

In vitro micropropagation and gas chromatography-mass spectrometry profiling of callus culture in *Pulicaria jaubertii* for conservation and metabolite production

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ABSTRACT

Pulicaria jaubertii is an aromatic and medicinal plant endemic to Yemen, currently facing habitat decline. This study aimed to evaluate its *in vitro* response in full-strength Murashige and Skoog (medium supplemented with different types and concentrations of plant growth regulators. Among the tested plant parts, only seed explants successfully initiated callus formation. Calli were subsequently subcultured in media containing 0.1 mg/L 1-naphthaleneacetic acid (NAA) with kinetin (Kin) at 0, 0.25, 0.5, or 1 mg/L. Additional experiments tested media with 0.1 mg/L 6-benzylaminopurine and indole-3-acetic acid (IAA) (0–1 mg/L), as well as 0.1 mg/L Kin with 2,4-dichlorophenoxyacetic acid (2,4-D) (0–1 mg/L). Growth parameters related to callus induction, root, shoot, and leaf production were assessed. Findings revealed that Kin had no significant effect on most growth parameters except callus colour ($P = 0.012$), with the best growth at 0.25 mg/L. Similarly, IAA significantly influenced callus induction ($P = 0.009$), with optimal results at 1.0 mg/L. In contrast, 2,4-D had no significant effect, but its highest concentration (1.0 mg/L) supported optimal growth. Gas chromatography-mass spectrometry (GC-MS) analysis identified 46 compounds in the ethanolic callus extract compared to 25 in the mother plant, which indicates a richer phytochemical profile in the callus. The 2-Ethoxyethylamine (85.60%) and Stigmasterol (58.79%) were most abundant in ethanolic and n-hexane extracts. In conclusion, *P. jaubertii* seeds are the most responsive explants for micropropagation, forming callus as an initial step. Interestingly, GC-MS profiling identified bioactive compounds with medicinal properties. Further studies should refine auxin and cytokinin ratios to enhance propagation efficiency.

1. INTRODUCTION

Pulicaria jaubertii Gamal Eldin, also known as *Pulicaria orientalis* Jaub., which is a member of the family *Asteraceae*, is one of the *Pulicaria* species endogenous to the Arabian Peninsula, particularly in Yemen. It is a perennial aromatic plant with erect branches that may reach 50 cm in height. In Arabic, it is officially called “Eter Elraee” [1,2]. However, it is known locally in Yemen as “Anssif” or “Alkhaoah” and is used as a spicing herb for making some food, such as laban (fermented milk) and soup. Traditionally, it is used as an

insecticide, antipyretic, diuretic, and for treating urogenital system disorders, colds, malaria, inflammation, and microbial infections [3-6].

P. jaubertii is a perennial aromatic herb with erect, tomentose, grey-green branches reaching 30–50 cm in height. The plant is strongly fragrant and partially woody at the base. Leaves are sessile, oblong to oblanceolate, measuring 1.5–4.5 cm in length and 0.4–2 cm in width, with toothed margins and an obtuse apex. The inflorescence consists of a few capitula (flower heads), each 1–2 cm in diameter, with yellow florets. Involucral bracts are oblanceolate and densely villous. The ray florets are 3–5 mm long. The fruit is an achene, setulose, and obscurely ribbed, containing small seeds adapted for wind dispersal [2,7].

Several studies revealed that *Pulicaria* species, including *P. jaubertii*, have been found to have various bioactive characteristics such as cytotoxic and anticancer [2,8-11], antioxidant [3,5,12-14], antibacterial [2,13,15], antifungal and immuno-regulatory [16], antiinflammatory and antihistaminic [9,10,17], antidiabetic [9],

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antihypertensive [8,18], and antispasmodic [19] activities.

Detailed agricultural statistics on this species are rare due to its large-scale harvesting rather than cultivation. Ethnobotanical surveys and herbarium records in Yemen indicate that *P. jaubertii* is seasonally collected because of its high demand for culinary and medicinal uses in rural areas [20,21]. In addition, it is highly sold in local markets; thus, formal data on annual harvest volumes are also lacking. Therefore, the emergence and worsening of habitats have increased concern for the stability of wild populations, which outlines the need for protective measures and further research [22].

P. jaubertii is not yet recorded on the International Union for Conservation of Nature Red List of endangered species. However, many factors like drought, overexploitation, uncontrolled overcutting, and overgrazing in Yemen and Saudi Arabia may put many native species, including *P. jaubertii*, at high risk and worsen this decline [7]. Therefore, conservation efforts should consist of protecting their natural habitats and preserving them in *ex-situ* environments through seed banks and botanical gardens [16]. However, some recent techniques, including the micropropagation technique, could be used to improve their production. This technique is an important *in vitro* tissue culture method that may be used to conserve a great number of threatened and rare crops, medicinal, and economically important plants [23–27]. It could be used to provide several benefits for the plant, such as producing a large amount of pathogen-free and healthy plants, as well as genetically improved crops within a relatively short time and small space [28–32]. Interestingly, the rate of proliferation and other variables could be affected by the use of diverse types and concentrations of plant growth regulators (PGRs), chiefly auxins and cytokinins [33,34]. These types of PGRs are commonly used in combination with plant tissue culture [35,36], and the research area for their action is still broad. Phytochemical composition in plants varies due to genetic, environmental, and methodological factors, which could influence their biological properties and therapeutic potential [37].

P. jaubertii has been widely investigated for its essential oils and secondary metabolites, which vary significantly with geographic origin, extraction method, and plant part used [2,5,13,38]. However, *in vitro* techniques such as callus culture remain unexplored for this species. Callus cultures offer a controlled environment for enhancing the production of bioactive compounds [16]; and understanding the biochemical and genetic factors influencing such variation may facilitate the synthesis of pharmacologically and industrially valuable phytochemicals [39]. To date, no study has reported a micropropagation protocol or gas chromatography-mass spectrometry (GC-MS)-based phytochemical profiling for *P. jaubertii*. This study, therefore, presents the first successful micropropagation strategy for

the species, employing seed-derived callus as the primary explant source. Although the approach follows standard protocols used in related *Asteraceae* species such as *Pulicaria incisa* and *Achillea* spp., its application to *P. jaubertii* addresses a critical gap. The developed protocol not only supports conservation but also establishes a reproducible platform for secondary metabolite production under controlled conditions, which aims to assess growth responses in Murashige and Skoog (MS) media supplemented with different types and concentrations of PGRs, specifically cytokinins and auxins.

2. MATERIALS AND METHODS

2.1. Chemicals

Cytokinins (kinetin [Kin] and 6-benzylaminopurine [BAP]) and Auxins (1-naphthaleneacetic acid [NAA] and 2,4-dichlorophenoxyacetic acid [2,4-D]), plant hormones, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol, n-hexane, and other chemicals used in the study were of the highest quality available.

2.2. Plant Material and Study Design

P. jaubertii [Figure 1a] was collected from Ibb City, Yemen, from March to April 2021 at GPS coordinates of 13° 57' 46.44" N and 44° 10' 23.88" E. The taxonomist, Dr. Esam Aqlan, identified the plant at the Biology Department, Faculty of Science, Ibb University. The experimental study was conducted in the Tissue Culture Laboratory, Department of Biology, Faculty of Sciences, Ibb University, in 2021.

2.3. Medium and Cultural Conditions

Several types of plant parts (leaves, nodes, and seeds) were surface-sterilised and inoculated in a full Murashige and Skoog [40] medium as performed by Salam *et al.* [41] [Figure 1b]. Preliminary trials also tested leaf and stem explants under various hormonal regimes, including high cytokinin and low auxin combinations; however, these explants failed to produce callus or shoots and often exhibited necrosis. Seeds, used with their coats intact, germinated successfully and induced callus without the need for decoating. The MS medium contains specific concentrations of nutrients necessary for most plant growth, but in hormone-free conditions, only seeds produce roots and shoots.

For micropropagation, seeds were supplemented with 0.5 mg/L Kin and 0.1 mg/L 1-naphthalene acetic acid (NAA) in MS medium. Callus was formed within 1–2 weeks [Figure 1c and d], followed by shoot formation and then root production after 3–4 weeks. Explants obtained from these calli were then subcultured with various types and concentrations of PGRs.

