ejpmr, 2018,5(5), 321-326

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

DEVELOPMENT AND VALIDATION OF RP-HPLC METHODS FOR DETERMINATION OF MARKERS IN POLYHERBAL FORMULATION NBIOTIC PREMIX

Kotagiri Ravikanth, Anirudh Sharma, Deepak Thakur* and Sameer Sapra

R & D Centre, AYURVET LIMITED, Village Katha, P.O. Baddi – 173205, District Solan, Himachal Pradesh, India.

Corresponding Author: Deepak Thakur

R & D Centre, AYURVET LIMITED, Village Katha, P.O. Baddi – 173205, District Solan, Himachal Pradesh, India.

Article Received on 07/03/2018 **Article Revised on 28/03/2018** **Article Accepted on 17/04/2018**

ABSTRACT

Objective: The objective is standardization of herbal formulation "Nbiotic Premix" a natural growth promoter, by using reverse phase high performance liquid chromatography (RP-HPLC)**. Materials and Methods:** RP-HPLC methods were prepared for the standardization of the Nbiotic Premix with respect to the biomarker compounds 6-gingerol and Thymol. The developed methods were validated in accordance with the statistical method of validation given in ICHQ2R1. **Results:** Developed methods were being successfully applied in identification and quantification of the phytoconstituents. The average recovery of the 6-gingerol (96.57%) and thymol (96.61 %) were computed from regression equation. RSD for inter day and intraday were also found to be less than 2.0 %. **Conclusion:** The present findings concluded that the RP-HPLC methods developed for standardization of formulation was found to be precise, simple, specific and accurate and can be used for the quality control of formulation and herbs used.

KEYWORDS: Nbiotic Premix, Natural growth promoter, Thymol, 6-gingerol, RP-HPLC, Standardization

1. INDRODUCTION

Growth promoters are chemical and biological substances, added in the pigs and poultry feed thereby improving overall growth, feed utilization, immune system stimulation, regulation of the intestinal micro-flora, and reduction in the morbidity and mortality rate due to numerous diseases[1] which in turn helps in better production and financial gains too. Antibiotic growth promoters (AGP) are now a day highly criticized because of deleterious effects on animal health, as the possibilities of residual effects on tissues long after withdrawal. Antibiotics act by altering the microbial metabolism which leads to the suppression of growth of pathogenic microbes present in the gut.[2]

The feed manufacturers are focusing towards natural feed additives as growth promoters which includes botanical additives mainly blend of herbs or extracts.[3,4] These herbs carry number of secondary plant metabolites such as terpenoids, alkaloids, flavonoids, bitters, mucilage, saponins and tannins. The biggest advantage of natural growth promoters (NGP) over AGP is that they do not bear any risk regarding bacterial resistance or undesired residues in animal products such as meat, milk or eggs.[5] It also helps in maintaining the balance of gut bacterial flora and reduces the nutrients availability for pathogens. The efficacy of any polyherbal formulation can only be maintained by standardizing the secondary plant metabolites which are responsible for pharmacological activity. Keeping the above facts in view the present study was designed to optimize the major constituents of herbs present in Nbiotic Premix.

Nbiotic Premix is a proprietary polyherbal formulation of AYURVET LIMITED, used as Natural growth promoter for pigs and poultry. It is a perfect blend of essential oils and other secondary plant metabolites.

Novel HPLC method was developed and validated for the quantification of 6-gingerol and thymol. Both the constituents are well documented for antibacterial activity against number of pathogenic bacteria.[6,7] The analytical methods are validated for linearity, accuracy and precision in accordance with the statistical method of validation mentioned in ICHQ2R1.[8,9] The average recovery of 6-gingerol and thymol was computed from the regression equation. Benchmark limits of two markers are set as quality control check points for batch to batch consistency of formulation in its efficacy. The method is simple precise, specific, accurate and has the potential for routine quality control of the formulation.

