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Method Development and Validation of Estimation of Eugenol in Herbal Formulation.

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ABSTRACT: The present study aims to develop a method and to validate the developed method for the estimation of the active phytoconstituent in the herbal formulation. It largely focuses on UV spectroscopic method, chemical tests and quantitative analysis of main active ingredient mentioned in label claim. Herbal formulation is reached extensive acceptability as therapeutic as well as nutritional agents. Hence, the need for development of authentic analytical methods which can reliably profile the phytochemical composition and quantitative analysis of phytochemicals and testing of marker/bioactive compounds and other major constituents present in the formulation. The present study is to develop a simple and precise UV spectroscopic method and to validate as per ICH guidelines for the estimation of the active constituents. The developed method was validated and can be used for routine analysis of herbal formulation. A simple, precise, rugged and rapid UV- visible spectrophotometric method was developed for estimation of active constituents in herbal formulations. The observations of the validation parameters such as linearity, range, precision, ruggedness reveal that the proposed method could be successfully applied in the routine analysis of quality control of herbal pharmaceutical dosage forms of semisolid dosage form Eugenol. The result obtained from the validation parameters meet the 4 parameters of ICH requirement as well as obeys BEER'S law. Eugenol was estimated from the marketed formulation and found matching with the label claimed.

KEYWORDS: Eugenol, UV spectroscopic method, ICH guidelines, Herbal Formulation.

I. INTRODUCTION

Herbal formulations are in great demand in the developed as well as in developing countries for primary health care because of their wide biological activities, higher safety margins and lesser costs. The recent global resurgence of interest in herbal medicines has led to an increase in the demand for them. But the most important challenges faced by these formulations arise because of their lack of complete standardization. Commercialization of the manufactured medicines and to meet this increasing demand has resulted in a decline in their quality, primarily due to a lack of adequate regulations pertaining to this sector of medicine. Herbal formulations are the synthesis of therapeutic experiences of generations of practicing physicians of indigenous systems of medicine for over hundreds of years.[1]. Herbal medicines are now in great demand in the developing world for primary health care not because they are inexpensive but also for better cultural acceptability, better compatibility with the human body and minimal side effects [2,3,4]. However, recent findings indicate that all herbal formulations may not be safe as severe consequences are reported for some herbal drugs. Most herbal products in the market today have not been subjected to drug approval process to demonstrate their safety and effectiveness. To be accepted as viable

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alternative to modern medicine, the same vigorous method of scientific and clinical validation must be applied to prove the safety and effectiveness of a therapeutical product [6,7,8]. Herbal formulations are still the mainstay of about 75 - 80% of the world population, mainly in the developing countries for primary health care. This is primarily because of the general belief that herbal drugs are without any side effects besides being cheap and locally available. According to the World Health Organization (WHO), the use of herbal remedies throughout the world exceeds that of the conventional drugs by two to three times [9,10,11]. The use of plants for healing purposes predates human history and forms the origin of much modern medicine. Extraction is the separation of medicinally active portions of plant using selective and standard procedures [12, 13]. Extraction methods include solvent extraction, distillation method, pressing and sublimation according to the extraction principle. Solvent extraction is the most widely used method. The extraction of natural products progresses through the following stages: (1) the solvent penetrates into the solid matrix; (2) the solute dissolves in the solvents; (3) the solute is diffused out of the solid matrix; (4) the extracted solutes are collected [14,15,16]. For the extraction procedures, solvents such as water, ethanol, chloroform, dichloromethane, hexane, ethyl acetate, methanol etc. are most commonly used. The conventional extraction methods generally use organic solvents and require a large volume of solvents and long extraction time. The modern extraction methods have also been applied in natural products extraction and they offer some advantages such as lower organic solvent consumption, shorter extraction time and improve extraction yield[17,18,19]. In this study to check the presence of active ingredient content which is mentioned on label of marketed herbal formulation the selected herbal formulation was Colgate Vedshakti containing Eugenol as an active material.

