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Article

An Efficient In Vitro Shoot Organogenesis and Comparative GC-MS Metabolite Profiling of *Gaillardia pulchella* Foug

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Abstract: *Gaillardia pulchella* Foug. is a widely studied plant because of its high pharmacological and ornamental value. The leaves of *G. pulchella* were used for inducing callus and subsequent plant regeneration as it is the primary source of phytocompounds. The purpose of the present investigation was to formulate an in vitro propagation method for *Gaillardia* by using leaf explants in MS (Murashige and Skoog) medium. The best callus induction was observed on high (2.0 mg/L) α-naphthalene acetic acid (NAA) and a low (0.5 mg/L) 6-benzylaminopurine (BAP) with callus induction frequency of 91.66%. The leaf callus also demonstrated high caulogenesis ability (95.83%), with an average 5.2 shoots/callus mass at 0.5 mg/L BAP and 2.0 mg/L NAA. Indole Acetic acid (IAA) at 1.0 mg/L had the maximum rooting percentage (79.17%) with 12.4 roots per shoot. Rooted plantlets were later transferred to greenhouse conditions, showing a survivability rate of 75–80%. The physiological parameters, i.e., phenolic compounds and the flavonoids' level, in the DPPH assay were higher in leaves obtained in vitro compared to callus formed from leaves and field-obtained (mother) leaves. Gas chromatography–mass spectrometry (GC–MS) analysis of methanol extracts of leaves (in vivo and in vitro) and leaf callus presented a wide array of compounds. In callus extract, some 34 phytocompounds were identified. Some of them were 3-hydroxy-2,3-dihydromaltol (25.39%), isoamyl acetate (11.63%), palmitic acid (11.55%), 4-methyloxazole (7.54%), and 5-methoxypyrrolidin-2-one (7.49%). Leaves derived in vivo and in vitro had 45 and 28 phytocompounds, respectively, belonging to different classes like lignans, phenols, terpenoids, alkaloids and fatty acids, etc. Those findings demonstrated that the leaf derived callus and the leaves are the potential stable source of several compounds with medicinal importance. The developed protocol may provide an alternative source of compounds without affecting wild flora.

Keywords: indirect organogenesis; biochemical; antioxidant; phytochemical fingerprinting; chromatography

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1. Introduction

Gaillardia pulchella Foug is an ornamental, short-lived, annual, flowering plant. It is commonly known as Indian blanket flower, sundance, or fire wheel [1]. *G. pulchella* is an indigenous species to North and South America and belongs to the Asteraceae family [2]. It is cultivated in several parts of India as one of the major commercial flower crops [3]. This plant thrives in dry, sunny regions and can spread to disrupted habitat [4]. Its easy cultivation, high tolerance to a variety of soil types and climates, extended flowering period, and vibrant colors (red, yellow, scarlet) make it one of the most popular flowers in India [5].

Due to the presence of a wide array of phytocompounds such as tannins, flavonoids, terpenoids, sterols, amino acids, polysaccharides, etc., with potential pharmacological uses, it is regarded as a therapeutically important plant [6]. *G. pulchella* has antitumoral, antiparasitic, chemotaxonomic indicator and cytotoxic properties [7]. Among several reported phytocompounds, pulchelloid A (sesquiterpene lactone) is a characteristic compound found in *Gaillardia* spp., which possess anti-mitotic activity [8]. Because of the immense phytocompounds present, classical breeding methods of *Gaillardia* spp. have recently been replaced by advanced biotechnological approaches [9]. Plant tissue culture is used for fast propagation of plants under a sterile environment, without harming natural vegetation [10]. It offers a number of advantages over conventional techniques like fast mass propagation, genetic transformation, procurement of microbe free healthy plants, important phytocompounds production, preservation of germplasm. [11,12]. Organogenesis (via direct or indirect pathway) is one of the methods by which plant parts like roots, shoots, and even flowers can be generated from cultured explants [13,14].

The regular sub-culturing, genetic makeup, extended exposure to plant growth regulators (PGRs), adverse physical environments like humidity and photoperiod are some of the factors that alter physio-biochemical attributes of cultured plantlets [15]. The biochemical changes may affect phenolic compounds' content, sugar level, flavonoid content, and excess accumulation of reactive oxygen species (ROS) [16]. Thus, such biochemical characteristics and other related phytochemical composition of in vitro raised tissues need regular monitoring. Gas chromatography–mass spectrometry (GC-MS) requires a small quantity of plant extracts and is one of the best, fastest, and most accurate methods for detecting a wide range of phytochemicals, including alcohols, alkaloids, nitro compounds, long chain hydrocarbons, organic acids, steroids, esters, and amino acids [17]. GC-MS investigation has recently been conducted in several medicinally important plants like *Tecoma stans*, *Pluchea lanceolate*, and others [18,19].

To date, there is only one report available regarding in vitro regeneration of *G. pulchella*, in which authors optimized PGRs level in bud breaking response [20]. This study, therefore, describes for the first time an efficient in vitro plant regeneration method via indirect organogenesis from leaf derivedcallus in *G. pulchella*. The current study describes and compares phytochemicals and biochemical attributes present in different tissues (leaf callus, in vivo and in vitro leaves) of *G. pulchella*. It enables the pharmaceutical industry to use in vitro cultivation technologies to obtain a steady supply of therapeutically significant bioactive compounds.

2. Materials and Methods

2.1. Plant Material and Surface Sterilization

Leaves of *Gaillardia pulchella* Foug were procured from the Jamia Hamdard herbal garden (identification number: JH/BOT/DAC/2023/01) and were used as a primary source of explants. Leaves were cut into pieces 1.5–2.0 cm in length and their surface disinfected by following the protocol described earlier [21] with some modifications. The excised leaves were washed with running tap water, later rinsed in Teepol B-300 (Reckitt Benkiser, Gurugram, India) (a liquid detergent) for 30 min, and stirred in running tap water for 4 to 5 min to physically eradicate microorganisms. The explants were dipped in 70% ethanol for 2–3 min, and finally washed with sterilized double-distilled water to reduce the adverse effect of ethanol. The cut leaves were later treated with 0.1% mercuric chloride ($HgCl_2$), for 2–3 min, and washed with sterilized double-distilled water 3–4 times to remove any traces of sterilizing solution.

2.2. Induction of Callus and Culture Conditions

Callus was induced from excised leaves (1.5–2.0 cm) when inoculated on MS (Murashige and Skoog) medium [22] comprising 3% sucrose as a carbon source and 0.7% agar (w/v) as a gelling agent. The medium was additionally supplemented with 0.5–2.0 mg/L of auxins [α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyactic acid (2,4-D)] and cytokinin [6-benzylamino purine (BAP)]. The medium pH was adjusted to 5.6 to 5.7 with 0.1 N sodium hydroxide (NaOH) or1 N hydrochloric acid (HCl), prior to autoclaving. The test tubes were kept in an incubation room with the temperature at 25 ± 2 °C, 55% relative humidity (RH), 60 μmolm^{-2} s^{-1} light intensity and 16/8 h photoperiod provided by producer bulbs. In control, no PGR was added to the medium. After four weeks, the calli underwent regular subculturing on the same PGR containing media. The callus formation frequency (%) and callus biomass (g) were defined after 4 weeks of culture.