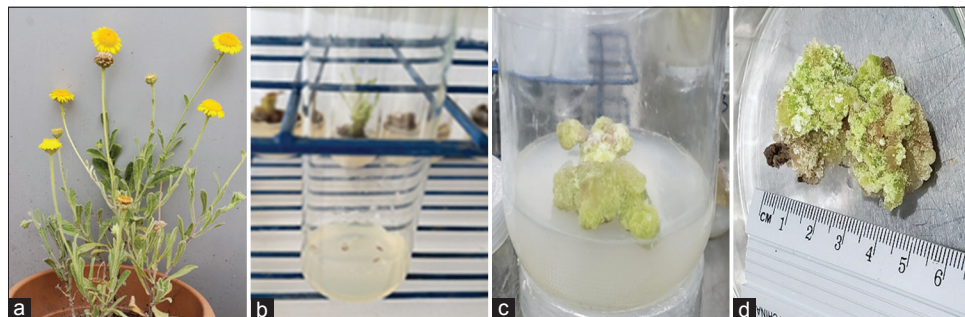


Figure 1: (a) Morphology of *Pulicaria jaubertii*; (b) Seeds; (c) Callus used as explants; (d) Harvested callus for extraction.

2.4. Hormonal Effects on Shoot, Root and Callus Regeneration

2.4.1. Shoot induction and multiplication

The explants' response to growth was analysed in full MS media with different concentrations of the cytokinin Kin (0, 0.25, 0.5, and 1 mg/L) and a fixed concentration of the auxin NAA (0.1 mg/L).

2.4.2. Root induction

The explants' response to growth was investigated in MS media supplemented with various concentrations (0, 0.25, 0.5, and 1 mg/L) of the auxin indole-3-acetic acid (IAA) and a fixed concentration of the cytokinin 6-benzylamino purine (BAP) (0.1 mg/L).

2.4.3. Callus induction

Likewise, explants' response was tested with different concentrations of growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) (0, 0.25, 0.5, and 1 mg/L) and a fixed concentration of Kin (0.1 mg/L).

The response included the regeneration of shoots, roots, and callus, as well as their related growth parameters. Cultures were kept at $25 \pm 2^\circ\text{C}$ in an air-conditioned environment under a 16-h light/8-h dark photoperiod. Since no previous micropropagation study has focused on *P. jaubertii*, a stepwise method was used to understand the basic hormonal needs for shoot and callus development. Each experiment changed one growth regulator (cytokinin or auxin) while keeping the concentration of the other constant to find effective baseline conditions.

2.4.4. Acclimatisation of in vitro derived plantlets

Acclimatisation was carried out on 50 rooted shoots from 35-day-old *in vitro* plantlets. These plantlets were moved to plastic pots filled with a soil-sand mixture (70:30, v/v) and kept under laboratory conditions ($25 \pm 2^\circ\text{C}$, 16-h photoperiod) for 4 weeks. The survival rate was noted, and 45 plantlets (90%) successfully acclimatised. Among these, 20 plantlets were later transplanted into larger pots and grown under outdoor garden conditions. All 20 plantlets survived and showed healthy growth (100%) after another 4 weeks.

2.5. Gas Chromatography-Mass Spectrometry (GC-MS) Analyses

Ethanol and n-hexane extracts were made from shade-dried powder obtained from the mother and callus derived from micropropagated explants of *P. jaubertii*. The four extracts were prepared using the cold maceration method and then concentrated at 45°C using a rotary evaporator (Büchi, Switzerland). We determined the volatile phytochemicals following the procedure by Yisak *et al.* [42] with some modifications. GC-MS analysis was done using an Agilent 7890A gas chromatograph coupled with a 5975C mass spectrometer (Agilent, USA) equipped with electron ionisation (EI) for phytochemical analysis. Chromatographic separations were carried out using an HP-5MS (19091S-433) capillary column that is 30 m long, has a 0.25 mm internal diameter, and a 0.25- μm column phase film thickness. The injection mode was split-less, Helium served as the carrier gas, and we injected 1 μL of the sample at a consistent flow rate of 1 mL/min into an inlet heated to 275°C . The initial oven temperature was 60°C with a 2-minute hold time, then increased to 200°C with a ramp of $10^\circ\text{C}/\text{min}$ and $3^\circ\text{C}/\text{min}$ to 240°C . The ion source temperature was set to 230°C for the mass spectrometer settings, and the quadrupole temperature was 150°C . The system ran in positive electron impact mode at 70 eV, scanning from 40 to 650 m/z. The total run time was 45 minutes. Phytochemical components were qualitatively identified by comparing their retention times and mass spectral data with those in the NIST98 library.

2.6. Statistical Analysis

Data were summarized in tables as frequencies and proportions. Crosstabulation and Fisher's exact test were used to study the effect of different concentrations of the hormones on the non-parametric markers of plant growth. One-way analysis of variance, followed by Duncan's Multiple Range test, was used to compare the means of the parametric values produced at various concentrations of the hormones. Data analysis was performed using the statistical software Statistical Package for the Social Sciences version 20.0, and $P \leq 0.05$ was taken as significant.

3. RESULTS

3.1. Hormonal Effects on Callus, Shoot, and Root Regeneration

Seeds were the only plant part that responded to growing in the media, whether supplemented or not with the tested PGRs. Shoot proliferation was first achieved from callus induced on MS medium supplemented with 0.5 mg/L Kin and 0.1 mg/L NAA. This medium served as the baseline for subsequent experiments in which Kin, IAA, and 2,4-D concentrations were varied. As shown in Table 1A and Figure 2a, adding Kin at any tested concentration (0, 0.25, 0.5, or 1 mg/L) to the MS medium containing 0.1 mg/dL of NAA induced rooting in some tubes ($n = 14$, 38.9%) and shooting and callus formation were noticed in most tubes ($n = 26$, 72.2%, and $n = 28$, 77.8%, respectively). Although the highest callus induction ($n = 9$, 100%) occurred at 1.0 mg/L kin, no significant differences were noticed between concentrations for callus induction, shooting, or rooting. In contrast, callus color varied significantly ($P = 0.012$) between treatments, with yellowish-brown calli being most frequent ($n = 12$, 33.3%), followed by greenish-brown calli ($n = 8$, 22.2%).

Furthermore, as shown in Table 1B and Figure 2b, it was observed that the addition of the auxin IAA at various concentrations (0, 0.25, 0.5, or 1 mg/L) to the MS medium containing a fixed concentration of BAP (0.1 mg/L) did not produce any significant difference in the shooting and rooting induction ($P > 0.05$). In addition, as revealed in Table 1C and Figure 2c, it was observed that the addition of auxin 2,4-D at various concentrations (0, 0.25, 0.5, or 1 mg/L) to the MS medium that contained a fixed concentration of Kin (0.1 mg/L) had no significant effect ($P > 0.05$) on shooting, rooting, and callus induction. However, the highest 2,4-D concentration (1.0 mg/L) resulted in the best shoot induction ($n = 7$, 77.8%). Root growth was observed in all tubes, regardless of the 2,4-D concentration. The best multiplication and growth performance were achieved at 1.0 mg/L 2,4-D.

As shown in Table 2A, the means of the number and length of shoots, length of roots, and the number of leaves formed on the calli showed no significant difference ($P > 0.05$) across various concentrations of Kin for each one of these parameters. However, the best multiplication and overall growth performance were observed at the lowest Kin concentration (0.25 mg/L). However, the highest concentration of IAA (1 mg/L) caused the highest shooting rate (88.89%). On the other hand, a significant difference in the callus formation ($P = 0.009$) was observed between tubes containing BAP alone without IAA and those supplemented with IAA. Callus formation was achieved 100% in all tubes with IAA, while only 55.56% of the tubes without IAA produced calli. Although calli exhibited different colours, no significant differences ($P > 0.05$) were detected across IAA concentrations. Similarly, as shown in Table 2B, the number and length of shoots, root length, and number of leaves did not significantly differ ($P > 0.05$) across IAA concentrations. These findings were confirmed by Duncan's test, which showed no

Table 1: Effect of different types and concentrations of cytokinins and/or auxins on the induction of roots, shoots, and callus and the colour and morphology of *Pulicaria jaubertii* callus.