II. PROCEDURE FOR METHOD DEVELOPMENT & VALIDATION FOR EUGENOL

Standard Eugenol was obtained from Central Drug House, New Delhi, India. Selection and Optimization of Solvent: - Different solvents like Chloroform, Acetone and Methanol were screened for solubility of Eugenol. Methanol was optimized from all the conditions based on solubility, peak quality and non-interference at the specified wavelength .

Preparation of solution for UV of Standard Eugenol: - Eugenol 10.0 mg was dissolved in 100 ml Methanol in volumetric flask. The dilutions of this stock solution were made by diluting the required amount of aliquot with methanol to obtain standard solution of in the range of (5-80 µg/mL). The absorbance of the resultant solution was determined at the λ max of 282 nm.

Extraction of Eugenol from sample.: - 1gm of toothpaste was taken in 10mL of methanol & then solvent extraction was performed by using stirring the solution on magnetic stirrer for 24 hours. The solution was filtered using Whatman filter paper.

Procedure for preparation of sample solution for UV: - 5ml of methanolic extract was taken in 5 ml volumetric flask and the solution was made in methanol up to 10mL. The absorbance of the resultant solution was determined at the λ max of 282 nm.

Preparation of standard calibration curve of Eugenol by UV Visible Spectroscopy: - The standard calibration curve of eugenol was obtained by measuring the absorbance of Eugenol solution in concentration range (5-80µg/mL) prepared from stock solutions in methanol at 282 nm in triplicate. Calibration curve of Eugenol was then plotted with absorbance on y-axis and eugenol concentration on x-axis.

Analytical Method Validation: - For the purpose of quantification of eugenol, development and validation of a UV spectrophotometric method in methanol was carried out as follows. The method was validated according to ICH guidelines, Q2(R1) (ICH, 2005) with respect to linearity and range, precision, detection limit (DL) and quantitation limit (QL). i) Linearity and range. The linearity of the analytical method was its ability to elicit test results which are directly proportional to analyte concentration in samples within a given range. The linearity was determined by analysing absorbance of the Eugenol standard concentrations (5-80 µg/mL) at 282 nm against methanol as blank. The calibration curve was plotted using concentration against absorbance. A regression equation and correlation coefficient were determined for eugenol standard concentrations (5-80µg/mL). The calibration curve was plotted using the concentration range of 5 - 80 µg/mL. The absorbance of the solutions was determined at 282 nm. A calibration curve was constructed by plotting absorbance vs. concentration of standard solution and the regression equation was determined. The experiment was carried out in triplicate. ii) Range In accordance with ICH Q2 (R1) guidelines, range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it

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has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. Results of range are shown in the following tables. iii) Precision Based on the ICH Q2 (R1) guidelines, precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Results of precision (repeatability and intermediate precision) are shown in Tables no. 6 and 7. Three concentrations of eugenol solution (10, 20 and 30 µg mL-1) were prepared. The precision of the method was assessed by analysing eugenol for repeatability and intermediate precision (a) Repeatability (intraday) was assessed by analysing eugenol in three different concentrations (10, 20 and 30 µg mL-1) of three times a day. The % RSD was calculated for absorbance thus obtained, to obtain the intra-day variation. (b) Intermediate precision Intermediate precision (inter-day) was established by analysing three different concentrations (10, 20 and 30 µg mL) of eugenol for three different days. The % RSD was calculated for absorbance thus obtained, to obtain the inter-day variation. iv) Ruggedness: Ruggedness was determined by carrying out analysing 5µg/mL concentration solution in methanol six times by two different analysts at 421nm. The results were indicated as %RSD.

III. METHOD DEVELOPMENT&METHOD VALIDATION

Choice of solvents for phytochemical- Eugenol: -. Different solvents like Chloroform, Methanol and Water were screened for solubility of Eugenol. The results are as tabulated in table no-1. As it can be seen in the table Eugenol was completely soluble in methanol individually. Hence, Methanol was optimized from all the conditions based on solubility, peak quality, non-interference at the specified wavelength and safety as the solvent to prepare the stock solutions. Selection of analytical wavelengths Standard solutions of Eugenol were (1 µg/mL, 10 µg/mL, 100 µg/mL respectively) were scanned in the range of 200-400 nm against blank. The resultant spectra are displayed in the graphs no 1.

