Jordan Journal of Pharmaceutical Sciences, Volume 17, No. 3, 2024

Phenolic Compounds and Antioxidant Activity of *Chiliadenus montanus* (Vhal.) Brullo. grown *in vitro*

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ABSTRACT

This study explores the in vitro cultivation of *Chiliadenus montanus* (Vhal.) Brullo (Asteraceae), focusing on callus multiplication, in vitro seed germination, phenolic compound production, and antioxidant activity. Callus induction was optimized, followed by multiplication using Murashige and Skoog (MS) media supplemented with 1.0 mg·L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) and 2.0 mg·L^{-1} 6-Benzylaminopurine (BAP). The highest in vitro germination rate of *C. montanus* seeds (11.6 ± 2.22%) was achieved using half-strength MS media supplemented with 0.5 mg·L^{-1} gibberellic acid (GA_3) and 1.0 mg·L^{-1} BAP. Methanol extracts from wild and in vitro samples were analyzed for Terpinen-4-ol, Eucalyptol (1,8-Cineole), and total phenolic content. In vitro microshoots exhibited an elevated Terpinen-4-ol concentration (0.01 ± 0.003 mg/g) compared to wild plants, while the concentrations of Eucalyptol (0.06 ± 0.001 mg/g) were similar in both microshoots and wild plants. Phenolic compound analysis revealed maximum levels in wild plants (30.67 ± 2.82 gallic acid equivalents [GAE]), followed by microshoots (22.81 ± 0.65 GAE), and the lowest in callus (6.37 ± 0.27 GAE). Antioxidant properties, evaluated via the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, indicated superior radical scavenging in wild plants (Inhibitory Concentration 50 [IC_{50}] 32.13 ± 0.83 µg/ml) compared to greenhouse plants (IC_{50} 221.04 ± 1.34 µg/ml). *C. montanus* emerges as a potential natural antioxidant source. In conclusion, an effective in vitro production system for phenolic compounds in *C. montanus* was established, offering a sustainable alternative to wild plant harvesting. The study highlights the potential benefits of *C. montanus* as a reservoir of bioactive substances and emphasizes the importance of in vitro cultivation for sustainable resource utilization.

Keywords: callus culture, microshoot, medicinal plant, *Chiliadenus montanus*, antioxidant activity, phenolic compounds.

1. INTRODUCTION

Chiliadenus montanus (Vhal.) Brullo, also known as *Jasonia montana*, *Varthemia montana* (Vahl.) Boiss., is an herbaceous plant of the Asteraceae family has been shown to contain diverse volatile organic compounds (VOCs) such as Alloaromadendrene, cis-Myrtanol, p-Cymene, α-pinene and β-Cyclocitral [1]. Phenolic compounds, recognized as prominent natural secondary metabolites in plants due to their significant physiological and biochemical activities, are exemplified by gallic acid (GA) [2]. These polyphenolic metabolites are known for their numerous roles, including antioxidant [3], antineoplastic [4], and apoptosis-inducing properties [5]. The antioxidative potential of phenolic compounds, by neutralizing oxygen radicals, addresses redox damage and associated health risks within cells [6,7].

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Received: 16/01/2024 Accepted: 18/05/2024.
DOI: https://doi.org/10.35516/jjps.v17i3.2248

Chiliadenus montanus, commonly referred to as Haneida, is a traditional medicinal plant indigenous to Jordan, Egypt, Palestine, and Saudi Arabia [8]. *C. montanus* is widely used in Mediterranean traditional healing practices for conditions such as diarrhea, kidney problems, stomachaches, and chest ailments [9-11]. Scientific investigations have confirmed its pharmacological activity, including antitumor [12], free radical scavenging [13], antiviral [14], cholestasis-reducing, and hypoglycemic properties [15], further emphasizing its biomedical significance [16].

In vitro cultivation of plants emerges as a crucial bioengineering tool for micropropagation, serving as a sustainable resource for management and conservation [17], as well as for the synthesis of natural products [18,19]. The induced formation of callus, an unorganized mass of cells, is a pivotal stage in in vitro culture, offering advantages such as reliable production of therapeutic metabolites without impacting wild plant populations [20,21]. In our previous studies on *C. montanus*, an efficient protocol for rapid micropropagation and secondary metabolite production was documented for the first time [1]. There is limited research on the phytochemical [10,22] and biological activities [12-16] of *C. montanus* extracts; therefore, this work enriches the understanding of the total phenolic content and antioxidant activity present in micropropagated plants.

This study was conducted to achieve the following objectives: to initiate in vitro cultivation of *C. montanus* using different hormone combinations for callus multiplication and in vitro germination of *C. montanus* seeds; to determine the volatile components Terpinen-4-ol and Eucalyptol (1,8-Cineole) using gas chromatography-mass spectrometry (GC-MS) analysis in micropropagated samples, and compare these outcomes with those of wild and greenhouse plants; to compare the total phenolic compounds, such as gallic acid, in wild and in vitro cultures; and to evaluate antioxidant properties using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

2. MATERIALS AND METHODS

2.1. Plant material and sterilization

C. montanus seeds were harvested from indigenous plants located in the Al-Kamsheh region within the Zarqa governorate, Jordan (32°07′28.4″ N; 35°53′22.1″ E) in November 2020 (Figure 1, A). After collection, the seeds underwent meticulous germination under controlled greenhouse conditions at the Agriculture Institute, The University of Jordan (Figure 1, B). Leaves obtained from the wild plants underwent a systematic disinfection procedure, involving a 30-second exposure to 70% (v/v) ethanol within a laminar airflow environment (ESCO Labculture® Class II Type A2 Biological Safety Cabinet, Esco Micro Pte. Ltd., Singapore). Following this, the plant material was subjected to a one-minute treatment with a 1% (v/v) solution of sodium hypochlorite (NaOCl) enriched with a small quantity of Tween® 20. Post-treatment, the explants underwent rigorous washing, involving six cycles with sterilized distilled water.

