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IN VITRO PROPAGATION OF ALGERIAN *LAVANDULA STOECHAS* AND ASSESSMENT OF BIOCHEMICAL COMPOSITION AND THEIR ANTIOXIDANT ACTIVITY

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The present research aimed to investigate the effect of plant growth regulators (PGRs) on the in vitro proliferation of Lavandula stoechas shoots and assessment of the antioxidant potential and total phenolic content of extracts obtained from in vitro cultures and wild plants of L. stoechas, as well as, the chemical analysis of the phenolic content of both extracts carried out using HPLC-DAD. The optimal shoot proliferation and biomass accumulation were achieved with the combination of 0.5 mg/l 6-benzyladenine (BA) and 0.5 mg/l gibberellic acid (GA3). The hydroalcoholic extract obtained from in vitro cultures contained the highest amount of phenolic compounds (83.18 mg GAE/g DW extract) compared to the total phenolic content of wild plants extract (32.33 mg GAE/g DW extract). Furthermore, the hydroalcoholic extract from in vitro cultures exhibited a higher antiradical effect against 2,2-diphenyl-1-picrylhydrazyl (DPPH) with IC50 value of 51.38 ± 0.7µg/ml and higher antioxidant capacity (757.26 mg GAE/g DW) observed by total antioxidant capacity (TAC) assay. The chemical profile of both extracts revealed the presence of naringenin, acacetin, rhamnetin, luteolin 3'- 7 diglucoside, and hesperetin. Overall, the results suggest that in vitro cultures of L. stoechas could serve as a potential source for producing active metabolites with high antioxidant properties.

Keywords: *Micropropagation, Phenolic compounds, free radical scavenging, Lavender, HPLC-DAD*

INTRODUCTION

The *Lavandula* genus, which belongs to the Lamiaceae family, comprises 39 species and around 400 cultivars[1]. *Lavandula* plants have been used since ancient times to flavor and preserve food and to treat diseases, including wound healing, sedative, antispasmodic, microbial, and viral infections[2]. *Lavandula stoechas* L. is an ornamental flowering plant from the *Lavandula* genus, commonly known as Lavender, originating from the Mediterranean Basin[3]. Ethnobotanical and phytopharmacological research has shown that *L. stoechas* is used in Algeria to treat rheumatic diseases, as an antispasmodic agent, and as a stimulant, sedative, diuretic, analgesic, and antiseptic[4–9]. Recently, *L. stoechas* has received considerable interest in the fields of medicine and pharmacology. Several studies have reported that *L. stoechas* is a valuable source of phenolic compounds, which can

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confer antioxidant, antibacterial, antidepressant, anti-inflammatory, and insecticidal effects[10–16].

Plant tissue cultures are promising for the large-scale production of valuable chemical compounds[17]. *In vitro* propagation offers many advantages compared to traditional approaches, including the absence of seasonal restrictions, predictable production, and efficient and prompt isolation of the compound of interest[18]. *In vitro* propagation techniques can potentially generate metabolites with pharmacological importance[19]. However, knowledge is scarce regarding the micropropagation of *L. stoechas*[20–22]. Therefore, the current study aimed to develop an efficient and speedy propagation technique for *L. stoechas* in order to produce a consistent raw material for large-scale extraction of medicinal compounds, as well as to perform a comparative analysis of the antioxidant potential and phenolic component composition of *in vitro* plant *L. stoechas* and wild plants.

MATERIAL AND METHODS

Chemicals

All macro- and micronutrients for tissue culture media and agar acquired from Duchefa Biochemie (Haarlem, The Netherlands), PGRs (Sigma Aldrich, Germany), Ethanol, and Methanol (Sigma Aldrich, Germany), Folin-ciocalteu phenol reagent (Sigma-Aldrich, Switzerland), Sodium Carbonate anhydrous (Honeywell, Fluka, Germany), Gallic acid (Titan biotech LTD., India), Aluminum chloride Hexahydrate (Biochem, Chemopharma, USA), Quercitin (Extrasynthese Genay, France), Vitamin C and butylated hydroxytoluene (BHT) (Honeywell, Fluka, Germany). All standards used for HPLC analysis (Honeywell, Fluka, Germany).

