Article

Indirect Organogenesis of *Calendula officinalis* L. and Comparative Phytochemical Studies of Field-Grown and In Vitro-Regenerated Tissues

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Abstract: *Calendula officinalis* L. is an important medicinal and ornamental plant possessing multiple bioactive compounds. The in vitro plant regeneration method has recently replaced traditional field cultivation practices of calendula due to its fascinating phytochemical profile. In this study, callus formation and indirect organogenesis were described to establish an effective in vitro propagation strategy in *C. officinalis*. Using a gas chromatography–mass spectrometry (GC–MS) approach, the phytochemical content of tissues developed in vitro and field-grown was studied, and the biochemical contents were quantified and compared in various tissues. The incidence of callus formation from leaf explants was highest (94.44%) on MS medium fortified with 1.0 mg/L BAP and 1.0 mg/L NAA, which later became organogenic. On MS, 1.0 mg/L BAP and 1.0 mg/L NAA showed the highest indirect shoot proliferation (88.88%) efficiency. After being sub-cultured, the regenerated shootlets were cultured onto rooting medium containing different IAA/IBA concentrations; the best rooting percentage (94.44%) was achieved with 1.0 mg/L IBA. The biochemical parameters, like total phenolic content, flavonoid content, and DPPH scavenging activity, were measured. When compared to callus and field-grown developed leaf (donor) samples, all the biochemical characteristics of in vitro-produced leaf were noted to be higher. The methanolic extracts of leaf-callus and field-grown and in vitro-developed leaf tissues were subject to GC–MS-based phytocompound investigation. More than 45 therapeutically significant bioactive chemicals, like n-hexadecanoic acid, vitamin E, stigmasterol, and squalene were found in these samples. These results showed that the callus that is formed from in vitro leaf is a reliable and powerful source of several bioactive compounds with a wide range of medicinal uses. The successful stimulation of callus development, indirect organogenesis, biochemical analysis, and GC–MS confirmation of the presence of significant phytocompounds are all described in this study. This work provides a different avenue for ongoing and sustained synthesis of chemicals without endangering the surrounding ecosystem or native vegetation.

Keywords: pot marigold; indirect organogenesis; biochemical; GC–MS; antioxidant; phytochemical profiling

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1. Introduction

Calendula officinalis L., widely known as pot marigold, is a member of the Asteraceae family possessing brightly orange-colored flowers [1]. It is an important medicinal and ornamental herbaceous plant, cultivated globally across North American and European regions, and is indigenous to the Mediterranean region [2]. *C. officinalis* generally blooms in sunny locations and all types of soil and is widely considered by skilled gardeners for its versatility in flower colors [3]. Pharmacologically, various parts of *C. officinalis*

(leaves, flower, root) are known to possess multiple bioactive compounds with diverse medicinal properties, such as antiseptic, anti-inflammatory, wound healing, diaphoretic, stimulant, anti-ulcer, anti-spasmodic, anti-pyretic, anti-bacterial, and anti-fungal activities, etc. [4]. This herb's phytochemical composition involves terpenoids (lupeol, calenduloside), flavonoids (quercetin, isorhamnetin), coumarins (esculetin, umbelliferone), volatile oils (cubenol, α-cadinol), and quinones (phylloquinone, α-tocopherol) [5].

Due to its intriguing phytochemical profile, the conventional field cultivation of calendula plants is recently being substituted by the in vitro plant regeneration technique [6]. Plant tissue culture plays a major role in the proffering of secondary metabolites, wherein different plant parts, such as leaves, stems, roots, meristems, etc., are cultured under sterile conditions to obtain microbe-free healthy plants on a larger scale for continuous production of important secondary metabolites [7]. A callus, being an undifferentiated cell mass, has the ability to redifferentiate into a complete plant through somatic embryogenesis/organogenesis [8]. Furthermore, numerous studies have suggested that callus cultures can produce natural phytochemical compounds [9]. Compared to traditional breeding methods, the in vitro culture technology has a number of advantages, including quick propagation, germplasm preservation, polyploid production, genetic transformation, and agricultural improvement [10]. Recently, optimization of plant tissue culture protocol has been done in various plants, e.g., *Tagetes* spp. [11], *Allium sativum* [12], and *Andrographis paniculata* [13].

Stresses in cultures are mostly caused by in vitro circumstances, such as PGR concentrations and combinations, light intensity, relative humidity, and aeration in the culture vessels, as well as osmotic alterations [14]. The in vitro cultures often stimulate certain physiological events, leading to the activation of a reaction series, including the generation of reactive oxygen species (ROSs) and the accumulation of important secondary metabolites, such as polyphenolics, alkaloids, terpenoids, etc. [15], which can be measured by assessing several biochemical parameters and the phytocompound profiling of in vitro-regenerated tissues.

Gas chromatography–mass spectrometry (GC–MS) has become a popular approach for analyzing therapeutic compounds, such as volatile essential oils, fatty acids, lipids, alkaloids, etc. [16]. This technique is very useful for determining the relative quantities of the significant metabolites in a single sample analysis, like amino acids, small soluble sugars, polyamines, and organic acids, and demonstrates the widespread use of the GC–MS technique in the biomedical field [17]. This technique has been applied in different plants for metabolite profiling [18,19]. Until now, multiple reports of phytochemical profiling of the flower of *C. officinalis* have been proposed by the GC–MS technique [20–23]. But to date, no report is available regarding the phytochemical composition of the in vitro-regenerated tissues (callus and leaf tissues) of *C. officinalis.*

In the current study, an endured in vitro plant propagation protocol is described through indirect organogenesis. The primary objective of this study was to investigate and juxtapose the phytochemical (metabolite) profiles and biochemical variability among distinct cultured tissues, such as leaf-derived callus and field-grown and in vitro-raised leaf tissues. The outcomes of this study have the potential to enhance the continuous provision of pharmacologically significant bioactive compounds in the pharmaceutical sector using in vitro culture techniques.

2. Materials and Methods

2.1. Explant Collection and Sterilization

Immature leaves of *C. officinalis* (2 months old) were collected at the flowering stage from the herbal garden of Jamia Hamdard, New Delhi, India, and were used as explants. These explants were then surface sterilized using the protocol described by [24]. Initially, the explants were soaked in a 25% Teepol (detergent) solution for approximately 12 min, then were kept under running water for a few min. The later steps were carried out in laminar flow, wherein the leaf explants were sterilized with 70% (*v*/*v*) ethanol and 0.1%

(*w*/*v*) $HgCl_2$ for two min each. Further, to remove any remaining sterilizing agents, these were then thoroughly rinsed thrice with autoclaved distilled water.

2.2. Callus Induction and Growth Conditions

Surface sterilized leaf sections of *C. officinalis* were inoculated onto Murashige and Skoog (MS) medium [25] supplemented with sucrose (3%; *w*/*v*) and agar (0.8%; *w*/*v*). The medium's pH was calibrated at 5.7 with 1 N HCl and/or 1 N NaOH prior to sterilization at 121 °C for 15 min. For callus induction, the MS medium was amended with varied concentrations and combinations of plant growth regulators, specifically auxins [α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D)] and cytokinins [6-benzylamino purine (BAP)]. The culture vessels were maintained at a temperature of 23 ± 2 °C under white fluorescent illumination (55 $\mu mol/m^2/s^{-1}$) for a 16–8 h light–dark cycle with 55–60% relative humidity. Subsequent to each four-week interval, the cultures were sustained through transferring the developed callus to fresh MS medium supplemented with the same plant growth regulators. Following a four-week culture period, the percentage of callus induction and the fresh weight of the callus (in grams) were recorded.

