

Chromatographic Fingerprinting and Quantification of Luteolin in *Arachis hypogaea* L. Peanut Shell Extract Using HPTLC and HPLC

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Abstract



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Peanut shell (*Arachis hypogaea* L.), a byproduct of peanut processing, is a rich source of various bioactive compounds, including luteolin, a prominent flavonoid known for its therapeutic properties. This study investigates the detection of luteolin in *Arachis hypogaea* shell extract using High-Performance Thin-Layer Chromatography and High-Performance Liquid Chromatography techniques. The *Arachis hypogaea* shell ethanolic extract was analysed using a CAMAG HPTLC system with a Linomat 5 applicator and Vision CAT software. Chromatographic separation was performed with a mobile phase of Hexane: ethyl acetate: formic acid (5:5:0.3), where luteolin had a retention factor (R_f) of 0.4. Densitometric scanning at 254 nm revealed the presence of 1.282 µg of luteolin in 5 mg of *Arachis hypogaea* shell extract. Additionally, HPLC analysis confirmed luteolin's presence in the extract, with 92.91 µg of luteolin found in 5 mg of *Arachis hypogaea* shell extract. This research provides valuable insights into quantifying luteolin in *Arachis hypogaea* shells, supporting their potential as a sustainable source of bioactive flavonoids.

Keywords: Peanut shell, HPTLC, HPLC, Luteolin, Densitometry

1. Introduction:

Peanuts (*Arachis hypogaea*), with their nutritional benefits and possible health benefits, are a crop that fascinates researchers in nutrition and medicine. Peanuts are the fourth-largest oilseed crop and the thirteenth-largest food crop in the world. The skin and seeds are included in the diet as part of the outer shells¹. Peanut seeds, as indicated by Yaw et al. (2008), are high in nutrients and comprise 20% protein and 50% oil, and are rich in vitamins and minerals. Peanuts, traditionally, were perceived as unhealthy because they were considered too oily. Peanut skins, while often considered a waste byproduct from the food industry, are rich in natural antioxidants². *Arachis hypogaea* shells, as a byproduct of agricultural waste, have attracted much attention because they contain high lignocellulosic content and, therefore, have the potential as a source for isolating bioactive compounds with pharmacological values. As a waste product produced in large quantities during peanut processing, *Arachis*

hypogaea shells are often underutilized and wasted, despite possessing bioactive properties³. Recent research has shown that these shells contain various phytochemicals, including flavonoids, phenolic acids, and lignans, which exhibit antioxidant, anti-inflammatory, and antimicrobial properties. The presence of these bioactive compounds makes *Arachis hypogaea* shell a promising material for the isolation of bioactive substances, such as luteolin, which can be identified and quantified using High-Performance Thin-Layer Chromatography (HPTLC) methods, leading to the sustainable utilization of agricultural waste. The use of HPTLC in fingerprinting luteolin in the extract of *Arachis hypogaea* L. is a powerful analytical technique for quantifying and profiling this flavonoid compound in extracts of *Arachis hypogaea* shells⁴. Luteolin is a naturally occurring flavonoid with excellent antioxidant and anticancer activities, and, as such, extraction from *Arachis hypogaea* shells is an area of increasing interest. The application of HPTLC, a sensitive and accurate

technique, enables the efficient separation and identification of luteolin and other co-occurring phytochemicals in *Arachis hypogaea* shells. Using this method, the amount of luteolin can be precisely quantified, enabling further elucidation of its therapeutic value as well as the valorisation of *Arachis hypogaea* shell biomass as a high-value resource in both pharmaceutical and nutraceutical sectors⁵. The pathway for flavonoid biosynthesis is integral to the biosynthesis of luteolin. It has been the subject of intensive studies aimed at understanding key developmental and physiological processes in plants. Luteolin can be synthesized via two pathways involving flavonoid molecules, such as eriodictyol or apigenin, which are components of the path⁶.

