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RESEARCH ARTICLE

Simultaneous Estimation of Eugenol and Scopoletin from the in House *Avipattikar Churna* by RP-HPLC Method and Estimation of Scopoletin from Different Extracts of *Jalap*

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Abstract

Background: Although standardising polyherbal medicine requires immediate attention, it is a tedious undertaking. Phytochemical profiling is a particularly useful tool for assessing the quality and effectiveness of polyherbal medicines, among various methods used for standardization. The proposal aimed to develop a precise RP-HPLC method for simultaneous estimation of eugenol and scopoletin in in-house *Avipattikar churna*. This method was also used to estimate scopoletin in various extracts of *Ipomoea turpethum*. **Methods:** The phytomarkers in *Avipattikar churna*, hydroalcoholic and alcoholic extracts of *Jalap* were estimated by RP-HPLC system. In this setup, RP-ODS C8 column was employed with methanol: water (30:70 v/v, 0.1% formic acid) at 1 ml/min for 0-10 minutes, and then with methanol: water (60:40 v/v) at 0.8 ml/min for 10.01-25 minutes. Detection was done at 280 nm for eugenol and 366 nm for scopoletin using a UV/VIS detector. The method was validated by performing validation parameters as per ICH guidelines. **Results**: The linearity of eugenol and scopoletin was performed, with correlation coefficients of 0.999 and 0.9969 respectively**.** In repeatability, % RSD was observed as 0.856 and 0.909 for eugenol and scopoletin correspondingly. The LOD (detection limit) of eugenol was 0.67 µg/mL and of scopoletin was 1.39 µg/mL. While LOQ (quantification limit) of eugenol was found as 2.04 µg/mL and 4.03 µg/mL for scopoletin. The % recovery was ranging from 102.96 - 100.45 % for eugenol and from 102.65 - 101.3 %w/w for scopoletin, after adding a pre-quantified amount (20 µg/mL) in the different concentrations of the standards. The eugenol and scopoletin were estimated 0.1366 %w/w and 0.0465 %w/w respectively in *Avipattikar churna*. The hydro alcoholic extract of *Jalap* showed presence of more scopoletin than in the alcoholic extract. **Conclusion:** The validated process is established as accurate, consistent and precise which results as a better standardization drive for *Ayurvedic* dosage forms.

Keywords: *Avipattikar churna*, Eugenol, *Ipomoea turpethum*, RP-HPLC, Scopoletin

Abbreviations

RSD : Relative Standard Deviation
LOD : Limit of Detection
LOQ : Limit of Quantification
SD : Standard Deviation
ICH : International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
GA : Gallic acid
R^2 : Correlation coefficient

1. Introduction

Ayurvedic formulations are composed of a wide range of individual ingredients with different phytoconstituents. Establishing a method of quantification from the various formulations is very challenging, so it is highly required to establish an analytical method for the quantification of phytochemicals from the plants or formulations for standardisation and in the evaluation of the effectiveness of the herbs[1].

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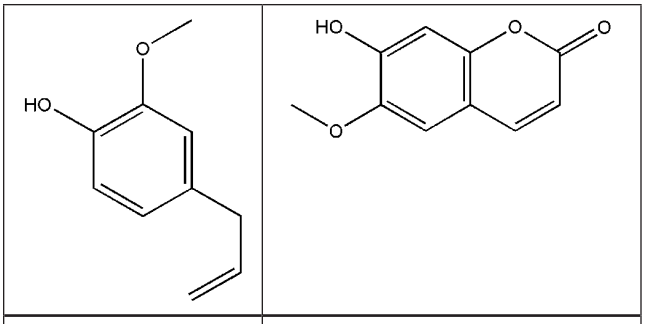
One of the classical *Ayurvedic* formulations, *Avipattikar churna*, contains fourteen herbs that are useful in curing acidity and its problems, such as headaches caused by aggravated *pitta*, nausea, indigestion, vomiting, headache, constipation, piles, and peptic ulcers. In *Ayurveda*, *Avipattikar churna* is recommended as an antacid and for calming the *pitta*. The unique attribute reduces intestinal hotness and proved to be an effective therapy for spicy meals[2,3]. The in-house *churna* was prepared as per the *Ayurvedic* Pharmacopoeia of India (2007). The *churna* is mainly composed of two major botanicals, clove (*Eugenia caryophyllus*) and *Jalap* (*Ipomoea turpethum*) in 8.33 % w/w and 33.33 % w/w, respectively out of 14 ingredients present. These two botanicals mainly contain eugenol and scopoletin, respectively[2,4]. The quantification of markers can be done by applying various analytical methods on the formulation and raw materials. Clove contains 18% of volatile oil as an active ingredient. Eugenol, a phenolic component, accounts for 89 % of the essential oil, along with other active compounds like α-humulen, 5-15 % of eugenol acetate and β-cariofileno[5-7]. It is well-documented that eugenol has a strong penetration augmentation feature and is a good option for several uses.

