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RESEARCH ARTICLE

QUANTITATIVE ANALYSIS OF TRIGONELLINE AND CUCURBITACIN-*E* WITH VALIDATED ISOCRATIC RP-HPLC METHODS FOR STANDARDIZING THE HERBAL FORMULATION: REPCHOL® PREMIX

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Abstract

Herbal formulations are complex mixtures of medicinal plants and great efforts are necessary to guarantee a constant and optimum quality. Since pharmacological response to the herbal formulations depends on the secondary metabolites present in the herbs, it is important to evaluate the phytoconstituents present in the formulation. Standardization has become a important part for the regulation of the herbal industry with optimum quality measures. The development of validated reverse phase - high performance liquid chromatographic (RP-HPLC) methods for the quantification of secondary metabolites trigonelline and cucurbitacin-*E* in herbal formulation Repchol® Premix are reported. The developed methods were validated following ICH guidelines for precision, linearity, accuracy, specificity, limit of detection, limit of quantification, and robustness. The outcome of the present investigation underlines the importance of the standardization of Ayurvedic formulations. This will ensure the batch to batch consistency in quality and efficacy. The amounts of trigonelline and cucurbitacin-*E* in Repchol® Premix a commercial herbal formulation, were successfully quantified by the developed RP-HPLC methods. The average content of these markers in different batches of the formulation was 0.079 % and 0.104 % (w/w) respectively. Our results offer new, sensitive and reliable tools for the standardization of herbal formulations containing *Trigonella foenum-graecum* and *Citrullus colocynthis*.

Introduction:-

Herbal formulations have a long tradition of use as feed supplements and medicines worldwide; they are increasingly used for the management of healthy growth and long-term illnesses [(1)].

The swine and broiler industry is gaining more approaches for the production of the organic type of meat. People also prefer meat produced from the feeding of swine and poultry without synthetic chemicals due to their health consciousness[(2-4)].

Constant research efforts are made to replace synthetic vitamins and enzymes with herbal preparations to avoid the side effects. Repchol® Premix a proprietary product of Ayurvet Limited, India, is a scientifically developed

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combination of herbs that mimics the action of choline in the body and improves the production and general health of swine and poultry. Repchol® Premix is a polyherbal formulation of seven herbs, where the key herbs like *Citrullus colocynthis*, *Trigonella foenum-graecum*, and *Sida cordifolia* majorly contribute to the biological properties of this herbal supplement. Repchol® Premix provides optimum activity of choline which further helps in liver functioning and control fatty liver syndrome. *Citrullus colocynthis* and *Trigonella foenum-graecum* pharmacologically donate for Betaine synthesis, results in improved growth, performance, and quality of the carcass[5-7].

Herbal formulations are chemically complex and contain multiple constituents, further polyherbal formulations add considerable complexity to their chemical and pharmacological properties[8,9]. Certain groups of secondary metabolite compounds of herbs were given greater priority because they are likely to be physiologically and pharmacologically active (for example, polyphenols, alkaloids, flavonoids, phytosterols, terpenes) based on the literature. Reliable quantitation of these bioactive components in the herb is important. Since it is impractical to analyze all the chemical components that may be present, the challenge is selecting a small group of chemical markers for quantification, which most aptly describes the quality, safety, and efficacy of the product[10].

Due to the variability of herbs and their potential use for serious clinical indications, it is important to develop and carry out adequate QA/QC on the finished herbal product, most especially where higher-order therapeutic claims are intended[11]. Some bioactive components of an herb have been well characterized in terms of their identity and concentration. Chemical quantification was done on the relevant part of the plant (for example, root, bark, leaves, or flowers) used in the formulation. In Repchol® Premix, *Citrullus colocynthis* and *Trigonella foenum-graecum* contain cucurbitacin-*E* (triterpene hydrocarbon) and trigonelline (alkaloid) respectively (Figugre 1). Validated analytical methods on RP-HPLC have been used for the quantification of trigonelline and cucurbitacin for standardization of Repchol® Premix.