2.3. Shoot Organogenesis via Indirect Method

To produce indirect organogenesis, proliferative calli obtained from leaf explants were cultured onto MS medium and treated with various concentrations and combinations of BAP (0.5–2.0 mg/L) and NAA (0.5–1.0 mg/L). After four weeks, the shoot induction rate (%) and average number of shoots per callus mass were determined.

2.4. Root Initiation and Acclimatization

In vitro regenerated shoots derived from organogenic callus were excised and transferred onto a root-inducing MS medium supplemented with varying concentrations and combinations of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). The number of roots generated per shoot and the percentage of root induction were recorded after a four-week incubation period, considering the effects of different auxin treatments. Subsequent to the removal of residual culture medium from the rooted plantlets, they underwent a cleansing process using sterile, double-distilled water before being transplanted into plastic pots filled with a sterile mixture of sand, soil, and soilrite in equal proportions. These potted plants were kept in a growth chamber set at 25 ± 2 °C, with a relative humidity of 70 ± 10% and a light intensity of 60 $\mu mol\ m^{-2}\ s^{-1}$ for two weeks. Thereafter, the plants were transferred to a growth chamber with controlled conditions of 25 ± 2 °C, humidity levels ranging from 55 to 60%, and a photoperiod lasting 11 to 12 h.

2.5. Preparation of Extracts

After being taken out, the leaf samples of *C. officinalis* that had both field-grown and in vitro-grown as well as leaf-derived callus were shade-dried for three days at ambient temperature. Using a mortar and pestle, around 1.0 g (dry weight) for each shade-dried sample was crushed into a fine powder. Each sample was then separately macerated using methanol solvent (10 mL) using a rotary shaker for 48 h. Next, Whatman No. 1 filter paper was used to filter the extracts. The filtered materials were then centrifuged for five min at 10,000 rpm, and the obtained supernatant was stored at 4 °C until it was needed.

2.6. Biochemical Attributes

2.6.1. Estimation of Total Phenolic Content

The Folin–Ciocalteu method [26] was employed for the determination of the total phenolic content (TPC) in the extracts. A mixture of 2.5 mL of 10% (*v*/*v*) Folin–Ciocalteu (FC) reagent (Sigma-Aldrich, St. Louis, MO, USA) and roughly 0.5 mL of the extract was utilized. The mixture was incubated at ambient temperature for approximately five to six minutes. Subsequently, 2.0 milliliters of a 7% solution of sodium carbonate were added, and it was incubated for 80 min. Following this, the absorbance was quantified at 765 nm

against the extract-free blank employing a UV–Vis spectrophotometer (Biolinkk, BL-295, Delhi, India). A calibration curve equation relating to the standard gallic acid solution was prepared and used to calculate the total phenolic content of the samples, which was assessed in triplicate. The results were expressed as gallic acid equivalents in milligrams per gram of dry weight (mg GAE/g DW).

2.6.2. Estimation of Total Flavonoid Content (TFC)

The procedure outlined by [27] was followed in order to measure the total amount of flavonoid (TFC). First, 1.0 mL of the extract solutions were combined with 0.2 mL of 10% aluminum chloride solution and 0.2 mL of 1 M potassium acetate solution. After 3.6 mL of distilled water was added and the mixture was allowed to sit at room temperature for 30 min, the reaction volume was increased to 5.0 mL. After fully mixing the solution, the absorbance of each sample at 415 nm was measured in comparison to a blank. Three copies of the measurement were made. Different quantities of quercetin (standard) were plotted against their relative absorbances on a graph. The TFC of the samples was reported in mg QE/g DW, or milligrams of quercetin equivalent per gm of dry weight.

2.6.3. Determination of Free Radical Scavenging Activity by DPPH Assay

The stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used to assess the scavenging activity of *C. officinalis* extract samples using the method described by [26]. Each test tube holding 3.0 mL of DPPH (0.024% *w*/*v*) and 0.1 mL of methanol combined with 3.0 mL of DPPH was used as a standard. A small amount of the extract solutions was applied to each test tube. The samples were later kept at room temperature for about 80 to 90 min in complete darkness. At 517 nm, the absorbance was finally measured.

$$\text{Scavenging activity \%} = (\mathrm{A_C} - \mathrm{A_S}/\mathrm{A_C}) \times 100$$

where $\mathrm{A_C}$ = absorbance of control and $\mathrm{A_S}$ = absorbance of sample.

2.7. GC–MS Analyses

Using the GC-MS-QP-2010 apparatus (Shimadzu, Tokyo, Japan), the GC–MS examination of methanolic extracts of leaf-derived callus was carried out in accordance with the program specifications. The beginning oven temperature was 100 °C, with a retention duration of 3 min, and it was progressively raised to 300 °C for 17 min. The helium gas carrier gas was maintained at a continuous flow of 1.21 mL/min; the injector temperature was set at 260 °C. In the GC–MS compound separation process, the Rxi-5Sil MS GC capillary column—30 m, 0.25 mm ID, 0.25 µm df—was employed as the column. The GC–MS operating period for all samples was 35 min, and the ion source and interfacial values were set to 220 °C and 270 °C, respectively. The identification of bioactive compounds in each sample was conducted through the utilization of the National Institute of Standards and Technology (NIST) mass spectral database. Additionally, retention indices, peak area, and peak area percentage were compared with previously determined phytocompounds using GC–MS solution software (Version 4.45 SP 1).

2.8. Statistical Analysis

A completely randomized design (CRD) was used for the in vitro tests. The information pertaining to how PGRs affect callus induction, somatic embryogenesis, and direct/indirect organogenesis on explants was presented as mean ± standard error. Every experiment was conducted twice, with three replicates for every experiment. Utilizing the program SPSS (version 15, Chicago, IL, USA), one-way ANOVA was used for the statistical analyses of the data. Duncan's multiple range test (DMRT) was used to determine the mean comparisons at $p < 0.05$ [28].

3. Results

3.1. Callus Induction and Proliferation

Leaf explants were cultured onto full-strength MS medium containing varying levels of BAP combined with NAA or 2,4-D (Figure 1A). A combination of 1.0 mg/L BAP and 1.0 mg/L NAA produced a high frequency of callus formation (94.44%), and the maximum fresh biomass (4.4 g/explant) was also achieved (Table 1, Figure 1B,C). On the contrary, the combined concentration of BAP (0.5 mg/L) and NAA (2.0 mg/L) generated a much lower amount of callus, with a frequency of 11.11% and a fresh biomass of 0.9 g/explant noted. The calli obtained were white, greenish, and friable in nature.

