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RESEARCH ARTICLE

VALIDATED ANALYTICAL METHODS FOR THE QUALITY STANDARDIZATION OF THE HERBAL MEDICINE JANOVA, AN ANESTROUS TREATMENT

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ABSTRACT

Background: The aim of the present study was to standardize Janova by two biomarkers Piperine and Gingerol-6 present in the herbs used for formulation by adopting sophisticated instruments High performance liquid chromatography and High performance thin layer chromatography methods. Janova is a very effective Ayurvedic polyherbal medicine used to treat anoestrus, one of the most common causes of infertility in cattle. Ingredients of commercial herbal medicines are assessed for quality primarily to ensure their safety. Janova contains four herbs including *Zingiber officinale, Piper nigrum and Piper longum*.

Methods: High performance liquid chromatography (HPLC) and High performance thin layer chromatographic (HPTLC) methods were prepared for the standardization of the Janova with respect to the biomarker compounds Gingerol-6 and Piperine of respective herbs. The developed methods were validated on parameters including linearity, selectivity, precision, recovery, LOD and LOQ in accordance with the statistical method of validation given in *ICHQ2R1*.

Results: Methods developed were being successfully applied in identification and quantification of phytotherapeutic constituents. The average recovery of (Gingerol-6) 99.47 % and (Piperine) 98.20 % were computed from regression equation. RSD for inter-day and intra-day variability were also found to be less than 1%.

Conclusion: The outcome of the present investigation underlines the importance of standardization of Ayurvedic formulations and how overall phytochemical consistency of herbal medicines is pivotal to their efficacy. Establishment of harmonized multilaboratory-validated analytical methods developed may be further used to standardize other formulation containing Zingiber *officinale, Piper nigrum and Piper longum*.

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INTRODUCTION

Quality standardization of complementary medicine is fundamental for industry and practice as it strengthens their quality, safety and efficacy. It is proposed that the identification of active ingredients, pharmacological activities and eventual clinical applications are required for a comprehensive quality standardization system. The findings of this article indicate that the combination of various chromatographic methods could advance the methodology of quality standardization and enhance the overall confidence in herbal medicine for the health practitioner (Kotagiri Ravikanth *et al.,* 2013; Kotagiri Ravikanth *et al.,* 2014). Anoestrus is one of the most common causes of infertility in cattle. Among the different classes of anoestrus, postpartum anoestrus is a common cause of infertility in cross bred cows, leading to prolonged inter calving period, delay in conception, reduction in milk yield and number of calves in life time and thus causing heavy economic losses. Thus to increase dairy economy, in anoestrus animals estrus induction has significant importance (Peter *et al.,* 2009; Berger *et al.,* 1981). Janova, a proprietary polyherbal medicine of AYURVET LIMITED. It includes herbs namely Zingiber officinale, Piper Longum, Piper nigrum and Citrullus colocynthis. This herbal medicine for cattle is a complete potent solution for Anoestrus treatment. Clinical trails indicated that by supplementing Janova herbal preparation to the cattle, resulted in resumption of cyclic activity and further shorten the postpartum estrus days and helped to achieve conception early (Bhattacharya *et al.,* 2001; Ahmed *et al.,* 2003; Singal, 1995; Patil *et al.,* 2010). Selection of abundant compounds as markers is currently a major approach for the quality control of herbal medicines. We proposed a universal strategy to identify the effective combinatorial markers (ECMs) that are representative of the

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bioactivities of herbal medicines, and took them as chemical markers for quality standardization. Fingerprinting and quantification were employed to find out the common components in various batches of medicines. The analytical methods were validated as per ICHQ2R1 guidelines (ICH Harmonised guidelines, 2005). Two most effective compounds, Piperine (Figure 1) and Gingerol-6 (Figure 2), were therefore proposed as ECMs. Chemical fingerprints obtained by chromatographic techniques, are strongly recommended for the purpose of quality control of herbal medicines, since they might represent appropriately the "chemical integrities" of the herbal medicines and therefore be used for authentication and identification of the herbal products. Based on the conception of phytoequivalence, the chromatographic fingerprints of herbal medicines could be utilized for addressing the problem of quality control of herbal medicines (Kotagiri Ravikanth *et al.,* 2006). Thus different chromatographic techniques commonly used in the instrumental inspection of Janova are first comprehensively reviewed.

