INTERNATIONAL JOURNAL OF PLANT, ANIMAL AND ENVIRONMENTAL SCIENCES
IJPAES
Volume-8, Issue-4 Oct-Dec-2018 Coden:IJPAJX-CAS-USA, Copyrights@2018 ISSN-2231-4490
*Received: 20th Oct-2018* *Revised: 5th Nov-2018* *Accepted: 11th Nov-2018*
*DOI: 10.21276/Ijpaes* *http://dx.doi.org/10.21276/ijpaes* *Research Article*

# PHYTOCHEMICAL STANDARDIZATION OF A POLYHERBAL UTERINE ECBOLIC

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**ABSTRACT:**
**Objectives**: Quality assurance is an integral part of medicine which in turn ensures quality medication. *Exapar Liquid* is a unique Ayurvedic formulation consisting of potent medicinal herbs extract that prevents retention of placenta and tones up uterus for better post-partum reproductive efficiency. As pharmacological properties of a herbal formulation depend upon phytochemical constituents present therein, development of authentic analytical methods which can reliably achieve the phytochemical quantification to maintain reproducible efficacy and safety of the formulation is paramount responsibility of the pharma industry. Standardization of traditional medicine involves a multidimensional approach that ensures the quality and concentration of chemical constituents for their bio-potency.
**Method**: Chemo-profiling-assisted characterization and quantification of biomarker compounds of the polyherbal formulation used as a complementary approach for the quality control and stability assessment which in turn decides its safety and efficacy. Quantification of therapeutically reported biomarkers under set optimized chromatographic conditions used as a quality control tool.
**Results**: Fingerprints and quantification of biomarkers have been used as tools for standardization of Exapar Liquid. Hence in the present study, HPTLC methods were developed and validated following ICH guidelines for the estimation of colchicine and harmaline in formulation prepared by Ayurvet Limited. Average content of colchicine and harmaline markers were found to be 0.03 mg/g and 0.051 mg/g respectively.
**Key words:** HPTLC, Exapar Liquid, Colchicine, Harmaline, Standardization

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## INTRODUCTION

Post-partum fertility has a profound impact on the economic viability of dairy industry. Post-partum period is the most crucial transitory phase in the life of a bovine female when various physiological, gynecological, biochemical and immunological changes are occurring. During this phase, cattle are exposed to increased risk of uterine infection, as during parturition the anatomical barriers are broached and remain open for several days [1]. Infection of the uterus is largely influenced by the balance between bacterial contamination and the local and systemic immune status during pregnancy and parturition. Infectious diseases are more prevalent during this period because of an impaired immune status before and immediately after parturition [2]. The major classes of immunoglobulins (IgM, IgA and IgG), either by passive diffusion or local production, play an important protective role in the uterus by acting as opsonins to enhance phagocytosis, stimulating the complement pathways or blocking pathogens from adhering to mucosal surfaces. Other cellular components activated in the uterus during this period include neutrophils, lymphocytes, eosinophils, mast cells and macrophages.

A scientific approach towards reproductive health management of animals during this crucial phase has always been emphasized by the scientific community and a lot of research has been performed to potentiate overall immune response of cows during postpartum phase [3]. Immune response at local sites with the help of cellular invasion such as B- and T- lymphocytes, neutrophils, macrophages and other polymorhonuclear cells is an important defense mechanism for cows against various post partum pathogenic infections.

Indiscriminate usage of antibiotics for treatment of uterine infections has led to emergence of resistant strains. As a result of overuse and misuse, attention is now focused towards alternative treatments viz. herbal medicine. Many herbs are known for their strong immunopotentiating properties. [4]

Exapar Liquid, a proprietary formulation of AYURVET LIMITED is known to have therapeutic efficacy. Exapar Liquid is capable of inducing myometrial contractions, potentiating immune response and is useful for hastening the expulsion or to prevent the retention. [5.6].

