

# Comprehensive HPTLC Fingerprinting and Quality Evaluation of Punicalagin in Pomegranate (*Punica granatum* L.) Aril Juice

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## ABSTRACT

**Objectives:** Pomegranate (*Punica granatum*) is a well-known source of bioactive phytochemicals, particularly punicalagin, which contributes to its antioxidant, anti-inflammatory, and antimicrobial properties. **Materials and Methods:** In this study, a robust High-Performance Thin-Layer Chromatography (HPTLC) method was developed to fingerprint and quantify punicalagin in aril juice. **Results:** The optimized mobile phase-chloroform: ethyl acetate: formic acid: methanol (4.0:5.2:0.6:0.2, v/v/v/v)-produced sharp, well-resolved bands, and densitometric scanning at 620 nm revealed a distinct punicalagin peak at (Retention factor)  $R_f=0.58\pm0.03$ , accounting for 5.32% of the total chromatographic area. Microbial evaluation confirmed the absence of *E. coli*, *S. aureus*, *Salmonella*, *Candida albicans*, and *Shigella*, with total aerobic and yeast/mould counts below 10 (Colony Forming Units) CFU/g. Elemental screening revealed trace levels of Pb, Cd, and Hg well below pharmacopeial limits, and nutritionally relevant minerals such as iron, zinc, and copper were present in appreciable amounts. **Conclusion:** These findings establish a reproducible HPTLC fingerprint for pomegranate aril juice and demonstrate that integrated chemical, microbial, and elemental profiling provides a comprehensive approach for quality control and authentication of pomegranate-derived nutraceuticals.

**Keywords:** HPTLC Fingerprinting, Nutraceutical Standardization, Pomegranate (*Punica granatum*), Punicalagin, Quality Control.

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## INTRODUCTION

Medicinal plants are an indispensable source of bioactive compounds that contribute to human health by preventing and managing chronic diseases. These plant-derived phytochemicals-including phenolic compounds, flavonoids, tannins, and alkaloids-exert diverse biological effects such as antioxidant, anti-inflammatory, antimicrobial, and anticancer activities, making them vital candidates for drug discovery and development (Mo *et al.*, 2022; Singh *et al.*, 2018). Rising antimicrobial resistance has renewed global interest in exploring plant-based therapeutics as alternative or adjunct treatments, offering solutions where conventional antibiotics have become ineffective (DEBIB *et al.*, 2022; Kiran *et al.*, 2024; Maslov *et al.*, 2025).

Pomegranate (*Punica granatum* L.) is a widely consumed fruit valued for its nutritional and therapeutic properties. Its peel, seeds, and arils are rich in phenolic compounds-including flavonoids (anthocyanins, anthoxanthins), tannins (ellagitannins such as punicalagin, punicalin, and pedunculagin), and phenolic acids (chlorogenic, caffeic, syringic, sinapic, p-coumaric, ferulic, ellagic, gallic, and cinnamic acids)-along with dietary fiber, minerals, and vitamins (Du *et al.*, 2025; Ranjha *et al.*, 2023; Siddiqui *et al.*, 2024; Singh *et al.*, 2018). Among these, punicalagin is the most abundant and pharmacologically significant ellagitannin, exhibiting potent antioxidant, anti-inflammatory, anticancer, cardioprotective, and antimicrobial activities (Shabir *et al.*, 2024; Venusova *et al.*, 2021; Wang *et al.*, 2024; Xu *et al.*, 2021). Mechanistic studies demonstrate that punicalagin modulates key signaling pathways-including Nuclear Factor kappa B (NF-Kb), Mitogen-Activated Protein Kinase (MAPK) and Phosphoinositide 3-kinase/Protein Kinase B/Mammalian Target of Rapamycin (PI3K/Akt/mTOR)-attenuates oxidative stress, induces apoptosis in malignant cells, and improves immune and endothelial function (Chen *et al.*, 2023; Ramlagan *et al.*, 2022; Tang *et al.*, 2022). These multifaceted bioactivities make punicalagin a promising candidate for nutraceutical and



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pharmaceutical applications, though its clinical translation requires consistent product characterization and standardized dosing (Shabir *et al.*, 2024; Xu *et al.*, 2021).

