

Induction of Callus from Date Palm (*Phoenix dactylifera* L. cv. Barhi) for Bioactive Compounds Production and Assessment of Cytotoxicity and Antioxidant Activities.

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Abstract

This study evaluated the biochemical and biological responses of callus cultures derived from shoot tip explants of *Phoenix dactylifera* L. cv. Barhi under *in vitro* conditions, following treatment with different combinations of jasmonic acid (JA) and plant growth F) were established, comprising three JA –regulators (PGRs). Six treatment groups (A concentrations (0, 2, and 4 mg/L) combined with varying concentrations of 2iP (2, 4-D; 8, 12 mg/L), and naphthalene acetic-dichlorophenoxyacetic acid (2,4-mg/L), 2,4 acid (NAA; 40, 60 mg/L). Callus tissues were extracted using 70% ethanol, and MS) identified -Mass Spectrometry (GC–chemical profiling via Gas Chromatography 138 distinct compounds across all treatments. The highest number of compounds was recorded in treatment B (50), followed by treatments D, E, and F (40 each), treatment C (35), and treatment A (32).

Antioxidant capacity, determined by the DPPH free radical scavenging assay at 1.0 mg/mL, was highest in treatments A (74.90%), B (74.70%), and D (74.21%), while treatments C, E, and F exhibited slightly lower activities (72.77%, 72.79%, and 72.80%, respectively). Cytotoxicity evaluation using the hemolysis assay with human red blood cells showed hemolysis rates below 2% at all tested concentrations (100, 200, and 300 µg/mL), indicating biosafety and non- hemolytic properties.

Statistical analysis using a Completely Randomized Design (CRD) and Least Significant Difference (LSD) test at $p \leq 0.05$ revealed significant differences among treatments in both chemical composition and biological activities. The results demonstrate that moderate JA levels, in combination with optimized PGR concentrations, enhance secondary metabolite accumulation and antioxidant activity without inducing cytotoxicity, supporting their potential use in pharmaceutical and nutraceutical applications.

Keywords: *Phoenix dactylifera* cv. Barhi, Callus culture, Jasmonic acid, Metabolite profiling, Antioxidant activity, Secondary metabolites.

Introduction

Phoenix dactylifera L., commonly known as date palm, is a prominent functional plant owing to its nutritional richness and therapeutic value [8,18]. In many developing countries, especially rural areas, it plays an essential role in food security and in combating malnutrition by supplying energy, vital minerals, and dietary fiber [24]. Numerous cultivars are cultivated across the Mediterranean basin, appreciated for their nutritional and biochemical attributes [21,51]. Among these, the 'Barhi' cultivar stands out due to its high fructose content, abundant micronutrients, strong antioxidant activity, and low anti-nutritional content. In a study involving 11 date palm genotypes from the UAE, 'Barhi' ranked among the top cultivars [39]. It is also extensively used in Saudi Arabia to counter mineral deficiencies [37]. However, its increasing demand and limited traditional propagation methods have raised its market value.

The bioactivity of *Phoenix dactylifera* is primarily due to its wealth of phytochemicals, especially antioxidants that neutralize reactive oxygen species (ROS) and reduce oxidative stress [4,11,30]. Traditionally, it has been used to treat gastrointestinal, cardiovascular, and neurodegenerative diseases [45]. Experimental studies have proven the antimicrobial efficacy of its extracts [23] and revealed anticancer potential through mechanisms like apoptosis and cell proliferation inhibition [16]. Additionally, preparations derived from this species have exhibited significant antioxidant and antimutagenic activities, contributing to oxidative damage prevention and mutation reduction [42].

Conventional propagation of date palm through offshoots is slow, with each plant producing a limited number [29]. This, combined with vulnerability to pests and pathogens, necessitates alternative propagation strategies. Plant tissue culture provides a reliable solution for mass production of uniform, disease-free plants under controlled *in vitro* conditions.

Callus induction is a crucial step in tissue culture, acting as a precursor for indirect organogenesis and somatic embryogenesis regardless of environmental variations [44]. Moreover, callus serves as a renewable source for secondary metabolite production [5]. Somatic embryogenesis (SE) is a well-established method for regenerating embryogenic biomass and examining developmental processes at various biological levels [14,17,32].

