

Purification and Characterization of α -methylene- γ -butyrolactone (Tulipalin A) from *Tulipa gesneriana* Callus Extracts

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Abstract

In this study, α -methylene- γ -butyrolactone (Tulipalin A) from *Tulipa gesneriana* L. callus extracts was purified and characterized via high-performance liquid chromatography (HPLC) and Fourier transform infrared (FTIR spectroscopy). Red callus generated in tissue culture was extracted with methanol, providing a crude extract with numerous components. HPLC analysis of the crude sample revealed three different peaks at 3.8, 4.4, and 6.0 minutes, indicating the presence of several metabolites. Following purification, a single strong peak was observed at 3.8 minutes, which was identical to that of the authentic Tulipalin A standard. The isolated compound's identification and purity were further validated by the co-injection test, which revealed only one co-eluted peak. FTIR examination of the purified fraction revealed γ -lactone absorption bands, such as a strong carbonyl band at approximately 1760 cm^{-1} , a conjugated C=C bond near 1660 cm^{-1} , and terminal vinyl group signals at $910\text{--}990\text{ cm}^{-1}$. These findings provide quantitative and qualitative evidence of the compound's identity and purity. Overall, this study shows that methanolic extraction and chromatographic purification are effective methods for isolating Tulipalin A from *Tulipa gesneriana* callus, and it emphasizes the importance of combining HPLC and FTIR for the structural characterization of bioactive metabolites.

Keywords: *Tulipa gesneriana*, red callus, Tulipalin A, HPLC, FTIR, purification, tissue culture.

1. Introduction

Tulip (*Tulipa gesneriana* L.), often known as the garden tulip, serves as a perennial ornamental plant in the family *Liliaceae* that is extensively cultivated for its beautiful spring blossoms (Kavaiya *et al.*, 2025). Tulip tissue, in addition to its aesthetic appeal, has several phytochemicals with traditional therapeutic effects such as antipyretic, anticancer, and laxative (Krzyńska, Gąsecka and Magdziak, 2020). Tulipalin A (α -methylene- γ -butyrolactone) represents a biosynthetic product formed from the precursor 6-Tuliposide A. Tulipalin A is kept as an inactive glucoside in the vacuoles of healthy tulip plant cells, away from the enzyme accountable for its conversion (Nomura, 2017).

Tuliposide A-converting enzyme (TCEA) was described as the first known lactone-forming carboxylesterase, which converts Tuliposide A to the active compound Tulipalin A by enzymatic transesterification without hydrolysis, and thus the conversion is enzyme-mediated and not spontaneous (Nomura *et al.*, 2015). When tissues are injured or exposed to a pathogenic attack, this enzyme is released from the plastids and reaches the substrate (Nomura *et al.*, 2015; Nomura *et al.*, 2017). Spatial separation of substrates and enzymes represents a subtle defense mechanism; Tulipalin A is produced in high quantities only upon cell rupture, protecting the plant from autotoxicity under normal conditions (Nomura, Ogita and Kato, 2012).

Tulipalin A is the main component responsible for the allergenic and bioactive effects of tulips (Nomura *et al.*, 2023; Phan *et al.*, 2025). It is the main cause of a phenomenon known as “tulip fingers,” a type of occupational contact dermatitis that affects tulip growers and florists (Hassan *et al.*, 2018). These compounds have a protective role within the plant, acting as natural antibiotics with fungicidal effects that protect the bulbs from pathogenic infections (McCluskey, Bourgeois and Harbison, 2014). A proteomic study demonstrated that Tulipalin A exerts immunostimulatory effects, activating stress response proteins and altering purine metabolism in human immune cell models (Zwicker *et al.*, 2016).

Plant tissue culture is a technique that involves growing plant tissue segments (explants) in the laboratory on artificial media under aseptic conditions (Hasnain *et al.*, 2022). It provides a sustainable source for active chemicals. Callus cultures and cell suspensions are commonly utilized to create secondary metabolites, ensuring consistent synthesis regardless of environmental conditions (Chandran *et al.*, 2020; Jdayea & Neamah, 2024; Abdelazeez *et al.*, 2025).