Equipements

Sensitive balance (Kern and Sohn Gmbh, Germany), Rotary evaporator (Büchi, R-200, France), UV-1800 spectrophotometer (Shimadzu, Japan), Centrifuge Germany (EBA 8, Hettich, Germany), HPLC UV-DAD (Agilent 1260, Canada).

Plant Material and Culture Conditions

L. stoechas plants were harvested during the flowering stage in the region of Beni Ourtilane in Algeria, at the geographical coordinates 36° 26′ 00″ N, 4° 54′ 00″ E. Shoots tips were excised from the donor mother plant, then were cleaned thoroughly by washing them with tap water mixed with a small amount of dish soap and then rinsed with water multiple times. Nodal segments with a 1.1-1.5 cm length were selected and subjected to surface sterilization under sterile conditions using 70% ethanol and sodium hypochlorite (13°). The explants were then placed in Murashige and Skoog[23] (MS) culture medium, which contained sucrose (20 g/l) and agar (6 g/l). The explants were kept at 20-25°C with 16 hrs of light exposure from cool white fluorescent lights that emitted a light intensity of (4000 Lux).

Effect of different concentrations of plant growth regulators on shoots proliferation

Shoots grown on MS medium were continuously transferred to new MS media until the sufficient stock was created. From these shoots, nodal segments were removed and cultivated on MS media with 6-benzyladenine (BA) concentrations at 0.25 and 0.5 mg/l with or without gibberellic acid (GA3) at 0.5 mg/l. After four weeks of cultivation, the number of buds, nodes per shoot, the height of the shoots, and the fresh weight of plant tissue (g) were all measured.

Extraction Procedure

The stems and leaves of *in vitro* shoots and wild plants were dried at 45 °C for 48 hrs. Then, 500 mg of the dried and powdered plant material was homogenized with 10 ml of ethanol:water (7:3 ratio) and macerated for 48 hrs with agitation (80 rpm) at room temperature. The resultant extracts were filtered using filter paper, and the filtrates were concentrated using a vacuum rotary evaporator at 45°C under reduced pressure. The resulting dried extracts were kept in the dark at + 4°C until use. This process was repeated three times for each sample, and the yields were calculated.

Determination of Total Phenolic Content

According to Singleton and Rossi (1965)[24], the Folin-Ciocalteu method was used to evaluate the total phenolic content (TPC) in

hydroethanolic extracts of *L. stoechas in vitro* shoots and wild plants. For this, 0.05 ml of the extracted solution with a concentration of 10 mg/ml was mixed with 3.95 ml of distilled water, followed by adding 0.25 ml of Folin-Ciocalteu reagent. After a 3-min reaction time, 0.75 ml of 20% sodium carbonate was added, and the mixtures were stirred and heated at 40°C for 40 min. The absorbance of the material was subsequently spectrophotometrically measured at 760 nm, and the blue coloring was observed. In order to calculate the amounts of phenolic compounds, the absorbance values were used in the following equation, which was derived from a conventional gallic acid curve **(Fig. 1)**: Absorbance = 0.1035 gallic acid (µg/ml) + 0.1046 (R2: 0.98). This process was performed three times for each sample.

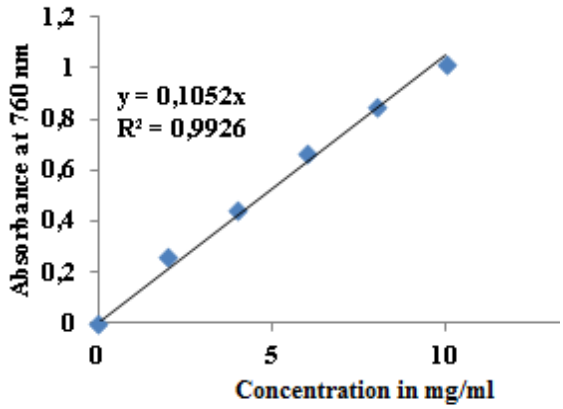

Fig. 1: The standard curve obtained using gallic acid for total phenolic content determination.

