

CHEMICAL FINGERPRINTING OF PIPERINE AND TANNIC ACID IN AN AYURVEDIC FORMULATION OF DASANAKANTHI CHURNAM USING RP-HPLC

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ABSTRACT

Dental diseases are one of the significant public health problems globally. Herbal medicines for managing oral diseases are considered an effective alternative to synthetic compounds due to their lower side effect. *Acacia catechu*, *Piper nigrum*, *Piper longum*, and *Piper cubeba* are vital components of Dasanakanthi Churnam used to control and prevent oral inflammations in dentistry. Tannic acid and Piperine are major markers for the chemical fingerprinting of Dasanakanthi Churnam. They serve as quality indicating parameters for Dasanakanthi Churnam because of their pharmacological action. Development and validation of a stability-indicating assay method using RP-HPLC become an integral part of simultaneous estimation of Tannic acid and Piperine in Dasanakanthi Churnam. Stability indicating the method is established by exposing markers to various stress conditions such as alkaline hydrolysis, photolytic, oxidative and thermal conditions. Stress testing of the markers can help identify the likely degradation products and validate the stability indicating the power of the analytical procedures used. Tannic acid and Piperine were effectively separated at 5.2 and 22.5 minutes respectively using a PDA detector without interference from any degradant and impurities. This research gives a scope to further carry out the stability studies such as accelerated, intermediate, and long-term studies for Dasanakanthi Churnam.

Keywords: Piperine, Tannic Acid, RP-HPLC, Dasanakanthi Churnam, Stability Indicating Method, Stress Studies.

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INTRODUCTION

There is a lack of data on the chemical fingerprinting of herbal formulations. The drawback of chemical fingerprinting is due to the lack of standard quality control profiles in the final product due to the complex nature of the chemical constituent.¹ In recent times, with the advent of oral diseases worldwide, there has been an upsurge in the use of herbal medicines for the management of oral diseases. The use of natural anti-inflammatory agents with minimal adverse effects has accelerated the development of traditional herbal medicine.² Dasanakanthi Churnam is used to treat dental problems by protecting the oral cavity. The pharmacological action of Dasanakanthi Churnam is exerted majorly by Tannic acid and Piperine. Tannic acid possesses antiviral and antibacterial effects whereas Piperine exerts anti-inflammatory effects.³⁻⁴ Development of an accurate analytical method becomes essential to maintain the quality of Ayurvedic preparations during storage and manufacturing conditions of an Ayurvedic formulation. ICH promotes the need for stress testing. It becomes an integral part of assessing the chemical stability of the formulation. It helps identify the likely degradation products, which in turn paves the to establish the degradation pathways, and chemical stability of the molecule and validates the stability indicating the

power of the analytical procedures used. Stability indicating the method is established by exposing markers to various stress conditions such as alkaline hydrolysis, photolytic, oxidative, and thermal conditions.⁵ So far, no stability- indicating assay method using RP-HPLC is available for simultaneous estimation of Piperine and Tannic Acid in Dasanakanthi Churnam.⁶⁻¹⁸ In this study, a routine stability-indicating assay method for simultaneous estimation of Tannic acid and Piperine was developed using RP-HPLC that serves the purpose of chemical fingerprinting of Dasanakanthi Churnam. It becomes an integral requirement that ensures the efficacy, stability, and safety of Dasanakanthi Churnam.

EXPERIMENTAL

Procurement of Experimental Materials

Dasanakanthi churna was procured from the Kottakal store at Manipal and Piperine (purity >97%) and Tannic acid (ACS Reagent) were purchased from Sigma Aldrich. Methanol and Acetonitrile (HPLC grade) were purchased from Finar.

Characterization of Markers

Piperine and Tannic acid from Sigma Aldrich, the samples were authenticated by infrared spectroscopy and UV spectroscopy. Samples were prepared by the pressed pellet technique for IR spectroscopy. The UV spectrum was taken to identify the λ_{max} of Tannic Acid and Piperine.

HPLC Method Development

Shimadzu LC-20 AT Prominence solvent delivery module equipped with SPD-M10Avp Prominence PDA detector. The separation was performed on HyperClone BDS C₁₈ Column (250cm x 4.6mm i.d, 5 μ particle size) Phenomenex by gradient program.

Validation of HPLC Method as per ICH Q2 (R1) Guideline

Specificity

A standard solution containing Tannic acid (100 μ g/ml) and Piperine (10 μ g/ml) were injected. The peak purity of the standard mixture solution of Tannic acid and Piperine was checked.