Tulipalin A and its precursors are isolated and identified from *T. gesneriana* using modern chromatographic and spectroscopic techniques. Tuliposides and Tulipalins are mostly separated from complicated plant extracts using high-performance liquid chromatography (HPLC) for analytical purposes (Nomura, Ogita and Kato, 2020). Fourier transform infrared (FTIR) spectroscopy is a crucial tool for identifying plant

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compounds. It quickly confirms the unique functional groups of Tulipalin A, including the α -methylene- γ -butyrolactone ring, which makes it an effective technique for confirming the metabolite's identity (Lee *et al.*, 2021). Although the biochemical pathway of Tulipalin A has been thoroughly described, the controlled production of Tulipalin A through *in-vitro* callus culture in *Tulipa gesneriana* has not been sufficiently investigated. This is because most studies have concentrated on extracting the compound from natural plant organs, which are subject to a wide variation depending on the environmental and seasonal conditions (Nomura, Ogita and Kato, 2012). Van Rossum, Alberda and van der Plas (1998) reported that tissue-cultured tulip explants can accumulate Tuliposide A and Tulipalin A up to ~1300% above their initial levels, supporting the superior capacity of *in-vitro* cultured tissues such as callus for enhanced Tulipalin production. The purpose of this study was to use tissue culture techniques in tulip (*T. gesneriana*) to yield homogeneous and sustainable plant material, then to extract and characterize some important secondary metabolites, especially Tulipalin A and its precursors Tuliposides, utilizing advanced analytical techniques such as high-performance liquid chromatography (HPLC) and Fourier transform infrared (FTIR).

2. Materials and Methods

This work was conducted in the tissue culture facilities of the University of Baghdad's College of Science for Women.

2.1. Laboratory Sterilization

Forceps, scalpels, and Petri dishes were sterilized in an autoclave at 121°C with 1.04 kg/cm² for 30 minutes. The instruments were then soaked in 96% ethanol to quickly sterilize and remove any impurities. They were then allowed to dry in a laminar airflow cabinet. To guarantee sterility, the cabinet was UV-sterilized for 30 minutes and then sprayed with 70% ethanol (Kareem, 2021).

2.2. Media Preparation

Standard Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) was prepared by dissolving 4.9 g/L MS powder (Himedia, India), 30 g/L sucrose, and 8 g/L agar (Biolife, India) in sterile distilled water. The pH was adjusted to 5.8 using HCl or NaOH. After sterilization, the medium was permitted to cool before being used.

2.3. Callus induction

T. gesneriana leaves were cut into 0.2 cm pieces under sterile conditions and cultivated on MS medium supplemented with plant growth regulators as follows: 2,4-Dichlorophenoxyacetic acid (2,4-D; Himedia, India) (1 mg/L) + Thidiazuron (TDZ; Himedia, India) (1 mg/L). The media were stored in a growth chamber at 25 ± 2°C, with a 16-hour light/8-hour dark cycle (Tahtamouni *et al.*, 2020).

2.4. 2.4. Extraction of the Active Compounds

Five grams of the collected callus (wet weight) were taken and mixed with 50 mL of 70% methanol (Alpha-Chemika, India). The samples were placed in a reflux apparatus under standard conditions at 60–80°C for 6 hours. After the extraction process was complete, the

samples were allowed to cool and then filtered using sterile filter paper. The crude extract was collected. The resulting extracts were stored at 4°C until chemical tests were performed (Alwasha, Shlahib and Hamad, 2018).