Method Validation for Eugenol: -

Calibration Curve of standard Eugenol (STD). The Beer Lambert's range was obtained for Eugenol by plotting calibration curves at predetermined wavelength. The calibration curve Data is given in the table no 3. The calibration plots are displayed below in graph

Linearity Curve of sample containing Eugenol. As can be seen, good correlation was obtained between absorbance values and concentration of the drugs in the concentration range of 10-60 µg/mL.

Range study: - As can be seen, good correlation was obtained between absorbance values and concentration of the drugs in the concentration range of 15- 105 µg/mL.

Precision study Precision analysis was performed by carrying out intraday and interday precision studies. The standard solution was analysed and the results are as below.

Intraday precision Intraday precision studies were carried out by recording the absorbance of the sample containing Eugenol in triplicate against blank, three times in a day. The results are depicted in table no 7.

Interday precision Inter-day precision studies were performed by carrying out analysis of standard solution on three consecutive days in triplicate. The results are as tabulated below in table no-7. As it can be seen % RSD for sample containing Eugenol for day 1, day 2 and day 3 was found to be < 2%. Hence the developed method complied with requirements of interday precision.

Ruggedness The robustness of the method was determined by introducing deliberate changes in experimental conditions during the analysis solution of drug by using different UV spectrophotometer, change of analyst and by altering the composition of the diluent (water: methanol, v/v). Ruggedness was determined by analysing 10 µg/mL concentration solution in methanol six times by two different analysts at 421nm. The results are as depicted in Table no 9.

As it can be seen that deliberate changes of Analyst introduced did not adversely affect the results of analysis as % purity was within the acceptance criteria of 90-110%. Hence, the UV method developed was found to be robust.

OBESERVATIONS AND CALUCLATIONS

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METHOD DEVELOPMENT

1.Choice of solvents for Phytochemicals

Table No -1-Choice of solvents for Eugenol

Standard Eugenol	Solvents	Solubility	Interference in the graph	Safety	Absorption
	Water	Soluble	More	Safe	Less Absorption
	Methanol	Very soluble	Less	Safe	Good Absorption & High Rwsolution
	Chloroform	Soluble	Less	Carcinogenic	Good Absorption & Low Rwsolution

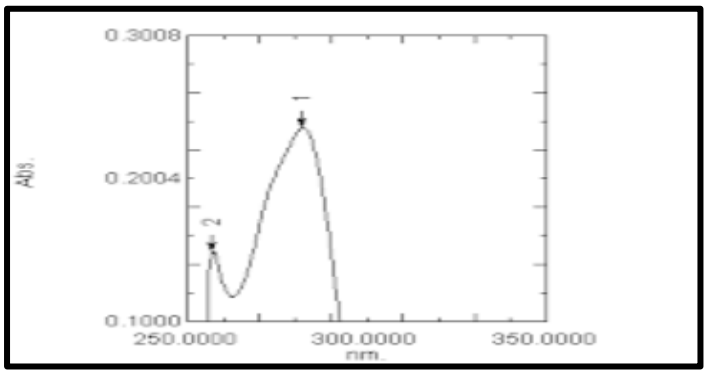
2. Choice of wavelength for Phytochemical

Selection of analytical wavelength

Standard solutions of Eugenol of concentration 1 µg/mL was scanned in the range of 200-400 nm against blank. It showed maximum absorption and sharp pointed spike in the spectrum at 282 nm. So, for Eugenol, 282 was chosen as λ max wavelength for UV spectroscopic experiment. The spectra was drawn as absorbance v/s wavelength. The resultant spectra are displayed in the graph no 1.