Antioxidant Activity

DPPH Radical Scavenging Activity Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) reduction assay was used to measure the antioxidant activity of each extract, following the method described by Braca et al. (2002)[25]. A freshly prepared DPPH solution in methanol (0.004% w/v) was mixed with different methanol dilutions of plant extract and fractions (5 µg/ml to 1000 µg/ml). The mixture was reacted for 30 minutes at room temperature in the dark before the absorbance of the sample was read at 517 nm. BHT was used as standard, and the absorbance of a blank sample containing methanol in the DPPH solution was measured. The percentage of radical scavenging activity was estimated as follows: % inhibition = [(AB - AS)/AB] %, where AB is the absorbance of the blank sample, and AS is the absorbance of the plant sample. A graph of the scavenging effect percentage versus extract concentration was used to calculate the concentration of the extract needed to scavenge 50% of the DPPH radicals (IC_{50}).

Total Antioxidant Capacity (TAC) Test

The total antioxidant capacity (TAC) test was adopted from a published protocol described by Prieto et al. (1999)[26]. The test combined 1.5 ml of reagent solution composed of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate with 25 µL of each extract. The mixture was incubated for 90 min at 95°C, and the optical density was measured at 695 nm using a spectrophotometer with a blank sample. A gallic acid calibration curve was used to measure the total antioxidant capacity in milligrams of gallic acid equivalent per gram of extract (mg GAE/g DW extract). The experiment was repeated three times.

HPLC-DAD analysis

Samples were characterized using high-performance liquid chromatography (HPLC) with an Agilent 1260 Infinity UV-VIS detector DAD. The analysis was conducted in reverse phase using a C18 column (5 µm, 250 × 4.6 mm) at a temperature of 22 ± 0.8°C. The injection volume was 5 µl, and the flow rate was 1 ml/min using HPLC grade solvents. The solvent system consisted of A (1% acetic acid dissolved in bi-distilled water) and B (methanol) with a gradient of 0 min: 95% A + 5% B; 55 min: 5% A + 95% B; 60 min: 95% A + 5% B. The detection was performed at 254 nm, 280 nm, and 320 nm. Compounds were identified by comparing retention times and UV spectra obtained with standards.

Statistical analysis

The mean and standard deviations of the results were estimated from three replicates. The data were analyzed using the one-way ANOVA method in the SPSS program (version 23.0) to determine significant differences between the various concentrations of PGRs on the growth of *L. stoechas in vitro* shoot cultures.

RESULTS AND DISCUSSION

Results

Shoot *in vitro* induction and propagation.

L. stoechas in vitro culture demonstrated bud proliferation **(Fig. 2)**, starting on the seventh (7th) day of culture. The highest number of axillary buds and nodes were observed in *L. stoechas* cultures grown on a medium (T3) supplemented with 5 mg/l of BA combined with 0.5 mg/l of GA3 **(Fig. 3)**, resulting in a significant increase of 104.13% and 127.02%, respectively, when compared to the control. Additionally, the same hormonal treatment led to a significant improvement of approximately 120.68% and 134.55% in shoot length and biomass, respectively, compared to the control. However, *L. stoechas* shoot cultured in a medium supplemented with 0.5 mg/l of BA (T1) showed a low growth performance compared to the medium containing the same concentration of BA combined with 0.5 mg/l of GA3 (T3), resulting in a development that was less than 50% and 57.81% in terms of biomass and shoot length, respectively, when compared to the control. In conclusion, the study shows that GA3 supplementation is required for optimum growth and proliferation.

Total Phenolic Content

The total phenolic content (TPC) was assessed using Folin-Ciocalteu's reagent and based on the gallic acid calibration curve in **Fig. 1**. The extraction yields, expressed as the weight of the extract versus to the weight of the initial plant material, are about 20% and 104% for *in vitro* and wild plants of *L. stoechas*, respectively. Notably, the TPC in extracts of *in vitro* shoot cultures was significantly higher than wild plants with 83.18 ± 3.07 mg GAE/g DW extract, which represented an increase of approximately 158% when compared to the TPC of wild plants extract (32.33 ± 0.90 mg GAE/g DW extract) **(Table 1).**