The following formula was used:

$$\text{Callus induction frequency (\%)} = \text{number of explants producing callus/total number of explants cultured} \times 100.$$

2.3. Indirect Shoot Organogenesis and Proliferation

After 3–4 weeks, the leaf-derived calli were placed into 0.5–1.0 mg/L BAP and 0.5–2.0 mg/L NAA containing MS for shoot induction. The shoots started to appear, and the shoot forming ability (%) and the average shoot number/callus mass were estimated after four weeks of incubation. The regenerated shoots were placed in a medium containing either 0.5–2.0 mg/L BAP or kinetin (Kn) for more proliferation of shoots. After five weeks, the average length of the regenerated shoots and shoot proliferation percentage (total number of shoots emerged/total explants $\times$ 100) per treatment were counted.

2.4. Induction of Roots and Acclimatization

The in vitro regenerated shoots measuring about 3–5 cm in length were removed and placed on a root-inducing MS medium containing different indole-3-acetic-acid (IAA),indole-3-butyric-acid (IBA)orNAA concentrations. After three weeks of culture, the average number of roots per shoot and the root induction frequency (%) were recorded. After washing with autoclaved double-distilled water to remove the adherent culture media, the rooted plantlets were transferred to plastic pots filled with an autoclaved 1:1:1 mixture of sand, soil, and soilrite. The potted plants were covered with clear plastic bags and kept in an incubation room at 25 $\pm$ 2 °C temperature, 70 $\pm$ 10% RH and 60 µmol m^{-2} s^{-1} light intensity, and were finally transferred to outdoor conditions.

2.5. Preparation of Extracts

The in vivo and in vitro grown leaves of *G. pulchella* and the leaf-derived callus were collected, and dried in the shade for three days at room temperature, and then 1.0 g samples (dry weight) were crushed into a fine powder using a mortar and pestle. Each sample was then separately extracted with 10 mL of methanol solvent in a rotary shaker for 48 h. Then, Whatman No. 1 filter paper was used to filter the methanolic extracts. The filtered samples were centrifuged at 10,000 rpm for five min, and the supernatant was kept at 4 °C before use.

2.6. Gas Chromatography–Mass Spectrometry Analysis

The GC–MS analysis of tissues (callus, in vivo and in vitro leaves) was performed with the use of a GC-MS-QP-2010 apparatus (Shimadzu, Tokyo, Japan) with the following program specifications: an oven temperature 100 °C for 3 min and then gradually increased to 300 °C, for 17 min. Helium was given as carrier gas at a continuous flow of 1.21 mL/min; the injection temperature was kept at 260 °C. For separation of compounds, Rxi-5Sil MS GC Capillary Column, 30 m, 0.25 mm ID, 0.25 µm df was used. Operating temperatures for the ion source and interface were 220 °C and 270 °C, respectively, with a 2.5 min solvent cut time and a GC–MS running time for every sample was 35 min. The GCMS solution software (Version 4.45 SP 1) was used to identify the bioactive compounds present in each sample by analyzing retention index, peak area, and area % with earlier identified phytocompounds and consulting mass spectral database with the National Institute of Standards and Technology (NIST) library.

2.7. Biochemical Parameters

2.7.1. Determination of Total Phenol Content

The Folin–Ciocalteu method [23] was used to determine the total phenolic content (TPC) of the extract. The tissue extract (0.5 mL) was thoroughly mixed with 2.5 mL (10%, *v*/*v*) of the Folin–Ciocalteu reagent (Sigma-Aldrich, New York, NY, USA). The mixture was kept at room temperature for five min; then, 2.0 mL sodium carbonate (7%) was added and the mixture was kept for 90 min. The absorbance was read at 765 nm against the extract-free blank using a spectrophotometer (BL-295 Biolinkk, Delhi, India). The calibration curve equation for the standard gallic acid solution was used to calculate the total phenolic content, which was measured in triplicate. The outcome information was presented as mg GAE/g Dw.

2.7.2. Determination of Flavonoid Level

The procedure described by Aryal et al. [24] was followed in order to measure the total flavonoid content (TFC). Firstly, 1.0 mL of extract solutions was mixed with 10% aluminum chloride (0.2 mL) and 1 M potassium acetate solution (0.2 mL). After an incubation time of 30 min at room temperature, 3.6 mL of distilled water was added to the final reaction volume, which was 5.0 mL. After mixing the solution, each sample's absorbance at 415 nm was measured in relation to a blank. There were three repetitions of the measurement made. Different quantities of quercetin were plotted against corresponding absorbances on a standard graph. The TFC of the samples was presented in mg quercetin per g dry weight (mg QE/g DW).

2.7.3. DPPH Assay for Scavenging Activity

With the help of stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), the free radical scavenging activity (FRSA) of *G. pulchella* sample extract was determined following the method described by Baliyan et al. [23]. DPPH (0.024%, *w*/*v*) 3.0 mL and 0.1 mL methanol was mixed and was used as standards, and 0.1 mL of extract solutions were immediately added to each test tube. Following that, the samples were incubated for 90 min at room temperature in complete darkness. The absorbance was measured at 517 nm. The following formula [10] was used to assess the antioxidant capacity of each sample: $AC - AS/AC \times 100$. AC is control absorbance and AS is sample absorbance, giving the scavenging activity percentage.

2.8. Statistical Analysis

A completely randomized design (CRD) was used for in vitro trials with eight explants per PGR treatment, conducted in triplicates. The biochemical analyses were also performed in triplicates. The mean ± standard error was used to express the data. The differences in the samples' means were estimated or calculated with Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$.

3. Results

3.1. Callus Induction

The leaves of *G. pulchella* were used as explants for inducing callus (Figure 1A). White friable callus began to emerge from the leaf after two weeks of culturing (Figure 1B). The control treatment lacking PGRs failed to produce any callus; the formation of callus was observed in all the media supplemented with PGRs. The NAA and BAP combinations (2.0 mg/L + 0.5 mg/L and 0.5 mg/L + 0.5 mg/L) were found to be more effective in inducing callus (91.66–79.17%) compared to treatments containing 2,4-D, which exhibited a callus induction range of 62.50%-8.33% (Table 1 and Figure 1C). The highest number of leaves produced callus (91.66%) on BAP (0.5 mg/L) and NAA (2.0 mg/L) fortified medium. The maximum callus biomass (3.8 g/explant) was achieved on this very medium. In contrast, very little callusing (8.33%) and biomass (0.9 g/explant) were observed on 2.0 mg/L 2,4-D.