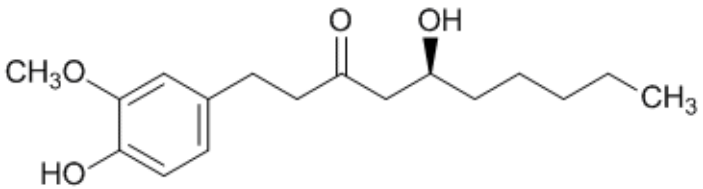


Fig 1: 6-gingerol.

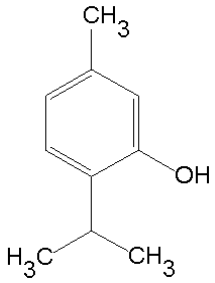


Fig 2: Thymol.

2. MATERIAL AND METHODS

2.1 Reagents and materials

All the reagents and solvents were of AR or HPLC grade as per requirement. The active compound 6-gingerol was isolated in our lab and structure was established by interpreting the 1H, 13C & 2D NMR spectra, Thymol standard was procured from SD Fine Chemical Limited. Latest controlled samples of Nbiotic Premix were obtained from the QA/QC department of AYURVET LIMITED, Baddi.

Preparation of standard solution of 6- Gingerol: Accurately weighed around 5 mg of standard dissolved in 50 ml of methanol to obtain stock concentrations of 100 µg/ml. Stock solution was further diluted to obtain the dilution range of 1 – 12 µg/ml and then injected in HPLC in order to prepare the calibration graphs and quantification of bioactive.

Preparation of standard solution of Thymol: Accurately weighed around 5 mg of standard dissolved in 50 ml of Acetonitrile: Water (50:50) to obtain stock concentrations of 100 µg/ml. Stock solution was further diluted with same solvent mixture to obtain the dilution range of 15 – 75 µg/ml and then injected in HPLC in order to prepare the calibration graphs and quantification of bioactive.

Preparation of test solution for 6-gingerol detection: For the quantification of 6-gingerol, 2 g Nbiotic Premix was refluxed with 50 ml of petroleum ether (60ºC – 80ºC) for 3 hours and filtered, repeated the process one more time. The defatted sample was extracted with 50 ml of methanol under reflux conditions for 3 hours and filtered, repeated the process twice. The final volume was made to 100 ml with methanol, filtered the solution through 0.45 µm membrane filter before injecting into HPLC.

Preparation of test solution for thymol detection: For the quantification of thymol, 2 g Nbiotic Premix was sonicated with 70 ml of Acetonitrile: Water (50:50) for 15 minutes, make volume up to 100ml with same solvent mixture. Then take 1 ml of the above stock and dilute to 10 ml in volumetric flask with same solvent mixture, filtered the solution through 0.45 µm membrane filter before injecting into HPLC.

2.2 High Performance Liquid Chromatography Apparatus and Conditions

6-gingerol and Thymol content were analyzed by High Performance Liquid Chromatography (WATERS, binary pump 515 with PDA 2996 detector, USA). The data was acquired on the Empower 2.0 controlling software. Separation was obtained on Phenomenex luna C18 column (250 mm x 4.6 mm, 5µm).

Selection and Optimization of chromatographic condition

To optimize the RP-HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for 6-gingerol (Fig. 1) and thymol (Fig. 2) were obtained by using Water : Acetonitrile in 45:55 v/v ratio and Water : Acetonitrile in 50 : 50 V/V ratio respectively as a mobile phase in isocratic mode. The mobile phases were filtered through 0.45 µm Millipore filter and degassed before use. The flow rate was adjusted to 1.0 ml/min for both markers. Injection volume was adjusted to 20 µl and detection was made at 280nm and 276nm respectively.

System suitability

The analytical results obtained by the method developed are only valid if the defined system suitability criteria are fulfilled. In this investigation, the experimental result indicates that the chromatographic system was suitable for intended analysis. Standard solution mixture containing known concentration of 6-gingerol and thymol were injected seven times, separately. RSD values for peak area and retention time of standard suggested the reproducibility for these parameters.