2.5. Chromatographic and Spectroscopic Methods

The analysis was carried out at the Scientific Research Authority, Environmental and Water Research Center, to detect Tulipalin A (α -methylene- γ -butyrolactone) using HPLC (SYKAM, Germany). A C18-ODS column (25 cm in length, 4.6 mm internal diameter) was used. The mobile phase consisted of distilled water and acetonitrile at a ratio of 20:80 (v/v), with a flow rate of 1.0 mL/min. Detection of the active compound was performed using a UV detector at 208 nm. Identification was confirmed by co-analysis with Tulipalin A standard (Santa Cruz Biotechnology, Dallas, TX, USA; Cat. No. sc-239165) (Harada *et al.*, 2002).

FTIR spectroscopy was also employed to confirm the functional groups of Tulipalin A. A known quantity of the sample intended for FTIR analysis was taken and mixed with potassium bromide (KBr) powder at a ratio of ten times the sample weight, to ensure proper homogenization and to minimize strong absorption effects. The mixture was extensively crushed in a porcelain mortar to produce a homogenous blend, which was then pressed into a specific die to generate a transparent pellet appropriate for spectroscopy (Das *et al.*, 2024). Prior to this operation, the sample was dried at room temperature to remove residual moisture, which could cause unwanted absorption peaks in the spectrum and interfere with the analysis of functional group bands. This procedure is commonly used to prepare KBr pellets for spectroscopic examination. Other studies have confirmed how absorbed moisture in KBr pellets can interfere with hydroxyl peaks, reducing the quality of the data (Dai *et al.*, 2023).

3. Results and Discussion

T. gesneriana leaf explants cultivated on MS medium supplemented with 2,4-D (1 mg/L) + TDZ (1 mg/L) demonstrated a unique reaction in callus formation, resulting in the production of a friable callus with a delicate texture. The callus formed as a result of this treatment has a distinct red coloration.

Active compounds were extracted utilizing methanol by using the conventional method. The extract obtained from the red-colored callus showed a red coloration, reflecting the chemical composition of the callus used.

To establish the identity and purity of the active ingredient recovered from the red callus, HPLC analysis was carried out as follows:

100 μ L of Tulipalin A standard (α -MGBL) was injected through the HPLC system to measure the retention time along with chromatographic properties. The resulting chromatogram showed a sharp and distinct peak at a retention time of 3.8 min, with a recorded peak area and height consistent with the standard specifications of the compound (Figure 1).

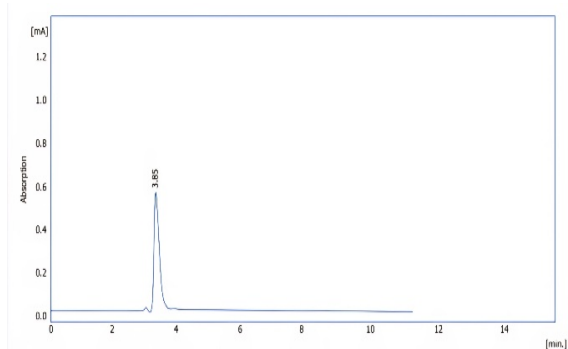


Figure 1: HPLC chromatogram of the Tulipalin A standard (α -MGBL) showing a single pure peak at a retention time of 3.8 min.

Additionally, 100 μ L of the crude sample was injected under the same chromatographic conditions. The chromatogram displayed several major peaks at different retention times, including a prominent peak at 3.8 min, in addition to other peaks that appeared at retention times beyond 4 minutes (Figure 2).

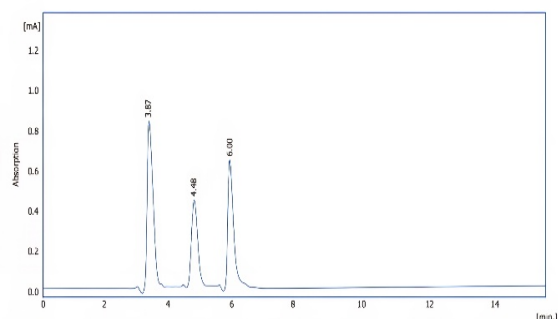


Figure 2: HPLC chromatogram of the crude extract showing three major peaks, one of which at 3.8 min.