Ethanol–water solvent reflux extraction, alkaline diluted alcohol extraction, supercritical fluid extraction, enzymatic extraction, and microporous resin adsorption are the principal methods currently employed to extract luteolin⁷. These traditional methods, however, are not without their limitations, such as high equipment costs, complex operating requirements, and the potential for active component degradation. Hence, developing an efficient and environmentally safe extraction method for luteolin remains a significant challenge⁸.

Over the past 20 years, increasing emphasis has been placed on utilizing metabolites derived from medicinal plants for the treatment and management of various diseases. Luteolin, a bioactive flavonoid metabolite derived from *Arachis hypogaea*, demonstrates a wide range of biological activities⁹. It is also recognized for its potent antioxidant and anti-inflammatory properties. In traditional Chinese medicine, plants rich in luteolin have been used to address a diverse range of ailments, including hypertension, various inflammatory disorders, and cancer¹⁰. *Arachis hypogaea* shells are known for having a high concentration of bioactive and functional compounds, primarily flavonoids and stilbenes, which are considered safe for human consumption¹¹. These substances are essential for defence systems against microbial contamination and physical harm, which makes peanuts a powerful immunostimulant with antioxidant, antiviral, and antibacterial qualities¹². *Arachis hypogaea* shells in conjunction with elevated amounts of neural differentiation marker-specific mRNA and proteins¹³. High-dose peanut tegument extract therapy decreased atherosclerotic plaques, and peanut tegument dietary intervention improved lipid metabolic disorders in ApoE-/- mice by raising HDL-C content and lowering blood TC and LDL-C levels. Interestingly, 16S rRNA gene sequence analysis showed that peanut tegument controlled the amounts of numerous metabolites in the liver, blood, and fecal samples and drastically changed the makeup of the gut microbial flora¹⁴.

2. Materials and Methods

2.1 Collection and authentication

Arachis hypogaea shells (*Arachis hypogaea*) were obtained as processed waste from a local cold-press oil processing unit in Nagpur, India, and authenticated by

Dr. Nitin Dongarwar, Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur.

2.2 Extraction

The *Arachis hypogaea* shells were collected and shade-dried for 2 days. The dried shells were powdered into a coarse powder using a mechanical grinder¹⁵. According to the literature, our study revealed that maceration is the most effective process for extracting phytochemicals. The 500 g of coarse powdered material was placed in a glass jar and macerated for 3-5 days using ethanol (3 volumes). The extract was filtered and concentrated using a rotary evaporator at a temperature of 50-60°C¹⁶.

2.3 Phytochemical Study

The Phytochemical screening was carried out on the ethanolic extract of *Arachis hypogaea* to identify the active phytoconstituents, including terpenoids, alkaloids, glycosides, flavonoids, steroids, and saponins, present in the extract, using the following standard phytochemical tests.

2.4 HPTLC Analysis

Instrumentation:

A CAMAG HPTLC system equipped with LINOMAT 5 applicator fitted with a 100 µl syringe with CAMAG TLC scanner and vision CAT software was used¹⁷.

Solvents and Chemicals

Standard luteolin was purchased from Sigma-Aldrich, St. Louis, MO, USA. All HPLC-grade solvents were used for HPTLC & HPLC analysis, and all chemicals of analytical grade were used in the above study.

Sample preparation

Dried *Arachis hypogaea* extract 5 mg was dissolved in 10 mL of methanol and then filtered.

Standard preparation

0.1 mg luteolin dissolved in 10 ml of methanol. The standard solution was sonicated using a sonicator and then filtered using filter paper.