Several *in vitro* propagation protocols have been developed for 'Barhi' using explants like shoot tips [3,7], mature inflorescences [26], and immature floral tissues [41]. Nevertheless, the biochemical and pharmacological responses of tissues during early development remain poorly characterized in many cultivars, including 'Barhi.'

Previous research has examined antioxidant profiles in embryogenic and non-embryogenic callus [13], somatic and zygotic embryos [17], cell suspensions [6], and degenerative embryogenic calli [48,49]. However, integrated phytochemical profiling through gas chromatography–mass spectrometry (GC-MS), combined with pharmacological assays, especially for antioxidant potential and enzyme inhibition are still limited in callus cultures of 'Barhi'.

Accordingly, the present study was designed to investigate the chemical composition of bioactive compounds present in callus tissues induced from shoot tip explants of *Phoenix dactylifera* L. cv. Barhi. GC-MS analysis was employed to characterize the metabolic profile of the ethanolic extract. Furthermore, the study aimed to evaluate the antioxidant activity and assess the cytotoxic potential of the extract under controlled *in vitro* conditions, thereby providing insight into the biological relevance of callus-derived metabolites. Therefore, the current study aimed to investigate the metabolic response of callus cultures derived from the Barhi cultivar in response to jasmonic acid and plant growth regulators (PGRs), with a focus on the production of bioactive compounds and the assessment of their biological effects.

Materials and Methods

Preparation of Explant Source from Date Palm Offshoots

The experimental procedures commenced in October 2024. Offshoots of *Phoenix dactylifera* L. cv. Barhi, aged between 3 and 4 years, were collected from healthy, disease-free mother plants cultivated in a private orchard located in Najaf Governorate, Iraq. The offshoots were initially cleaned by thorough rinsing under running tap water for several minutes to eliminate soil particles and surface impurities, serving as a preparatory step prior to surface sterilization and explant dissection.

Surface Sterilization of Explants

Explants were subjected to a sequential surface sterilization procedure under strictly sterile conditions within a laminar airflow

cabinet. Initially, the explants were immersed in 70% (v/v) ethanol for 30 seconds to disrupt the cuticular barrier and preliminarily reduce the surface microbial load. This was followed by treatment with 2.5% (v/v) sodium hypochlorite (NaOCl) solution supplemented with a few drops of Tween-20 as a surfactant, and maintained for 10 minutes. Subsequently, a final disinfection step was performed using 0.1% (w/v) mercuric chloride (HgCl₂) for 3 minutes to ensure deep tissue sterilization and the elimination of any persistent contaminants (Figure 1). After each sterilization step, the explants were rinsed thoroughly three to five times with sterile distilled water to remove residual sterilizing agents and prevent phytotoxic effects.



Figure 1. Surface sterilization steps of shoot tip explants prior to *in vitro* culture initiation.

Preparation of Culture Medium

The culture medium was prepared based on the Murashige and Skoog (MS)[33], formulation at a concentration of 4.44 g/L. Sucrose was added as a carbon source at 30 g/L. The final volume was adjusted to 1 L using double-distilled water. The pH of the medium was carefully adjusted to 5.7 using 1 N NaOH or 1 N HCl, as appropriate. To solidify the medium, agar was added at a concentration of 8

ISSN 2072-3857

g/L. The medium was then heated and stirred continuously using a magnetic stirrer until all components were fully dissolved. Subsequently, 10 mL aliquots of the prepared medium were dispensed into sterile culture tubes.

Hormonal Treatments and Medium Sterilization

A series of hormonal treatments was designed to assess the interactive effects between plant growth regulators and jasmonic acid in promoting the biosynthesis of bioactive compounds. The treatments involved three concentrations of jasmonic acid (0, 2, and 4 mg/L), in combination with specific concentrations of growth regulators, namely: 2-isopentenyladenine (2iP) at 2,4 mg/L, 2,4-dichlorophenoxyacetic acid (2,4-D) at 8, 12 mg/L, and α -naphthaleneacetic acid (NAA) at 40,60 mg/L, as detailed in Table (1). Based on these combinations, six distinct experimental groups (labeled A through F) were established. Following the addition of the appropriate hormonal components to the MS culture medium, each formulation was sterilized by autoclaving at 121 °C and 1.04 kg/cm² of pressure for 21 minutes. The sterilized media were then cooled to room temperature under aseptic conditions and subsequently used for tissue culture procedures [20].

