ISSN: 0975-833X

Available online at http://www.journalcra.com

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

International Journal of Current Research
Vol. 14, Issue, 01, pp.20267-20270, January, 2022

DOI: https://doi.org/10.24941/ijcr.42837.01.2022

RESEARCH ARTICLE

RP-HPLC & HPTLC ANALYSES AND STANDARDIZATION OF PAYAPRO™ PREMIX-A POLYHERBAL VETERINARY GALACTOGOGUE

Anirudh Sharma, Uma Ranjan Lal, Pushap Lata and Deepak Thakur*

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

ARTICLE INFO

Article History:
Received 07th October, 2021
Received in revised form
16th November, 2021
Accepted 14th December, 2021
Published online 28th January, 2022

Keywords
Apigenin, Glycyrrhizin,
RP-HPLC, HPTLC,
Payapro™ Premix.

Corresponding author:
Anirudh Sharma

ABSTRACT

Objective: The aim of the present study was to standardize Payapro™ Premix by quantitative estimation of apigenin and glycyrrhizin by reverse phase high performance liquid chromatography (RP-HPLC) and high-performance thin layer chromatography (HPTLC) methods. Payapro™ Premix is a polyherbal formulation used as galactogogue containing seven herbs including *Cuminum cyminum* and *Glycyrrhiza glabra*. **Materials and methods:** RP-HPLC and HPTLC methods were developed for the standardization of Payapro™ Premix by quantitative estimation of apigenin and glycyrrhizin, the bio-active constituents of *Cuminum cyminum* and *Glycyrrhiza glabra* respectively. The developed methods were validated on various parameters, including linearity, precision, accuracy, LOD, and LOQ as per ICH guidelines. **Results:** The RP-HPLC and HPTLC analysis methods were selective for the polyherbal formulation. Both the methods had specific linearity range with regression coefficient ≥ 0.995. The apigenin and glycyrrhizin estimation methods were precise (% RSD 0.7 and 0.4), accurate (average recovery values were 96.28 % and 95.83 %), LOD (sensitive) (0.0054 µg/mL & 0.016 µg spot^{-1}), LOQ (reliable) (0.0162 µg/mL & 0.048 µg spot^{-1}), respectively. Methanolic extracts of polyherbal formulation showed the presence of significant amount of apigenin (50 µg/g) and glycyrrhizin (1200 µg/g) using developed methods. The comparison of different batches for these markers was found to be uniform which ensures their quality. **Conclusion:** The present work emphasized on standardization of the polyherbal formulation by determination of bio-active marker constituents. The developed methods can be used to standardize other samples containing *Cuminum cyminum* and *Glycyrrhiza glabra*.

Citation: Anirudh Sharma, Uma Ranjan Lal, Pushap Lata and Deepak Thakur. *"*RP-HPLC & HPTLC analyses and standardization of Payapro™ Premix-a polyherbal veterinary galactogogue*", 2022. International Journal of Current Research, 14, (01), 20267-20270.*

INTRODUCTION

Milk is an essential commodity, being consumed worldwide. Global milk production reached nearly 906 million tonnes in year 2020[1]. India is the highest producer of milk in the world. According to *statista research department* Oct-2021 milk production amounted to about 198 million tonnes in fiscal year 2020. However, the milk productivity per animal in developing countries, in particular in India is still very low as compared to global average[2]. This lower productivity might be attributed to many factors, including the genetic and environmental factors such as non-availability of good quality feed resources, low protein content, poor husbandry management practices, high incidence of mastitis, and the small-scale dairy production units. In order to restore animal productivity and to optimize the milk production in individual animals for better profits, various drugs, herbal preparations, hormones, mineral supplements and feed additives have been tried with variable results[3-5]. Ayurveda, the traditional system of medicine in India, is gaining importance throughout the world and many of the herbal formulations are now clinically tested and being accepted for manufacturing in present scenario. Payapro™ Premix an herbal galactogogue is one such proprietary medicine of Ayurvet Limited[6-7]. It is a clinically tested formulation which improves milk production and let down process, while maintaining the alveolar size. It is a non-hormonal galactogogue with no interference on reproductive system of animal. This formulation is a blend of herbs like *Cuminum cyminum, Asparagus racemosus, Glycyrrhiza glabra, Leptadenia reticulata, Pueraria tuberosa, Nigella sativa, and Foeniculum vulgare*[8]. Payapro™ Premix relieves heat stress in dairy cows and thus improve their productivity. It also restores the altered milk constituents and increased the milk production in cattle with sub-clinical mastitis. The herbal ingredient in Payapro™ Premix, like *Cuminum cyminum* and *Glycyrrhiza glabra* have shown to have beneficial effects in