Chromatographic Conditions:

A 10 x 20.0 cm pre-coated silica gel 254 HPTLC plate (E. Merck) was used for the HPTLC densitometric analysis, with no plate modification or pre-washing. A CAMAG Linomat applicator with a 100 µl syringe was used to apply the sample solution on the plate in the form of a band. Samples were applied to the TLC plates as 8 mm bands using a Camag Automatic TLC Sampler 4 (ATS4) sample applicator (Switzerland) fitted with a Camag microlitre syringe. A constant application rate of 150 nL/s was used. Linear ascending development of the plates to a distance of 70 mm was performed with Hexane: Ethyl acetate: Formic acid (5:5:0.3 v/v/v) previously saturated for 20 min and the luteolin standard and sample-loaded plates were developed in an automated development chamber.

2.5 HPLC Analysis

Preparation of the Mobile Phase

Acetonitrile, 0.1 % Ortho Phosphoric Acid in distilled water were mixed at a ratio of (1:1) respectively, then passed through a nylon membrane filter having a pore size of 0.45µm and sonicated to degas.

Preparation of Reference Standards

The stock solution was prepared by accurately weighing 5.0 mg of luteolin and transferring it to a 10 mL volumetric flask. Mobile phase was added to complete the volume to 5 mL (Final Concentration 0.60 mg/mL). From the stock solution, solutions with concentrations of 5, 10, 15, 20, and 25 µg/ml were prepared, and a standard calibration curve was plotted.

Sample Preparation

The dry extract was transferred to a 25 mL volumetric flask, and 15 mL of diluent (mobile phase) was added, followed by sonication for 20 minutes. The volume was completed with diluent (mobile phase) and filtered through a 0.45 µm Nylon filter.

Method Validation

The analytical method used for the quantification of Luteolin in the *Arachis hypogaea* extract was validated for LOD and LOQ.

LOD=3.3 (S.D/S)

LOQ=10 (S.D/S)

Chromatographic conditions

Chromatographic measurements were performed using an Agilent Tech. 1100 with an auto injector, which included a Quaternary Gradient (G130A) S.NO.DE9180834) and a UV G13148 S.NO. DE71365875, which was adjusted to 347 nm. Other HPLC equipment comprised a Knauer K-1500 solvent organizer and a Knauer K-500 degasser. The utilized column was a C18 (4.6 × 150 mm, 5 µm) from Waters. The mobile phase was composed of Acetonitrile:0.1% OPA water (50:50). In gradient mode, elution was carried out at a flow rate of 0.7 mL/min with an injection volume of 20 µL.

The mobile phase composition (Hexane: Ethyl acetate: Formic acid, 5:5:0.3 v/v/v) was selected after optimization trials to achieve adequate separation, peak resolution, and reproducible retention factors for luteolin. The detection wavelength of 254 nm for HPTLC was selected based on the UV absorption characteristics of luteolin, which exhibits significant absorbance in the UV region, enabling sensitive detection and quantification. Similarly, the HPLC detection wavelength of 347 nm was selected based on the absorption maxima of luteolin and provided optimal sensitivity and selectivity for analysis.

3.0 Results

The *Arachis hypogaea* peels were subjected to ethanolic extraction by using maceration. The extract was then evaporated to dryness using a rotary evaporator and concentrated for phytochemical analysis.

The polyphenolic compounds were found in the extract based on the above qualitative phytochemical study. The extract was assessed using HPTLC and fingerprinting with Luteolin as a standard. The outcomes are shown in Table 1. The chromatograms were obtained upon scanning at a UV wavelength of 254 nm. Figures 1, 2 & 3 depicted for Luteolin standard. The R_f values, area percentage, and peak height of luteolin were calculated.