Table No -2-Choice of wavelengths for Phytochemicals

Sr. No	Phyto chemicals	Selected Wavelength λ max
1	Standard Eugenol	282 nm



Graph no-1 Standard Eugenol Graph

3. Calibration Curves of Standard Drug

Calibration Curve of standard Eugenol

The Beer Lambert's range was obtained for Eugenol drug Standard by plotting calibration curves at selected wavelength. The Sets of dilution of solution ranging from concentration 5-80 µg/ml were measured at 282 nm. The calibration curve Data is given in the table no 3. Calibration curve was obtained by plotting the graph of Absorbance v/s Concentration in µg/mL. Linearity is found in the absorbance of concentration range of 5-80 µg/with standard deviation $R^2 = 0.999$. The calibration plots are displayed above graph no.-1 The linearity is found in the concentration range of 5-80 µg/mL of standard Eugenol .

Table No. 3- Calibration Curve of standard Eugenol (STD)

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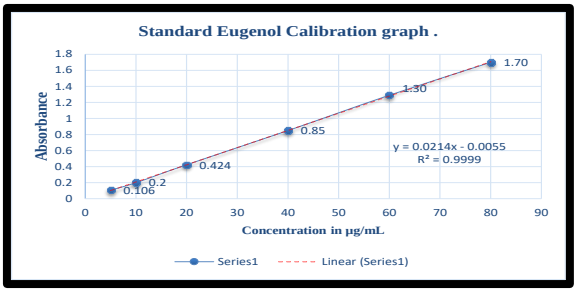
Sr. No	Concentration	Absorbance
1	5 µg/mL	0.106
2	10 µg/mL	0.200
3	20 µg/mL	0.424
4	40 µg/mL	0.850
5	60 µg/mL	1.290
6	80 µg/mL	1.650



Graph no -2. Graph showing standard Eugenol calibration graph

4.Linearity Curves of sample containing Phytochemicals

Table No.4 – Linearity Data for sample containing Eugenol

Sr. No	Concentration	Absorbance
1	5 µg/mL	0.104
2	10 µg/mL	0.213
3	20 µg/mL	0.410
4	40 µg/mL	0.835
5	60 µg/mL	1.278
6	80 µg/mL	1.698

Graph no. -3.- Linearity curve for sample containing Eugenol

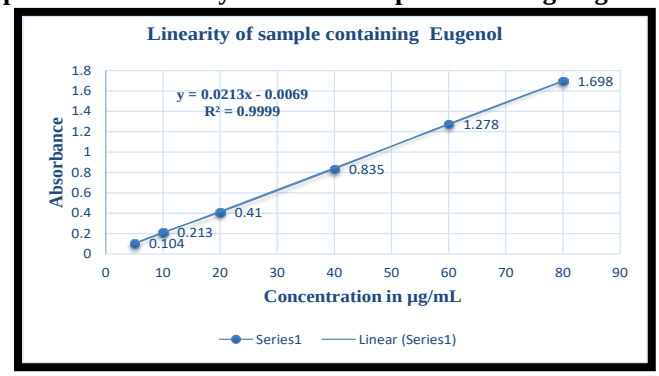


Table no.5- Summary of linearity data for sample containing eugenol

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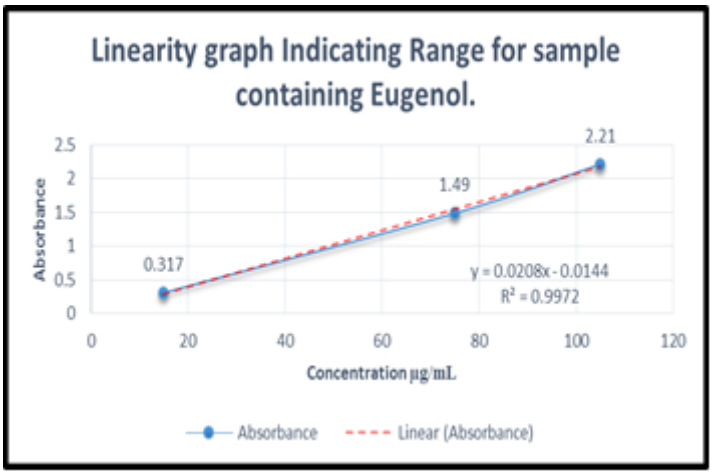
Parameters	At 204nm
Beer's law range	10-60 µg/mL
Regression equation	y=0.047x-0.021
Slope (b)	0.047
Intercept (c)	-0.021
Correlation coefficient (r^2)	0.999

As can be seen, good correlation was obtained between absorbance values and concentration of the drugs in the concentration range of 10-60 µg/mL.