Exapar Liquid is a unique combination of potent medicinal herbal extracts that tone up the uterus for better post-partum reproductive efficiency. It is a combination of herbs with documented activity profile e.g. *Plumbago zeylanica* [7], *Aloe barbadensis* [8], *Aristolochia indica* [9], *Gloriosa superba* [10], *Peganum harmala, Lepidium sativum* [11] and others.

Major difference in the assessment of quality, safety and efficacy would hinder free circulation of herbal medicinal products and may represent a risk for consumers. Standardization of the product with respect to the bioactive phyto-constituents was taken to ensure batch to batch consistency in efficacy. New HPTLC methods were developed for the quantification of two main ingredients of the formulation i.e. *Gloriosa superba* with respect to biomarker alkaloid Colchicine (Figure 1, I) and *Peganum harmala* with respect to biomarker indole alkaloid Harmaline (Figure 1, II). The analytical methods were validated as per ICHQ2R1 guidelines [12-13]. Limits of the biomarkers were set as check points for the formulation efficacy.

## 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. In this method, 20 × 10 cm aluminum 60F254 TLC plates (E-Merck-Germany) were used with stationary phase silica gel and layer thickness 0.2 mm.

### Reagents and materials

Chemicals and reagents used were of analytical reagent grade. Ethyl acetate, formic acid, ammonia, chloroform, methanol and water were purchased from Rankem. colchicine and harmaline were isolated in house and characterized by different spectroscopic methods before use. TLC plates were purchased from Merck (Darmstadt, Germany). Controlled samples of Exapar Liquid were obtained from the QA/QC department of Ayurvet Limited, Baddi.

### Chromatographic conditions

Chromatography 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) onto the TLC plate under a flow of nitrogen gas. The application parameters were identical for all the analysis performed and the delivery speed of the syringe was 10 s/µl. For fingerprint profile development & standardization of the clinically validated product two mobile phases were optimized, **a**. Chloroform : methanol : 10% ammonia :: 9 : 1 : 0.5, **b**. Ethyl acetate : Methanol : Water : Formic acid :: 10 : 1.3 : 0.5 : 0.5 for resolution of spots. Each TLC plate was developed to a height of about 9.0 cm, under laboratory conditions. For quantification of colchicine and harmaline the spots were scanned at 357 nm and at 325 nm respectively with a slit size of 6 × 0.3 mm.

### Preparation of sample & standard solutions

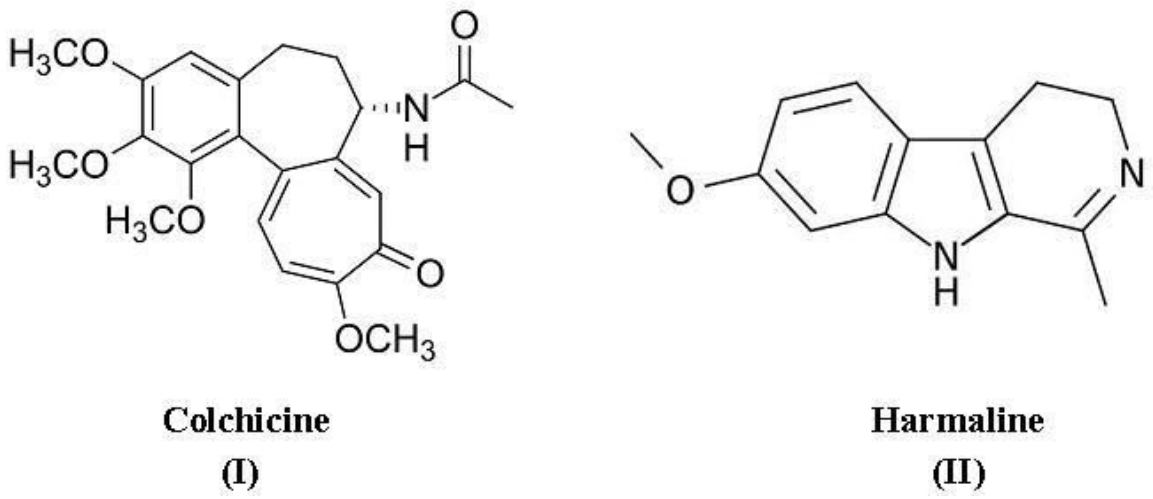
### Preparation of standard solutions

Stock solutions (~20.0 µg/mL) of standard (marker compound) colchicine in chloroform and stock solutions (~100.0 µg/mL) of standard (marker compound) harmaline were prepared in methanol, different concentrations were spotted onto TLC plates in order to prepare the calibration graphs and quantification of bioactives.