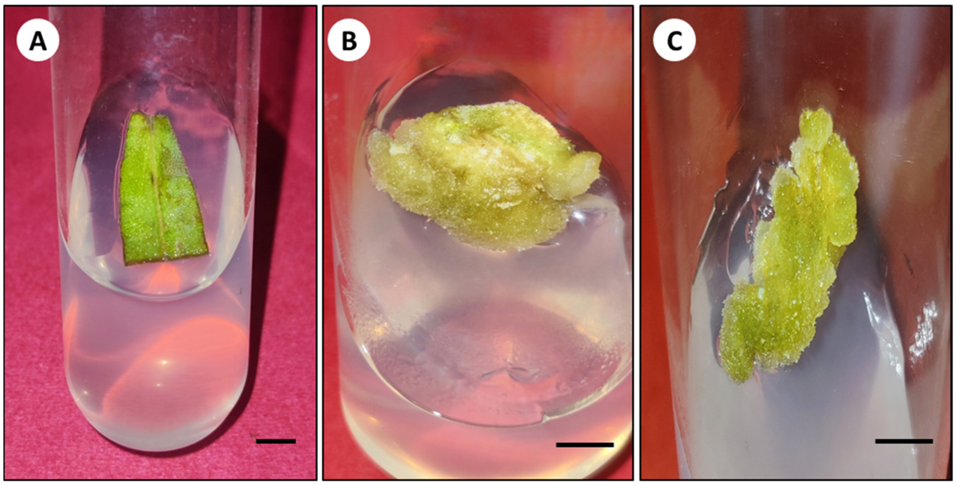

Figure 1. Callus induction and proliferation from leaf explant of *G.pulchella.* (**A**) Leaf explant inoculated on PGR (2.0 mg/L NAA and 0.5 mg/L BAP) containing MS medium. (**B**) Callus initiation after 2-week period. (**C**) Callus proliferation after 4-week period (bars (**A**) = 1.0 cm, (**B**,**C**) = 0.5 cm).

Table 1. Effects of various PGR's concentrations on callus induction from leaves in *G. pulchella*.

PGRs	Concentration (mg/L)	Callus Induction Percentage (%)	Callus Biomass (g)
Control	0	0^{e}	0^{d}
NAA + BAP	0.5 + 0.5	79.17 ± 4.17^{ab}	3.3 ± 0.5^{a}
	0.5 + 1.0	58.33 ± 8.33^{c}	2.6 ± 0.4^{ab}
	1.0 + 2.0	54.17 ± 4.17^{c}	2.7 ± 0.3^{ab}
	2.0 + 0.5	91.66 ± 8.33^{a}	3.8 ± 0.8^{a}
2,4-D	0.5	62.50 ± 7.22^{bc}	2.9 ± 0.4^{ab}
	1.0	45.83 ± 4.16^{cd}	1.8 ± 0.2^{bc}
	1.5	33.33 ± 11.02^{d}	1.1 ± 0.2^{c}
	2.0	8.33 ± 4.16^{e}	0.9 ± 0.1^{c}

Values represent means ± S.E. of eight replicates of three experiments. Within each column, means followed by different letters are significantly different at $p \leq 0.05$ according to DMRT. The superscripts represent ranking, i.e., 'a' indicates the best response and 'e/d' indicates the worst response.

3.2. Indirect Shoot Organogenesis and Proliferation

The MS medium without PGRs was ineffective for shoot induction, but the inclusion of NAA and BAP in media successfully transformed the callus into an organogenic one (Figure 2A), which induced shoots from the calli. Friable callus on the MS medium containing NAA (0.5–2.0 mg/L) and BAP (0.5–2.0 mg/L) induced a shoot (indirect) within 3–5 weeks' time (Figure 2B,C). Among various tested BAP and NAA levels, the 0.5 mg/L BAP and 2.0 mg/L NAA was the most efficient treatment for producing maximum numbers of shoots. The callus showed a shoot regeneration ability of 95.83%, with an average 5.2 shoot numbers/callus mass at 0.5 mg/L BAP and 2.0 mg/L NAA concentration (Table 2). At higher BAP level, a significant reduction in shoot number was noted. The lowest shoot formation rate (33.33%) with an average 1.8 shoot number/callus mass was recorded on BAP (1.0 mg/L) and NAA (2.0 mg/L) containing an MS medium.

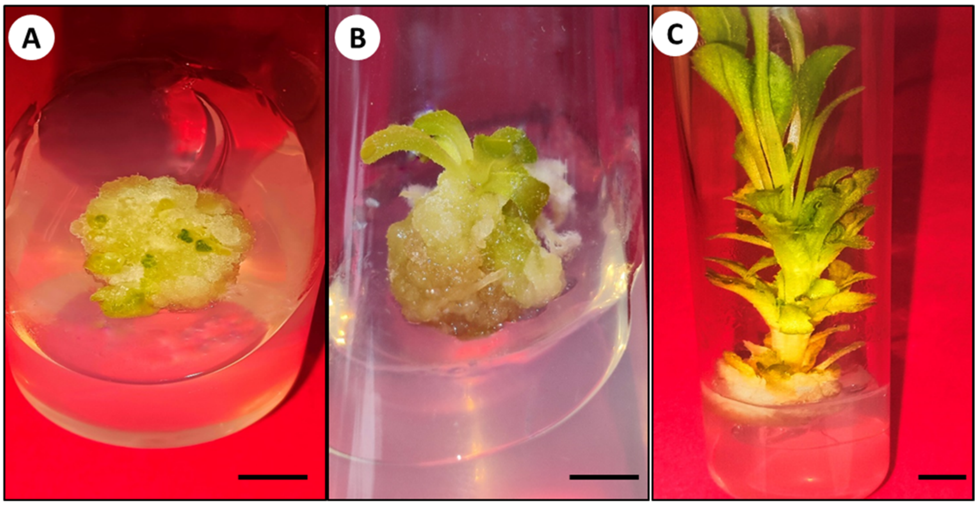

Figure 2. Indirect shoot organogenesis from callus of *G. pulchella* on MS media supplemented with 2.0 mg/L NAA and 0.5 mg/L BAP. (**A**) Organogenic callus. (**B**) Indirect shoot formation. (**C**) Elongated shoot after 6 weeks of culture (bars (**A**,**B**) = 0.5 cm, (**C**) = 1.0 cm).

Table 2. Influence of BAP and NAA on callus shoot formation in *G. pulchella*.

PGRs	Concentration (mg/L)	Shoot Regeneration Percentage (%)	Shoot Numbers/Callus Mass
Control	0	0^{d}	0^{c}
BAP + NAA	0.5 + 1.0	79.17 ± 11.02^{ab}	4.9 ± 0.8^{a}
	0.5 + 2.0	95.83 ± 4.16^{a}	5.2 ± 0.5^{a}
	1.0 + 0.5	54.17 ± 11.02^{bc}	4.1 ± 0.2^{a}
	1.0 + 1.5	37.50 ± 7.21^{c}	2.6 ± 0.3^{b}
	1.0 + 2.0	33.33 ± 11.02^{c}	1.8 ± 0.4^{b}

Values represent mean ± S.E. of eight replicates of three experiments. Within each column, means followed by different letters are significantly different at $p \leq 0.05$ according to DMRT. The superscripts represent ranking, i.e., 'a' indicates the best response and 'c/d' indicates worst response.

3.3. Induction of Root and Acclimatization

The regenerated shoots were subsequently transferred to a root induction medium. Two auxins, namely IBA and IAA, were added to the MS medium at three different concentrations (0.5, 0.75, and 1.0 mg/L) in order to achieve rooting of regenerants. With the exception of control, in every rooting media, the roots were formed at the ends of the shoots. In terms of average root numbers per shoot and root induction percentage, IAA treatments outperformed IBA. Furthermore, 1.0 mg/L IAA caused the maximum rooting percentage (79.17%) with 12.4 roots per shoot (Figure 3A), whereas 0.75 mg/L and 1.0 mg/L IBA allowed us to obtain the lowest rooting percentage with mean number of roots per shoot (Table 3). Plants treated with IAA formed longer roots, while shoots produced in IBA formed thin and lower numbers of roots. Healthy plantlets were later transferred to a greenhouse for acclimatization and had a survivability rate of 75–80% (Figure 3B).