2.2. Callus Induction and Culture Medium

In our previous published paper, we provided detailed information on the specific media compositions used for callus culture induction. optimal culture conditions were observed when disinfected leaves were aseptically cultured on (MS) media enriched with 0.5 $mg \cdot L^{-1}$ (2, 4-D) and 1.5 $mg \cdot L^{-1}$6-Furfurylaminopurne (Kin) , along with 30 g sucrose per liter, 100 $mg \cdot L^{-1}$ myo-inositol. The pH of the medium was ranged between 5.7-5.8, and 6 g/l agar (Bacto-agar, Difco, India) was added following established protocols [1]. The resulting medium was subjected to autoclaving at 121 ◦C and 1.06 $kg \cdot cm^{-2}$ for 15 minutes, and subsequently, 25 mL aliquots were distributed to Petri dishes. The aseptically treated explants were horizontally positioned on the medium and maintained in complete darkness at 25 ± 2 ◦C for eight weeks.

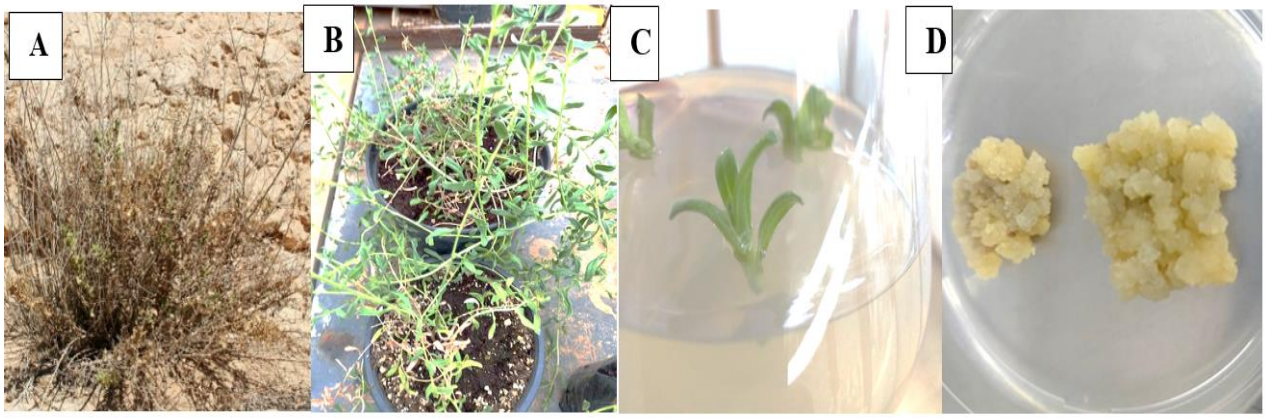

Figure 1. (a) Wild plant (WP) *C. montanus* (Vahl.) Brullo in the natural site; (b) Green house plant of *C. montanus*; (c) Germinated plant *in vitro*, and (d) callus culture.

2.3. Callus Multiplication

Following callus induction, 0.5 g of callus was transffered to (MS) media supplemented with varying combinations of (BAP), Thidiazuron (TDZ), or (Kin) at concentrations of 0.5, 1, and 2 mg·L^{-1}, along with 1.0 mg·L^{-1} (2, 4-D). Incubation occurred in total absence of light at 24 °C, resulting in the proliferation of calli, which were then utilized in subsequent experimental procedures. Employing a completely randomized design (CRD), each experimental condition was replicated 10 times (each replicate comprised two 0.5 g callus pieces within individual culture plates). Upon completion of the incubation period, measurements were taken for callus diameter, fresh weight, texture, and color. Statistical analyses were conducted utilizing SAS software (version 9, SAS Inc., Cary, NC, USA), employing analysis of variance (ANOVA) for each experiment. Mean separation was performed at a significance level of 0.05 using Tukey's Honestly Significant Difference (HSD) test. The growth regulators utilized in this study were sourced from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. The optimal callus multiplication medium, determined by superior callus growth characterized by increased fresh weight and morphological alterations, was subsequently employed for callus proliferation.

2.4. *In vitro* Shoot Germination

To determine the optimal basal medium formulation for maximizing seed germination, four distinct formulations were assessed: (1) Half-strength (MS) medium supplemented with 15 g sucrose, 2.2 g MS salts, 0.5 mg·L^{-1} (GA_3), and 1 mg· L^{-1} (BAP).(2) Full-strength MS medium containing 30 g sucrose, 4.4 g MS salts, and 2 mg· L^{-1} GA_3.(3) Full-strength MS medium supplemented with 0.1 mg· L^{-1} α-naphthaleneacetic acid (NAA) and 1.0 mg· L^{-1} BAP.(4) Full-strength MS medium supplemented with 0.5 mg· L^{-1} (NAA) and 2.0 mg· L^{-1} BAP. Prior to solidification with 8 g agar (Bacto-agar, Difco, India), the pH of each medium was adjusted to 5.8. Petri dishes containing inoculated seeds were incubated in a culture room under a 16-hour photoperiod with an irradiance of 56 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by cool white fluorescent lamps (Philips, Germany) at 25 ± 2°C. A CRD was employed, with each treatment replicated 10 times, and each replicate comprising ten seeds in a Petri dish. Following a 4-week incubation period, the percentage of seed germination was calculated using the formula: [Seed germination percentage] = [Number of seeds germinated] \ [Total number of seeds] times [100%]. Data were analyzed statistically as described in callus culture experiments.

2.5. Preparation of Extracts

This study utilized four distinct samples of *C. montanus* plant extracts, including extracts from the wild plant collected in November 2020 from Alkamsheh, Zarqa, one-month-old microshoots cultivated in shoot maintenance media (comprising half-strength MS media, 0.5 $mg \cdot L^{-1}$ GA^3, 1.0 $mg \cdot L^{-1}$ BAP, and 0.1 $mg \cdot L^{-1}$ NAA), callus grown in callus maintenance media (consisting of full-strength MS media, 0.5 $mg \cdot L^{-1}$ 2, 4-D, and 1.5 $mg \cdot L^{-1}$ Kin), and greenhouse plants (Figure 1).

