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RESEARCH ARTICLE

STANDARDIZATION OF PHYTOLEAN™ POWDER A PHYTOGENIC FEED SUPPLEMENT FOR LEAN MEAT PRODUCTION

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ABSTRACT

Nowadays Ayurvedic herbal formulations have gained acceptance and demand globally due to their safe consumption and minimal side effects. This has gained acceptance in the veterinary market in recent years. This rise in the use of herbal products has also given rise to various adulteration, so it become necessary to develop standardization protocols for safety, efficacy, and quality control. These protocols must have reliable, specific, and sensitive quality control methods. Phytolean™ Powder is a herbal formulation for lean meat production containing herbs like *Curcuma longa* and *Trigonella foenum graecum*, as prime ingredients. The present study was undertaken to develop standardization parameters for Phytolean™ Powder. Reverse-phase high-performance liquid chromatography equipped with a photodiode array detector (RP-HPLC-PDA) was employed quantification of dominant biomarkers in this herbal formulation. The RP-HPLC-PDA Rt-value of Trigonelline 4.85 and Curcuminoids i.e., Curcumin, Bisdemethoxycurcumin, Demethoxycurcumin are 22.50±0.15, 24.43±0.03, and 27.15±0.05 minutes in the formulation and reference standards were found comparable under UV light at 265 nm and 420 nm respectively. The percentage of Trigonelline and Curcuminoids were 0.120±0.85 % and 0.220±0.92 % present in the Phytolean™ Powder formulation. Bioactive markers are characteristic of the ingredients or botanicals to identify the presence of ingredients in formulation easily. The quantification of biomarkers by HPLC is the best way to identify and evaluate the quality of the finished formulation in the course of the development of a standardization protocol for quality control of Ayurvedic formulation.

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INTRODUCTION

Meat fat contains saturated fat and cholesterol which may increase the risk of atherosclerosis & other associated diseases. Nutritional guidelines that follow scientific evidence recommend a reduction in total fat intake, particularly of saturated fatty acids (SFAs) that are associated with an increased risk of obesity and cardiovascular diseases (Sacks *et al*., 2017). Although meat is the main source of SFAs in the human diet, it is also an important source of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) that have properties beneficial to human health (Ander *et al*., 2003; Qian *et al*., 2016). The modulation of fatty acid profiles in meat is thought to be a compelling approach in preventing cardiovascular diseases via the reduction of SFAs, as well as promoting positive effects that occur via increases in PUFA levels (Bessa *et al*., 2015). Recently, the ability of natural products to positively modulate meat fatty acid profile has been examined, primarily because most of them have no deleterious effects and are well accepted by consumers (Shahidi and Ambigaipalan, 2015). The herbs like *Zingiber officinale, Curcuma longa*, and *Trigonella foenum graecum* has been found effective in modulating the fatty acid profile of meat (Mancini *et al*., 2019; Galli *et al*., 2013; Bhatt *et al*., 2021). These herbs are scientifically known to reduce cholesterol & saturated fat in the body. These herbs not only decompose a greater portion of fat but also reduce the synthesis of fat as well (Joanna *et al*., 2003; Sahoo *et al*., 2013). Biomarkers curcuminoids and trigonelline are considered functional molecules that can be added to animal feed (Jaguezeski *et al*., 2018; Marchiori *et al*., 2019; Rafiq *et al*., 2021). Curcuminoids also exhibit several pharmacological properties, including antioxidant, anti-inflammatory, anticancer, and antimicrobial activities (Liu *et al*., 2017). Jaguezeski *et al*. 2018 also reported that the addition of 80 mg curcumin kg^{-1} of feed exerted positive effects on milk fatty acid profiles of dairy sheep via augmentation of MUFA levels in milk. Furthermore, the addition of 30 mg curcumin kg^{-1} of feed exerted positive effects on quail egg yolk fatty acid profiles via reduction of SFA levels, as well as increases in MUFA and PUFA levels (Marchiori *et al*., 2019). Phytolean™ Powder is a herbal feed supplement for lean meat production and is a proprietary formulation of Ayurvet Limited. It is a clinically tested formulation that effectively reduced saturated fatty acids and increases the MUFA and PUFA levels in meat (Sahoo *et al*., 2013). The efficacy of the Phytolean™ Powder was also studied in poultry effectively (Verma *et al*., 2021). Further, Phytolean™ Powder was found to be safe for usage and has efficient methane-mitigating potential along with the added benefit of improvement in digestion, nutrient utility, and performance traits in sheep, pigs, and poultry (Naga *et al*., 2020; Pankaj *et al*., 2013).

