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,"ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FORDETERMINATION OF FLAVONOIDS BY RP-HPLC"

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Abstract : This study focused on the analytical method development and validation for the determination of flavonoids in Artocarpus heterophyllus using reverse-phase high-performance liquid chromatography (RP-HPLC). The organoleptic evaluation confirmed the plant's dark green color and foliaceous texture. Aqueous extraction yielded 10.9% of the extract, which was subjected to phytochemical screening, revealing the presence of alkaloids, flavonoids, phenols, proteins, carbohydrates, and saponins. TLC analysis indicated the presence of multiple flavonoid compounds, with Rf values suggesting the presence of quercetin. Quantitative analysis demonstrated total phenolic and flavonoid contents of 0.221 mg/100 mg and 0.763 mg/100 mg, respectively. Method validation showed excellent linearity (r2 = 0.999), recovery rates near 100%, and high precision and robustness. The mean quercetin content in the extract was found to be 0.810%. These findings highlight the potential of Artocarpus heterophyllus as a source of bioactive flavonoids, supporting its application in nutraceuticals.

***IndexTerms* -. Artocarpus heterophyllus, flavonoids, RP-HPLC, Phytochemical screening, TLC analysis, method validation, bioactive compounds, quercetin, aqueous extract.**

Introduction

Flavonoids are a diverse group of polyphenolic compounds widely distributed in the plant kingdom, known for their significant health benefits, including antioxidant, anti-inflammatory, and anticancer properties. Their presence in various dietary sources, such as fruits, vegetables, and beverages, has garnered increasing interest in both nutritional and pharmaceutical research.
to quantify flavonoid content accurately, reliable analytical methods are essential.Reverse-phase high-performance liquid chromatography (RP-HPLC) has emerged as one of the most effective techniques for the separation and analysis of flavonoids. This method offers several advantages, including high resolution, sensitivity, and the ability to analyze complex mixtures without extensive sample preparation. The development and validation of analytical methods for flavonoid determination are crucial for ensuring the accuracy and reproducibility of results.

The importance of method validation is emphasized by regulatory bodies such as the International Conference on Harmonisation (ICH), which outlines guidelines for analytical method validation, covering parameters like specificity, linearity, accuracy, precision, and robustness (ICH, 2005). These parameters ensure that the developed method can be reliably used for the quantification of flavonoids in various matrices, contributing to a better understanding of their biological effects and potential health benefits.

Recent studies have focused on optimizing RP-HPLC conditions to enhance the separation of flavonoids, employing various stationary phases, mobile phase compositions, and detection techniques. For example, the use of C18 columns and gradient elution has proven effective in separating flavonoids in herbal extracts (Mojiiö et al., 2019). Furthermore, the integration of mass spectrometry with HPLC (LC-MS) has facilitated the identification of flavonoid compounds with greater accuracy and specificity (Gao et al., 2020).
In summary, the development and validation of RP-HPLC methods for the determination of flavonoids is essential for advancing research in nutrition and pharmacology. This paper aims to outline the procedures for method development and validation, with a focus on optimizing separation conditions and ensuring compliance with regulatory standards.

Material and Methods

The study utilized a range of chemicals sourced from reputable suppliers to facilitate the analytical processes involved in flavonoid determination. Key reagents included potassium mercuric iodide, iodine, potassium iodide, and potassium bismuth

iodide from Loba Chemie Pvt. Ltd. and Thomas Baker, essential for various chemical reactions. Additionally, picric acid, sodium nitroprusside, sodium hydroxide, and pyridine, sourced from S. D. Fine Chem. Ltd., played essential roles in the analytical procedures. Ferric chloride and gelatin, also from S. D. Fine Chem. Ltd. and Thomas Baker, respectively, were used for complexation studies. Solvents such as methanol, ethanol, and chloroform from Qualigens Fine Chemicals were employed for sample preparation and extraction. The study also included reagents like the Folin-Ciocalteu reagent and Fehling's solution, sourced from Loba Chemie Pvt. Ltd. and Central Drug HouseLtd., respectively, for specific analytical techniques.

Methods

Collection of plant material

Leaves of Artocarpus heterophyllus were collected from the local area of Bhopal (M.P), in the months of February 2024.

Organoleptic characters

A small amount of powdered drug was spread on a white tile and physically examined for general appearance i.e. color, taste, texture, etc.

