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Extraction And Purification Of Pithecellobium Dulce Bioactive Compounds By HPLC And Flash Chromatography

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Abstract

Phytochemicals, being secondary metabolites made by plants, are well-known for their many uses in medicines and help a lot in finding and producing new drugs. Being widely known as jungle jalebi, Pithecellobium dulce (Pd) is a tropical fruit plant and is valued for its many medicinal properties. This work was focused on analyzing the phytochemical content of P. dulce fruit extract and improving the methods used to obtain its main bioactive components. Ethanol was used as a solvent in the Soxhlet extraction to get the crude plant extract. A preliminary screening of phytochemicals found that the plant contains alkaloids, flavonoids, phenols, tannins, terpenoids and saponins. HPLC confirmed the presence of gallic acid, known for its antioxidant and anti-inflammatory properties, after 2.8 minutes of analysis and showed a high degree of linearity when compared to the standard ($R^2 = 0.993278$, $p < 0.05$). The solution was then purified using flash chromatography to isolate gallic acid which appeared as well-separated peaks and was readily isolated according to its polarity and absorption profile at 280 nm. The results suggest that the purified gallic acid meets the required standards for pharmacological studies. The study suggests that P. dulce contains promising therapeutic compounds and using optimized methods like Soxhlet extraction, HPLC and flash chromatography plays an important role in separating and identifying them. The results point to the potential of P. dulce fruit in creating plant-based drugs and natural remedies, encouraging further study in laboratories as well as animal and human experiments.

Key words: *Phytochemicals; Pithecellobium dulce; Soxhlet; HPLC; flash chromatography, gallic acid.*

1. INTRODUCTION

Phytochemical analysis in chemical science is gaining popularity, with researchers focusing on the structure, synthesis and impact of plant compounds on the body [1,2]. Many industries such as pharmaceutical, medical, food and cosmetic make use of naturally occurring compounds due to their medical and health benefits [3]. Over the years, bioactive compounds have been valued in many traditional medical practices. Due to their phytochemicals and secondary metabolites such as alkaloids, flavonoids, phenolic acids, tannins, saponins, steroids and terpenoids, these plants are recognized for their therapeutic and nutraceutical potential. These bioactive compounds are found in edible and non-edible parts of the plants and trees including fruits, leaves, bark, stem, and root [5]. Their metabolites have several applications as antioxidants, anti-inflammatory, anti-cancer and anti-bacterial properties [6]. The increasing demand for natural remedies with health supplements and alternatives have acerated interest in less explored bioactive compounds.

One of the promising and alternative, Pithecellobium dulce (p. dulce), commonly known as jungle jalebi, manila tamarind, and vijayati Babula, with many other names in different regions, the tree belongs to the Fabaceae family. Originally native to tropical regions of the Americas, it was found in the plain of the India. The fruits of P. dulce contain wide variety of bioactive compounds with significant therapeutic properties [7,8]. The utilization of bioactive compounds from P. dulce is challenging, requiring the adoption of advanced techniques for the efficiently extracting, isolating, and purifying its phytochemicals. The process is inherently challenging and sensitive due to very minimal concentration of phytochemicals and bioactive compounds generally small amount and it's essential to extract without damaging critical and minor components in the raw material [9]. Furthermore, reasonable extraction procedures should ensure that molecules of interest are quickly separated

from the appropriate solvents [10]. Many conventional methods have been adopted for the extraction and purification of compounds from medicinal plants. Notably Soxhlet extraction, which remains a highly used and efficient technique for phytochemical analysis and its ability to extract broad range of constituent by continuous recycling [11,12]. The isolation and quantification of bioactive compounds is a demanding and increasingly popular area in analytical science, with High-Performance Liquid Chromatography (HPLC) and Flash Chromatography being among the most widely used techniques for quantification and purification. isolation and quantification of bioactive compounds is another one demanding and advanced gaining popularity in the field of analytical techniques among them high-performance liquid chromatography (HPLC) and Flash chromatography, which are consideration the most popular techniques for quantifications and purification [13]. HPLC provides high sensitivity and precision in identifying and quantifying phytochemicals, and flash chromatography is particularly advantageous forth rapid purification of targeted fractions [14]. The selection of appropriate extraction protocol focuses on many more factors, like nature of plant material, solvent purity, pH, temperature, and the solvent to sample ratio, all which influence the efficiency and quality of the extracted compounds [15].