The key herbs in Phytolean™ Powder formulation include *Trigonella foenum graecum* and *Curcuma longa***.** Keeping in view the greater acceptance and demand of herbal products by virtue of their better safety profile and efficacy, herbal products should be standardized and validated for batch-to-batch consistency and quality optimization. The present study represents an efficient and rugged analytical approach, which strengthens to scientific validation of the product quality by standardizing the Phytolean™ Powder for two phytoconstituents i.e. trigonelline and curcuminoids [curcuminoid-B (bisdemethoxycurcumin), curcuminoid-C (curcumin), curcuminoid-D (demethoxycurcumin)] (Figure 1) by RP-HPLC-PDA. Satisfactory separations were achieved within a short analysis time without any interference from the matrix co-extracted constituents. The proposed methods were validated following the ICH guidelines.

MATERIALS AND METHODS

Reagents and materials: All the reagents and solvents were of AR or HPLC grade as per requirement. The reference standard curcuminoids were isolated in our lab and its structure was established by interpreting the 1H, 13C & 2D NMR spectra, trigonelline standard was procured from Sigma Aldrich. Controlled samples of Phytolean™ Powder were obtained from the QA/QC department of Ayurvet Limited.

Preparation of standard solution of trigonelline: 2.5 mg of the trigonelline standard was accurately weighed and dissolved in 50 ml of methanol to obtain stock concentrations of 50 µg/ml. The stock solution was further diluted to obtain the dilution range of 10 – 50 µg/ml and then injected in HPLC to prepare the calibration graphs and quantification of bioactive.

Preparation of standard solution of total curcuminoids: 5 mg of the curcuminoids standard was accurately weighed and dissolved in 25 ml of methanol to obtain stock concentrations of 200 µg/ml. The stock solution was further diluted to obtain the dilution range of 10 – 150 µg/ml and then injected in HPLC to prepare the calibration graphs and quantification of bioactive.

Preparation of test solution (Phytolean™ Powder): For the quantification of trigonelline and curcuminoids, 5 g Phytolean™ Powder was refluxed with 70 ml of methanol under reflux conditions for 1 hour and filtered, the process was repeated twice. The final volume was made to 200 ml with methanol and filtered through a 0.45µ membrane filter before injecting into HPLC.

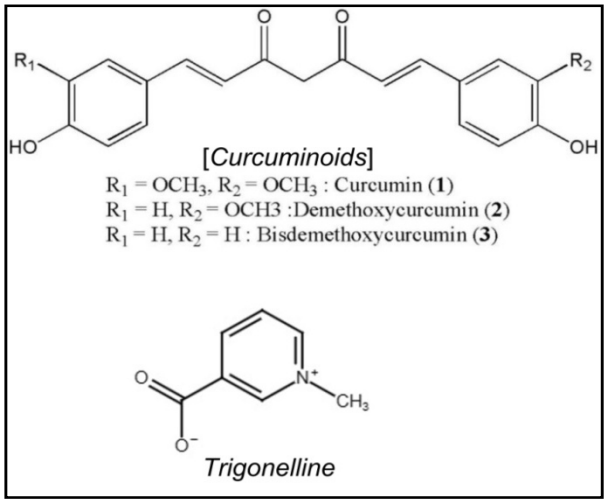

Figure 1. Curcuminoids (B, C, and D) and Trigonelline

High-Performance Liquid Chromatography apparatus and conditions: Trigonelline and curcuminoids content were analyzed by High-Performance Liquid Chromatography (WATERS, binary pump-1525, 2707-auto sampler with PDA-2998 detector).