Table 1. Concentrations of jasmonic acid and growth regulators (mg/l) added to the MS medium.

Treatment	JA	2iP	2,4-D	NA A
A(control)	0	2	8	40
B	2	2	8	40
C	4	2	8	40
D	0	4	12	60
E	2	4	12	60
F	4	4	12	60

Callus Induction under Controlled Growth Conditions

Following surface sterilization, shoot tip explants were aseptically inoculated into glass culture tubes containing 10 mL of MS medium supplemented with specific concentrations of plant growth regulators and jasmonic acid, according to the designated six treatments (A–F). Each tube received a single shoot apex (10 replicates per treatment).

The inoculated cultures were incubated in a controlled-environment growth chamber set at $25 \pm 2^\circ\text{C}$, under a 16-hour light and 8-hour dark photoperiod. Illumination was provided by 40-watt cool-white fluorescent lamps, arranged to ensure uniform light distribution across all culture vessels (Figure 2).



Figure 2. Tissue culture growth room during date palm callus induction.

Extraction and GC-MS Analysis of Callus Metabolites

Callus tissues derived from each hormonal treatment were initially dried in a hot air oven at a controlled

temperature until constant weight was achieved. The dried samples were then finely ground using a mechanical grinder to obtain a uniform powder. A defined quantity of the powdered material was subjected to ethanol extraction using 70% (v/v) ethanol. Following the extraction process, the crude extracts were filtered through Whatman No.1 filter paper, and the resulting filtrates were collected and stored under appropriate conditions for chemical analysis [34].

The chemical profiling of the extracts was carried out using Gas Chromatography–Mass Spectrometry (GC-MS) to identify the bioactive constituents present in the ethanolic extracts. The analysis was performed using a Shimadzu GC-MS instrument (Shimadzu, Japan) at the laboratories of the College of Science, University of Al-Qadisiyah. The mass spectra of the detected compounds were compared against entries in the National Institute of Standards and Technology (NIST) spectral database for accurate identification of the constituents.

Cytotoxicity Assay

The hemolysis assay was conducted to evaluate the cytotoxic potential of ethanolic extracts derived from the callus tissue of *Phoenix dactylifera L.* cv. Barhi, using human red blood cells (RBCs) as a model system. Fresh human blood (obtained from a healthy donor with blood group A+) was collected into a sterile 15 mL Falcon tube and centrifuged at 1500 rpm for 5 minutes to separate the serum. The pelleted erythrocytes were washed three times with chilled, sterile phosphate-buffered saline (PBS). For the assay, 180 μ L of the RBC suspension was mixed with 20 μ L of

the extract at concentrations of 100, 200, and 300 μ g/mL in sterile 2 mL Eppendorf tubes. The mixtures were incubated at 37 °C for 1 hour, followed by centrifugation at 1500 rpm for 5 minutes. Subsequently, 100 μ L of the supernatant was transferred to a new tube containing 900 μ L of cold PBS, and the absorbance was measured at 576 nm using a UV-Vis spectrophotometer. Phosphate-buffered saline served as the negative control, while 1% Triton X-100 was used as the positive control for maximum hemolysis [40].

Antioxidant Activity Assay (DPPH Radical Scavenging Test)

The antioxidant activity of the ethanolic extracts obtained from the callus tissues of *Phoenix dactylifera L.* cv. Barhi—subjected to treatments with jasmonic acid and various growth regulators—was evaluated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay. This non-enzymatic method was conducted based on the protocol described by Lee et al. [28], with minor modifications adapted to the specific nature of the plant extract.

DPPH solution was prepared in methanol, and 200 μ L of this solution was added to the first well of a 96-well microplate, serving as the positive control. For the remaining wells, 100 μ L of the DPPH solution was mixed with 100 μ L of the ethanolic extract at four different concentrations: 1000, 500, 250, and 125 μ g/mL. Ascorbic acid was used as the standard reference antioxidant for comparison. The microplate was then incubated in the dark at room temperature for 30 minutes to ensure full interaction between the DPPH radicals and the

bioactive compounds within the extract.

After incubation, absorbance was measured at 514 nm using an ELISA microplate reader (TECAN, Graz, Austria). Pure methanol (100%) was used as the blank for calibration purposes.