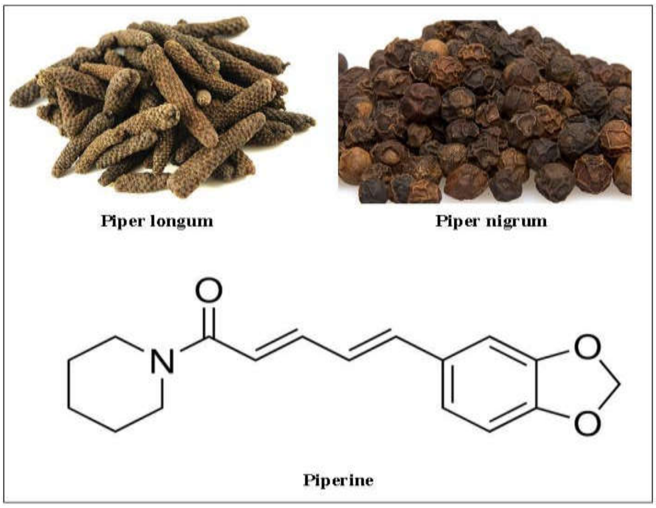


Figure 1. Piperine, a biomarker compound of Piper longum and Piper nigrum

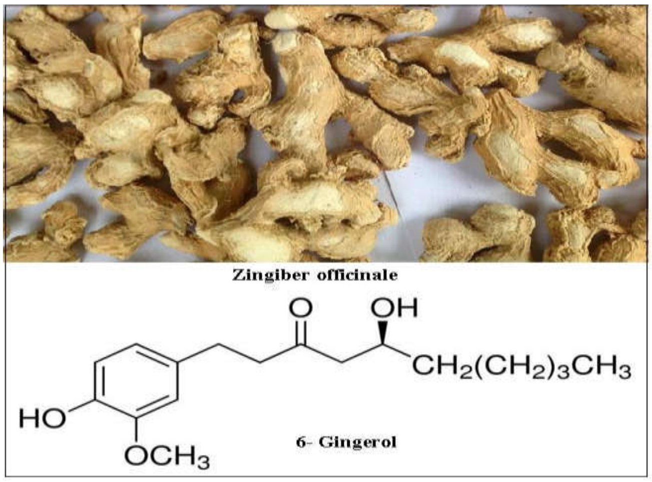


Figure 2. 6-Gingerol, a biomarker compound of zingiber officinale.

MATERIALS AND METHODS

Apparatus

HPTLC was performed with Camag HPTLC equipment (Muttenz, Switzerland) comprising Linomat-V auto sample applicator, Camag Scanner-III, Camag flat bottom and twin trough developing chamber, and UV cabinet with dual wavelength UV lamp. 20 × 10 cm aluminum 60F254 TLC plates (E-Merck-Germany) with stationary phase silica gel and layer thickness 0.2 mm were used for the resolution of chemical constituents. HPLC was performed with WATERS, USA having binary pump 515 with PDA 2996 detector. 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, 5 µm).

Reagents and materials

Chemicals and reagents used were of analytical reagent or HPLC grade as per requirement. Toluene, Diethyl ether, Dioxane, Acetonitrile and Water were purchased from RANKEM. 6-Gingerol was purchased from Sigma-Aldrich and Piperine was isolated in our lab and structure was established by interpreting the 1H, 13C and 2D NMR spectra. TLC plates were purchased from Merck (Darmstandt, Germany). Controlled samples of Janova were obtained from the QA/QC department of AYURVET LTD, Baddi.