Pomegranate juice, particularly from the arils, is a major dietary source of anthocyanins and other polyphenols, contributing to its strong antioxidant capacity (Habib *et al.*, 2023; Karagecili *et al.*, 2023). *In vitro* and *in vivo* studies have demonstrated its ability to inhibit pathogenic bacteria, including Multi Drug-Resistant (MDR) strains such as Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Extended-Spectrum B-Lactamase (ESBL)-producing *Enterobacteriaceae*, by disrupting microbial growth and resistance mechanisms (Alghamdi *et al.*, 2024; Gosset-Erard *et al.*, 2021; Maslov *et al.*, 2025). These findings support the potential of pomegranate-derived products as natural antimicrobials, functional foods, and adjunct therapies to combat antibiotic resistance.

Reliable quantification and profiling of bioactive compounds are essential for ensuring the authenticity, reproducibility, and quality of pomegranate-based products. High-Performance Thin-Layer Chromatography (HPTLC) is a robust and cost-effective technique that allows simultaneous analysis of multiple phytochemicals with minimal sample preparation, producing a reproducible chemical “fingerprint” for qualitative and quantitative assessments (Venkatesham *et al.*, 2021). HPTLC is particularly suitable for detecting marker compounds such as punicalagin, distinguishing genuine from adulterated products, and enabling high-throughput quality control in both research and industry (Farang *et al.*, 2020; Riolo *et al.*, 2025; Skrt *et al.*, 2022). While High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography-Mass Spectrometry (LC-MS) provide higher resolution and sensitivity, HPTLC offers a rapid and economical alternative for routine screening, especially where large-scale analysis is required (Farang *et al.*, 2020; Riolo *et al.*, 2025; Skrt *et al.*, 2022).

Despite growing evidence of pomegranate's therapeutic potential, several gaps remain. Quantitative standardization of punicalagin and other bioactive compounds across different juice preparations is inconsistent due to variability in plant parts, extraction methods, and analytical protocols (Magangana *et al.*, 2020; Yisimayili and Chao, 2022). The lack of validated, comprehensive fingerprinting methods hinders reliable product comparison and clinical translation (Aziz *et al.*, 2020; Laurindo *et al.*, 2022). Large-scale, well-controlled clinical trials are still needed to substantiate health claims (Mohan *et al.*, 2024; Mokgalaboni *et al.*, 2023).

Therefore, the present study aims to develop and validate a HPTLC-based fingerprinting method for the quantification of punicalagin in pomegranate aril juice. This standardized approach will facilitate routine quality control, ensure product authenticity,

and enable reproducibility in research and nutraceutical applications.

## MATERIALS AND METHODS

### Plant Material and Sample Collection

Fresh pomegranate (*Punica granatum L.*) fruits were collected from mature trees. Only fruits without visible defects, decay, or microbial contamination were selected to ensure sample integrity. The fruits were washed twice with Double-Distilled Water (ddH<sub>2</sub>O) and disinfected using a 150 µL/L sodium hypochlorite solution for 1 min, followed by thorough rinsing with sterile ddH<sub>2</sub>O to remove residual chlorine (Cite: Standard disinfection and preparation protocols). The arils were manually separated from the peel and seeds under aseptic conditions and processed immediately for juice extraction.

### Sample Extraction

Approximately 1 kg of fresh pomegranate arils was macerated with 1 L of a hydroalcoholic solvent mixture (50:50, v/v, distilled water: methanol) to obtain a homogeneous slurry. The mixture was filtered through a double-layered muslin cloth to remove seeds and coarse pulp. The filtrate was concentrated to dryness using a water bath at controlled temperature (<50°C) to prevent thermal degradation of phenolic compounds. The resulting dry extract was stored in a desiccator until constant weight was achieved and subsequently weighed to determine the yield.