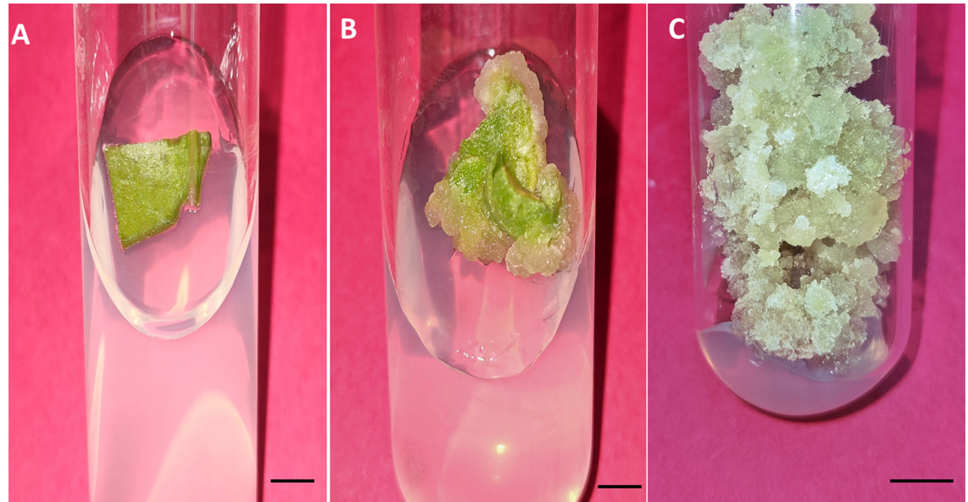

Figure 1. Callus induction and proliferation from leaf explant of *C. officinalis* onto MS medium supplied with 1.0 mg/L BAP and 1.0 mg/L NAA. (**A**) Leaf explant inoculated on PGR-containing MS medium, (**B**) callus initiation following 2-week period, and (**C**) callus proliferation following 4 weeks (Bars (**A**,**B**) = 1.0 cm, (**C**) = 0.5 cm).

Table 1. Effect of combinations of cytokinin (BAP) and auxins (2,4-D/NAA) on callus-inducing percentage and biomass growth using leaf explants of *C. officinalis* following 4 weeks of culture.

PGRs	Concentrations (mg/L)	Callusing Frequency (%)	Fresh Biomass (g)
Control	0	0^{f}	0^{e}
NAA + BAP	0.5 + 0.5	88.89 ± 5.56^{ab}	3.67 ± 0.8^{ab}
	0.5 + 0.1	61.11 ± 5.55^{bcd}	2.70 ± 0.6^{bcd}
	0.5 + 2.0	55.55 ± 14.69	2.50 ± 0.3^{bcde}
	1.0 + 0.5	72.22 ± 14.69^{abc}	3.30 ± 0.6^{abc}
	1.0 + 1.0	94.44 ± 5.56^{a}	4.40 ± 0.4^{a}
2,4-D + BAP	0.5 + 0.5	38.89 ± 11.11^{def}	1.60 ± 0.7^{cde}
	0.5 + 1.0	27.78 ± 5.56^{ef}	1.30 ± 0.6^{de}
	0.5 + 2.0	11.12 ± 5.55^{f}	0.90 ± 0.2^{e}
	1.0 + 0.5	27.78 ± 14.69^{ef}	1.10 ± 0.5^{de}
	1.0 + 1.0	44.44 ± 5.56^{cde}	1.93 ± 0.1^{cde}

Each given value represents means ± standard errors (n = 6/treatment) of three repeated experiments. Mean values followed by different letters within each column are significantly different from each other according to DMRT at $p \leq 0.05$ level.

3.2. Shoot Organogenesis via Indirect Method

After continuous subculturing of leaf-derived callus for about four weeks in the same PGR-amended medium, the shoot formation was noted with a varied frequency of 27.77% to 88.88% (Table 2, Figure 2). The best medium for both callus induction and shoot organogenesis was found to be the same, i.e., 1.0 mg/L BAP and 1.0 mg/L NAA showing the highest shoot induction (88.88%) ability with 3.33 mean shoot number/explant. On the other hand,

the lowest shoot induction percentage (27.77%) and 0.66 mean shoot number/explant was recorded in BAP- (0.5 mg/L) and NAA- (2.0 mg/L) amended MS medium.

Table 2. Effect of BAP and NAA combination treatments on indirect shoot organogenesis from leaf-derived callus in *C. officinalis*.

PGRs	Concentration (mg/L)	Frequency of Organogenesis (%)	Mean No of Shoot /Callus Mass
Control	0	0	0
NAA + BAP	0.5 + 0.5	0^{d}	0^{d}
	0.5 + 1.0	55.55 ± 05.5^{bc}	1.67 ± 0.3^{c}
	0.5 + 2.0	27.77 ± 11.1^{cd}	0.67 ± 0.3^{d}
	1.0 + 0.5	72.22 ± 20.1^{ab}	2.67 ± 0.3^{b}
	1.0 + 1.0	88.89 ± 05.6^{a}	3.33 ± 0.3^{a}

Each given value represents means ± standard errors (n = 6/treatment) of three repeated experiments. Mean values followed by different letters within each column are significantly different from each other according to DMRT at $p \leq 0.05$ level.

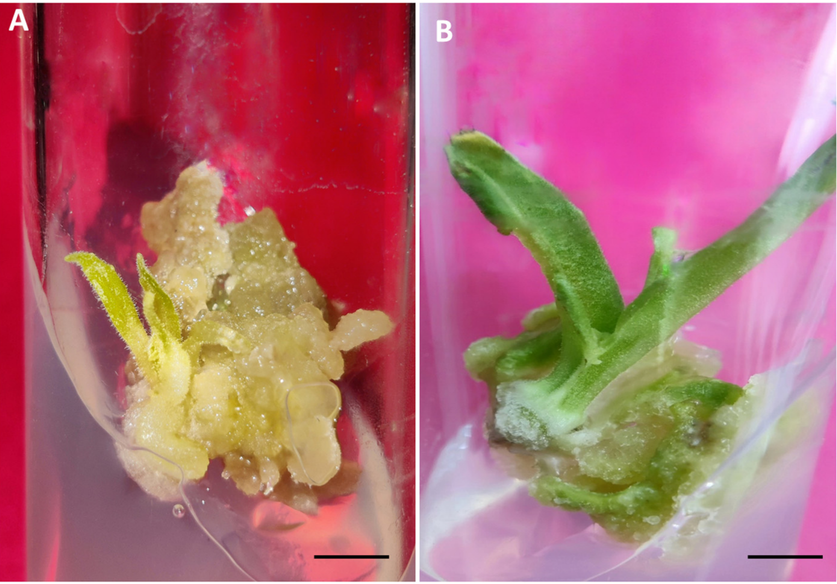

Figure 2. Indirect shoot organogenesis from leaf-derived callus of *C. officinalis* onto MS medium supplied with 1.0 mg/L BAP and 1.0 mg/L NAA. (**A**) Indirect shoot induction after 4 weeks of subculture (Bar = 0.5 cm), (**B**) shoot growth following 6-week period (Bar = 0.5 cm).

3.3. Rooting and Acclimatization

To achieve rooting of regenerants, two distinct auxins, viz., IBA and IAA, at different concentrations, were added to MS medium. In all rooting treatments, roots developed from the base of the shoots within three to four weeks. IBA treatments had a greater influence than IAA in terms of root induction percentage and average root numbers per shoot (Table 3, Figure 3A,B). A concentration of 1.0 mg/L IBA had the maximum rooting percentage (94.44%), with 12.3 roots per shoot, whereas 2.0 mg/L IAA had the lowest rooting percentage (22.21%), with 2.1 mean roots per shoot. Thicker roots were seen in IBA treatment, whereas fine and narrow roots were noted in IAA treatment. The in vitro-regenerated plants were later transferred to greenhouse conditions and showed a 75–80% survivability rate (Figure 3C).

Table 3. Effect of different PGR concentrations on rooting in *C. officinalis*.

