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RESEARCH ARTICLE

"Standardization of clinically validated polyherbal formulation".

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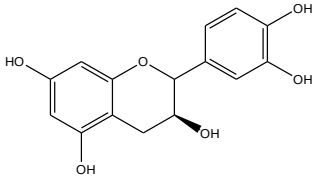
Abstract

Since both EU & US now have mandatory GMPs in place for all the products coming onto the market, there is significant recognition by the herbal drug industry for the absolute standardization to ensure batch to batch consistency. The concept of phyto equivalence is also in practice in order to ensure consistency in efficacy of herbal products. According to this concept, a chemical fingerprint profile of efficacy proven herbal product should be constructed which will serve as the reference for the quality control at commercial scale. An exercise was carried out to establish the phyto equivalence by generating the fingerprint profile of the clinically validated product and simultaneously standardizing the two antidiarrheal herbs *Acacia catechu* & *Berberis aristata* with their respective bioactive markers to ensure quality and efficacy of the product Diaroak, a proprietary polyherbal formulation of AYURVET.

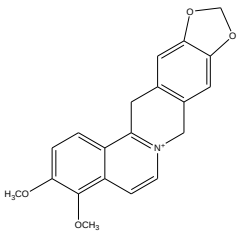
Introduction:-

Clinical validation of herbal products represents a significant step forward in the scientific integration of herbal preparations into modern therapy. Their general accepted use will only take place when the tested herbal products are authentic, standardized and quality controlled. Since both EU & US now have mandatory GMPs in place for all the products coming onto the market, there is significant recognition by the herbal drug industry for the absolute standardization to ensure batch to batch consistency (1). The concept of phyto equivalence is also in practice in order to ensure consistency in efficacy of herbal products. According to this concept, a chemical fingerprint profile of efficacy proven herbal product should be constructed which will serve as the reference for the quality control at commercial scale.

Diarrhea is a very common problem in animals associated with inflammation of the intestinal mucosa, varying degree of dehydration & acid base imbalance caused by bacterial infection, viral infection, worm infestation, protozoans, poor hygiene, stress etc. "Diaroak" a proprietary polyherbal formulation of AYURVET is a scientific blend of herbal extracts that maintains and restores the Gastro-Intestinal (GI) functions thus preventing and arresting the episodes of diarrhea, checks diarrhea of variable etiology, minimizes the loss of water and nutrients & repair the damaged GI mucosa. An exercise was carried out to establish the phyto equivalence by generating the fingerprint profile of the clinically validated product and standardizing the two antidiarrheal herbs *Acacia catechu* & *Berberis aristata* with their respective bioactive markers to ensure batch to batch consistency of the product in quality and efficacy.



(I)



(II)

Figure 1. Structure of Catechin (I) & Berberine (II)

Experimental:-

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) with stationary phase silica gel and layer thickness 0.2 mm were used.

Reagents and materials:-

Chemicals and reagents used were of analytical reagent grade. Propan-1-ol and formic acid, chloroform, methanol and water were purchased from Rankem. Catechin & Berberine were isolated in house and characterized by different spectroscopic methods before use. TLC plates were purchased from Merck (Darmstadt, Germany). Controlled samples of Diaroak were obtained from the QA/QC department of AYURVET LTD, 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 : Acetone : Formic Acid:: 75: 16.5 : 8.5, b. Ethyl acetate : Formic acid : Acetic acid : Water :: 100 : 11: 11: 27 for resolution of spots. Each TLC plate was developed to a height of about 9.0 cm, under laboratory conditions. Observation of spots was carried out by dipping the plate in iodine chamber and at 366 nm for mobile phase a & b respectively. Whereas, for quantification of Catechin & Berberine the spots were scanned at 254 nm and at 366 nm respectively with a slit size of 6 × 0.3 mm.

Preparation of sample & standard solutions:-

Preparation of standard solutions:-

Stock solutions (~ 0.5 mg/mL) of standards (marker compounds) **I** and **II** 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:-

Weighed accurately around 5 g of Diaroak and transferred to a 100ml round bottom flask. Added 50 ml of methanol, refluxed for 1 hour and filtered, repeated the process one more time and made up the volume to 100ml, if required. Clear resulting solution obtained after filtering it from 0.45µ syringe filter was used for TLC fingerprint profile generation & quantification of I & II.

