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Title: **HPLC-Based Elucidation of Tannins from the Tissue and Callus Culture Extracts of Selected Medicinal Plants**

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HPLC-Based Elucidation of Tannins from the Tissue and Callus Culture Extracts of Selected Medicinal Plants

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ABSTRACT

Background. Optimized HPLC profiling is a powerful and effective analytical tool to standardize plant samples and authenticate plant materials. In this study, three selected medicinal plants namely: *Achyranthes aspera*, *Ipomoea hederacea*, and *Ocimum basilicum* were subjected to callus induction following seedling, leaf, and stem germination.

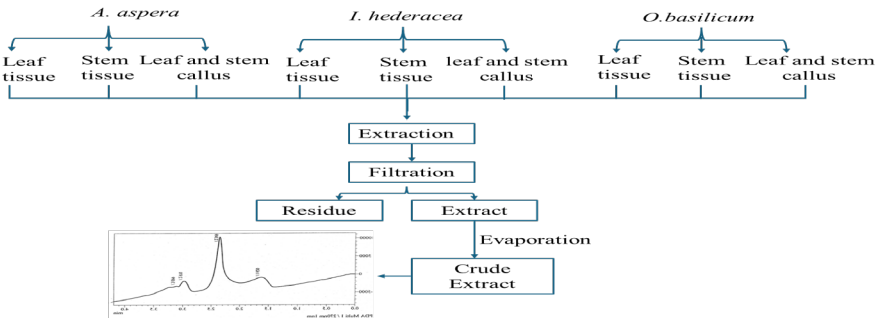
Methods. The induced callus was subsequently dried, finely ground, and extracted using methanol and water for HPLC analysis. A validated procedure was employed to identify and separate the tannin content in seedling leaf, stem, and callus culture extracts. HPLC fingerprinting was performed using a Shimadzu LC-20A system with a retention time of 2.9 minutes at 270 nm. The aim was to ensure quality and consistency in tannin analysis across different plant parts and callus culture samples.

Results. The highest callogenic response occurred in *A. aspera* leaf explants on MS medium with 2.0 mg/L 2,4-D and 4.0 mg/L NAA, producing green, granular callus. The lowest was in *I. hederacea* stem explants with 0.5 mg/L 2,4-D and BAP, yielding brown, granular callus. *O. basilicum* leaf callus extract showed the largest sample area (9365.56) and tannin content (2.66), with superior precision in tannin analysis for *O. basilicum* and *A. aspera* (7.81).

Conclusion. HPLC profiling proved to be an accurate, efficient, and precise method for evaluating tannin content in selected plant samples. It is a crucial method to standardize the quality of medicinal plant compounds.

Keywords: analytical tool, callus culture, explants, HPLC, medicinal plant

GRAPHICAL ABSTRACT



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1. INTRODUCTION

Callus is a mass of mostly unorganized and undifferentiated cells. The plant tissue culture method provides a continuous and reliable source of natural products. The standardization of plant products is critical considering the growing demand for natural goods as medicines [1]. The production of secondary metabolites in cell culture relies on the amount/types of PGRs, carbon source, and other climatic conditions including light, temperature and gas composition [2].

Chromatographic fingerprinting presents a practical and effective solution to the global need for a stronger quality assessment method for traditional medicine [3]. This optimized technique enables the representation of chemical constituents distributed in plant materials, creating a "chemical database" that can be referenced in future research [4]. Tannins, also known as tannic acid, play an important biological role and have diverse applications. Their anticarcinogenic and antimutagenic effects are believed to stem from their antioxidant properties, which help protect cells from oxidative damage, including lipid peroxidation. Tannins also exhibit significant antibacterial activity. They suppress the growth of different microorganisms such as fungi, yeasts, bacteria, and viruses. Notably, propyl gallate and tannic acid but not gallic acid have been shown to inhibit the growth of aquatic bacteria, and microbes responsible for producing undesirable flavors [5].

The hydrolysis of ester linkages between gallic acid and polyols hydrolyzed during the maturing of any edible fruit is presumably linked to their antimicrobial capabilities. Tannins in these fruit, therefore, act as a natural barrier against microbial diseases [6]. Tannins demonstrate additional physiological effects, including lowering serum cholesterol, blood pressure, increasing blood coagulation and altering immune response [7]. The selected medicinal plants contain tannins, which have traditionally been used to treat a range of ailments. To demonstrate their effectiveness, it is essential to conduct both their qualitative analysis and quantification of tannin compounds present in various plant parts, using systematic scientific methods and comparisons with standard tannin compounds.

Achyranthes aspera L. belongs to the family Amaranthaceae. *A. asperas* is used to treat diarrhea, piles, heart disease, dyspepsia, vomiting, ascites, abdominal enlargement, and enlargement of the cervical gland [8-10]. *Ipomoea hederacea* of Convolvulaceae are known as ivy leaf morning glory or kaladana and habbunil. It is used to cure abdominal diseases, bronchitis, eye inflammation, gout, scabies, headache, constipation, fever, flatulence, leucoderma, eye disorders, splenopathy and hepatopathy [11]. *Ocimum basilicum* of Lamiacae/Labiatae is commonly known as basil or niazbo. It is used to treat stomachache and diarrhea due to its diuretic, demulcent and antipyretic properties [12]. While callus cultures provide a reliable source of secondary metabolites, there is limited standardized methodology employed for optimizing culture conditions with the aim to maximize tannin production. Moreover, despite the known medicinal potential of tannins in selected plants, comprehensive chromatographic profiling and quantitative analysis using HPLC remains underexplored for quality assurance and therapeutic validation. Hence, this study aims to use High Performance Liquid Chromatography (HPLC) to identify tannin compounds in selected medicinal plants by testing different mobile phase gradients and run times.