Chromatographic conditions

HPTLC was performed using commercially-prepared, pre-activated (110°C) silica gel 60 F254 TLC plates. A Linomat V (Camag, Muttenz, Switzerland) automatic TLC applicator was used to apply samples and standards (marker compounds) on pre-activated (110°C) silica gel 60 F254 TLC plates under a flow of nitrogen gas and the delivery speed of the syringe was 10 s/ µl. Each TLC plate was developed to a height of about 9.0 cm, under laboratory conditions. Toluene: Diethyl ether: Dioxane (62.5: 21.5: 16 v/v/v) was the mobile phase developed for the resolution of and quantification of Piperine (Figure 1). Quantitative determination of Piperine spots were done by Camag TLC Scanner 3 at 340 using deuterium lamp with a slit size of 6 × 0.3 mm. HPLC was performed with WATERS, USA having binary pump 515 with PDA 2996 detector. 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, 5 µm). The mobile phase was filtered through 0.45 µm Millipore filter and degassed. To optimize the RP-HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for 6-Gingerol (Figure 2) was obtained with a mobile phase Acetonitrile: Water: 55: 45. Flow rate of 1.0 ml/min was set to get better reproducibility and repeatability. Selecting 280.0 nm as the detection wavelength resulting in acceptable responses and enabled the detection of compound under investigation.

Preparation of sample and standard solutions

Preparation of standard solutions

Stock solutions (0.05 mg/ml) of standards (marker compounds) A and B were prepared in methanol, different concentrations were spotted/injected in order to prepare the calibration graphs and quantification of bioactives.

Preparation of sample solution for 6-Gingerol and Piperine estimation

Weighed accurately around 3.0 g of Janova capsule powder and transferred to a 100 ml round bottom flask. Added 30 ml of methanol reflux for 1 hour on water bath filter the extract, repeat the process 2 more times. Transfer the combined extract to 100ml volumetric flask and adjust the volume with methanol. Filter the solution through 0.45 u filter before injecting into HPLC or application of spots on TLC.

RESULTS 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. Experimental studies have already been carried out to evaluate effectiveness of Janova in the anoestrus. 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 three 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.

Method validation

Validation parameters

The methods were validated according to ICH guidelines for linearity, precision, accuracy, selectivity, limit of detection and limit of quantification.

Calibration curve (Linearity)

The method was validated in accordance with the statistical method of validation given in ICHQ2R1 (ICH Harmonised guidelines, 2005). Two independent calibration equations were obtained. Linear regression analysis was used to calculate the slope, intercept, and coefficient of determination/regression coefficient (r2) for each calibration plot. Responses were linear in the concentration ranges investigated. Quantification was on the basis of peak area.

Calibration

The marker compounds in the formulation were quantified using calibration curves established with the dilutions of the standard at concentrations ranging from 100 – 600 ng/spot for the Piperine and 1-12 µg/ml for 6-Gingerol standard compounds (Table 1; Figures 3c and 4c). The corresponding peak area in the formulation was plotted against the concentrations of the standard injected. Peak identification was achieved by comparison of both the Rf/RT and UV absorption spectrum with those obtained for standard.

Linearity

Linear regression analysis was used to calculate the slope, intercept, and coefficient of determination/regression coefficient (r2) for calibration plot. Linearity was determined by using five concentration of the standard solution. The calibration curve was obtained by plotting the area versus the concentrations of the standard solution. Responses were linear in the concentration ranges investigated (Table 1; Figures 3c and 4c).

Rang

Range is the interval between upper and the lower concentration (amount) of analyte in sample for which it has been demonstrated that the analytical method has suitable level of precision, accuracy and linearity. The linear responses were observed at 280 nm / 340 nm over a range of 1 - 12 µg/ml for 6-Gingerol and 100 – 600 ng/spot for the Piperine standard compounds respectively.

Accuracy (% Recovery)

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.