System Suitability

A standard solution containing Tannic acid (100 μ g/ml) and Piperine (10 μ g/ml) were injected into the system to monitor the peak parameters retention time, area, tailing factor, resolution, and theoretical plates.

Linearity

A Linearity standard solution for Tannic acid and Piperine was prepared in from 50-250 μ g/ml for Tannic acid and 2-10 μ g/ml for Piperine.

Limit of Detection and Limit of Quantification

The LOD and LOQ were calculated based on the standard deviation of response and slope.

Accuracy

The accuracy was performed based on the recovery of known amounts of analyte by the standard spiking method. A known amount of the marker was spiked and its recovery was calculated. Accuracy was performed at three levels 80%, 100%, and 120% of the standard concentration. The three concentration levels were 3.2 μ g/ml, 4 μ g/ml, and 4.8 μ g/ml for Piperine and 80 μ g/ml, 100 μ g/ml, and 120 μ g/ml for Tannic acid.

Precision

Intraday and Interday precision was performed. 6 injections of the standard mixture were injected. The retention time and peak area of markers were analyzed. Mean, SD and RSD were calculated.

Assay of Marketed Formulation

3g of formulation Dasanakanthi Churnam powder was taken in a 10ml volumetric flask and volume is made up with methanol, sonicated for 30 minutes by frequent stirring for every 5 minutes, and centrifuged for 10 minutes at 1000 rpm. 1ml of the supernatant solution was withdrawn for analysis and % recovery was calculated.

Stress Testing

Forced degradation studies were carried out at a concentration of 100µg/ml of Tannic acid and 10µg/ml of Piperine as a mixture to detect the degradation products. The drug samples were analyzed by the same chromatographic conditions with a photo-diode array detector for the establishment of peak purity of the drug substance in the presence of degradant peaks to monitor the degradation products.

Acid Hydrolysis

The hydrolysis of Tannic acid and Piperine in an acidic medium was carried out under different conditions. Initial trials were conducted with 0.1 M hydrochloric acid at room temperature and there was less than 10% degradation. Hence, further trials were conducted at a heated temperature. 5mg of Piperine and 50mg of Tannic acid were accurately weighed and transferred into 50ml round bottom flask and 50ml of 0.1M hydrochloric acid was added (This becomes 100µg/mL of Piperine and 1000µg/mL of Tannic acid). Piperine stock solution and tannic acid stock solution were diluted further. Adjusted the pH of the solution to 7.0 with 0.1M NaOH. The final solution of Piperine (10µg/ml) and Tannic acid (100µg/mL) was injected into the HPLC system.

Alkali Hydrolysis

Preparation is the same as that of in acid hydrolysis but instead 0.1 M 0.1M NaOH was used in alkali hydrolysis and pH was adjusted with 0.1 M HCl. The final solution of Piperine (10µg/ml) and Tannic acid (100µg/mL) was injected into the HPLC system.

Oxidative Degradation

The hydrolysis of Tannic acid and Piperine in an acidic medium was carried out under different conditions. Initial trials were conducted with 3%v/v H₂O₂, and 5%v/v H₂O₂ at ambient conditions for 24 hours but no degradation was observed up to 24 hours, so further it was continued by using 30%v/v hydrogen peroxide solution for 24 hours. The final solution of Piperine (10µg/ml) and Tannic acid (100µg/mL) was injected into the HPLC system.

Photolytic Degradation

Approximately 15mg each of Piperine and Tannic acid were placed in a petri dish and exposed to direct sunlight for 24 hours. 10mg of Piperine and Tannic acid were weighed and the volume was made up to with MeOH (1000µg/mL). Piperine stock solution and tannic acid stock solution were diluted further. The final solution of Piperine (10µg/ml) and Tannic acid (100µg/mL) was injected into the HPLC system.

Thermal Degradation

Approximately 15mg each of Piperine and Tannic acid were mounted on Petri plates and stored in a hot air oven at a temperature of 80°C for a duration of 24 hours. The final solution of Piperine (10µg/ml) and Tannic acid (100µg/mL) was injected into the HPLC system.

RESULTS AND DISCUSSION

Characterization of Markers

Piperine and Tannic acid from Sigma Aldrich, samples were authenticated by infrared spectroscopy which provides knowledge about the functional groups present in the markers.

HPLC Method Development

Reverse phase HPLC was selected for separation. The optimized condition for chromatography is shown in Table-1. The chromatogram of the optimized condition is shown in Fig.-1 and 2.