Comparisons variables	A					B					C				
	Concentration of the cytokinin: Kinetin ^a					Concentration of the auxin: IAA ^b					Concentration of auxin: 2,4-D ^c				
	Total <i>n</i> (%)	0 ml/L <i>n</i> (%)	0.25 mg/L <i>n</i> (%)	0.5 mg/L <i>n</i> (%)	1 mg/L <i>n</i> (%)	Total <i>n</i> (%)	0 ml/L <i>n</i> (%)	0.25 ml/L <i>n</i> (%)	0.5 ml/L <i>n</i> (%)	1 ml/L <i>n</i> (%)	Total <i>n</i> (%)	0 ml/L <i>n</i> (%)	0.25 ml/L <i>n</i> (%)	0.5 ml/L <i>n</i> (%)	1.0 ml/L <i>n</i> (%)
Root induction															
No	22 (61.1)	4 (44.4)	7 (77.8)	5 (55.6)	6 (55.7)	2.333 (0.668)	13 (36.1)	8 (88.9)	6 (66.7)	7 (77.8)	6 (66.7)	1.739 (0.833)	0 (0)	0 (0.0)	0 (0.0)
Yes	14 (38.9)	5 (55.6)	2 (22.2)	4 (44.4)	3 (44.4)		23 (63.9)	1 (11.1)	3 (33.3)	2 (22.2)	3 (33.3)		36 (100)	9 (100.0)	9 (100.0)
Shoot induction															
No	10 (27.8)	2 (22.2)	2 (22.2)	4 (44.4)	2 (22.2)	1.634 (0.784)	27 (75)	4 (44.4)	3 (33.3)	5 (55.6)	1 (11.1)	4.201 (0.317)	19 (52.8)	4 (44.4)	6 (66.7)
Yes	26 (72.2)	7 (77.8)	7 (77.8)	5 (55.6)	7 (77.8)		9 (25)	5 (55.6)	6 (66.7)	4 (44.4)	8 (88.9)		17 (47.2)	5 (55.6)	3 (33.3)
Callus induction															
No	8 (22.2)	3 (33.3)	2 (22.2)	3 (33.3)	0 (0.0)	4.108 (0.297)	4 (11.1)	4 (44.4)	0 (0)	0 (0)	0 (0)	8.521 (0.009*)	4 (11.1)	0 (0.0)	3 (33.3)
Yes	28 (77.8)	6 (66.7)	7 (77.8)	6 (66.7)	9 (100.0)		32 (88.9)	5 (55.6)	9 (100)	9 (100)	9 (100)		32 (88.9)	9 (100.0)	6 (66.7)
Colour of callus															
No callus produced	8 (22.2)	3 (33.3)	2 (22.2)	3 (33.3)	0 (0.0)	20.388 (0.012**)	4 (11.1)	4 (44.4)	0 (0)	0 (0)	0 (0)	14.277 (0.139)	4 (11.1)	0 (0.0)	3 (33.3)
Green	6 (16.7)	3 (33.3)	0 (0.0)	2 (22.2)	1 (11.1)		17 (47.2)	3 (33.3)	4 (44.4)	6 (66.6)	4 (44.4)		14 (38.9)	6 (66.7)	2 (22.2)
Yellowish Brown	12 (33.3)	2 (22.2)	6 (66.7)	3 (33.3)	3 (11.1)		1 (2.8)	0 (0)	0 (0)	1 (11.1)	0 (0)		16 (44.4)	2 (22.2)	3 (33.3)
Dark Brown	2 (5.6)	0 (0.0)	1 (11.1)	0 (0.0)	1 (11.1)		12 (33.3)	2 (22.2)	4 (44.4)	2 (22.2)	4 (44.4)		1 (2.8)	1 (11.1)	0 (0.0)
Greenish brown	8 (22.2)	1 (11.1)	0 (0.0)	1 (11.1)	6 (66.7)		2 (5.6)	0 (0)	1 (11.1)	0 (0)	1 (11.1)		1 (2.8)	0 (0.0)	1 (11.1)

Values are expressed as *n* (%); (*n*=36 for each hormonal mixture and *n*=9 for each concentration). **P*≤0.01, ***P*≤0.05. ^aWith a fixed concentration of the cytokinin, Kin. ^bWith a fixed concentration of the auxin, IAA. ^cWith a fixed concentration of the cytokinin, BAP. NS: No statistics were computed because root induction is constant. IAA: Indole-3-acetic acid, 2,4-D: 2,4-dichlorophenoxyacetic acid.

Table 2: The effect of various concentrations of the cytokinin Kin and the auxin NAA on the number and lengths of shoots, length of roots, and number of leaves of *Pulicaria jaubertii*.

Growth parameter	A					B					C				
	Concentration of the cytokinin: Kinetin ^a					Concentration of auxin: IAA ^b					Concentration of auxin: 2,4-D ^c				
	Total <i>n</i> (%)	0 mg/L <i>n</i> (%)	0.25 mg/L <i>n</i> (%)	0.5 mg/L <i>n</i> (%)	1 mg/L <i>n</i> (%)	Total <i>n</i> (%)	0 mg/L <i>n</i> (%)	0.25 mg/L <i>n</i> (%)	0.5 mg/L <i>n</i> (%)	1 mg/L <i>n</i> (%)	Total <i>n</i> (%)	0 mg/L <i>n</i> (%)	0.25 mg/L <i>n</i> (%)	0.5 mg/L <i>n</i> (%)	1.0 mg/L <i>n</i> (%)
Number of shoots	1.89±0.54	2.56±0.68	1.44±0.56	2.11±0.65	0.569	0.639	0.252	1.56±0.41	1.44±0.44	1.22±0.49	1.56±0.34	0.136	0.938	0.620	0.501
Length of shoots	2.06±0.58	2.06±0.47	1.06±0.37	1.34±0.42	1.181	0.333	0.177	1.22±0.38	1.28±0.40	0.89±0.30	1.06±0.38	0.230	0.875	0.460	0.196
Length of roots	1.00±0.35	0.39±0.26	0.72±0.30	0.61±0.32	0.668	0.578	0.214	0.56±0.56	1.11±0.59	0.78±0.52	1.22±0.62	0.286	0.835	0.529	0.620
Number of leaves	15.33±5.60	19.11±5.73	7.78±3.19	10.89±3.65	1.129	0.352	0.127	9.33±4.15	7.78±2.93	3.33±1.15	6.67±2.69	0.753	0.529	0.196	0.620

Values are expressed as mean±standard error of the mean. (*n*=36 for each hormonal mixture and *n*=9 for each concentration). IAA: Indole-3-acetic acid, NAA: 1-naphthaleneacetic acid, Kin: kinetin.

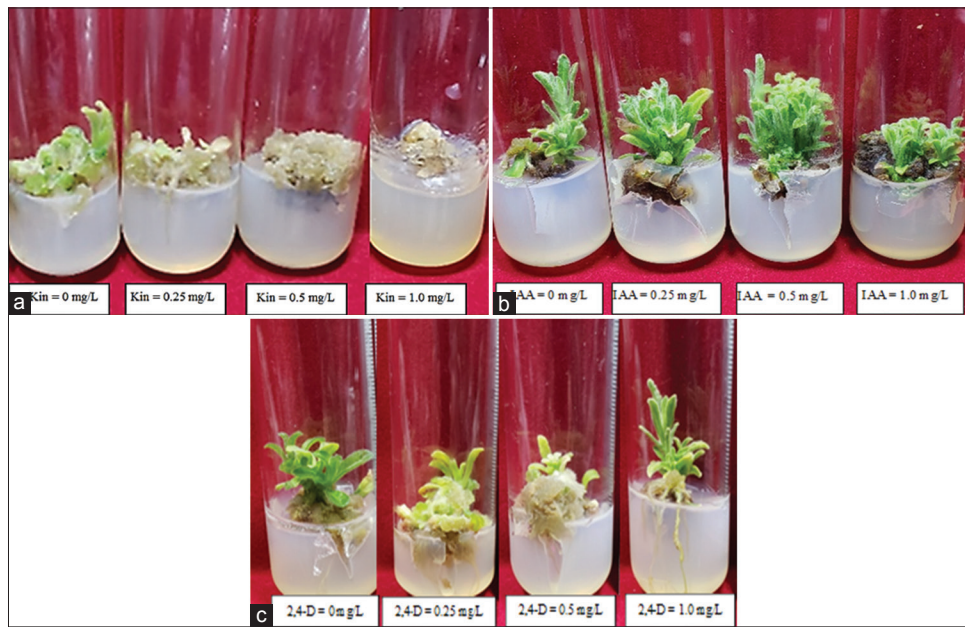


Figure 2: Growth of *Pulicaria jaubertii* in Murashige and Skoog media: (a) Growth at different concentrations of kinetin (Kin) and 0.1 mg/L of 1-naphthaleneacetic acid; (b) Growth at different concentrations of indole-3-acetic acid and 0.1 mg/L of 6-benzylaminopurine; (c) Growth at different concentrations of 2,4-dichlorophenoxyacetic acid and 0.1 mg/L of Kin ($n=36$ for each hormonal mixture and $n=9$ for each concentration).

significant differences in growth means across hormone treatments. However, the best overall induction and growth performance was achieved at the highest IAA concentration (1.0 mg/L).

3.2. Acclimatisation of *In vitro* Derived Plantlets

A total of 50 plantlets were acclimatised under optimal laboratory conditions in a soil-sand mixture (70:30, v/v) following successful shoot and root inductions. After 4 weeks, 45 plantlets survived, showing a 90% survival rate. Twenty of these acclimatised plantlets were then transferred to larger pots and grown under outdoor garden conditions, where all survived and exhibited healthy growth after an additional 4 weeks (100% survival), as shown in Figure 3. These results confirm the feasibility of acclimatising regenerated *P. jaubertii* plantlets for *ex vitro* establishment.