Antioxidant activity

The antioxidant activity of both extracts was evaluated using DPPH and TAC assays. The DPPH radical scavenging activity test is widely used to determine the ability of plant extracts and compounds to perform as free radical scavengers or hydrogen donors[27]. In this study, the extracts were demonstrated to be effective DPPH radical scavengers. The data from **Table 1** showed that the *in vitro* shoot extract exhibited higher potency to scavenge DPPH radicals with a lower IC_{50} value (51.38 ± 0.7 µg/ml) than wild plants extract and BHT (reference antioxidant). The results obtained by TAC assay also revealed that the highest antioxidant capacity was displayed by the *in vitro* shoots extract (757.26 ± 67.7 mg GAE/g DW extract), which was 2.25 times greater than that of the wild plant's extract (335.63 ± 3.60 mg GAE/g DW extract) **(Table 1)**.

Fig. 2: *In vitro* propagation of *L. stoechas* on MS Medium (A) Culture showing multiple shoots at 45 days of culture, (B) Morphology of induced shoots.

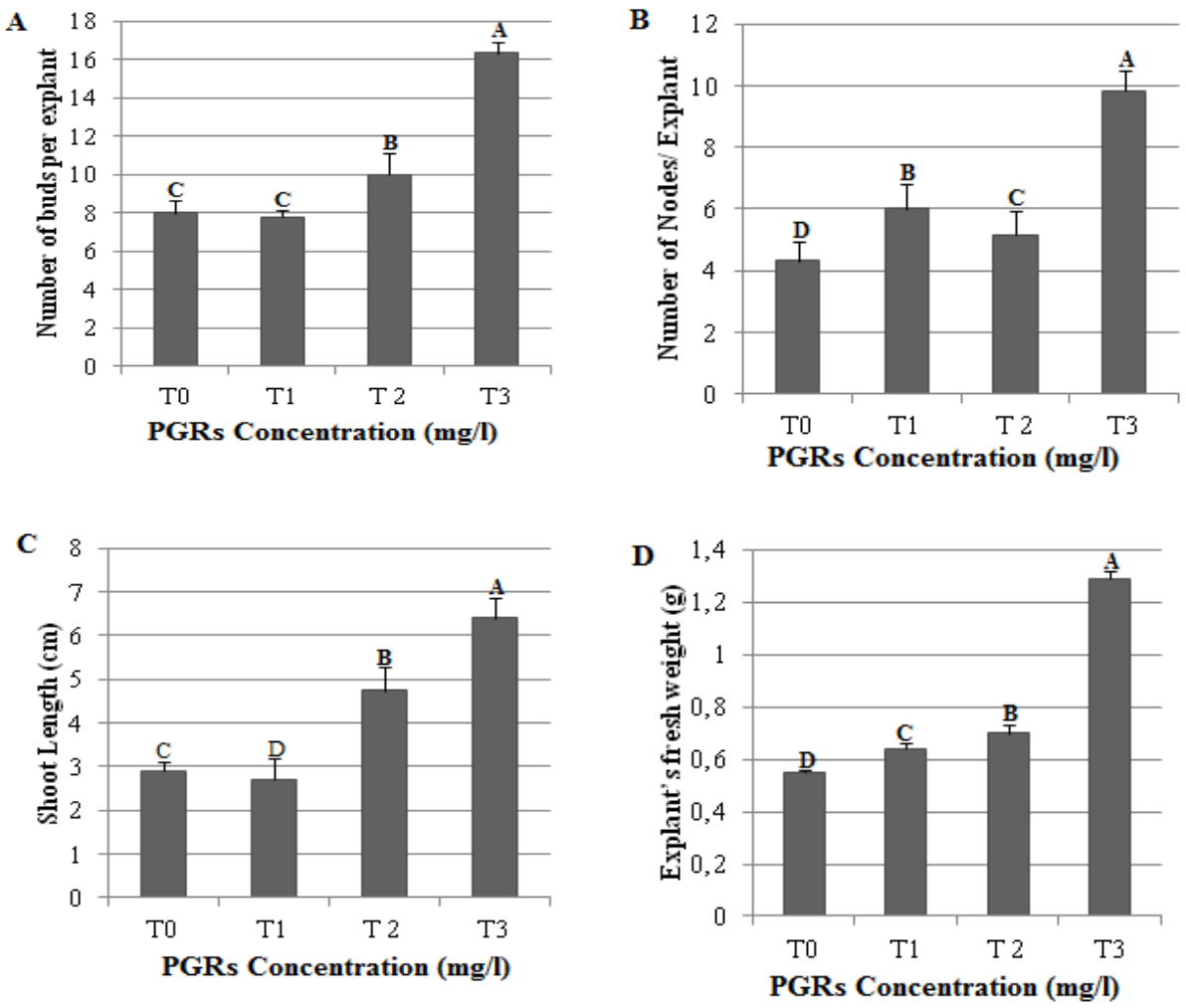

Fig. 3: Effect of different concentrations of PGRs on the growth *L. stoechas* shoots.

(A) Number of buds in shoots grown on MS medium supplemented with different levels of BA and GA3. (B) A number of nodes in shoots grown on MS medium supplemented with different levels of BA and GA3. (C) Length of shoots grown on MS medium supplemented with different levels of BA and GA3. (D) The fresh weight of shoots grown on MS medium was supplemented with different levels of BA and GA3.

