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DEVELOPMENT AND VALIDATION OF NOVEL METHOD FOR ESTIMATION OF HERBAL DRUG- CAJANIN

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ABSTRACT

Many Natural drugs have been extensively used recently for the treatment of different diseases and health problems including cancers. The use of natural drug has been well written in the history of Indian medicine however, extraction of the medicinal drug from the crude plant parts are difficult. Therefore, there is a requirement for the development of a method for extraction and evaluation of plant-based drugs. Our research is based on the development of a method for extraction of anticancer drug from *Cajanus cajan* and validation of the developed method. The plant was obtained from the local market and dried under shade for 15 days. After proper drying extraction was performed by the process of maceration with methanol as a solvent for 72 hours. Different physicochemical and phytochemical parameters were evaluated spectrometrically using HPLC. The developed method of extraction and evaluation was validated for linearity, precision, accuracy, specificity, robustness, ruggedness, and a few others. Results drawn from the study indicated the amount of the sample extracted to be 24.69mg and the percentage purity was found to be 98.76%. Linearity was determined in the range of 5 - 25 µg/ml. The correlation coefficient value was >0.99. The developed method was found to be accurate and precise from the % RSD values determined. The LOD and LOQ value for cajanin was found to be 0.044 µg/ml, and 0.134 µg/ml, respectively. This resembles that the proposed method was sensitive.

Keywords: Anticancer, Apoptosis, Validation, LOD, Cajanin, Extraction

INTRODUCTION

Cancer is one of the leading non-communicable disease causing the highest deaths and illnesses globally. World's one-third population is affected by one or the other type of cancer. According to the figures from the survey of WHO, cancer is responsible for one in every eight death occurring globally, which is even bigger than the deaths occurring due to AIDS, malaria, and tuberculosis combined[1]. Survey reports total cancer cases in the year 2012 were approximately 14.1 million causing 8.2 million deaths globally,

and these numbers are estimated to rise upto 11.2 million upto 2030. Despite of advance diagnostic methods and therapies developed across the years, cancer still terrifies individuals more than any other disease reason lying the diagnosis of most of the cancer is possible in later stages when chemotherapy is the most relied treatment [2]. Tobacco, viral infection, chemicals, radiation, environmental factors, and nutritional variables are among the causes of cancer. The majority of cancer patients are typically diagnosed after the disease has progressed to the point where treatment is no longer effective [3].

The effectiveness of chemotherapy in the treatment of different tumor growth can be a slow and temporary solution due to the development of drug resistance by the cancerous cells. In addition to developing drug resistance, these treatment remedies have huge side effects [4]. Due to such problems associated with the remedies, there is a critical requirement to develop a novel, effective, and much safer therapy with reduced side effects for the treatment of cancerous cells. The use of medicinal plants has a long history in the traditional method of treatment of cancer, the use of such medicinal plants provides a much safer way of treatment. Recent studies conducted on the use of the medicinal plant for cancerous treatment give a supporting conclusion for the presence of antioxidants in the medicinal plant that contributes toward their anticancer activity [5].

Owing to the need for the development of novel therapy for cancer treatment with minimal side effects, plant-based therapy can act as one of the based alternative treatments. For decades plants have been used for the treatment of various ailments, including cancer with over 60% of modern-day medication for cancer being plant-based [6]. Plants, animals, marine creatures, and microbes all contribute to medications that might be used as anti-cancer agents, making nature an appealing source of novel therapeutic entities. Various researches are conducted on the efficacy of treatment with naturally occurring minerals such as Vinca alkaloids, taxanes, podophyllotoxin, camptothecin, and many other plant-based secondary metabolites [7]. These therapies also provide an advantage by re-establishing the physiological homeostasis and conditioning the body tissues. Several studies were conducted on the presence of antioxidants in natural drugs which can be a chief product for the treatment of different types of cancer. Even compared to conventional therapy, plant-based drugs are readily available, less expensive, and have the least toxicity [8]. Thus this research is based on developing a novel method for extraction and evaluation of anticancer properties from *Cajanus cajan*.