2. MATERIALS AND METHODS

2.1. Collection and Identification of Selected Plants

Healthy seeds of *Achyranthes aspera L.* (Voucher No. GC. Herb. Bot. 3492*), Ipomoea hederacea (L.) Jacquin.* (Voucher No. GC. Herb. Bot. 3493), and *Ocimum basilicum L.* (Voucher No. GC. Herb. Bot. 3491) were purchased from the market and identified by a taxonomist from GC University Lahore. The entire study was carried out at the Plant Biotechnology Laboratory, GC University of Lahore.

2.2. Seed Surface Sterilization and Callus Induction

To prevent microbial contamination during *in vitro* culture, seeds were subjected to sterilization using 3% sodium hypochlorite solution for 1 minute and then rinsed thrice with sterile distilled water. A total of 10 seeds from each species were aseptically placed in sterile Petri plates containing moist cotton pads to facilitate germination in the growth room.

Following successful germination, young seedling tissues (leaf and stem) were excised under sterile conditions and used for callus induction. Murashige and Skoog (MS) medium was supplemented with various concentrations of auxins (e.g.NAA, 2-4D) and cytokinins (BAP, KIN) to establish callus biomass production. Cultures were maintained at 25 ± 2°C under a 16-hour light/8-hour dark photoperiod provided by cool white; fluorescent lights to mimic optimal physiological conditions for callus development.

2.3. Drying and Maceration of Callus

Developed callus tissues, along with the original leaf and stem tissues, were harvested and oven-dried at 40°C for one week to remove moisture while preserving bioactive compounds. Dried samples were ground into fine powder using a mechanical grinder. One gram of each powdered sample (leaf, stem, and corresponding callus) was weighed for tannin extraction, following the HPLC protocol outlined by reference [13].

2.4. HPLC profiling

2.4.1. Preparation of Mobile Phase. A binary mobile phase consisting of methanol and water in a 1:1 ratio (50:50 mL) was prepared. The solvent mixture was thoroughly filtered using a 0.2 µm syringe filter to remove the particulate matter. Then, it was subjected to sonication for 20 minutes to ensure degassing and homogeneity, which improved peak resolution during chromatographic separation [14]

2.4.2. Preparation of Standard. Tannic acid was used as a standard and dissolved into 10 ml of mobile phase to form stock solution. A series of dilution (20 µg/ml, 30 µg/ml, 40 µg/ml and 50 µg/ml) was prepared and diluted with 10 ml of mobile phase. A calibration curve was plotted against the area.

2.4.3. Preparation of Sample. One gram of each plant tissue and macerated callus of leaf and stem were soaked in 10 mL of mobile phase and kept for 12 hrs with stirring. Then extracts were filtered using 0.2 µm syringe filters and subjected to 20 minutes of sonication to enhance solubility and remove trapped air. The resulting clear extracts were injected into the HPLC system for profiling and quantification.

Table 1. Chromatographic Instrumentation of Quantitative Determination of Tannins

Parameter	Chromatograph Instrumentation
HPLC system	Shimadzu LC20A
Injector	Rheodyne

Parameter	Chromatograph Instrumentation
Pump	LC20AT
Column	A Hiber C18 Pore size 5µm.
Mobile phase	Methanol: water (50:50)
Wavelength	270
Flow rate	20µl
Rentation time	2.9 min

3. RESULTS

The leaf and stem explants of *A. aspera, I. hederacea* and *O. basilicum* were grown on MS media containing different combinations of plant growth regulators (PGRs). The effects of different concentrations of PGRs were recorded for the callogenic response of different explants of *I. hederacea,* that is, leaf, stem and root. The maximum callogenic response was exhibited by MS medium supplemented with 2.0 mg/l 2,4-D in combination with 4.0 mg/l NAA for the leaf of *A. aspera* as compared to other explants as the leaf callus had green colour and granular morphology (Figure 1A). The minimum callogenesis response was observed in MS medium supplemented with 0.5 mg/l 2,4-D, in combination with 0.5 mg/l BAP, for the stem of *I. hederacea* as compared to other explants. Stem callus was brown and had granular morphology. Stem explants of MSDB and MSDK of *I. hederacea* and *O. basilicum* respectively responded for callus induction with a low callus induction percentage (Table 2).