2.3 Validation of the Proposed Method: The proposed methods were validated for the determination of 6-gingerol and thymol using following parameters as per ICH guidelines.

a. **Calibration:** The marker compounds in the formulation were quantified using a calibration curve established with five dilutions of the standard. The corresponding peak area in formulation was plotted against the concentrations of the standard injected. Peak identification was achieved by comparison of both the retention time (RT) and UV absorption spectrum with those obtained for standard.

b. Linearity: Linear regression analysis was used to calculate the slope, intercept, and /regression coefficient (r2) for calibration plot. Linearity was determined by using five concentrations of the standard solution. Response were found to be linear in the concentration ranges investigated (Fig. 3;d, Fig. 4; d, Table 1).

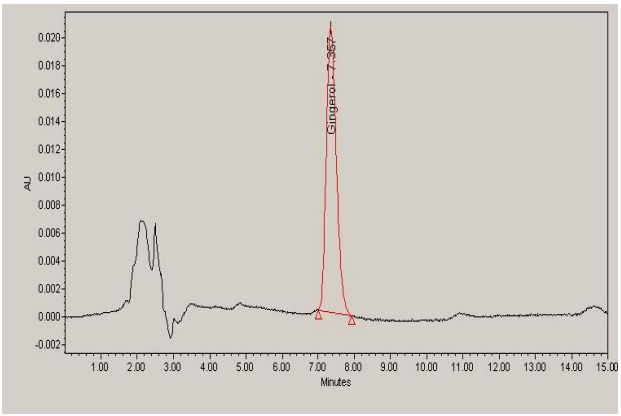
c. Range: Range is the interval between upper and lower concentration of analyte in sample for which it has been demonstrated that the analytical method has suitable level of precision, accuracy and linearity. The linear response were observed over a range of 9-45 ppm (Fig. 3; d, Table 1) for 6-gingerol and 15-75 ppm for thymol (Fig. 4; d, Table 1).

d. Precision: Three different concentrations of marker compound solution in triplicates were injected on three different times within the same day and repeating the same on three different days to record intra-day and inter-day variations in the results. The low % RSD values of intraday and interday (Table 1) for the marker compounds 6-gingerol and thymol reveals that the proposed methods are precise.

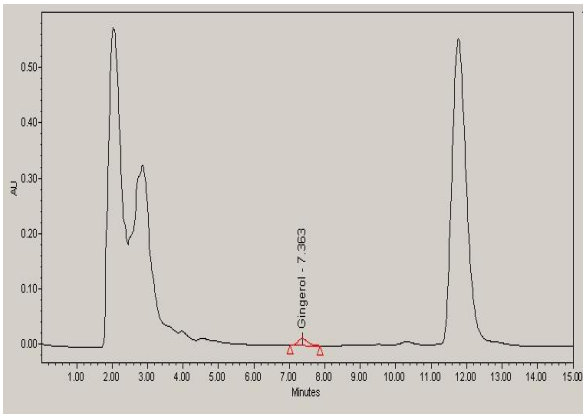
e. Limit of Detection (LOD) and Limit of Quantification (LOQ): For determination of limits of detection and quantification, different dilutions of the markers were injected with mobile phase as blank and determined on the basis of signal to noise ratio 3:1 and 10:1 respectively. The LOD and LOQ for the standard compounds were calculated and tabulated (Table 1).

f. Selectivity: The retention time of 6-gingerol and thymol and their counterpart in the formulation were 7.36±0.02 and 5.25±0.02 minute respectively. The UV-Vis spectrum of marker compounds were compared with their counterpart in formulation at three different positions, the peak start, peak center, and peak end. There was good correlation between spectra obtained at each of the three positions. The 6-gingerol and thymol peaks were, therefore, not masked by any peak of other compound present in the formulation (Figures 3,4 ; c), which was indicative of peak purity.