Cajanin is a novel chemical extracted from Cajanus cajan (Pigeon Pea) that possesses anticancer activities, though the bioactivity of cajanin from this plant has not been extensively studied due to the low quantity of extract and difficulty in the extraction process [9]. *Cajanus cajan* is a legume plant extensively grown in tropical and subtropical areas. Its therapeutic properties have long been

documented. Sickle cell anemia, plasmolysis, and diabetes are all treated using extracts from *C. cajan* leaves. The predominant active components in *C. cajan* leaves were phenolics (flavonoids and stilbenes), according to chemical and pharmacological studies [10]. The antioxidant activity of *C. cajan* leaves and their components have been thoroughly reported in earlier studies. Compound's cytotoxic property makes it a potent drug in the treatment of cancer. Many studies are conducted on the cytotoxic activity of cajanin however the extracting mechanism behind the cytotoxic activity is still not understood completely [11].

Materials and method

Collection and authentication of plant:

The plant material used for method development and validation is dried fruits of *Cajanus cajan* which were collected from the local market and authenticated. They were dried under shade for 15days, powdered and passed through sieve no.40 and stored in well closed airtight containers for further use. Powder prepared from plant part purchased from local market of New Delhi was labelled as Powder. Powder of the plant purchased from supplier was labeled as powder.

Process of Extraction:

The shade dried coarsely powdered plant part was extracted with 80% aqueous methanol as solvent by maceration process at room temperature for 72 hrs. Extract obtained for maceration was filtered, concentrated to dried in a rotavapor under reduced pressure and controlled temperature (40-50°C). The residue was then stored in a dessicator. Hydroalcoholic extract prepared from plant parts was marked as extract I and standard extract purchased from supplier was marked as extract II.

Physicochemical Parameters:

In physiochemical evaluations, different extract-related parameters are evaluated such as total ash value, acid-soluble value, water-soluble ash value, and alcohol soluble extractive value. The ash value represents the inorganic salt present in the drug.

Ash value:

Ash values are most helpful in determining the quality and purity of the respective sample. Hence this test has remarkable importance in the evaluation of crude drugs. There are two types of ash values namely total ash value and acid-insoluble ash. The total ash mostly consists of inorganic contents like carbonates, sulfates, and silicates of potassium, sodium, and magnesium. Sulfated ash is nothing but the treatment of plant samples with concentrated sulphuric acid and filtering of the solution. But most of the variables are soluble in conc. Sulphuric acid. For this reason, acid insoluble ash is determined to overcome the

problem of soluble ash. For the determination of various ash values viz. total ash and acid insoluble ash all the powdered plant materials were passed through sieve no 40 and used.

Total ash:

1 gram of powdered extract was taken in the previously ignited tarred silica crucible and weighed. The powdered drug was scattered into a fine layer at the bottom of the crucible and incinerated gradually by increasing the heat not exceeding dull red heat, cooled, and weighed. The obtained charred mass was exhausted with hot water and the residue was collected on an ashless filter paper. The process was continued until a weight difference of more than 0.5mg was found. The percentage of ash concerning air-dried drugs was calculated and tabulated [12].

5.3.2 Acid insoluble ash:

The total ash obtained was washed from the crucible and boiled for 5 minutes with 25ml of dilute HCl and filtered through a Whatman filter paper No 42. The insoluble ash obtained was washed with hot water and cooled in a dessicator. The remaining residue was weighed and the acid insoluble value was calculated [13].

A) Loss on drying:

The weight loss in percentage w/w due to water and volatile materials of any sort that can be driven off under specific circumstances is known as a loss on drying. A shallow glass stoppered bottle was weighed that had been dried in the same conditions to be employed in the determination. Nearly 1 gm of the sample was transferred to the bottle, closed, and accurately weighed. The samples were distributed as evenly as practicably gently sidewise shaking to a depth not exceeding 10 mm. The sample-loaded bottle was placed in a drying chamber by removing the stopper. The sample was dried to a constant weight, the drying chamber was opened and the bottle was closed and allowed to cool. The bottle and the contents were weighed. The process was repeated until the successive weights differed not more than 0.5 mg (Drying to constant weight). The percentage loss of weight was calculated and tabulated [13].

Extractive value:

Alcohol soluble extractive

About 5 gms of dried coarse powder with 100 ml of 90 % methanol was kept in a closed flask for 24 hrs, flask was shaken frequently for 6 hours and allowed to stand for 18 hours. Then it was filtered immediately to prevent loss of methanol. 25 ml of the filtrate was evaporated to dryness in a tarred shallow dish. The residue was dried at 105 ˚C and weighed. The percentage of alcohol soluble extractive was calculated with reference to air dried drug and tabulated [13].