Table-1: Optimized Chromatographic Condition

Stationary Phase	C ₁₈ Phenomenex HyperClone BDS (250×4.6mm, 5µ)
Mobile Phase	Methanol: pH 4.7 Phosphate buffer (10mMol) of ratio 20:80 as Solution A and Acetonitrile: pH 4.7 Phosphate buffer (10mMol) of ratio 60:40 as Solution B.
Column temperature	30° C
Mode of flow	Gradient

Flow rate	1 mL/min
Injection volume (μL)	10
Total Run time (min)	30
Retention time (min)	Tannic acid : 5.2 Piperine : 22.5
λ_{max}	Tannic acid-276nm Piperine-341nm

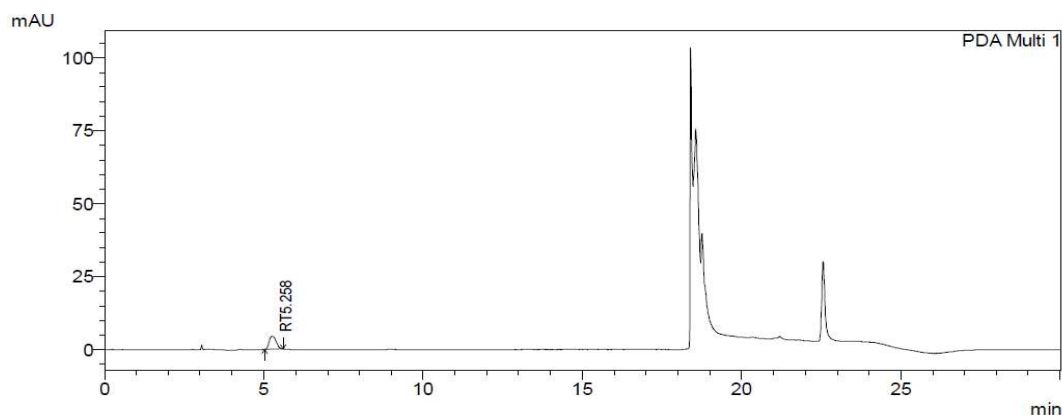


Fig.-1: Chromatogram of Tannic Acid at 5.2 min

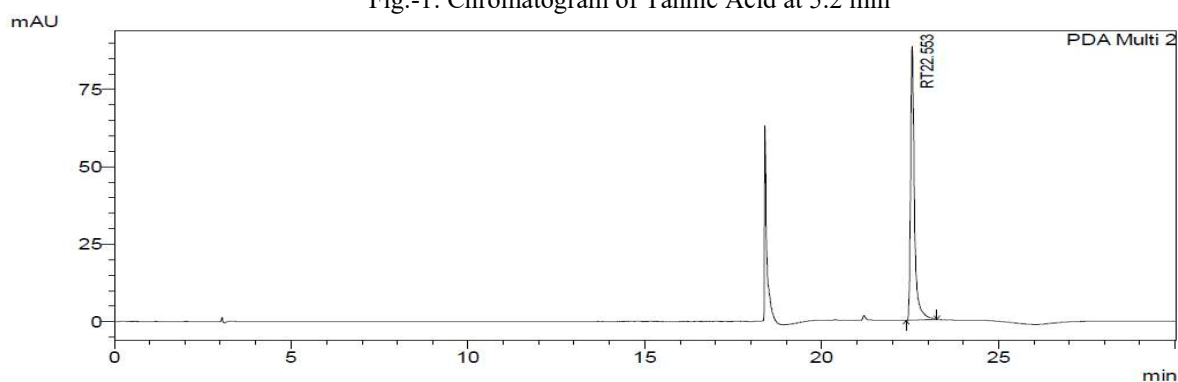


Fig.-2: Chromatogram of Piperine at 22.5 min

HPLC Method Validation as Per ICH Q2 (R1) Guideline

Method validation results can be used to assess the efficiency, reliability, and accuracy of analytical results; it is an essential component of any successful analytical technique. Validation was performed as per ICH Q2AR1. The method was specific as the peak purity index was 0.999 and 0.997 for Tannic acid and Piperine respectively which was well within the limit. This indicates that there was no interference from other compounds at that particular retention time. The system suitability parameters were evaluated for the suitability of the proposed method and the values were complying with acceptance criteria, which indicate that the method is suitable for the system. The linearity results indicated that the method was linear over a concentration range of 2-10 μg/ml for Piperine and 50-250 μg/ml for Tannic acid, as the correlation constant was found to be 0.9962 and 0.9996 for Piperine and Tannic acid respectively. The calculated LOD and LOQ values of the described method are as follows. 0.74 μg/ml and 1.25 μg/ml for Piperine whereas 6.17 μg/ml and 18.69 μg/ml for Tannic acid. The accuracy of the method was performed based on the recovery of known amounts of analyte by the standard spiking method. A known amount of the standard drug was spiked to the pre-analyzed standard samples and the recovery of the drug was calculated. Accuracy was performed at 3 levels of 80%, 100%, and 120% of the standard concentration. The three concentration levels were 3.2 μg/ml, 4 μg/ml, and 4.8 μg/ml for Piperine and 80 μg/ml, 100 μg/ml, and 120 μg/ml for Tannic acid. The recovery values at three levels were found to be within the range of 95% to 105% as per ICH guidelines. Therefore, the HPLC method was found to be accurate. 6 injections of standard mixture solution were introduced into the system and the retention time and peak area of the