The percentage of DPPH radical inhibition, used as an indicator of antioxidant capacity, was calculated according to the following equation:

$$\text{scavenging (\%)} = [\text{control} - \text{sample} / \text{control}] \times 100.$$

Higher inhibition percentages reflect a greater ability of the extract to neutralize free radicals, indicating stronger antioxidant potential.

Experimental Design and Statistical Analysis

The experiment was conducted using a Completely Randomized Design (CRD) with two independent factors. The first factor was jasmonic acid (JA) applied at three concentrations (0, 2, and 4 mg/L). The second factor consisted of plant growth regulators, 2-isopentenyladenine (2iP), 2,4-dichlorophenoxyacetic acid (2,4-D), and naphthaleneacetic acid (NAA), applied in three combination levels: (2, 4 mg/L), (8, 12 mg/L), and (40, 60 mg/L), respectively. Treatment means were compared using the Least Significant Difference (LSD) test at a significance level of $P \leq 0.05$. Statistical computations and graphical outputs were performed using SPSS software.

Results and discussion

GC-MS Analysis of Ethanolic Callus Extracts

The gas chromatography–mass spectrometry (GC-MS) analysis of

ethanolic extracts derived from *Phoenix dactylifera* L. cv. Barhi callus tissues—originating from six distinct hormonal treatments (A–F) demonstrated notable variation in the number of bioactive compounds detected across treatments. A total of 138 chemical constituents were identified among all samples.

Treatment A exhibited the lowest compound richness, with 32 compounds (Figure 3) identified, followed by treatment C, which contained 35 compounds (Figure 5). Treatments D, E, and F each showed an identical total of 40 detected compounds (Figure 6,7,8). Notably, treatment B yielded the highest number of constituents, with 50 distinct compounds identified (Figure 4).

These variations reflect the differential influence of growth regulator and jasmonic acid combinations on the metabolic profile of the callus tissues, suggesting treatment-dependent modulation of secondary metabolite biosynthesis. Statistical analysis using the Least Significant Difference (LSD) test confirmed that these differences were significant at the $P \leq 0.05$.

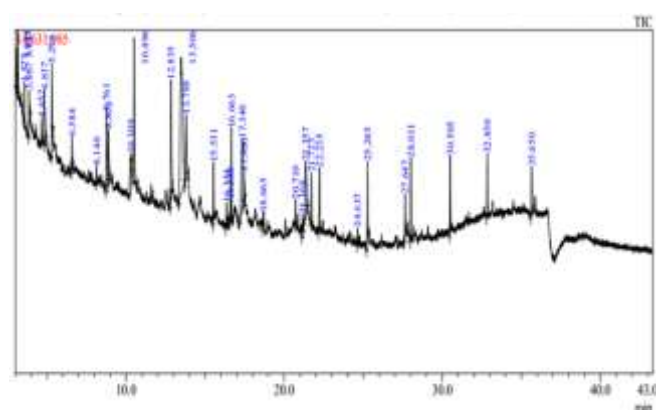


Figure 3. Profile of chemical constituents identified through GC-MS analysis in the ethanolic extract of callus tissue from *Phoenix*