This study uses appropriate solvents to evaluate standard and rapid, time-efficient extraction methods, such as Soxhlet extraction. Additionally, it involves phytochemical screening, quantifying targeted bioactive compounds using standard HPLC analytical techniques, purification via flash chromatography, and identifying bioactive compounds in Pithecellobium fruits.

2. MATERIAL AND METHODS

2.1 Collection of Pithecellobium dulce fruits

Fresh white aril fruits of Pithecellobium dulce were procured from the local market in Vijayapura, Karnataka, India, during the peak harvest season between March and April 2023. And authenticated by the Department of Dravya Guna, BLDE Association's AVS Ayurveda Mahavidyalaya, Vijayapura, Karnataka, India. The fruits were initially inspected to remove any spoiled or damaged ones to ensure sample integrity. To eliminate surface contaminants such as dust, microbes, or pesticide residues, the fruits were thoroughly washed under running tap water, followed by rinsing with distilled water. After cleaning, the fruits were spread out on clean trays and air-dried under shade for 3–5 days to retain maximum phytochemical content by avoiding direct sunlight exposure, which may degrade heat- and light-sensitive compounds.

Once adequately dried, the arils were separated, and the fruit pulp was subjected to coarse grinding using a mechanical grinder. The resulting material was then further pulverized using a laboratory-grade grinder to obtain a fine, homogenous powder. Approximately 100 grams of this powdered fruit material were stored in airtight, amber-colored containers at room temperature (25 ± 2°C) to prevent oxidation and moisture absorption until further use. Ethanol (500 mL) was selected as the extraction solvent due to its efficiency in extracting a broad range of polar phytochemicals, ensuring maximum recovery during Soxhlet extraction.

Fig. 1. Collection, Cleaning, Drying, and Powdering Process of Pithecellobium dulce Fruits.

Figure 1 illustrates the sequential steps involved in the preparation of Pithecellobium dulce fruit samples for phytochemical analysis. The process includes fruit collection, washing with tap and distilled water to remove impurities, shade drying to preserve bioactive compounds, and mechanical grinding to obtain a fine powder. This processed material served as the basis for solvent extraction in the subsequent experimental procedures.

2.2. Soxhlet Extraction Method

The extraction was conducted using a Soxhlet extractor, which is a standard and simple handling method for processing plant materials. The apparatus made up of a glass material with a spherical bottom flask, an extraction chamber connected by a siphon tube, and a condenser connected on top of the extractor. We have taken around 80grams of plant material and crushed by using a mortar and pestle, packed in filter paper, placed in a thimble within the extraction chamber. About 500ml of ethanol was poured into 500ml round bottom flask [16]. The apparatus was seated properly solvent was heated by providing 70-80°C temperature the ethanol was evaporated and pass through the condenser [17]. The condensed solvent dropped into the extraction chamber, where it come and contact with the plant material reached the siphon tube, the solvent along with extracted compounds flow back into the round bottom flask. This cycle was repeated continuously at the same temperature. The process was conducted over 48 hrs, approximately around 20 cycles have processed until the extraction was complete and all soluble compounds were extracted from the plant material [18].

2.3. Rotary Evaporator

Plant extract was processed using a rotary evaporator under vacuum conditions to separate ethanol from the extract. The vacuum system allowed ethanol to evaporate at a lower boiling point, which was then collected as the vapour condensed back into liquid form [19]. This sensitive method focused the concentration of the plant's importance without applying high temperatures [20]. The rotary evaporator was set to operate at 50°C and 80 revolutions per minute (rpm) to separate the ethanol from the plant sample.