Ipomoea turpethum majorly contain coumarins like betulin, scopoletin, lupeol, β-sitosterol, cycloartenol, lanosta-5-ene and resins such as α- and β-turpethein, turpethinic acids A, B, C, D, and E, etc. Many secondary phytoconstituents such as saponins, flavonoids, phenols, glycosidic resins, phenols, ethereal oils and sugars were found in the roots and rhizomes of *Jalap*[8]. In addition to their main role in the treatment of gastric ulcers and esophagitis, the main ingredients of *churna* also have significance in addressing neurological diseases. Scopoletin, one of these ingredients, has demonstrated various pharmacological actions, including the reduction of inflammation, scavenging of free radicals, and anti-bacterial activity[9,10].

In an *in vivo* study, aspirin and pyloric ligation-induced ulcers in male albino rats showed antiulcer effectiveness in methanolic and hydroalcoholic extracts from the stem of *Operculina turpethum*[11]. Rajashekar *et al.,* proved gastroprotective effects in *Jalap* and its polyherbal formulation through experimental models[12]. Clove and *Jalap* both demonstrated gastroprotective activity, either directly or indirectly through various mechanisms. However, no HPLC method is reported for the quantification of eugenol and scopoletin from the major ingredients of the *Avipattikar churna* and different extracts of *Jalap*. So, the objective of the current study is to perform simultaneous estimation of eugenol and scopoletin from manually prepared *Avipattikar churna* and amount of scopoletin from *Jalap* by a precise and validated RP-HPLC method. The chemical structures of eugenol and scopoletin are shown in Figure 1.



4-Allyl 2 methoxyphenol (Eugenol)	7 hydroxy 6 methoxy chromen 2-one (Scopoletin)

Figure 1. Structures of Phyto constituents.

2. Materials and Methods

2.1 Plant Materials

Ginger, *pippali*, *mari*, *behda*, *harde*, *amla*, *vaj*, salt, cardamom, vidanga, clove, *tamal patra*, *Jalap* and *sarkara* were purchased from the local market, Ahmedabad, India and identified by botanists (Gujarat University).

2.1.1 Instruments

UV/VIS (VWD) is used as a detector in Agilent's RP-HPLC (Infinity 1200) apparatus.

2.1.2 Chemicals

The phytomarkers (eugenol and scopoletin) were bought from Yucca Enterprises, Mumbai. All the reagents used were of HPLC grade. Sample solutions were prepared in HPLC-grade reagents.

2.2 Preparation of In-house *Avipattikar Churna*

According to the method mentioned in API (*Ayurvedic* Pharmacopoeia of India, 2007), the *churna* was

prepared. All the ingredients mentioned in the plant materials were weighed separately, ground, and mixed in 1 part, except clove, *Jalap*, and sugar, which are mixed in 11, 44, and 66 parts, respectively. The regimen of the *Avipattikar churna* is recommended at 10g with water for treating illnesses related to gastrointestinal.

2.3 Estimation of Eugenol and Scopoletin in Manually Prepared *Avipattikar churna* and Different Extracts of *Jalap* by RP-HPLC Method

2.3.1 Chromatographic Conditions

Agilent's HPLC (Infinity 1200) with UV/VIS (VWD) detector was used to perform the estimation of scopoletin and eugenol content in the in-house *Avipattikar churna*.

2.3.2 Preparations of Solutions

2.3.2.1 Preparation Standard Stock Solution of Eugenol

A methanolic solution of eugenol was prepared to obtain 1000 µg/mL. Further, this prepared solution was diluted to obtain 10 µg/mL.