Water soluble extractive

6gm of coarse powder dissolved in 100 ml of water in a stoppered flask, heated at 80 ˚C, shaked well and allow to stand for 10 minutes. After cooling down extract 2 gm of kieselghur was added, filtered and transferred to 5 ml of the filtrate to a tarred evaporating dish, and solvent was evaporated on a water bath and the residue was weighed. The percentage of water soluble extractive was calculated with reference to air dried drug and tabulated [12].

pH

A 10% w/v solution is prepared using water as a solvent. The values for both the sample extract and standard powder were determined and tabulated.

Qualitative analysis of phytochemical parameters:

The plant extract was subjected to following tests for qualitative determination of various phytochemical constituents.

Test for carbohydrates:

A) Molisch's test

The extract was dissolved in 4 ml of distilled water and filtered. The filtrate was treated with 2-3 drops of 1 % alcoholic β-naphthol and 2ml of concentrated sulphuric acid was added along the sides of the test tube. Appearance of brown ring at the junction of two liquids shows the presence of carbohydrates [14].

B) Test for glycosides:

Presence of glycoside was evaluated using Legal's Test. A small portion of extract was treated with dilute hydrochloric acid for few hours on a water bath and to the hydrolysate 1ml of pyridine and few drops of sodium nitroprusside were added and sodium hydroxide solution to make it alkaline. Presence of pink to red colour would shows the presence of glycosides.

C) Detection of fixed oils and fats:

Few drops of 0.5 N alcoholic potassium hydroxide was added to small quantity of extract along with a drop of phenolphthalein. The mixture was heated on a water bath for 1-2 hours. Formation of soap will indicates the presence of fixed oils and fats.

D) Test for proteins and aminoacids:

Protein and amino acid presence was evaluated using Ninhydrine test. The extract was treated with Ninhydrine reagent. Purple colour produced shows the presence of proteins and free amino acids.

E) Test for phenolic compounds and tannins:

Small quantity of the extract was taken in water and test for the presence of phenolic compounds and tannins was carried out with 5% Ferric chloride solution. Formation of violet colour shows the presence of tannins and phenolic compounds.

F) Test for alkaloids:

Small quantity of extract was separately treated with few drops of dilute hydrochloric acid and filtered. The filtrate was treated with Wagner's reagent. Formation of reddish brown precipitate shows the presence of alkaloids.

G) Test for flavanoids:

Small quantity of extract was dissolved in aqueous sodium hydroxide. Appearance of yellow colour indicates the presence of flavonoids.

METHOD DEVELOPMENT

A) Apparatus and chromatographic conditions:

Spectroscopic analysis was performed using UV on a Perkin-Elmer UV-VIS Double Beam Spectrophotometer. HPLC analysis was performed on a chromatographic system of Waters 2695 equipped with an auto injector with UV/Visible detector (UV-2489). A chromatographic separation was achieved on merck C18 analytical column (5 µm, 250 mm x 4.6 mm, i.d). Data acquisition was made with Empower 3 software. Analytical Balance (BSA224S-CW, Sartorius), Ultra Sonicator (Fast Clean) from Shimadzu were used for the study.

B) Preparation of standard and sample solutions:

As the compound is freely soluble in ethanol standard stock solutions of standard were prepared in ethanol at a concentration of 100µg/ml. The aliquots of standard were prepared by using mobile phase. 100 mg of extract was dissolved with 10 ml ethanol in a 100 ml volumetric flask. The solution was sonicated for about 10mins and then made up to volume with mobile phase. 10ml was pipette out from stock solution into separate 100ml volumetric flask and made the volume up to the mark with mobile phase.

C) Selection of mobile phase:

The method development and validation of Piperine extract (PE) requires greater resolution. Hence different solvent systems were tried with varying flow rate between 0.8 ml to 1ml/min

Two different composition of solvents were taken for analysis, solvent containing methanol: water in ratio 7:3 and another solvent with ratio 19:1. Both the solvents were used to prepare different standard

solutions containing 25 mg of standard extract dissolved in 25 ml of solvent. Standard solution was further diluted by taking 0.5ml of solution and diluting it upto 10ml with solvents.

Method Validation:

The chromatographic conditions were validated by evaluating linearity, recovery, method and system precision, limit of detection (LOD), limit of quantification (LOQ) and robustness in accordance with ICH guidelines Q2 (R1).