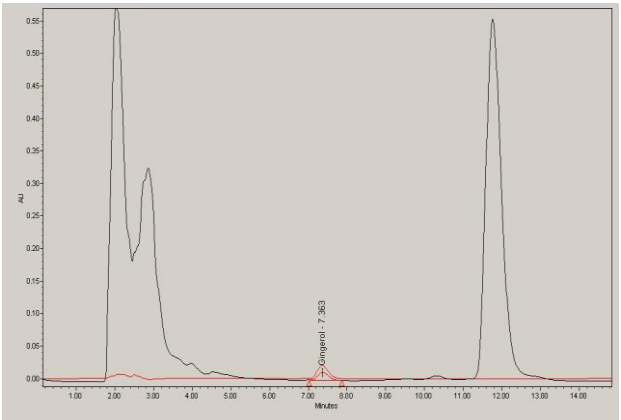
g. Accuracy: Recovery experiments were conducted to check for the presence of positive or negative interferences from other ingredients/excipients present in the formulation and to study the accuracy of the method. Recovery was determined by the standard addition method. 6-gingerol and thymol standard were added to the formulation at two different concentrations, extraction and analysis were performed as described above. Recovery was calculated for each standard at each concentration (Table 2). The low value of relative standard deviation indicates that the proposed methods are accurate.



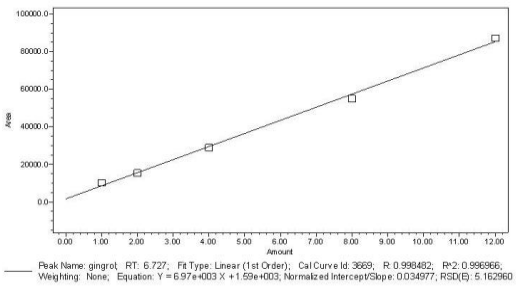
(a)



(b)



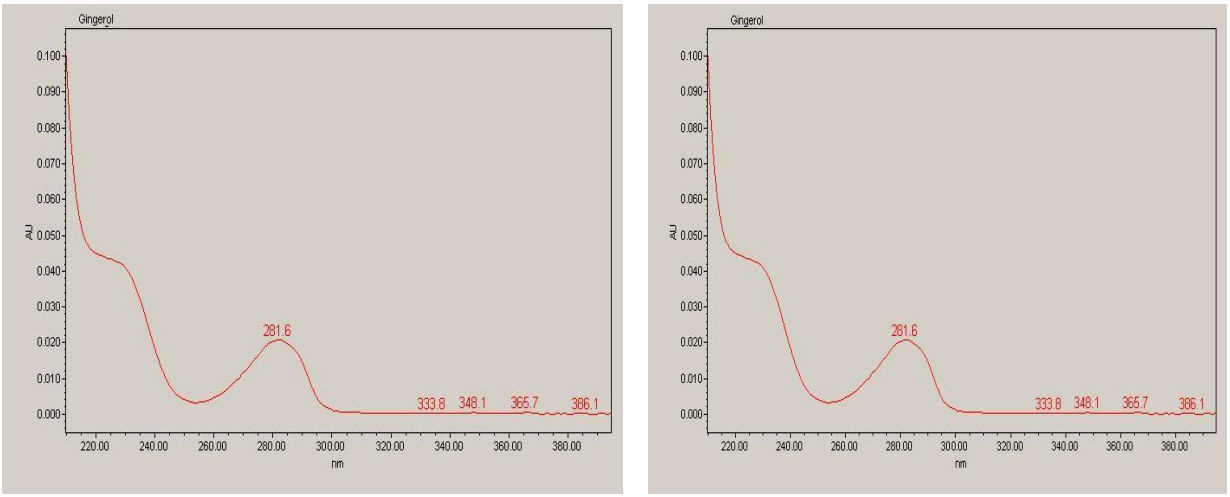
(c)