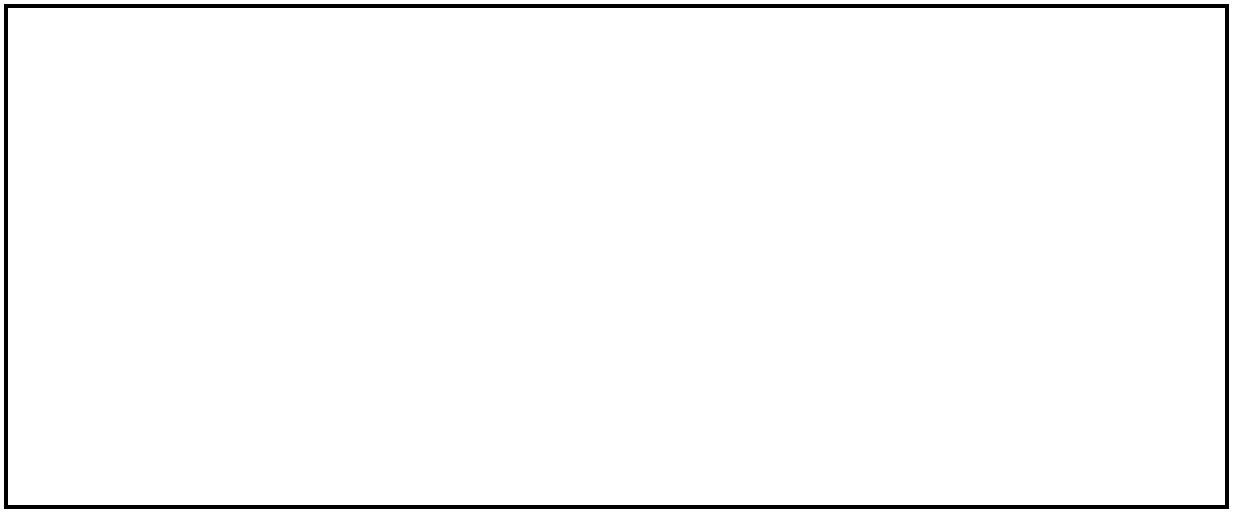


Figure 1:- Trigonelline and cucurbitacin-*E*.

Material And Methods:-

Reagents and materials

All the reagents and solvents were of AR or HPLC grade as per requirement. The active compound cucurbitacin-*E* was isolated in our lab and its structure was established by interpreting the 1H, 13C & 2D NMR spectra, Trigonelline hydrochloride (CAS number: 6138-41-6) standard was procured from Sigma Aldrich. Controlled samples of Repchol® Premix were obtained from the QA/QC department of Ayurvet Limited.

Preparation of standard solution of trigonelline:

2.5 mg of 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 5 – 50 µg/ml and then injected in HPLC to prepare the calibration graphs and quantification of bioactive.

Preparation of standard solution of cucurbitacin-*E*:
5 mg of 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 20 – 200 µg/ml and then injected in HPLC to prepare the calibration graphs and quantification of bioactive.

Preparation of test solution (Repchol® premix):
For the quantification of trigonelline and cucurbitacin, 5 g Repchol® Premix was refluxed with 50 ml of petroleum ether (60 – 80ºC) for 3 hours and filtered, the process was repeated once. The defatted sample was extracted with 50 ml of methanol under reflux conditions for 3 hours and filtered, process was repeated twice. The final volume was made to 100 ml with methanol and filtered through 0.45µ membrane filter before injecting into HPLC.

High-Performance Liquid Chromatography
Apparatus and conditions
Trigonelline and cucurbitacin-*E* content were analyzed by High-Performance Liquid Chromatography (USA-WATERS, binary pump-1525, 2707-auto sampler with PDA-2998 detector). The data was acquired on the Empower 3.0 controlling software. Separation was obtained on the Phenomenex luna C18 column (250 mm x 4.6 mm, 5µm).