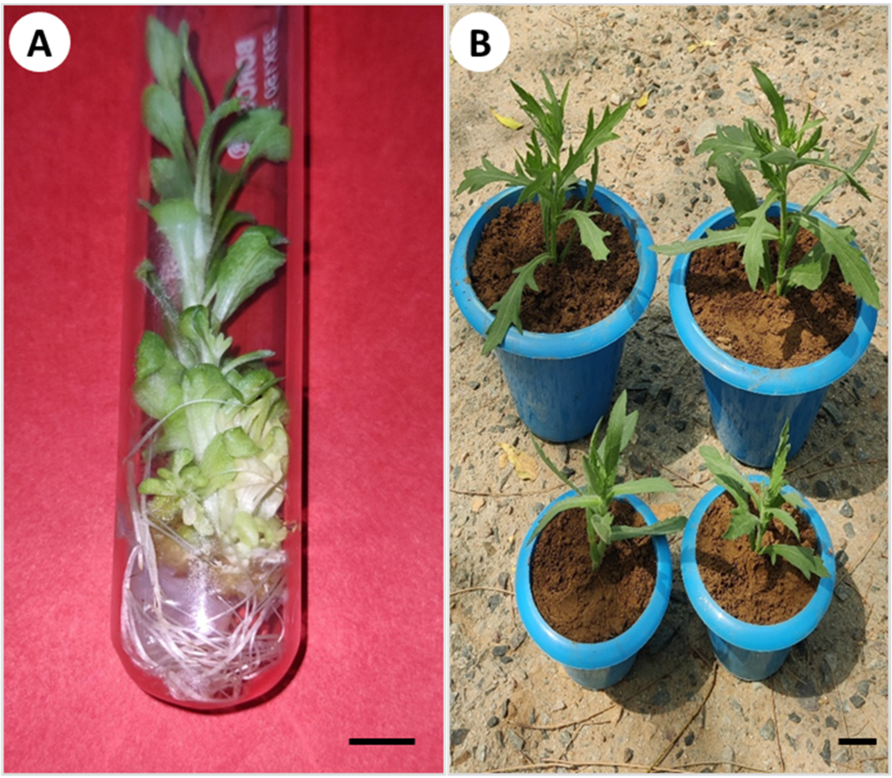

Figure 3. Root induction and transfer of in vitro derived shootlets of *G. pulchella*, (**A**) development of roots in 1.0 mg/L IAA fortified MS medium, (**B**) potted plants in outdoor (bars (**A**) = 1.0 cm, (**B**) = 2.5 cm).

Table 3. Effect of IBA and IAA on rooting of in vitro developed *G. pulchella* shoots. The data were scored after 3 weeks of cultivation.

PGRs	Concentration (mg/L)	Root Induction Frequency (%)	Root Numbers/Shoot
Control	0	0^{d}	0^{e}
IAA	0.5	54.16 ± 11.02^{abc}	9.7 ± 0.4^{bc}
	0.75	70.83 ± 15.02^{ab}	10.6 ± 0.6^{b}
	1	79.17 ± 8.33^{a}	12.4 ± 0.2^{a}
IBA	0.5	41.66 ± 4.16^{bc}	8.4 ± 0.4^{c}
	0.75	29.16 ± 11.02^{c}	6.1 ± 0.5^{d}
	1	29.17 ± 4.17^{c}	5.8 ± 0.5^{d}

Values represent mean ± S.E. of eight replicates of three experiments. Within each column, means followed by different letters are significantly different at $p \leq 0.05$ according to DMRT. The superscripts represent ranking, i.e., 'a' indicates the best response and 'e/d' indicates worst response.

3.4. GC-MS Analysis

The phytocompounds present in methanolic extracts of in vivo and in vitro grown leaves, and callus of *G. pulchella* were identified by using GC–MS. The phytocompounds with their retention time (RT), retention area (%),molecular formula and molecular weight are presented in Tables 4–6. The NIST library consulted and the GC–MS chromatograms are presented in Figures 4–6. The phytochemical composition of *G. pulchella's* leaf-derived callus was identified and examined. Upon analyzing the methanolic callus extract, 34 phytocompounds were identified (Table 4 and Figure 4), several of which were found in trace amounts. Compounds like 3-hydroxy-2,3-dihydromaltol (25.39%), isoamyl acetate (11.63%), palmitic acid (11.55%), 4-methyloxazole (7.54%), 5-methoxypyrrolidin-2-one (7.49%) were observed as the major phytocompounds present in higher quantities.

Table 4. Phytocompounds identified in leaf-derived callus extract of *G. pulchella* through GC-MS.

Peak#	R. Time (min)	Area%	Name	Molecular Formula	Molecular Weight
1	4.160	3.38	pyranone	$C_6H_8O_4$	144
2	4.552	7.54	4-methyloxazole	C_4H_5NO	83
3	5.103	1.93	levulinic acid	$C_5H_8O_3$	116
4	5.311	0.05	2,5-dimethyl-3(2h) furanone	$C_6H_8O_3$	128
5	5.675	3.28	cyanuramide	$C_3H_6N_6$	126
6	5.910	4.03	1-aminocyclopropane-1-carboxylic acid	$C_4H_7NO_2$	101
7	6.344	0.52	3-methyloctane	C_9H_{20}	128
8	6.645	25.39	3-hydroxy-2,3-dihydromaltol	$C_6H_8O_4$	144
9	6.837	0.69	di-2-ethylhexyl amine	$C_{16}H_{35}N$	241
10	6.957	0.69	neopentyl acetate	$C_7H_{14}O_2$	130
11	7.172	7.49	5-methoxypyrrolidin-2-one	$C_5H_9NO_2$	115
12	7.843	11.63	isoamyl acetate	$C_7H_{14}O_2$	130
13	8.159	1.56	glycerol 1-acetate	$C_5H_{10}O_4$	134
14	8.749	1.04	2-methylpentyl propyl ether	$C_9H_{20}O$	144
15	8.975	0.38	1-methylpiperidine	$C_6H_{13}N$	99
16	10.183	1.03	bis(2-ethylhexyl) amine	$C_{16}H_{35}N$	241
17	11.032	3.39	di-sec-butyl oxalate	$C_{10}H_{18}O_4$	202
18	11.703	0.77	1,2,3,5-cyclohexanetetrol	$C_6H_{12}O_4$	148
19	12.696	0.73	2-aminobenzoyl azide	$C_7H_6N_4O$	162
20	13.165	1.65	methyl hexopyranoside	$C_7H_{14}O_6$	194
21	13.640	0.47	santalol	$C_{15}H_{24}O$	220
22	14.510	0.94	caprinic acid	$C_{10}H_{20}O_2$	172
23	15.238	0.34	(−)-cis-pinan	$C_{10}H_{18}$	138
24	15.729	0.33	(s)-(+)-3-methyl-1-pentanol	$C_6H_{14}O$	102
25	16.590	11.55	palmitic acid	$C_{16}H_{32}O_2$	256
26	18.215	0.79	cyclooctene, 3-methyl-	C_9H_{16}	124
27	18.467	0.56	cyclohexyl laurate	$C_{18}H_{34}O_2$	282
28	19.473	0.25	bis(2-(dimethylamino)ethyl) ether	$C_8H_{20}N_2O$	160
29	20.493	1.11	neophytadiene	$C_{20}H_{38}$	278
30	21.205	1.18	cis-z-.alpha.-bisabolene epoxide	$C_{15}H_{24}O$	220
31	21.531	0.76	1,2-benzenedicarboxylic acid, diisooctyl e	$C_{24}H_{38}O_4$	390
32	23.909	2.16	squalene	C30H50	410
33	31.099	1.97	1,16-dichlorohexadecane	$C_{16}H_{32}Cl_2$	294
34	32.623	0.40	6(e),9(z),13(e)-pendectriene	$C_{15}H_{26}$	206

Table 5. GC–MS analysis of in vivo grown plant's leaves of *G. pulchella* showing phytocompounds.