Peak: gingrol

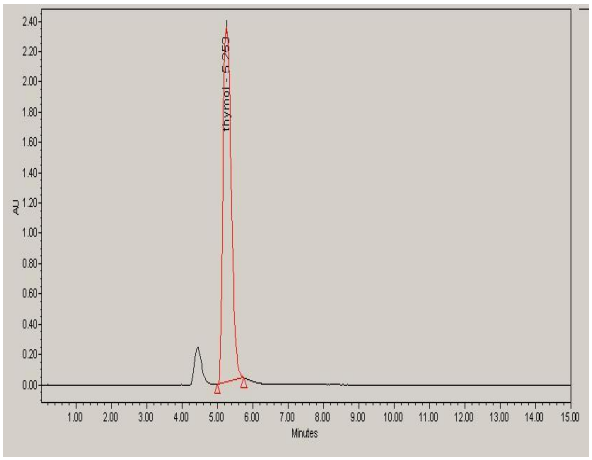
	Sample Name	Result Id	Peak Name	X Value	Response	Calc. Value	% Deviation	Manual
1	Gingerol std 1 ppm	3684	gingrol	1.000	10126.775	1.225	22.52	No
2	Gingerol std 2 ppm	3681	gingrol	2.000	15338.046	1.972	-1.39	No
3	Gingerol std 4 ppm	3678	gingrol	4.000	28804.145	3.903	-2.42	No
4	Gingerol std 8 ppm	3675	gingrol	8.000	54885.762	7.644	-4.46	No
5	Gingerol std 12 ppm	3673	gingrol	12.000	87047.083	12.256	2.13	No

(d)

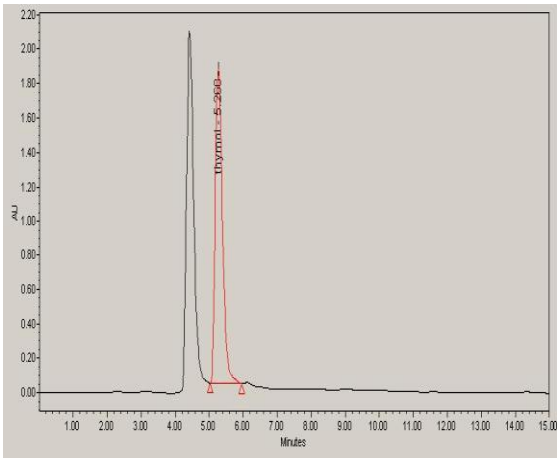


(e) **(f)**

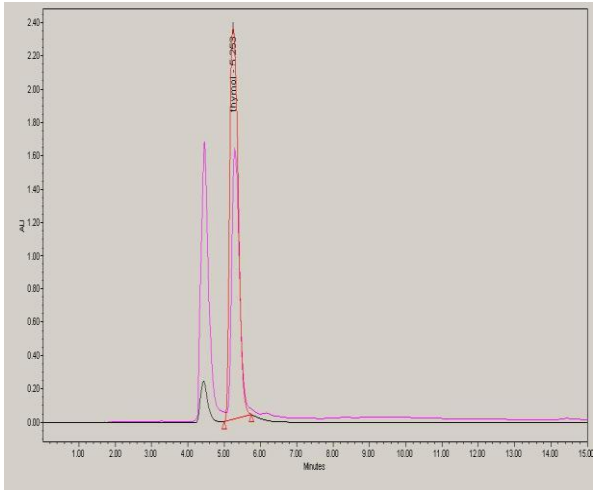
Figure 3: Chromatograms showing the resolution of marker compound in the formulation Nbiotic Premix. (a) Chromatogram of standard 6-gingerol. (b) Chromatogram of sample Nbiotic Premix. (c) Overlay of the 6-gingerol chromatograms i.e. sample against standard. (d) Calibration plot for 6-gingerol standard. (e) Spectral scan of standard 6-gingerol. (f) Spectral scan of 6-gingerol in Nbiotic Premix.