Linearity:

Linearity of the method was analysed using HPLC chromatogram. Stock solution of concentration 0.01gm/ml or 100 ppm was prepared, which was used to prepare 6 different dilutions of 2 ppm, 4 ppm, 6 ppm, 8 ppm, 10 ppm, and 12 ppm. 20µl of each dilution were analysed in HPLC and chromatogram was obtained using methanol: water (19:1) as mobile phase. The flow rate was maintained at 0.8ml/min. Total run time was fixed as 20 minutes. Detector wavelength was fixed at 343nm. A calibration curve was plotted for concentration v/s peak area and the correlation coefficient was calculated.

Accuracy:

Accuracy of developed method was evaluated by calculating the percentage recovery of the extract from three different solution of 50%, 100%, and 150% concentration. Twenty micro liters of the above prepared solution was injected through auto injector and the chromatogram was developed using methanol: water (95:5), as a mobile phase and keeping the spectrometric conditions same as other methods. The standard solution 50%, 100%, 150% solutions were injected. Individual recovery and mean recovery values were calculated

Precision:

Preciseness for developed method was analysed chromatographically by preparation of dilute extract solution of concentration 10 ppm. Chromatogram were obtained for five similar dilution with methanol: water (19:1) as solvent at a flow rate of 0.8ml/min and 25°C column temperature. 343 nm wavelength was fixed for obtaining the chromatogram. The % RSD for the area of five replicate injections was found to be within the specified limits.

Robustness:

Robustness of method was evaluated with varying flow rate and changing wavelength of analysis. Flow rate was varied between 0.6ml/min and 1ml/min, with two different varying wavelength of 290nm and 294 nm. Retention time value of extract and effect of varying wavelength was evaluated.

Ruggedness:

Ruggedness of the method analysed by using a solution of concentration 0.005mg/ml and chromatogram was obtained in HPLC. The sample was run through suing methanol: water (95:5) as solvent with a flow rate of 0.8ml.min at 25°C column temperature. The total run time for analysis was 20 min and sample was analysed at 292 nm. The standard solution (50ppm) was injected by different analysts and the area for injections in HPLC was measured. The %RSD for the area of replicate injections was found to be within the specified limits.

Limit of detection (LOD) & limit of quantification (LOQ):

The Limit of Detection (LOD) can be defined as the smallest level of analyte that gives a measurable response. The Limit of Quantification (LOQ) can be defined as the smallest concentration of analyte which gives a response that can be accurately quantified. The LOD & LOQ was determined by the formula:

$$LOD = 3.3\ \sigma / S$$

$$LOQ = 10\ \sigma / S$$

The values of LOD & LOQ were found to be 0.1269ppm and 0.3847ppm respectively.

Specificity:

Specificity was evaluated by injecting the blank, placebo and the control sample solution prepared as per the proposed method to check for the interference if any peak at the retention time of plant extract.

RESULT & DISCUSSION

Physico-Chemical Parameters of Cajanin

Table 1 Physico-Chemical Parameters of Cajanin

S.No	Parameters	Powder (%w/w)*		Extract (%w/w)*	
		1	2	1	2
1	Total Ash	2.56	1.02	2.56	2.03
2	Acid Insoluble Ash	0.78	0.88	0.57	0.67
3	LOD	3.71	5.43	3.67	5.25
4	Alcohol soluble extractive	13.96	11.82	22.17	19.29
5	Water soluble extractive	10.83	6.57	18.33	17.48
6	pH	5.21	3.56	4.81	4.53

Qualitative Analysis of Phytochemical Parameters:

Table 2 Qualitative Analysis of Phytochemical Parameters

S.No	Parameters	Present	Absent
1	Alkaloids	√	-
2	Glycosides	-	√
3	Flavonoids	-	√
4	Tannins	√	-
5	Phenols	√	-
6	Carbohydrates	√	-
7	Amino-acids	-	√
8	Fixed Oils & Fats	-	√

Microbial Analysis

Microbial analysis was performed to determine the level of contamination in the samples. This occurs mainly during the collection of samples or during their storage. Hence it is important to determine the values and find out whether they fall into limit or not according to WHO. The results obtained were tabulated as below in table 3.