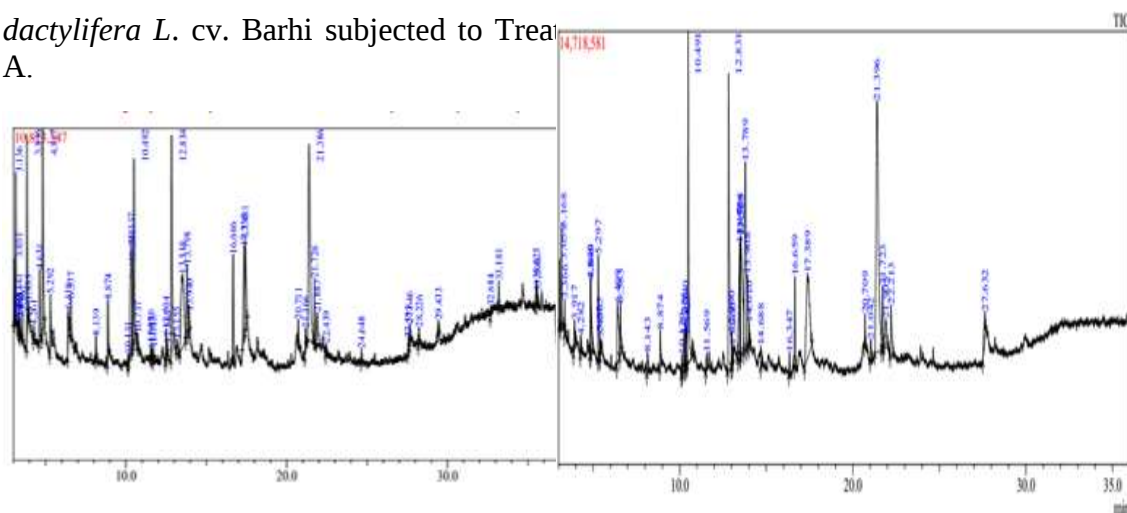


Figure 4. Profile of chemical constituents identified through GC-MS analysis in the ethanolic extract of callus tissue from *Phoenix dactylifera* L. cv. Barhi subjected to Treatment B.

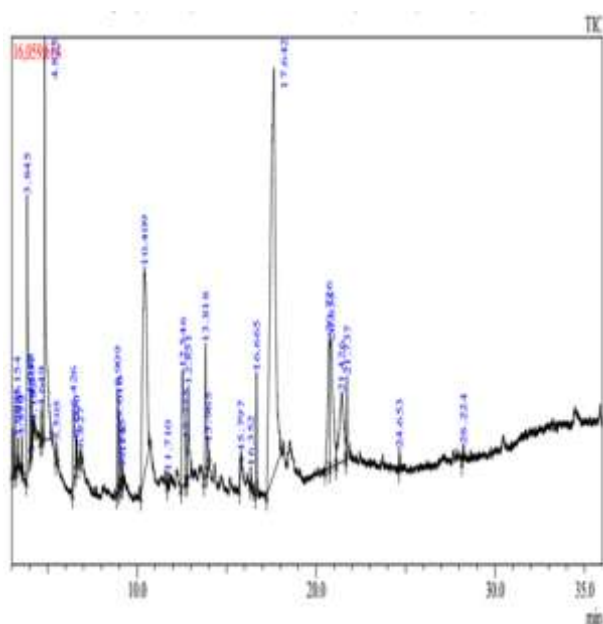


Figure 6. Profile of chemical constituents identified through GC-MS analysis in the ethanolic extract of callus tissue from *Phoenix dactylifera* L. cv. Barhi subjected to Treatment D.

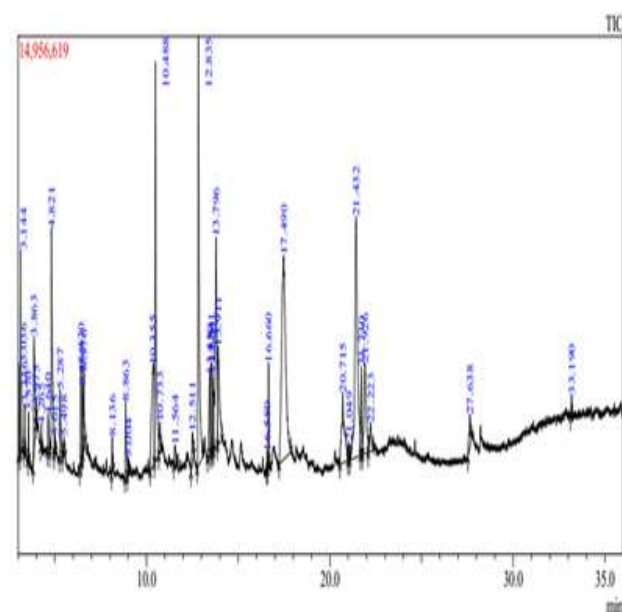


Figure 5. Profile of chemical constituents identified through GC-MS analysis in the ethanolic extract of callus tissue from *Phoenix dactylifera* L. cv. Barhi subjected to Treatment C.