**Preparation of sample solutions**

**Analysis of Colchicine:**

Accurately around 25 g of Exapar Liquid weighed and transferred in a 250 ml separating funnel and partition with 30 ml of chloroform. Organic layer was separated. Process was repeated three more times. Combine organic layer was pass through anhydrous sodium sulphate. Organic layer was concentrated on rotary evaporator to 25 ml and transfer to a 25 ml volumetric flask. Made up the volume using chloroform and filtered solution through 0.45µ syringe filter was then used for TLC fingerprint profile generation & quantification of colchicine.




**Colchicine** **Harmaline**

**(I)** **(II)**

**Figure 1: Biomarker compounds**

**Analysis of Harmaline**

Weighed and transferred around 10 g of Exapar Liquid in a 50ml volumetric flask. Added 30 ml of methanol, sonicated for 10 minutes and make up volume with methanol. Filtered solution through 0.45µ syringe filter was used for TLC fingerprint profile generation and quantification of harmaline.

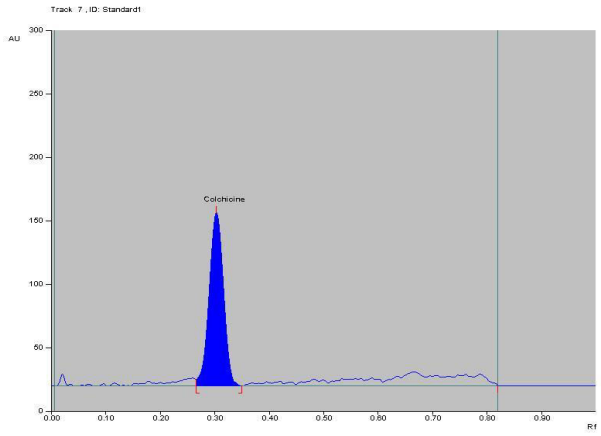
**Method validation**

**Calibration curve (Linearity)**

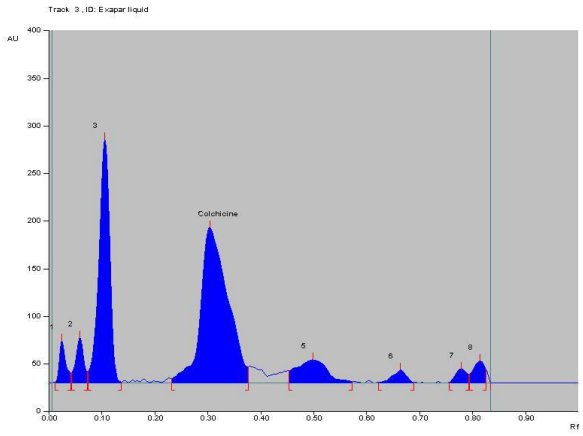
The method was validated in accordance with the statistical method of validation given in ICHQ2R1 [12-13]. Two independent calibration equations for two marker compounds were obtained. Linear regression analysis was used to calculate the slope, intercept, and coefficient of determination/regression coefficient (r2) for each calibration plot. Response was linear in the concentration ranges investigated (Table 1; Figures 2(e,f) and 3(e,f). Evaluation was on the basis of peak area.

**Accuracy (% Recovery)**

Recovery experiments were conducted to check 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. colchicine and harmaline standards were added to the formulation at two different concentrations, extraction and analysis was performed as described above for sample solution. Recovery was calculated for each standard at each concentration. The results obtained are listed in Table 2.