3.3. GC-MS Analyses

The GC-MS analysis allows the identification of the main secondary metabolites and provides insights into their composition and variability. This study investigates the phytochemical differences between the ethanolic and n-hexane extracts of the mother plant and *in vitro*-derived callus of *P. jaubertii* to assess their metabolic range and potential pharmaceutical significance. The GC-MS analysis of ethanolic extracts identified 25 compounds [Table 3 and Figure 4] in the mother plant extract and 46 in the callus extract [Table 4 and Figure 5], which indicates that the callus contains more phytochemicals. The most abundant volatile components found in the ethanolic extract of the mother plant are Formic acid, 1-methylethyl ester (39.95%), 2-Ethoxyethylamine (26.5%), Formic acid (13.11%), Boron, trihydro(N-methylmethanamine)-, (T-4)- (6.55%), 2-Decanol (3.46%), and 2-Cyclohexen-1-one, 2-methyl-5-(1-methylethyl)-, (S)- (2.95%). However, the analysis showed the presence of many compounds in small amounts. Similarly, the ethanolic extract of the callus exhibited the presence of high quantities of 2-Ethoxyethylamine (85.60%), Methyl serine (4.39%), n-Hexadecanoic acid (2.83%), and

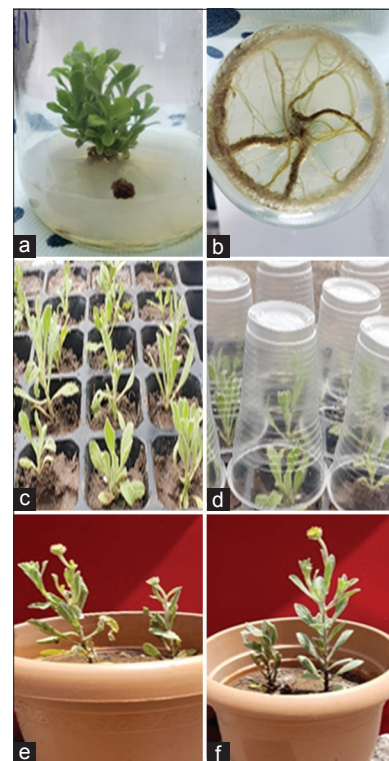


Figure 3: Acclimatisation of *Pulicaria jaubertii* plantlets: (a) Rooted shoots; (b) Root initiation; (c) Plantlets grown in soil-rite mixture; (d) Vessels covered with transparent containers; (e and f) Successful growth *In vitro*-derived plantlets in the garden after 4 weeks of acclimatisation.

Pentadecanoic acid, ethyl ester (2.22%).

In contrast, the n-hexane extracts contained 25 compounds in the mother plant and 14 in the callus. The n-hexane of this mother

Table 3: Volatile compositions of ethanolic extract of *Pulicaria jaubertii* mother plant.

No.	Compounds	Retention time	Relative area (%)	Molecular formula	Molecular weight (g/mol)
1	2-Decanol	1.249	3.46	C ₁₀ H ₂₂ O	158.167
2	Formic acid	1.476	13.11	CH ₂ O ₂	48.005
3	Boron, trihydro (N-methylmethanamine-, (T-4)-	1.564	6.55	C ₂ H ₇ BN	46.022
4	2-Ethoxyethylamine	1.832	26.50	C ₄ H ₁₁ NO	89.084
5	Formic acid, 1-methylethyl ester	1.954	39.95	C ₄ H ₈ O ₂	88.052
6	Propanamide, 2-hydroxy-	3.452	0.07	C ₃ H ₇ NO ₂	89.048
7	Phenol, 4-(2-aminopropyl)-	5.259	0.14	C ₉ H ₁₃ NO	151.100
8	2-Octanol, (R)-	5.405	0.01	C ₈ H ₁₈ O	130.136
9	2-Formylhistamine	5.970	0.12	C ₆ H ₉ N ₃ O	139.075
10	Xanthatin, 8-[4-[[isopropylamino] carbonyl] methoxy] phenyl]-1,3-dipropyl	6.063	0.01	Unknown	Unknown
11	2-Cyclohexen-1-one, 2-methyl-5-(1- methylethyl)-, (S)-	6.110	2.95	C ₁₀ H ₁₈ O	152.120
12	Pyrazole[4,5-b] imidazole, 1-formyl-3-ethyl-6-.beta.-d-ribofuranosyl	7.474	0.01	C ₁₂ H ₁₆ N ₄ O ₅	296.279
13	(R)-(-)-14-Methyl-8-hexadecyn-1-ol	12.848	0.28	C ₁₇ H ₃₂ O	252.245
14	1,2-Benzenediol, 4-[2-(methylamino) ethyl]-	13.186	0.05	C ₉ H ₁₃ NO ₂	167.095
15	1-(3,5-Dimethyl-1-adamantanoyl) semicarbazide	13.425	0.06	C ₃ H ₄ N ₂ O ₄	265.179
16	1,2-Ethanediamine, N-(2-aminoethyl)	14.672	0.03	C ₄ H ₁₃ N ₃	103.166
17	Hexadecanoic acid, ethyl ester	14.818	0.12	C ₁₈ H ₃₆ O ₂	284.272
18	Benzeneethanamine, N-methyl-	16.671	0.05	C ₁₀ H ₁₅ N	135.105
19	Benzenemethanol, 3-hydroxy-.alpha. [(methylamino) methyl]-	16.771	0.07	C ₉ H ₁₃ NO ₂	167.095
20	Cyanacetyl urea	16.945	0.03	C ₄ H ₅ N ₃ O ₂	194.044
21	Methylpent-4-enylamine	19.592	0.03	C ₆ H ₁₃ N	99.105
22	Cyclobutanol	20.116	0.02	C ₄ H ₈ O	72.058
23	1-Octadecanamine, N-methyl-	21.363	0.02	C ₁₉ H ₄₁ N	283.324
24	2-Hexanamine, 4-methyl	23.817	0.02	C ₇ H ₁₇ N	115.136
25	Phenethylamine p,α-dimethyl	31.972	0.05	C ₁₀ H ₁₅ N	149.12

All reported compounds were identified with reference compounds in the NIST 98 library.

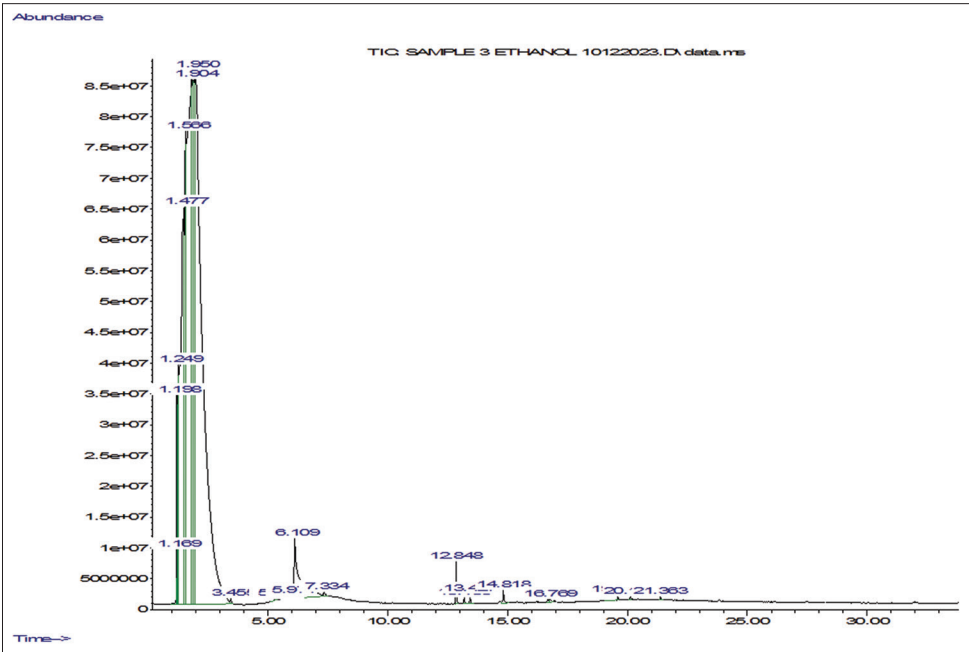


Figure 4: Gas chromatography-mass spectrometry chromatogram of ethanolic extract of *Pulicaria jaubertii* mother plant.

Table 4: Volatile compositions of ethanolic extract of *Pulicaria jaubertii* callus.