T0 = Control, T1 = 0.5 mg / L BA, T2 = 0.25 mg / L BA + 0.5 mg / L GA3, T3 = 0.5 mg / L BA + 0.5 mg / L GA3. Error bars represent standard error (n = 3). Means with different lowercase letters are considered significantly different according to Tukey's range test at $p < 0.05$.

Table1: Antioxidant activity and total phenolic content of hydroalcoholic extracts of–*in vitro* shoot cultures and wild plants of *L. stoechas*

Samples	TPC (mg GAE/g DW extract)	IC_{50} / DPPH assay (ug/ml)	TAC (mg GAE/g DW extract)
Wild plant	32.33 ± 0.90	106.66 ± 0.62	335.63 ± 3.60
***In vitro* culture**	83.18 ± 3.07	51.38 ± 0.7	757.26 ± 67.7
BHT		72.16 ± 0.1	

Each value was expressed as the mean ± standard deviation (n = 3); TPC was expressed as mg gallic acid equivalents/g dry weight of extract; TAC = total antioxidant capacity; IC_{50} in µg/ml represents a concentration of extract corresponding to inhibit 50% of DPPH free radical.

Chemical analysis of *L. stoechas* extracts by HPLC-DAD.

The *in vitro* shoot cultures and wild plant extracts from *L. stoechas* were analyzed using by HPLC-DAD technique. The profiles obtained were generally similar **(Fig. 4).** The phenolic compounds were identified by comparing the retention time values and UV-VIS spectra of the standards and peaks in the chromatograms **(Table 2).** The presence of naringenin, acacetin, and rhamnetin was confirmed in both extracts. The luteolin 3'- 7 diglucoside was only detected in the *in vitro* plant extract. Otherwise, hesperetin was exclusively present in the wild plant extracts. Additionally, compared to wild plants, *the in vitro* plants had a greater surface area (%) derived from most chemicals.