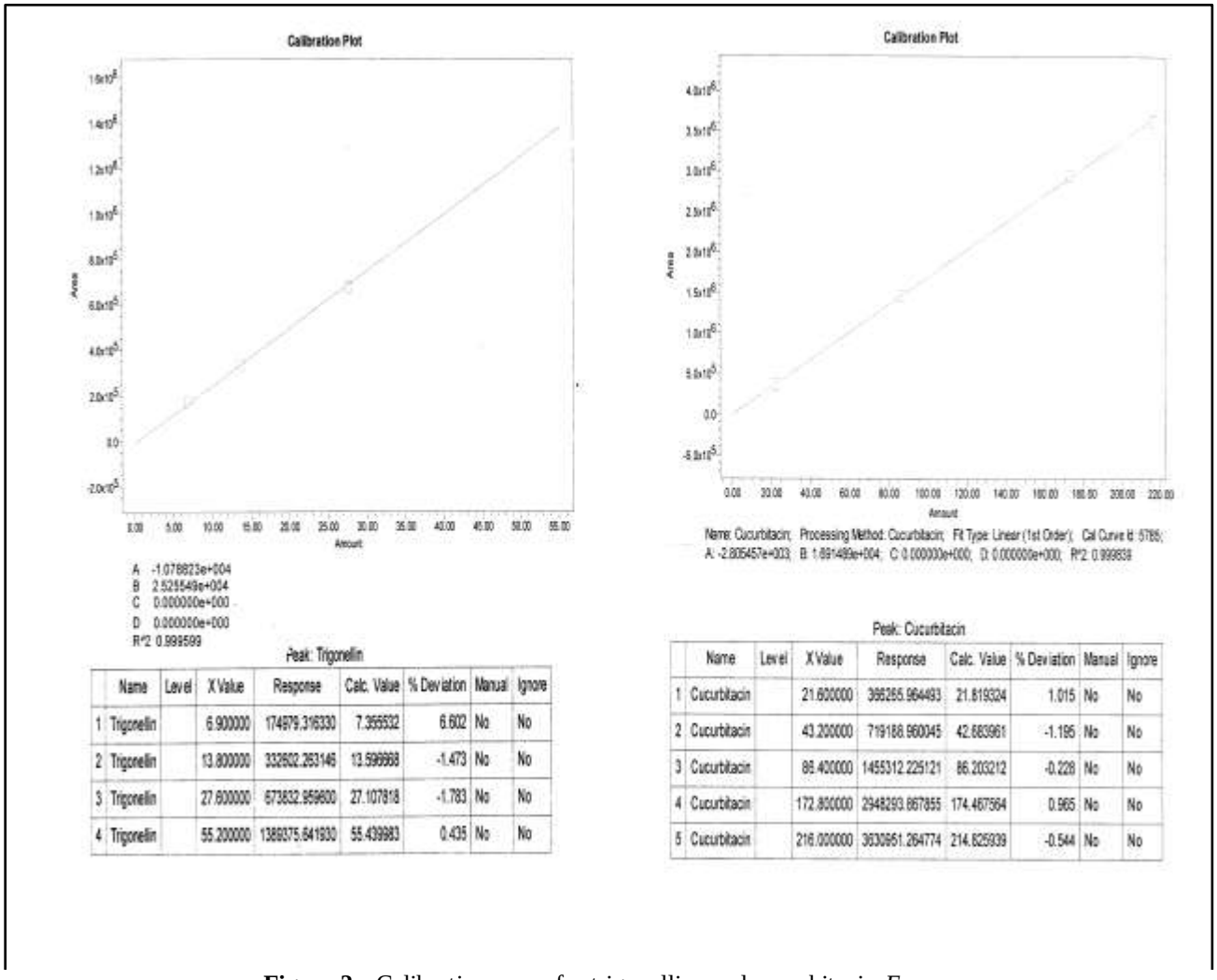
Selection and optimization of chromatographic condition
Several mobile phase compositions were tried to optimize the RP-HPLC parameters. A satisfactory separation and good peak symmetry for trigonelline (Figure 3) and cucurbitacin-*E* (Figure 4) were obtained by using acetonitrile : water (0.1 % acetic acid) :: 20 : 80 V/V ratio and water : acetonitrile in 50:50 v/v ratio respectively as a mobile phase in isocratic mode. The mobile phases were filtered through 0.45 µ filter and degassed before use. The flow rate was adjusted to 0.6 mL/min for both markers. Injection volume was adjusted to 20 µL and detection was made at 237nm and 265nm, respectively.

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 intended analysis. Standard solution mixture containing a known concentration of trigonelline and cucurbitacin-*E* were injected seven times, separately. RSD values for peak area and retention time of standard suggested the reproducibility for these parameters[12].