No.	Compounds	Retention time	Relative area (%)	Molecular formula	Molecular weight (g/mol)
1	2-Ethoxyethylamine	1.826	85.60	C ₄ H ₁₁ NO	89.084
2	Methoxymethyl isothiocyanate	3.440	0.06	C ₃ H ₅ NOS	103.009
3	2-Formylhistamine	4.944	0.09	C ₆ H ₉ N ₃ O	139.075
4	Acetic acid, (aminooxy)-	5.259	0.07	C ₂ H ₅ NO ₃	91.027
5	Propanoic acid, 2-(aminooxy)	5.684	0.17	C ₃ H ₇ NO ₃	105.043
6	2-O-Mesyl arabinose	5.754	0.07	C ₆ H ₁₂ O ₇ S	228.03
7	N-Methoxy-1-ribofuranosyl-4-carboxylic amide	5.830	0.04	C ₇ H ₁₂ N ₂ O ₆	273.096
8	Phenol, 4-(2-aminopropyl)-	5.947	0.10	C ₉ H ₁₃ NO	151.100
9	2-Cyclohexen-1-one, 2-methyl-5-(1-methylethyl)-, (S)-	6.244	0.11	C ₁₀ H ₁₈ O	152.12
10	Azetidin-2-one 3,3-dimethyl-4-(ethyl-1-amino)-	6.436	0.10	C ₇ H ₁₄ N ₂ O	142.111
11	Benzenemethanol, 3-hydroxy-.alpha. [(methylamino) methyl]-	10.767	0.07	C ₁₀ H ₁₅ NO ₃	167.095
12	Benzenethanamine, N-methyl-	10.919	0.07	C ₁₀ H ₁₅ N	135.105
13	Methyl serine	11.123	4.39	C ₃₀ H ₃₀ O ₈	119.058
14	Allantoic acid	11.933	0.16	C ₄ H ₈ N ₄ O ₄	176.055
15	l-2,4-Diaminobutyric acid	12.242	0.07	C ₄ H ₁₀ N ₂ O ₂	118.074
16	Benzocycloheptano[2,3,4-I, j] isoquinoline, 4,5,6,6a-tetrahydro-1,9-dihydroxy-2,10-dimethoxy-5-methyl-	12.533	0.21	C ₂₀ H ₂₃ NO ₄	341.163
17	Imidazole-5-carboxylic acid, 2-amino-	12.585	0.36	C ₅ H ₅ N ₃ O ₂	127.038
18	p-Hydroxynorephedrine	12.848	0.04	C ₉ H ₁₃ NO ₂	167.095
19	2H-Pyran-2,6 (3H)-dione	12.964	0.06	C ₅ H ₆ N ₂ O ₂	112.016
20	Tetraacetyl-d-xylic nitrile	13.104	0.06	C ₁₄ H ₁₇ NO ₉	343.09
21	4-Amino-1-pentanol	13.425	0.20	C ₅ H ₁₃ NO	103.100
22	Octanoic acid, ethyl ester	13.588	0.07	C ₁₀ H ₂₀ O ₂	172.146
23	3,4-Furandiol, tetrahydro-, trans-	14.136	0.05	C ₄ H ₈ O ₃	104.047
24	n-Hexadecanoic acid	14.544	2.83	C ₁₆ H ₃₂ O ₂	256.24
25	Hexadecanoic acid, ethyl ester	14.794	0.20	C ₁₈ H ₃₆ O ₂	284.272
26	Pentadecanoic acid, ethyl ester	14.812	2.22	C ₁₇ H ₃₄ O ₂	270.256
27	Benzenethanamine, 2-fluoro-.beta.,5-dihydroxy-N-methyl-	15.377	0.07	C ₉ H ₁₂ FNO ₂	185.085
28	l-Threitol	15.780	0.04	C ₄ H ₁₀ O ₄	122.058
29	1,7-Diaminoheptane	16.153	0.15	C ₇ H ₁₈ N ₂	130.147
30	3,3-Dimethyl-4-methylamino-butan-2-one	16.269	0.08	C ₇ H ₁₅ NO	129.115
31	2-Amino-4-hydroxypteridine-6-carboxylic acid	16.450	0.77	C ₇ H ₅ N ₅ O ₃	207.039
32	Benzyl alcohol, .alpha.-(1-aminoethyl)-m-hydroxy-, (-)-	16.450	0.77	C ₉ H ₁₃ NO ₂	167.095
33	Linoleic acid ethyl ester	16.631	0.72	C ₂₀ H ₃₆ O ₂	308.272
34	Oleic Acid	16.683	0.95	C ₁₈ H ₃₄ O ₂	282.256
35	Octadecanoic acid, ethyl ester	16.922	0.33	C ₂₀ H ₄₀ O ₂	312.303
36	o-Veratramide	17.925	0.03	C ₉ H ₁₁ N ₃ S	181.074
37	4-Fluorohistamine	18.484	0.08	C ₉ H ₈ FN ₂	129.07
38	3-Piperidinol	18.746	0.06	C ₅ H ₁₁ NO	101.084
39	Hexanedioic acid, bis (2-ethylhexyl) ester	18.816	0.32	C ₂₂ H ₄₂ O ₄	370.308
40	bicyclo[2.2.1], heptane-5-(ethyl-1-amine)	19.481	0.07	C ₂₀ H ₂₇ N	139.136
41	Benzenepropanamine, N-(1,1-dimethylethyl)-.alpha.-methyl-.gamma.-phenyl	19.889	0.10	C ₉ H ₁₇ N	281.214
42	1-[a-(1-Adamantyl) benzyldiene] thiosemicarbazide	19.988	0.06	C ₁₈ H ₂₃ N ₃ O	313.161
43	Bis (2-ethylhexyl) phthalate	20.099	0.11	C ₂₄ H ₃₈ O ₄	390.277
44	Benzenemethanol, .alpha.-(1-aminoethyl)-, (R*, S*)-(./-)-	21.369	0.06	C ₉ H ₁₃ NO	151.1
45	Methylpent-4-enylamine	22.488	0.03	C ₆ H ₁₃ N	99.105
46	Cathinone	32.012	0.82	C ₉ H ₁₁ NO	149.084

All reported compounds were identified with reference compounds in the NIST 98 library. The asterisk indicates undefined stereochemistry; (R*, S*) denotes mixed stereoisomers; (+/-) indicates a racemic mixture.

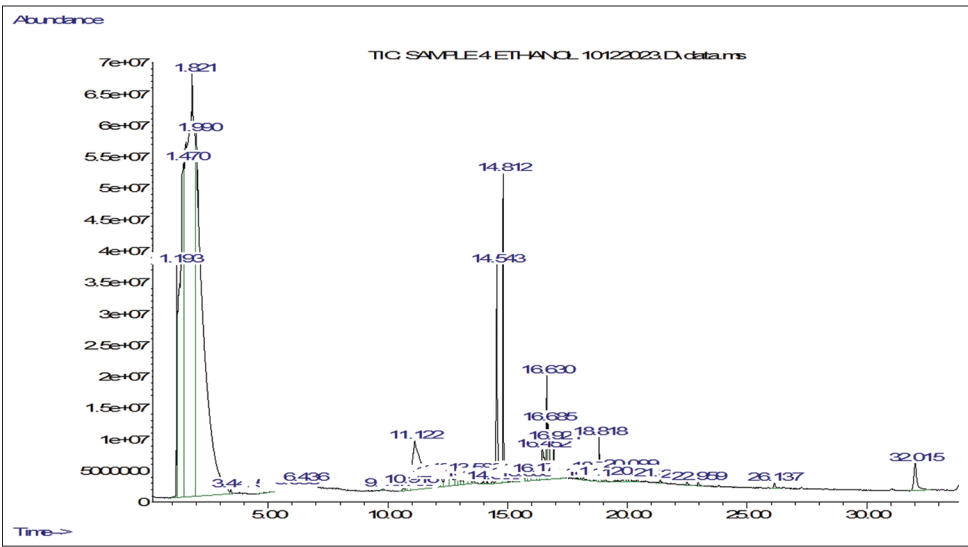


Figure 5: Gas chromatography-mass spectrometry chromatogram of ethanolic extract of *Pulicaria jaubertii* callus.

Table 5: Volatile compositions of n-hexane extract of *Pulicaria jaubertii* mother plant.