The preparation of all plant extracts involved soaking 1.3 g of dried plant samples in 100 ml of HPLC-grade methanol (80%, technical quality, Algol, Espoo, Finland), with subsequent agitation in darkness for one week. Following filtration through a 110 mm filter paper (Whatmann), the filtrate obtained was moved into a 250 ml round-bottom flask for each material. Methanol was then evaporated from the filtrate using a rotavapor (Heidoph machine LABORATA 4000, Germany) operating at 60 rpm and 62 °C until complete evaporation, leaving only the extract in the flask. The net weight of the derived concentrate was determined and subsequently solubilized using 10 ml of methanol.

2.6. Determination of Terpinen-4-ol and Eucalyptol (1, 8-Cineole) Content

Identification of the key compounds, Terpinen-4-ol and Eucalyptol (1, 8-Cineole), within the essential oil of *C. montanus* from the four samples were performed using gas chromatography-mass spectrometry (GC-MS), as described previously [1]. GC-MS analyses were conducted utilizing a Varian chrompack CP-3800 C/MS/MS-200 instrument (Saturn, The Netherlands), equipped with a DP-5 (5% diphenyl, 95% dimethyl polysiloxane) GC capillary column (30 m × 0.25 mm × 0.25 µm film thickness). Peak identifications, along with specific retention times, were determined employing a mixture of (C8–C20) alkanes in hexane (Sigma Aldrich, Saint Louis, MI, USA), which was injected under the same conditions outlined for GC-MS analysis. Retention indices were subsequently calculated. External reference standards for Terpinen-4-ol and Eucalyptol (1, 8-Cineole) were procured from Sigma Standard SAFC supply solution, USA. The identification process involved matching the recorded mass spectrum of these compounds with the NIST library of mass spectral database (National Institute of Standards and Technology, Standard Reference Data Program, Gaithersburg, MD 2089, USA). The relative abundance of each identified compound was determined by dividing the peak area of the specific compound by the total area encompassing all identified peaks [24]. To quantify the area under the peak, a standard curve was employed. Each plant sample was assayed by GC-MS in three replicates.

2.7. Assessment of Total Phenolic Content

The Folin–Ciocalteau assay, as outlined by Singleton et al. (1999), was employed to assess the total phenolic content within extracts obtained from callus, wild plant, and microshoots of *C. montanus*. briefly, 0.5 ml of each methanol concentrate (mg/ml) was aliquoted into a 10 ml volumetric flask. Subsequently, 2.5 ml of deionized water was added, followed by the introduction of 2.5 ml Folin–Ciocalteu reagent 0.2 N (Sigma-Aldrich Chemie, Stieinheim, Germany), with thorough mixing for 3 minutes utilizing a Vortex (IKA Genius350Hz Vortex Mixer Genius3 50Hz 500 - 2500rpm). Following this, 0.5 ml of 10% sodium carbonate Na_2Co_3 (Labosi, Paris, France) was incorporated, and the volume was adjusted to 10 ml with deionized water, followed by incubation in darkness for two hours. The resultant green-blue complex was spectrophotometrically measured at 765 nm (Model UVD-2950; Labomed Inc., Los Angeles, CA, USA), and the wavelength was standardized to 765 nm using a blank sample containing 0.5 ml of methanol without extract. a calibration curve was generated using gallic acid serving as the standard (Figure 2).

The overall phenolic compound contents (mg/g) were

quantified as gallic acid equivalent (GAE) per gram of extract, utilizing the regression model derived from the established standard curve:

[y = 0.0741x - 0.0114, \ R^2 = 0.9955]. In this context, Y represents absorbance, and x denotes gallic acid concentration in ppm. All measurements were conducted in triplicate.

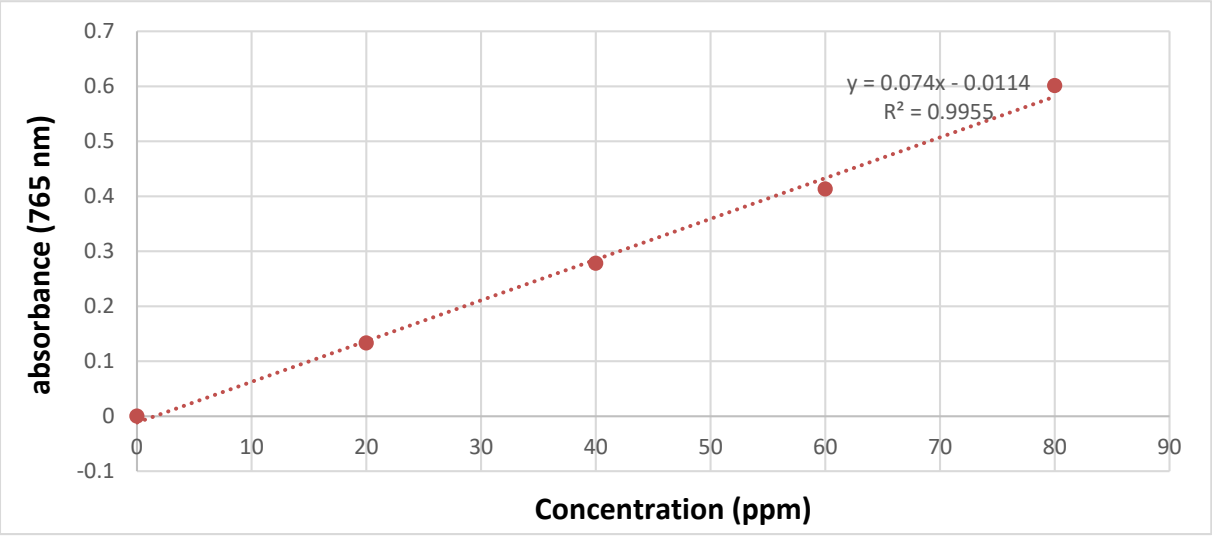

Figure 2: Gallic acid calibration curve using concentrations: 0, 20, 40, 60, and 80 ppm. The regression model was derived by correlating absorbance values at 765 nm with gallic acid concentration.