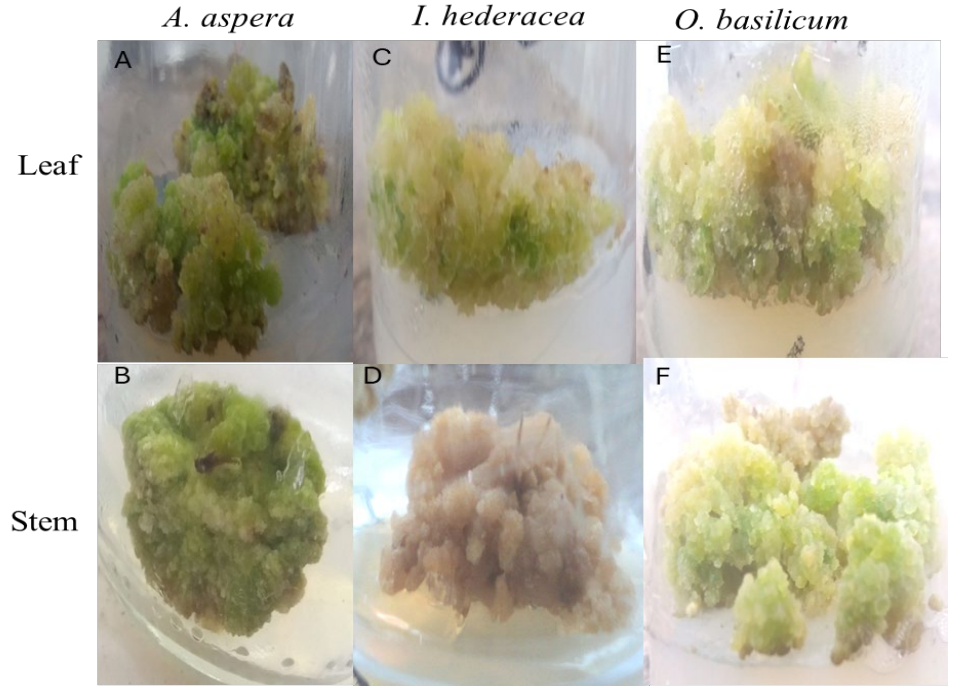

Figure 1. Effects of Different Concentrations of PGRs on Callus Induction (A) Leaf Explant of *A. aspera* on MSDN Medium (B) Stem Explant of *A. aspera* on MSDN Medium (C) Leaf Explant of *I. hederacea* on MSDB Medium (D) Stem Explant of *I. hederacea* on MSDB Medium (E) Leaf Explant of *O. basilicum* on MSDK Medium (F) Stem Explant of *O. basilicum* on MSDK Medium.

The combination of media is given in table 2 below.

Table 2. Effect of Different PGRs on Callus Induction of Different Parts of Selected Plants

Medium Code	MS Media Compositions	Explants	Callus Induction (%)	Callus Induction Duration (Days)	Callus Index	Callus Weight (g)	Callus Morphology
MSDN	(2,4-D mg/l+ NAA mg/l) 2.0+4.0	Leaf	60	17	70	2.18±0.00	Green and granular
	(2,4-D mg/l+ NAA mg/l) 6.0+8.0	Stem	60	17	70	0.32±0.00	Green and compact
MSDB	(2,4-D mg/l + BAP mg/l) 0.5+1.5	Leaf	40	17	200	0.56±0.03	Light green and granular
	(2,4-D mg/l + BAP mg/l) 0.5+0.5	Stem	20	15	100	0.29±0.00	Brown and granular
MSDK	(2,4-D mg/l + KIN mg/l) 2.0+4.0	Leaf	40	17	100	1.55±0.00	Light green and granular
	(2,4-D mg/l + KIN mg/l) 0.5+2.0	Stem	20	14	60	0.39±0.005	Light green and granular

3.1. HPLC-Based Comparison of Tannin Extraction from Leaf, Stem, and Callus Tissue *of A. aspera, I. hederacea,* and *O. basilicum*

Chromatogram of selected plants showed the retention time of 2.9 minutes at 270 wavelengths for tannin using methanol and water mobile phase. The tannins compound was analyzed for 5 minutes run time. The maximum area of sample was shown by *O. basilicum* callus culture extract, that is, 9365.56 among all explants and callus culture samples. The highest tannin content was exhibited by *O. basilicum* leaf callus culture extract, that is, 95.04 (Table 3).

Table 3. Quantitative Estimation of Tannin Content in Leaf and Stem of Selected Medicinal Plants

Plant Sample	Appearance	Area of Sample (a.u)	Percentage of Tannins
Achyranthes aspera leaf explants	Brown	2547.088	82.17
Achyranthes aspera leaf callus	Dark brown	3231.69	78.27
Ocimum basilicum leaf explants	Brown	7406.16	81.88
Ocimum basilicum leaf callus	Dark brown	9365.56	95.04
Iopomea hederacea leaf explants	Light brown	9287.89	83.16
Iopomea hederacea leaf callus	Light brown	18741.95	87.12
Achyranthes aspera stem explants	Yellowish brown	1241.350	93.06
Achyranthes aspera stem callus	Dark brown	8788.20	91.08
Iopomea hederacea stem explants	Dark yellow brown	3036.814	76.23

Plant Sample	Appearance	Area of Sample (a.u)	Percentage of Tannins
Iopomea hederacea stem callus	Light brown	4397.62	91.08
Ocimum basilicum stem explants	Dark yellow	6513.52	85.14
Ocimum basilicum stem callus	Light yellow	9291.56	81.18

A. aspera leaf seedling extract showed the highest area (1,245,086), indicating abundant bioactives. *Ocimum basilicum* leaf extract had the highest concentration (22.90 mg/mL), suggesting strong extractability. *A. aspera* stem tissue showed maximum recovery (135.45%), Further, its callus culture had the highest average signal (101.25), pointing to enhanced *in vitro* metabolite production. Conversely, *Ipomoea hederacea* stem seedling had the lowest area (41,976), indicating low phytochemical yield. The highest standard deviation (32.19) and RSD (23.97%) in *I. hederacea* extracts indicated low precision, requiring optimization (Table 4).