Peak#	Retention Time (min)	Retention Area (%)	Phytocompounds	Formula	Molecular Weight
1	7.811	6.28	Isoamyl Acetate	$C_7H_{14}O_2$	130
2	11.061	0.38	(−)-Globulol	$C_{15}H_{26}O$	222
3	11.307	0.07	4iodobis(Bicyclo [2.2.1])Hexane	$C_{12}H_{17}I$	288
4	11.349	0.25	2-Tridecynyl2,6-Difluorobenzoate	$C_{20}H_{26}F_2O_2$	336
5	11.543	0.11	(3e)-6-Methyl-3,5-Heptadien-2-One	$C_8H_{12}O$	124
6	15.170	0.12	(5e)-3-Methyl-5-Undecene	$C_{12}H_{24}$	168
7	15.239	3.63	3-Methylene-7,11,15-Trimethylhexadec-1-Ene	$C_{20}H_{38}$	278
8	15.494	0.72	Neophytadiene	$C_{20}H_{38}$	278
9	15.688	1.15	3,7,11,15 Tetramethyl-2-Hexadecen-1-Ol	$C_{20}H_{40}O$	296
10	15.800	0.32	1-Hexadecyloctahydro-1h-Indene	$C_{25}H_{48}$	348
11	16.575	0.77	1,4-Cyclohexanediol, (Z)-, Tms Derivative	$C_{16}H_{32}O_2$	256
12	16.798	0.15	Pentadecanal	$C_{15}H_{30}O$	226
13	17.954	3.64	Phytol	$C_{20}H_{40}O$	296
14	19.000	0.12	14-Heptadecenal	$C_{17}H_{32}O$	252

Table 5. *Cont.*

Peak#	Retention Time (min)	Retention Area (%)	Phytocompounds	Formula	Molecular Weight
15	20.299	1.14	Vitamin A Acetate	$C_{22}H_{32}O_2$	328
16	20.554	1.23	2-Methyl-1-Phenyl-1-Butanol	$C_{11}H_{16}O$	164
17	20.613	0.44	6-Epi-Shyobunol	$C_{15}H_{26}O$	222
18	20.808	0.37	Xanthinin	$C_{17}H_{22}O_5$	306
19	20.896	0.45	Limonene Dioxide	$C_{10}H_{16}O_2$	168
20	20.942	1.25	Isoborneol, Allyldimethylsilyl Ether	$C_{15}H_{28}OSi$	252
21	21.187	13.23	Stanolone Acetate	$C_{21}H_{32}O_3$	332
22	21.260	34.53	1-Heptatriacotanol	$C_{37}H_{76}O$	536
23	21.467	0.35	5,8,11-Eicosatrienoic Acid, (Z)-, Tms Derivative	$C_{23}H_{42}O_2Si$	378
24	21.539	0.40	5-Hydroxy-4-Nitro-1-Decalinone	$C_{10}H_{15}NO_4$	213
25	21.719	0.20	Diazoprogesterone	$C_{21}H_{30}N_4$	338
26	22.453	0.48	Cis-Z-.Alpha.-Bisabolene Epoxide	$C_{15}H_{24}O$	220
27	22.657	0.12	Cyclopropanecarboxylic Acid, 1-Amino-	$C_4H_7NO_2$	101
28	22.740	0.95	Periplogenin	$C_{23}H_{34}O_5$	390
29	22.824	0.42	2-Bromo Dodecane	$C_{12}H_{25}Br$	248
30	23.162	12.26	Chrysanin	$C_{20}H_{24}O_5$	344
31	23.503	0.27	1,2-Dibromoethane	$C_2H_4Br_2$	186
32	23.702	0.34	N,N′-Di(2-Propenyl)-1,2,4,5-Tetrazin-3,6-Diamine	$C_8H_{12}N_6$	192
33	23.774	0.77	2-Hydroxy-1,1,10-Trimethyl-6,9-Epidioxy-7-Octalin	$C_{13}H_{20}O_3$	224
34	23.912	2.08	Squalene	$C_{30}H_{50}$	410
35	24.386	0.27	1-Chlorooctadecane	$C_{18}H_{37}Cl$	288
36	24.699	0.38	1-Heptatriacotanol	$C_{37}H_{76}O$	536
37	24.834	1.20	1-Tridecyn-4-Ol	$C_{13}H_{24}O$	196
38	25.287	1.06	Bromocyclohexane	$C_6H_{11}Br$	162
39	27.054	0.29	.Gamma.-Tocopherol	$C_{28}H_{48}O_2$	416
40	28.402	1.87	Vitamin E	$C_{29}H_{50}O_2$	430
41	30.647	0.64	2,2,4, 4-Tetramethyl-3-Pentanone	$C_9H_{18}O$	142
42	30.998	1.00	2,6-Dimethyl-6-Nitro-2-Hepten-4-One	$C_9H_{15}NO_3$	185
43	31.598	2.08	Isocitronellol	$C_{10}H_{20}O$	156
44	32.616	1.28	Sitosterol	$C_{29}H_{50}O$	414
45	32.864	0.97	(6e)-2,4-Dimethyl-2,6-Octadiene	$C_{10}H_{18}$	138