rumen fermentation, milk production, and also balances milk fatty acid (FA) composition [9-10]. *Glycyrrhiza glabra* have a positive role in modification of chemical and physical properties of cow cheese. It reduces lipid oxidation and induce changes in color and flavor which is good for consumer acceptability[11]. Keeping in view the greater inclination for herbal products by virtue of their better safety profile and efficacy, the herbal products should be standardized and validated for batch-to-batch consistency and quality optimization. It also minimizes trouble for adulteration with low grade of exhausted plant material. It also ensures their acceptability in masses. The present study gives strength to scientific validation of the product quality by standardizing the Payapro™ Premix for two phytoconstituents i.e. apigenin and glycyrrhizin with respect to *Cuminum cyminum* and *Glycyrrhiza glabra* by RP-HPLC and HPTLC.

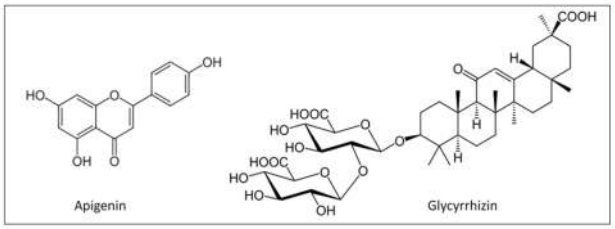

Figure 1. Structural description of apigenin and glycyrrhizin

MATERIAL AND METHODS

Chemicals and reagents: All the reagents and solvents were of analytical or HPLC grade as per requirement. The marker compounds apigenin and glycyrrhizin were isolated in Phytochemistry lab of Ayurvet Limited and structures were established by comparing the ^{1}H, ^{13}C, and 2D- NMR spectra with literature values. Latest controlled samples of Payapro™ Premix (obtained from the QA/QC department of Ayurvet Limited, Baddi) were used for analysis of marker constituents.

Instrumentation: The HPLC system consisted of WATERS binary pump 515 with PDA 2996 detector, USA. Separation was obtained on Phenomenex Luna C-18 column (250 mm × 4.6 mm, 5 µ). The data were acquired on the Empower 3.0 controlling software (all equipment from Waters, Milford). The HPTLC system consisted of CAMAG-HPTLC system with Scanner III, Linomat V, twin trough chambers and WIN-CATS software Ver.1.4.1.

Preparation of test solution: Weigh accurately 5 g of each Payapro™ Premix samples and transfer in to 100 mL round bottom flask add 70 mL methanol and reflux it on water bath using reflux condenser, repeat the process for two more times, filter and concentrate up to 100 mL using rotavapor and transfer in to a 100 mL volumetric flask, make up the volume with methanol. Filter the solution through 0.45 µ before injecting into HPTLC and RP-HPLC analysis.

Preparation of Standard solutions

Apigenin: Weigh accurately 2 mg of apigenin reference standard and transfer to 100 mL volumetric flask. Add 70 mL methanol and sonicate for 5 minutes and make up the volume with above solvent. Filter the solution through 0.45 µ before injecting into RP-HPLC.

Glycyrrhizin: Weigh accurately 5 mg of glycyrrhizin reference standard and transfer to 50 mL volumetric flask. Add 35 mL methanol and sonicate for 5 minutes and make up the volume with above solvent. Filter the solution through 0.45 µ before use in HPTLC.