Results & discussion:-

In one of the investigation, cases of diarrhoea of bacterial, protozoal or non-specific aetiology in goats and sheep , 88 & 53 in number respectively , of various ages occurring sporadically at an organized farm were treated with the polyherbal preparation Diaroak. The cases ranged in severity from mild to very severe diarrhoea of 1-2 days duration, before start of treatment. Response to the therapy was measured in terms of time taken for full recovery.

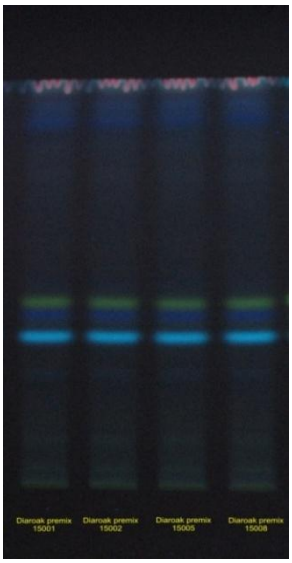
All the time 88 goats suffering from diarrhoea responded to Diaroak treatment. Overall, 69 (78.4%) of the goats were fully recovered in three days, while 19 (21.6%) of the adult goats required four days treatment for recovery. Diaroak was even more effective in sheep, all 53 of cases recovered within an average of 1.5 to 2.5 days. Diaroak, the antidiarrhoeal dry suspension, by virtue of its constituent herbs, has soothing action on the intestines through reduced peristalsis, absorption of enterotoxins and protective coating over mucosa, in addition to anti-secretory activities (2).

New HPTLC methods were developed to generate the fingerprint profile and standardization of product. The analytical methods were validated for linearity, accuracy, and precision in accordance with the statistical method of validation given in ICHQ2R1 (3)..

T1 T2 T3 T4

Tracks-
T1 – T4 different batches of DIAROAK
Detection: Spots developed in iodine chamber

(A)

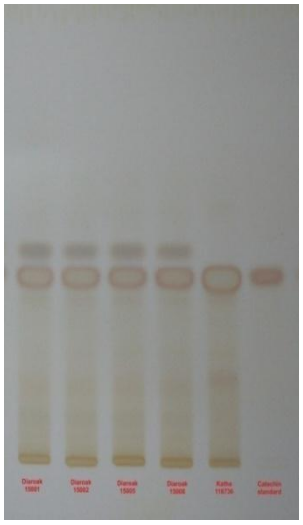


T1 T2 T3 T4

Tracks-
T1 – T4 different batches of DIAROAK
Detection: at 366 nm

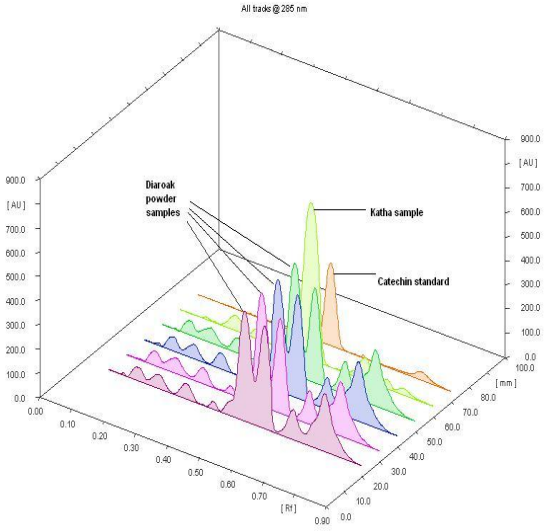
(B)

Fig. 2 Fingerprint profile (A) – using mobile phase a. & (B) – using mobile phase b.



T1 T2 T3 T4 T5 T6

(a)