Table 4. HPLC Profiling of Selected Medicinal Plants

Extracts	Area (mAU.s)	Concentration recovered	Recovery (%)	Average	STD	RSD (%)
A. aspera leaf seedling tissue extract	1245086	74.30921219	99.07894959	85.85068182	11.81251798	5.282718638
A. aspera leaf callus (MSDN) extract	8429952	20.31446254	125.4297502	101.5226771	14.47479398	6.814965447
I.hederacea leaf seedling tissue extract	554041	11.91786102	95.34288814	49.19339767	30.93091877	13.8327274
I.hederacea leaf callus culture (MSDB) extract	799874	18.93644721	75.74578884	37.65602505	32.18195984	14.39220997
O.basilicum leaf seedling tissue extract	1386038	229.0315448	101.7917977	104.8058182	8.739828576	3.908570161
O.basilicum leaf callus culture (MSDK) extract	365051	225.6780551	100.3013578	60.21710659	5.947601166	2.659848102
A. aspera stem seedling tissue extract	668138	20.31446254	135.4297502	103.5226771	17.47479398	7.814965447
A. aspera stem callus culture	563397	6.522154971	25.21772398	98.04397409	20.60680005	9.215641143

Extracts	Area (mAU.s)	Concentration recovered	Recovery (%)	Average	STD	RSD (%)
(MSDN) extract						
I.hederacea stem seedling tissue extract	706150	16.70171649	111.3447766	99.42438963	53.55927427	23.95243562
I.hederacea stem callus culture (MSDB) extract	439762	35.67155827	19.02483108	110.1427622	50.8640405	22.74709043
O.basilicum stem seedling tissue extract	563397	24.87683255	82.92277516	95.54590879	15.47717828	6.921604547
O.basilicum callus stem callus culture (MSDK) extract	497742	1386038	31.64734768	50.6357563	70.91843929	7.814965447

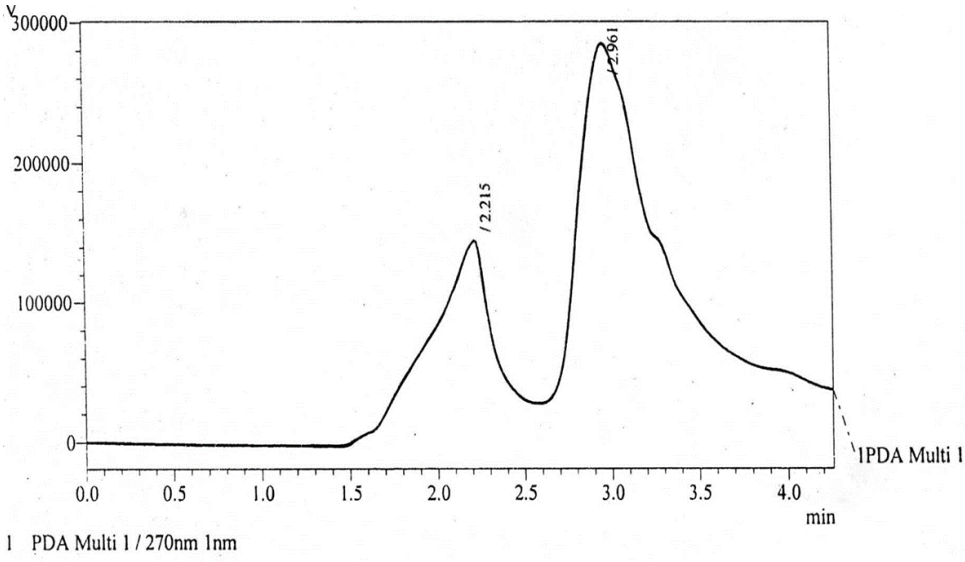

Figure 2. HPLC Profile of Standard Drug (Tannic Acid)

HPLC chromatograms at 270 nm display the tannin profiles extracted from the leaf, stem, and callus tissues of *Achyranthes aspera, Ipomoea hederacea,* and *Ocimum basilicum,* revealing distinct differences in the tannin content across tissue types and species. In all three plants, leaf extracts consistently exhibited the highest and sharpest peaks, particularly around the

retention time of ~2.6 minutes, indicating a greater concentration of tannins (Figure 3). Stem extracts showed moderate peak intensities, reflecting lower tannin levels as compared to leaf tissues (Figure 4). In contrast, callus cultures displayed slightly reduced peak intensities, suggesting minimal tannin accumulation under *in vitro* conditions. Among the species, *O. basilicum* leaf extract showed the highest peak intensity, indicating it as the richest tannin source, followed by *A. aspera* and *I. hederacea*. These findings highlight that natural, differentiated tissues, especially leaves are superior to stem and callus tissues for tannin extraction and confirm species- and tissue-specific variation in tannin biosynthesis.