Extraction procedure by maceration process

50-gram shade dried plant materials were subjected to extraction with petroleum ether (60-80 ^{0}C) using maceration. The extraction was continued till the defatting of the material had taken place (Mukherjee•, 2007).

Defatted dried plant material of Artocarpus heterophyllus were extracted with aqueous. The extract was evaporated above their boiling points. The dried crude concentrated extract was weighed to calculate the extractive yield then transferred to glass vials (6 cm) and stored in a refrigerator (4^{0}C), till used for analysis.

Determination of Percentage yield

The extraction yield is evaluated of the solvent's efficiency to extracts bioactive components from the selected natural plant samples and it was defined as quantity of plant extracts recovered in mass after solvent extraction compared with the initial quantity of plant samples. After extraction, yield of the plant extracts obtained was calculated in grams and then converted it into percentage. Following formula was adopted for determination of percentage yield of selected plant materials:

$$\text{Percentage yield} = \frac{\text{Weight of extract}}{\text{Weight of powdered drug taken}} X100$$

Qualitative phytochemical tests

Phytochemical screening is a process in which the extraction, identification and screening of phytochemicals can be done easily for variety of medicinal plants. As these phytochemicals are precursors for the preparation of various new drugs. Hence, the plant extracts obtained by aqueous and ethanol extraction of all selected plant samples were subjected to various qualitative screening to detect the presence of plant bioactive constituents present in them. Preliminary phytochemical screening is primarily an important aspect for establishing profile of given extract for its chemical compounds produced by plant. Phytochemical examinations were carried out extracts as per the following standard methods (Kokate; 1994).

Qualitative chromatographic analysis by thin-layer chromatography

Thin layer chromatography is based on the adsorption phenomenon. In this type of chromatography mobile stage containing the dissolved solutes passes over the exterior of the stationary phase:

Detection and Calculation of Rf Value

Once the chromatogram was developed the Rf Value of the spot was calculated using the formula:

$$\mathbf{Rf} = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

Quantitative studies of bioactive constituents

Estimation of total phenolic content

The total phenolic content of the extract was determined by the modified Folin-CiocaIteu method. 10 mg Gallic acid was dissolved in 10 ml methanol, various aliquots of 10- 50pg/ml was prepared in methanol. lgm of dried powder of drug was extracted with 100 ml methanol, filter, and make up the volume up to 100 ml. One ml (Img/ml) of this extract was for the estimation of Phenol. 1 ml of extract or standard was mixed with 5 ml of Folin-Ciocalteu reagent (previously diluted with distilled

water 1:10 v/v) and 4 ml (7.5g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 30min for colour development. The absorbance was measured at 765 nm using a spectrophotometer (Parkhe and Bharti; 2019).

Estimation of total flavonoid content

Determination of total flavonoid content was based on the aluminum chloride method. 10 mg quercetin was dissolved in 10 ml methanol, and various aliquots of 5- 25gg/ml were prepared in methanol. 10 mg of dried extract was dissolved in 10 ml methanol and filter. Three ml (Img/ml) of this extract was for the estimation of flavonoids.l ml of 2% AIC13 solution was added to 3 ml of extract or each standard and allowed to stand for 15min at room temperature; absorbance was measured at 420 nm (Mishra et al., 2017).

Identification of marker compound (Quercetin) by HPLC

Instrumentation

The HPLC system (Waters) consisted of a pump, a U.V. Visible detector, a Thermo CIB (250 X

4.6 mm, -Sym) column, a Data Ace software.

Chromatographic conditions

The chromatographic analysis was performed at ambient temperature on a RP-C18 analytical column with a mobile phase composed of Acetonitrile: Methanol (50:50 v/v) and was isocratically eluted at a flow rate of 1 mL min-I. A small sample volume of 20 YL was used for each sample run, being injected into the HPLC system. The chromatogram was monitored with
UV detection at a wavelength of 256 nm (Acharya et al., 2019).

Preparation of standard solution

10mg of Quercetin was weighed accurately and transferred to a 10ml volumetric flask, and the volume was adjusted to the mark with the methanol to give a stock solution of 1000ppm. From stock solutions of Quercetin 1 ml was taken and diluted up to 10 ml. from this solution 0.5, 1.0,
1.5, 2.0, 2.5 ml solutions were transferred to 10ml volumetric flasks and make up the volume up to 10 ml with mobile phase, gives standard drug solution of 5, 10, 15, 20, 25gg/ ml concentration.