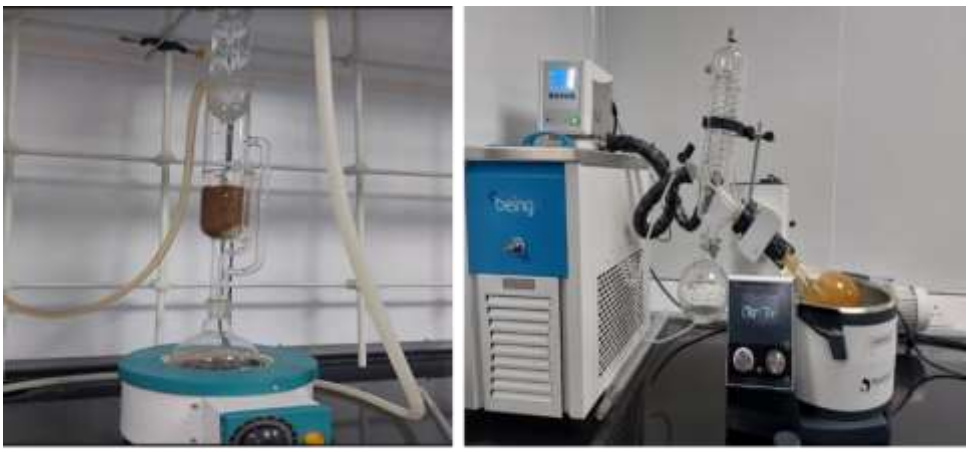

Fig. 2. Rotary Evaporation Setup for Concentration of Pithecellobium dulce Ethanolic Extract.

Figure 2 presents the equipment used for reducing the concentration of the ethanolic Pithecellobium dulce extract. The setup uses a flask that turns inside a vacuum, a water bath held at 50°C and a condenser for capturing the ethanol as it evaporates. With this technique, solvents can be removed under mild conditions, sparing sensitive active components from heat damage. Rotary evaporation helps maintain bioactive compounds and allows for a more concentrated extract to support phytochemical analysis and purification.

2.4. Phytochemical Screening

The preliminary phytochemical analysis of the extracts was performed using aqueous and ethanolic extracts utilising established techniques to identify the primary components [21].

Table.1. Photochemical qualitative analysis.

SI NO	Phytochemical	Test	Reagents	Positive indication
1	Tannins	Dissolve extract in warm water and filter. Add 5% ferric chloride drops in 90% alcohol to the filtrate	5% ferric chloride in 90% alcohol	Bluish-black or greenish-black precipitate
2	Alkaloids	Stir 0.4 g of extract with 8ml of 1% HCL, warm and filter. To 2 ml of filter, add (a) Mayers reagent or (b) Dragendroffs reagent.	1% HCL Mayers reagent or (b) Dragendroffs reagent.	Yellow PPT
3	Flavonoids (Ferric Chloride Test)	Boil 0.5g of extract with ml of water then filter. Add 10% ferric Chloride solution to 2ml of filtrate	10% ferric Chloride solution	Yellow, orange, or red colour
4	Saponins	Boil 1g of extract with 5ml of water and filter. Add 3ml of water to the filtrate and shake vigorously for 5 minutes	water	Frothing (foam formation)
5	Terpenoids	Dissolve the extract in chloroform and add a few drops of Conc. Sulfuric acid	Chloroform, Conc. Sulfuric acid.	Red, pink, or purple colour
6	Phenols	Dissolve 500mg of extract in 5 ml of water. Add a few drops of neutral 5% ferric chloride solution	5% Ferric chloride (neutral ferric Chloride solution)	Dark green colour

Table 1 displays the preliminary qualitative testing results for the Pithecellobium dulce fruit extract. Several groups of phytochemicals were examined, including tannins, alkaloids, flavonoids, saponins, terpenoids and phenols. It describes the specific tests conducted which reagents are applied and how these can indicate the presence of each chemical group. The appearance of bluish black for tannins or yellow for alkaloids was used to determine if a reaction was positive. According to the table, P. dulce contains a range of phytochemicals, especially phenols, flavonoids and terpenoids, suggesting it may be helpful in treating health conditions.