drugs were analyzed on the same day for Repeatability or intra- day precision and on different days for Intermediate or inter-day precision. Mean, SD and RSD were calculated The RSD was found to be less than 2 in both intra-day and inter-day precision. So, the method was found to be precise and reproducible. Validation results are tabulated in Table-2.

Table-2: HPLC Method Validation Summary

Parameters	Acceptance criteria	Piperine	Tannic acid	Inference
Specificity	(a) Blank interference Identification of active ingredient peaks and diluents (b) Forced degradation Identification of active ingredient peak and impurity/ degradant peak by Retention time.	Marker peak and degradant peaks Are well resolved.	Marker peak and degradant peak Are well resolved.	Passes
Linearity	The correlation coefficient of the linear regression line should not be less than 0.99	2-10 μ g/ml ($r^2=0.9962$)	50-250 μ g/ml ($r^2=0.9996$)	Passes
Accuracy (95.0% to 105.0%)	80% 100% 120%	99.59 101.24 100.41	98.86 99.54 99.76	Passes
Precision	The RSD should not be more than 2% (i) Intra-day (ii) Inter-day	0.84% 1.34%	1.54% 1.53%	Passes
LOD	-	0.74 μ g/ml	6.17 μ g/ml	-
LOQ	-	1.25 μ g/ml	18.69 μ g/ml	-
System suitability	The RSD should not be more than 2%	0.84%	1.54%	Passes

Assay of Marketed Formulation

The recoveries of Tannic acid and Piperine were found to be 101.3% and 100.5% respectively which indicates that the method can be used as stability indicating method for assay of Tannic acid and Piperine in Dasanakanthi Churnam. Mean recovery in Dasanakanthi Churnam is shown in Table-3. The chromatogram of Tannic acid and Piperine in Dasanakanthi Churnam is shown in Fig.-3.

Stress Testing

In forced degradation studies, there may be a chance of co-elution of degradation peaks along with drug peaks. Thus, the PDA detector was used to establish the purity and spectral homogeneity of the peaks at the λ_{max} of Tannic Acid and Piperine which was 276nm and 341nm respectively. Results obtained from the analysis of samples show that there was no other peaks co- eluting or interfering with active constituents, degradants or impurities even under variable stress conditions. The method was specific for the estimation of Tannic acid and Piperine in presence of various degradant. The percentage degradation was calculated by using the area normalization method. The percentage degradation is mentioned in the table-4. The chromatogram of stress studies is shown in Fig.-4 to 8.