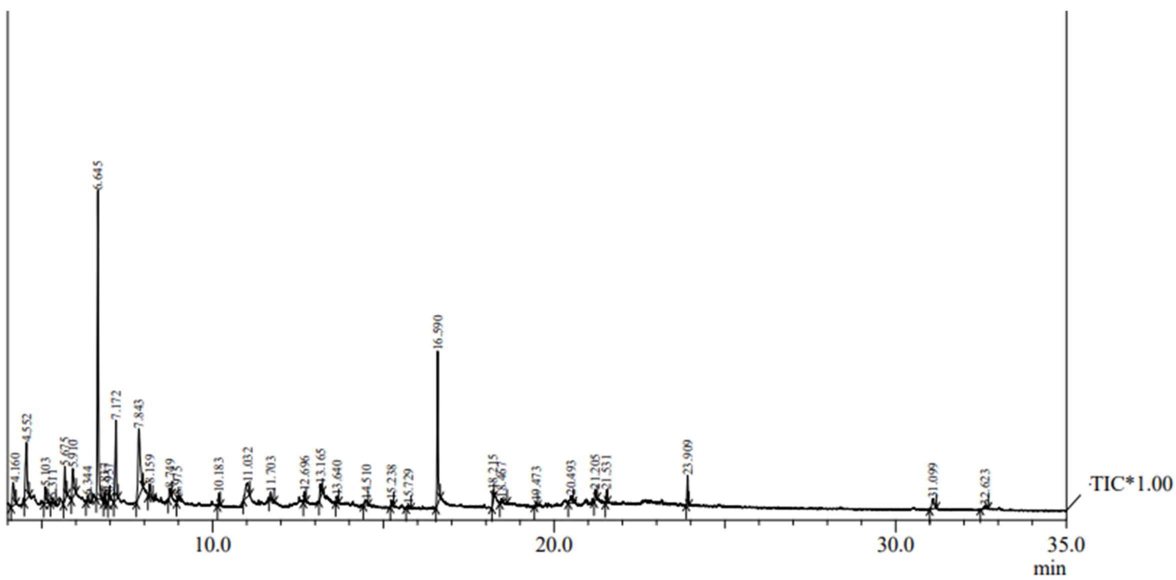

Figure 4. GC-MS chromatogram of methanolic extract of leaf-derived callus extract of *G. pulchella* revealing phytoconstituents with retention time.

Table 6. GC–MS analysis of in vitro grown plant's leaves showing phytocompounds.

Peak#	Retention Time (min)	Retention Area (%)	Phytocompound	Formula	Molecular Weight
1	7.573	1.70	Pyranone	$C_6H_8O_4$	144
2	11.856	15.72	Nitroisobutylglycerol	$C_4H_9NO_5$	151
3	13.636	0.41	1,4-Cyclohexanediol, (Z)-, TMS Derivative	$C_9H_{20}O_2Si$	188
4	16.225	3.20	Phytol, Acetate	$C_{22}H_{42}O_2$	338
5	16.477	0.28	3-Eicosyne	$C_{20}H_{38}$	278
6	16.675	1.39	Neophytadiene	$C_{20}H_{38}$	278
7	17.129	0.28	Octadecanoic Acid, Methyl Ester	$C_{19}H_{38}O_2$	298
8	18.284	0.93	Cholestan-3,26-Diol-22-One	$C_{27}H_{46}O_3$	418
9	18.637	1.74	2-Methylpropionic Acid, 3,4-Dichlorophenyl Ester	$C_{10}H_{10}Cl_2O_2$	232
10	18.831	0.37	Methyl Petroselinate	$C_{19}H_{36}O_2$	296
11	19.447	0.43	Hexane, 3,3-Dimethyl	C_8H_{18}	114
12	20.467	0.50	Octanoic Acid, 2-Dimethylaminoethyl Ester	$C_{12}H_{25}NO_2$	215
13	21.880	0.46	2-Decanone	$C_{10}H_{20}O$	156
14	21.953	1.09	Propylhexedrine	$C_{10}H_{21}N$	155
15	22.088	6.43	Longifolenaldehyde	$C_{15}H_{24}O$	220
16	22.165	13.16	Isocembrol	$C_{20}H_{34}O$	290
17	23.837	4.27	Tetracontane	$C_{40}H_{82}$	562
18	24.030	2.31	Chrysanin	$C_{20}H_{24}O_5$	344
19	24.598	1.31	Squalene	$C_{30}H_{50}$	410
20	25.415	14.56	17,18,19,20-Tetrahydro-16,21-Di-T-Butyl-1,6-Methano-32-Annulene	$C_{41}H_{44}$	536
21	26.447	6.48	Hexatriacontane	$C_{36}H_{74}$	506
22	26.865	0.90	Carbonic Acid, 2-Ethylhexyl Pentadecyl Ester	$C_{24}H_{48}O_3$	384
23	27.175	2.17	(+)-.Alpha.-Tocopherol	$C_{29}H_{50}O_2$	430
24	28.395	1.10	11-Hydroxypregn-4-Ene-3,20-Dione	$C_{21}H_{30}O_3$	330
25	28.695	2.90	6-Nitrocholesteryl Acetate	$C_{29}H_{47}NO_4$	473
26	29.222	3.98	2-Methylhexacosane	$C_{27}H_{56}$	380
27	29.465	4.98	Ergost-5-En-3-Ol, (3.Beta.)-	$C_{28}H_{48}O$	400
28	32.471	3.02	Eicosane	$C_{20}H_{42}$	282

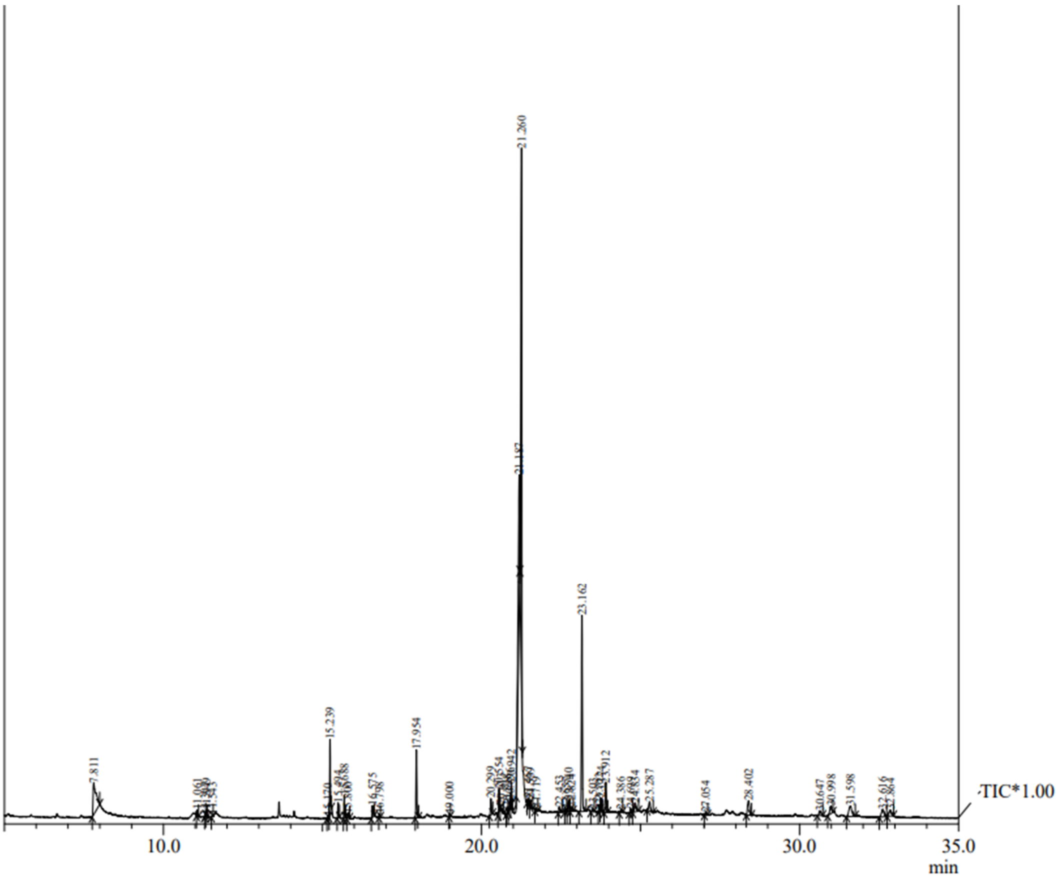

Figure 5. GC-MS chromatogramic view of methanolic extract of in vivo grown leaves of *G. pulchella* revealing phytoconstituents with retention time.