PGRs	Concentration	Rooting (%)	Mean Root Numbers/Shoot
control	0	0 ^{e}	0 ^{e}
IBA	0.5	77.77 ± 5.56 ^{ab}	9.4 ± 1.4 ^{ab}
	1	94.44 ± 5.56 ^{a}	12.3 ± 0.5 ^{a}
	2	55.55 ± 14.70 ^{bc}	8.2 ± 1.1 ^{b}
IAA	0.5	49.01 ± 9.62 ^{c}	6.7 ± 1.8 ^{bc}
	1	38.89 ± 5.56 ^{cd}	4.4 ± 0.9 ^{cd}
	2	22.21 ± 5.55 ^{de}	2.1 ± 0.1 ^{d}

Each given value represents mean ± standard error (n = 6/treatment) of three repeated experiments. Mean values followed by different letters within each column are significantly different from each other according to DMRT at $p \leq 0.05$ level.

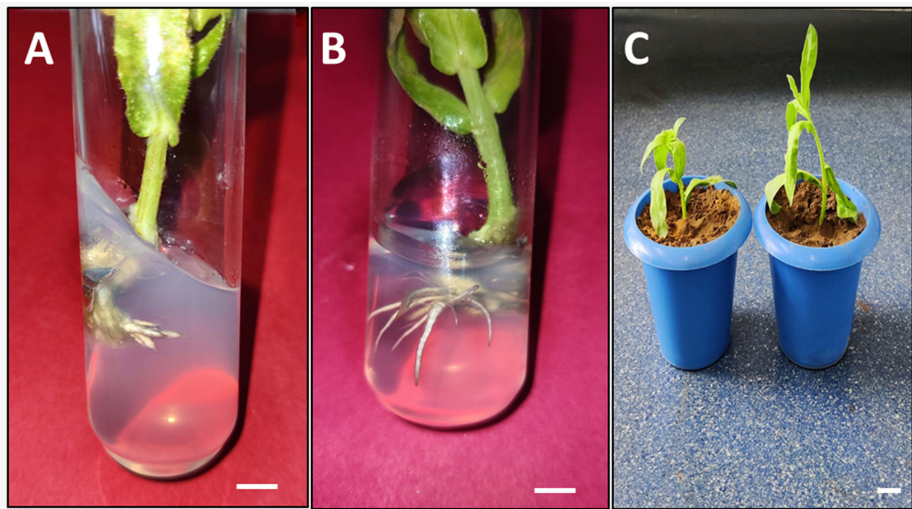

Figure 3. Root induction and acclimatization of in vitro-derived shoots of *C. officinalis*. (**A**) Initiation of rooting in MS medium containing 1.0 mg/L IBA (Bar = 1.0 cm), (**B**) further root development of in vitro-derived plantlets (Bar = 1.0 cm), and (**C**) transferred plantlets in pots (Bar = 2.5 cm).

3.4. Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and DPPH-Scavenging Activity

Using gallic acid as a reference, the Folin–Ciocalteu technique was employed for the quantification of the total phenolic content in each of the specimens. The highest phenolic content was observed in the leaf tissue cultured in vitro, followed by the leaf grown in the field, with the callus extract exhibiting the lowest content (Table 4). Specifically, the TPC value of the field-grown leaf extract was recorded at 8.51 ± 0.2 mg GAE/g DW, while the in vitro-grown leaf extract showed a TPC value of 10.28 ± 0.1 mg GAE/g DW. Conversely, the callus extract displayed the lowest TPC value of 1.43 ± 0.04 mg GAE/g DW.

Table 4. Content of total phenolic and flavonoid, and DPPH-scavenging activity of callus and leaf tissues of *C. officinalis*.

Sample Type	Total Phenolic Content (mg GAE/g DW)	Total Flavonoid Content (mg QE/g DW)	DPPH Scavenging Activity (%)
Callus	1.43 ± 0.04 ^{c}	8.25 ± 0.2 ^{b}	22.72 ± 4.11 ^{b}
Field-grown leaf	8.51 ± 0.2 ^{b}	15.55 ± 0.3 ^{a}	28.07 ± 3.11 ^{ab}
In vitro leaf	10.28 ± 0.1 ^{a}	16.04 ± 0.2 ^{a}	39.93 ± 4.81 ^{a}

Quercetin equivalent (QE), gallic acid equivalent (GAE), and dry weight (DW). The values show the mean ± standard error of three separate experiments. Mean values with different letters within the same column are significantly different from each other according to DMRT at $p \leq 0.05$ level.

Quercetin was utilized as the standard in the aluminum chloride method to determine the total flavonoid content across the different samples. The results were expressed as quercetin equivalent (QE) per gram of dry weight. Notably, the flavonoid content in the extracts exhibited a variation of approximately two-fold, ranging from 8.25 to 16.04 mg QE/g DW (Table 4). The leaf-derived calli presented the lowest TFC value of 8.25 mg QE/g DW, while the in vitro- and field-grown leaf extracts showcased TFC values of around 16.04 and 15.55 mg QE/g DW, respectively.

The DPPH free radical scavenging test was used to evaluate the antioxidant activity of the three extracts. The results, as demonstrated in Table 4, showed a similar trend observed in TPC and TFC. Notably, the leaf cultivated in vitro exhibited superior scavenging activity (39.93%) compared to the field-grown leaf (28.07%). Conversely, the callus extract displayed the least scavenging activity at 22.72%.

3.5. GC–MS Analysis

The GC–MS method was used in the current investigation to undertake metabolite profiling of tissues. The field-grown and in vitro-derived leaves and leaf calli of *C. officinalis* were used for the presence of phytocompounds. It was observed that all the samples, i.e., field-grown, in vitro-leaf, and leaf calli, contained more than 40 phytocompounds. In total, 55 phytocompounds were detected in the methanolic callus extract result (Table 5, Figure 4), many of which were present in minimal quantities when the phytochemical profiling of *C. officinalis* leaf callus was carried out. Phytocompounds including 5-hydroxymethylfurfural (30.23%), 2-palmitoylglycerol (9.95%), *n*-hexadecanoic acid (7.66%), pyranone (7.66%), stigmasterol (4.47%), squalene (0.42%), and vitamin E (0.41%) were detected as the versatile phytocompounds present in considerable concentrations in methanolic callus extract.

Table 5. GC–MS analysis revealed below phytocompounds in methanolic extract of callus of *C. officinalis*.

S. No.	R. Time	Area%	Name	Molecular Formula	Molecular Weight
1	4.763	0.52	1,2-butanolide	$C_4H_6O_2$	86
2	5.02	0.19	2-propylheptanol	$C_{10}H_{22}O$	158
3	5.551	0.92	5-methyifurfural	$C_6H_6O_2$	110
4	5.83	0.56	pyranone	$C_6H_8O_4$	144
5	6.186	0.43	1,4-diazabicyclo[2.2.2]octane	$C_6H_{12}N_2$	112
6	6.933	0.68	ethyl methylacetoacetate	$C_7H_{12}O_3$	144
7	7.604	3.66	melamine	$C_3H_6N_6$	126
8	8.006	0.25	levoglucosenone	$C_6H_6O_3$	126
9	8.568	6.35	pyranone	$C_6H_8O_4$	144
10	9.153	0.26	5-methoxypyrrolidin-2-one	$C_5H_9NO_2$	115
11	9.397	0.22	isoamyl trimethylacetate	$C_{10}H_{20}O_2$	172
12	9.927	30.23	5-hydroxymethylfurfural	$C_6H_6O_3$	126
13	10.193	0.4	3-hexene-2,5-dione	$C_6H_8O_2$	112
14	11.132	1.21	ethyl 3-hydroxy-4-pentenoate	$C_7H_{12}O_3$	144
15	13.119	1.69	xanthosine	$C_{10}H_{12}N_4O_6$	284
16	13.717	3.3	levoglucosan	$C_6H_{10}O_5$	162
17	15.043	1.48	1,6-anhydro-beta-d-glucofuranose	$C_6H_{10}O_5$	162
18	16.506	0.29	tetradecanoic acid	$C_{14}H_{28}O_2$	228
19	17.26	0.23	neophytadiene	C_20H_{38}	278
20	18.17	0.22	methylpalmitate	$C_{17}H_{34}O_2$	270
21	18.396	0.82	palmitoleic acid	$C_{16}H_{30}O_2$	254
22	18.609	7.66	*n*-hexadecanoic acid	$C_{16}H_{32}O_2$	256
23	19.108	0.47	1,4-naphthalenedione, 2-hydroxy-3-(1-propenyl)	$C_{13}H_{10}O_3$	214
24	19.813	0.41	linoleic acid, methyl ester	$C_{19}H_{34}O_2$	294
25	19.871	0.38	methyl petroselinate	$C_{19}H_{36}O_2$	296