### Preparation of Test Solution

1 g of the dried pomegranate extract was dissolved in 10 mL of HPTLC-grade methanol to prepare a stock solution. Aliquots of 3, 6, and 9 µL from this stock solution were used for HPTLC analysis. This methanolic extract served as the test solution for fingerprinting and quantitative determination of punicalagin (Bhargava *et al.*, 2021).

### HPTLC Fingerprinting and Quantification

The schematic plan of HPTLC finger printing was depicted in Figure 1.

### Chromatographic Conditions

HPTLC analysis was achieved on pre-coated silica gel 60 F254 aluminum plates (Merck, Germany). Using a CAMAG Linomat 5 applicator, 3, 6, and 9 µL of the methanol extract were applied as 7 mm-wide bands with a constant application rate under a nitrogen stream. The chromatograms were developed in a twin-trough glass chamber pre-saturated with a mobile phase consisting of chloroform: ethyl acetate: formic acid: methanol in the ratio of 4.0:5.2:0.6:0.2 (v/v/v/v). After development, plates were air-dried and visualized under short-wave (Ultraviolet) UV (254 nm) and long-wave UV (366 nm). Plates were then derivatized with

anisaldehyde-sulphuric acid reagent, heated at 105°C until color development, and scanned densitometrically at 620 nm using a CAMAG Scanner 4 with winCATS software.

### Statistical Analysis

Quantitative data obtained from densitometric scanning were expressed as mean  $\pm$  Standard Deviation (SD). All HPTLC analyses were performed in triplicate to ensure reproducibility. Retention factor ( $R_f$ ) values and peak areas were calculated using winCATS software (CAMAG, Switzerland).

### Microbial and Elemental Analysis

Microbial analysis of pomegranate juice was conducted according to pharmacopeial guidelines (Cite: Indian/United states/British Pharmacopoeia). Tests included Total Aerobic Microbial Count (TAMC), Total Yeast and Mold Count (TYMC), and presence/absence of common foodborne pathogens (*E. coli*, *Salmonella* spp., *S. aureus*). Elemental examination was performed using standard pharmacopeial procedures to regulate the existence and concentration of essential minerals, including potassium, sodium, calcium, and trace elements (Popovici *et al.*, 2021).

## RESULTS

### HPTLC Fingerprinting Profile

The methanolic extract of *Punica granatum* aril juice was successfully analyzed using High-Performance Thin-Layer Chromatography (HPTLC) to generate a characteristic fingerprint profile. The optimized mobile phase system-chloroform: ethyl acetate: formic acid: methanol (4.0:5.2:0.6:0.2, v/v/v/v)-produced well-resolved, sharp, and symmetrical peaks without overlapping bands. The densitometric scan at 620 nm revealed four distinct peaks, with Peak 3 corresponding to punicalagin ( $R_f=0.58\pm0.03$ ). This peak accounted for 5.32% of the total chromatographic area, confirming its significant presence in the extract (Figure 2 and Table 1).

### Chromatogram and Photo-Documentation

The HPTLC chromatogram displayed clear separation of components, as indicated by discrete peaks across the  $R_f$  range (0.01-0.72). Photodocumentation of the developed plates under short-wave UV (254 nm), long-wave UV (366 nm), and white light (after derivatization with anisaldehyde-sulphuric acid) confirmed the presence of multiple phytoconstituents with distinct fluorescence and color intensities (Figure 3). The band corresponding to punicalagin was prominent across all applied concentrations (3, 6, and 9  $\mu$ L), supporting the specificity and reproducibility of the method.

### Specificity and Quantification

Specificity of the HPTLC method was verified by comparing the  $R_f$  value and densitometric profile of the sample with the

punicalagin reference standard under identical chromatographic conditions. The results demonstrated no co-elution with other constituents, confirming the method's selectivity for punicalagin quantification.