2.5. Quantification of Pithecellobium dulce by High performance of liquid chromatography.

(HPLC)

Quantification of bioactive compounds using High-Performance Liquid Chromatography (HPLC) is increasingly recognized as one of the most important analytical techniques for herb quality control and fingerprinting research [22]. Plants are primarily analysed for non-volatile chemicals such as higher terpenoids, various phenolics, alkaloids, lipids, and sugars [23]. HPLC works best for substances detectable in the ultraviolet or visible ranges of the spectrum. Natural products are typically separated after evaluating a relatively crude extract in biological experiments to accurately characterize the active component [24,25]. HPLC results are interpreted by examining chromatograms that display the chemical separation in a sample. This approach involves comparing the chromatogram of a standard to that of a plant extract to quantify gallic acid. In our study, we have used the "JASCO AUTOSAMPLER" instrument with a reversed-phase C18 column (silica powder as the

stationary phase). Analytical procedures were performed at a wavelength of 280 nm with an injection volume of 20 µL. The mobile phase solvents consisted of HPLC-grade methanol and water. This setup allowed for precise measurement of flow rate, retention time, and wavelength. The retention time (RT) for both the standard and the extract was 2.8 minutes. This indicates that the chemicals were detected based on their travel time across the chromatographic column, which facilitated their identification by comparison with the standard.

2.6 Purification of Gallic acid by Flash chromatography

Flash chromatography combines medium and short-column chromatography with air pressure to achieve quick separation [26]. It is commonly used to separate molecular mixtures into distinct components, making it valuable for drug discovery and plant metabolite purification [27,28]. Flash chromatography differs from conventional methods in two significant ways: it uses slightly smaller silica gel particles (250–400 mesh), and to compensate for the reduced solvent flow caused by these smaller particles, pressurized gas (around 10–15 psi) is applied to push the solvent through the stationary phase column. This process results in fast and high-resolution chromatography, commonly referred to as "flash" chromatography [29,30,31].

Our study used the Combi Flash RF^{+} Lumen instrument to purify gallic acid. The ethanol extract was mixed with 4 grams of solid silica powder and loaded into the flash chromatography column. A solvent mixture of methanol and water was used, and the wavelength was set to 280 nm to detect gallic acid based on standard references. The sample was processed over 31.5 minutes.

Elution occurred when the mobile phase was pushed through the column under pressure, causing the compounds to move at different speeds through the stationary phase, depending on their interaction with both phases. Less polar compounds moved faster, while more polar compounds moved slower. Fractions were collected at predetermined intervals as the compounds eluted from the column.

Fig.3. Instrumentation used for phytochemical analysis: (A) JASCO HPLC Autosampler for quantification of gallic acid; (B) Combi Flash RF+ Lumen system for purification of bioactive compounds.

Figure 3 illustrates the advanced analytical instruments used in this study for the quantification and purification of bioactive compounds from Pithecellobium dulce fruit extract. Image (A) shows the JASCO HPLC Autosampler system equipped with a reversed-phase C18 column, which was employed for the precise detection and quantification of gallic acid based on retention time and peak area. Image (B) displays the Combi Flash RF+ Lumen chromatography system used for rapid purification of gallic acid from the crude extract. The use of these high-resolution instruments ensured accurate profiling and separation of targeted phytochemicals, enhancing the reliability and efficiency of the analytical process.

3. RESULTS AND DISCUSSION:

3.1. Soxhlet Extraction and Rotary Evaporator Yield

The Soxhlet extraction method successfully separated plant material using ethanol as the solvent. This approach efficiently extracted compounds from plant materials partially soluble in ethanol and those containing insoluble contaminants. Around 31% of the sample was extracted from the Soxhlet setup using 100 grams of Pithecellobium dulce fruits. However, the method was noted to be unsuitable for thermolabile plant materials due to the heating involved in the process.

Rotary evaporator approximately 31% of a brown-coloured liquid extract was obtained from the plant material after processing. The collected sample was stored at 4°C for further use.

3.2 Phytochemical screening

Primary phytochemical screening analysis was performed by using various qualitative tests included tannins, alkaloids, Flavonoids, saponins, terpenoids and phenols in compare with the aqueous extract the ethanol extract sample has shown good presence phenols, flavonoids and terpenoids.