2.3.2.2 Preparation of Scopoletin Standard Solution (50 ppm)

After accurate weighing, add 5 mg of scopoletin in 50 ml methanol to prepare 100 µg/mL. Further, this solution was diluted to obtain 50 µg/mL.

2.3.2.3 Preparation of Mixture of Eugenol and Scopoletin

1 ml 10 PPM of eugenol and 50 PPM of scopoletin were added into a 10 mL volumetric flask and further methanol was used to make up the volume up to the mark.

2.3.2.4 Preparation of Test Extract

5 g of in-house *Avipattikar churna* was refluxed with 50 mL of alcohol for 30 mins. The solution was filtered and diluted up to 50mL with alcohol (methanol). The 20 µl of extract was injected.

2.3.2.5 Preparation of Different Extracts of Jalap

5 g of *Jalap* was refluxed with 50 mL each of hydro alcohol (70:30) and alcohol (methanol) individually for half an hour. The solutions were filtered and diluted up to 50 mL with the respective solvents. This solution was used for the quantification of scopoletin.

2.3.3 Method Development

After a number of trials and runs, the resolution symmetry peak was obtained in a mixture of standards in the mobile phase methanol: water with 0.1 % formic acid (30:70 v/v) at 0-10 mins with the flow rate 1 ml/min and methanol: water with 0.1% formic acid (60:40 v/v) with the flow rate 0.8 ml/min at 30 °C. Analysis was conducted using a RP-ODS C8 (250*4.5 mm). From the literature, UV detection and injection volume (20 µl) were optimized by conducting various trials[13-15].

2.3.4 Method Validation

As per the recommendation of ICH (2005) Q2R(1), different validation parameters like linearity, precision, accuracy, LOD and LOQ were performed.

2.3.4.1 Linearity and Range

By plotting peak areas vs. drug concentration, calibration curves with regression equations for eugenol and scopoletin were generated. The linear plots were developed (constructed) for 10–50 µg/mL of eugenol and 20–100 µg/mL of scopoletin. Eugenol (1–5 ml) and scopoletin (2, 4, 6, 8, and 10 ml) from the stock solutions were taken and diluted in 10 ml of volumetric flask with mobile phase to obtain desired concentration of 10 - 50 and 20–100 µg/mL respectively. Using the above-mentioned chromatographic conditions (2.3.3), 20 µL of each solution was injected.

2.3.4.2 Method Precision (% Repeatability)

% RSD was determined from 3 measurements of the same concentration of solutions that contain the same amount of eugenol and scopoletin (20 µg/mL) and chromatograms were documented.

2.3.4.2.1 Intraday Precision

The solutions of eugenol (10, 20 and 50 µg/mL) and scopoletin (40, 60 and 80 µg/mL) were evaluated and readings were taken in triplicates for each in a day. % RSD was determined.

2.3.4.2.2 Interday Precision

The solutions of eugenol (10, 20 and 50 µg/mL) and scopoletin (40, 60 and 80 µg/mL) were evaluated for the 3 different days. The experiments were performed in triplicates. % RSD was determined.

2.3.4.3 Accuracy (% Recovery)

For determining accuracy, the conventional addition technique was used to calculate the recovery of eugenol and scopoletin. Pre-quantified, separate solutions of eugenol (20 µg/mL) and scopoletin (20 µg/mL) were added to standard solutions containing different concentrations of eugenol (10.0, 20.0, and 50.0 µg/mL) and scopoletin (20, 40, and 60 µg/mL). The regression equations were used to compute the amounts and rates of recovery of the standard solutions.

2.3.4.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of Detection (LOD) is mainly calculated for the detection of analytes, but the exact value cannot be quantified. It can be calculated as per the following formula.

The limit of Quantification (LOQ) is used to quantify the minimum amount of drug present in a sample. It can be calculated by following the following equation.

Where σ is the standard deviation of the peak areas of the drug in linearity and S is the mean of the slope of the linearity curve for that drug.