Analysis of extract

10 mg extract was taken in 10 ml volumetric flask and dilute upto the mark with Methanol; resultant solution was filtered through Whatmann filter paper and finally volume made up to mark with same solvent to obtain concentration of 1000 pg/ml. The resulting solution was again filtered using 0.45» membrane filter and then sonicated for 10 min.

Validation of developed method

Linearity

Linearity of analytical procedure is its ability (within a given range) to obtain test, which are directly proportional to area of analyte in the sample. The calibration plot was contracted after analysis of five different (from 1 to 5pg/ml) concentrations and areas for each concentration were recorded three times, and mean area was calculated. The regression equation and correlation coefficient of curve are given and the standard calibration curve of the drug is shown in table. From the mean of AUC observed and respective concentration value, the response ratio (response factor) was found by dividing the AUC with respective concentration (Teasdale et al.,
2018).

Specificity

Specificity of the method was carried out to assess unequivocally the analyte presence of the components that might be expected to be present, such as impurities, degradation products and matrix components.

Accuracy

Recovery studies were performed to validate the accuracy of developed method. To preanalysed sample solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed.

Precision

The precision are established in three differences:

Repeatability

The repeatability was performed for five replicate at five concentrations in linearity range 1, 2, 3, 4 and 5 gg/ml for Quercetin indicates the precision under the same operating condition over a short interval time.

Intermediate Precision Day To Day Precision

Intermediate precision was also performed within laboratory variation on different days in five replicas at five concentrations.

Robustness

As per ICH norms, small, but deliberate variations in concentration of the mobile phase were made to check the method's capacity to remain unaffected. The ratio of the mobile phase was changed from, methanol: acetonitrile (50:50 % v/v), to (45:55 % v/v).

Detection Limit and Quantitation Limit

The LOD and LOQ of the developed method were calculated based on the standard deviation of response and slope of the linearity curve.

Analysis of Quercetin in herbal extract

10mg of extract was taken in a 10mI volumetric flask. The volume is made up to the mark by mobile phase and filtered by Whatman filter paper (no. 41) and the filtrate was used to prepare samples of 10pg/ml. This solution is injection 20111 records the peak area and calculates the percentage using the calibration curve method.

Results and Discussion

The organoleptic characterization of Artocarpus heterophyllus revealed that the plant exhibited a dark green color with a green and foliaceous texture (Table 1). This morphological assessment is essential as it provides a preliminary identification that can aid in the standardization of herbal materials.

The percentage yield of the aqueous extract was found to be 10.9% (Table 2), indicating a moderate extraction efficiency. This yield is significant for subsequent phytochemical analyses, as it suggests the potential presence of various bioactive compounds.

Phytochemical screening (Table 3) showed that the aqueous extract contained several important constituents. The presence of alkaloids and flavonoids, indicated by positive results in Wagner's and alkaline reagent tests, respectively, suggests potential therapeutic properties. However, glycosides and tannins were absent, which may indicate a selective profile of bioactive compounds in the extract. The positive results for phenols, proteins, carbohydrates, and saponins further highlight the extract's nutritional and pharmacological potential. Notably, the presence of saponins is particularly important as these compounds are known for their various health benefits, including cholesterol-lowering effects.

Thin-layer chromatography (TLC) analysis (Table 4) of the extract showed multiple spots under UV light, indicating the complexity of the extract. The Rf values for the aqueous extract, particularly at 0.45, 0.53, 0.62, and 0.80, suggest the presence of various flavonoids, including quercetin, which was used as a standard. The separation of compounds is essential for further quantitative analyses.

Quantitative analysis revealed the total phenolic content to be 0.221 mg/100 mg and total flavonoid content to be 0.763 mg/100 mg in the aqueous extract (Table 5). These values suggest that Artocarpus heterophyllus is a valuable source of bioactive compounds, which may contribute to its therapeutic efficacy.

The linearity of the quercetin standard (Table 6) was demonstrated with a correlation coefficient of 0.999, confirming the reliability of the method for quantifying flavonoids. The high slope and consistent intercept further support the method's robustness.

Recovery studies (Table 7) indicated excellent accuracy across different concentration levels, with mean recoveries close to 100%. This high level of recovery underscores the reliability of the analytical method employed.

Precision results (Table 8) demonstrated good repeatability and intermediate precision, confirming that the method is consistent and reproducible over time and across different conditions. The robustness of the method was also affirmed, with values indicating that slight variations in analytical conditions do not significantly affect results (Table 9).