**(a)**

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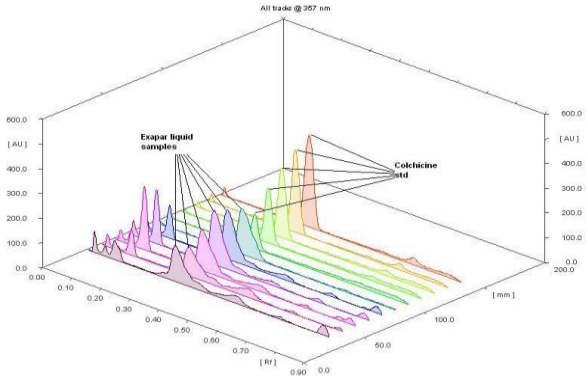
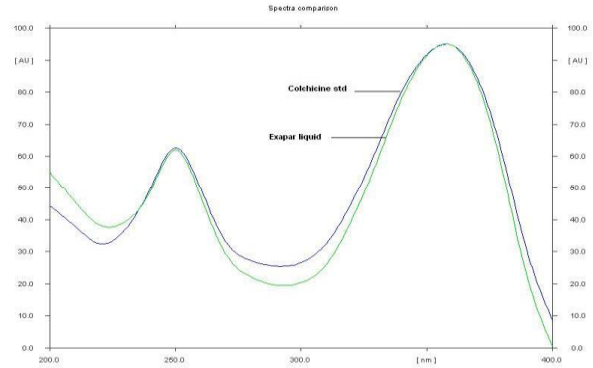
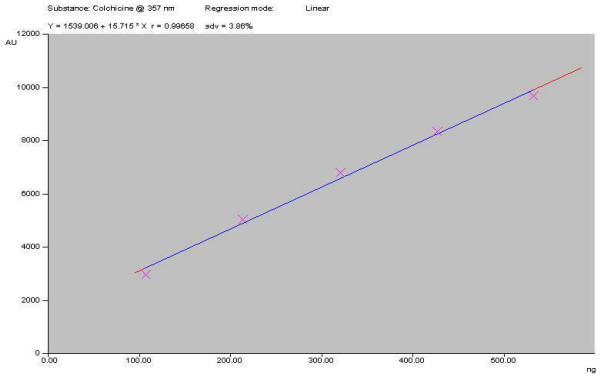


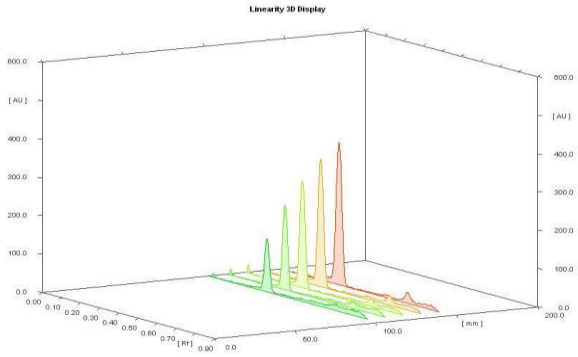

**(c)**




**(d)**




**(e)**




**(f)**

**Figure 2: Chromatograms showing the resolution of marker compound in the formulation Exapar Liquid: (a) Chromatogram of colchicine standard (I). (b) Chromatogram of formulation Exapar Liquid. (c) 3D overlay chromatogram of formulation and standards (I). (d) Overlay of spectra of colchicine with its counter part in formulation. (e) Calibration plot for Colchicine standard. (f) 3D overlay pattern of calibration curve.**

## Precision

a. Method precision (Repeatability)

The precision of the instrument was checked by repeated scanning of the same spot ($n$ = 7) of colchicine (300.0 ng/spot) and harmaline (150 ng/spot) without changing the position of the plate for the HPTLC method.

b. Intermediate precision (Reproducibility).

To study precision of analytical methods, three different concentrations of standard solutions in triplicates were applied to the TLC plates on three different times within the same day and repeated on three different days to record intra-day and inter-day variations in the results, respectively. The lower RSD for colchicine and harmaline suggested that proposed method is robust (Table 1).