2.3.5 Analysis of Phytoconstituents from the Different Samples

The two phytoconstituents were estimated in the extract of *churna*, and scopoletin was also estimated from the hydroalcoholic and alcoholic (methanolic) extracts of *Jalap* by using the mentioned chromatographic techniques. The developed method mentioned above was used to generate chromatograms of standards and the test extract.

3. Results

3.1 Method Development

From the resolution symmetry, the peak was obtained in the above-mentioned mobile phase run in the RP-ODS C8 (250*4.5 mm) column. The injection volume was kept at 20 µl for the above-developed method. The detection was done at 280 nm for eugenol and 366 nm for scopoletin. Retention Time (RT) was found for eugenol as 26.42 minutes and for scopoletin as 9.613 minutes. The chromatograms of individual standards and mixtures of standards are represented in Figure 2 and Figure 3 respectively.

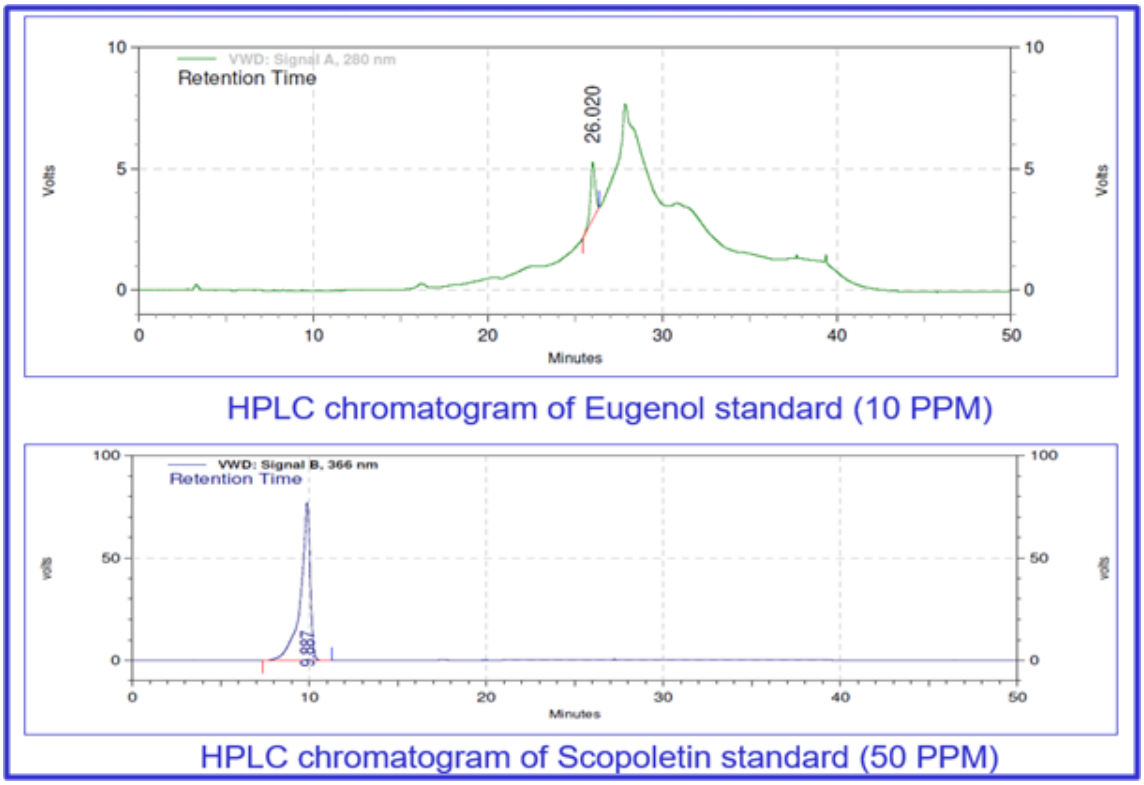


Figure 2. HPLC chromatograms of individual standards.