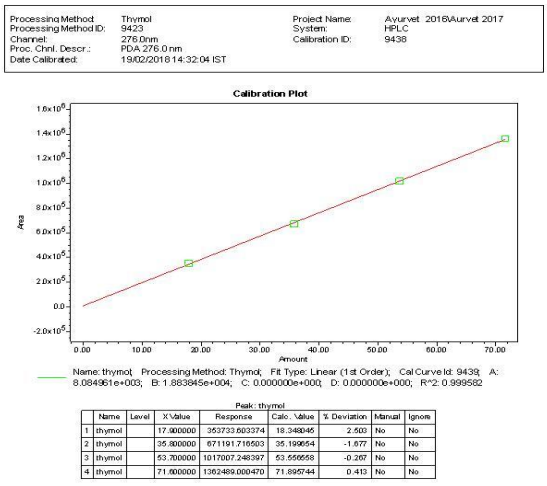
(a)



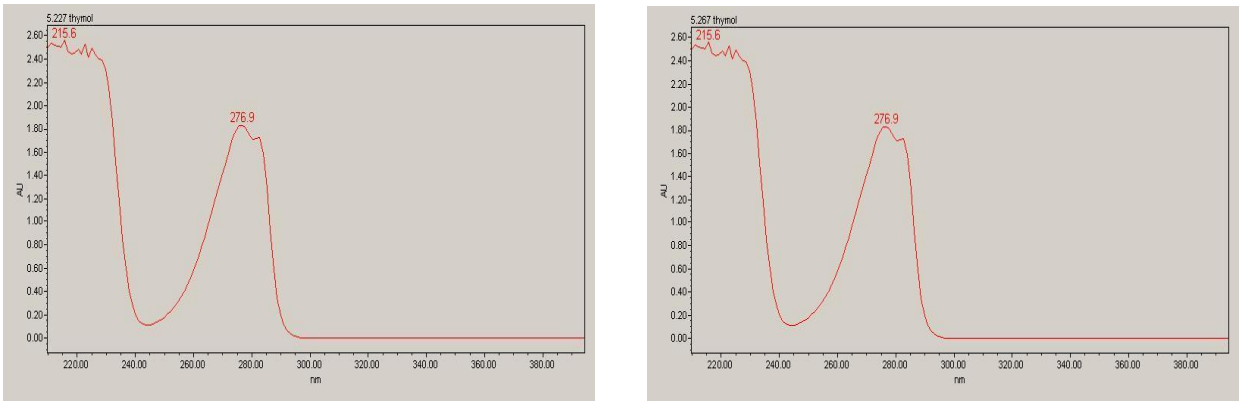
(b)



(c)



(d)



(e) **(f)**

Figure 4: Chromatograms showing the resolution of marker compound in the formulation Nbiotic Premix. (a) Chromatogram of standard Thymol. (b) Chromatogram of sample Nbiotic Premix. (c) Overlay of the thymol chromatograms i.e. sample against standard. (d) Calibration plot for thymol standard. (e) Spectral scan of standard thymol. (f) Spectral scan of Thymol in Nbiotic Premix.

Table 1: Results of precision, LOD, LOQ, linear regression analysis and their correlation coefficient for quantitative analysis of different marker compounds.

Sr. no.	Parameters	6-Gingerol	Thymol
1	Concentration range [µg ml^{-1}]	6 - 48	15 - 75
2	Regression equation	Y = 6461.x + 3058	Y=18838x + 8085
3	Correlation Coefficient (r2)	0.997	0.999
4	Amount of marker compound in Nbiotic Premix [%] (w/w)a	0.05	1.72
5	Method precision (Repeatability) – RSD %	1.7	0.5
6	Intermediate precision (Reproducibility) - RSD [%] Intraday 1 Interday 3	 1.2 1.5	 0.79 0.81
7	LOD	0.039 µg ml^{-1}	0.062 µg ml^{-1}
8	LOQ	0.120 µg ml^{-1}	0.186 µg ml^{-1}

y = peak area response
x = amount of marker compound
a = Mean ± SD, n=6

Table 2: Results from determination of recovery.