2.8. Assessing antioxidant Activity Measurement through DPPH Radical Scavenging assay

The assessment of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging was conducted to evaluate the antioxidant capacity in the methanol extract from four plant samples, following the experimental procedure outlined by Piątkowska et al. (2022) with a modification of 30-minutes of exposure at room temperature in the dark, deviating from the original 24-hour duration. Methanolic DPPH was prepared by dissolving 24 milligrams of DPPH (Sigma Aldrich, Saint Louis, MI, USA) in 100 ml of methanol HPLC grade 80%. Subsequently, 100 µL of various concentrations (12.5-400 µg/ml) of the plant extracts, along with positive and negative controls, were introduced to 100 µL of methanolic DPPH in a 96-well microplate. Ascorbic acid served as the positive control in the experimental setup. Initially, 10 mg of ascorbic acid powder was solubilized in 1 mL of PBS (phosphate-buffered saline), followed by mixing thoroughly. Subsequently, triplicate volumes of 100 µL each were prepared for ascorbic acid concentrations of 2, 6, 10, 17, 22 µg/ml. The mixture was homogenized using a pipette tip, and following 30-minutes exposure in darkness at room temperature, absorbance values were quantified at 517 nm using a UV-VIS spectrophotometer in a microplate reader (BIO-TEK-UQANT-MXQ200). The percentage of DPPH radical inhibition was computed using the formula: Inhibition % = [(Abs control - Abs sample/standard) / (Abs control)] x 100, where Abs control is the absorbance of the control reaction (0.1 ml of methanolic DPPH), and Abs sample is the absorbance of the sample reaction (0.1 ml sample dispersed in methanolic DPPH).

The DPPH scavenging activity of the extracts was represented through the IC_{50} value, indicating the essential

oil concentration required to scavenge 50% of DPPH free radicals, in accordance with the approach outlined by Brand-Williams et al. (1995). Determination of IC_{50} for each sample involved plotting percentages of inhibition against sample concentrations, using a nonlinear regression model through GraphPad Prism 8. The experiment was conducted in triplicate for robustness and reliability.

3. RESULTS AND DISCUSSION

3.1. Initiation of *C. montanus* cultures

C. montanus green house plants were grown from seeds sourced from (WP). Microshoots, derived from *in vitro* germinated seeds, were subcultured every four weeks to generate extracts. Leaves from wild plants were cultured for two months, producing friable callus utilized for extract preparation (Figure 1 a-d).

3.2. Callus multiplication

Callus proliferation was achieved by subjecting 0.5 g of fresh callus to a blend of phytohormones, as detailed in Table 1. Optimal results, including the maximum fresh weight (1.59 g) and diameter of the callus (3.08 cm) exhibiting a yellow color and friable texture, were noted on MS media enhanced with 1.0 mg·L^{-1} 2, 4-D and 2.0 mg·L^{-1} BAP. Subsequently, MS media enriched with 1.0 mg·L^{-1} 2,4-D and 2.0 mg·L^{-1} Kin yielded friable and yellow-brown callus, presenting a fresh mass of 1.45 g and a callus diameter of 2.6 cm. Other callus induction intervention displayed limited growth, featuring pale brown, compact-textured calli. The synergistic influence of cytokinins and auxins played a pivotal role in fostering callus formation [28]. Various factors, such as explant source, medium composition, and *in vitro* culture conditions (temperature, light, humidity), contribute to the intricacies of callus induction [29].

In the context of *A. adenophora* (Asteraceae), leaves served as explants, and effective callus induction was attained among of 0.5 mg·L^{-1} 2,4-D and 2.0 mg·L^{-1} BAP [30]. Similarly, optimal callus development was achieved in *Dieffenbachia* cv. Camouflage leaf explants through treatment of 1.0 mg·L^{-1} 2, 4-D and 2.0 mg·L^{-1} BAP [31]. Successful callus initiation in *Ruta graveolens* was demonstrated on MS media fortified with 1.0 mg·L^{-1} 2, 4-D and 1.5 mg·L^{-1} Kin [32]. The *Elephantopus scaber* plant, affiliated to the Asteraceae family, necessitated a synthesis of 2,4D and Kin for successful callus induction [33].

Table 1. The influence of 1.0 mg·L^{-1} 2, 4-D in combination with different concentrations of BAP, TDZ, or Kin (0.5, 1, 2 mg·L^{-1}) on callus multiplication from leaf explants of *C. montanus* (Vhal.) Brullo was assessed four weeks post-induction.

Growth regulators(mg·L^{-1})	Callus weight (g)	Callus diameter (cm)	Callus color	Callus texture
Free	0.10 g	0.36 g	Brown	Compact
1.0 (2,4-D) +0.5(kin)	1.19 d	2.12 cd	Yellow	Friable
1.0 (2,4-D) +1.0(kin)	1.15 d	2.06 d	Yellow+brown	Compact
1.0 (2,4-D) +2.0(kin)	1.46 b	2.60 b	Yellow+light brown	Friable
1.0 (2,4-D) +0.5(BAP)	1.27 c	2.25 c	Yellow+light brown	Friable
1.0 (2,4-D) +1.0(BAP)	1.14 d	2.06 d	Yellow+light brown	Friable
1.0 (2,4-D) +2.0 (BAP)	1.59 a*	3.08 a*	Yellow	Friable
1.0 (2,4-D) +0.5 (TDZ)	0.76 f	1.08 f	Yellow+brown	Compact
1.0 (2,4-D) +1.0 (TDZ)	0.87 e	1.56 e	Yellow+brown	Compact
1.0 (2,4-D) +2.0 (TDZ)	0.71 f	1.04 f	Yellow+brown	Compact

* Mean values with different letters are significantly different according to Tukey's HSD test at $p < 0.05$.