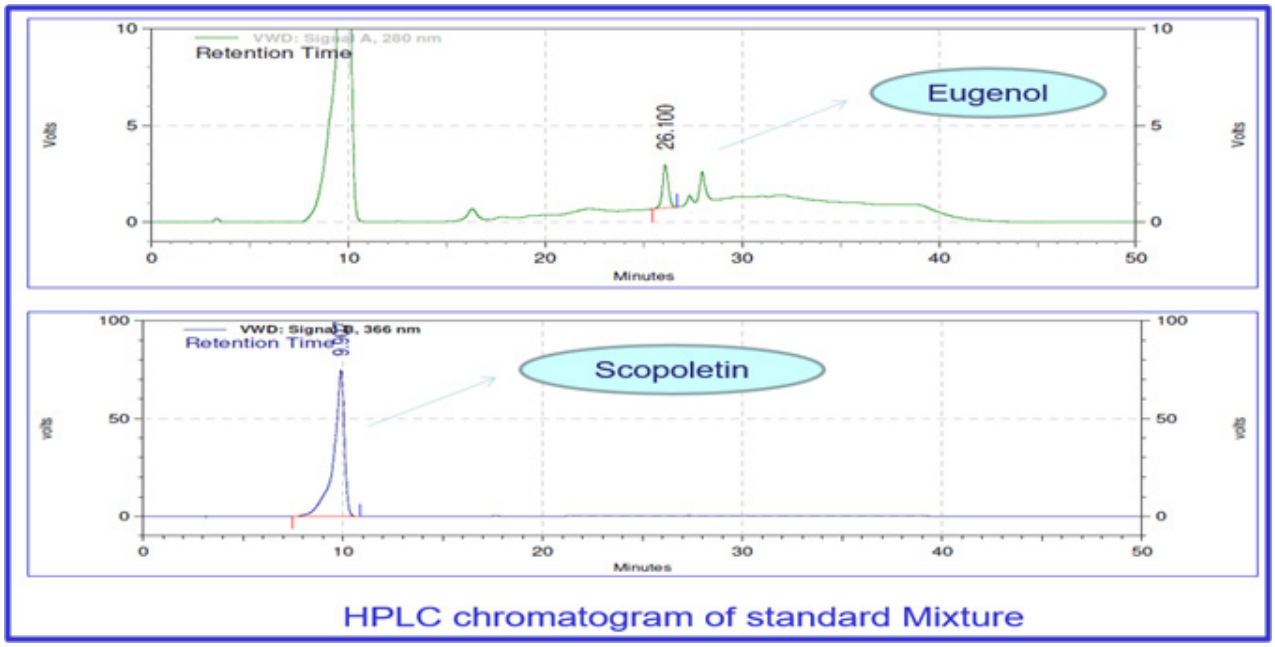


Figure 3. HPLC chromatogram of the standard mixture.

3.2 Method Validation

3.2.1 Linearity and Range

Both the markers, eugenol and scopoletin exhibited a direct connection between peak area and drug concentrations. The calibration curve and equation on the chart were constructed from the readings of peak area and concentration (Figure 4). The R^2 was observed to be 0.999 for eugenol and 0.9969 for scopoletin. The slope and intercept of regression equations are represented in Table 3.

3.2.2 Method precision (% Repeatability)

The repeatability study was performed with 20 µg/mL for both marker compounds. The % RSD (Relative Standard Deviation) was calculated as 0.856 for eugenol and 0.909 for scopoletin. The % RSD