Graph no -4. Linearity graph indicating Range for sample curve for sample containing

Range data for sample containing Eugenol

Range Study

In the Range study which was carried out between the concentration range of 15-105 µg/mL. It was found that this method is linear in the range of 15-105 µg/mL. Linearity graph for Range study was plotted with absorbance against Concentration in µg/mL with standard deviation of acceptance value of 0.999. Hence this method can be applied for UV spectroscopy study in the range of 15-105 µg/mL of Eugenol in the Eugenol containing sample.

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Table no – 6 Range study for the sample containing Eugenol

Sample Dilution	Concentration	Absorbance
1 ml methanolic extract diluted up to 10 mL with methanol	15 µg/ mL	0.317
5 ml methanolic extract diluted up to 10 mL with methanol	75 µg/ mL	1.49
7 ml methanolic extract diluted up to 10 mL with methanol	105 µg/ mL	2.210

As can be seen, good correlation was obtained between absorbance values and concentration of the drugs in the concentration range of 15- 105 µg/mL.

Precision study

Precision analysis was performed by carrying out intraday and interday precision studies. The standard solution was analysed and the results are as below:

Precision Study

Precision analysis was performed by carrying out intraday and interday precision studies. The sample containing solution was analysed and the results are as below:

Intraday precision

Intraday precision studies were carried out by recording the absorbance of the sample containing Eugenol in triplicate that is in 3 different concentration of sample containing Eugenol against blank, three times in a day. The results are depicted in table 6. As can be seen in the table % RSD for Eugenol was found to be < 2%. Hence the developed method complied with requirements for intraday precision

Interday precision

Inter-day precision studies were performed by carrying out analysis of sample containing Eugenol on three consecutive days in triplicate. The results are as tabulated below in table no 7.

As can be seen % RSD for sample containing Eugenol for day 1, day 2 and day 3 was found to be < 2%. Also, in the system precision studies the concentration of 20 µg/mL of sample containing Eugenol was analysed. It was that % RSD for sample containing Eugenol found to be < 2%. Hence the developed method complied with requirements of interday precision.

Intraday precision

Intraday precision studies were carried out by recording the absorbance of the sample containing Eugenol in triplicate against blank, three times in a day. The results are depicted in table no 7.

Table no -7 Intraday Precision Data

Precision Eugenol	Intraday 1			Intraday 2			Intraday 3		
Concentration / Sr. No.	10 µg/ml	20 µg/ml	30 µg/ml	10 µg/ml	20 µg/ml	30 µg/ml	10 µg/ml	20 µg/ml	30 µg/ml
1	0.207	0.420	0.631	0.210	0.417	0.630	0.209	0.419	0.631
2	0.210	0.421	0.629	0.211	0.419	0.631	0.211	0.420	0.627
3	0.211	0.417	0.635	0.209	0.420	0.627	0.208	0.421	0.635
4	0.209	0.419	0.631	0.211	0.421	0.635	0.207	0.418	0.631
5	0.210	0.420	0.632	0.210	0.418	0.631	0.210	0.420	0.629
6	0.208	0.421	0.628	0.207	0.419	0.632	0.212	0.421	0.632
Average	0.209	0.420	0.631	0.210	0.419	0.631	0.210	0.420	0.631
SD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% RSD	0.70	0.36	0.39	0.72	0.34	0.41	0.89	0.28	0.43

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As seen above % RSD for Eugenol was found to be < 2%. Hence the developed method complied with requirements for intraday precision.

Interday precision

Inter-day precision studies were performed by carrying out analysis of mixed standard solution on three consecutive days in triplicate. The results are as tabulated below in table no 8.