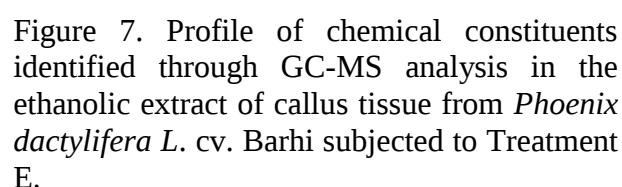


Figure 7. Profile of chemical constituents identified through GC-MS analysis in the ethanolic extract of callus tissue from *Phoenix dactylifera* L. cv. Barhi subjected to Treatment E.

in the induced callus. This effect can be attributed to their ability to create a favorable intracellular environment by stimulating cell division and promoting protein and amino acid biosynthesis, thereby enhancing the metabolic pathways involved in secondary metabolite production [15,38]. Additionally, PGRs contribute to enhancing enzymatic activity and increasing cell proliferation rates, thus improving the overall biosynthetic capacity of cultured tissues [1]. Furthermore, they function as essential signaling molecules that actively participate in the synthesis of plant secondary metabolites while also regulating plant growth and development [51].

These findings are consistent with previous studies on date palm, where a balanced application of 2iP and 2,4-D was shown to promote the production of secondary compounds. The highest concentrations of phenolics and flavonoids were observed when cells were treated with 5 mg/L 2,4-D and 2.5 mg/L 2iP [6].

Similarly, Naik and Al-Khayri [36] demonstrated that treating *Phoenix dactylifera* L. suspension cultures with 1.5 mg/L 2iP and 10 mg/L NAA stimulated the accumulation of important phenolic and flavonoid compounds such as catechin and caffeic acid. These findings underscore the significance of growth regulator balance and hormonal environment optimization as critical factors for enhancing the metabolic pathways responsible for bioactive compound production.

Moreover, the current results indicate a complex interactive relationship between jasmonic acid and PGR concentrations in modulating

secondary metabolite production in tissue-cultured plant cells. The treatment containing moderate levels of jasmonic acid combined with intermediate PGR concentrations (Treatment B) resulted in a clear increase in the number of identified chemical constituents. Conversely, high concentrations of growth regulators appeared to stimulate metabolic activity to a lesser extent unless accompanied by jasmonic acid. The addition of JA under these conditions did not produce marked differences, suggesting potential interference in hormonal signaling at elevated PGR levels. This potential reduction in detected compound diversity may be attributed to the synthesis of novel metabolites at higher concentrations, altering the relative abundance of existing compounds in the sample [10].

Cytotoxicity Evaluation of Ethanolic Extracts Using the Hemolysis Assay

The hemolysis assay was performed to assess the cellular safety of ethanolic extracts derived from the callus tissues of *Phoenix dactylifera* L. cv. Barhi, using human red blood cells (RBCs) as a biological indicator. The results showed that all six samples (A to F), at the tested concentrations (100, 200, and 300 mg/mL), did not induce significant hemolysis in RBCs, with hemolysis rates for all treatments remaining below 2%.

These findings are based on the comparison of the optical absorbance values of the samples with those of the negative control (PBS) and the positive control (Triton X-100). The absorbance values of all tested samples remained close to that of the negative control, confirming the absence of substantial hemolytic activity.

According to the international standard ASTM F756-17, which is globally recognized for classifying hemolytic response, substances that induce less than 2% hemolysis are considered non-hemolytic.

Evaluation of Antioxidant Activity of Plant Extracts

The results of the DPPH assay revealed that all six investigated plant treatments exhibited varying degrees of free radical scavenging activity. A clear positive correlation was observed between the extract concentration and the percentage of inhibition, as antioxidant efficacy increased progressively with the rise in concentration from 0.12 to 1.0 mg/mL, as shown in Table (2) and Figure (9). This concentration-dependent behavior suggests that the higher the extract concentration, the greater the availability of bioactive compounds capable of interacting with and neutralizing free radicals.

Among the tested treatments, A, B, and D showed the highest antioxidant activities across all concentrations, particularly at the maximum concentration (1.0 mg/mL). This indicates that these treatments may be rich in phenolic and flavonoid compounds known for their strong antioxidant properties. In contrast, treatments C, E, and F exhibited comparatively lower activity, which

may be attributed to lower concentrations or altered profiles of active constituents.

Collectively, these findings suggest that the nature of the hormonal or chemical treatments significantly influenced the accumulation of secondary metabolites responsible for antioxidant activity in *Phoenix dactylifera* L. cv. Barhi callus. The results support the potential use of specific treatments as promising sources of natural antioxidants with potential pharmaceutical or nutritional applications.

