COMPARATIVE ANALYSIS OF SOME PHENOLIC ACIDS OF 
IN VITRO AND IN VIVO GROWN PLANT LEAVES OF SALVIA HISPANICA 

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ABSTRACT

Several plant species could be produce bioactive compounds, which play a key role in protecting human health, Chia is one of these plant species which has been gaining growing popularity among the traditional medicine groups. In order to sustainably produce plant biomass and its phytochemical content. Numerous biotechnological approaches need to be employed, and elicitation has proven to be a very effective method for increased secondary metabolite production in various in vitro culture. The current research involves the application of various concentrations of SA as an elicitor with 2,4-D and BAP in callus cultures, and the main aim was to stimulate the accumulation of biomass and phytochemical contents. The results showed that the highest concentration of keamferol and gallic acid compounds in callus of S. hispanica were occurred in the treatment with 2 mg l⁻¹ 2,4-D and 2 mg l⁻¹ SA in the presence of 0.5 mg l⁻¹ BA.

Keyword: Chia, SA, secondary metabolite production, human health

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INTRODUCTION
Salvia hispanica L., better known as chia, which belongs to the genus Salvia (2), is a plant food alternative, is an annual herbaceous plant, native from northern Guatemala southern and Mexico, belonging to the Lamiaceae family. More recently, chia was grown in Argentina, Ecuador Colombia, Australia, Peru, Bolivia and Paraguay for commercial purposes (5). Various parts of this plant are now available commercially for human consumption worldwide, as food supplements. Chia seed is known for its high concentration of dietary fiber, proteins, omega 3 (n-3) Alpha Linolenic Acid (ALA) and phytochemicals, including phenolic compounds (3,8,17). In the absence of effective liver-protective drugs in the modern system of all opathic medical practices, herbal medicines play an important role in health care programs worldwide and interest in herbal treatment of various hepatic conditions is resurgent (25). Phenolic compounds are one of the large and widely distributed classes of secondary metabolites in plants (23), which play a protective role for plants against insects and other organisms. Studies have found that the dietary consumption of bioactive components as phenolic compounds from chia is correlated with a decreased risk of cardiovascular disease and hepatoprotective function (21), and a protective impact against plasma oxidative and obesity-related disease (9,16). It has been documented that modulation of plant growth regulator concentration in culture medium can alter antioxidant properties in plant extracts (20). In addition, information on Salvia hispanica is still quite scarce and, to date, there is a lack of studies on this plant species, in particular the tissue culture research. In the light of that, this was study aimed to extract and purify phenolic content and to characterize the comparative antioxidants activities of both leaf extracts of in vivo and in vitro callus of S. hispanica to assess their nutraceutical.

MATERIALS AND METHODS
This study was carried out from February 2019 to January 2020 in the plant tissue culture laboratory, Genetic Engineering Institute, Baghdad University. Chia seeds were collected from homegrown at Baghdad gardens. Leaf explants were obtained from 30-day old chia seedlings grown under green house.

Explant sterilization and callus induction
The explants were thoroughly washed under running tap water, treated for 5 minutes with 1% NaOCl and 3 drops of Tween 20, followed by distilled sterile rinsing, and then by rinsing with distilled sterile water for 3 times under the cabinet of laminar airflow. The explants of the surface sterilized leaf were taken and trimmed into 1.5 cm pieces.

Induction and measuring of fresh and dry weight of callus: In order to induce the calli, 2 explants were inoculated into each test tube containing 10 ml MS media (19) fortified with 3% sucrose and various concentrations of 2,4-D (1, 2, 3) mg/l and or SA (1, 2) mg/l which they added with the presence of BA (0.5 mg/l) for all treatments. The medium was solidified with 0.7% agar, then pH was adjusted to 5.7 ± 0.1 prior to autoclaving. The culture were maintained at a temperature of 24±1ºc under dark. After six weeks, we harvested the calli samples and calculated the fresh weight, later oven dried twenty four hours at 50ºC, then stored for farther analysis

Sample extraction: Enhanced callus with 100 mg from optimized treatment [2,4-D(2mg/l) plus SA (2mg/l)] that gave good fresh and dry weight of callus, and 3.0 g from dried leaves of greenhouse plants were powdered and extracted with methanol in shaker at 30 ºC for 48 hours, in order to produce a crude sample, methanol was evaporated and traces dissolved in water. Those extracts were then used to assess the efficacy of antioxidants (20, 24).

HPLC quantification: Chromatographic quantification of phenolic acid was performed with the HPLC method according to Gupta et al. (12). The separation was performed on liquid chromatography with binary delivered pump. Under the optimum conditions and on C18-ODS column, the main compounds were separated. Standard compounds of Apiginine, Catechine, Kaemferol, Quercetine and Gallic acid were used, and by applying the following equation, we can quantify the concentrations of active compounds (4): Concentration of compound= {area of sample/(area of standard) X (concentration of standard) X (dilution factor)}. 
Statistical analysis
Data was analyzed statistically with SAS (Statistical Analysis System). Results are represented as means of ten replicates using a Completely Randomized Design (CRD), means have been compared using the Least Significant Difference test (LSD) at 5% level.