To obtain the active compound in a purified form, multiple injections were performed and fractions were collected using a fraction collector. When the collected fraction was reinjected into the HPLC system, the resulting chromatogram showed a single peak at 3.8 min, with no additional peaks detected in the profile (Figure 3).

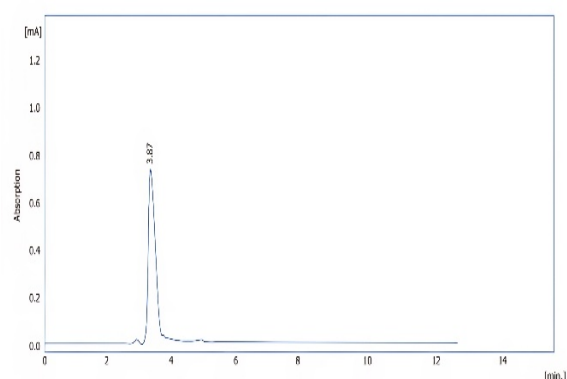


Figure 3: HPLC chromatogram of the isolated compound showing a single peak at 3.8 min.

An additional test was conducted by mixing 50 μ L of the isolated compound with 50 μ L of the Tulipalin A standard (α -MGBL) and injecting the mixture into the system. The resulting chromatogram revealed a single peak at 3.8 min, with nearly double the peak area compared with the individual injections of either the standard or the isolated compound (Figure 4).

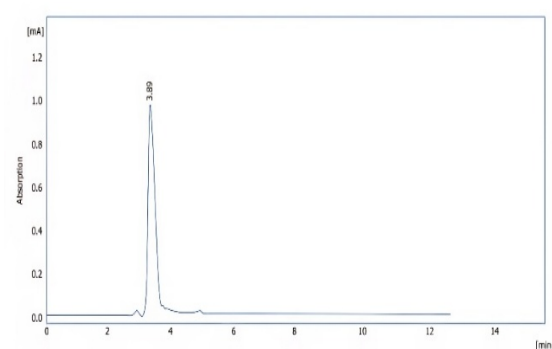


Figure 4: HPLC chromatogram of co-injection (isolated compound + standard) showing a single peak at 3.8 min with doubled area.

The findings obtained from HPLC were reinforced by FTIR analysis to verify the functional groups and structural identity of the compound (Figure 5), which illustrates a comparative representation of the FTIR spectra of the Tulipalin A standard (a) and the isolated compound (b), showing the distribution of characteristic absorption bands across the spectral range (4000–500 cm^{-1}). The FTIR spectrum of the Tulipalin A standard (α -MGBL), shown in Figure (5a), revealed several characteristic absorption bands distributed across the range of 4000–500 cm^{-1} . The major peaks were observed at 3506, 2938, 1880, 1632, 1439, 1270, 1116, 1024, and 767 cm^{-1} . The FTIR spectrum of the isolated compound Figure (5b) demonstrated a pattern of absorption bands within the same spectral range (4000–500 cm^{-1}). The main absorption bands appeared at 3512, 2936, 1883, 1630, 1432, 1272, 1118, 1022, 823, and 764 cm^{-1} .

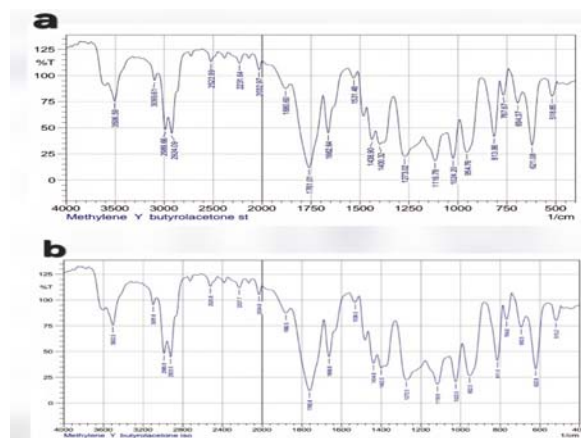


Figure 5: FTIR spectra of Tulipalin (α -methylene- γ -butyrolactone) standard and the isolated compound, showing the major absorption bands in the range of 4000–500 cm^{-1} . (a) FTIR spectrum of Tulipalin standard. (b) FTIR spectrum of the isolated compound.