Sr.no	Parameter	6-Gingerol			Thymol		
1	Initial concentration in formulation [mg g-1]	0.5	0.5	0.5	17.2	17.2	17.2
2	Concentration added [mg g-1]	0	2.0	4.0	0	40.0	80.0
3	Total concentration [mg g-1]	0.5	2.5	4.5	17.2	57.2	97.2
4	Concentration found [mg g-1]	0.48	2.41	4.38	16.80	54.20	94.68
5	RSD [%] (n=7)	0.98	0.95	0.95	0.97	0.96	0.98
6	Recovery [%]	96.0	96.4	97.3	97.67	94.75	97.41
7	Mean recovery [%]	96.57			96.61		

3. RESULT AND DISCUSSION

The exercise was carried out to ensure the consistency in the desired pharmacological effect by establishing the lowest possible limit for two of its most relevant bioactive phytoconstituents. Developed methods were being successfully applied in identification and quantification of the phytoconstituents. The average recovery of the 6-gingerol (96.57%) and thymol (96.61 %) were computed from regression equation. RSD for inter day and intraday were also found to be less than 2.0 %. Standardization of these phytotherapeutic constituents with validated analysis methods will ensure the batch to batch consistency in efficacy of the product on commercial scale.

As two herbs mentioned under experimental investigation are among the main active ingredients in the polyherbal formulation, quantifying them with their respective bioactive markers and setting the limits will help us in ensuring authenticity and efficacy of the product in turn.

4. CONCLUSION

New HPLC methods were developed for the fine resolution of two phyto constituents of the product.

Nbiotic Premix, a proprietary polyherbal feed supplement of AYURVET LIMITED. Standardization of phytotherapeutic constituents '6-Gingerol and Thymol' with validated analysis methods will help in ensuring the batch to batch consistency in quality & efficacy of the product on commercial scale. Further, methods reported here are simple, precise, accurate and are suitable for the routine analysis and quantification of the active constituents in formulation containing them.

5. CONFLICTS OF INTEREST

All authors have no conflicts of interest to declare.

6. ACKNOWLEDGEMENT

We thank Mr. Mohanji Saxena, Managing Director, AYURVET LIMITED, for providing necessary facilities, help and guidance.

7. REFERENCES

1. Thomke S and Elwinger K. "Growth promotants in feeding pigs and poultry ii; mode of action of antibiotic growth promotants". Annales de Zootechnie, 1998; 47: 153-167.
2. Chattopadhyay MK. "Use of antibiotics as feed additives: a burning question". Front Microbiol, 2014; 5: 334.
3. Newcomb MD. "Herbs as a source of nutrition versus herbs as a source of drugs: A matter of claims, biology and regulations". Proc. of Alltech's 15th Annu. Symp., Biotehn. in the Feed Industry. Alltech Technical Publications, Nottingham University Press. Nicholasville, KY, 1999; 295-300.
4. Wezyk S, Poltowicz K and Sosnowka-Czajka E. "Effect of replacing antibiotic growth stimulants with herbs on performance and meat quality of chicken broilers". Proc. of XXI World's Poult. Cong. Montreal, Canada, 2000; (August) 20-24.
5. Sethiya NK. "Review on Natural Growth Promoters Available for Improving Gut Health of Poultry: An Alternative to Antibiotic Growth Promoters". Asian Journal of Poultry Science, 2016; 10(1): 1-29.
6. Park M, Bae J, Lee DS. "Antibacterial activity of [10]-Gingerol and [12]-Gingerol isolated from ginger rhizome against periodontal bacteria". Phytother Res, 2008; Nov; (11) 22.
7. Encun D, Liping G, Zhui L, Weiwei W, Dan L, and Yuming G. "In vitro antibacterial activity of thymol and carvacrol and their effects on broiler chickens challenged with Clostridium perfringens". J Anim Sci Biotechnol. 2015; 6: 58.
8. ICH Harmonised guidelines, 2005. Q2 (R1), International conference on harmonization, Geneva, 1-13.
9. ICH harmonized tripartite guideline, 2009. Q8 Guidelines for pharmaceutical Development Revision-2, Geneva, Switzerland, 12-14.