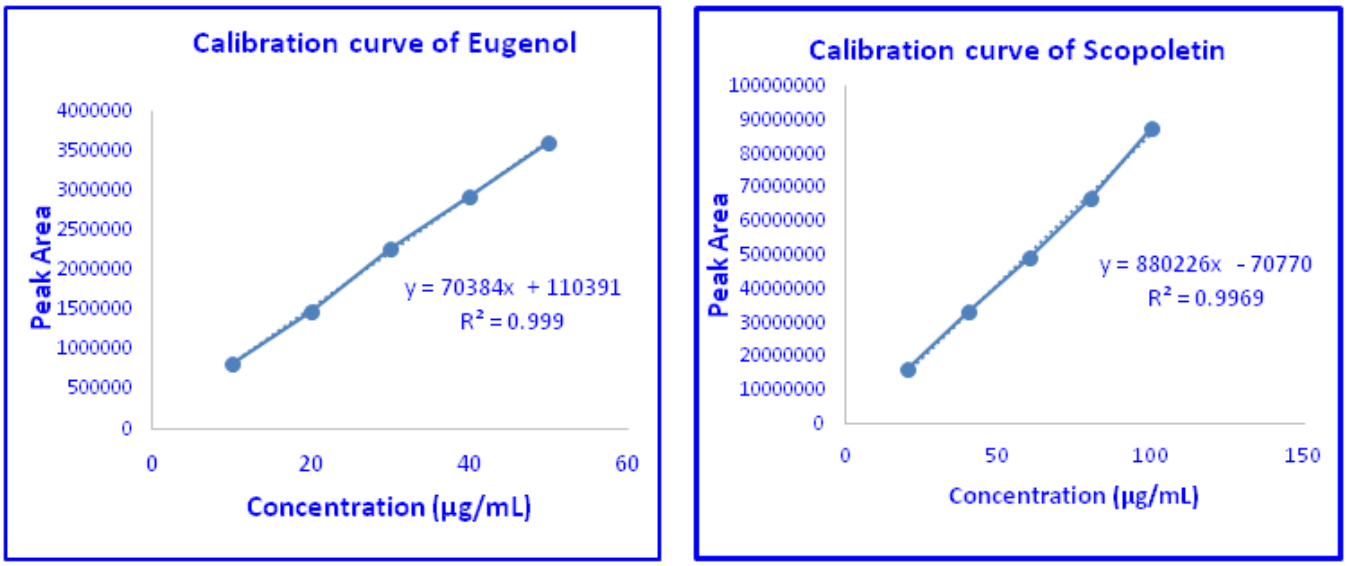


Figure 4. Linearity of eugenol and scopoletin.

values were found to be less than two for the three different concentrations of both marker compounds during the intraday and interday experimental work (Table 1).

3.2.3 Accuracy (% Recovery)

Pre-quantified amount was added in different concentrations of the standards and % recovery was found ranging from 102.96 - 100.45 % for eugenol and 102.65 - 101.3 %w/w for scopoletin. The RSD was found 1.29 % and 1.065 % for eugenol and scopoletin respectively (Table 2).

3.2.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

From the equations (refer 2.3.4.4), the limits of detection and quantification were derived. Eugenol was found to have an LOD and LOQ of 0.67 µg/mL and 2.04 µg/mL, respectively, whereas scopoletin had an LOD and LOQ of 1.39 µg/mL and 4.03 µg/mL, respectively. Table 3 presents the summary results of validation parameters.

3.3 Quantification of Phytoconstituents

Validated HPLC method was used to quantify the active phytoconstituents present in in-house *Avipattikar*

Table 1. Results of precision

	Drug concentration (µg/mL)						
Marker	**Repeatability**	**Intra day**			**Inter day**		
Eugenol (280nm)	20	10	20	50	10	20	50
%RSD	0.856	1.245	0.997	1.221	0.845	1.297	1.217
Scopoletin (366nm)	20	40	60	80	40	60	80
%RSD	0.909	0.9341	1.321	1.114	1.135	0.879	0.956

Table 2. Results of % recovery for eugenol and scopoletin

Name of Marker	Test amount (µg/mL)	Quantity of drug taken (µg/mL)	Overall Quantity (µg/mL)	Total quantity recovered (µg/mL)	% Recovery	% RSD
Eugenol	10	20	30	30.89	102.96	1.29
	20	20	40	40.41	101.025	
	50	20	70	70.32	100.45	
Scopoletin	20	20	40	41.05	102.62	1.065
	40	20	60	60.29	100.48	
	60	20	80	81.04	101.3	

Table 3. Summary results of method validation

Parameters	**RP-HPLC method**	
	Eugenol	**Scopoletin**
Range of concentration (µg/mL)	10-50	20-100
Slope	70384	880226
Intercept	110391	70770
R^2	0.999	0.9969
LOD (µg/mL)	0.67	1.39
LOQ(µg/mL)	2.04	4.03
% Recovery (Accuracy, n = 3)	101.47 %	101.46 %
Repeatability (% RSD, n = 3)	0.856	0.909
Precision (%RSD)		
Intraday (*n* = 6) **Intderday (*n* = 6)**	0.997-1.245 0.9341-1.114	0.845-1.297 0.879-1.135

churna. Scopoletin was estimated from the alcoholic (methanolic) extract and hydroalcoholic extract of *Jalap*. From the results, it was observed that eugenol and scopoletin were marked as 0.1366 %w/w and 0.0465 %w/w respectively. The hydroalcoholic extract of *Jalap* showed more scopoletin than the methanolic extract. The data and chromatograms are represented in Table 4 and Figures 5 and 6.