Table 5. *Cont.*

S. No.	R. Time	Area%	Name	Molecular Formula	Molecular Weight
26	19.98	0.21	phytol	$C_{20}H_{40}O$	296
27	20.245	0.61	9,12-octadecadienoic acid (z,z)-	$C_{18}H_{32}O_2$	280
28	20.296	0.81	13-tetradecenal	$C_{14}H_{26}O$	210
29	20.48	1.17	podocarpan-12-ol	$C_{17}H_{30}O$	250
30	20.667	0.13	2-piperidinemethanol	$C_6H_{13}NO$	115
31	21.292	1.48	sclareolide	$C_{16}H_{26}O_2$	250
32	21.796	0.28	palmidrol	$C_{18}H_{37}NO_2$	299
33	21.858	0.26	11-hexadecenal, (z)-	$C_{16}H_{30}O$	238
34	22.642	0.48	sclareolide lactol	$C_{16}H_{28}O_2$	252
35	22.943	0.29	3-aminoheptane	$C_7H_{17}N$	115
36	23.084	0.62	4-cyanobenzoic acid, undec-10-enyl ester	$C_{19}H_{25}NO_2$	299
37	23.255	1.05	1-heptacosanol	$C_{27}H_{56}O$	396
38	23.45	9.95	2-palmitoylglycerol	$C_{19}H_{38}O_4$	330
39	23.826	3.28	copalic acid	$C_{20}H_{32}O_2$	304
40	24.683	1.24	oleoyl chloride	$C_{18}H_{33}ClO$	330
41	24.885	2.29	17-pentatriacontene	$C_{35}H_{70}$	490
42	25.131	3.19	glycerin 1-monostearate	$C_{21}H_{42}O_4$	358
43	25.697	0.32	9-octadecenamide	$C_{18}H_{35}NO$	281
44	25.975	0.42	squalene	C_30H_{50}	410
45	26.413	0.29	1-cyclohexene-1-butyraldehyde, 2,6,6-trimethyl-	$C_{13}H_{22}O$	194
46	27.313	0.19	hexacosanoic acid, methyl ester	$C_{27}H_{54}O_2$	410
47	28.631	0.15	stigmasta-4,7,22-trien-3.alpha.-ol	$C_{29}H_{46}O$	410
48	29.568	0.15	stigmasterol acetate	$C_{31}H_{50}O_2$	454
49	30.478	0.41	vitamin e	$C_{29}H_{50}O_2$	430
50	32.617	0.25	ergost-5-en-3-ol	$C_{28}H_{48}O$	400
51	33.221	4.47	stigmasterol	$C_{29}H_{48}O$	412
52	34.745	2.82	gamma-sitosterol	$C_{29}H_{50}O$	414
53	35.153	0.38	fucosterol	$C_{29}H_{48}O$	412

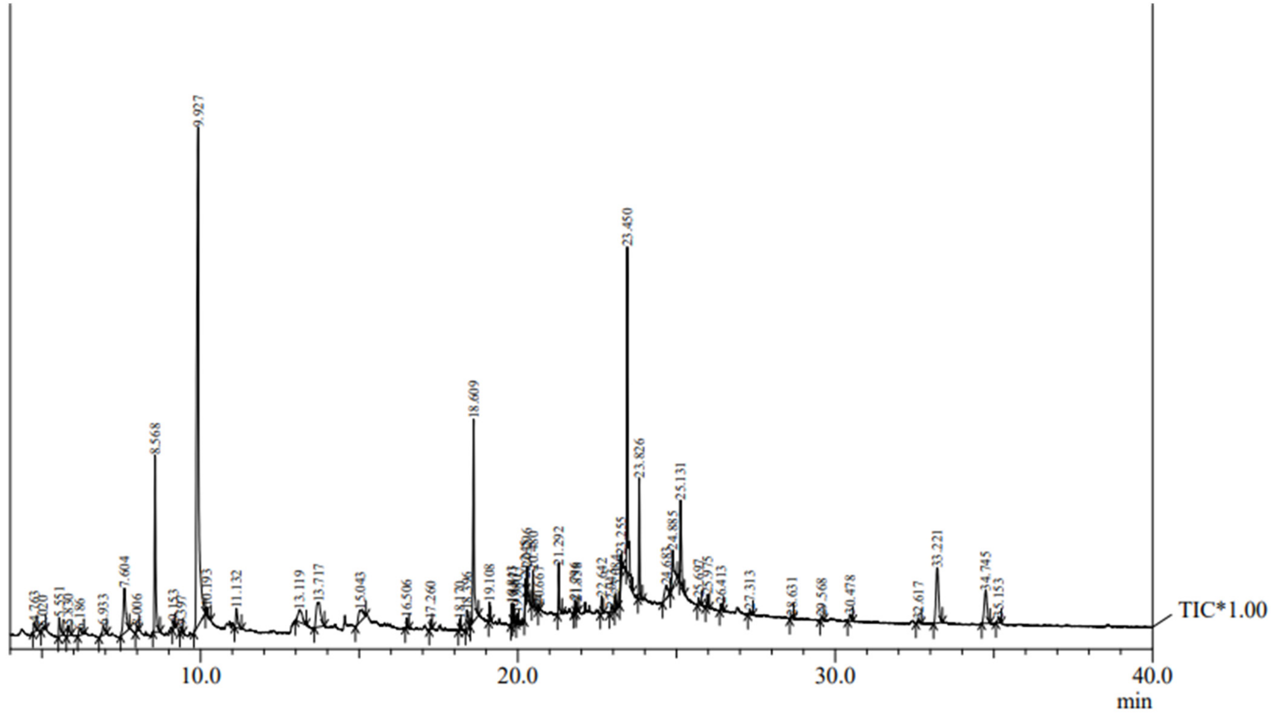

Figure 4. GC–MS analysis presenting total ion chromatogram (TIC) of leaf-derived callus of *C. officinalis*, displaying the retention time of each phytocompound detected.