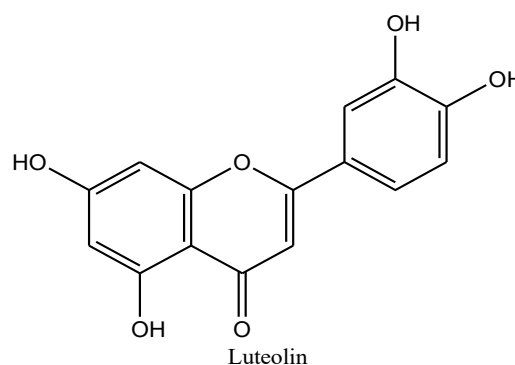


Fig 1: Structure of Luteolin

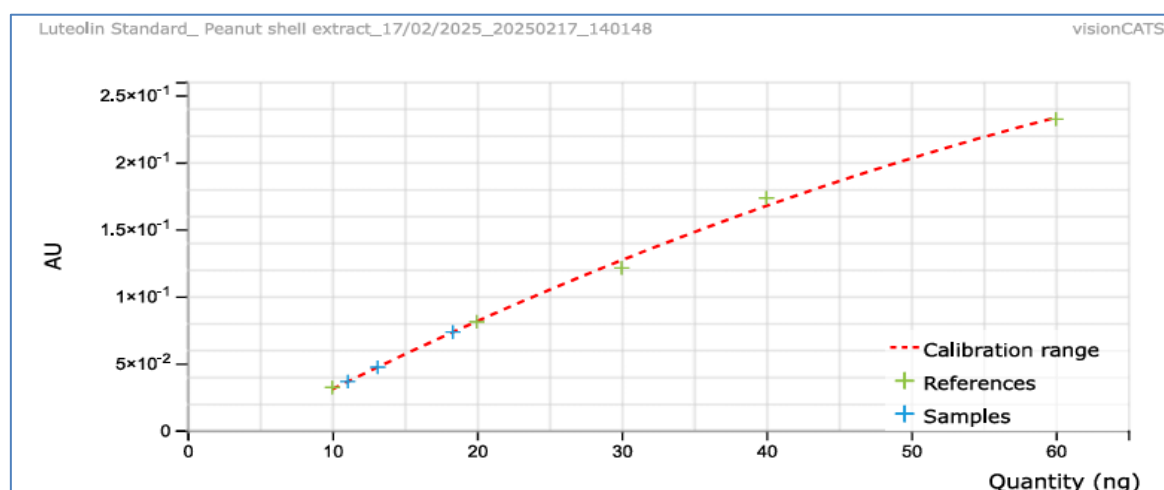


Fig. 2 Calibration curve of Luteolin standard

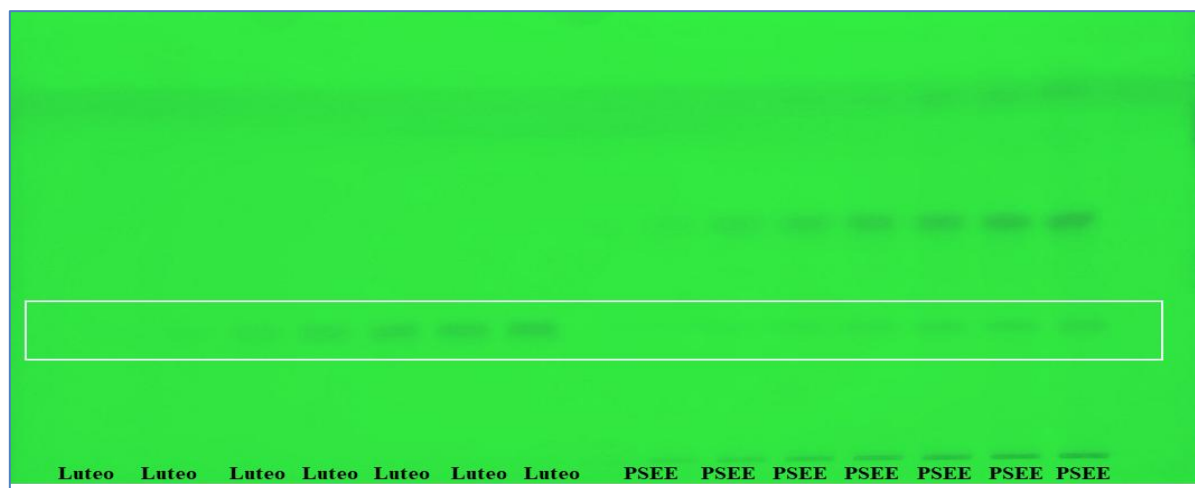


Fig. 3 Scanned image of Luteolin and PSEE at 254 nm

After performing the phytochemical study, it was revealed that the polyphenolic compounds are abundantly present. The same extract was then analyzed using High-performance thin-layer chromatography (HPTLC) and fingerprinted with luteolin as a standard. The results are displayed in