Validation of the proposed method:
The proposed methods were validated for the determination of trigonelline and Cucurbitacin-*E* using the following parameters as per ICH guidelines.
a. 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 (RT) and UV absorption spectrum with those obtained for standard.
b. 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 (Figure. 2, Table 1).
c. Range: Range is the interval between the upper and lower concentration of analyte in 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 5-50 ppm for trigonelline and 20-200 ppm for Cucurbitacin-*E* (Figure. 2, Table 1).
d. Precision: Three different concentrations of marker compound solution in triplicates were injected at 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. The low % RSD values of intraday and interday (Table 1) for the marker compounds trigonelline and cucurbitacin-*E* reveals that the proposed methods are precise.
e. 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).
f. Selectivity: The retention time of trigonelline and cucurbitacin-*E* and their counterpart in the formulation were 4.70±0.02 and 7.80±0.02 minutes 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 cucurbitacin-*E* peaks were, therefore, not masked by any peak of another compound present in the formulation (Figures 3 & 4) which was indicative of peak purity.

g. 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 cucurbitacin-*E* 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).



Peak: Trigonellin

	Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	Trigonellin		6.900000	174879.316330	7.355532	6.602	No	No
2	Trigonellin		13.800000	332602.263146	13.599868	-1.473	No	No
3	Trigonellin		27.600000	673832.959600	27.107818	-1.783	No	No
4	Trigonellin		55.200000	1389375.641930	55.439983	0.435	No	No

Peak: Cucurbitacin

	Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	Cucurbitacin		21.600000	366265.964493	21.819324	1.015	No	No
2	Cucurbitacin		43.200000	719188.960045	42.663961	-1.195	No	No
3	Cucurbitacin		86.400000	1455312.225121	86.203212	-0.228	No	No
4	Cucurbitacin		172.800000	2948293.867855	174.467564	0.965	No	No
5	Cucurbitacin		216.000000	3630951.264774	214.625939	-0.544	No	No

Figure 2:- Calibration curve for trigonelline and cucurbitacin-*E*.