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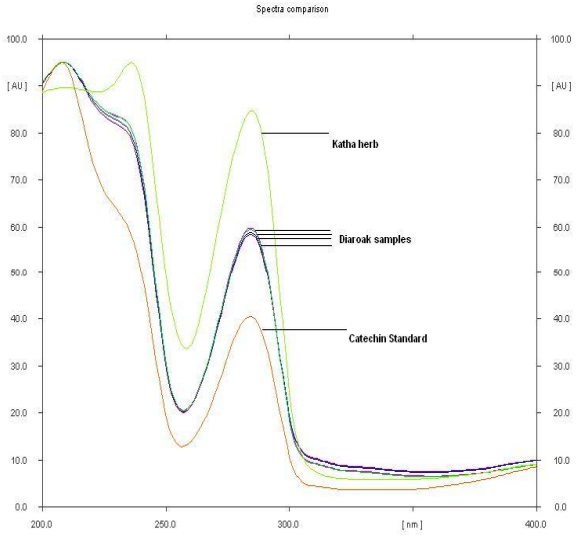
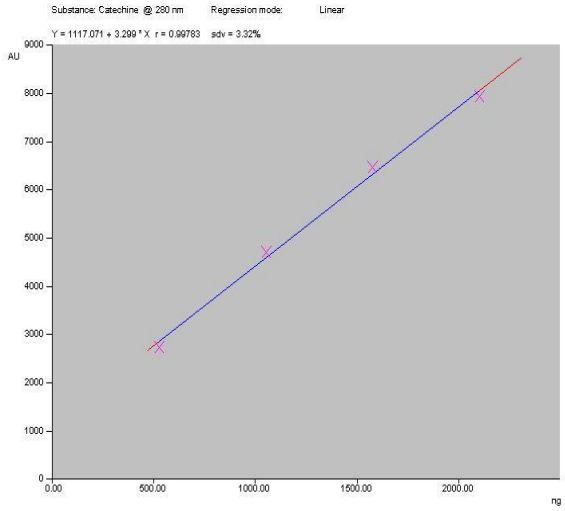
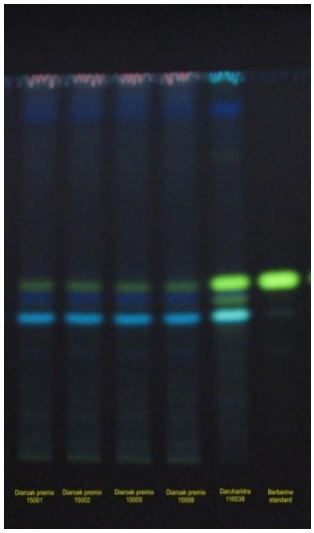




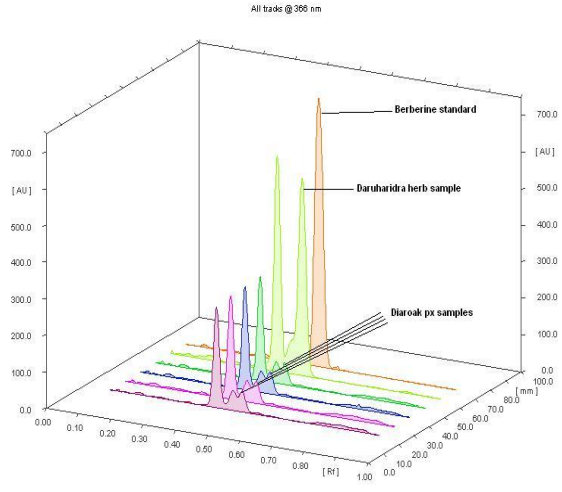
(c) **(d)**

Figure 3: Chromatograms showing the resolution of marker compound in the formulation Diaroak - (a) TLC profile of Diaroak, Acacia catechu herb & Catechin standard (I) ; Tracks- T1 – T4 different batches of DIAROAK, T5 – *Acacia catechu* herb, T6 – Catechin standard(b) TLC densitometric chromatogram overlay (c) Overlay of spectra of Catechin standard with its counterpart in formulation & herb (d) Calibration plot for Catechin standard.



T1 T2 T3 T4 T5 T6

(a)



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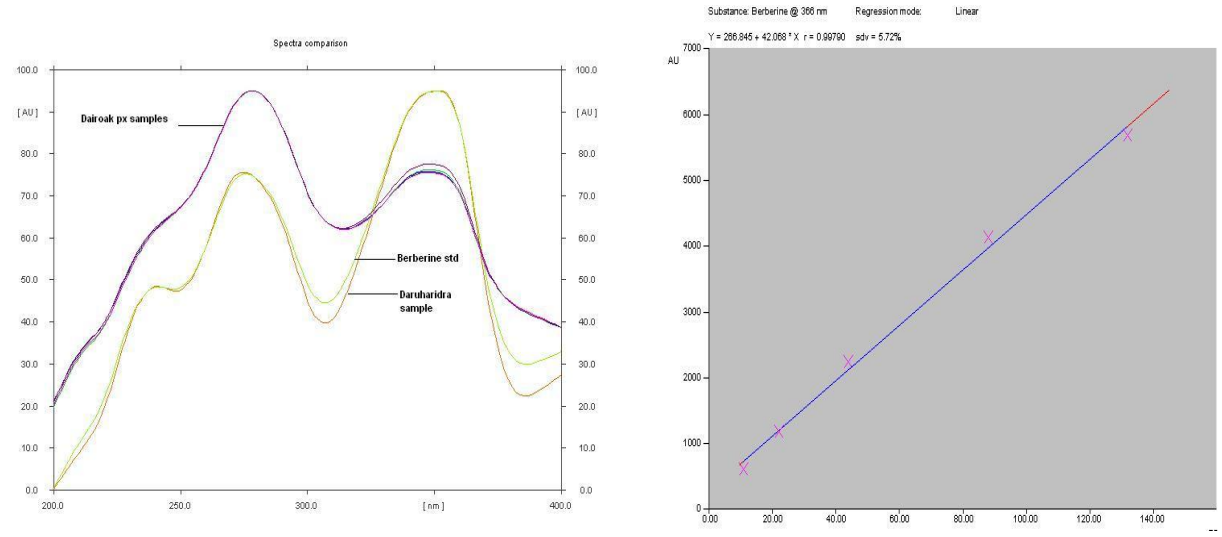