3.3. *In vitro* seeds germination

In the present study, the highest *in vitro* germination of *C. montnus* seeds (11.6 ± 2.22 %) was achieved using half strength (MS) media supplemented with 0.5 mg·L^{-1} (GA_3), and 1 mg·L^{-1} (BAP). Very low seed germination rates were obsreved with the following media; (MS media + 2.0 mg·L^{-1} GA_3) (5.2± 0.79 %), (MS with 0.1 mg·L^{-1} NAA and 1.0 mg·L^{-1} BAP) (2.3±0.67 %) and (0.9± 0.47 %) recorded for seeds inoculated in (MS media + 0.5 mg·L^{-1} NAA and 2.0 mg·L^{-1} BAP) (Table 2).

Table 2. Effect of different media types on seed germination (n = 10) after four weeks of *in vitro* culture of *C. montanus*.

Medium	Seeds germination percentage (%)
MS 1⁄2 +0.5 mg·L^{-1} GA_3+1.0 mg·L^{-1} BAP	11.6 ± 2.22 a*
MS +2.0 mg·L^{-1} GA_3	***5.20 ±0.79 b***
MS +0.1 mg·L^{-1} NAA+1.0 mg·L^{-1} BAP	***2.30 ±0.67 c***
MS +0.5 mg·L^{-1} NAA+2.0 mg·L^{-1} BAP	***0.90 ±0.47c***

* Mean values with different letters are significantly different according to tukey's hsd test at $p < 0.05$ ± standard deviations.

Even today, there are no reports concerning in vitro seed germination of C. montanus or other members of Chiliadenus species. This delay could be related to the poor in vitro seed germination percentage encountered during our study. The Murashige and Skoog formulation is the most commonly used medium in plant tissue culture experiments [34]. In vitro germination experiments for some Asteraceae species observed that the seeds of Centaurea arifolia were very difficult to germinate, with a germination percentage of around 25% after three weeks when seeds were inoculated on half strength (MS) media [35].

This result is in agreement with Okay and Günöz (2015) who showed that germination percentage of *C. tchihatcheffii* seeds were quite low. Yüzbaşıoğlu, et al. (2012) compared different types of MS media with or without BAP, NAA, IBA, and 2,4-D at different concentrations and combinations, and show that half strength (MS) media was the best for seed germination. The low germination percentage of *C. montanus* seeds could be related to deep dormancy resulting from physiological factors such as hormones or other environmental factors [37]. The failure of full-strength MS media could be linked to osmotic pressure, thus the need for low nutrients [38]

3.4. Determination of Terpinen-4-ol and Eucalyptol (1, 8-Cineole) Content

A calibration curve was established for Terpinen-4-ol and Eucalyptol (1,8-Cineole) reference standard solutions, facilitating the quantification of Terpinen-4-ol and Eucalyptol content in volatile oils obtained from wild, microshoot, and callus samples (Figure 3). The elution times for Terpinen-4-ol and Eucalyptol were 19.8 min and 10.4 min, respectively (Figure 4, 5). A three-point linear calibration curve spanning the range of 0.005-0.02 µg/mL (0.005, 0.01, and 0.02 ppm) resulted in r2 values of 0.9878 and 0.997 for Terpinen-4-ol and Eucalyptol, respectively.

Microshoots exhibited a higher Terpinen-4-ol content at 0.01 ±0.003 mg/g compared to the minimal concentration in wild plants at 0.001 ± 0.0002 mg/g, while callus samples showed no detectable Terpinen-4-ol. Regarding Eucalyptol concentration, *in vitro* microshoots demonstrated a comparable level (0.06 ±0.001 mg/g) to wild plants, whereas callus tissues exhibited significantly lower potential for Eucalyptol production at 0.003 ±0.0002 mg/g (Table 3).

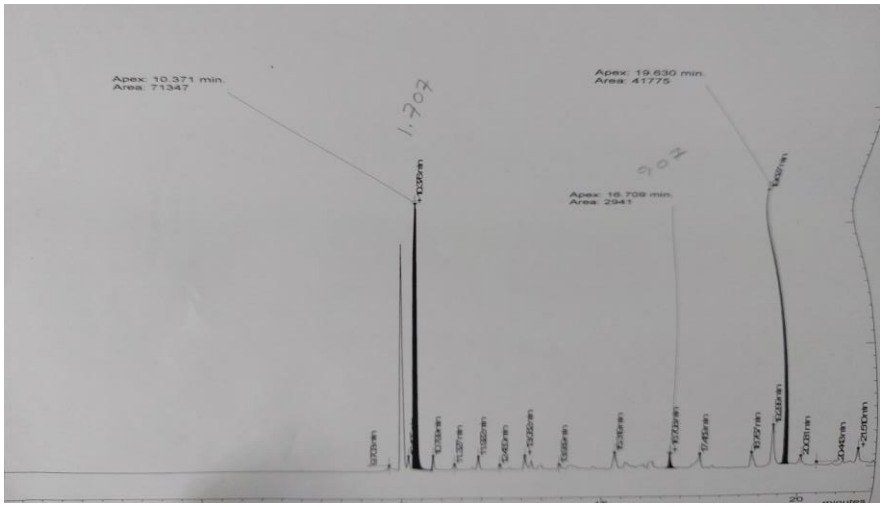

Figure 3. GC-MS chromatogram for standard Eucalyptol and Terpinen-4-ol.

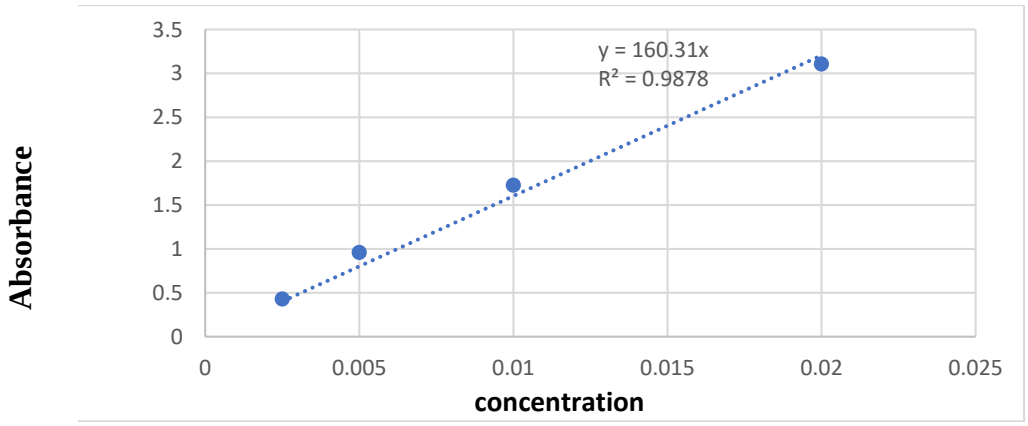

Figure 4: Standard calibration curve of Terpinen-4-ol.