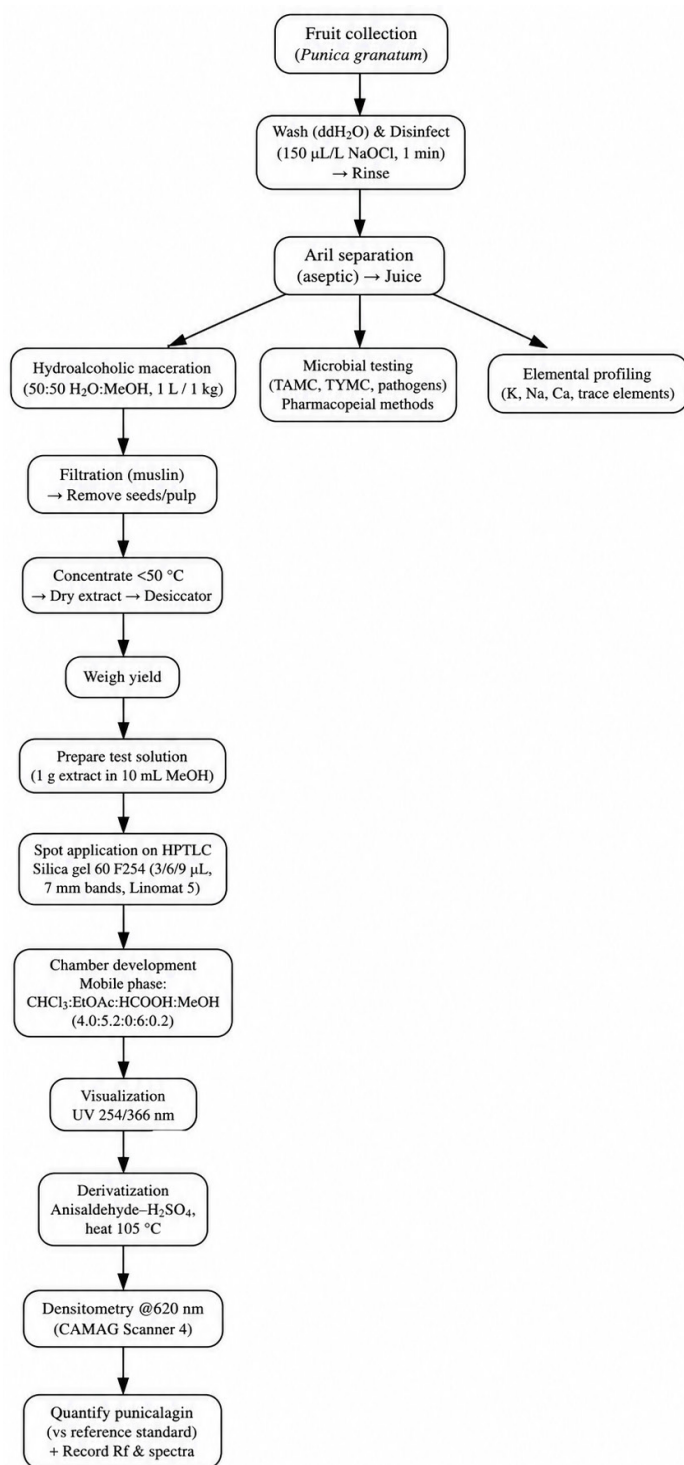


**Figure 1:** Schematic presentation of Workflow for the preparation, HPTLC fingerprinting, quantitative analysis of punicalagin, and microbial/elemental assessment of pomegranate (*Punica granatum*) aril juice.