Tables 2 & 3, respectively. The chromatograms were obtained upon scanning at a UV wavelength of 254 nm. Figure 7 shows the UV chromatographic overlay of luteolin standard & PSEE, while Figure 8 shows the UV overlay spectra of PSEE extract at 254 nm.

Table 1: Tracks of Luteolin & PSEE ethanolic extract

Track no	Ref/Samp	ID X (mm)	Y (mm)	Appl. Volume	Rf	Conc
1	Luteo	20	36.6	1.0 μ l	0.461	1 mg/ml
2	Luteo	31.4	36.4	2.0 μ l	0.458	1 mg/ml
3	Luteo	42.8	36.1	3.0 μ l	0.453	1 mg/ml
4	Luteo	54.2	36.1	4.0 μ l	0.453	1 mg/ml
5	Luteo	77	36	6.0 μ l	0.452	1 mg/ml
6	Luteo	88.4	35.8	7.0 μ l	0.452	1 mg/ml
7	Luteo	99.8	34.5	2.0 μ l	0.448	1 mg/ml
8	AHSEE	111.2	34.7	4.0 μ l	0.427	5mg/ml
9	AHSEE	122.6	34.5	6.0 μ l	0.431	5mg/ml
10	AHSEE	134	34.3	8.0 μ l	0.431	5mg/ml
11	AHSEE	145.4	34.6	10.0 μ l	0.427	5mg/ml
12	AHSEE	156.8	34.3	12.0 μ l	0.424	5mg/ml
13	AHSEE	168.2	34.5	14.0 μ l	0.427	5mg/ml
14	AHSEE	179.6	34.7	16.0 μ l	0.431	5mg/ml

Luteo – Luteolin, PSEE – Peanut Shell ethanolic extract

Table 2: Calibration curve parameters of Luteolin Standard

Parameters	Values
Regression mode	Polynomial
Range deviation	5.00%
Related substances	None
Assignment mode	Single
Number of references	6
Calibration function	$y = -2.53 \times 10^{-11}x^2 + 5.8213 \times 10^{-6}x - 2.494 \times 10^{-2}$

Coefficient of variation	2.94%
Correlation coefficient	R=0.998544

The UV spectra overlay displays both the Luteolin standard and PSEE with coincident flavone bands: an intense $\pi \rightarrow \pi^*$ band around 255–270 nm and an $n \rightarrow \pi^*$ band near 345–360 nm. The similar λ_{max} and spectral shape suggest the presence of Luteolin in PSEE, with a slight bathochromic shift and decreased intensity, indicating matrix effects and a lower concentration compared to the standard reference solution.