RESULTS AND DISCUSSION

Callus induction
Tables 1, 2 and 3 show inoculated explants on the control medium, with no supplement of any GPRs (the control), did not show any callus initiation. As reported and in order to initiate the calli formation, most explants had exogenous requirements of one or more growth regulators (6). Callus enhancement from S. hispanica leaf explants with various 2,4-D concentrations alone or in combinations with SA with presence of BA (0.5 mg/l) showed different results, callus induction was noted on 2,4-D alone or combined with SA after 30 days of cultivation. Even though, MS medium supported with 2,4-D (1, 2, 3) mg/l or SA (1, 2) mg/l were effective in callus induction, MS media supplemented with 2,4-D (2 mg/l) interacted with SA (2 mg/l) produced friable and creamy callus and showed significant increase (70% callus induction, FW=7.316 mg, DW=0.334 mg). Results of the other researches (7,18,20) suggested that the best stimulated callus was in leaves, also, the combination of auxins and cytokinins led to best stimulation of calli than an individual PGRs. The variance in the content of the PGRs found in plant internal sections may affect in the reaction of plant parts grown by the addition of plant growth regulators, which affects the optimum concentration of cytokinin and auxin mediated callus, or perhaps the both, when applied to the media (11, 13, 22).

Table 1. Effect of 2,4-D and SA and their interaction on the % callus induction, after inoculating explants on MS medium for six weeks

| 2,4-D (mg/l) | SA (mg/l) |
|------------|-----------|
| 0.0000     | 0.0000    |
| 0.3000     | 0.3000    |
| 0.0000     | 0.0000    |
| 0.3000     | 0.3000    |
| Mean       | 0.2250    |

LSD: 2,4-D=0.2382 SA=0.2063 2,4-D*SA=0.4126

Table 2. Effect of 2,4-D and SA and their interaction on mean callus fresh weight (mg), after inoculating explants on solid MS medium for 6 weeks

| 2,4-D (mg/l) | SA (mg/l) |
|------------|-----------|
| 0.0000     | 0.0000    |
| 0.0614     | 0.0924    |
| 0.2825     | 2.1775    |
| Mean       | 0.2395    |

LSD: 2,4-D=0.3864 SA=0.3346 2,4-D*SA=0.6693

Table 3. Effect of 2,4-D and SA and their interaction on mean callus dry weight (mg), after inoculating explants on solid MS medium for 6 weeks

| 2,4-D (mg/l) | SA (mg/l) |
|------------|-----------|
| 0.0000     | 0.0000    |
| 0.00451    | 0.01686   |
| 0.00248    | 0.1211    |
| Mean       | 0.01572   |

LSD: 2,4-D=0.0827 SA=0.0716 2,4-D*SA=0.1432
**Total phenolic analysis**

Sustainable production of phytochemicals among the most studied groups of compounds regardless to their immense ability to protect against pathogenic attacks, to signal molecules and to control essential biochemical processes like antioxidants (15 and 26). The callus antioxidant activity and extraction of leaves belong to *in vivo* grown plants were calculated by using HPLC and applying the equation mentioned above. Results in Tables 4, 5, 6; Figure 1 and Figure 2 reveal to the highest concentration of keamferol and gallic acid compounds in callus of *S. hispanica* which occurred in the treatment with 2 mg l\(^{-1}\) 2,4-D and 2 mg l\(^{-1}\) SA in the presence of 0.5 mg l\(^{-1}\) BA.

**Table 4. In vitro and in vivo comparison antioxidant activity**

|                | Gallic acid | Catechin | Apiginine | Keamferol | Querceti | The compounds part of Plant |
|----------------|------------|----------|-----------|-----------|----------|-----------------------------|
| In vitro       | 70.539     | 514.860  | 92.589    | 346.26    | 1173.941 |                             |
| In vivo        | 40.345     | 1212.95  | 313.747   | 40.345    | 2947.54  |                             |
| LSD 0.05       | 17.28 *    | 146.02 * | 67.39 *   | 55.62 *   | 128.07 * |                             |

**Table 5. Phenolic acids and flavonoids of leaves from in vivo grown plants**

| Compound         | Retention time (minute) | Area     |
|------------------|-------------------------|----------|
| Apiginine        | 8.083                   | 810.642  |
| Catechine        | 9.567                   | 1571.922 |
| Keamferol        | 11.343                  | 1956.910 |
| Quercetine       | 12.333                  | 2474.006 |
| Gallic acid      | 13.557                  | 288.926  |

**Figure 1. HPLC analysis of phenolic acids and flavonoids of leaves from in vivo grown plants**
It is very important to highlight that SA play a key role in phenolic and flavonoid compounds production and stimulates the plant endogenous enzymes (1 and 14), as for example, SOD (Super Oxide Dismutase) which is a defensive system in plant for Reactive Oxygen Species (ROS)(10). Also, it should be noticed that a low concentrations of catechin, apiginine and querceti compounds was observed in in vivo tissues in the presence of PGRs and SA compared to the in vivo leaves which lacked to PGRs and SA. The explanation for the great response in case of in vivo compared with in vitro might be that SA is the regulator of plant growth and it’s exogenous application stimulates the main growth (26) and had (in the presence of PGRs) a negative response on the production of those three phenolic compounds.

CONCLUSION
The results of this study showed, it can be concluded that the experimental conditions used in current research allowed the putative detection of polyphenolic compounds of in vitro and in vivo grown plant leaves extract of Salvia hispanica based on their mass fragmentation trend, retention time and UV spectrum. Five phenolic acid compounds derivatives were identified in the crude extract, namely catechin, apiginine, querceti, keamferol and gallic acid their concentrations vary between in vitro and in vivo. Future
studies may be conducted to develop and research secondary metabolites in the in vivo and in vitro culture of S. hispanica, with the goal of further understanding and discovering the medicinal functions and property of this plant species.

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