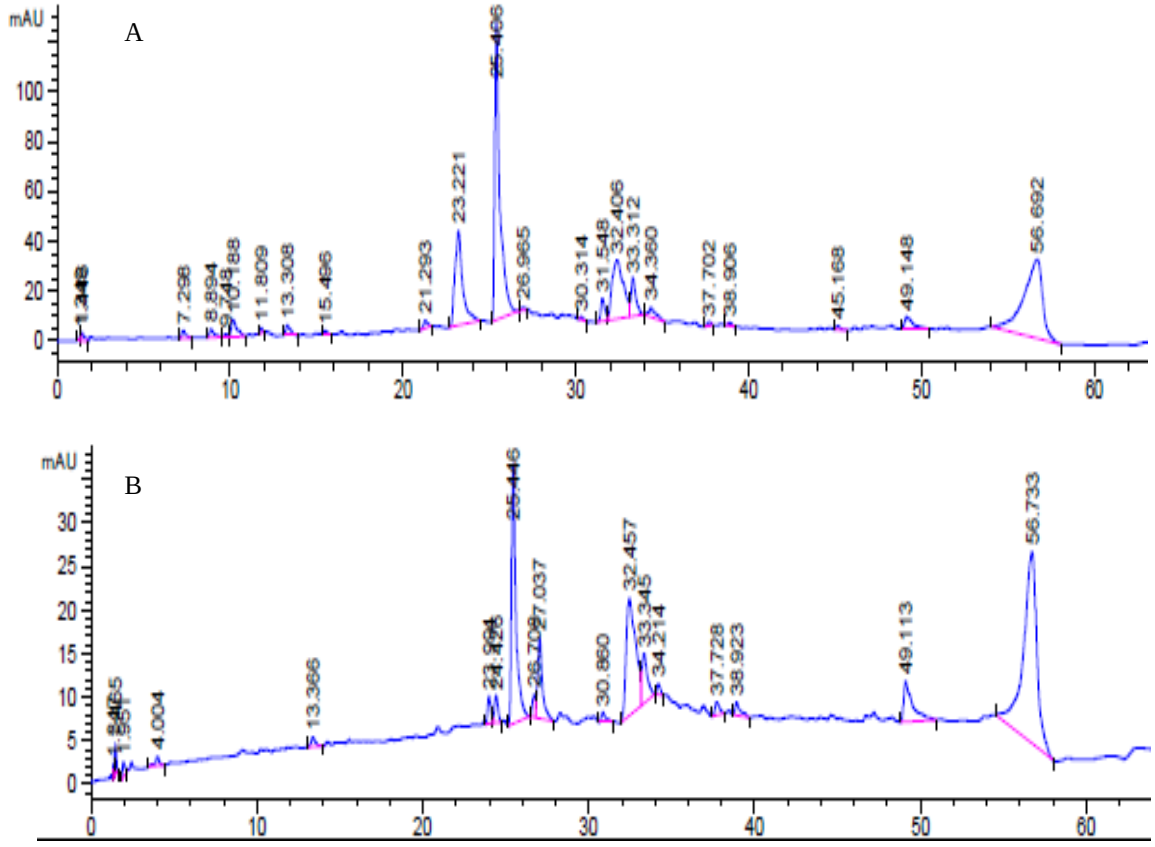

Fig. 4: Lavandula stoechas hydroalcoholic extract phenolic profile measured at 254 nm.

A: Chromatogram of wild plant extract. B: Chromatogram of in vitro culture extract.

Table 2: The phenolic compounds identified by HPLC-DAD in hydroalcoholic extracts of *in vitro* shoot cultures and wild plants of *L. stoechas*.

Standars	**Wild plant Area%**	***In vitro* plant Area %**	**RT (min)**
Luteolin 3'- 7 diglucoside	-	1.42	24.253
Hespertin	0.2	-	28.284
Naringenin	12.56	16.85	32.461
Rhamnetin	0.39	1.2	36.818
Acacetin	1.94	5.54	49.153

(–) not detected.

Discussion

L. stoechas in vitro propagation has received little attention, with only a few studies on the issue[20,22,28,29]. However, this study successfully established a simple and effective methodology for the micropropagation of *L. stoechas*. Plant tissue culture techniques are advantageous for large-scale plant propagation in less time than traditional approaches[30]. Additionally, *in vitro* propagation reduces the occurrence of diseases in propagated plants[31].

Previous academic studies have revealed that the composition of the culture media is a crucial component regulating Lavender micropropagation, influencing plant tissue's growth, shape, and phytochemical qualities[32–36]. This study's achievement in establishing a micropropagation protocol for *L. stoechas* could have significant implications for the large-scale production of this plant species. Further research may be necessary to optimize the protocol and investigate various applications of the propagated plants.

This research aimed to evaluate the effect of different concentrations of 6-benzyladenine (BA), both individually and in combination with gibberellic acid (GA3), on the propagation of *L. stoechas* explants and their morphological characteristics.

The study demonstrated that combining GA3 and BAP was the most effective technique for increasing shoot proliferation and optimizing biomass, with a ratio of 1:1 (v:v), at a concentration of 0.5 mg/l.