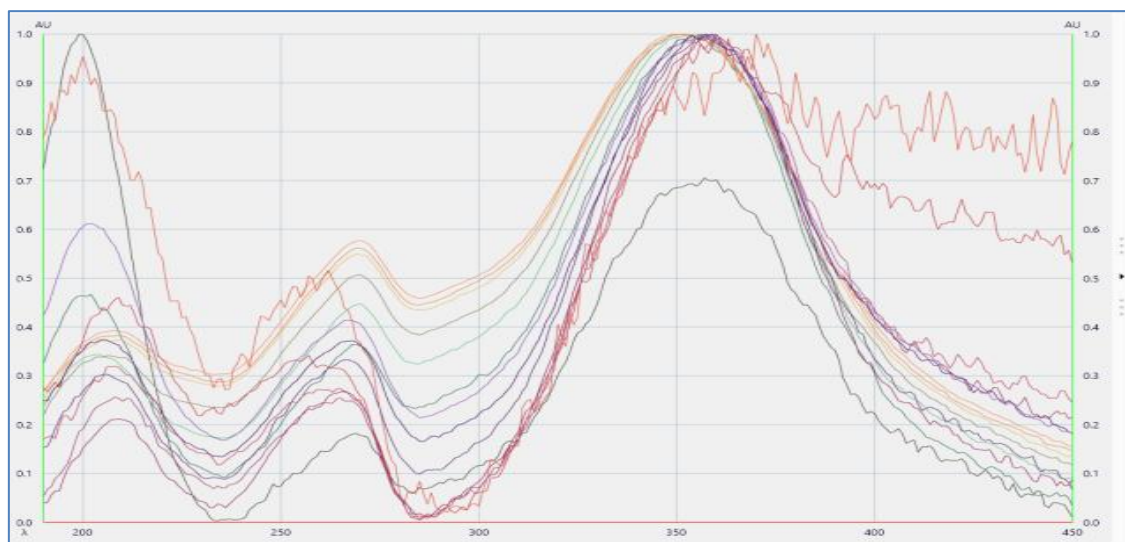


Figure 4: UV overlay of Luteolin standard & PSEE

The polyphenolic compounds were found in the extract based on the above qualitative phytochemical study. The extract was assessed using HPLC and fingerprinting with Luteolin as a standard. The outcomes are shown in

Fig 5 and 6. The calibration curve for luteolin and HPLC Fingerprinting was established. The R_f values, area percentage, and peak height of luteolin were calculated.