4. Discussion

To assure the quality, safety, and effectiveness of an *Ayurvedic* formulation, it is important to establish analytical procedures to standardise the many phytoconstituents found in *Ayurvedic* formulations. One of the most accurate analytical methods is marker-based standardisation of herbal formulation by RP-HPLC[16]. For the treatment of peptic ulcers, *Avipattikar churna* is best treatment available in *Ayurveda*. To recognise the efficacy of *churna*, a method should be established for the quantification of phytoconstituents. The *churna* was standardized and reported with the results of various preliminary physicochemical and phytochemical studies. The researchers have already outlined the estimation of GA (gallic acid) in *churna* by HPTLC and UV spectroscopic methods at 271 nm. Moreover, a HPLC method was also established as per the report for performing stability studies by comparing and verifying the presence and peak areas of eugenol after 45 days[17,18]. Shah *et al.*, reported the first derivative UV spectroscopic process for the quantification of eugenol and scopoletin at 296 nm and 278.66 nm correspondingly in manually prepared *Avipattikar churna*[19]. The process was also validated as per ICH guidelines[19]. Here, in this study, the quantification of the scopoletin was also performed by HPLC method in hydroalcoholic and alcoholic (than methanolic)

Table 4. Estimation of phytoconstituents

Sample	% Eugenol (%w/w)	% Scopoletin (%w/w)
Avipattikar churna	0.1366%	0.0465%
***Jalap* Hydroalcoholic extract**	-	0.1215%
***Jalap* alcoholic extract**	-	0.063%

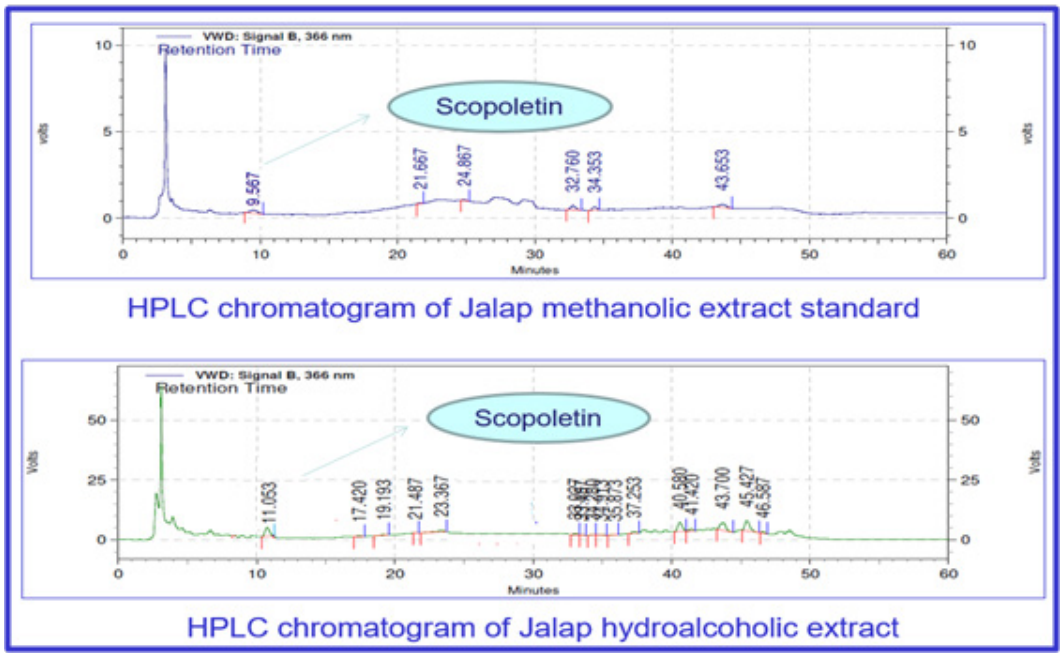


Figure 5. HPLC chromatogram of different extracts of *Jalap*.