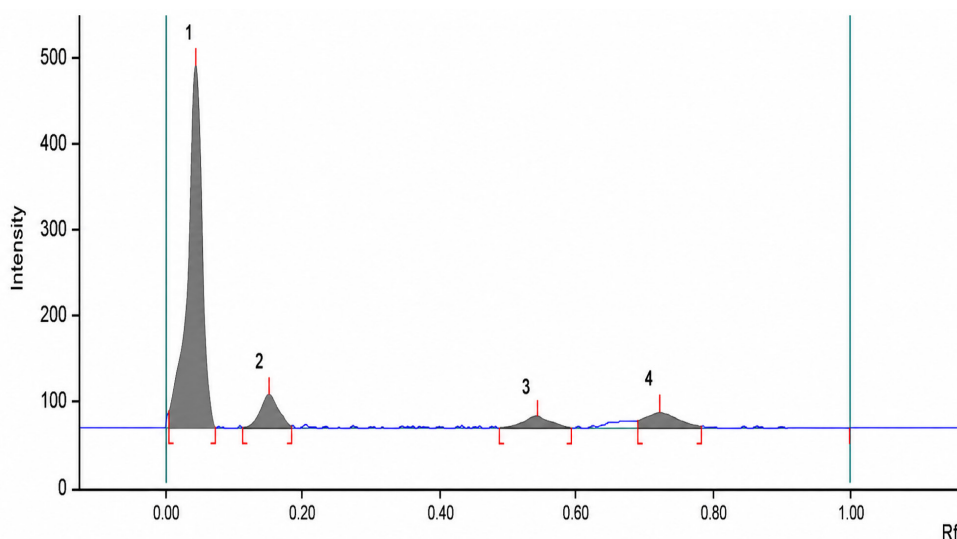


**Figure 2:** HPTLC densitometric chromatogram of *Punica granatum* aril juice extract (6  $\mu$ L) scanned at 620 nm, showing four major peaks. Peak 3 ( $R_f=0.58\pm0.03$ ) corresponds to punicalagin.

### Densitometric data analysis

The four distinct peaks measured, with Peak 1 accounting up most (78.16% area). Punicalagin occupied 5.32% of the total peak area and was found with an  $R_f$  value of 0.55 in Peak 3.

### Densitometric Profiling extract

After development chromatographic development, plates were derivatized and scanned densitometrically at 620 nm to assess the (Start, Maximum End)  $R_f$  values, maximum peak height and relative percentage of identified compounds. The four well defined peaks were found showing  $R_f$  values of 0.05, 0.16, 0.55 and 0.72 indicates the confirming different phytochemical components in the prepared extract (Figure 4).

### Microbial Quality Assessment

Microbiological analysis of the dried pomegranate aril juice extract was performed according to pharmacopeial guidelines. The results specified that the sample met the microbiological quality criteria for herbal preparations. Total Aerobic Bacterial Count (TAMC) and Total Yeast and Mold Count (TYMC) were both found to be below 10 CFU/g, indicating minimal microbial load. No pathogenic organisms-including *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* spp., *Candida albicans*, or *Shigella* spp.-were detected in the sample, confirming its microbiological safety for consumption (Table 2).

### Elemental Analysis

Elemental screening of the dried pomegranate aril juice powder was performed according to pharmacopeial guidelines to determine the presence of essential minerals and potential toxic heavy metals. The results demonstrated that the concentrations of lead, cadmium, and mercury were well below permissible limits, indicating no risk of heavy metal contamination. Among

the essential elements, iron and zinc were found in appreciable amounts, followed by copper, suggesting that the juice contributes beneficial micronutrients (Table 3).

## DISCUSSION

Chromatographic fingerprinting provides a unique chemical profile of complex matrices and is widely recognized as a powerful tool for identity testing and quality assessment of herbal medicines and food products (Meena *et al.*, 2021). In the present study, an optimized High-Performance Thin-Layer Chromatography (HPTLC) method was developed for the fingerprinting and quantification of punicalagin in *Punica granatum* aril juice. The chosen mobile phase, chloroform: ethyl acetate: formic acid: methanol (4.0:5.2:0.6:0.2, v/v/v/v), produced shawell-resolved bands without interference from other phytoconstituents, confirming the specificity of the system. Densitometric analysis revealed four major peaks, with the peak corresponding to punicalagin appearing at an  $R_f$  value of  $0.58\pm0.03$ , accounting for 5.32% of the total chromatographic area. Reporting the  $R_f$  value is particularly significant because most published HPTLC studies on punicalagin quantification focus on concentration data but omit  $R_f$  information, which is essential for reproducibility and inter-laboratory comparisons (Shahkoomahally *et al.*, 2023). The presence of a distinct punicalagin band across all applied concentrations (3, 6, and 9  $\mu$ L) further supports the robustness of the established method and its suitability for repetitive quality control applications.