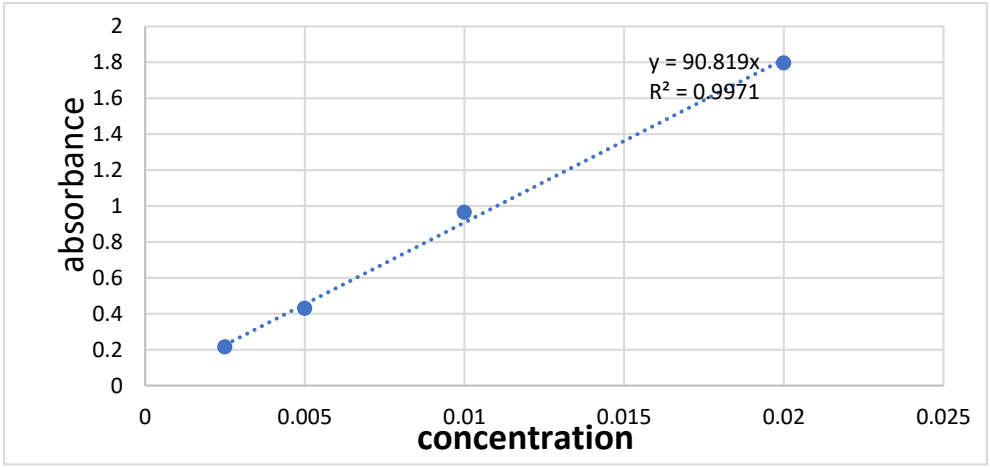

Figure 5. Standard calibration curve of Eucalyptol (1, 8-Cineole).

Table 3. Average Terpinen-4-ol and Eucalyptol (1, 8 Cineol) content (ppm) of different *C. montanus* plant extracts.

Extracts	Concentration (ppm)	
	Terpinen-4-ol	Eucalyptol(1, 8 Cineol)
Callus	0.0 ±0 c	0.003 ±0.0002 b
Wild Plant	0.001 ± 0.0002 b	0.06 +0.001 a
Microshoot	0.01 ±0.003 a	0.06±0.001 a

* Mean values with different letters are significantly different according to Tukey's HSD test at $p < 0.05$ ± standard deviations.

Zito et al. (2013) investigated the volatile oils derived from the leaves and flowers of the wild plant *Chiliadenus lopadusanus* through hydrodistillation and GC-MS analysis, revealing a notable concentration of 1,8-cineole (3.8%). In the Jordan Valley, essential oils from *Chiliadenus iphionoides* were extracted using hydrodistillation, and subsequent GC-MS analysis identified 44 components, with 1,8-cineole (8.4%) and camphor (3.7%) as the principal constituents [40].

Variations in essential oil content among different plant samples may be attributed to the auxins or cytokinins employed in in vitro culture media [41]. Various factors impact the chemical constituents of essential oils in the field, including abiotic factors such as water, light, temperature, soil pH, and salinity, as well as biotic factors encompassing soil microorganisms [42, 43]. Postharvest handling also plays a role in the quantity and quality of secondary metabolite production, with research indicating that drying plant material before extraction enhances oil yield [44, 45]. Moreover, the extraction procedure itself can impact the chemical constituents of the extracted essential oil [46].

Genetic factors exert a significant influence on the observed variations between wild plants and those cultivated in vitro [47]. In vitro cultivation techniques frequently introduce genetic alterations, arising from the manipulation of tissue culture conditions and hormone combinations [48]. These alterations induce changes in gene expression, metabolic pathways, and, consequently, the biosynthesis of secondary metabolites such as Terpinen-4-ol and Eucalyptol. Wild plants have developed within specific ecological habitats, resulting in a natural genetic makeup. Conversely, in vitro cultivation enforces artificial growth conditions [49].

In vitro propagation techniques often involve clonal propagation methods, which may reduce genetic diversity compared to sexually reproducing wild populations [50]. This reduction in genetic variability can significantly influence the secondary metabolite profile of in vitro cultivated plants relative to their wild counterparts [51].

3.5. Total Phenolic Content

Distinct concentrations of phenolic compounds, expressed as gallic acid, were obtained through methanol extracts from wild, green house and *in vitro* samples (callus and microshoots) of *C. montanus*. The wild plant exhibited the highest yield of phenolic compounds, reaching 30.67 ±2.82 gallic acid equivalent (GAE) per gram of dry mass, followed by *in vitro* microshoots at 22.81± 0.65 of GAE per gram of dry mass, with the lowest amount recorded in callus at 6.37 ±0.27 of GAE per gram of dry weight (Table 4). Analogous findings were reported by Al Khateeb et al. (2012), who observed that wild *Cichorium pumilum* Jacq. produced more phenolic compounds compared to *in vitro* samples. Barral-Martinez et al. (2021) also documented similar results, noting that wild *Achillea millefolium* contained 110 mg/g dry extract of phenolic compounds using heat-assisted extraction. Additionally, Eissa et al. (2013) indicated a total phenolic content of 107.4 mg/g sample (GAE) in hydroalcoholic extracts of wild *C. montanus* using the Folin-Ciocalteau method.

Table 4. Average total phenolic content GAE (mg/g) of different *C. montanus* plant extracts.

Extracts	Phenolic Content
Callus	6.37 ±0.27 d
Wild	30.67 ±2.82 a
Microshoot	22.81± 0.65 c
Green house	26.38 ±2.11 b

* Mean values with different letters are significantly different according to Tukey's HSD test at $p < 0.05$± standard deviations.