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

Parameters	Piperine	6 - Gingerol
Concentration range	100 – 600 ng/spot	1 - 12 µg/ml
Regression equation	y = 39.31 x + 742.59	y = 6.97 x – 1.59
Correlation Coefficient (r2)	0.993	0.997
Amount of marker compound in Janova [%w/w] [a]	0.24 %w/w	0.0143 %w/w
Method precision (Repeatability) – RSD %	0.93	0.95
Intermediate precision (Reproducibility) - RSD [%]		
Intraday 1	0.96	0.92
Interday 3	0.93	0.91
LOD	0.02 µg $spot^{-1}$	0.12 µg ml^{-1}
LOQ	0.06 µg $spot^{-1}$	0.36 µg ml^{-1}

y = peak area response; x = amount of marker compound; [a] = Mean, n=5

Table 2. Results from determination of recovery

Parameter	6 - Gingerol			Piperine		
Initial concentration in formulation [mg g-1]	0.143	0.143	0.143	2.4	2.4	2.4
Concentration added [mg g-1]	0	2.0	4.0	0	2.0	4.0
Total concentration [mg g-1]	0.143	2.143	4.143	2.4	4.4	6.4
Concentration found [mg g-1]	0.141	2.140	4.141	2.4	4.3	6.2
RSD [%] (n=7)	0.94	0.96	0.98	0.92	0.90	0.95
Recovery [%]	98.60	99.86	99.95	100	97.72	96.87
Mean recovery [%]	99.47			98.20		

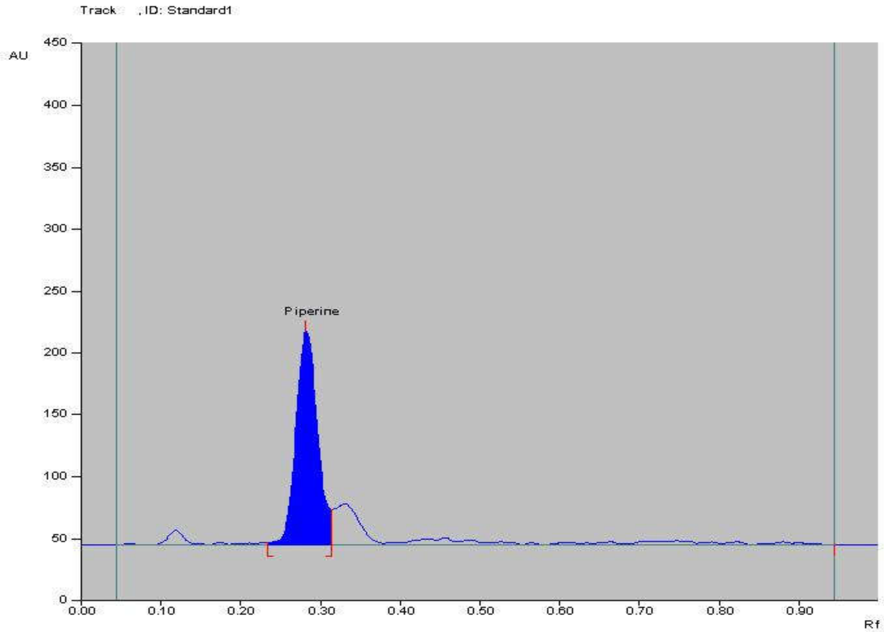


Figure 3a.