Table-3: Mean Recovery in Formulation

S. No.	Tannic acid	Piperine
1.	101.65%	101.78%
2.	101.30%	99.70%
3.	101.16%	100.08%
Mean	101.37	100.52
S.D	0.252	1.107

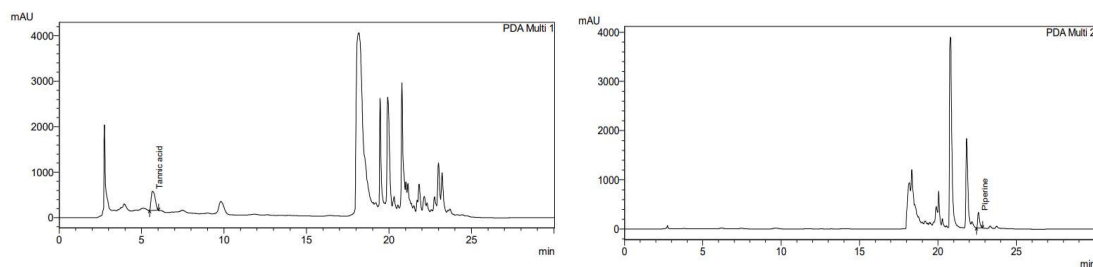


Fig.-3: Chromatogram of Tannic acid and Piperine in Dasanakanthi Churnam

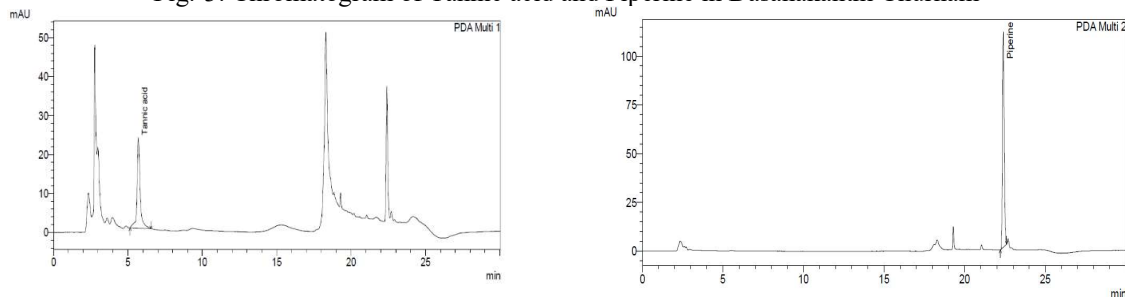


Fig.-4: Chromatogram of Acid hydrolysis

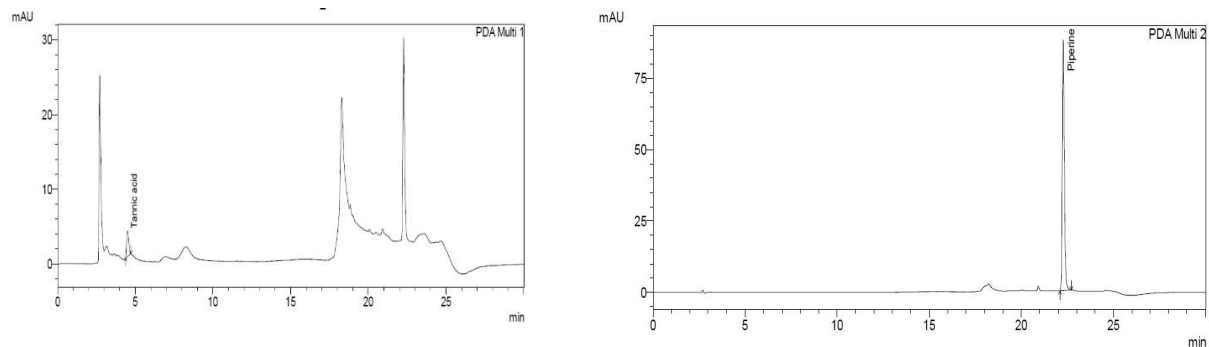


Fig.-5: Chromatogram of Alkali hydrolysis

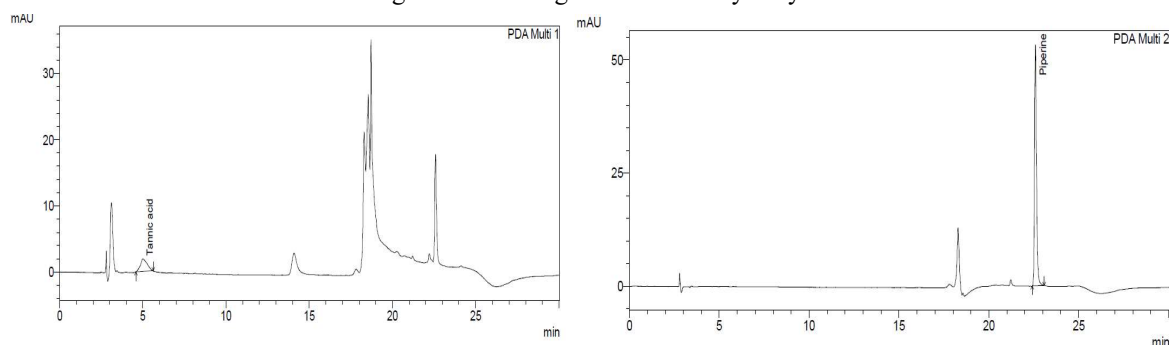


Fig.-6: Chromatogram of Oxidative Degradation

Table-4: Stress Testing

S. No.	Forced Degradation	Conditions	% Degradation Observed		Peak Purity
			Piperine	Tannic acid	
1.	Acid Hydrolysis	0.1M Hydrochloric acid (80°C, 6 hours)	30.84%	27.09%	Passes
2.	Alkali Hydrolysis	0.1M Sodium Hydroxide (80°C, 6 hours)	55.1%	25.12%	Passes
3.	Oxidative Degradation	30% v/v H ₂ O ₂ (Room temperature, 24 hours)	55.31%	38.76%	Passes
4.	Thermal degradation	80°C for 24 hours in a hot air oven	14.26%	19.56%	Passes

5.	Photolytic degradation	4 hours of exposure to direct sunlight	19.05%	32.01%	Passes