Chromatographic conditions

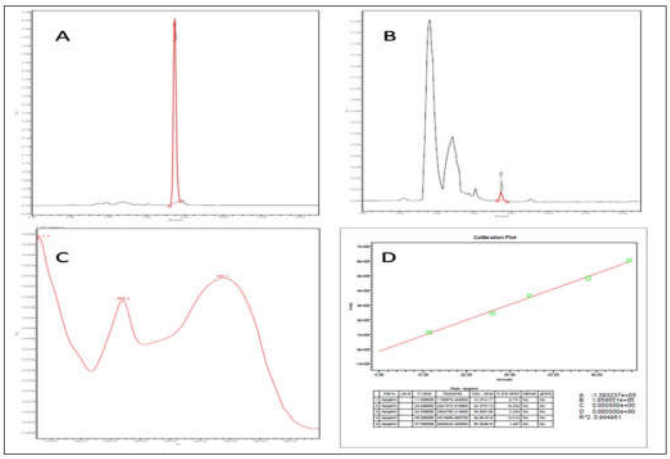

Figure 2. RP-HPLC analysis data (λ max, 336 nm) of Payapro™ Premix samples. (A) Chromatogram of standard apigenin. (B) Chromatogram of Payapro™ Premix samples showing presence of apigenin. (C) Chromatogram of spectral scan (λ max, 267 & 336 nm) of apigenin standard and Payapro™ Premix samples. (D) Chromatogram of linearity curve for apigenin standard

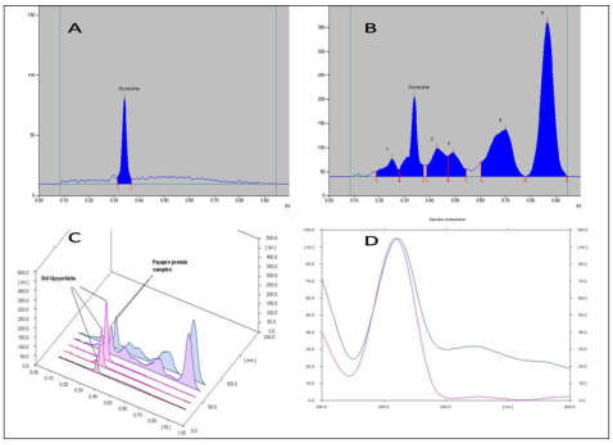

Figure 3. HPTLC analysis data (λ_{max}, 254 nm) of Payapro™ Premix samples. (A) Chromatogram of standard glycyrrhizin. (B) Chromatogram of Payapro™ Premix samples showing presence of glycyrrhizin. (C) Three-dimensional overlay chromatogram of overlay of peaks of standard and Payapro™ Premix samples. (D) Chromatogram of spectral scan (λ_{max}, 254 nm) of glycyrrhizin standard and Payapro™ Premix samples.

Apigenin analysis by RP-HPLC: Initial trials were performed by a gradient mode of analysis using the mobile phase, which consisted of a gradient solvent system of water (containing 0.2% acetic acid) and acetonitrile (from 50:50 to 100:0 over 20 min). Experiments concluded lack of resolution of a complex mixture of different phytoconstituents and time consuming using the gradient approach of analysis. The simple isocratic mode was opted comprising of water and acetonitrile in 50:50 ratio. The elution was clear and well-separated peaks of apigenin with a flow rate of 1 mL/min over a run time of 20 min.

The eluent was monitored at 336 nm. The mobile phase was filtered through 0.45 µ Millipore membrane filter and degassed before use. The injection volume was 20 µL and all analyses were performed at ambient temperature.

Glycyrrhizin analysis by HPTLC: Applied 10 µL of Payapro™ Premix test samples and 3, 6, 9, 12, 15 µL of standard glycyrrhizin on TLC plate pre-coated with Silica gel 60F $_{254}$ using linomat applicator. TLC plate then dipped in saturated twin trough chamber containing the mobile phase of n-butanol: water: Glacial acetic acid in 70:20:10 ratio. Eluted TLC plate then scanned in CAMAG-HPTLC scanner III under Deuterium lamp at 254 nm in absorbance mode. Peaks were integrated and areas were determined. Spectral scan was taken of all peaks to confirm that spot in samples and standard track are similar.

Analytical method validation: Both the analytical methods of RP-HPLC and HPTLC were validated for linearity, the limit of detection (LOD), the limit of quantification (LOQ), accuracy, intraday and inter-day precision, and repeatability according to the International Conference on Harmonization (ICH) guidance for Q2B validation of analytical procedures[12-13]. Namely, linearity was evaluated by the coefficient of determination (r^2) of the calibration curve in the tested linear range of each compound. LOD and LOQ values were calculated, based on the standard deviation of the y-intercept in each calibration curve and the slope of the calibration curve (s). The linearity of an analytical method is its ability to elicit a test result. The linearity of the both marker standards were observed by taking five different concentrations and measuring their correlation coefficient (r^2). Accuracy, tested as percentage recovery, was determined by using the standard addition method and calculated as:

Recovery (%) = (recorded concentration – original concentration) / spiked concentration × 100.