Many studies have highlighted the synergistic effect of PGR combinations on the process of organogenesis[37–40]. The individual application of cytokinin BAP increases the bud's formation. Moreover, GA3 stimulates cell elongation and causes plants to grow taller[41]. Kousalya & Narmatha Bai (2016)[42] found that applying GA3 and BAP combination in a ratio of 1:1 increases shoot elongation but also shoot multiplication of *Canscora decussate*. This was also supported by *in vitro* cultures of *Citrus jambhiri* explants which had a high frequency of multiple shoot proliferation after growing in the presence of GA3/ BAP ratio was 1:1 in the culture media[43]. Nevertheless, in many works on the *in vitro* culture of the Lavender species, the most effective method for shoot propagation was MS media supplemented with BA hormone alone[33,,44,45].

According to Khateeb et al. (2017)[46], the best medium for propagating *L. coronopifolia* shoot cultures was MS medium supplemented with 0.5 mg/l BA. In another study, the highest shoot proliferation from shoot tip explants of *Lavandula officinalis* was observed in media containing 0.4 mg/l BAP. In addition, combining cytokinin BA with low concentrations of auxins (IAA, NAA, or IBA) has resulted in favorable rates of shoot proliferation in several Lavender species, including *L. stoechas*[22], *L. dentata*[47,48], and *L. angustifolia*[49].

Previous research has shown that Lavender species have a significant quantity of total phenolic chemicals in hydroethanolic extracts, which *L. stoechas* having an exceptionally high phenolic content[50]. In the present study, the hydroethanolic extract from *in vitro* cultures of *L. stoechas* exhibited the highest concentration of phenolic compounds, with a value of 83.18 ± 3.07 mg GAE/g DW extract. However, the hydroethanolic extract from wild *L. stoechas* plants contained less than half the phenolic content observed in the *in vitro* extract. This difference could be attributed to the seasonal constraints in wild plants. At the same time, cell and tissue cultures offer the benefit of producing phenolic compounds throughout the year, allowing for reliable and predictable[18].

Previous studies found similar findings in *L. viridis* plants grown *in vitro*, where a greater concentration of phenolic compounds was detected compared to wild plants[18]. Furthermore, using a water/ethanol extract from *Thymus lotocephalus in vitro* cultures resulted in more phenolic compounds than those found in the original plant's leaves[51]. Plant extracts with high levels of phenolic content have a high antioxidant capacity[52]. Our findings align with a previous finding that showed a positive relationship between total phenolic and flavonoid contents and antioxidant activity in *Salvia chudaei*[53]. In the present study, the ability of *L. stoechas* extracts to scavenge free radicals was studied using the DPPH scavenging assay and the total antioxidant capacity (TAC) test. Both extracts exhibited high antioxidant activity. However, the hydroethanolic extracts from *in vitro*

cultures were better than the extract from wild plants. A previous study by Costa et al. (2013)[18] evaluated the antiradical activity of the water/ethanol extract from the wild plant and *in vitro* cultures of *L. viridis* and reported high antiradical potency in the extract from *in vitro* cultures.

Furthermore, similar results have been reported in other plant species, where extracts from *in vitro* cultures exhibited significant antioxidant potential compared to wild plants. This was observed in *L. angustifolia*[54], *L. viridis*[18], *Thymus lotocephalus*[51], *Canscora decussata*[42], and *Perovskia abrotanoides*[55]. On the other hand, our findings are relatively more significant when compared to those of earlier research that examined the antiradical potency of *L. stoechas* extract[12,56,57].

Phenolic compounds such as caffeic acid, rosmarinic acids, quercetin, naringenin, acacetin, hesperidin, and luteolin are commonly found in *Lavandula* species contribute to their biological activity[3,10,,13,58,59]. Using HPLC-DAD analysis, we identified five flavonoid compounds in the crude extracts of *L. stoechas*, including Luteolin 3'- 7 diglucoside, naringenin, rutin, acacetin, rhamnetin, and hesperidin. Interestingly, Luteolin 3'- 7 diglucoside was only detected in the extract from *L. stoechas in vitro* cultures, while hesperetin was only found in the *L. stoechas* wild plant extract.