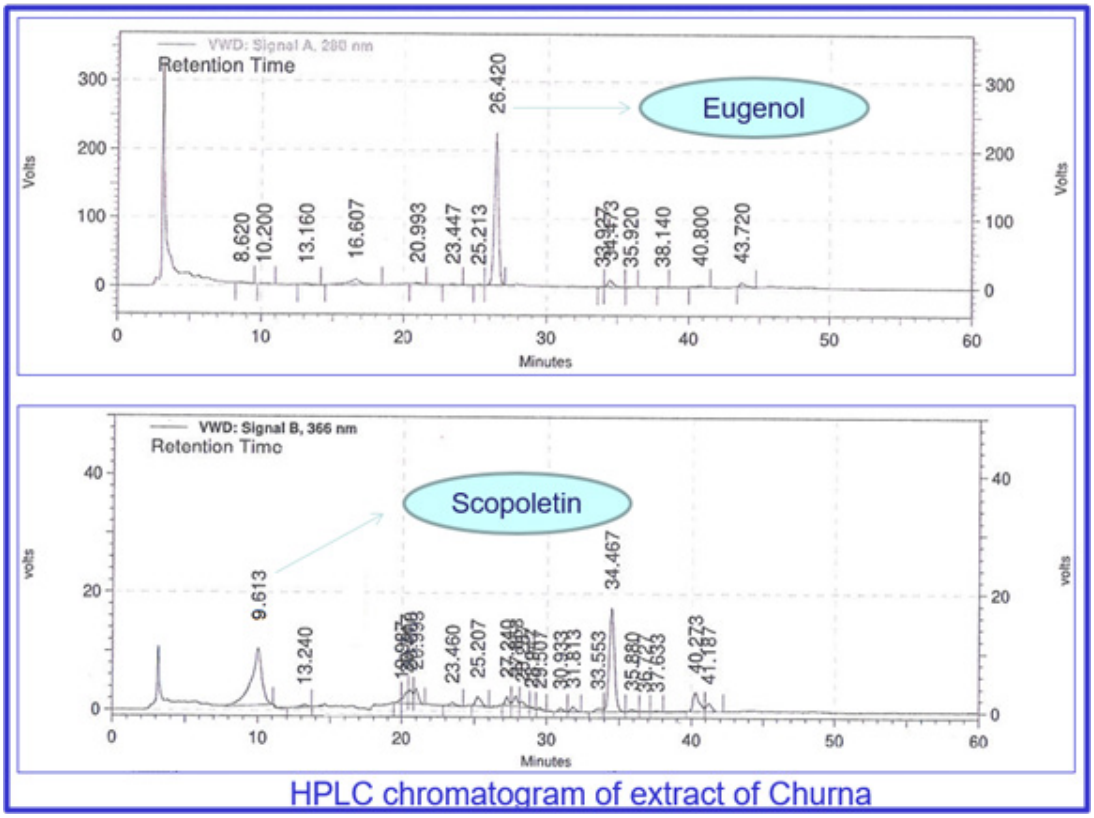


Figure 6. HPLC chromatogram of *Avipattikar churna*.

extracts of *Jalap*. From the results, it was concluded that hydroalcoholic extract contains more scopoletin than alcoholic (methanolic) extract. For one of the works, the author has revealed that hydroalcoholic extract showed significantly higher gastroprotective activity than methanolic activity[11]. The developed RP-HPLC technique for simultaneous estimation of standard samples of eugenol and scopoletin is acute and cost-effective. For concurrent quantification of scopoletin and eugenol, this method is apposite.

5. Conclusion

Because of the complexity of an *Ayurvedic* formulation, a scientific method has not been previously defined for confirming the quality of the final product. The investigation of this research article estimates the phytoconstituents from *churna* with the help of the HPLC method for the treatment of peptic ulcer. The established HPLC method will aid in the standardisation of *churna* by using chemical markers responsible for biological significance. Here, the HPLC analytical method has been employed for the quantification of eugenol and scopoletin from *churna* and is found to be replicable, precise and accurate. There has been a tilt towards acceptance of herbal drugs as promising medicine eventually increases in usage of herbal formulations. Hence, developing and establishing the RP-HPLC method for standardization will aid in ensuring the quality of an *Ayurvedic* formulation.

6. Acknowledgements

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