Intraday precision for RP-HPLC was determined by analyzing a single sample five times within a day and inter-day precision was determined by measuring the sample on three consecutive days, whereas for HPTLC three different concentrations of standard were applied in triplicate on TLC plate and then analyzed within a day and inter-day precision was determined by measuring the same on three consecutive days. concentration. Repeatability was evaluated by calculating the relative standard deviation (RSD) and calculated by the following equation.

RSD (%) = standard deviation (SD) / mean×100.

RESULTS AND DISCUSSION

Standardization of polyherbal formulation with bioactive markers is an important tool to assess their quality and efficacy. Selection of the markers is equally important. Payapro™ Premix is an herbal galactogogue containing seven herbs based on the traditional system of medicine (Ayurveda). *Cuminum cyminum* (rich in flavonoids like apigenin) and *Glycyrrhiza glabra* (rich in triterpene glycosides like glycyrrhizin) are important constituents of the formulation. Apigenin and glycyrrhizin have galactogogue properties[14-15]; pharmacologically they increase the milk production in the early stages of lactation by reducing the serum prolactin[16-18]. Other herbs mentioned in this herbal formulation act as adaptogens. Quantification of selected markers by RP-HPLC and HPTLC in Payapro™ Premix polyherbal formulation has been performed to ensure its efficacy as setting their limits will help in ensuring quality. This is the first report for the quantitation of bioactive markers in veterinary medicinal product which is scares in the literature.

Analytical studies of apigenin and glycyrrhizin in Payapro™ Premix by RP- HPLC and HPTLC.

Different concentrations of apigenin and glycyrrhizin standards were prepared and injected in RP-HPLC and HPTLC, respectively. Calibration curves were established for peak area verses the concentration of standards applied. The calibration peak summary has been tabulated in Table 1 and the calibration curve has been depicted in Figure 2. The marker compound apigenin is exhibited in RP-HPLC at a retention time of 7.456 minutes for standard and 7.455 minutes for formulation in the chromatogram (Figure 2). From the calibration curve, the correlation coefficient 'r^2' value was found to be 0.995 (Figure 2).

Table 1. Method development parameters and results for apigenin and glycyrrhizin

S. no	Parameters	Apigenin (RP-HPLC)	Glycyrrhizin (HPTLC)
1	Concentration range for linearity	10 – 60 µg/mL	0.3 – 1.5 µg spot^{-1}
2	Regression equation	y = 1.06x – 1.39	y = 2.41x + 325.5
3	Correlation coefficient (r^2)	0.995	0.999
4	Amount of marker compounds in Payapro™ Premix (average of five different batches)	50 µg/g	1200 µg/g
5	Method precision (Repeatability) % RSD	0.70	0.40
6	Intermediate precision (Reproducibility) %RSD		
	Intra-day 1	0.54	2.03
	Inter-day 3	0.61	1.44
7	LOD	0.0054 µg/mL	0.016 µg spot^{-1}
8	LOQ	0.0162 µg/mL	0.048 µg spot^{-1}

Table 2. Results from determination of recovery

S. no	Parameters	Apigenin			Glycyrrhizin		
1	Initial concentration in formulation (mg/g)	0.05	0.05	0.05	1.2	1.2	1.2
2	Concentration added (mg/g)	0.0	1.5	3.0	0.0	2.0	4.0
3	Total concentration (mg/g)	0.05	1.55	3.05	1.2	3.2	5.2
4	Concentration found (mg/g)	0.048	1.49	2.95	1.13	3.14	4.95
5	Recovery (%)	96.0	96.13	96.72	94.17	98.12	95.19
6	Mean recovery		96.28			95.83	

value was found to be 0.999. Quantification of both marker compounds was performed based on peak area and found to be 50.0 µg/g and 1200.0 µg/g (average of five batches of Payapro Premix™). Determination of limit of detection (LOD), limit of quantification (LOQ), repeatability, and recovery studies (accuracy) parameters for both markers in RP-HPLC and HPTLC were done for method validated as per ICH guidelines (Table 1, 2). Methods were successfully developed and found to be much simpler and reliable as compared to previously used methods for the quantification of apigenin and glycyrrhizin in literature.

CONCLUSION

Herbal veterinary formulation Payapro™ Premix was standardized by standard analytical techniques RP-HPLC and HPTLC. The characterization parameters presented in this communication may serve as a standard reference for quality control analysis of Payapro™ Premix to ensure the batch-to-batch consistency with respect to the main phytoconstituents.