No.	Compounds	Retention time	Relative area (%)	Molecular formula	Molecular weight (g/mol)
1	1,4-Eicosadiene	12.830	0.16	C ₂₀ H ₃₈	278.297
2	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	13.407	0.10	C ₂₀ H ₄₀ O	296.308
3	n-Hexadecanoic acid	14.526	0.20	C ₁₆ H ₃₂ O ₂	256.424
4	Hexadecanoic acid, ethyl ester	14.794	0.10	C ₁₈ H ₃₆ O ₂	284.272
5	Linoleic acid, ethyl ester	16.613	0.20	C ₂₀ H ₃₆ O ₂	306.483
6	9,12,15-Octadecatrienoic acid, ethyl ester, (Z, Z, Z)-	16.677	0.21	C ₂₀ H ₃₄ O ₂	306.256
7	9,12,15-Octadecatrien-1-ol, (Z, Z, Z)-	16.694	0.34	C ₁₈ H ₃₂ O	264.245
8	9,12,15-Octadecatrienoic acid, methyl ester, (Z, Z, Z)-	16.694	0.34	C ₁₉ H ₃₂ O ₂	292.240
9	Heptadecanoic acid, 15-methyl-, ethyl ester	16.910	0.04	C ₂₀ H ₄₀ O ₂	298.504
10	Tridecane, 7-hexyl-	19.574	0.25	C ₁₉ H ₄₀	268.313
11	Eicosane, 10-methyl-	19.579	0.25	C ₂₁ H ₄₄	296.344
12	Octadecane	20.401	0.04	C ₁₈ H ₃₈	254.297
13	Pentatriacontane	21.345	0.31	C ₃₅ H ₇₂	492.563
14	Octacosane	22.453	0.07	C ₂₈ H ₅₈	394.454
15	Trichothec-9-en-4-ol, 7,8:12,13-diepoxy-, 2-butenolate, [4.beta.(Z),7.beta.,8.beta.]-	22.925	0.07	C ₁₉ H ₂₄ O ₅	332.162
16	Eicosane, 9-octyl-	23.799	0.64	C ₂₈ H ₅₈	394.454
17	Eicosane	25.420	0.11	C ₂₀ H ₄₂	282.329
18	Heptadecane	25.420	0.11	C ₁₇ H ₃₆	240.471
19	Tetracosane	27.512	2.04	C ₂₄ H ₅₀	338.391
20	Hexadecane	29.978	0.17	C ₁₆ H ₃₄	226.266
21	Stigmastan-6,22-dien, 3,5-dedihydro-	31.907	0.29	C ₂₉ H ₄₆	394.360
22	Hexatriacontane	33.213	1.05	C ₃₆ H ₇₄	506.579
23	Stigmasterol	33.213	1.05	C ₂₉ H ₄₈ O	412.371
24	Docosane, 9-butyl-	33.213	1.05	C ₂₆ H ₅₄	366.423
25	5-Methyl-2-phenylindolizine	33.755	0.01	C ₁₅ H ₁₃ N	207.105

All reported compounds were identified with reference compounds in the NIST 98 library.

extract contained Tetracosane (2.04%), Hexatriacontane (1.05%), Stigmasterol (1.05%), and Docosane, 9-butyl- (1.05%) as the most abundant ones [Table 5 and Figure 6]. The n-hexane extract of callus [Table 6 and Figure 7] has the lowest list of compounds,

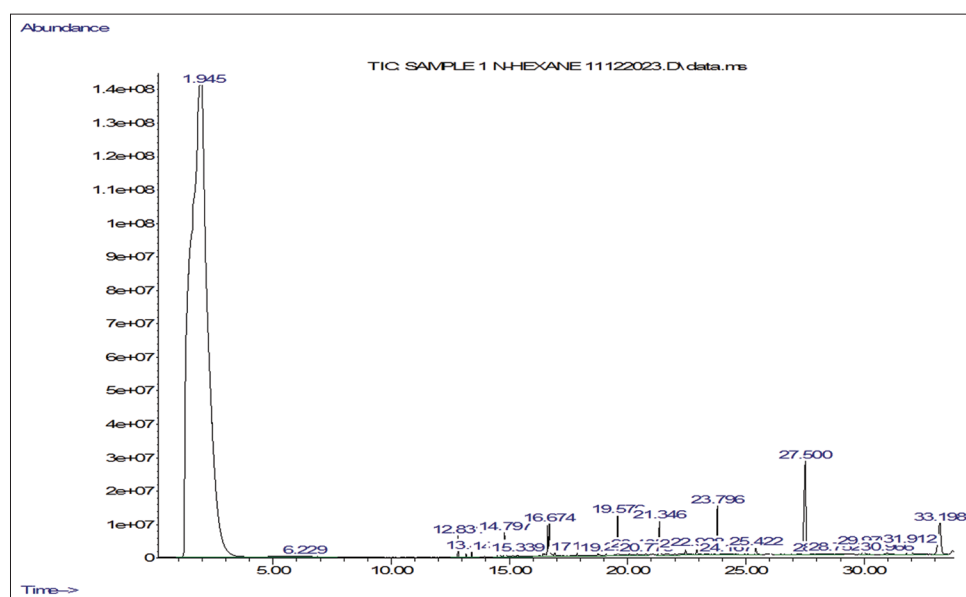


Figure 6: Gas chromatography-mass spectrometry chromatogram of n-hexane extract of *Pulicaria jaubertii* mother plant.

Table 6: Volatile compositions of n-hexane extract of *Pulicaria jaubertii* callus.

No.	Compounds	Retention time	Relative area (%)	Molecular formula	Molecular weight (g/mol)
1	Methylpent-4-enylamine	14.503	1.48	C ₆ H ₁₃ N	99.105
2	Hexadecanoic acid, ethyl ester	14.794	16.20	C ₁₈ H ₃₆ O ₂	284.272
3	p-Hydroxynorephedrine	16.630	2.21	C ₉ H ₁₃ NO ₂	167.095
4	Benzeneethanamine, N-methyl-	16.665	0.52	C ₉ H ₁₃ N	135.105
5	Amphetamine	16.927	1.82	C ₉ H ₁₃ N	135.105
6	2-Aminononadecane	17.866	1.34	C ₁₉ H ₄₁ N	283.324
7	Benzenepropanamine, .alpha.-methyl	18.746	1.27	C ₁₄ H ₁₄ N ₄ O ₄	149.12
8	Imidazole-5-carboxylic acid, 2-amino-	19.579	1.57	C ₄ H ₅ N ₃ O ₂	127.038
9	1,2-benzenedicarboxylic acid diisooctyl ester	20.087	4.30	C ₂₄ H ₃₈ O ₄	390.556
10	L-Alanine, N-(3-methyl-1-oxobutyl)-, methyl ester	21.351	1.77	C ₃₄ H ₄₆ N ₂ O ₈	187.121
11	Benzenemethanol, 3-hydroxy-.alpha. [(methylamino) methyl]-	23.794	2.92	C ₆ H ₁₃ N	99.105
12	Phenethylamine, p,.alpha.-dimethyl	27.466	4.77	C ₁₀ H ₁₅ N	149.12
13	Stigmasterol	31.954	58.79	C ₂₉ H ₄₈ O	412.371
14	Benzenemethanol, .alpha.-(1-aminoethyl)-, (R*, S*)-(./-.)-	33.766	1.03	C ₉ H ₁₃ NO	151.100

All reported compounds were identified with reference compounds in the NIST 98 library. The asterisk indicates undefined stereochemistry; (R*, S*) denotes mixed stereoisomers; (±) indicates a racemic mixture.

although it contains high amounts of Stigmasterol (58.79%), Hexadecanoic acid, ethyl ester (16.20%), Phenethylamine, p,.alpha.-dimethyl (4.77%), and 1,2-benzenedicarboxylic acid diisooctyl ester (4.32%). Furthermore, some volatile components have been shown in both ethanolic extracts (e.g., 2-Ethoxyethylamine, Phenol, 4-(2-aminopropyl)-, 2-Formylhistamine, and 2-Cyclohexen-1-one, 2-methyl-5-(1-1-methylethyl)-, (S)-. Hexadecanoic acid, ethyl ester was found in all extracts, while Benzeneethanamine, N-methyl-, and

Benzenemethanol, 3-hydroxy-.alpha. [(methylamino)methyl]- were not found in the n-hexane extract of the callus. Overall, these findings suggest that the ethanolic extract of callus possesses the richest phytochemical profile among the tested extracts and highlight it as a promising source for bioactive compound production. These compounds may serve as starting materials, intermediates, or derivatives in pharmaceutical production, thereby contributing to drug development and therapeutic applications.