Finally, the drug content analysis (Table 10) showed a mean quercetin content of 0.810%, with low standard deviation and relative standard deviation values, suggesting that the extraction process was efficient and that the quantification method is reliable.

Table 1: Organoleptic characters of plant

S. No.	Plant	Color	Texture
1.	Artocarpus heterophyllus	Dark green	Green and foliaceous

Table 2: Result of percentage yield of extract

S. No.	Solvent	Extract weight	Percentage Yield (%)
1.	Aqueous	5.45	10.9%

Table 3: Result of phytochemical screening of Artocarpus heterophyllus extract

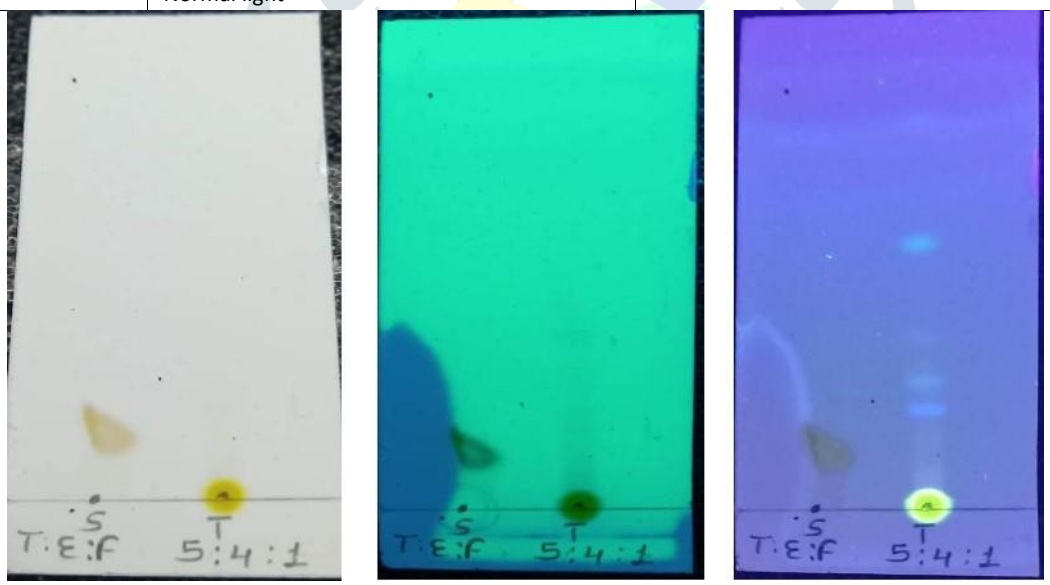
S. No.	Constituents	Aqueous extract
1.	Alkaloids(Wagner's test Hager's test)	Positive
2.	Glycosides (Legal's test)	Nagative
3.	Flavonoids Lead acetate	Positive

4.	Phenol Ferric chloride tes	Positive
5.	Proteins Xanthoproteic test	Positive
6.	Carbohydrates Fehling's test Benedict's test	Positive
7.	Saponins Foam test	Positive
8.	Diterpenes Copper acetate test	Positive
9.	Tannins Gelatin Test	-ve
10.	Sterols Salkowski Test	-ve

[+ve— positive; -ve= negative]

Table 4: TLC of Artocarpus heterophyllus extract

S. No.	Extract	Rf Value
	Mobile phase (Toluene: Ethyl acetate: Formic acid; 5:4:1)	
1	Quercetin Artocarpus heterophyllus extract No. of Spot Long UV Short UV Normal light	0.43 4 1 0
2	Rf Value Long UV Short UV Normal light	 0.45, 0.53, 0.62, 0.80 0.54



Light Short UV Long UV Normal

1 st spot Quercetin standard

2nd spot Aqueous extract of Artocarpus heterophyllus

Figure 1: Thin layer chromatography (Quercetin)

Table 5: Total bioactive constituents' content of Artocarpus heterophyllus

S.No	Extract	Total phenol	Total Flavonoid
		mg/100mg	
1	Aqueous extract	0.221	0.763