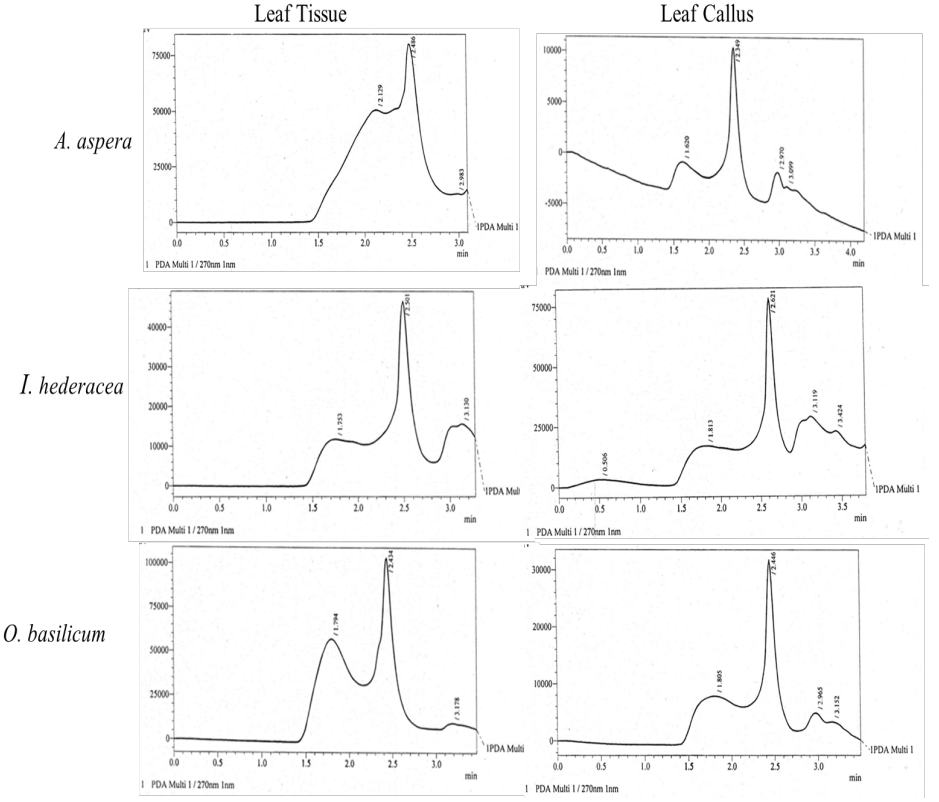

Figure 3. HPLC Profile of Seedling Leaf Tissue (Left Side) and Callus Culture (Right Side) of *A. aspera, I. hederacea* and *O. basilicum.* Leaf Extracts Exhibit Higher and Sharper Peaks, Especially Around ~2.6 Min, Indicating Greater Tannin Concentrations

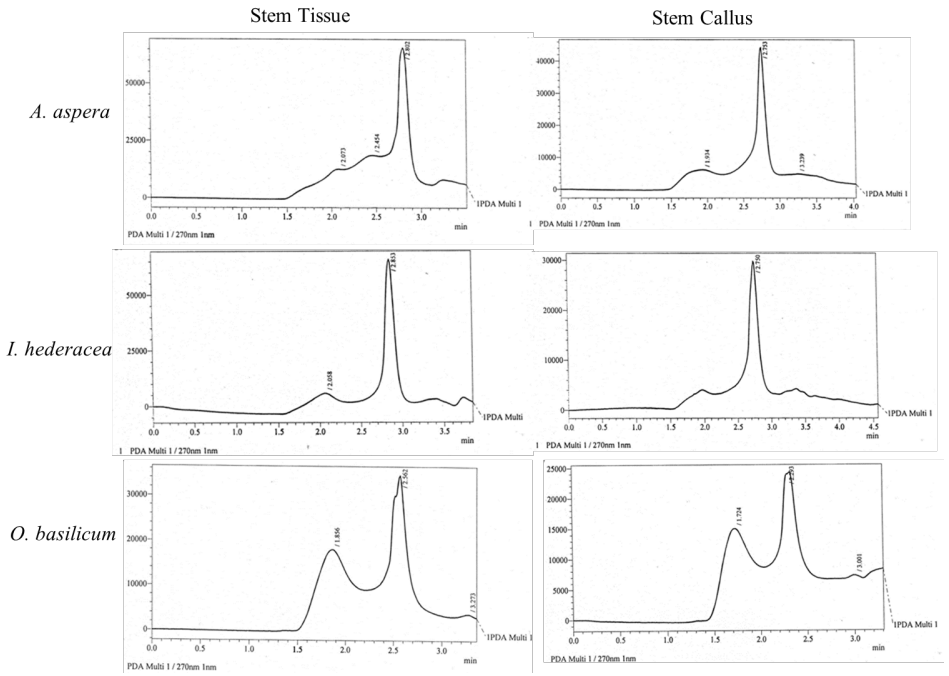

Figure 4. HPLC Profile of Seedling Stem Tissue (Left Side) and Callus Culture (Right Side) of *A. aspera, I. hederacea* and *O. basilicum.* Stem Extracts Show Moderate Peak Intensities