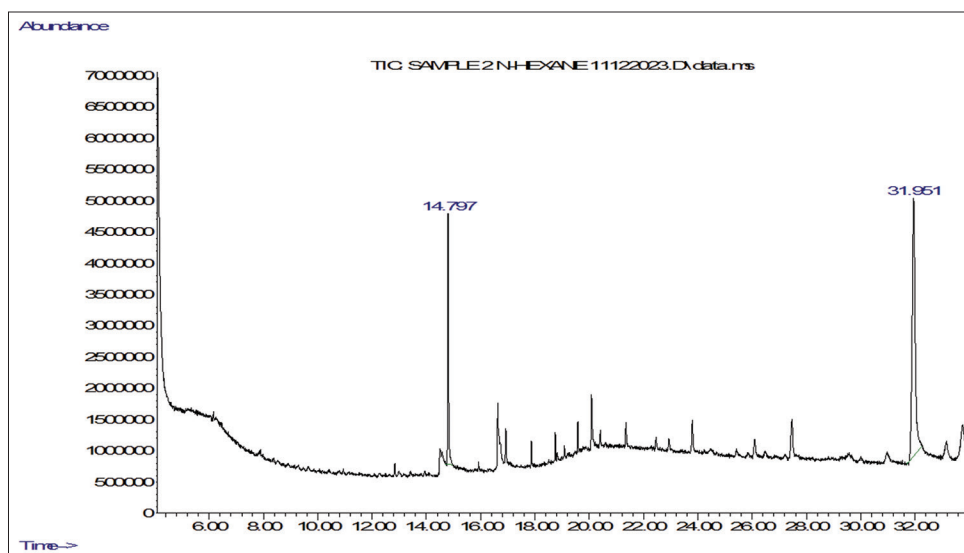


Figure 7: Gas chromatography-mass spectrometry chromatogram of n-hexane extract of *Pulicaria jaubertii* callus.

4. DISCUSSION

Our study showed that the plant seeds successfully responded to growth on the media, and callus induction was greater for plant multiplication with all the studied hormones. This was consistent with those of other studies. For instance, Ghareb [43], in her research on another species of *Pulicaria* named *P. incisa*, reported that a combination of various concentrations of the auxin NAA and the cytokinin 2-isopentenyladenine produced a large amount of callus and the lowest number of shoots per explant. The study also showed that similar observations were made with several plants belonging to other genera and/or families, e.g., *Acacia chundra* [27], *Acacia mangium* [44], *Acacia auriculiformis* [45], *Pogostemon cablin* [46], and *Pterocarpus santalinus* [47]. The combination of cytokinins and auxins was hypothesised to play an important role in callus induction and increasing its percentage [48].

In this study, auxin IAA significantly enhanced callus induction in all tested concentrations. Consistent with this, previous studies have highlighted the important role of auxins in callus induction [36,49]. Our findings revealed that the highest concentrations of Kin produced callus in all the tubes. This observation is comparable with results from Younes *et al.* [50], who declared that *Achillea fragrantissima* (Family: *Asteraceae*) had increased callus formation at higher kin concentrations. Similarly, Sivanesan and Jeong [51] observed callus production in *Pentanema indicum* (Family: *Asteraceae*) with high cytokinin and low auxin levels, which is also seen in *Vicoa indica* (*Asteraceae*) [52].

This current study observed successful callus formation and plant proliferation using NAA and Kin. Kamili *et al.* [53] reported comparable findings on *Artemisia annua* L. (Family: *Asteraceae*). The callus was successfully induced from leaf explants on MS medium with NAA. In addition, NAA alone promoted root regeneration, while a mixture of NAA and Kin was required for shoot regeneration. Equally, Juan *et al.* [54] noted that callus induction and plant proliferation in *Atractylodes macrocephala* Koidz (Family: *Asteraceae*) were achieved using leaf explants cultured on MS medium supplemented with Kin and NAA. Sánchez-Ramos *et al.* [55] reported that a combination of NAA and Kin could significantly increase callus production in *Ageratina pichinchensis* (Family: *Asteraceae*) within 15 days.

Similar findings extend to other plant families. Manasa *et al.* [56] reported that callus induction in *Mussaenda frondosa* L. (Family: *Rubiaceae*) was achieved on MS medium supplemented with 2 mg/L NAA and 4 mg/L Kin, whereas the highest callus formation rate (81.7%) was achieved with 1.0 mg/L NAA and 0.1 mg/L Kin. Our results indicate an inverse relationship between Kin concentration and growth parameters. Under a fixed NAA concentration, optimal plant proliferation was achieved at the lowest Kin concentration. Consistently, Mehta and Subramanian [57] noted that *Asparagus adscendens* Roxb. (Family: *Asparagoideae*) produced multiple shoots after 3 weeks on MS medium with 0.46 μ M NAA and 0.27 μ M Kin, though higher concentrations of both hormones did not yield better results.

In addition, BAP combined with IAA significantly induced callus, followed by plant multiplication, with higher IAA concentrations yielding the best results. Sivanesan and Jeong [51] noted that *P. indicum* Ling. (Family: *Asteraceae*) callus produced the highest number of shoots on MS medium with 4.0 mg/L BAP and 1.0 mg/L IAA. Pramanik *et al.* [58] reported that *Pluchea indica* (Family: *Asteraceae*) produced the most leaves at 0.087 μ M IAA, with a count of 55%. However, higher concentrations at 1.312 μ M caused a slight decrease to 49%. Despite this reduction, they still encouraged callus formation at 275 mg/L.

Noticeably, our results showed that increasing the 2,4-D concentration did not significantly affect plant growth parameters, including callus formation. Still, the highest concentration produced the best results for most parameters. In contrast, Rouane *et al.* [59] noted that *P. incisa* capitula performed better in a medium with 2,4-D and Kin. Similarly, numerous studies pointed out the positive effects of 2,4-D and Kin on callus induction [60-63]. However, consistent with our findings, Ali and Afrasiab [64] and Dangash *et al.* [65] observed reduced callogenic responses at higher 2,4-D concentrations across various plant species. Uddin *et al.* [66] reported that *Stevia rebaudiana* (Family: *Asteraceae*) produced maximum callus at 3.0 mg/L 2,4-D, while higher concentrations resulted in poor callus formation.

Callus colour and shape variations observed in this study were also noted by Rouane *et al.* [59], who reported similar changes in *P. incisa* calli over time and with different PGR concentrations. Sari and Kusuma [67] similarly observed friable callus formation in *Myrmecodia tuberosa* (Family: *Rubiaceae*). According to Elias *et al.* [68], these

variations are derived from interactions between endogenous and exogenous PGRs, the type of explant, and environmental conditions.

Our results showed that different hormone concentrations had no significant positive effect on shoot and root induction, as new shoots only developed on pre-existing callus. Similar outcomes were found by Sivaram and Mukundan [69]. In their study, *Stevia rebaudiana* leaf explants did not produce shoots when using Kin along with IAA or NAA. Tamura *et al.* [70] also observed slow shoot proliferation in *Stevia rebaudiana* on a high-Kin medium. Likewise, Jin *et al.* [46] reported that the addition of NAA to *Pogostemon cablin* (Family: Lamiaceae) cultures induced callus formation but slowed shoot growth compared to cytokinin alone. Singh *et al.* [71] further reported that NAA with Kin promoted callus formation but did not induce shoot formation. Ghareb [43] suggested that lower shoot multiplication might happen because of too much callus formation at the base of shoot buds.

The high survival rates of plantlets were achieved during acclimatisation in this study, which evidences the strong adaptability of regenerated *P. jaubertii* plantlets to *ex vitro* conditions. These results are similar to, or even better than, those seen in other micropropagated *Asteraceae* species. Survival during acclimatisation is often a major challenge in large-scale propagation [41,50]. Successful acclimatisation is an important procedure of any micropropagation protocol, as it determines whether *in vitro* plantlets can transition to soil-based growth and maintain healthy development under ambient conditions. The current findings show that our protocol works well for inducing callus and regenerating shoots. It also helps produce strong plantlets that can survive in uncontrolled environments. This supports both conservation and possible cultivation efforts for this species.

Organic solvents used in this study facilitated the extraction of a broad spectrum of phytochemicals from *P. jaubertii*. Ethanol solvent could extract both polar and moderately lipophilic compounds, such as phenolics and alkaloids, while n-hexane targets non-polar lipophilic constituents, such as terpenes and sterols. The use of different solvents allows for a more comprehensive phytochemical analysis by capturing a wide range of compounds with different solubilities in both the mother plant and *in vitro*-derived callus samples. GC-MS analysis revealed unique volatile components in the callus. It also identified compounds that have been reported in the same species [2,5,12,13] or other *Pulicaria* species [72-74]. This supports our observation that the *in vitro*-derived callus showed a richer phytochemical profile than the mother plant.