Table 3 Microbial Analysis for Cajanin

Sl .No	Microbial Analysis	Powder (%w/w)		Extract (%w/w)	
		1	2	1	2
1	Bacteria	118	128	108	123
2	Yeast & Mold	45	56	38	52

Selection of wavelength:

The solution of standard plant extract scanned though a range of 200 nm to 400 nm. The maximum absorbance found to be at 292 nm with characteristic peak. This wavelength was taken for further analysis. The known concentration of Plant extract was weighed separately and dissolved in volumetric flasks using methanol. The resulting solution was scanned in the range of 200nm to 400nm. The maximum absorbance was found at 292nm with characteristic peak.

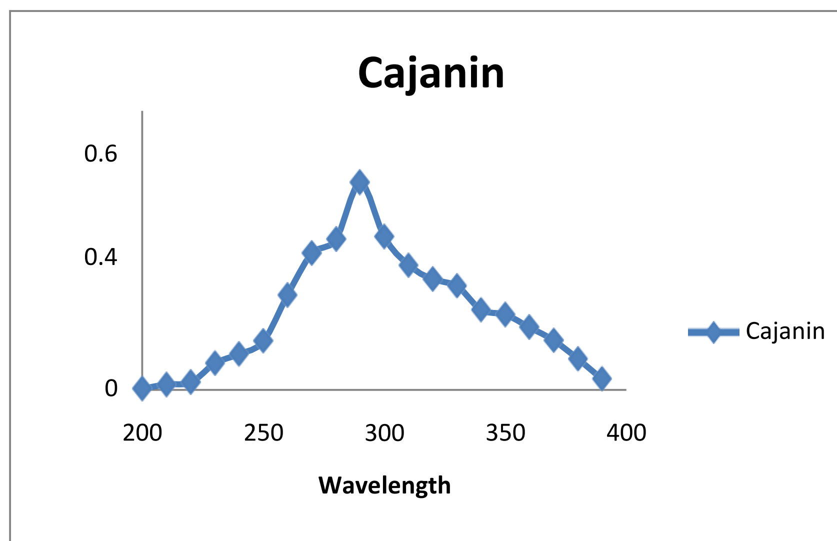


Figure 1: UV spectra of Cajanin

Chromatographic condition:

Twenty micro liters of the sample was injected through auto injector and the chromatogram was developed using Methanol: water (95:5) as a mobile phase. The flow rate was maintained at 0.8 ml/min and column temperature was maintained at 25°C. Total run time was fixed as 20 minutes. Detector wavelength wasfixed at 292nm. The resultant chromatogram was shown in **figure 2**

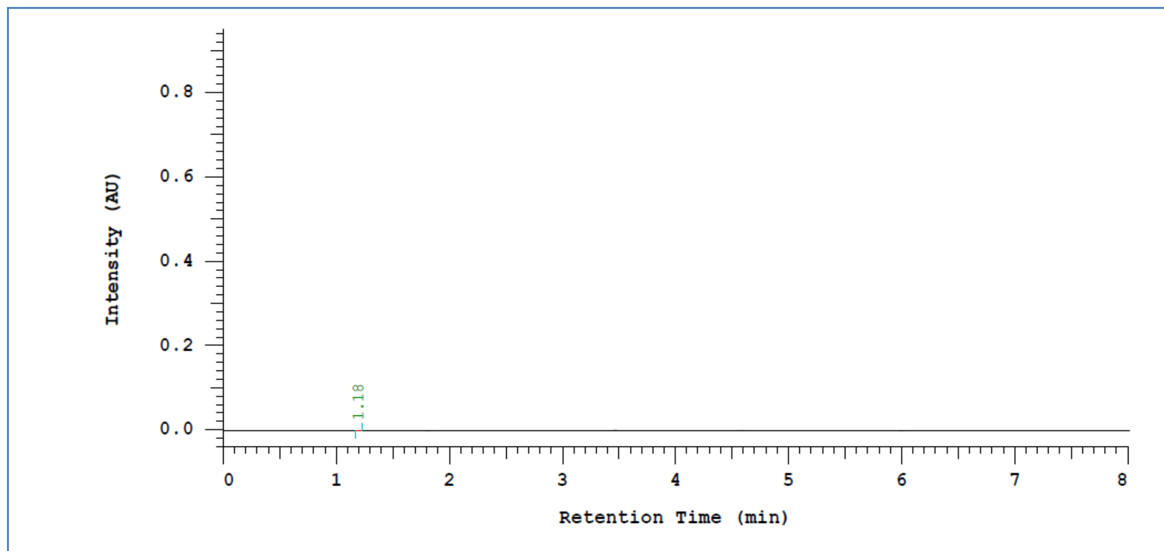


Figure 2: HPLC Chromatogram of Cajanin (Trail 1)