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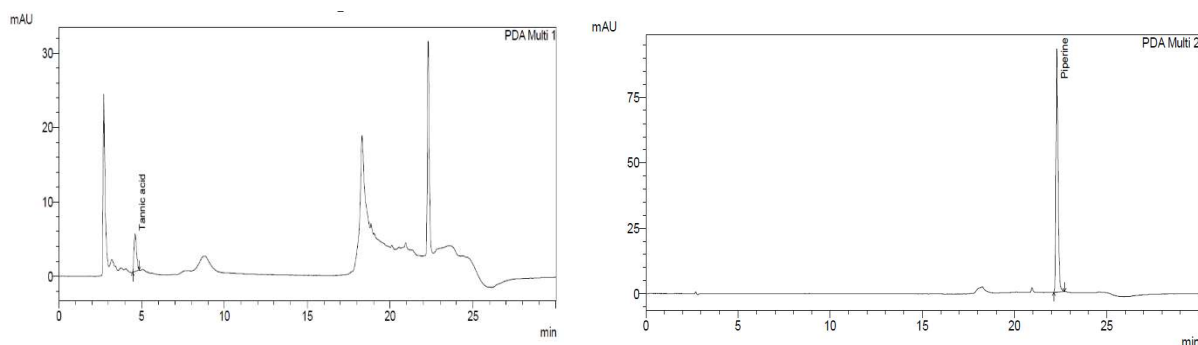


Fig.-7: Chromatogram of Photolytic Degradation

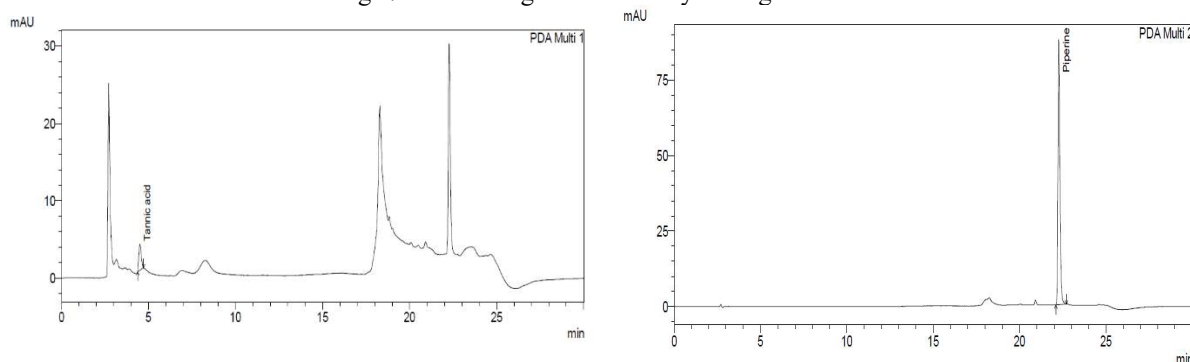


Fig.-8: Chromatogram Thermal Degradation

CONCLUSION

A routine stability-indicating assay method by RP-HPLC for simultaneous estimation of Tannic acid and Piperine in the formulation was established. The results obtained from the stress studies show that there was no interference with the active peak, degradant, or impurities due to variable stress conditions. The method can be specifically used for the estimation of Tannic acid and Piperine in presence of various degradant. The method can be used as stability indicating method for assay of Tannic acid and Piperine from an Ayurvedic formulation. The stability-indicating analytical method which will be developed can be used for the routine stability of the formulations. The information of the research gives a scope to further carry out the stability studies such as accelerated, intermediate, and long-term studies for the Ayurvedic formulation Dasanakanthi Churnam.

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