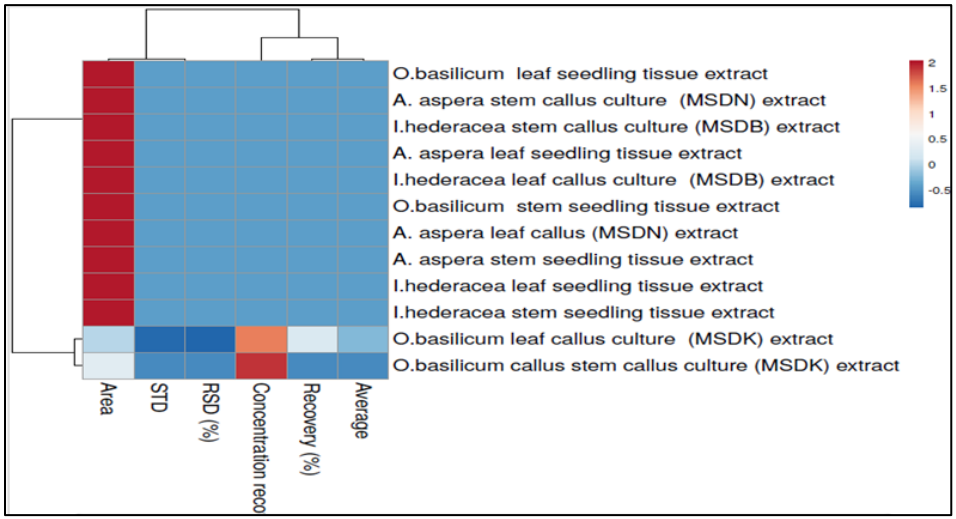

Figure 5. Comparative Analysis of the Heat Maps of HPLC Parameters of Selected Plants. The Colors Blue and Red Show the Value from the Lowest to the Highest, respectively.

The heat map illustrates the relative recovery or concentration of tannin compounds across various medicinal plant extracts, as analyzed by HPLC. Each row represents a specific extract from *Ocimum basilicum*, *Achyranthes aspera*, and *Ipomoea hederacea*, differentiated by plant part (leaf or stem) and tissue type (seedling tissue or callus culture). The color gradient from blue (low) to red (high) indicate the abundance of target compounds. Notably, the extracts from seedling tissues, particularly *O. basilicum* leaf and *I. hederacea* stem callus cultures, show higher compound recovery, while callus cultures, especially from *A. aspera*, generally exhibit lower recovery levels. This suggests that differentiated plant tissues may be more effective sources of bioactive tannins than callus cultures under the tested conditions (Figure 5).

4. DISCUSSION

The influence of different combinations of PGRs on the callogenic response of explants (leaf and stem) of the selected plants was assessed. Among the tested combinations, the maximum callus biomass production was noted in the leaf explants *of Achyranthes aspera* on MS medium fortified with 2.0 mg/L 2,4-D and 4.0 mg/L NAA. The resulting callus was green with a granular texture (Figure 1A), highlighting a robust morphogenic response as compared to other explants. Previous studies also highlighted the effectiveness of leaf explants in callus induction for *A. aspera.* Reference [15] reported successful callus formation using leaf explants cultured on MS medium supplemented with various concentrations of 2,4-D and NAA. The most pronounced callogenic response was achieved with a combination of 2 mg/L 2,4-D, IBA, BAP, IAA, and 4 mg/L NAA. Similarly, [16] also demonstrated efficient callus induction using leaf explants of *A. aspera* on MS medium enriched with varying concentrations of 2,4-D and NAA, further reinforcing the potential of this explant-PGR combination for the *in vitro* morphogenesis. In contrast, the lowest callogenic response was noted in stem explants of *I. hederacea* cultured on MS medium supplemented with 0.5 mg/L 2,4-D and 0.5 mg/L BAP. This callus exhibited a brown coloration with a granular morphology, indicating limited cellular proliferation. Callus initiation was typically observed within 13 to 17 days of incubation. Notably, stem explants from MSDB and MSDK variants of *I. hederacea* and *O. basilicum* demonstrated only modest callus induction, reflected by a relatively low callus formation percentage.

Chromatography, central to phytochemistry, is essential to obtain pure compounds for therapeutic development by enabling the separation, identification, and structural analysis of bioactive substances. It also plays a vital role in quality control and standardization of phytotherapeutics, primarily through HPTLC/HPLC fingerprinting and quantification of specific chemical markers. These techniques not only help in species identification and classification but also support the isolation and characterization of marker compounds, estimate genetic diversity, and offer real-time analyte detection through spectrum scanning and post-chromatographic derivatization[17-20].

HPLC settings were optimized to produce excellent chemical information and chromatograms with the best separation of neighboring peaks, which could be used to quantify the bioactive chemicals under investigation. The influence of different column temperatures, such as 25°C, 30°C, and 35°C on the separation process was also investigated. At 35°C, the majority of the peaks in HPLC chromatograms were well

resolved. As a result, this temperature was chosen as the fingerprint analysis column temperature [21].