The current study found many volatile components in the ethanolic extract of *P. jaubertii* callus compared to the extract from the mother plant, which could be attributed to the dedifferentiated nature and metabolic plasticity of callus culture. In a lab setting, PGRs like auxins and cytokinins could stimulate biosynthetic pathways of the plant. This results in producing a wider range of secondary metabolites that are not usually found in mature cultures [75,76]. Furthermore, the simple structure and loose cells of callus allow solvents to penetrate more easily and improve the extraction of metabolites. Unlike the mother plant, the callus lacks specialised structures like oil glands and cuticular wax layers that compartmentalise lipophilic constituents, allowing free diffusion of intracellular compounds. The ethanolic extract of the callus produced several polar and semi-polar compounds, including nitrogen-containing molecules, amino alcohols, alkaloid derivatives, and sugar alcohols, not detected in the mother plant [77,78]. In contrast, the n-hexane extract from callus had fewer lipophilic compounds compared to the extract from the mother plant. This is likely due to the limited accumulation of non-polar metabolites such as long-chain hydrocarbons, terpenoids, and triterpenoids in undifferentiated tissues. As a non-polar solvent,

n-hexane primarily extracts terpenoids, fatty acids, and sterols. In the structurally mature mother plant, these compounds accumulate in specialised tissues such as glandular trichomes and cuticular layers, which are either absent or poorly developed in callus. This anatomical and biochemical disparity explains the reduced abundance of lipophilic constituents in the callus-derived hexane extract [76].

Importantly, GC-MS analysis revealed that the ethanolic callus extract yielded several compounds reported for the first time in *P. jaubertii*, including Methoxymethyl isothiocyanate, 2-O-Mesyl arabinose, N-Methoxy-1-ribofuranosyl-4-carboxylic amide, Azetidin-2-one 3,3-dimethyl-4-(ethyl-1-amino)-, Allantoic acid, 1,7-Diaminoheptane, 1-2,4-Diaminobutyric acid, 2-Amino-4-hydroxypteridine-6-carboxylic acid, Benzocycloheptano [2,3,4-I,j]isoquinoline derivative, p-Hydroxynorephedrine, 2H-Pyran-2,6(3H)-dione, Tetraacetyl-d-xylonic nitrile, 1-Threitol, and Cathinone. In the n-hexane callus extract, new components included 2-Aminononadecane, Amphetamine, L-Alanine, N-(3-methyl-1-oxobutyl)-, methyl ester, and 1,2-Benzenedicarboxylic acid diisooctyl ester. These compounds were not previously reported in previous phytochemical studies of *P. jaubertii* [2,5,9,13,16,18,38], which primarily examined essential oils and solvent extracts from aerial parts, roots, or flowers. Their detection in callus culture shows the importance of tissue culture systems as alternative ways to discover new plant-based molecules that could have medical uses.

In our analysis, 2-Ethoxyethylamine was identified for the first time, while Stigmasterol was abundant in the current n-hexane extracts [5,16] and has been previously reported in this species. Some identified compounds were also found in other plant families, including *Dipterygium glaucum* (Capparidaceae) [79] and *Urginea maritima* (L.) (Asparagaceae) [80]. These findings suggested possible biochemical convergence across other plant families and highlighted the diverse secondary metabolite production in callus cultures.

Phytochemical variation among *P. jaubertii* samples has been consistently reported. The oxygenated monoterpene named carvotanacetone (2-Cyclohexen-1-one, 2-methyl-5-(1-methylethyl)-, (S)-) was found in low amounts (2.95%) in the ethanolic extract of the mother plant but reached 98.34% in plants from Sana'a, Yemen [12], 63.957% in Hajja Province, Yemen [5], 93.5% in Lahj Province, Yemen [13], and 98.59% in Jazan Province, Saudi Arabia [2]. However, these studies looked at essential oils taken through hydrodistillation from leaves or flowers. In contrast, this research examined ethanolic and n-hexane extracts of the whole plant and callus extracted by maceration. In line with our findings, Alharthi *et al.* [16] did not detect carvotanacetone in the methanolic extract of *P. jaubertii* from Ibb, Yemen. Phytochemical content variation can result from many factors, such as plant genotype, geographical location, climate, season, reproductive stage, and extraction methods [37].

These findings underscore the potential metabolic differences between the callus and mother plant, which provide new insights into the biosynthetic potential of callus cultures and their relevance for pharmacological and biotechnological applications. PGRs can stimulate the production of secondary metabolites by changing the activity of important regulatory genes in metabolic pathways. Cytokinins and auxins, in particular, have been shown to influence the expression of genes like phenylalanine ammonia-lyase and chalcone synthase. These genes play a crucial role in the biosynthesis of flavonoids, terpenoids, and phenolics. Several identified compounds possess therapeutic properties. Carvotanacetone and other oxygenated monoterpenes exhibit antimicrobial activity [2,13], n-Hexadecanoic

acid has antioxidant, antiandrogenic, antimicrobial, and hypocholesterolemic properties, while phytol (3,7,11,15-Tetramethyl-2-hexadecen-1-ol) is known for its antioxidant, antimicrobial, anti-inflammatory, neuroprotective, anticancer, and diuretic effects [81]. While these results highlight the phytochemical richness and morphogenetic potential of *P. jaubertii* callus cultures, the study presents several limitations that warrant further investigation.

4.1. Study Limitations and Future Directions

A primary limitation of this study is its non-factorial experimental design. While the stepwise approach allowed the initial assessment of hormonal effects on callus induction and organogenesis, it did not fully explore interactive effects among PGRs. Future studies should assume factorial designs to evaluate hormone combinations and optimise regeneration efficiency systematically. Another limitation concerns the explant type. Although we tested leaf and stem explants with high cytokinin and low auxin levels, they did not start callus or morphogenesis. This response likely happened because of their higher differentiation status, the buildup of inhibitory secondary metabolites, or differences in hormonal response specific to the genotype. The study, therefore, focused on seeds, the only explant type that consistently responded. Additional investigations using pre-treatments or rejuvenation techniques may enhance regeneration from mature tissues in *P. jaubertii*. Although gene expression profiling was not investigated in the present study, the observed increase in metabolite diversity in callus culture may be partially attributed to PGR-mediated activation of these pathways [75]. Future studies are recommended to include molecular tools such as reverse transcription quantitative polymerase chain reaction to explore the correlation between hormonal treatments and the expression of biosynthetic genes responsible for secondary metabolite accumulation.

GC-MS identified volatile compounds qualitatively, but no quantification was done with authentic standards. This limits the pharmacological relevance of the findings. Future efforts should include validated quantification techniques, e.g., high-performance liquid chromatography or GC-MS with calibration curves, to determine absolute concentrations of key metabolites like Stigmasterol or 2-Ethoxyethylamine [79]. In addition, this study successfully achieved callus induction and shoot/root emergence, as well as plantlet acclimatisation, with high survival rates under both laboratory (90%) and garden (100%) conditions. These inductions are critical components of any propagation protocol designed for conservation or commercial application. However, a genetic fidelity assessment using molecular markers such as random amplified polymorphic DNA or inter-simple sequence repeat was not conducted, and this remains a limitation. Future studies should focus on this part to support the conservation and commercial growth potential of *P. jaubertii*. The current study serves as a pilot framework toward establishing a complete and genetically validated micropropagation protocol [53,64].

The callus culture protocol offers a useful platform for both conservation and biotechnological exploitation of *P. jaubertii*. Scaled-up *in vitro* propagation could support the sustainable supply of plant material, reducing pressure on wild populations affected by overharvesting and habitat degradation. Furthermore, callus culture can serve as a renewable source of bioactive compounds under controlled conditions. Future research should focus on enhancing metabolite production via elicitation, precursor feeding, or metabolic engineering. Gene expression studies linked to secondary metabolite pathways could clarify how PGRs influence biosynthesis. Finally, it is recommended to use bioactivity-guided fractionation and pharmacological screening

to confirm the therapeutic potential of high quantities of identified metabolites, especially from hormonally enriched callus extracts.

5. CONCLUSION

To conclude, *P. jaubertii* seeds were the only plant part that successfully grew in MS medium, first inducing callus formation before plant vegetation. Since 2,4-D showed no significant effect on callus induction or growth parameters, while IAA achieved optimal growth at its highest concentration and Kin was most effective at its lowest concentration, we propose an optimised micropropagation protocol. This protocol supplements the growth medium with 0.25 mg/L Kin and 1.0 mg/L IAA to ensure efficient callus induction and optimal growth. Further studies are needed to establish a standardised protocol based on these findings.

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7. AUTHOR'S CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

8. CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve any experiments on animals or human participants.

10. FUNDING

There is no funding to report.

11. DATA AVAILABILITY

All data supporting this study are available from the authors and could be provided upon request.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors confirm that no artificial intelligence (AI) tools were used for writing or editing this manuscript, and no images were generated or manipulated using AI.

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