Pérez-del Palacio et al. (2020)[59] identified Luteolin 3'-7 diglucoside in the hydroalcoholic extract of *L. stoechas* using LC-QTOF/MS analysis. This glycosylated flavone is a luteolin analog known for exhibiting potent antioxidant activity[60] and other pharmacological properties, such as anticancer, anti-inflammatory, and neuroprotective effects[60,61]. Thus, we suggest that Luteolin 3'- 7 diglucoside Luteolin 3'- 7 diglucoside was the most crucial compound contributing to the high antioxidant efficacy of *in vitro* shoot culture extract. On the other hand, we suggest that flavanone compounds in *L. stoechas* extract, such as naringenin and acacetin, may increase its antioxidant activity. These flavanones have been previously extracted from different parts of the *L. stoechas* plant[58,62]. They are acknowledged for their vigorous biological activities, such as anti-inflammatory, anti-viral, antimicrobial, antidepressant, anti-obesity, and anticancer effects[63–65].

These findings suggest that the *in vitro* cultures of *L. stoechas* can produce active metabolites in high amounts, which may be responsible for the antioxidant activity found in the extract and may play an important role in therapeutic efficiency.

Conclusions

In conclusion, our findings reveal that *L. stoechas in vitro* cultures contain more phenolic compounds than wild plants, making them a significant source of bioactive molecules with antioxidant activity. Further investigation is required to enhance the production of molecules with a high antioxidant activity using biotic and abiotic elicitors. The high antioxidant activity of *L. stoechas* makes it a promising candidate for developing new antioxidant supplements or natural products. However, supplementary researches are needed to identify the specific bioactive compounds responsible for antioxidant activity and to evaluate their potential medicinal applications. These findings highlight the potential of *in vitro* plant cultures as an alternative technique to produce bioactive compounds with medicinal properties.

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الإكثار المخبري لنبات *Lavandula stoechas* الجزائري وتقييم التركيب الكيميائي الحيوي ونشاطه كمضاد للأكسدة

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تهدف هذه الدراسة إلى معرفة تأثير منظمات نمو النبات (PGRs) على الإكثار المخبري لنبات *Lavandula stoechas* ، وكذا تقييم إمكانات مضادات الأكسدة والمحتوى الفينولي الكلي للمستخلصات المأخوذة من المزارع المخبرية والنباتات البرية لنبات *L. stoechas* ، بالإضافة إلى التحليل الكيميائي للمحتوى الفينولي لكلا المستخلصين باستخدام HPLC-DAD. أظهرت النتائج أن الإكثار المخبري الأمثل لنبات *L. stoechas* وكذا تراكم الكتلة الحيوية كان باستخدام الوسط الذي يحتوي على ٠,٥ مجم / لتر من ٦- بنزيل أدينين (BA) و ٠,٥ مجم / لتر حمض الجبريليك (GA3). يحتوي المستخلص المائي الكحولي الذي تم الحصول عليه من المزارع المخبرية على أعلى كمية من المركبات الفينولية (٨٣,١٨ مجم GAE/جم من الوزن الجاف للمستخلص) مقارنة بالمحتوى الفينولي الكلي لمستخلص النباتات البرية (٣٢,٣٣ مجم GAE/ جم من الوزن الجاف للمستخلص). علاوة على ذلك، أظهر المستخلص المائي الكحولي للمزارع المخبرية أنه يمتلك قدرة كبيرة في تفخيخ الجذور الحرة لمركب DPPH بقيمة IC_{50} تبلغ ٥١,٨٣ ميكروغرام / مل وقدرة أعلى لمضادات الأكسدة (٧٥٧,٧٢مجم GAE/جم من الوزن الجاف للمستخلص) حسب اختبار القدرة الكلية المضادة للأكسدة (TAC). كشف التحليل الكيميائي لكلا المستخلصين باستخدام HPLC-DAD عن وجود نارينجين ، وأكاسيتين ، ورامنيتين ، ولوتولين ٣'-٧-دي-جلوكوزيد ، وهسبريتين. بشكل عام، تشير النتائج إلى أن المزارع المخبرية لـ *L. stoechas* يمكن أن تكون بمثابة مصدر مهم لإنتاج مستقلبات فعالة ذات خصائص عالية مضادة للأكسدة.