CONFLICT OF INTEREST: Authors have no conflict of interest.

ACKNOWLEDGMENTS

We thank Ayurvet Limited for providing necessary facilities, help and guidance.

REFERENCES

1. Roman M. Spatial integration of the milk market in Poland. Sustainability. 2020 Jan;12(4):1471.
2. Taylor Preciado A, Orozco Hernandez JR, Contreras Carranza A, Carranza de la Mora V, Rocha Chavez G. Use of an herbal galactogogue on milk quality and yield. Asian Journal of Animal and Veterinary Advances. 2011;6(3):297-300.
3. Patel VK, Joshi A, Kalma RP, Parmar SC, Damor SV, Chaudhary KR. Shatavari (*Asparagus racemosus*), Jivanti (*Leptadenia reticulata*) and Methi (*Trigonella foenum-graecum*): the herbal galactogogues for ruminants. Journal of Livestock Sciences. 2016 Jan 1; 7:231-7.
4. Tesfa A, Garikipati DK. Genetic and non-genetic parameter estimates of dairy cattle in Ethiopia: A review. Online Journal of Animal and Feed Research. 2014;4(4):83-90.
5. Niraj K, Alemayehu E, Abreha T, Yizengaw HA. Productive performance of indigenous and HF crossbred dairy cows in Gondar, Ethiopia. Veterinary World. 2014;7(3):177-81.
6. Kumar S, Kumar B. Comparative assessment of different herbal galactogogue preparations on milk production and economics of lactating crossbred cows. Journal of Pharmacognosy and Phytochemistry. 2018;7(5):2508-12.
7. M. Kalyana Chakravarthi et al. Potential of herbal galactogogue in augmenting milk production. World Journal of Pharmaceutical Research, Volume 6, Issue 2, 817-821.
8. Bhatt N, Singh M, Ali A. Effect of feeding herbal preparations on milk yield and rumen parameters in lactating crossbred cows. International Journal of Agriculture and Biology. 2009 Nov 1;11(6):721-6.
9. Patil AK, Baghel RP, Nayak S, Malapure CD, Govil K, Kumar D, Yadav PK. Cumin (*Cuminum cyminum*): As a feed additive for livestock. Journal of Entomology and Zoology Studies. 2017;5(3):365-9.
10. Ghafari M, Shahraki AF, Nasrollahi SM, Amini HR, Beauchemin KA. Cumin seed improves nutrient intake and milk production by dairy cows. Animal Feed Science and Technology. 2015 Dec 1; 210:276-80.
11. Bennato F, Ianni A, Innosa D, Martino C, Grotta L, Pomilio F, Verna M, Martino G. Influence of licorice root feeding on chemical-nutritional quality of cow milk and Stracciata cheese, an Italian traditional fresh dairy product. Animals. 2019 Dec;9(12):1153.
12. Guideline IH. Validation of analytical procedures: Text and Methodology. Q2 (R1). 2005 Nov;1(20):05.
13. Guideline, ICH Harmonised Tripartite. "Pharmaceutical development." Q8 (2R). As revised in August (2009).
14. Silva FV, Dias F, Costa G, Campos MD. Chamomile reveals to be a potent galactogogue: the unexpected effect. The Journal of Maternal-Fetal & Neonatal Medicine. 2018 Jan 2;31(1):116-8.
15. Le Moli R, Endert E, Fliers E, Mulder T, Prummel MF, Romijn JA, Wiersinga WM. Establishment of reference values for endocrine tests II: Hyperprolactinemia. The Netherlands journal of medicine. 1999 Aug 1;55(2):71-5.
16. Chao J, Ko CY, Lin CY, Tomoji M, Huang CH, Chiang HC, Yang JJ, Huang SS, Su SY. Ethnobotanical Survey of Natural Galactagogues Prescribed in Traditional Chinese Medicine Pharmacies in Taiwan. Frontiers in pharmacology. 2021 Feb 12; 11:2443.
17. Cuzzolin L, Zaffani S, Benoni G. Safety implications regarding use of phytomedicines. European journal of clinical pharmacology. 2006 Jan;62(1):37-42.
18. Brodribb W, Academy of Breastfeeding Medicine. ABM Clinical Protocol# 9: Use of galactogogues in initiating or augmenting maternal milk production, second revision 2018. Breastfeeding Medicine. 2018 Jun 1;13(5):307-14.

****