Previous reports have quantified punicalagin in pomegranate peel and commercial herbal preparations using HPTLC, with concentrations ranging from 807.6  $\mu$ g to 3.207 mg/g of sample (Gadkari and Daharwal, 2022). whereas most recent studies employ HPLC or UHPLC methods, reporting 5.2-130.32  $\mu$ g/mL in peel extracts and up to 238.7  $\mu$ g/mL in juice, depending on the

cultivar and extraction method (Kumar *et al.*, 2023; Maharana *et al.*, 2022; Mahmood and Jabar, 2023). Although direct comparison of area percentages with concentration data is challenging due to methodological differences, the present study demonstrates that HPTLC can reliably detect and quantify punicalagin in aril juice, offering a cost-effective screening tool prior to more advanced HPLC or LC-MS-based analysis. This is consistent with WHO and pharmacopeial recommendations that advocate the use of HPTLC as a reproducible and regulatory-compliant tool for authentication, standardization, and detection of adulteration in herbal materials (El-Waey *et al.*, 2023; Frommenwiler *et al.*, 2022).

Punicalagin is one of the most pharmacologically significant ellagitannins in pomegranate, exhibiting antioxidant, anti-inflammatory, anticancer, cardioprotective, and antimicrobial properties (10,11,12). It modulates several key signaling pathways, including NF- $\kappa$ B, MAPK, and PI3K/Akt/mTOR, reduces oxidative stress, and improves immune and endothelial function (Chen *et al.*, 2023; Ramlagan *et al.*, 2022; Tang *et al.*, 2022). Therefore, its detection and quantification are critical for establishing the nutraceutical value of pomegranate juice and ensuring batch-to-batch consistency in commercial formulations. The results of microbial analysis further strengthen the quality assessment of the juice powder, as total aerobic count and yeast/mold count were <10 CFU/g and no pathogens

(*Escherichia coli*, *Staphylococcus aureus*, *Salmonella spp.*, *Candida albicans*, or *Shigella spp.*) were detected, these findings indicate great microbial quality and are in accordance with better microbial resilience commonly associated with freeze-dried fruit powders with low moisture levels (Howell and D'Souza, 2013). Similarly, elemental analysis revealed that concentrations of toxic heavy metals (Pb, Cd, Hg) were well below pharmacopeial limits, while nutritionally relevant minerals such as iron, zinc, and copper were present in appreciable amounts. These findings are in line with reports that identify potassium and iron as dominant minerals in pomegranate juice and confirm the nutritional relevance of the analyzed material (Nuncio-Jáuregui *et al.*, 2015).

Collectively, these results demonstrate that HPTLC fingerprinting coupled with microbial and elemental profiling provides a comprehensive approach for the authentication and quality control of pomegranate aril juice. The reported  $R_f$  value for punicalagin can serve as a reference parameter for future studies, and the established fingerprint profile offers a baseline for comparison with other varieties or commercial products. Future work should include full validation of the method-covering precision, accuracy, Limit of Detection (LOD), and Limit of Quantification (LOQ)-and comparative studies using HPLC or LC-MS to benchmark the sensitivity and specificity of the approach. Large-scale screening of pomegranate juices from different cultivars and processing methods would