The results of this study indicated that the formation of red callus in tulip tissue culture was associated with an increased accumulation of bioactive secondary metabolites. This interpretation agrees with the findings of (Lai *et al.*, 2022), who discovered that red callus produced by *Vitis davidii* contained excessive levels of anthocyanins and that the amount of red pigmentation raised significantly under blue and violet light, with important pigments such as cyanidin-3-glucoside as well as peonidin-3-glucoside accumulating beneath these conditions. Similarly, Yao *et al.* (2023) reported that

culture factors-including sugar source, light quality, and temperature-significantly enhanced anthocyanin and phenolic accumulation in red pear callus. Sucrose supplementation, paired with red-blue light and relatively low temperature ($\sim 15^{\circ}\text{C}$), resulted in increased production of anthocyanins and secondary metabolites, as well as activation of biosynthetic genes. Compared with our findings, the red coloration of tulip callus serves as a clear indicator of secondary metabolic activation, supporting the concept that pigmentation is linked to the accumulation of other defense-related metabolites such as Tulipalin.

In terms of extracting and purifying the active ingredient, methanolic extraction from the red callus allowed for the isolation of Tulipalin A, which was compatible with the findings of (Christensen and Kristiansen, 1999). They observed that Tuliposide A is the major precursor to Tulipalin and that both chemicals are plentiful in tulip tissues, accounting for more than 1% of the fresh weight. Likewise, Nomura and colleagues (Nomura, Ogita and Kato, 2020) demonstrated that Tuliposide A quickly transforms into Tulipalin after cell injury or during extraction, and chromatographic examination of tulip extracts revealed the existence of several Tuliposides (A, B, D, and F). In a more recent study (Lee *et al.*, 2021) isolated three new Tuliposides (H–J) alongside known compounds from a methanolic extract of *Amana edulis* bulbs (a close relative of tulip in the Liliaceae family), using spectroscopic analyses to confirm their identities. Therefore, the findings on the isolation of pure Tulipalin from the red callus agree with these studies and further highlight the efficiency of methanolic extraction in releasing the active compound from tulip tissues.

3.1. Retention Time Matching with the Standard

The chromatogram of the crude tulip extract displayed three distinct peaks at retention times of 3.8, 4.4, and 6.0 minutes. The presence of multiple peaks in the crude extract indicates the coexistence of several compounds. Indeed, previous studies have reported that α -methylene- γ -butyrolactone (commonly known as Tulipalin A) typically appears as one of the major peaks within complex plant extracts. Following the isolation and purification steps, a single sharp peak was obtained at a retention time of 3.8 minutes, which matched exactly with that of the authentic standard (reference compound). Such correspondence of retention time between the isolated compound and the standard, under identical HPLC conditions, provides strong evidence of the compound's identity. In chromatographic analysis, this retention-time matching is considered a decisive criterion for confirming that the unknown and the standard are the same compound. Accordingly, the agreement of the isolated sample's peak with that of the Tulipalin A standard strongly supports that the purified compound is indeed α -methylene- γ -butyrolactone.

3.2. Purity of the Isolated Compounds and Removal of Impurities

The appearance of a single chromatographic peak for the isolated compound directly indicates its high purity. In HPLC analysis, a pure sample yields a single sharp peak, whereas the presence of impurities is manifested by additional peaks, shoulders, or peak tailing adjacent to the main peak. In this case, the two peaks initially observed at

4.4 and 6.0 minutes in the crude extract completely disappeared after purification, confirming the removal of these unwanted components. The HPLC profile of the purified sample showed one dominant peak occupying nearly the entire signal area, suggesting that the target compound now represents the overwhelming majority of the sample. Quantitatively, the peak area of the target compound (at 3.8 minutes) increased from being only a fraction of the total chromatographic area in the crude extract to accounting for nearly 100% of the total area in the purified sample. This clearly demonstrated that the isolation procedure concentrated the desired compound while eliminating most other constituents. Similarly, in a comparable study on the purification of a natural metabolite, the chromatographic analysis of the purified fraction displayed a single retention-time peak identical to that of the standard, with a purity approaching 99%, thereby confirming the efficiency of purification in eliminating impurities and achieving a high degree of compound purity.