The phytochemical profiles for field-grown and in vitro leaf samples of *C. officinalis* were analyzed in a similar manner. The methanolic extract of field-grown leaf samples revealed 41 phytocompounds at varied levels (Table 6, Figure 5), which include 2-palmitoglycerol (16.06%), neophytadiene (13.94%), phytol isomer (12.41%), guanosine (6.40%), 1-heptacosanol (4.62%), stigmasterol (3.75%), n-tetracosanol-1 (3.08%), vitamin E (2.09%), etc. In addition to this, the in vitro-grown leaf sample also showed the presence of about 40 phytocompounds (Table 7, Figure 6), out of which 2-monopalmitin (23.59%), guanosine (12.18%), neophytadiene (10.47%), phytol (7.18%), 2-ethylbutyric acid, and eicosyl ester (5.94%) were recorded to be available in higher quantities. Guanosine was seen to be nearly twice as high (12.18%) in lab-grown leaf tissue as compared to field-grown leaf (6.40%) of *C. officinalis.* Similarly, 4-cyanobenzoic acid-undec-10-enyl ester was found to be in greater amounts in in vitro leaf samples (3.35%) when compared with field-grown leaf samples (0.90%); squalene in field-grown leaf tissue was 2.38% and 2.52% in in vitro leaf tissues. On the contrary, certain phytocompounds have been found to be accumulated more in field-grown leaf samples than laboratory-grown tissues, such as vitamin E (2.09%) in field-grown leaf tissue; in the in vitro sample, the content was 0.90%. Similarly, stigmasterol in field-grown leaf tissue was about 3.75%, and in in vitro tissue it was 1.36%.

Table 6. GC–MS analysis revealed phytocompounds in field-grown leaf of *C. officinalis*.

S. No.	R. Time	Area%	Name	Molecular Formula	Molecular Weight
1	8.570	1.72	pyranone	$C_6H_8O_4$	144
2	13.083	6.40	guanosine	$C_{10}H_{13}N_5O_5$	283
3	13.360	0.98	2-tridecynyl 2,6-difluorobenzoate	$C_{20}H_{26}F_2O_2$	336
4	15.060	0.25	megastigmatrienone 4	$C_{13}H_{18}O$	190
5	15.554	0.16	4,6,6-trimethyl-bicyclo[3.1.1]heptan-2-ol	$C_{10}H_{18}O$	154
6	15.982	0.17	tetradecanal	$C_{14}H_{28}O$	212
7	16.508	0.14	undecanoic acid	$C_{11}H_{22}O_2$	186
8	17.193	0.37	tetrahydrogeranyl acetate	$C_{12}H_{24}O_2$	200
9	17.265	13.94	neophytadiene	$C_{20}H_{38}$	278
10	17.321	0.41	hexa-hydro-farneso	$C_{15}H_{32}O$	228
11	17.715	3.24	neophytadiene	$C_{20}H_{38}$	278
12	18.173	0.19	pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270
13	18.370	0.30	11,14,17-eicosatrienoic acid, methyl ester	$C_{21}H_{36}O_2$	320
14	18.595	3.44	pentadecanoic acid, 14-methyl-, methyl ester	$C_{16}H_{32}O_2$	256
15	19.749	0.36	phytol isomer	$C_{20}H_{40}O$	296
16	19.817	0.32	linolic acid	$C_{18}H_{32}O_2$	280
17	19.876	0.35	6-octadecenoic acid, methyl ester, (z)-	$C_{19}H_{36}O_2$	296
18	19.983	12.41	phytol isomer	$C_{20}H_{40}O$	296
19	20.230	0.33	9,12-linoleic acid	$C_{18}H_{32}O_2$	280
20	20.302	1.15	9,12-octadecadienoic acid	$C_{18}H_{32}O_2$	280
21	21.291	1.24	2-formylhexadecane	$C_{17}H_{34}O$	254
22	21.513	1.46	3-cyclopentylpropionic acid, 2-dimethylaminoethyl ester	$C_{12}H_{23}NO_2$	213
23	21.793	0.23	hexadecanoyl-chloride-	$C_{16}H_{31}ClO$	274
24	22.938	0.73	3-cyclopentylpropionic acid, 2-dimethylaminoethyl ester	$C_{12}H_{23}NO_2$	213
25	23.001	2.92	3-cyclopentylpropionic acid, 2-dimethylaminoethyl ester	$C_{12}H_{23}NO_2$	213
26	23.088	0.90	4-cyanobenzoic acid, undec-10-enyl ester	$C_{19}H_{25}NO_2$	299
27	23.260	3.08	n-tetracosanol-1	$C_{24}H_{50}O$	354
28	23.455	16.06	2-palmitoylglycerol	$C_{19}H_{38}O_4$	330
29	23.859	0.60	globulol	$C_{15}H_{26}O$	222
30	24.689	1.69	oxalic acid, monoamide, n-allyl-, hexadecyl ester	$C_{21}H_{39}NO_3$	353
31	24.890	4.62	1-heptacosanol	$C_{27}H_{56}O$	396
32	24.965	0.65	ethyl linolate	$C_{20}H_{36}O_2$	308
33	25.137	4.31	octadecanoic acid, 2,3-dihydroxypropyl ester	$C_{21}H_{42}O_4$	358

Table 6. *Cont.*

S. No.	R. Time	Area%	Name	Molecular Formula	Molecular Weight
34	25.709	0.44	9-octadecenamide	$C_{18}H_{35}NO$	281
35	25.978	2.38	squalene	$C_{30}H5_{0}$	410
36	26.971	2.32	1-heptacosanol	$C_{27}H_{56}O$	396
37	28.267	1.31	8,14-cedrane oxide	$C_{15}H_{24}O$	220
38	29.143	1.01	gamma-tocopherol	$C_{28}H_{48}O_2$	416
39	30.495	2.09	vitamin e	$C_{29}H_{50}O_2$	430
40	33.227	3.75	stigmasterol	$C_{29}H_{48}O$	412
41	34.758	1.58	gamma-sitosterol	$C_{29}H_{50}O$	414

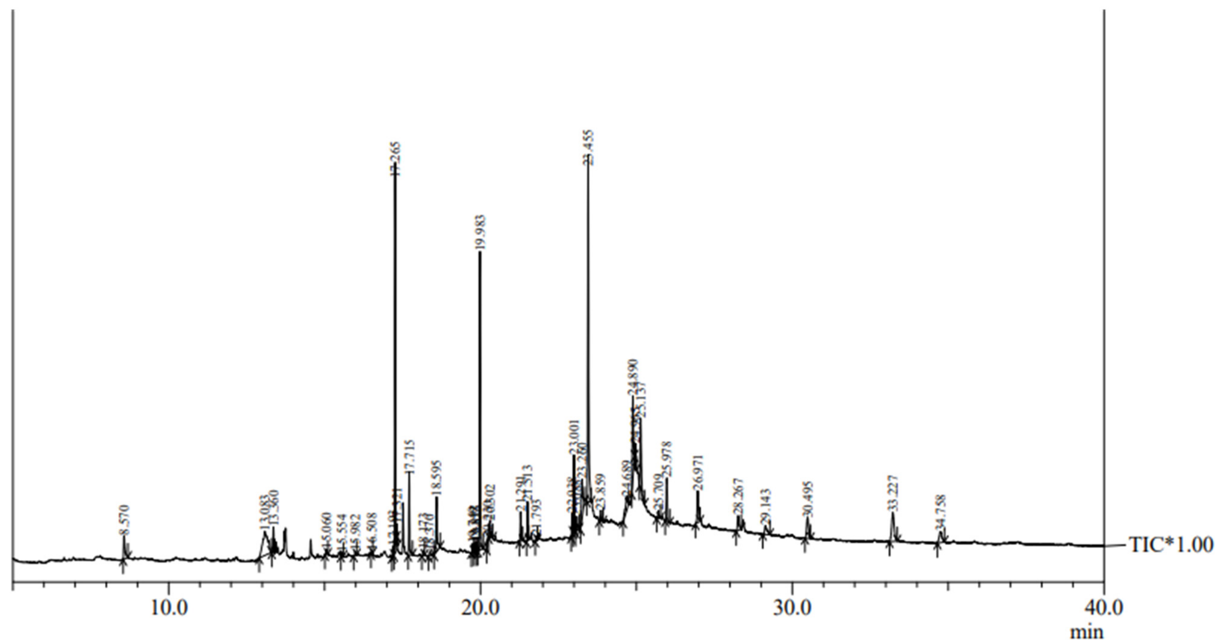

Figure 5. GC–MS analysis presenting total ion chromatogram (TIC) of field-grown leaf of *C. officinalis*, displaying the retention time of each phytocompounds detected.