Discrepancies in gallic acid equivalent (GAE) values obtained from methanolic extracts between wild, greenhouse, and in vitro cultures may be associated with variations in the chemical constituents of plant samples, likely influenced by environmental conditions [56]. The elevated concentration of total phenolic content could be explained by differences in growth conditions, extraction methods, and solvents used during extraction [46].

Understanding the interaction between genetic and environmental factors is essential for explaining the mechanisms of phenol content variation in both wild and in vitro cultured plants [57]. Genetic factors include genotypic variation among plant populations as well as differential expression of genes involved in phenolic compound biosynthesis [47]. Environmental factors, on the other hand, affect phenol content by influencing plant metabolism and enzymatic activities. Factors such as light intensity, temperature fluctuations, nutrient status, and soil composition profoundly influence phenol content by affecting plant physiological responses and metabolic pathways [58].

3.6. Antioxidant Activity

In experimental conditions, reference ascorbic acid was examined at six varying concentrations, demonstrating scavenging effects on DPPH radicals, as indicated by its IC50 value (18.71±0.41 µg/ml) (Figure.6)

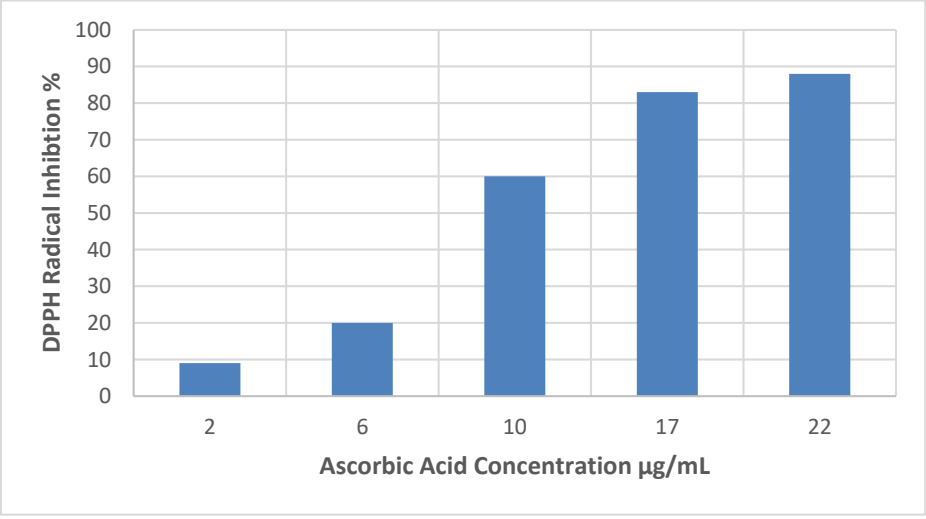

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Figure 6. Ascorbic acid's inhibition of DPPH radicals. The reported are triplicates mean values.

In the current study, the antioxidant functionality of methanolic extracts from various samples of *C. montanus*, including wild, greenhouse-grown, and in vitro samples (callus and microshoots), was investigated using the DPPH scavenging assay. The findings are presented in Table 5. The antioxidant potential was assessed using the DPPH assay as described by Gulcin and Alwasel (2023). In this assay, the stable DPPH free radical, initially deep violet-

purple, reacts with the methanol plant extract, causing a reduction and conversion to the more stable product DPPH-H, resulting in a pale yellow or colorless solution. The discoloration was measured at an absorbance of 517 nm using a spectrophotometer (Figure 7) [60].

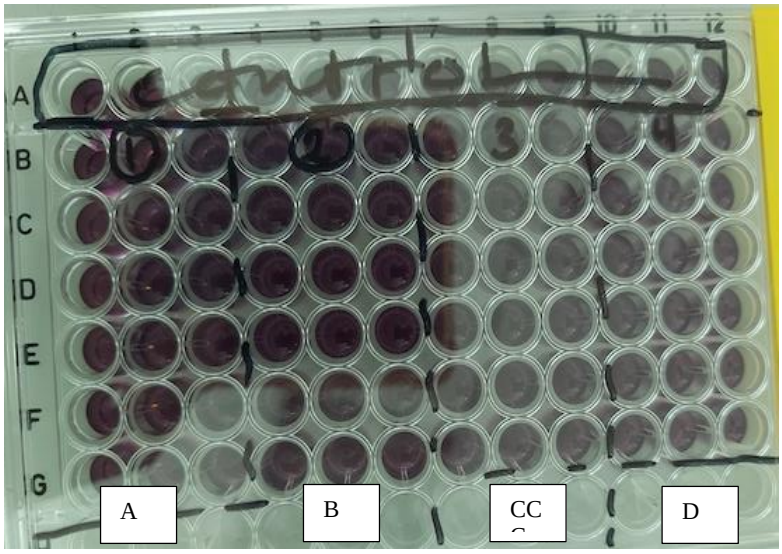

Figure 7: 96 well microplates, showing the changes in *C. montanus* methanolic extract color. A) callus, B) microshoots, C) green-house plant, D) wild plant.

A decreased IC50 value corresponds to heightened antioxidant activity, representing the compound's effectiveness in inhibiting the DPPH free radical by 50%. The same equation employed in the previous DPPH method was employed to determine the free radical scavenging capacity IC50. The results demonstrate significant variation in free radical scavenging activity among different *C. montanus* plant samples (Table 5). The DPPH activity of wild *C. montanus* methanolic extract (IC50 32.13 ±0.83 µg/ml) exhibited greater effectiveness compared to greenhouse-grown plants (IC50 221.04 ±1.34 µg/ml), while *in vitro* samples (microshoots and callus) showed low activity.

Table 5. Antioxidant activity represented as IC_{50} values (µg/ml) of *C. montanus* methanolic extract, values are mean of three replicates.