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

| Parameters | Colchicine | Harmaline |
|---|---|---|
| Concentration range [µg spot-1] | 100.0 ng – 500.0 ng | 20.0ng - 200.0 ng |
| Regression equation | y = 15.71x + 1539 | y = 116.09x + 1207.87 |
| Correlation Coefficient ( r2) | 0.997 | 0.998 |
| Amount of marker compound in Exapar Liquid [%] (w/w)a | 0.003 ± 0.002 | 0.0051 ± 0.002 |
| Method precision ( Repeatability ) – RSD % | 0.91 | 0.83 |
| Intermediate precision (Reproducibility) - RSD [%]<br>Intraday 1<br>Interday 3 | <br>0.88<br>0.85 | <br>0.78<br>0.91 |
| LOD | 6.5 ng spot-1 | 4.5 ng spot-1 |
| LOQ | 19.5 ng spot-1 | 13.5 ng spot-1 |

**y = peak area response**

**x = amount of marker compound**

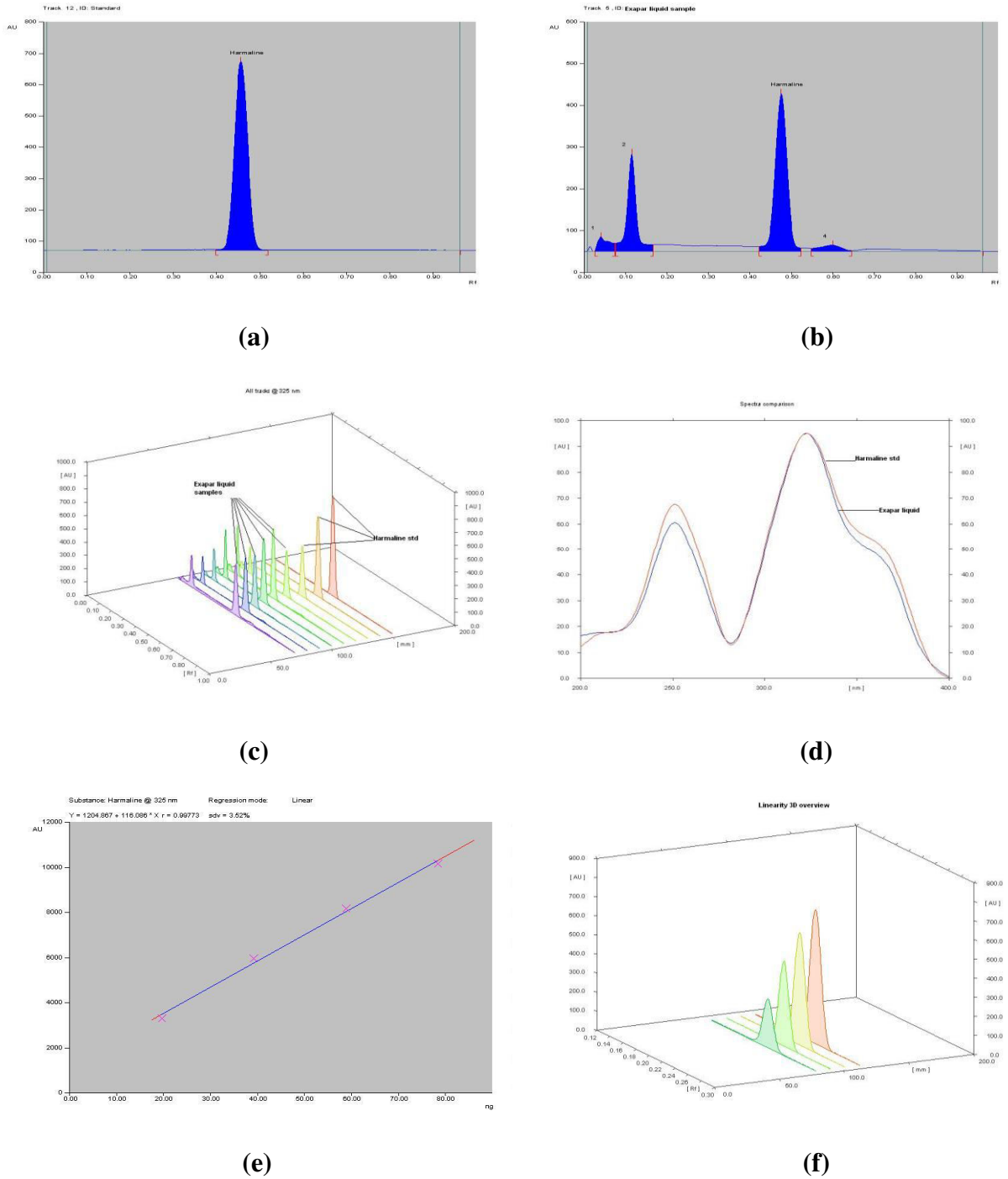
**a = Mean ± SD, n=6**

**Table 2: Results from determination of recovery.**

| Parameter | Colchicine | | | Harmaline | | |
|---|---|---|---|---|---|---|
| Initial concentration in formulation [mg g-1] | 0.03 | 0.03 | 0.03 | 0.051 | 0.051 | 0.051 |
| Concentration added [mg g-1] | 0 | 2.0 | 4.0 | 0 | 2.0 | 4.0 |
| Total concentration [mg g-1] | 0.03 | 2.03 | 4.03 | 0.051 | 2.051 | 4.051 |
| Concentration found [mg g-1] | 0.029 | 1.95 | 3.83 | 0.048 | 1.95 | 3.85 |
| Recovery [%] | 96.67 | 96.06 | 95.04 | 94.12 | 95.07 | 95.04 |
| Mean recovery [%] | 95.92 | | | 94.75 | | |

## 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 colchicine and harmaline peaks separately were, therefore, not masked by any peak of other compound present in the formulation (Figures 2d and 3d), which indicated respective peak purity.




**(a)** **(b)**

**(c)** **(d)**

**(e)** **(f)**

**Figure 3: Chromatograms showing the resolution of marker compound in the formulation Exapar Liquid: (a) Chromatogram of harmaline standard (II). (b) Chromatogram of formulation Exapar Liquid. (c) 3D overlay chromatogram of formulation and standards (II). (d) Overlay of spectra of harmaline with its counter part in formulation. (e) Calibration plot for harmaline standard. (f) 3D overlay pattern of calibration curve.