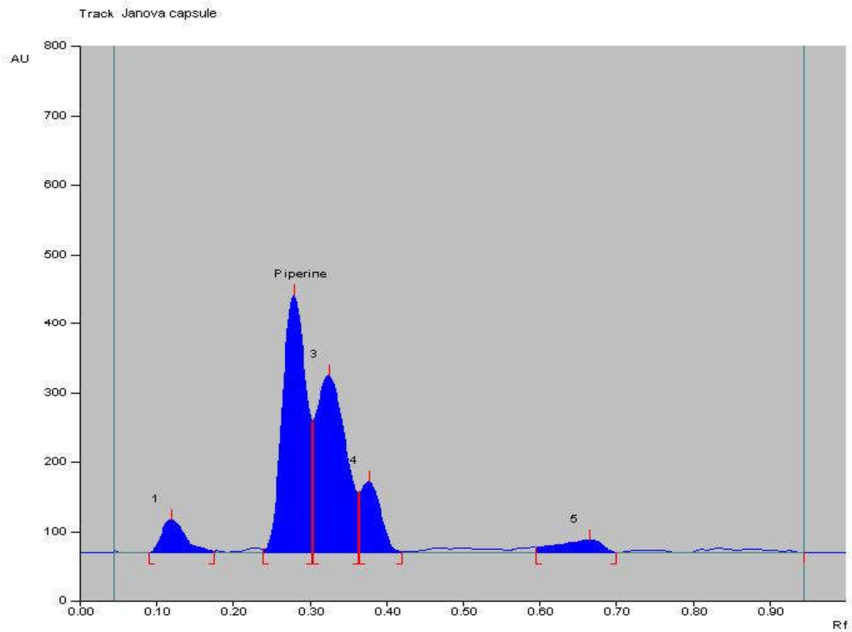


Figure 3b.

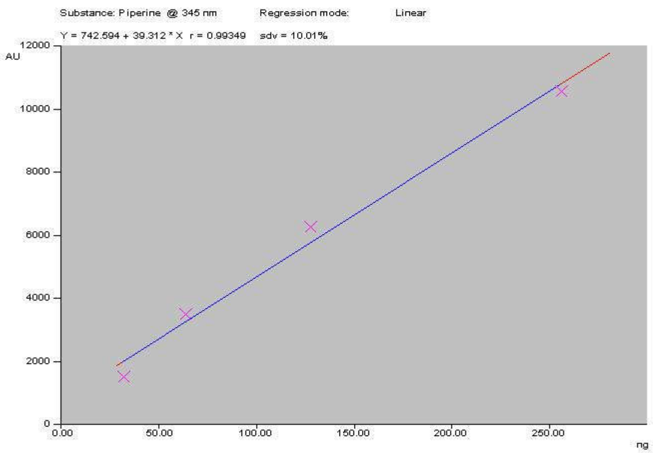


Figure 3c.

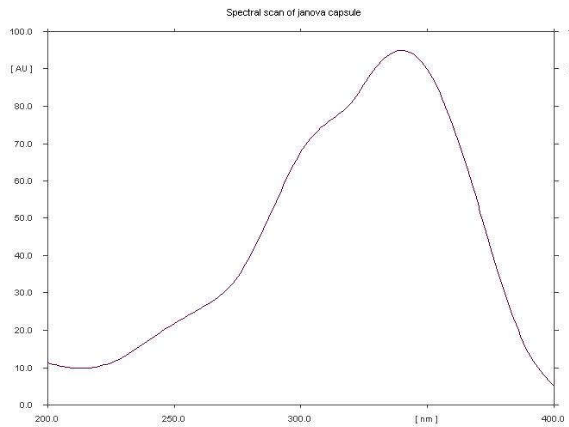


Figure 3d.

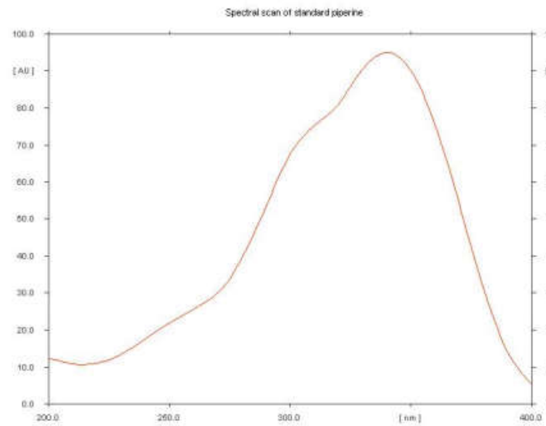


Figure 3e.

