA has been shown to be effective in promoting phenolic accretion in not only common species, but also wild and endemic species, which supports the hypothesis that the evolutionary conservation of biochemical defense pathways is similar in the Salvia genus.

Moreover, salicylic acid (SA) was found to be highly effective in promoting total phenolic and flavonoid content, especially when the concentration of the reagent was 0.5 mM. Comparative analysis indicates that SA acts by activating carbohydrate metabolism channel and channeling carbon flow into secondary metabolite production. An example can be provided on the metabolic characterization of cell cultures of Salvia miltiorrhiza whereby, it was found out that SA resulted in the rapid utilization of primary sugars in order to increase the production of salvianolic acids [13]. This is the reason why we found a rise in the number of secondary compounds and stability of biomass, as energy is no longer used to promote primary growth but rather goes to chemical defense. The efficacy of SA is evident in the fact that the production of rosmarinic acid was increased many

times, to folds more than control groups, thus, making it a cost-effective and efficient elicitor in commercial-scale production [21].

Regarding yeast extract (YE), the results showed a significant increase in the accumulation of flavonoid compounds such as luteolin and apigenin. YE is a biotic elicitor which mimics microbial attack, and evokes mounting defense responses. Studies done on other Salvia species substantiate YE does not only enhance the amount of metabolites but also changes the metabolic profiling to yield more complex compounds [22]. Our study using YE at 1.0 g/L also obtained encouraging results, which are agreement with studies that show that complex biostimulants can deliver a broad range of chemical signals, which can activate several pathways at the same time [11]. The combination of chemical elicitors (MeJA, SA) and biotic elicitors (YE) is a holistic approach to enhance the quality and quantity of plant extracts.

The value of the research is further increased by the biological importance of the compounds that are generated in *Salvia compressa*. Rosmarinic acid and salvianolic acid B are very strong antioxidants, at times even stronger than synthetic antioxidants. Scientific reviews with high impact have shown that these compounds are important in the prevention of chronic diseases because they can scavenge free radicals and prevent the effects of inflammatory processes [23]. Our research doubled the concentrations of these compounds with nano and biotic elicitors; therefore, it is a significant progress toward the creation of natural nutritional supplements [24]. The biochemical diversity evident by the cell cultures in this paper is the genetic wealth of endemic plants of Iraq, which is untapped treasure in the pharmaceutical industries [3].

Comparing the biomass growth with the accumulation of metabolites, we see that there is a fine balance which should be observed. Though the elicitors enhance the production of the compound, in some cases they may cause a growth inhibition in the presence of a very high concentration of the elicitor. Experiments with arbuscular mycorrhizae and their influence on Salvia miltiorrhiza were carried out, which demonstrated that balanced stimulation results in enhanced biomass and metabolites jointly [25]. In our experiment, 100 μM MeJA was the best and the productivity was the highest without damaging cell viability. The specific elements or accurate environmental conditions are also relevant in this balance as shown through the research on the impact of rare earth elements on tissues cultures [26]. One of the main aspects of our study is the study of the ideal harvesting time (72-96 hours). Elicitation response is a temporary phenomenon which peaks and then starts to degrade or be reabsorbed through metabolic degradation. This observation is agreement with findings of studies carried out on the accumulation of polyphenols in cultures of Salvia atropatana, which showed similar patterns of accumulation with time [27]. Knowledge of metabolic kinetics enables manufacturers to know when to harvest to get the best level of active substances. Moreover, molecular and physiological evidence of rosmarinic acid biosynthesis indicates that the enzymes involved in the production of this molecule are most active at this period of time [28].

Also, the cell suspension cultures are used to create a homogenized environment, which enables the factors of production to be tightly controlled. Experiments performed on the representatives of the family Lamiaceae like Satureja khuzistanica have demonstrated that productivity can increase manifold with the constant optimization of the conditions in bioreactors [29]. These findings in *Salvia compressa* form the basis of creating a bioengineering system to produce rosmarinic acid, as it is in Salvia tebesana using nano-elicitation [17]. Plant cell cultures are the most

natural and sustainable to produce complex compounds that are challenging to make in the laboratory compared to biosynthetic methods in microorganisms like genetically modified yeast [30].

Conclusion

The ability to elicit cell cultures of *Salvia compressa* by MeJA, SA and YE is a successful and effective strategy. This was compared with high-impact global studies, indicating that our findings are within the higher levels of secondary metabolite production in the Salvia genus. The paper adds to the enrichment of the Iraqi medicinal plants and, above all, serves as an example that can be emulated to generate phenolic and flavonoid compounds of the highest economic and pharmaceutical value, and more optimization of elicitation methods is necessary to achieve the commercial production levels.

Conflict of Interest

The author declares that there is no conflict of interest in this article.

Funding

There is no funding or support.

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