Table 2: Phytochemical qualitative analysis

SI No.	Test	Aqueous	Ethanol
1	Tannins	+	+
2	Alkaloids	++	+
3	Flavonoids	++	++
4	Saponins	-	+
5	Terpenoids	++	++
6	Phenols	+++	+++

Note: High +, Highest +++, Note present -

Table 2 summarizes the results of the qualitative phytochemical screening of Pithecellobium dulce fruit extracts prepared using both aqueous and ethanolic solvents. The presence of major phytochemical groups—such as tannins, alkaloids, flavonoids, saponins, terpenoids, and phenols—was evaluated based on colorimetric or precipitate-forming tests. The table compares the intensity of these compounds in both extracts using a relative scale, where "+" denotes low presence, "++" moderate, and "+++" high. The results reveal that the ethanolic extract showed a stronger presence of phenols, flavonoids, and terpenoids compared to the aqueous extract, indicating ethanol as a more effective solvent for extracting these bioactive compounds. This supports the selection of ethanol for further extraction and analytical procedures.

3.3 Quantification of Pithecellobium dulce by High performance of liquid chromatography.

(HPLC)

The HPLC method effectively separated and quantified the bioactive compound gallic acid from the plant extract. The standard peak height for 200 µg/ml was 114171 µV, with an area of 1714707 µV-sec. The retention time for both the standard and the extract was 2.8 minutes, indicating proper identification. A strong linear correlation was observed with an R-squared value of 0.993278, and the P-value was < 0.05, confirming statistical significance. The regression curve for absorbance showed a direct proportionality between concentration and peak response, ensuring accurate quantification

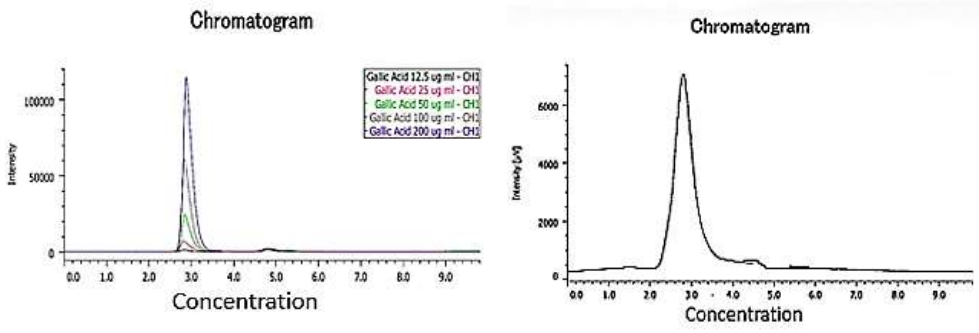

Fig. 4. HPLC Chromatograms of Standard Gallic Acid (Left) and Extracted Pithecellobium dulce Sample (Right).

Figure 4 displays the chromatographic profiles obtained through High-Performance Liquid Chromatography (HPLC) for the quantification of gallic acid. The left panel shows overlay chromatograms of standard gallic acid at varying concentrations (12.5–200 µg/mL), all exhibiting a sharp peak around the 2.8-minute retention time, indicating consistency and linearity of detection. The right panel presents the chromatogram of the ethanolic extract of Pithecellobium dulce, which also shows a prominent peak at approximately 2.8 minutes, corresponding to gallic acid. The similarity in retention times confirms the presence of gallic acid in the plant extract, validating the analytical method. The peak intensity further suggests a measurable concentration suitable for quantitative analysis, supporting the effectiveness of the extraction and detection protocol.

Figure 5 illustrates the standard calibration curve used for quantifying gallic acid concentration in Pithecellobium dulce fruit extract using High-Performance Liquid Chromatography (HPLC). The graph plots intensity (µV·sec) against known concentrations of standard gallic acid (µg/mL), showing a strong linear relationship. The linear regression equation ($y = 1084.7x - 13734$) and high coefficient of determination ($R^2 = 0.9817$) confirm the accuracy and reliability of the method. This standard curve was used to interpolate the concentration of gallic acid present in the plant extract based on its chromatographic peak area, supporting precise quantification of this bioactive compound.