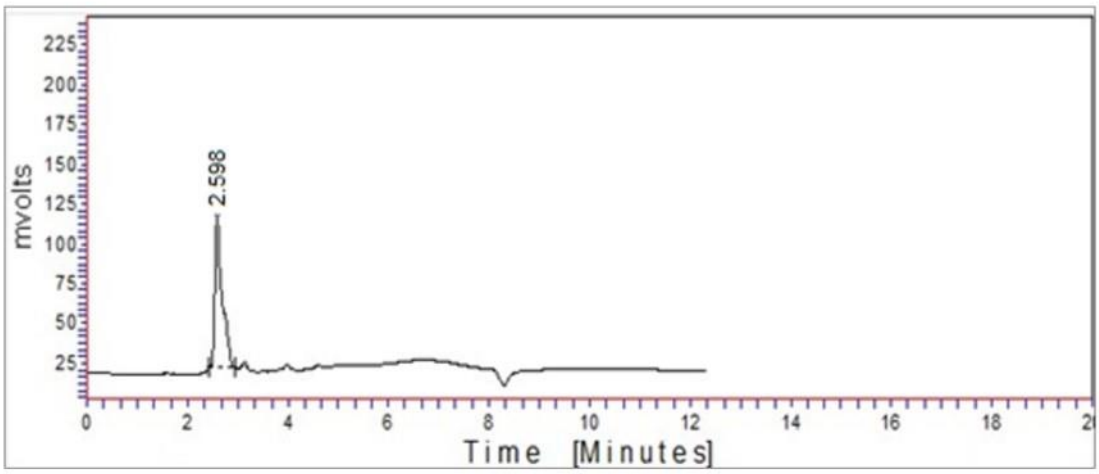


Figure 2: Chromatogram of standard Quercetin

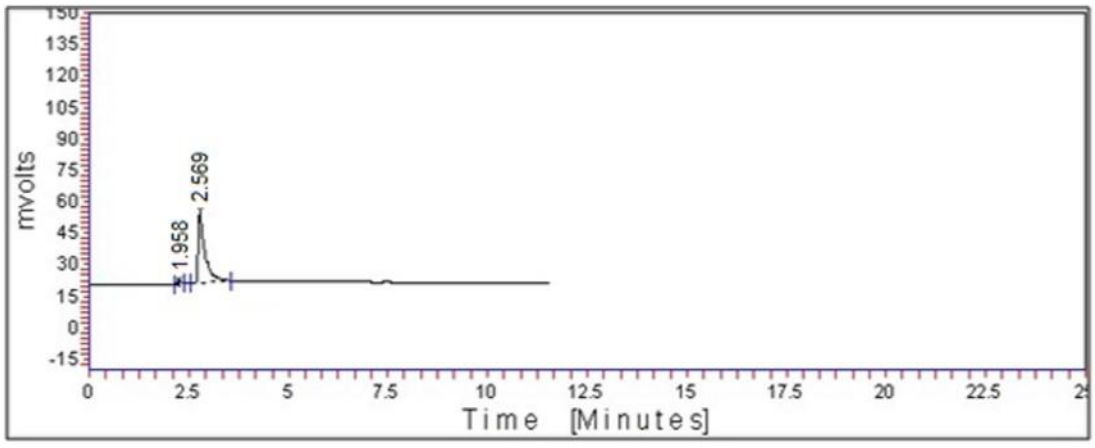


Figure 3: Chromatogram of aqueous extract of Artocarpus heterophyllus

Table 6: Results of linearity of Quercetin

Parameter	Quercetin
Concentration(gg/ml)	5-25
Correlation Coefficient	0.999
Slope (m)*	106.43
Intercept (c)*	26.032

Table 7: Results of recovery study

% Level	% Mean±SD*
80%	99.44±0.112
100%	99.12±0.125
120%	98.85±0.132

*Value of three replicate and three concentrations

Table 8: Results of Precision

Parameter	% Mean±SD*
Repeatability	98.32±0.120
Intermediate precision	
Day-to-day precision	99.65±0.115

*Value of five replicate and five concentrations

Table 9: Results of robustness

Parameter	% Mean±SD*
Robustness	98.11±0.085

*Value of five replicate and five concentrations

Table 10: Drug content of extract

S. No.	Parameter	Quercetin (%)
1.	Mean	0.810
2.	S.D	0.015
3.	% RSD	1.851

Conclusion

In conclusion, the results indicate that Artocarpus heterophyllus possesses significant bioactive constituents, particularly flavonoids, which can be effectively quantified using validated RPHPLC methods. This study contributes valuable insights into the phytochemical profile of the plant, supporting its potential use in nutraceuticals and pharmaceuticals. Further research could explore the bioactivity of these compounds and their therapeutic applications.

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