Extracts	**IC_{50} µg/ml**
Callus	>8000 ±5.2 c
Wild	32.13 ±0.83 a
Microshoot	>8000 ±5.2 c
Green-house plant	221.04 ±1.34 b
Ascorbic acid (standard)	18.71±0.41

* Mean values with different letters are significantly different according to tukey's hsd test at $p < 0.05$± standard deviations.

This finding was analogous to results obtained previously by Eissa et al., 2014, who have studied the antioxidant activity of *C. montanus* essential oil using ORAC assay. Eissa et al. (2014) founds that *C. montanus* have scavenging activity (39±0.18 µg/ml). Researches showed that using methanol extract produce the maximum DPPH radical scavenging activity compared to water extract [61]. The methanolic extracts of *Grammosciadium platycarpum* (Asteraceae) displayed (IC50 45±2.1 µg/ml) high level of antioxidant scavenging activity of DPPH [62]. Antioxidant activity of *Pluchea indica* (Asteraceae) was determined using DPPH assay (16.66 ±0.08µg/ml) [63].

The difference in antioxidant activity of phenolic compounds between in vitro-cultured plants and wild plants can be attributed to various factors, including altered growth conditions, stress responses, and genetic variations [46,47]. In vitro conditions may lack certain environmental stresses that contribute to the development of phenolic compounds in wild plants [64]. Additionally, the tissue culture process can influence gene expression patterns, leading to variations in secondary metabolite production [65,66].

Finally, considering that our experiments were conducted using only a low dose of the compounds (1.3 g/100 ml methanol) for the antioxidant evaluation of phenolic compounds, it is recommended to conduct further studies at higher doses using different techniques, such as the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay, Ferric Reducing Antioxidant Power (FRAP) assay, Total Antioxidant Capacity (TAC) assay, and Oxygen Radical Absorbance Capacity (ORAC) assay, to obtain a comprehensive understanding of the antioxidant potential of plant extracts.

4. CONCLUSION

Conclusively, this study successfully established a productive in vitro production system for phenolic compounds in *Chiliadenus montanus*. The callus culture multiplication method was optimized, and in vitro shoots exhibited elevated concentrations of Terpinen-4-ol. Phenolic compound analysis revealed varying amounts between wild and in vitro cultures, with the highest phenolic content observed in wild plants. The antioxidative potential, assessed through the DPPH radical scavenging assay, indicated superior radical scavenging activity in wild plants compared to greenhouse-grown plants. *C. montanus* emerges as a potential natural antioxidant source, providing a sustainable alternative to wild plant harvesting. The study offers valuable insights into callus tissue culture, phenolic compound synthesis, and antioxidant activities in *C. montanus*, suggesting a promising technique for in vitro culture and bioactive compound synthesis**.**

Acknowledgments :We sincerely acknowledge Prof. Dr. Mohanad Akash, Prof. Dr. Maher Al-Dabbas, Prof. Dr. Yasser Bustanji, Ismail Abaza, Basha'er Abu Irmaileh, Shireen Qasrawi, Saida Abu Maluh, Noor Alhourani and hayat alobedat for their invaluable assistance.

Conflicts of Interest: The authors declare no conflict of interest.

Funding: The Deanship of Scientific research\The University of Jordan to DAD (Grant number: 19-2020-99).

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المركبات الفينولية والنشاط المضاد للأكسدة في نبات الهنيدة البري *Chiliadenus montanus* (Vhal.) Brullo.

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ملخص

تستكشف هذه الدراسة زراعة نبات الهنيدة البري (العائلة النجمية) *Chiliadenus montanus* (Vhal.) Brullo مركزة على تكاثر الكالوس ،إنتاج مركبات الفينولية ،والنشاط المضاد للأكسدة. تم تحسين تحفيز الكالوس، عن طريق زراعة فرعية في وسط موراشيغ وسكوج المزود ب1.0 ملغم/ لتر حمض 2,4-ثنائي كلورو فينسكي الاسيتيك (2,4-D) و2.0 ملغم/ لترحمض 6–بنزيل أمينو بيورين (BAP). تم تحليل استخراجات الميثانول من النباتات البرية وعينات الاكثار الدقيق لتيربينين–4–أول ،يوكاليبتول (1،8–سينيول) ومحتوى الفينول الكلي . أظهرت البراعم الدقيقة تركيزًا مرتفعًا للتيربينين–4–أول (0.003± 0.01 ملغ/غ) مقارنة بالنباتات البرية، بينما كانت لديها مستويات متماثلة من اليوكالبتول (1،8–سينيول) (0.001± 0.06 ملغ/غ). أظهر تحليل المركبات الفينولية أعلى مستويات في النباتات البرية (2.82± 30.67 مكافئ حمض الجاليك) تلتها البراعم الدقيقة في الانابيب (0.65 ±22.81 مكافئ حمض الجاليك) وأدنى كمية في الكالوس (0.27± 6.37 مكافئ حمض الجاليك). أشارت الخصائص المضادة للأكسدة ،المقيمة من خلال اختبار (1,1–diphenyl-2- picrylhydrazyl DPPH)، الى فعالية فائقة في النباتات البرية (0.83± 32.13 IC50ميكروغم/مل) مقارنة بالنباتات الدفيئة (1.34± 221.04 IC50ميكروغم/مل) يظهر *C. montanus* مصدرًا محتملاً لمضادات الأكسدة الطبيعية ويعمل بمثابة مزيل فعال للجذور الحرة. في الختام، تم العثور على نظام فعال لانتاج المركبات الفينولية في المختبر لنبات *C. montanus*، مما يقدم بديلاً مستداماً لاستخدام النباتات البرية. تسلط الدراسة الضوء على الفوائد المحتملة لنبات *C. montanus*، كمخزن للمواد الحيوية الفعالة وتؤكد أهمية زراعته في المختبر للاستفادة المستدامة من الموارد.

الكلمات الدالة: زراعة الكالوس، البراعم الدقيقة، النبات الطبي، النشاط المضاد للأكسدة، المركبات الفينولية، *Chiliadenus montanus*.

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تاريخ استلام البحث 2024/01/16 وتاريخ قبوله للنشر 2024/05/18.