Figure 4: Chromatograms showing the resolution of marker compound in the formulation Diaroak - (a) TLC profile of Diaroak, Acacia catechu herb & Berberine standard (I) ; Tracks- T1 – T4 different batches of DIAROAK, T5 – *Berberis aristata* herb, T6 – Berberine standard(b) TLC densitometric chromatogram overlay (c) Overlay of spectra of Berberine standard with its counterpart in formulation & herb (d) Calibration plot for Berberine standard.

Method validation:-

Calibration curve (Linearity):-

The method was validated in accordance with the statistical method of validation given in ICHQ2R1 (3). 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 3d and 4d). 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. Catechin & Berberine 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.

Precision:-

Method precision (Repeatability):-

The precision of the instrument was checked by repeated scanning of the same spot (n = 7) of Catechin (1.0 µg/spot) and Berberine (350 ng/spot) without changing the position of the plate for the HPTLC method.

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 repeating the same on three different days to record intra-day and inter-day variations in the results, respectively.

The lower RSD for Catechin & Berberine suggested that proposed method is robust (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 Catechin & Berberine peaks separately

were, therefore, not masked by any peak of other compound present in the formulation (Figures 3c and 4c), which indicated respective peak purity.

LOD & LOQ:-

For determination of limits of detection and quantification different dilutions of the standard solutions of Catechin & Berberine were applied to the plates with methanol as blank and determined on the basis of the signal-to noise ratio. 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 Catechin & Berberine was 0.15 µg spot-1 and 1.5 ng spot-1 respectively, whereas, the LOQ was 0.45 µg spot-1 and 4.5 ng spot-1, respectively.

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

Name of marker	Catechin	Berberine
Concentration range [µg spot-1]	526.0 ng - 2.104ug	11.0ng - 176 ng
Regression equation	y = 42.07x + 266.8	y = 3.3x + 1117.17
Correlation Coefficient (r2)	0.998	0.998
Amount of marker compound in Diaroak [%] (w/w)a	0.4 ± 0.05	0.01 ± 0.002
Method precision (Repeatability) – RSD %	0.92	0.87
Intermediate precision (Reproducibility) - RSD [%] Intraday 1 Interday 3	 0.89 0.81	 0.79 0.90
LOD	0.15 µg spot-1	1.5 ng spot-1
LOQ	0.45 µg spot-1	4.5 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	**Catechin**			**Berberine**		
Initial concentration in formulation [mg g-1]	4.0	4.0	4.0	0.1	0.1	0.1
Concentration added [mg g-1]	0	2.0	4.0	0	0.1	0.2
Total concentration [mg g-1]	4.0	6.0	8.0	0.1	0.2	0.3
Concentration found [mg g-1]	3.75	5.60	7.59	0.092	0.185	0.279
RSD [%] (n=7)	0.9	0.95	0.95	1.0	1.1	1.0
Recovery [%]	93.75	93.33	94.87	92.0	92.5	93.0
Mean recovery [%]	93.98			92.5		

Conclusion:-

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

Establishing the phytoequivalence & standardization of clinically validated batch has helped us in ensuring the quality control and efficacy of the product on commercial scale.

Acknowledgement:-

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References:-

1. Evidence-Based Validation of Herbal Medicine, edited by Pulok K. Mukherjee, Elsevier publication, 2015.
2. Singh, Z., 1996, Indian Vet. Med. Jour. Vol.20, 311-313.
3. ICH Harmonized Tripartite Guideline-Q2R1, (2005): Validation of Analytical Procedures, Text and Methodology, Geneva, 1-13.