To examine the number of peaks and their spacing, detection wavelengths of 280, 254, and 360 nm were used. Finally, the wavelength of 280 nm was chosen, since this wavelength yielded more peaks than 254 or 360 nm. According to the current study, HPLC fingerprinting involves the characterization of bio active compounds. Mobile phase and column play an important role in isolating the compounds. Standards and extracts from seedling leaf, stem tissue, and callus cultures of selected plants showed a consistent baseline and a distinct peak characteristic of tannins, with 270 nm identified as the optimal wavelength for their detection. The tannin content of seedling leaf, stem tissue, callus culture extracts of selected plants exhibited a peak and retention time between 0 to 15 minutes. The HPLC profiling of seedling leaf, stem tissue and callus culture extracts of the leaf and stem of selected plants established a significant peak of tannin fraction at 2 to 2.9 retention time (Figure 3 and 4), (Table 1, 2). A previous study demonstrated that *P. tuberosa* showed best peak of tannic acid at 270 nm wavelength [22]. The presence of additional minor peaks further supports the occurrence of diverse polyphenolics, such as catechol and benzoic acid, as commonly reported in similar phytochemical analyses. Such findings were observed in the HPLC analysis of whole plant extracts of *E. campestre*, which revealed the presence of various polyphenolic compounds such as catechol and benzoic acid, further supporting the identification of tannins and related phenolics at the optimal detection wavelength of 270 nm [14]. The HPTLC analysis of ethanolic extracts from *C. nilgirensis, C. gigantea*, and *C. crinita* also revealed remarkable results, highlighting the presence of a diverse range of phytochemicals, including phenolics, flavonoids, and tannins. These results coincide with the current observations [23]. Another study [24] also confirmed the presence of polyphenols in the methanolic leaf extract of *Synsepalum dulcificum*. This finding also coincides with the current results.

A. aspera leaf tissue extract exhibited the highest chromatographic area (1,245,086), highlighting a rich abundance of bioactive compounds and confirming its potential as a valuable phytochemical source. In contrast, *O. basilicum* leaf extract recorded the highest concentration recovered (22.9031 mg/mL), suggesting it is chemically potent and highly extractable. Remarkably, *A. aspera* stem seedling tissue demonstrated the highest recovery percentage (135.45%), while its callus culture produced the strongest average signal (101.25), both pointing toward enhanced metabolite accumulation, likely due to favorable *in vitro* culture conditions stimulating biosynthesis. On the other hand, *I. hederacea* stem seedling tissue showed the lowest area (41,976), indicating a low phytochemical yield from that specific tissue. The highest standard deviation (32.19) and relative standard deviation (RSD) (23.97%) were observed for *I. hederacea* leaf callus and stem seedling extracts, respectively. These value reflected low analytical precision and signaled the need for optimization to improve reproducibility (Table 4).

The heat map shows that tannin compound recovery varied across different plant extracts, with higher levels found in seedling tissues especially in *Ocimum basilicum* leaves and *Ipomoea hederacea* stem callus cultures. In contrast, callus cultures, particularly from *A. aspera*, showed lower recovery. This indicates that differentiated tissues may be more suitable for

extracting bioactive tannins, as compared to undifferentiated calluses under the conditions tested. These findings highlight the importance of tissue type in optimizing compound yield for medicinal plant research (Figure 5).

The HPLC fingerprints of these standard phenolic compounds acquired using the procedures outlined above might be used as standards for future research. Comparison with such standard chromatograms would provide both qualitative and quantitative analysis of the real phenolic compounds present in any unknown plant sample. This would allow the identification and confirmation of the presence of any of these 9 typical phenolic compounds in the study sample [25]. [26] used HPLC to extract condensed tannins from the bark of four tree species in Mexico; namely *Arbutus xalapensis* Kunth, *Prunus serotina* Ehrh., *Quercus crassifolia* Humb. & Bonpl, and *Quercus laurina* Humb. & Bonpl. In that study, the total tannin content was also isolated from the *Citrus limon* extract using HPLC, which aligns with the methodology employed in the current study.

4.1. Conclusion

The current HPLC data revealed distinct trends among species and tissue types. *A. aspera* emerged as a metabolically rich plant, especially in its seedling and stem tissues, while *O. basilicum* displayed exceptional reproducibility and high chemical recovery, particularly in its callus forms. *I. hederacea*, although showing potential, demonstrates higher variability, especially in its stem extracts. This profiling not only underscores the phytochemical potential of these plants but also identifies which culture conditions and plant parts yield the best results, paving the way for future standardized extraction and pharmacological applications.

Author Contribution

Madieha Ambreen: conceptualization, investigation, methodology, writing – original draft. **Safdar Ali Mirza:** supervision, visualization, writing – review & editing. **Zahida Bano:** software formal analysis, data curation.

Conflict of Interest

The authors declare that they have no conflict of interest regarding the publication of this manuscript.

Data Availability Statement

Data sharing is not applicable to this article as no new data were generated during the study.

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Generative AI Disclosure Statement

The authors did not used any type of generative artificial intelligence software for this research.

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   Steward et al. demonstrated that freely suspended plant cells in culture can develop organized structures, highlighting the totipotency of plant cells. This study laid foundational work for modern plant tissue culture and regeneration techniques.