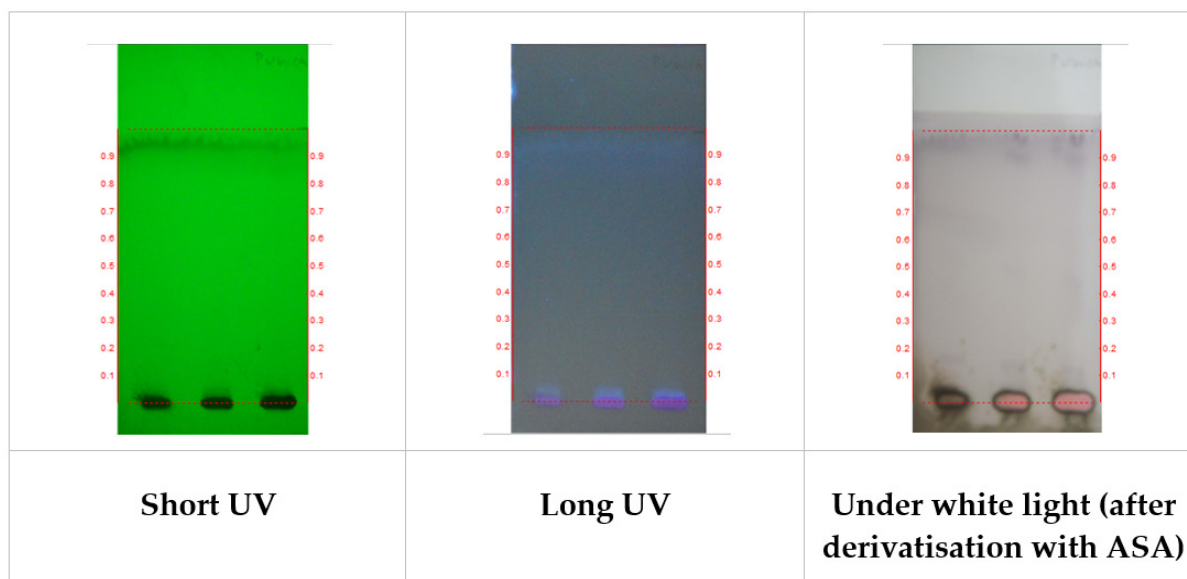


**Figure 3:** HPTLC photodocumentation of *Punica granatum* hydroalcoholic extract under (A) short-wave UV (254 nm), (B) long-wave UV (366 nm), and (C) white light after derivatisation with anisaldehyde-sulphuric acid. Tracks: 1=3  $\mu$ L, 2=6  $\mu$ L, 3=9  $\mu$ L.

**Table 1:** HPTLC Densitometric Data of *Punica granatum* hydroalcoholic Extract (6  $\mu$ L).

| Peak No.        | Start Position ( $R_f$ ) | Max Position ( $R_f$ ) | End Position ( $R_f$ ) | Max Height (AU) | Area (AU) | Area % |
|-----------------|--------------------------|------------------------|------------------------|-----------------|-----------|--------|
| 1               | 0.01                     | 0.05                   | 0.08                   | 422.0           | 6331.1    | 78.16  |
| 2               | 0.11                     | 0.16                   | 0.19                   | 38.6            | 734.4     | 9.07   |
| 3 (Punicalagin) | 0.49                     | 0.55                   | 0.59                   | 14.9            | 431.2     | 5.32   |
| 4               | 0.69                     | 0.72                   | 0.78                   | 14.3            | 603.4     | 7.45   |

| Track 2 ID: Punica Granatum Extract |                |              |              |            |        |              |            |          |         |
|-------------------------------------|----------------|--------------|--------------|------------|--------|--------------|------------|----------|---------|
| Peak                                | Start Position | Start Height | Max Position | Max height | Max %  | End Position | End Height | Area     | Area %  |
| 1                                   | 0.01 Rf        | 23.2 AU      | 0.05 Rf      | 422.0 AU   | 86.16% | 0.08 Rf      | 1.0 AU     | 6331.1AU | 78.16 % |
| 2                                   | 0.11 Rf        | 0.8 AU       | 0.16 Rf      | 38.6 AU    | 7.88%  | 0.19 Rf      | 3.0 AU     | 734.4 AU | 9.07%   |
| 3                                   | 0.49 Rf        | 0.6 AU       | 0.55 Rf      | 14.9 AU    | 3.04%  | 0.59 Rf      | 0.2 AU     | 431.2 AU | 5.32%   |
| 4                                   | 0.69 Rf        | 10.6 AU      | 0.72 Rf      | 14.3 AU    | 2.92%  | 0.78 Rf      | 2.5 AU     | 603.4 AU | 7.45%   |