Table 7. GC–MS analysis revealed phytocompounds in in vitro-raised leaf of *C. officinalis*.

S. No.	R. Time	Area%	Name	Molecular Formula	Molecular Weight
1	12.999	12.18	guanosine	$C_{10}H_{13}N_5O_5$	283
2	13.365	0.82	2,6-difluorobenzoic acid	$C_{20}H_{26}F_2O_2$	336
3	13.717	0.10	1-[2-bromoethenyl] adamantane	$C_{12}H_{17}Br$	240
4	13.749	0.10	gamma-cadinene	$C_{15}H_{24}$	204
5	15.984	0.09	tridecanal	$C_{13}H_{26}O$	198
6	17.192	0.28	3,7-dimethyloctyl acetate	$C_{12}H_{24}O_2$	200
7	17.265	10.47	neophytadiene	$C_{20}H_{38}$	278
8	17.320	0.26	hexa-hydro-farnesol	$C_{15}H_{32}O$	228
9	17.520	1.81	neophytadiene	$C_{20}H_{38}$	278
10	17.715	2.60	3,7,11,15-tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296
11	18.175	0.33	pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270
12	18.590	0.83	*n*-hexadecanoic acid	$C_{16}H_{32}O_2$	256
13	19.816	0.60	9,12-octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294
14	19.875	0.54	6-octadecenoic acid, methyl ester, (z)-	$C_{19}H_{36}O_2$	296
15	19.985	7.18	phytol	$C_{20}H_{40}O$	296
16	20.294	0.60	9,12-octadecadienoic acid (z,z)-	$C_{18}H_{32}O_2$	280

Table 7. *Cont.*

S. No.	R. Time	Area%	Name	Molecular Formula	Molecular Weight
17	20.677	0.12	9,12-octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294
18	21.291	0.66	11-dodecen-2-one	$C_{12}H_{22}O$	182
19	21.512	0.99	octanoic acid, 2-dimethylaminoethyl ester	$C_{12}H_{25}NO_2$	215
20	21.630	0.44	glycidyl palmitate	$C_{19}H_{36}O_3$	312
21	22.938	0.55	fumaric acid, 2-dimethylaminoethyl nonyl ester	$C_{17}H_{31}NO_4$	313
22	22.999	1.58	3-cyclopentylpropionic acid, 2-dimethylaminoethyl ester	$C_{12}H_{23}NO_2$	213
23	23.086	3.35	4-cyanobenzoic acid, undec-10-enyl ester	$C_{19}H_{25}NO_2$	299
24	23.259	5.94	2-ethylbutyric acid, eicosyl ester	$C_{23}H_{46}O_2$	354
25	23.455	23.59	2-monopalmitin	$C_{19}H_{38}O_4$	330
26	23.857	0.21	alpha-selinene	$C_{15}H_{24}$	204
27	24.180	0.56	glycerol.beta.-palmitate	$C_{19}H_{38}O_4$	330
28	24.685	4.12	4-cyanobenzoic acid, tridecyl ester	$C_{21}H_{31}NO_2$	329
29	24.889	3.36	eicosyl heptafluorobutyrate	$C_{24}H_{41}F_7O_2$	494
30	25.030	2.04	1-monolinolein	$C_{21}H_{38}O_4$	354
31	25.139	5.13	octadecanoic acid, 2,3-dihydroxypropyl ester	$C_{21}H_{42}O_4$	358
32	25.705	0.77	9-octadecenamide, (z)-	$C_{18}H_{35}NO$	281
33	25.976	2.52	squalene	$C_{30}H_{50}$	410
34	26.969	1.02	1-heptacosanol	$C_{27}H_{56}O$	396
35	27.326	0.36	eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	326
36	28.257	0.42	cysteamine sulfonic acid	$C_2H_7NO_3S_2$	157
37	29.138	0.42	gamma-tocopherol	$C_{28}H_{48}O_2$	416
38	30.491	0.90	vitamin e	$C_{29}H_{50}O_2$	430
39	33.218	1.36	stigmasterol	$C_{29}H_{48}O$	412
40	34.759	0.78	beta-sitosterol	$C_{29}H_{50}O$	414

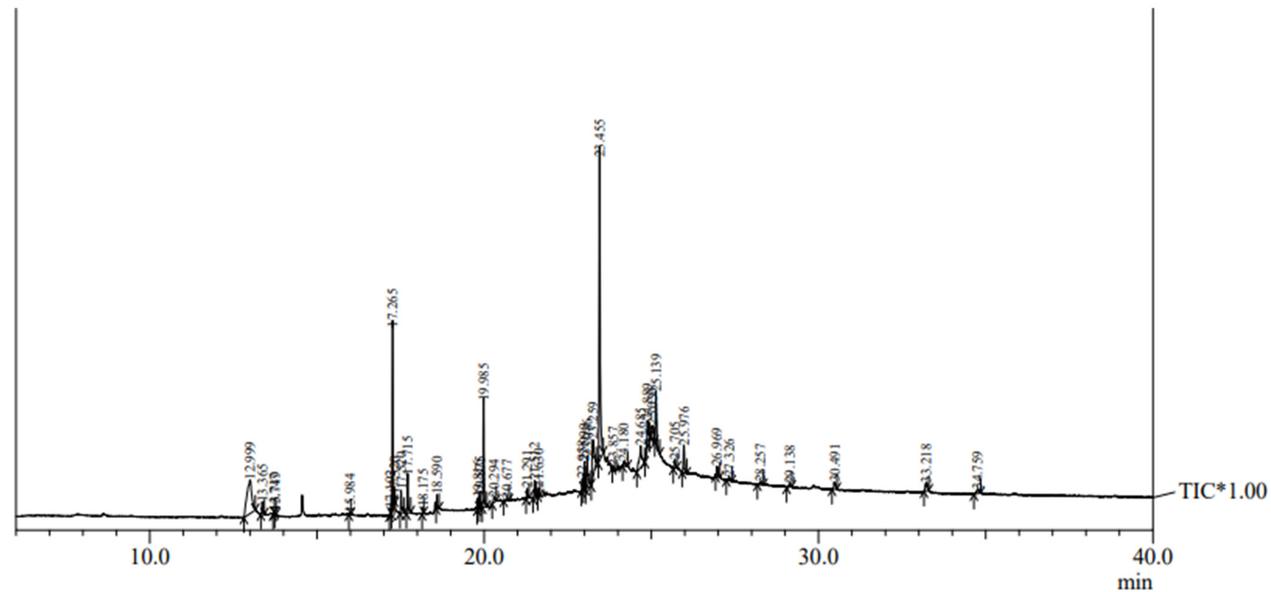

Figure 6. GC–MS analysis presenting total ion chromatogram (TIC) of in vitro-grown leaf of *C. officinalis*, displaying the retention time of each phytocompounds detected.