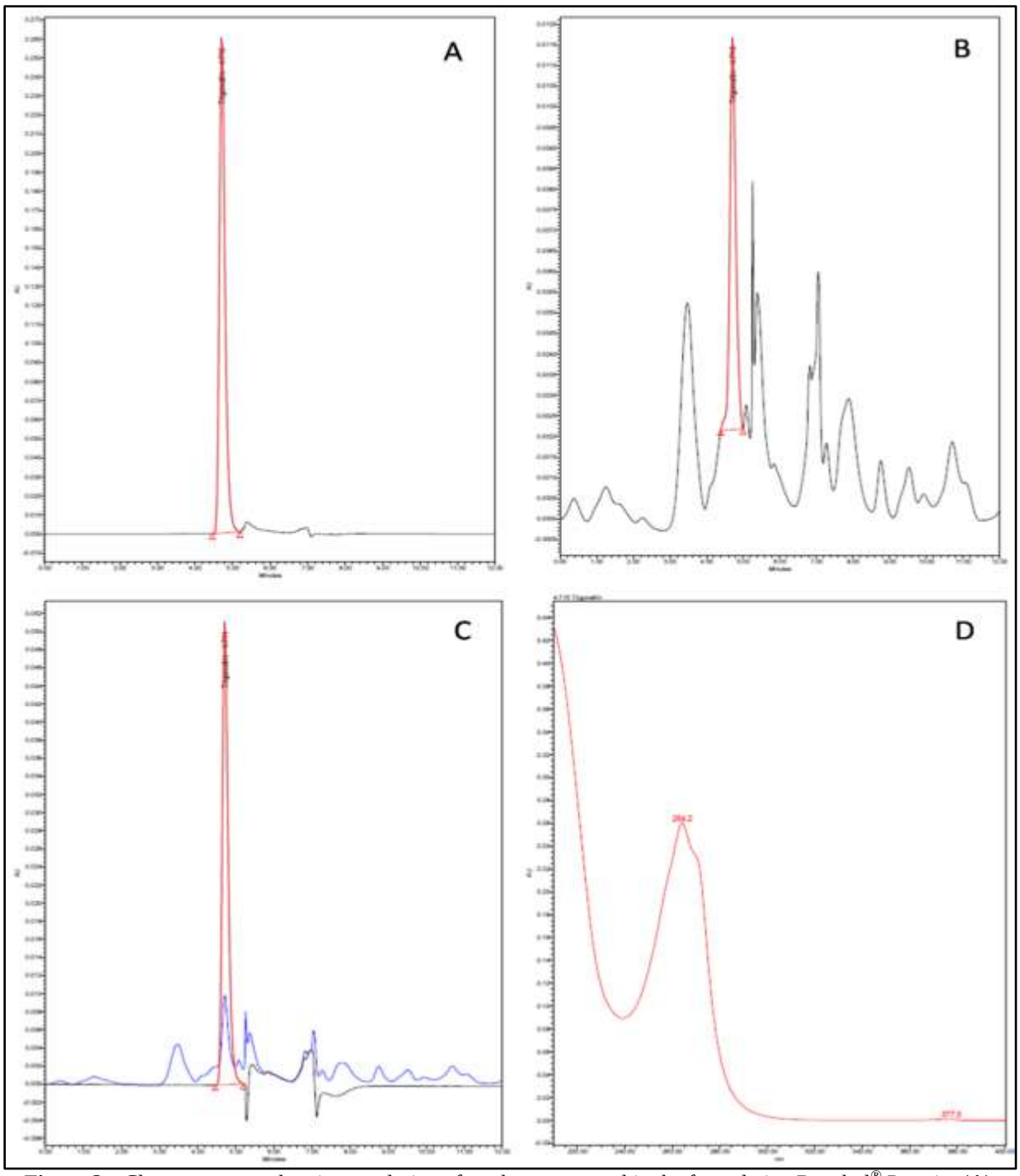


Figure 3:- Chromatograms showing resolution of marker compound in the formulation Repchol® Premix. (A) Chromatogram of the marker compound trigonelline. (B) Chromatogram of the formulation Repchol® Premix. (C) Chromatogram overlay comparison of standard and formulation. (D) Spectral scan of the trigonelline marker.