Figure 3. HPTLC Chromatograms showing the resolution of the marker compounds in the formulation Janova. (a) Chromatogram of marker compound Piperine (Fig.1). (b) Chromatogram of the formulation Janova. (c) Calibration plot for Piperine standard. (d) Spectral chromatogram of the Piperine in formulation Janova. (e). Spectral chromatograms of Piperine standard

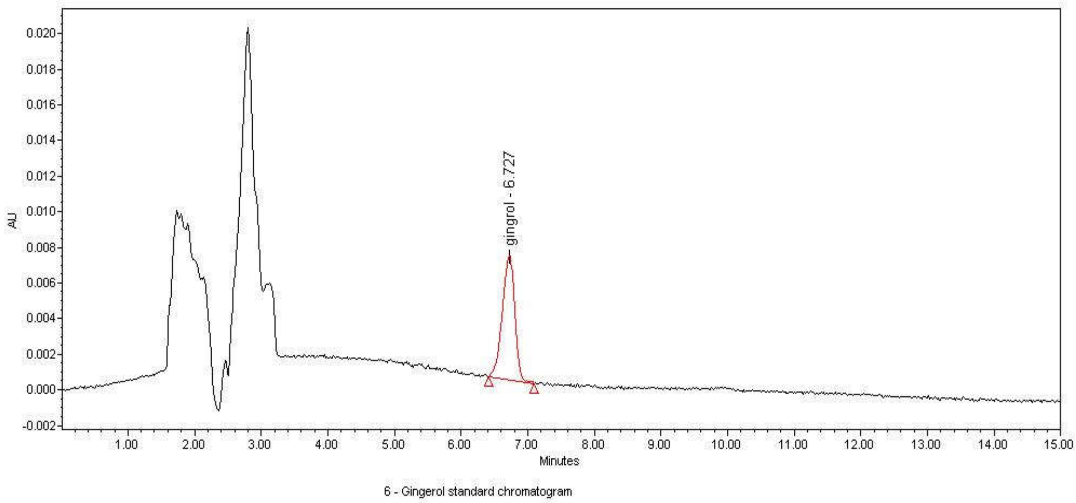


Figure 4a.