Certain phytocompounds were found exclusively in each of the sample. Out of 53 compounds, the methanolic callus extract showed the presence of 36 phytocompounds, such as melamine, levoglucosenone, xanthosine, palmidrol, oleoyl chloride, etc., which were not being found in any other samples. Similarly, the field-grown leaf extract showed 18 exclusive

phytocompounds, including 9,12-linoleic acid, globulol, 2-palmitoylglycerol, undecanoic acid, and so on. Among 40 compounds detected, the in vitro-derived leaf extract displayed 22 exclusive bioactive compounds, like glycidyl palmitate, 2-monopalmitin, beta-sitosterol, 1-monolinolein, etc.

4. Discussion

The current study attempted to set out a systematic in vitro plant regeneration protocol via organogenesis. In the present examination, callus induction and organogenesis were successfully carried out in *C. officinalis* using and optimizing PGRs (NAA and BAP). The phytochemical and biochemical profiling of the regenerated tissues in vitro was further analyzed. Initially, the leaf explants were cultured on MS medium containing varying concentrations of PGRs to induce callus formation. The results indicate that a combination of BAP and NAA promoted robust callus proliferation, resulting in the highest fresh biomass yield compared to when BAP and NAA were used individually. The highest frequency of callus induction was observed when BAP (1.0 mg/L) and NAA (1.0 mg/L) were combined, in comparison with other treatments. In tissue culture practice, callus was induced with auxins, but in combination with cytokinins, callus was produced in a high quantity [29]. Similar results have been described in other members of Asteraceae, such as *Artemisia vulgaris* [30] and *Rhaponticum carthamoides* [31]. An effective strategy for producing in vitro plants by organogenesis or with embryogenesis is with the formation of callus. This study includes induction of callus from leaf tissue in MS medium containing BAP and NAA at 1.0 mg/L each. In *C. officinalis*, callus induction was accomplished using several explants [6,7]. The organogenic capacity of leaf callus was also investigated. Cytokinins have the ability to stimulate shoot development and proliferation in vitro [32]. In this instance, BAP and NAA both showed modest impacts in causing shoots. Auxins, in addition to cytokinin, are frequently helpful in stimulating shoot formation since these signaling components are widely known to counteract cytokinin's dominating effects [33]. Auxin and cytokinin have been shown to have a promotive influence on shoot production in a number of other plant species, like *Thapsia garganica* [34] and *Ficus religiosa* [35]. Afterwards, the shoots were transferred to a rooting medium with varying levels of IAA and IBA concentrations. Compared to IAA, shoots cultivated on MS supplemented with IBA exhibited a greater rooting rate. IBA has been shown to have a better influence than other auxin treatments on promoting roots in *C. officinalis* shoots [36]. Similar effects of IBA on roots were observed in other plant species, like *Vaccinium corymbosum* [37] and *Dracaena sanderiana* [38], when studied in vitro. IBA's stability and ease of translocation to various tissues are thought to be responsible for its high rate of root induction [39].

In vitro conditions frequently cause stress in cell lines and regenerated tissues, which lowers the survival rate [40]. It is essential to evaluate the cellular physiology by routinely observing the biochemical characteristics of the tissues. The biochemical and antioxidant properties of tissues obtained in vitro were examined and compared with those of the donor plant. Various factors, such as different PGRs employed in culture, influence the up- and down-regulation of phenolics and flavonoid synthesis [41]. In this study, in vitro leaf tissues exhibited elevated levels of phenolic and flavonoid compounds. This finding aligns with previously documented biochemical analysis conducted across various plant species [42,43]. Three antioxidant assays (TPC, TFC, and DPPH) were employed to evaluate the antioxidant capacity of in vitro-propagated tissues. The findings from these assessments indicated that the leaf tissue derived in vitro displayed superior antioxidant properties compared to the field-grown leaf and callus samples. Adverse environmental conditions lead to elevated production of reactive oxygen species (ROS) in plant tissues. Antioxidant enzymes, such as catalase, glutathione reductase, superoxide dismutase, and peroxidase, can also lower this level of ROS generation [44,45]. When it takes an electron or another free radical, DPPH, a dark-colored, stabilized, organic free radical, changes to a light-yellow color, signifying the scavenging action [46,47]. The higher level of antioxidant potential shown in this study's laboratory-grown leaf tissue is attributable to the positive link between

antioxidant activity and phenolics and flavonoids, which give free radicals hydrogen atoms to deactivate them [27]. Different plants, such as *Thalictrum foliolosum* [48], *Zingiber officinale* [49], *Salvia hispanica* [50], *Tylophora indica* [51], etc., showed comparable antioxidant potential results. The aforementioned data clarifies the enormous pharmacological potential of in vitro-derived tissues, including callus and leaves, in terms of phytoconstituents.

Many phytochemicals, including volatile compounds, long-chain hydrocarbons, sterols, sugar alcohols, esters, phenolics, alkaloids, flavonoids, and saponins, among others, can be detected through GC–MS, a widely utilized analytical method [52]. Furthermore, by identifying variations in relative peak area percentage in the metabolite profiling of regenerants and their wild counterparts, this approach also provides valuable insights into the influence of various in vitro factors on plant growth and development [53]. The chromatographic results derived from this investigation revealed that each examined sample contained over 45 notable bioactive compounds. Different levels of identified phytocompounds were found in the in vitro-grown and field-grown leaf tissues when their metabolite profiles were compared. When compared to intact in vitro plant tissues, the field-grown-generated leaf produced more phytocompounds. This difference in phytocompound production could be caused by a number of variables, including temperature, photoperiod, genotype, hormone levels, and media composition [15,54]. It might therefore be a more dependable and powerful source of phytocompounds for pharmaceutical applications. In a number of plants, like *Amomum nilgiricums* [55], *Tanacetum sinaicum* [56], and *Catharanthus roseus* [57], the identification and quantification of bioactive chemicals have recently been reported through GC–MS. Samples of *C. officinalis* revealed a large number of phytoconstituents with potential medicinal use. Terpenoid squalene has a variety of biological properties, it shows anti-oxidant, anti-cancerous, detoxifying, and moisturizing properties [58]. Stigmasterol has been linked to callus samples showing anti-tumor, anti-osteoarthritis, immunomodulatory, anti-parasitic, antibacterial, anti-oxidant, anti-fungal, and neuroprotective qualities [59]. Vitamin E compounds have strong antioxidant qualities; these are employed extensively in pharmacological studies [60]. Similarly, n-hexadecanoic acid, which was only present in the leaf tissue produced in vitro, had anti-inflammatory, antibacterial, and antioxidant qualities [61,62]. The presence of important phytochemicals reported in flower tissues of *C. officinalis* could be helpful for numerous herbal formulations, demonstrating antibacterial and antifungal properties [63].

5. Conclusions

The investigations described the biochemical and antioxidant evaluations of callus, field-grown, and in vitro-grown leaf tissues. The field-grown leaf showed greater quantities of flavonoids and phenolics and greater antioxidant capacity. The metabolites of *C. officinalis* leaf tissues, produced field-grown and in vitro, were compared using the GC–MS technique. Numerous phytocompounds, such as alkaloids, flavonoids, phenolics, terpenoids, sugars, and sterols, were found in the investigations. There were 55 phytochemicals in leaf callus, and each one has a different use. This study shows that in vitro plant tissues synthesize a variety of beneficial bioactive compounds that the pharmaceutical industry can utilize.

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

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