Standard Calibration Curve of Luteolin

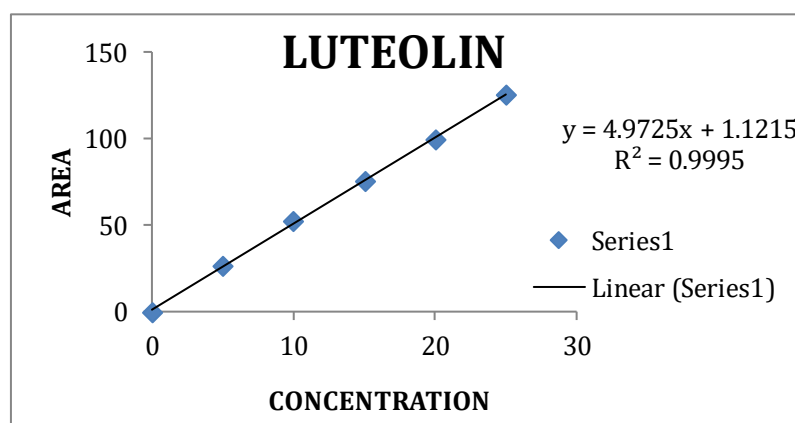


Figure 5: Standard Calibration curve of Luteolin

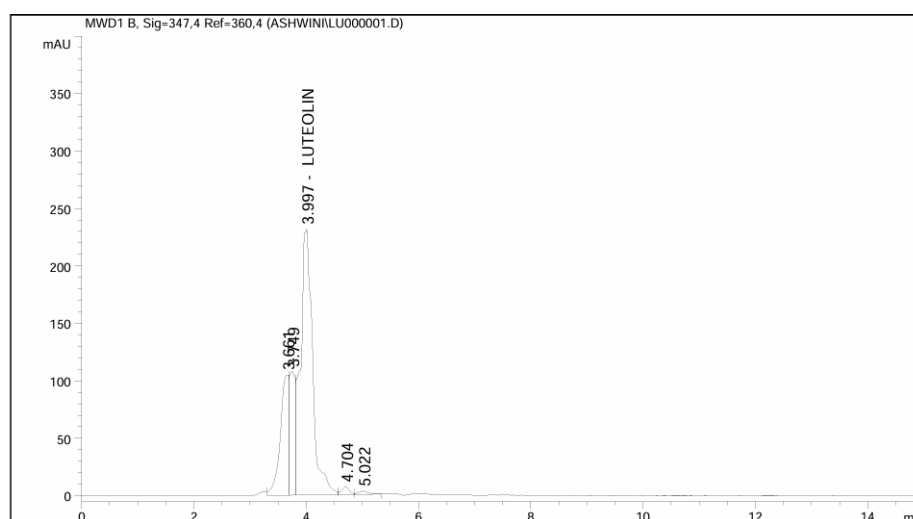


Figure 6: HPLC chromatogram of Luteolin in *Arachis hypogea* shell extract

4. Discussion:

Arachis hypogaea shells are a waste product that contains different chemical constituents, which are responsible for various therapeutic potential. Luteolin is a significant flavonoid present in *Arachis hypogaea* shell peel. In the present study, the HPTLC analysis of *Arachis hypogaea* shell extract was performed, and the densitometric analysis of the ethanolic extract of *Arachis hypogaea* shell was carried out using a CAMAG HPTLC system and Vision CAT software. The results were observed in the form of chromatograms scanned at 254 nm. The R_f values of Luteolin were found to be 0.4 with a mobile phase of Hexane: ethyl acetate: formic acid (5:5:0.3). 1.282 micrograms of luteolin were found in 1 mg of *Arachis hypogaea* shell extract. The HPLC analysis of luteolin in *Arachis hypogaea* shell extract. 5 mg of *Arachis hypogaea* shell extract contains 92.9068705 mcg of Luteolin.

5. Conclusion:

Validated HPTLC and HPLC methods were developed and applied for the quantification of luteolin in *Arachis hypogaea* shells (*Arachis hypogaea* L.). HPTLC densitometric analysis (CAMAG system; detection at 254 nm) produced a luteolin R_f of 0.40 using hexane: ethyl acetate: formic acid (5:5:0.3) as the mobile phase, and indicated a luteolin content of 1.282 $\mu\text{g}\cdot\text{mg}^{-1}$ of *Arachis hypogaea* shell extract ($\approx 0.128\%$ w/w). HPLC analysis of the same extract measured 92.9069 μg luteolin in 5 mg of extract (equivalent to 18.581 $\mu\text{g}\cdot\text{mg}^{-1}$, $\approx 1.858\%$ w/w).

Although both chromatographic approaches confirmed the presence of luteolin in *Arachis hypogaea* shell waste, the absolute amounts obtained by HPTLC and HPLC differ substantially. This discrepancy may reflect method-dependent factors, such as differences in sensitivity and specificity, sample preparation and dilution, calibration procedures, detector response, or potential co-eluting matrix components that affect peak integration. These results, therefore, underscore the importance of cross-validation and orthogonal confirmation (for example, spike-recovery experiments or the use of an internal standard) when reporting absolute concentrations.

Overall, the study demonstrates that *Arachis hypogaea* shells, an abundant agricultural waste product, contain measurable luteolin and thus represent a promising, low-cost source for the recovery of this bioactive flavonoid. Future work should focus on (i) harmonizing and further validating the quantification procedures across platforms, (ii) expanding sampling to multiple batches and geographic sources to assess variability, (iii) optimizing extraction/isolation for preparative recovery, and (iv) evaluating the biological activity of the recovered luteolin to support value-added utilisation of *Arachis hypogaea* shell biomass.

Author's contribution

AG: Conceptualization, methodology, investigation, data collection, formal analysis, interpretation of results, and writing-original draft preparation.

SM: Methodology, experimental investigation, data analysis, and interpretation of results.

AB: Investigation, data collection, analytical work, and validation.

MM: Investigation, data analysis, literature review, and validation.

PI: Conceptualization, supervision, project administration, scientific guidance, critical revision of the manuscript, and final approval of the version to be published.

All authors contributed to the review and editing of the manuscript and approved the final version for publication.

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