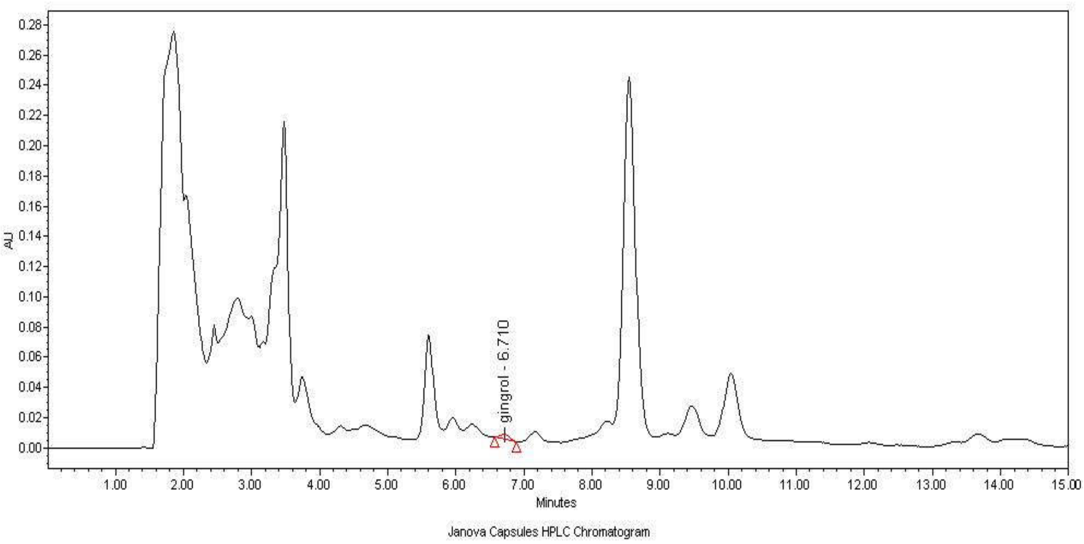
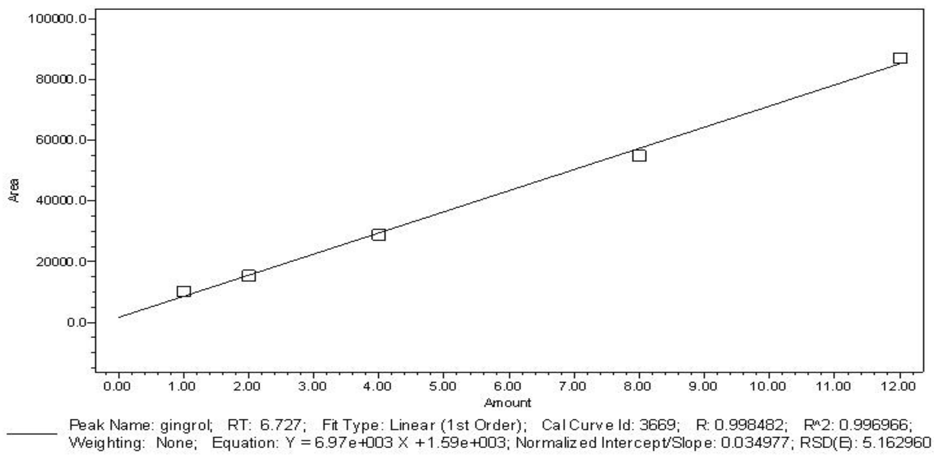


Figure 4b.



Peak: gingrol

	Sample Name	Result Id	Peak Name	X Value	Response	Calc. Value	% Deviation	Manual
1	Gingerol std 1 ppm	3684	gingrol	1.000	10128.775	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

Figure 4c.

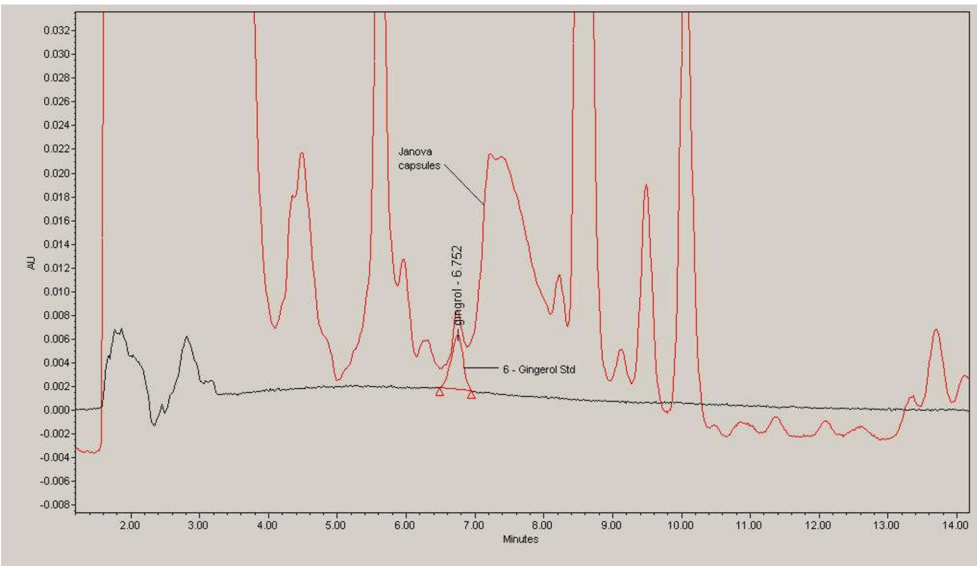


Figure 4c.