Trial-2

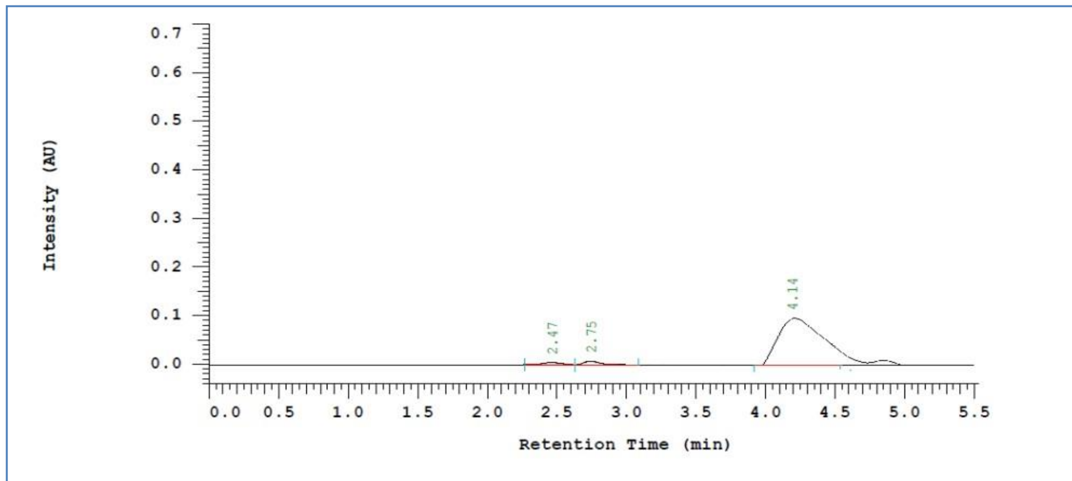
Preparation of mobile phase: Mixture of Phosphate buffer 800ml and 200ml of ACNwas taken and degassed in ultrasonic water bath for 5 minutes. The solution was filtered through 0.45 µ filter under vacuum filtration.

Preparation of Standard Solution: About 25mg of Plant extract was weighed and transferred into a 25ml clean dry volumetric flask containing the mobile phase. The solution was sonicated for about 20mins and then made up to volume with the mobile phase. From this 0.5ml of Plant extract was taken into a separate 10ml volumetric flask and made up the volume to the mark with diluent.

Chromatographic condition:

Twenty microliters of the sample were injected through an auto-injector and the chromatogram was developed using Phosphate buffer: ACN (80:20) as a mobile phase. The flow rate was maintained at 0.8 ml/min and the column temperature was maintained at 25°C. The total run time was fixed as 20 minutes. The detector wavelength wasfixed at 292nm. The obtained chromatogram is shown in figure 3.

Figure 3: HPLC chromatogram of Cajanin



Determination of amount present:

The amount of extract present and its percentage purity was calculated using the following formula.

$$\text{Amount present} = \frac{\text{Sample absorbance}}{\text{Standard absorbance}} \times \frac{\text{Standard dilution}}{\text{Sample dilution}} \times \frac{\text{Average weight}}{100} \times \text{Potency}$$

$$\text{Percentage purity} = \frac{\text{Amount present}}{\text{Label claim}} \times 100$$