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Sen et al. optimized in vitro protocols for callus induction and plantlet regeneration of Achyranthes aspera, a medicinally important plant. Their findings support conservation and large-scale propagation through tissue culture techniques.

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   Chung et al. provided a comprehensive review on tannins, discussing their sources, biological activities, and effects on human health. The study highlighted both beneficial and adverse impacts, depending on dosage and dietary context.

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antimicrobial efficacy, highlighting its potential in natural therapeutics.

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    Senthilmanickam et al. examined the effects of 2,4-D and NAA on callus induction in Achyranthes aspera and analyzed its secondary metabolites. The study demonstrated hormone-specific responses influencing callus formation and bioactive compound production.

16. Kayani S, Zia M, Sarwar S, Chaudhary MF. Callogenic studies of Achyranthes aspera leaf explant at different hormonal combinations. *Pak J Biol Sci*. 2008;11(6):950–952. https://doi.org/10.3923/pjbs.2008.950.952

    Kayani et al. investigated callus induction in Achyranthes aspera leaf explants using various hormonal combinations. The study identified optimal conditions for callogenesis, aiding in tissue culture-based propagation of the species.

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18. Edeoga HO, Okwu DE, Mbaebie BO. Phytochemical constituents of some Nigerian medicinal plants. *Afr J Biotechnol*. 2005;4(7):685–688. https://doi.org/10.5897/AJB2005.000-3127

    Edeoga et al. analyzed the phytochemical constituents of selected Nigerian medicinal plants, identifying key compounds like alkaloids, flavonoids, and tannins. The study supports the therapeutic potential of these plants in traditional medicine.

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    Kpoviéssi et al. validated a capillary gas chromatography method for analyzing sterols and triterpenes in the aerial parts of Justicia anselliana. The study ensures accurate phytochemical profiling for quality control in herbal formulations.

20. Faiyazuddin MDA, Rauf N, Ahmad. A validated HPTLC method for determination of terbutaline sulfate in biological samples: application to pharmacokinetic study. *Saudi Pharmaceut J*. 2011;19(3):185–191. https://doi.org/10.1016/j.jsps.2011.03.004

    Faiyazuddin et al. developed and validated an HPTLC method for detecting terbutaline sulfate in biological samples. The method was successfully applied in pharmacokinetic studies, offering a reliable tool for drug monitoring.

21. Ahmed R. High-performance liquid chromatography (HPLC): principles, applications, versatility, efficiency, innovation and comparative analysis in modern analytical chemistry and in pharmaceutical sciences. *Clinic Invest*. 2024;14(9):524–535. https://doi.org/10.20944/preprints202409.0057.v1

Ahmed provided an in-depth overview of High-Performance Liquid Chromatography (HPLC), highlighting its principles, efficiency, and wide-ranging applications in analytical chemistry and pharmaceutical sciences. The study emphasizes HPLC's innovation and versatility in modern research.

22. Durgawale TP, Durgawale PP, Khanwelkar CC. Quantitative estimation of tannin by HPLC. *Der Pharm Lett*. 2016;8(3):123–126.

Durgawale et al. conducted quantitative estimation of tannins using HPLC, demonstrating an accurate and efficient method for tannin analysis. The study supports its application in standardizing tannin-rich herbal formulations.

23. Mradu GS, Saumyakanti, Sohini M, Arup M. HPLC profiles of standard phenolic compounds present in medicinal plants. *Int J Pharm Phytochem Res*. 2012;4(3):162–167.

Mradu et al. presented HPLC profiles of standard phenolic compounds in various medicinal plants, enabling accurate identification and quantification. The study aids in quality control and validation of plant-based formulations.

24. Obafemi TO, Akinmoladun AC, Olaleye MT, et al. High performance liquid chromatography (HPLC) fingerprinting, mineral composition and in vitro antioxidant activity of methanol leaf extract of Synsepalum dulcificum (Sapotaceae). *J Appl Pharmaceut Sci*. 2017;7(10):125–131.

Obafemi et al. performed HPLC fingerprinting and mineral analysis of Synsepalum dulcificum leaf extract, revealing significant antioxidant activity. The study supports its pharmacological potential and use in herbal medicine.

25. Ruiz-Aquino F, Feria-Reyes R, Rutiaga-Quiñones JG, Robledo-Taboada LH, Gabriel-Parra R. Characterization of tannin extracts derived from the bark of four tree species by HPLC and FTIR. *Forest Sci Technol*. 2023;19(1):38–46. https://doi.org/10.1080/21580103.2023.2166593

Ruiz-Aquino et al. characterized tannin extracts from the bark of four tree species using HPLC and FTIR techniques. Their findings provided detailed insights into the chemical composition and potential applications of these natural extracts.

26. Rathod ZR, Sarita S, Saraf MS. Identification and estimation of total tannins from Citrus limon L. Burm. f. (lemon) and its endophytes. *Curr Trends Biomed Eng Biosci*. 2022;20(5):1–12.

Rathod et al. identified and quantified total tannins from Citrus limon and its associated endophytes. The study highlights the role of endophytes in enhancing bioactive compound production for potential therapeutic applications.