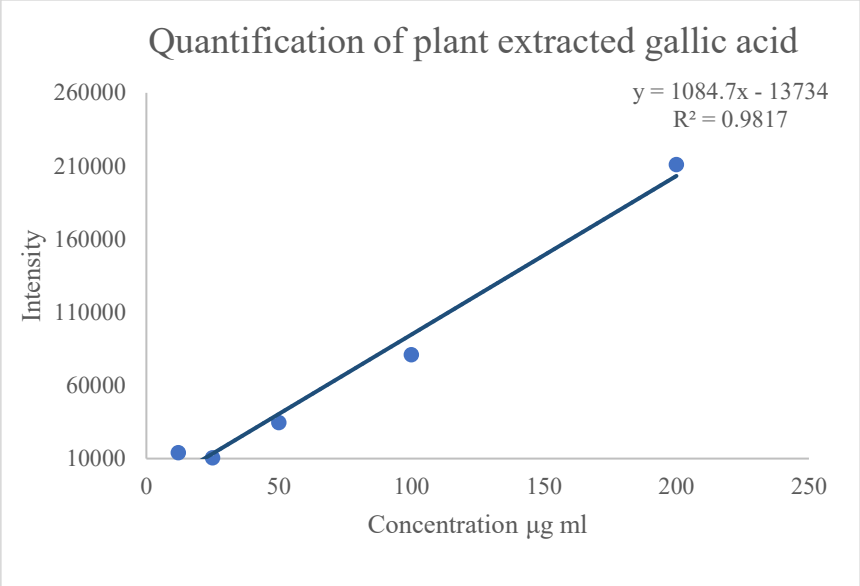

Fig. 5. Calibration Curve for Quantification of Gallic Acid by HPLC.

Table 3. A. Standard gallic acid concentration and intensity. B. Plant extracted gallic acid concentration.

Sl. No.	Sample Type	Concentration (µg/mL)	Retention Time (min)	Intensity (Area in µV·sec)
1	Standard Gallic Acid	12.5	2.8	13,997
2	Standard Gallic Acid	25.0	2.8	105,209
3	Standard Gallic Acid	50.0	2.8	346,113
4	Standard Gallic Acid	100.0	2.8	859,005

5	Standard Gallic Acid	200.0	2.8	1,714,707
6	P. dulce Extract	34.28 (Interpolated)	2.7	211,061

Table 3 consolidates both standard and plant extract HPLC data for gallic acid quantification. Rows 1–5 represent standard gallic acid solutions at known concentrations (12.5–200 µg/mL), all showing consistent retention times (2.8 min) and increasing peak intensities with concentration, confirming linearity. Row 6 presents the data for gallic acid isolated from the Pithecellobium dulce extract, which showed a similar retention time (2.7 min) and a peak area of 211,061 µV·sec. By comparing this intensity to the standard curve, the gallic acid content in the extract was interpolated to be approximately 34.28 µg/mL. This validates the successful quantification and confirms the presence of gallic acid in the plant extract.

3.4. Purification of Gallic acid by Flash chromatography

The process yielded three distinct peaks in the chromatogram, indicating different concentrations of the separated compounds. The fractions were collected into three tubes (labelled 1, 2, and 3), based on the peaks. The second tube contained the highest concentration of gallic acid, with 10 mL of the sample collected. The sample was then analysed using high-performance liquid chromatography (HPLC). A chromatogram showed the separation of components over time with a flow rate of 18 mL/min, a peak width of 30 seconds, and the absorbance of gallic acid at 280 nm measured at 0.20 AU. Flash chromatography proved effective in isolating the desired compound.

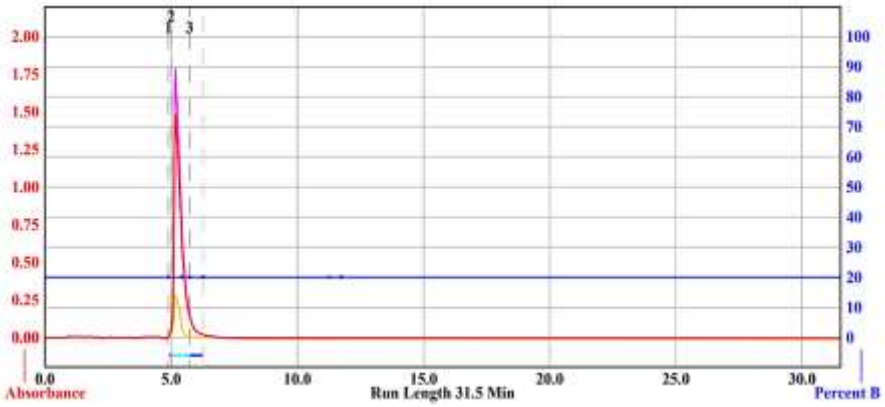

Figure 6. Flash chromatography gallic acid peaks.