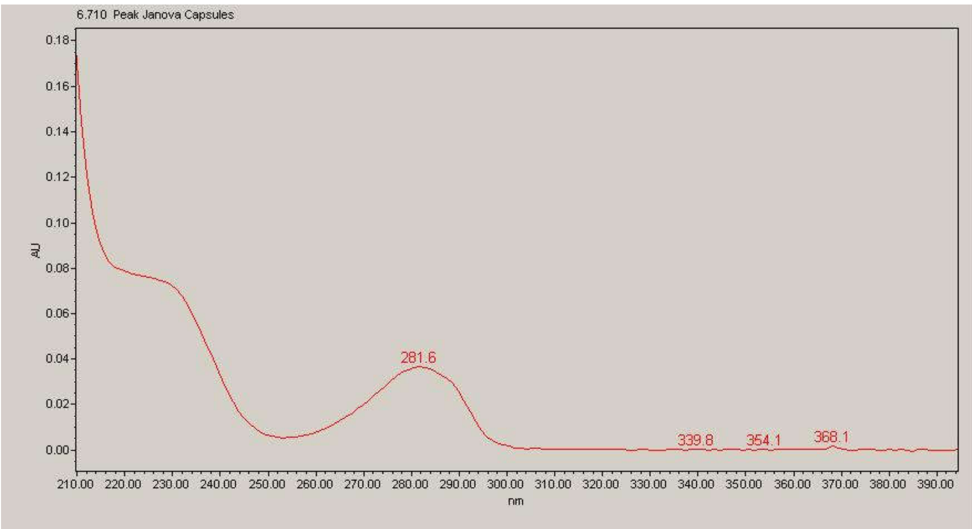


Figure 4c.

Figure 4c.

Figure 4. HPLC Chromatograms showing the resolution of the marker compounds in the formulation Janova. (a) Chromatogram of marker compound 6-Gingerol (Fig. 2). (b) Chromatogram of the formulation Janova. (c) Calibration plot for 6-Gingerol standard. (d) Overlay of chromatograms of Piperine standard with its counterpart in formulation. (e) Spectral chromatogram of the Piperine in formulation Janova. (f) Spectral chromatogram of Piperine standard

6-Gingerol and Piperine standards were added to the formulation at two different concentrations, extraction and analysis was performed as described in preparation of sample solution. Recovery was calculated for each standard at each concentration. The results obtained are listed in Table 2.

Precision

Three different concentration of the 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 variation in the results. The low %RSD values of intraday and inter day for marker compound reveals that the proposed method is precise (Table 1).

Selectivity

The selectivity of the respective method was determined by comparing the retention factor and absorbance spectrum of the standards and the corresponding peaks obtained from the extracts of the formulation. The UV-Vis spectra of both the compounds were compared 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 & Piperine peaks separately were, therefore, not masked by any peak of other compound present in the formulation (Figures 3 a,b,d,e and 4 a,b,d,e,f) which indicated respective peak purity.

LOD and LOQ

The LOD, defined as the amount of compound required to produce a signal at least three times the noise level. The LOQ, defined as the amount of compound required to produce a signal at least ten times the noise level. The LOD for Piperine and 6-Gingerol were 20 ng spot^{-1} and 0.12 µg ml^{-1} respectively, whereas, the LOQ was 60.0 ng spot^{-1} and 0.36 µg ml^{-1} respectively. Quantification of 6-Gingerol and Piperine in clinically efficacious batches helped in standardization of the product with respect to its phytotherapeutic constituents. The newly developed method ensures the batch to batch consistency in efficacy of the product on commercial scale.

Conclusion

New HPLC and HPTLC methods were developed for the fine resolution of two phyto constituents of the product. Janova, a proprietary polyherbal medicine of Ayurvet Limited for cattle is a complete potent herbal medicine for Anoestrus treatment. Standardization of phototherapeutic constituents' 6-Gingerol and Piperine 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.

Conflicts of interest

All authors have no conflicts of interest to declare.

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