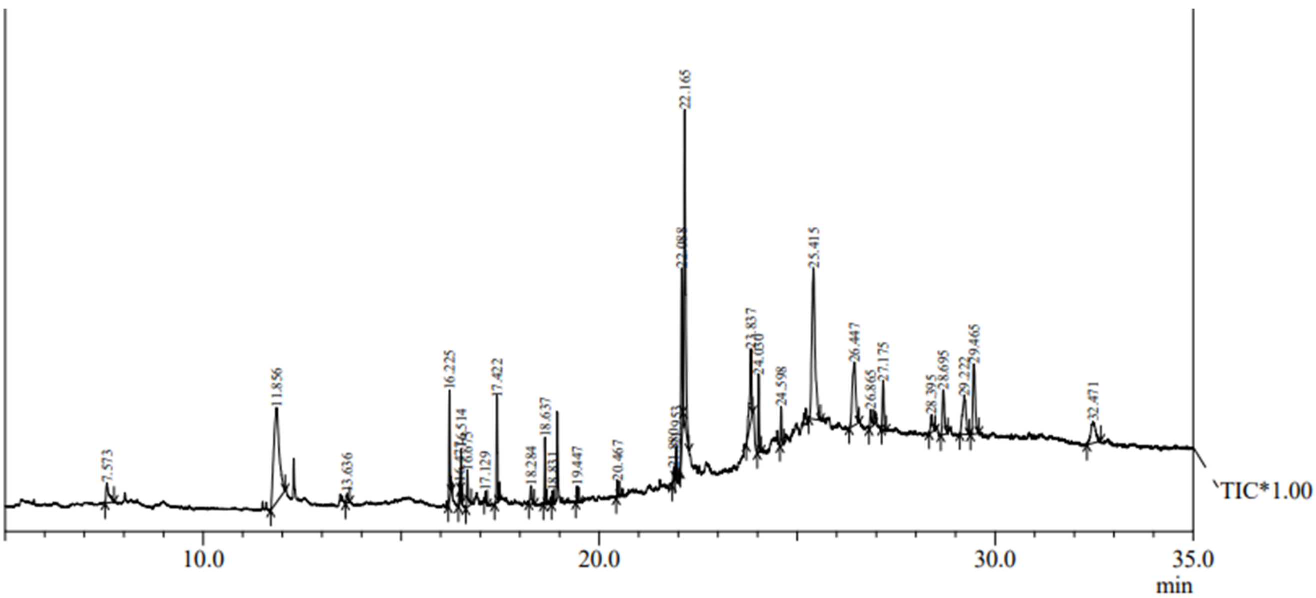

Figure 6. GC-MS chromatogram of methanolic extract of in vitro derived leaf tissue of *G. pulchella* revealing the phytoconstituents with their retention time.

Leaves derived in vivo and in vitro were exposed to chromatographic examination, in which 45 and 28 phytocompounds were identified, respectively, that belong to different classes like lignans, phenols, terpenoids, alkaloids and fatty acids, etc. Various medicinally important phytocompounds were found in methanolic extract of in vivo leaves samples such as stanolone acetate (13.23%), isoamyl acetate (6.28%), phytol (3.64%), isocitronellol (2.08%), vitamin E (1.87%), sitosterol (1.28%) vitamin A acetate (1.14%), (−)-globulol (0.38%), pentadecanal (0.15%) (Table 5 and Figure 5). Similarly, several bioactive compounds were exclusively found in in vitro regenerated leaf tissues in varied quantities. They were like nitroisobutylglycerol (15.72%), isocembrol (13.16%), hexatriacontane (6.48%), longifolenaldehyde (6.43%), ergost-5-en-3-ol (4.98%), tetracontane (4.27%), 2-methylhexacosane (3.98%), phytol acetate (3.20%), eicosane (3.02%), (+)-.alpha.-tocopherol (2.17%), and pyranone (1.70%) (Table 6 and Figure 6). In both extracts, the most common bioactives found were squalene, chrysanin, neophytadiene and 1,4-Cyclohexanediol, (Z)- and TMS derivative. The amount of chrysanin was found to be more than five times higher (12.26%) in in vivo grown leaves compared to in vitro grown ones (2.31%). Similarly, squalene content in in vivo grown leaves was nearly twice higher (2.08%) than in in vitro grown leaves (1.31%). Neophytadiene also showed a comparable variation, with field-grown leaves' amount of 0.72% and in vitro derived leaves' of 1.39%. A similar difference was noted for 1,4-Cyclohexanediol, (Z)-, TMS derivative (0.77% in field-grown and 0.41% in tissue-culture-obtained leaves).

3.5. Determination of Total Phenolic and Flavonoid Content and DPPH Activity

The Folin–Ciocalteu method was used to assess the total phenolic content of tissues (the callus and leaves extracts), with gallic acid serving as the standard. The in vitro leaves had the highest phenolic content, followed by in vivo leaves and the callus (Table 7). The in vitro developed leaves had a TPC (total phenolic content) value of 5.32 ± 0.1 mg GAE/g DW, while the field-grown leaves had a TPC value of 3.49 ± 0.1 mg GAE/g DW. The callus had the lowest TPC, which was 0.95 ± 0.1 mg GAE/g DW. The aluminum chloride method was used to quantify the total flavonoid content (TFC) of the tested samples. The values were represented as QE/gDW. The extracts contained flavonoids and the yield varied from 6.56 to 13.19 mg QE/g DW. Among all the samples tested, the in vitro leaves had the highest flavonoid content, followed by in vivo grown leaves and leaf-derived callus, respectively (Table 7). The in vitro developed leaves had a TFC value of 13.19 ± 0.1 mg QE/g DW, while the field ones grown had a TFC value of 11.20 ± 0.4 mg QE/g DW. The callus extract had the lowest TFC value 6.56 ± 0.2 mg QE/g DW. Using a DPPH free radical scavenging experiment, the antioxidant activity of each extract was measured, and the results are shown in Table 7. Just like TPC and TFC, DPPH demonstrated a similar trend in antioxidant potential of the tested tissues. The in vitro grown leaves' extract was found to have a greater level of scavenging activity (50.64%) in comparison to that of the in vivo leaves (36.22%). The callus extract had the lowest level of scavenging activity (30.28%).

Table 7. Total phenolic content (mg GAE/g DW), total flavonoid content (mg QE/g DW) and DPPH scavenging activity (%) of different tissues in *G. pulchella*.

Tissue Type	TPC	TFC	DPPH Scavenging Activity
Callus	0.95 ± 0.1 ^{c}	6.56 ± 0.2 ^{c}	30.28 ± 1.0 ^{c}
Leaves (in vivo)	3.49 ± 0.1 ^{b}	11.20 ± 0.4 ^{b}	36.22 ± 0.9 ^{b}
Leaves (in vitro)	5.32 ± 0.1 ^{a}	13.19 ± 0.1 ^{a}	50.64 ± 0.6 ^{a}

Values represent means ± S.E. of three replicates. Within each column, means followed by different letters are significantly different at $p \leq 0.05$ according to DMRT. Abbreviations used: TPC: total phenolic content, TFC: total flavonoid content, GAE: gallic acid equivalent, QE: quercetin equivalent, DW: dry weight.