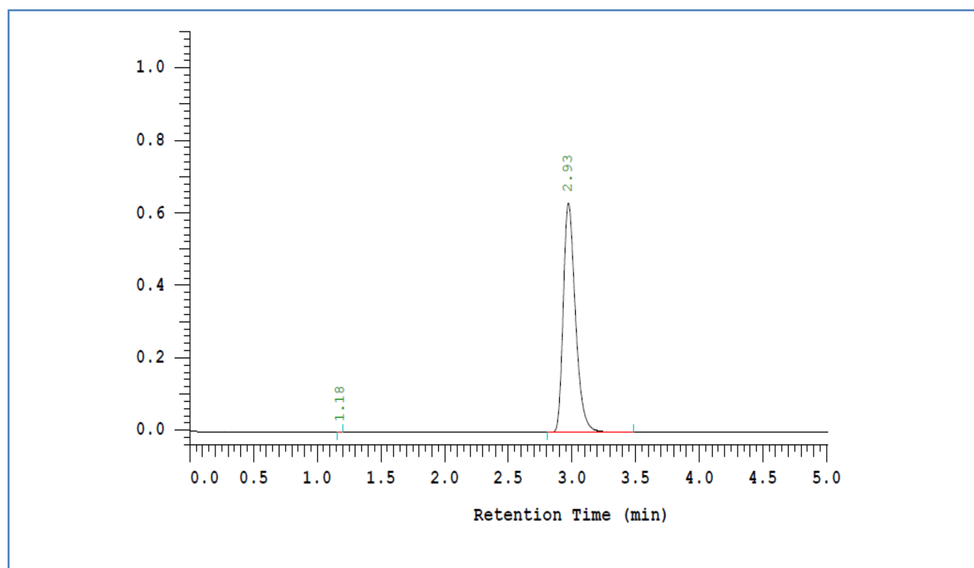


Figure 4: HPLC Chromatogram of Cajanin

METHOD VALIDATION:

The chromatographic conditions were validated by evaluating linearity, recovery, method and system precision, limit of detection (LOD), limit of quantification (LOQ), and robustness in accordance with ICH guidelines Q2 (R1).

Linearity:

The data from the evaluation of linearity of the method is summarized in the table. And graph was plotted for concentration vs peak area. The result obtained from the graph gives a supporting conclusion for linearity of the method with an R^2 value of 0.9966

Table 4: Linearity of Cajanin

Sl.No.	Concentration level (%)	Conc. (ppm)	Peak Area
1	50	5	118289
2	100	10	198537
3	150	15	308833
4	200	20	388927
5	250	25	469456

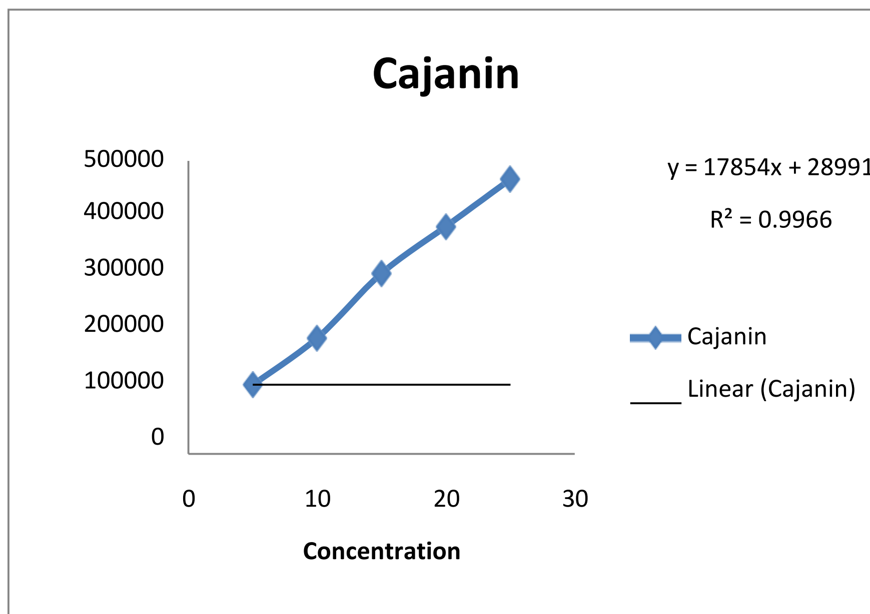


Figure 5. Curve for linearity of the method for Cajanin

Accuracy:

Accuracy of the method was evaluated by analyzing three different solutions of 50%, 100%, and 150% compared with the standard stock solution. Individual recovery and mean recovery values were calculated and the results were discussed in **table 5.**

Table 5: Accuracy of Cajanin

Level	Amount added (ppm)	Total amount (ppm)	Amount found	% recovery
50%	5	15	14.55	97.02
100%	10	20	20.02	100.13
150%	15	25	24.65	98.62

Precision:

The standard solution was injected five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits. The results were illustrated in table 6. The result obtained from the analysis concludes the method to be precise.

Formula:

$$\% \text{ RSD} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

Table 6. Precision results for Cajanin

Parameter	Mean	SD	%RSD
Inter-day	199123	504.53	0.253
Intra-day	198175	558.33	0.281

Robustness:

The samples were analyzed for the change in flow rate and varying wavelength of analysis and Relative standard deviation was calculated for the area under graph. %RSD for the change in flow rate and change wavelength was summarized in table 7. The result of % RSD found to be within the acceptable limits indicating the method to be robust.