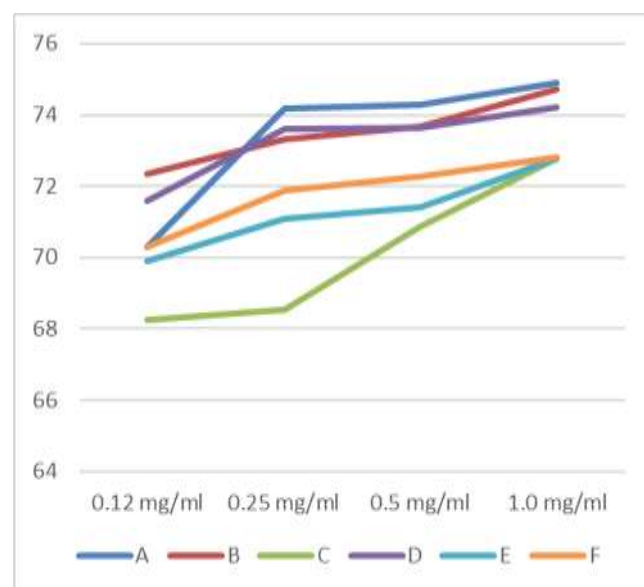


Figure 9. Dose-dependent increase in antioxidant activity of the plant extracts.

Table 2. DPPH Radical Scavenging Activity (%) of Ethanolic Callus Extracts from *Phoenix dactylifera* L. cv. Barhi at Various Concentrations.

Sample	0.12 mg/ml	0.25 mg/ml	0.5 mg/ml	1.0 mg/ml
A(Conterol)	70.314	74.166	74.287	74.901
B	72.361	73.300	73.685	74.708
C	68.26	68.524	70.856	72.77
D	71.59	73.589	73.649	74.215
E	69.917	71.073	71.422	72.794
F	70.302	71.879	72.276	72.803

The DPPH method is a widely accepted and reliable assay for evaluating the *in vitro* free radical scavenging ability of plant extracts or compounds. DPPH is a stable and highly reactive free radical that acts through accepting electrons or hydrogen atoms from antioxidants, leading to its reduction and transformation into a non-radical form. This reaction is visually indicated by a color change from deep violet to pale yellow, serving as a measurable marker for the antioxidant capacity of the test compound [31]. At the molecular level, jasmonic acid (JA)—after conjugation to isoleucine (JA-Ile)—activates the SCF^{COI1} receptor complex, leading to ubiquitination and degradation of JAZ repressors and the consequent release of MYC-family transcription factors. This upregulates genes of the phenylpropanoid pathway (PAL, C4H, 4CL) and the flavonoid branch (CHS, CHI, F3H), increasing the

Conclusions

The present study demonstrated that the application of jasmonic acid (JA) in combination with specific concentrations of plant growth regulators (2iP, 2,4-D, and NAA) significantly influenced the biochemical behavior of *Phoenix dactylifera* L. cv. Barhi callus. Treatment B (2 mg/L JA + low PGRs) was particularly effective, yielding the highest number of chemical compounds (50) and exhibiting superior antioxidant activity, along

accumulation of phenolics and flavonoids capable of hydrogen/electron donation, thereby enhancing the antioxidant activity measured by the DPPH assay in JA-containing treatments [46].

These findings are consistent with the results reported by Ammar *et al.*[12], who demonstrated that callus tissues of the Barhi cultivar exhibit notable antioxidant activity. Their bioassays confirmed that the callus extract has a pronounced ability to inhibit free radicals. Additional studies have also shown that Barhi seed extracts display considerable antioxidant activity in validated bioassays, further supporting its effectiveness in countering oxidative stress. This is largely attributed to the high phenolic content present in the cultivar. Therefore, Barhi date palm may serve as a valuable natural source of antioxidant compounds suitable for therapeutic or food industry applications [22, 48].

with complete biosafety as indicated by hemolysis rates below 2%. GC-MS analysis revealed 138 distinct compounds across treatments, including several bioactive constituents commonly associated with antioxidant and antimicrobial properties. The study also highlighted a concentration-dependent response in DPPH radical scavenging activity, with treatments A, B, and D showing the strongest inhibition at 1.0

mg/mL. These findings underscore the potential of moderate JA elicitation to enhance secondary metabolite production in date palm

callus cultures, suggesting their future utility in pharmaceutical and nutraceutical applications

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