Table No -8 Interday Precision study of Sample containing Eugenol

Precision Eugenol	System Precision	Interday 1			Interday 2			Interday 3		
Concentration / Sr. No	20 µg/ml	10 µg/ml	20 µg/ml	30 µg/ml	10 µg/ml	20 µg/ml	30 µg/ml	10 µg/ml	20 µg/ml	30 µg/ml
1	0.424	0.211	0.424	0.633	0.212	0.421	0.629	0.213	0.421	0.635
2	0.425	0.210	0.423	0.631	0.207	0.422	0.63	0.209	0.418	0.631
3	0.424	0.208	0.421	0.629	0.211	0.425	0.631	0.211	0.424	0.628
4	0.423	0.212	0.42	0.635	0.213	0.424	0.632	0.210	0.419	0.634
5	0.425	0.210	0.422	0.631	0.209	0.423	0.626	0.207	0.426	0.63
6	0.424	0.211	0.424	0.628	0.209	0.419	0.628	0.210	0.425	0.633
Average	0.424	0.210	0.422	0.631	0.210	0.422	0.629	0.210	0.422	0.632
SD	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% RSD	0.38	0.65	0.39	0.41	0.65	0.40	0.44	0.65	0.41	0.42

As seen above % RSD for sample containing Eugenol for day 1, day 2 and day 3 was found to be < 2%. Hence the developed method complied with requirements of interday precision.

Ruggedness

The robustness of the method was determined by introducing deliberate changes in experimental conditions during the analysis solution of drug by using different UV spectrophotometer, change of analyst and by altering the composition of the diluent (water: methanol, v/v). Ruggedness was determined by analysing 10 µg/mL concentration solution in methanol six times by two different analysts at 421nm.The results are as depicted in table no 9.

Ruggedness

Ruggedness was determined by analysing 10 µg/mL concentration solution in methanol six times by two different analysts at 225 nm. The results are as depicted in table no 9. As seen the deliberate changes of analyst introduced did not adversely affect the results of analysis as % RSD was found to < 2% and within the acceptance criteria. Hence, the UV method developed was found to be robust.

Table No- 9 Ruggedness data

Concentration (µg/ mL)	Absorbance by Analyst 1		Absorbance by Analyst 2	
10	0.0211	SD=0.00039 R.S. D=0.017 %R.S.D.=1.76%	0.0211	SD=0.00039 R.S. D=0.017 %R.S.D.=1.76%
10	0.0212		0.0212	
10	0.0212		0.0212	
10	0.0211		0.0212	
10	0.0211		0.0211	
10	0.0212		0.0211	

As seen above, the deliberate changes of Analyst introduced did not adversely affect the results of analysis as % purity was within the acceptance criteria of 90-110%. Hence, the UV method developed was found to be robust.

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Analysis of sample containing Eugenol by chemical test

Table no -10 Test for Eugenol by chemical test

Sr. no.	Name of test	Observation	Inference
1	Marketed product add sudan red III solution	Red color obtained with globules	Present of Volatile oil
2	Marketed product add few drops of tincture alkane	Red color indicates	Presence of Volatile oil
3	Drug product + ferric chloride (5%) solution were mixed	Blue coloration	Presence of Volatile Oil-Eugenol

IV. CONCLUSION

An analytical method was developed for marketed herbal formulation containing Eugenol, and validated by UV method as per 4 ICH guidelines.

A titrimetric method was developed for the determination of Vit. C in the marketed formulation.

The new analytical procedure described for phytochemicals was linear, precise, and rugged and is suitable for determination of Eugenolin bulk and pharmaceutical dosage forms.

The observations of the validation parameters such as linearity, precision, rugudness , shows that the developed methods can be employed for routine analysis of semisolid dosage form of Eugenol.The result obtained from the validation parameters met the ICH requirement as well as obeys BEER'S law.

Determination and estimation of the content of active constituent in product to check its authenticity by UV, chemical tests was successfully done .

On Verification of the presence and amount of content of API stated on label complies with stated amount for Eugenol.Completion of estimation and determination of the herbal drugs in marketed preparation showed the presence of the drugs as per the label claims.

Ultraviolet Spectroscopy method adopted for the determination of Eugenol in toothpaste and carrying out further calculations ,successful results were obtained as per the label claim on the marketed preparation.

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