Variables		Mean	SD	%RSD
Flow rate	0.6ml/min	198565	498.73	0.251
	1ml/min	199257	508.05	0.254
Wavelength	290nm	198537	546	0.275
	294nm	197532	555.97	0.281

Ruggedness:

The method of analysis was run by different analysts and different laboratories to check the ruggedness of the developed method. The %RSD values were calculated and were found to be within the specified limits.

Limit of detection (LOD) & limit of quantification (LOQ):

The LOD & LOQ was determined by the formula:

$$LOD = 3.3\ \sigma / S$$

$$LOQ = 10\ \sigma / S$$

The values of LOD & LOQ were found to be 0.1269ppm and 0.3847ppm respectively.

Specificity:

It is evaluated by injecting the blank, placebo, and the control sample solution prepared as per the proposed method to check for the interference if any peak at the retention time of plant extract. Figure 6 explains the specificity of Cajanin.

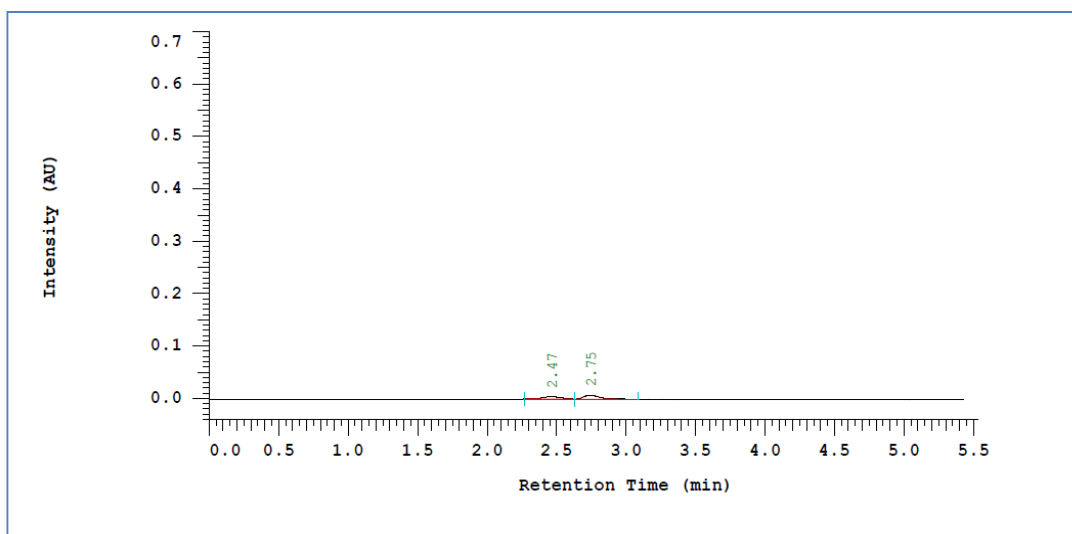


Figure 6 Specificity of Cajanin

CONCLUSION

After the extraction of Cajanin from the source plant part and successful evaluation the following conclusion was drawn indicating, that the amount of the sample extracted was 24.69mg and the percentage purity was found to be 98.76%. Linearity was determined in the range of 5 - 25 µg/ml. The correlation coefficient value was >0.99. Typically, the regression equation for the calibration curve was found to be y = 17854x + 28991. The results presented in table 5 of our study showed the percentage recovery of cajanin was found to be 97.02%, 100.139%, and 98.623% from 50%, 100%, and 150% sample solutions respectively. The obtained percentage recovery of the drug was found to be within the range which indicates that the proposed method was more accurate than the existing methods. Both inter-day and intra-day precision were carried out and the %RSD for cajanin was found to be 0.2534% and

0.2817% respectively. The % RSD value indicated a good degree of precision within the specified range. The results of precision studies were shown in table 6. The LOD and LOQ value for cajanin was found to be 0.044 µg/ml, and 0.134 µg/ml, respectively. This resembles that the proposed method was sensitive. The results obtained in the present study also indicate the method is robust. The ruggedness of the method was investigated under a variety of conditions including different analysts. There is no significant change which indicates that the proposed method is having ruggedness. The specificity of the proposed method was illustrated in figure 6 which shows that there is no interference of any peak at the retention time of cajanin in the chromatogram of blank solution. Thus the proposed method was specific and selective.

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