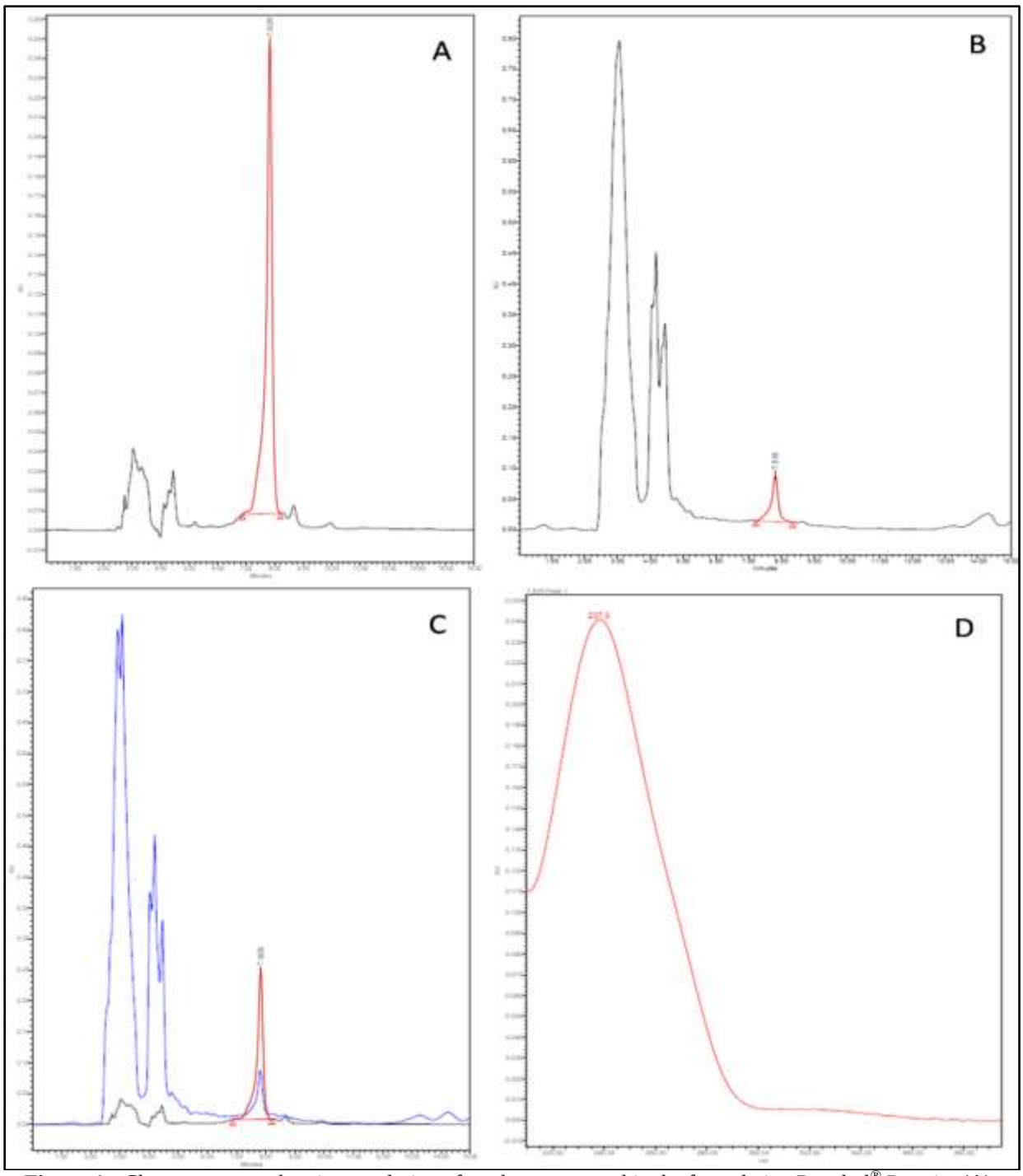


Figure 4:- Chromatograms showing resolution of marker compound in the formulation Repchol® Premix. (A) Chromatogram of the marker compound cucurbitacin -*E*. (B) Chromatogram of the formulation Repchol® Premix. (C) Chromatogram overlay comparison of standard and formulation. (D) Spectral scan of the cucurbitacin-*E* marker.

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

Sr. no.	Parameters	Trigonelline	Cucurbitacin-*E*
1	Concentration range [µg ml-1]	5 - 50	20 - 200
2	Regression equation	y = 2.5255x – 1.078	y = 16915x - 2806

3	Correlation Coefficient (r2)	0.999	0.999
4	Amount of marker compound in Repchol® Premix [%] (w/w)a	0.079	0.104
5	Method precision [Repeatability (Seven replicates)] – Area RSD %	0.3	0.5
6	Intermediate precision (Reproducibility) - RSD [%] Intraday 1 Interday 3	 0.42 0.51	 0.72 0.78
7	LOD	1.77 µg ml-1	0.06 µg ml-1
8	LOQ	5.31 µg ml-1	0.18 µg ml-1

y = peak area response
x = amount of marker compound
a = Mean ± SD, n=6

Table 2:- Results from determination of recovery.

Sr. no.	Parameter	Trigonelline			Cucurbitacin-*E*		
1	Initial concentration in formulation [mg g-1]	0.79	0.79	0.79	1.04	1.04	1.04
2	Concentration added [mg g-1]	0.0	2.0	4.0	0.0	2.0	4.0
3	Total concentration [mg g-1]	0.79	2.79	4.79	1.04	3.04	5.04
4	Concentration found [mg g-1]	0.74	2.70	4.48	1.0	2.88	4.83
5	Recovery [%]	93.67	96.77	93.53	96.15	94.74	95.83
6	Mean recovery [%]	94.66			95.57		

Result 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 being successfully applied in the identification and quantification of the phytoconstituents. A linearity range of 5 -50 µg ml-1 for trigonelline and 20 – 200 µg ml-1 for cucurbitacin-*E* shown good coffiecint of correlation (r^2) of 0.999 each.The average recovery of trigonelline (94.66%) and cucurbitacin-*E* (95.57 %) 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 Repchol® Premix 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. Repchol® Premix, a proprietary polyherbal feed supplement of Ayurvet Limited. Standardization of phytotherapeutic constituents trigonelline and cucurbitacin-*E* 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, methods reported here are simple, precise, accurate, and are 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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