**

## LOD & 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. For determination of limits of detection and quantification different dilutions of the standard solutions of Colchicine and Harmaline were applied to the plates with chloroform and methanol as blank and determined on the basis of the signal-to noise ratio. The LOD for colchicine and harmaline were 6.5 ng/spot and 4.5 ng/spot respectively, whereas, the LOQ were 19.5 ng/spot and 13.5 ng/spot, respectively.

## RESULTS & DISCUSSION

The uterine cleansing and restoration activity of Exapar Liquid can be attributed to the various herbal constituents present in the formulation which makes it a unique choice for veterinarians and farmers for enhancing local immunity and prevention of various reproductive disorders in post- partum dairy cattle.

New HPTLC methods were developed to generate the fingerprint profile and standardize the product. The developed methods are being successfully applied in identification and quantification of the phytoconstituents. The two herbs mentioned under experimental investigation are among the main active ingredients of the polyherbal formulation, quantifying them with their respective biomarkers and setting the limits help us in ensuring authenticity and efficacy of the product in turn. The analytical methods were validated for linearity, accuracy, and precision in accordance with the statistical method of validation given in ICHQ2R1 [12-13].

## CONCLUSION

To ensure the quality control the phytoequivalence study and standardization of clinically validated batch was performed. New HPTLC methods were developed for the purpose and two bioactive marker compounds i.e. colchicine & harmaline were used for standardization.

Establishing the phytoequivalence & standardization of formulation has helped us in ensuring the quality and efficacy of the product at a commercial scale.

## ACKNOWLEDGEMENT

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

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INTERNATIONAL JOURNAL OF PLANT, ANIMAL AND ENVIRONMENTAL SCIENCES

ISSN 2231-4490

# International Journal of Plant, Animal and Environmental Sciences