**Figure 4:** HPTLC densitometric peak table of the hydroalcoholic extract of *Punica granatum* fruit arils at 620 nm.

**Table 2:** Microbial Quality Analysis of *Punica granatum* Aril Juice Extract.

| Sl. No. | Test Parameter                       | Observed Value |
|---------|--------------------------------------|----------------|
| 1.      | Total Aerobic Count (CFU/g)          | <10            |
| 2.      | Yeast and Mould Count (CFU/g)        | <10            |
| 3.      | <i>Escherichia coli</i> (CFU/g)      | Negative       |
| 4.      | <i>Staphylococcus aureus</i> (CFU/g) | Negative       |
| 5.      | <i>Salmonella spp.</i> (CFU/g)       | Negative       |
| 6.      | <i>Candida albicans</i> (CFU/g)      | Negative       |
| 7.      | <i>Shigella spp.</i> (CFU/g)         | Negative       |

**Table 3:** Elemental Analysis of *Punica granatum* Aril Juice Powder.

| Sl. No. | Element      | Observed Value (ppm) |
|---------|--------------|----------------------|
| 1.      | Lead (Pb)    | 0.23                 |
| 2.      | Cadmium (Cd) | 0.004                |
| 3.      | Mercury (Hg) | 0.002                |
| 4.      | Copper (Cu)  | 9.077                |
| 5.      | Iron (Fe)    | 39.8                 |
| 6.      | Zinc (Zn)    | 12.34                |

further strengthen the applicability of this technique for routine quality assurance and support the development of standardized, evidence-based nutraceutical formulations.

## CONCLUSION

This investigation successfully developed an HPTLC-based fingerprinting method for the qualitative and quantitative analysis of punicalagin in *Punica granatum* aril juice, reporting an  $R_f$  value of  $0.58 \pm 0.03$  and confirming its significant presence through densitometric analysis. The combined use of microbial and elemental profiling further established the safety and nutritional relevance of the sample, providing a comprehensive quality assessment framework. These findings support the use of HPTLC as a robust, cost-effective tool for routine authentication, standardization, and quality control of pomegranate-derived nutraceuticals. Future work focusing on full analytical validation and comparative studies with HPLC/LC-MS will enhance the reliability and regulatory acceptance of this approach.

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## ABBREVIATIONS

**CFU:** Colony-Forming Unit; **ddH<sub>2</sub>O:** Double-distilled water; **ESBL:** Extended-Spectrum  $\beta$ -Lactamase; **HPLC:** High-Performance Liquid Chromatography; **HPTLC:** High-Performance Thin-Layer Chromatography; **LC-MS:** Liquid Chromatography-Mass Spectrometry; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **MAPK:** Mitogen-Activated Protein Kinase; **MDR:** Multidrug-Resistant; **MRSA:** Methicillin-Resistant *Staphylococcus aureus*; **NF- $\kappa$ B:** Nuclear Factor kappa B; **PI3K/Akt/mTOR:** Phosphoinositide 3-kinase/Protein Kinase B/Mammalian Target of Rapamycin; **R<sub>f</sub>:** Retention factor; **SD:** Standard Deviation; **TAMC:** Total Aerobic Microbial Count; **TYMC:** Total Yeast and Mold Count; **USP/BP:** United States Pharmacopeia/British Pharmacopoeia; **UV:** Ultraviolet.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS CONTRIBUTION

**RPC:** Conceptualization, study designing, data collection, manuscript drafting and resources. **BU:** Conceptualization and study design. **AKAK:** Manuscript drafting and data analysis. **TJ:** Supervision and approval of final draft of manuscript.

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