4. Discussion

The current investigation described the influence of PGRs on in vitro morphogenic processes like the induction of callus, the formation of shoots and the rooting of *Gaillardia pulchella* shoots. In vitro generated tissue's photochemical and biochemical profiles were also examined. The leaves were first inoculated on MS with varying concentrations of PGRs for induction of callus. The response obtained suggests that NAA(2.0 mg/L) and

BAP (0.5 mg/L) synergistically induced prolific callus with maximum fresh biomass as compared to the 2, 4-D treatments. In recent years, Manokari et al. [20] noted in vitro shoot regeneration on MS medium supplemented with IAA and BAP in *G. pulchella*. The beneficial effects of NAA and BAP were observed on several other members of Asteraceae, like *Saussurea obvallata* [25] and *Artemesia annua* [26]. After further subculture of callus in the presence of the same PGRS, the shoots were formed and proliferated. Similar effects of BAP and NAA on callus-mediated shoot induction were observed in various plant species, such as *E. urophylla* × *E. grandis* and *Dendrocalamus latiflorus* [27,28], which is in accordance with the present study. Auxins, along with cytokinins, are frequently used in triggering shoots since these signaling components are known to influence cytokinins' overbearing effects [29]. Earlier reports on plant species like *Curcuma zedoaria* [30], *Basella rubra* [31], and *Santalum album* [32] demonstrated a similar promotive action of auxin with cytokinin in shoot production. Afterwards, in our study, the shoots were moved to a rooting medium with varying IAA and IBA concentrations. The rate of rooting was greater in shoots raised on IAA than IBA. Sholikhah et al. [33] reported the promotive effect of IAA compared to other auxin treatments in producing roots of in vitro derived Cavendish banana shoots. On the contrary, IBA was equally effective in promoting roots, observed in plants like *Dracaena sanderiana* [34], *Adenostyles alpina* [35] and many others.

Regenerated tissues and cell lines are under stress in in vitro culture environments, which affect growth, development and survival rate [36]. It is thus essential to evaluate cellular physiology periodically. In *G. pulchella*, the biochemical attributes of tissue-culture-derived plant tissues were studied and compared with the mother plant. There are several elements that affect up- and down-regulation of phenolic and flavonoid production, such as different PGRs used during cultivation [37]. In our investigation, it was found that in vitro leaf tissues had higher levels of phenolic and flavonoid contents than the mother plants. This is inconsistent with previously published biochemical research carried out on several plant species [38,39]. The data from the present investigations demonstrated that in vitro leaf tissues had enriched level of antioxidant activities than the other two tested samples. The antioxidant activities of in vitro raised tissues were assessed through DPPH assays. When DPPH, an organic free radical with a dark color, absorbs an electron or another free radical, it changes to a light-yellow color, signifying the scavenging action [40]. According to this study, the higher level of antioxidant potential found in laboratory-grown leaves is due to phenolics and flavonoids, which positively correlate with antioxidant activity. These molecules deactivate free radicals by donating hydrogen atoms to them [24]. Numerous plants like *Salvia hispanica* [41], *Tylophora indica* [42], etc., showed similar antioxidant ability.

Many phytoelements, like volatile oils, hydrocarbons, sugar alcohols, esters, alkaloids, flavonoids, and saponins, can be easily identified by GC–MS, a commonly used analytical approach [18]. By identifying variations in peak area percentage in the metabolite profiles of in vitro regenerated plants and their wild (mother) counterparts, this method offers significant insights into the impact of plants' growth and development. Different plants are frequently used for medicinal purposes like *Catharanthus roseus* [43], and *Amomum nilgiricums* [17] and were recently subject to the GC–MS technique. The chromatographic data obtained from the present research analysis demonstrated the presence of over 25 significant bioactive chemicals in all examined samples. Different levels of phytocompounds were found in methanolic leaf extracts produced in vivo and in vitro. The in vitro derived leaves tissues produced a nearly similar amount of phytocompounds when compared to the in vivo grown leaves. The in vitro derived leaves also produced pharmaceutically important phytocompounds that were not present in in vivo leaves and leaf-derived callus. This phenomenon could be attributed to a number of factors like genotype, temperature, photoperiod, media composition and PGRS concentration [44]. A range of phytochemicals, such as n-hexadecanoic acid, phytol, cyclopropaneoctanoic acid, limonene, platambin, etc., were previously reported to be present in field-grown leaves of *G. pulchella* [45]. Thus, it represents a more potent and dependable source of phytocompounds for pharmacological applications. *G. pulchella* contains a variety of phytoconstituents with therapeutic application. In this study, several phytocompounds were exclusively found in methanolic leaves extracts of in vivo grown plants. These were vitamin E, beta-sitosterol, vitamin A acetate, andisocitronellol, etc. Vitamin E acts as a liposoluble antioxidant and is used in the treatment of cardiovascular, neurological and aging-related diseases [46]. Beta-sitosterol is a phytosterol showing many beneficial effects against different diseases; it lowers urinary tract infection, hypercholesteremia, immunosuppression and inflammation, rheumatoid arthritis and androgenetic alopecia [47]. Some phytocompounds are exclusively found in methanolic leaf extract of in vivo grown plants, viz., eicosane, phytol acetate, 2-Methyl hexacosane, etc. Eicosane is a very strong anti-inflammatory, analgesic, and antipyretic agent [48]. Several phytocompounds such as squalene, neophytadiene, etc., were found in all methanolic samples. The terpenoid, squalene has several biological properties; it presents antioxidant, anticancer, detoxifying and moisturizing properties [49]. Neophytadiene is a diterpene, and it plays a role as an anti-inflammatory, antimicrobial, antipyretic agent, and is frequently exploited against headaches, rheumatism and in skin issues [50]. This observation shows that in vitro grown tissues like callus and leaves have enormous pharmacological potential, which is in agreement with previous observation [51]. Finding these compounds may make it possible to develop new drugs more quickly and in a cheaper way. Future research could be conducted to understand protein–ligand action of important bioactives/drugs by employing techniques like molecular docking and bio-prospecting.

5. Conclusions

The micropropagation method was successfully used to induce callus, shoots and roots in *G. pulchella*, an important medicinal plant. A combination of NAA and BAP was proven to be efficient in inducing callus from leaf explants with subsequent shoot formation (indirect organogenesis) on the same medium. The in vitro regenerated tissues were then analyzed through GC-MS for identifying phytocompounds. The present study identified a large number of phytocompounds including alkaloids, flavonoids, and terpenoids of medicinal uses. When tissues of different sources were compared for biochemical and antioxidant activities, the in vitro leaves demonstrated a higher level of phenolics and flavonoids and antioxidant capacity than the other studied tissues. A variety of therapeutically important bioactive compounds were found in in vitro grown plant tissues. The pharmaceutical industry can use these compounds on a large-scale by collaborating in vitro culture techniques as the cultures can be proven to be a continuous source of such important phytocompounds. Future studies like structural characterization and modification, investigations on ligand–protein interaction, and toxicological analyses under in vivo models can be performed for better development of novel drugs.

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Conflicts of Interest: The authors declare that there are no conflicts of interest.

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