The data was acquired on the Empower 3.0 software. Separation was obtained on the Phenomenex luna C18 column (250 mm x 4.6 mm, 5µm). In the selection and optimization of chromatographic conditions, several mobile phase compositions were tried to optimize the RP-HPLC parameters. A satisfactory separation and good peak symmetry for trigonelline (Figure 3) and curcuminoids (Figure 2) were obtained by using acetonitrile : water (0.1 % acetic acid) :: 20 : 80 V/V ratio and water (0.1 % ortho-phosphoric acid): acetonitrile in 50:50 v/v ratio respectively as a mobile phase in isocratic mode. The mobile phases were filtered through a 0.45 µ filter and degassed before use. The flow rate was adjusted to 0.6 mL/min and 1.0 mL/min for trigonelline and curcuminoids markers respectively. The injection volume was adjusted to 20 µL and detection was made at 265 nm and 420 nm, respectively.

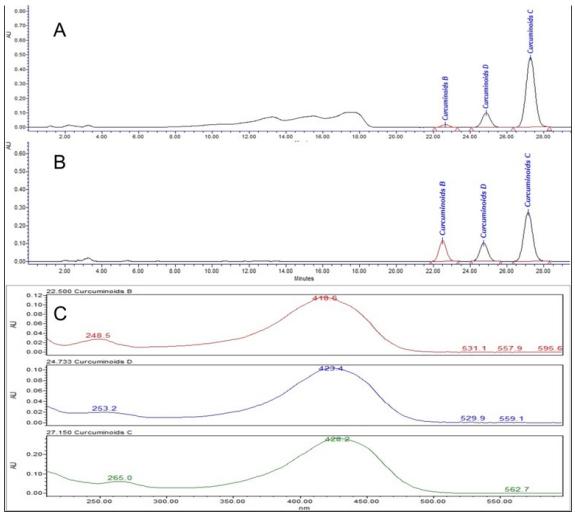

Figure 2. RP-HPLC-PDA analysis of total Curcuminoids (Curcuminoid-B, Curcuminoid-D, Curcuminoid-C) in Phytolean™ Powder: A) Chromatogram of the standard total Curcuminoids. B) Chromatogram of Phytolean™ Powder sample. C) Spectrum index plot of total Curcuminoids.

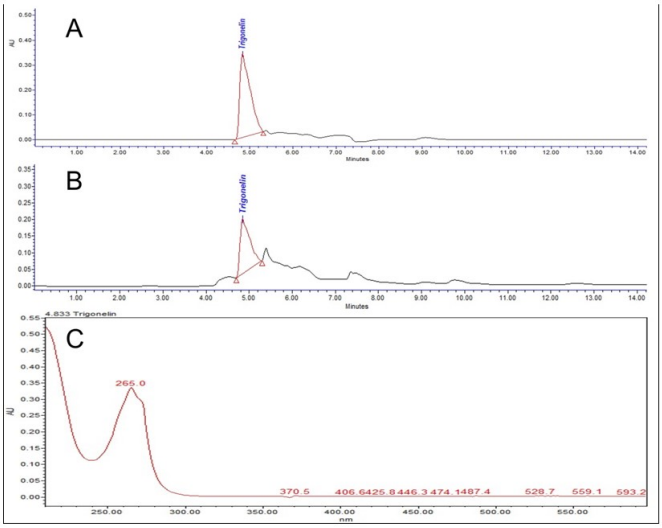

Figure 3. RP-HPLC-PDA analysis of Trigonelline in Phytolean™ Powder: A) Chromatogram of the standard Trigonelline. B) Chromatogram of Phytolean™ Powder sample. C) Spectrum index plot of Trigonelline

SYSTEM SUITABILITY

The analytical results obtained by the method developed are valid only if the defined system suitability criteria are fulfilled. In this investigation, the experimental result indicates that the chromatographic system was suitable for the intended analysis.

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

S.no.	Parameters	Trigonelline	Curcuminoids
1	Concentration range [µg/mL]	10 - 50	10 - 150
2	Regression equation	Y=2.5255x-1.078	Y=240750x-59691
3	Correlation Coefficient [r^2]	0.999	0.999
4	Amount of marker compounds in Phytolean™ Powder [% w/w][a]	0.122±1.01	0.22±0.78
5	Method precision [repeatability study of seven replicates-area % RSD]	0.3	0.045
6	Intermediate precision [reproducibility-% RSD]	0.42	
	Intraday 1	0.51	0.071
	Intraday 3		0.082
7	LOD [µg/mL]	1.77	0.02115
8	LOQ [µg/mL]	5.31	0.06345

y = peak area response; x = amount of marker compound; a = Mean±SD, n=7; Table 2: Results of recovery studies.