To provide additional confirmation of the compound's identity, a co-injection test was performed by introducing a mixture of the isolated compound and the standard into the HPLC system. If the isolated compound had been different from the standard, two distinct peaks or a change in peak shape would have been expected. Instead, the analysis yielded a single, sharp peak at 3.8 minutes with no overlap or splitting. This behavior demonstrates that the isolated compound and the reference standard co-elute under identical chromatographic conditions, indicating a perfect match of identity. Therefore, this test provided unequivocal evidence that the compound isolated from the tulip extract was pure α -methylene- γ -butyrolactone, as its retention peak was identical to that of the standard with no detectable trace of other components.

FTIR analysis of the standard and the isolated sample from tulip extract showed a clear match in the positions of the absorption bands, confirming that the compound isolated after purification is α -methylene- γ -butyrolactone (Tulipalin A) with high purity, as no additional peaks indicative of impurities were observed. The main bands detected included a strong absorption band at $\sim 1760\text{ cm}^{-1}$ corresponding to the carbonyl group of the γ -lactone ring, and a band at $\sim 1660\text{ cm}^{-1}$ indicative of an α , β -unsaturated double bond conjugated with the carbonyl. Additional features comprised the region at $2850\text{--}3000\text{ cm}^{-1}$ due to stretching vibrations of both saturated and unsaturated C–H bonds, a band at $\sim 1450\text{ cm}^{-1}$ associated with methylene scissoring vibrations, characteristic absorptions between $1100\text{--}1200\text{ cm}^{-1}$ assigned to C–O stretching in the lactone ester, and distinct bands at 910 and 990 cm^{-1} corresponding to out-of-plane vibrations of the terminal vinyl group ($=\text{CH}_2$), which serve as a fingerprint for the compound. These values are fully consistent with previous reports describing α -methylene- γ -lactones, where recent studies (Smith, 2016; Graur *et al.*, 2022) highlighted that the combination of a strong carbonyl band near 1760 cm^{-1} , a conjugated double bond around 1660 cm^{-1} , and vinyl group signals at $910\text{--}990\text{ cm}^{-1}$ provides conclusive evidence of this structural unit. Such spectral concordance strongly supports the earlier HPLC findings, which revealed a single peak at a retention time identical to the standard, thereby providing combined quantitative and qualitative evidence that the isolated compound is pure

Tulipalin A. This confirms the success of the purification process and validates the reliability of the sample for subsequent biological and analytical applications.

4. Conclusion

This study demonstrated that red callus of *Tulipa gesneriana* is an in vitro source of secondary metabolites. Methanolic extraction enabled the successful isolation of Tulipalin A (α -methylene- γ -butyrolactone). HPLC analysis confirmed the identity and purity of the compound, as the isolated fraction yielded a single peak at 3.8 minutes, identical to the standard, with co-injection further validating the match. FTIR spectra revealed characteristic absorption bands consistent with Tulipalin A, including the γ -lactone carbonyl and terminal vinyl group. Together, these findings provide strong quantitative and qualitative evidence of the compound's identity and purity, highlighting the efficiency of red callus culture for producing valuable bioactive metabolites.

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Conflict of interest

There are no conflicts of interest in this paper.

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Author contribution

- Israa Adnan Salman: Conceptualization, study design, supervision, Data analysis, figure preparation, manuscript writing.
- Sumaya F. Hamad: Methodology, experiments, manuscript editing, sample collection.

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