Figure 6 illustrates the elution profile obtained during flash chromatography used for the purification of gallic acid from Pithecellobium dulce ethanolic extract. The x-axis represents the total run time (31.5 minutes), while the y-axes display absorbance (left, red line) and solvent composition (% Solvent B, right, blue line). Three distinct peaks were observed between approximately 4.5 and 6.0 minutes, corresponding to different compounds separated during the process. Among these, Peak 2 showed the highest absorbance, indicating the fraction with the highest concentration of gallic acid, as confirmed by further HPLC analysis. The gradient elution employed a mixture of methanol and water, with the solvent B composition gradually increasing over time (blue curve), aiding in the efficient separation of polar and semi-polar compounds. The narrow peak widths and clear resolution indicate successful and high-efficiency separation. This chromatographic profile validates the use of flash chromatography as a rapid and effective method for isolating bioactive phytochemicals.

Table 4 gathers the main points from the research done on Pithecellobium dulce. This process involved analyzing what was obtained from extraction, performing a qualitative screening, evaluating gallic acid levels quantitatively with HPLC and purifying it via flash chromatography. These methods were tested using specific observations

that proved their usefulness for isolating and analyzing gallic acid, a well-known therapeutic substance. Using this structure makes the main findings of the study more straightforward to understand.

Table 4. Summary of Results and Key Observations from Extraction, Phytochemical Analysis, and Compound Quantification of Pithecellobium dulce.

Sl. No.	Parameter	Method	Key Findings / Observations
1	Extraction Yield	Soxhlet Extraction (Ethanol)	Yielded approximately 31% extract from 100 g fruit powder; ethanol proved efficient solvent.
2	Crude Extract Concentration	Rotary Evaporator	Brown viscous extract concentrated at 50°C under vacuum; stored at 4°C for further analysis.
3	Preliminary Phytochemical Screening	Reagent-based qualitative tests	Positive for phenols (+++), flavonoids (++), terpenoids (++); ethanol extract showed higher yield.
4	Gallic Acid Quantification	HPLC	Retention time: 2.8 min; concentration in extract: 34.28 µg/mL; high correlation (R^2 = 0.9817).
5	Standard Calibration Curve	HPLC (Standard Series)	Linearity observed from 12.5 to 200 µg/mL with regression: y = 1084.7x – 13734.
6	Gallic Acid Purification	Flash Chromatography	Three peaks observed; Peak 2 showed highest gallic acid purity at 280 nm with sharp elution.

CONCLUSION

This research points out that the use of various analytical methods helps efficiently pull out, measure and clean bioactive substances from the fruits of Pithecellobium dulce. When used together, Soxhlet extraction, HPLC and Flash Chromatography resulted in successful and efficient separation of our target phytochemicals, mainly gallic acid. Ethanol in the Soxhlet extraction recovered numerous polar compounds and HPLC was used to accurately measure the amount of gallic acid with consistent results. The gallic acid product was purified further through flash chromatography and the purity was confirmed by reviewing the chromatography results.

The tests revealed that P. dulce fruit has high levels of gallic acid, a phenolic substance known to protect against disease and diabetes. This research highlights the potential of using P. dulce as a source of nutraceuticals and plant-based bioactives in therapy. The approach used in this research could guide future studies on medicinal plants and their active chemicals.

However, while the in vitro quantification and purification results are promising, further research involving in vitro biological assays and in vivo preclinical studies is essential to validate the pharmacological efficacy, safety profile, and therapeutic relevance of gallic acid derived from P. dulce. Such comprehensive evaluations could pave the way for its application in drug development and functional food formulations.

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