S.no.	Parameters	Trigonelline			Curcuminoids		
1	Initial concentration in formulation [mg g-1]	1.22	1.22	1.22	2.20	2.20	2.20
2	Concentration added [mg g-1]	0.0	1.0	2.0	0.0	1.0	2.0
3	Total concentration [mg g-1]	1.22	2.22	3.22	2.20	3.20	4.20
4	Concentration found [mg g-1]	1.14	2.15	3.07	2.12	3.04	4.05
5	Recovery [%]	93.44	96.85	95.34	96.36	95.0	96.43
6	Mean recovery [%]	95.21			95.93		

Each standard solution containing a known concentration of trigonelline and curcuminoids was injected seven times, separately. RSD values for peak area and retention time of standard suggested the reproducibility of these parameters (WHO, 2007; ICH Guideline 2009).

Validation of the proposed method: The proposed methods were validated for the determination of trigonelline and curcuminoids using the following parameters as per ICH guidelines.

- ***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 the formulation was plotted against the concentrations of the standard injected. Peak identification was achieved by comparison of both the retention time (R_t) and UV absorption spectrum with those obtained for standard.
- ***Linearity:*** Linear regression analysis was used to calculate the slope, intercept, and regression coefficient (r^2) for the calibration plot. Linearity was determined by using five concentrations of the standard solution. The response was found to be linear in the concentration ranges investigated (Table 1).
- ***Range:*** Range is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical method has a suitable level of precision, accuracy, and linearity. The linear response was observed over a range of 10-50 ppm for trigonelline and 10-150 ppm for curcuminoids (Table 1).
- ***Precision:*** Three different concentrations of marker compound solution in triplicates were injected at three different times within the same day and repeated 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 trigonelline and curcuminoids reveal that the proposed methods are precise.
- ***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 based on signal-to-noise ratio 3:1 and 10:1 respectively. The LOD and LOQ for the standard compounds were calculated and tabulated (Table 1).
- ***Selectivity:*** The retention time of trigonelline was 4.83±0.02 minutes and 22.50±0.15, 24.43±0.03, and 27.15±0.05 minutes retention time were observed for curcuminoid-B, curcuminoid-D, and curcuminoid-C in the formulation respectively. The UV-Vis spectrum of marker compounds was compared with their counterpart in the formulation at three different positions, the peak start, peak center, and peak end. There was a good correlation between spectra obtained at each of the three positions. The trigonelline and curcuminoids peaks were, therefore, not masked by any peak of another compound present in the formulation (Figures 2 & 3) which was indicative of peak purity.
- ***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. Trigonelline and curcuminoids 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).

RESULTS AND DISCUSSION

The exercise was performed to ensure consistency in the desired pharmacological effect by establishing the lowest possible limit for two of its most relevant bioactive phytoconstituents. Developed RP-HPLC methods were successfully applied in the identification and quantification of the phytoconstituents. A linearity range of 10 -50 µg ml-1 for trigonelline and 10 – 150 µg ml-1 for curcuminoids showed a good coefficient of correlation (r^2) of 0.999 each. The average recovery of trigonelline (95.21%) and curcuminoids (95.93 %) were computed from the regression equations. RSD for inter-day and intraday was also found to be less than 2.0 %. The low value of relative standard deviation indicates that the proposed methods are accurate. Standardization of these phytotherapeutic constituents with validated analysis methods will ensure batch-to-batch consistency in the efficacy of the product on a commercial scale. As two herbs mentioned under experimental investigation are among the main active ingredients in the Phytolean™ Powder formulation, quantifying them with their respective bioactive markers and setting the limits will help us in ensuring the authenticity and efficacy of the product in turn.

CONCLUSION

New RP-HPLC methods were developed for the fine resolution of two phytoconstituents of the product. Phytolean™ Powder, a proprietary herbal feed supplement of Ayurvet Limited. Standardization of phytotherapeutic constituents trigonelline and curcuminoids with validated analysis methods will help in ensuring the batch-to-batch consistency in quality and efficacy of the product on a commercial scale.

Further, the methods reported here are simple, precise, accurate, and suitable for the routine analysis and quantification of the active constituents in a formulation containing them.

Conflicts of Interest: All authors have no conflicts of interest to declare.

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