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RESEARCH ARTICLE

Establishing the phyto equivalence of an antidiarrhoeal and gut function modulator feed additive - Salcochek

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Abstract

The concept of phyto equivalence is 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. Pharmacological properties of an herbal formulation depend on phytochemical constituents present therein, development of authentic analytical methods which can reliably profile the phytochemical composition, including quantitative analyses of marker/bioactive compounds and other major constituents, is a major challenge to scientists. Without consistent quality of a phytochemical mixture, a consistent pharmacological effect is not expected. New HPTLC methods were developed for the quantification of two main ingredients of the formulation i.e. *Acacia catechu* with the active tannin Catechin and *Berberis aristata* with bioactive alkaloid berberine. The analytical methods were validated for linearity, accuracy, and precision in accordance with the statistical method of validation given in ICHQ2R1. The average recovery of Catechin (100.28%) and Berberine (98.84%) was computed from the regression equation. The methods are simple, precise, specific, and accurate and have the potential for use in the routine quality control of the formulation. Benchmark limits were set rationally as check points for the two bioactive markers in the formulation as testimonials for its effectiveness.

Introduction

The recent global resurgence of interest in herbal medicines has led to an increase in the demand for them. Commercialization of the manufacture of these medicines to meet this increasing demand has resulted in a decline in their quality, primarily due to a lack of adequate regulations pertaining to this sector of medicine. The need of the hour is to evolve a systematic approach and to develop well-designed methodologies for the standardization of herbal raw materials and herbal formulations.

In recent years, there is upsurge in therapeutic trials of herbal medicines against various infectious diseases in poultry (1). Contamination of feed ingredients with microbes affects gut health and nutrient utilization. Occurrence and load of pathogenic bacteria in poultry feed has increased in the recent past which results in poor gut health and nutrient utilization. Pathogens like *Clostridium perfingens* produce such toxins which damages the mucosal lining of digestive tract. Performance and profitability of poultry operation depends not only on the quality of feed offered but also the gut health of the birds. Further, contamination of feed ingredients with microbes affects gut health and nutrient utilization. The product for efficient gut management is not only required to curb pathogenic bacteria viz; *Salmonella, E.coli* population but also friendly action on useful bacteria and inactivation mechanism of toxins.

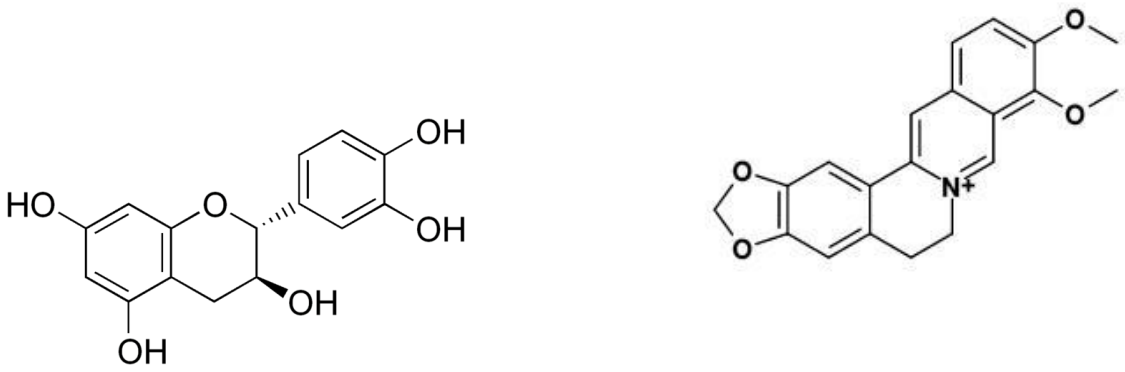
Salcochek, a proprietary polyherbal formulation of AYURVET for poultry is a scientifically prepared herbal formulation enriched with prestandardized herbs and electrolytes. Its constituent herbs adsorb and inactivate enterotoxins; hence control damage of GI mucosa. Its herbal ingredients impart hepatoprotective, gastroprotective, antibacterial and antiviral activity. Different Phytoactive constituents work in synergy and helps bird to maintain optimum gut health.

A variety of molecular techniques including restriction fragment length polymorphism, random amplification of polymorphic DNA and DNA sequencing are used to authenticate plant material and detect adulterated plant species. Each technique has advantages and disadvantages in terms of cost, accuracy, reproducibility, time and taxonomic level of identification.

For standardization of natural product drugs, single chemical entities, "marker compounds," may be used as potency standards in high performance thin layer chromatography (HPTLC) analysis. Using well-characterized marker compounds, conventional pharmaceutical manufacturing criteria for assay and content uniformity may be applied. These marker compounds may be used to help identify herbal materials, set specifications for raw materials, standardize botanical preparations during all aspects of manufacturing processes, and obtain stability profiles. HPTLC analysis for marker compounds may provide additional information in the form of "chromatographic fingerprints."

As pharmacological properties of an herbal formulation depend on phytochemical constituents present therein, development of authentic analytical methods which can reliably profile the phytochemical composition, including quantitative analyses of marker/bioactive compounds and other major constituents, is a major challenge to scientists. Without consistent quality of a phytochemical mixture, a consistent pharmacological effect is not expected. The concept of phyto equivalence is 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.

Among various experimental techniques chromatographic methods are highly recommended to generate the fingerprint profile of herbal products because of their high separation ability. Standardization of the product with respect to the bioactive phyto constituents was taken up to ensure the batch to batch consistency in efficacy. Fingerprint profile of the efficacious batch was generated and the new HPTLC methods were developed for the quantification of two main ingredients of the formulation i.e. *Acacia catechu* with the bioactive Catechin (Fig.1, I) & *Berberis aristata* with bioactive Berberine having hepatoprotective activity (2, 3), (Fig.1, II). The analytical methods were validated for linearity, accuracy, and precision in accordance with the statistical method of validation given in ICHQ2R1 (5). The average recovery of Catechin (100.28 %) and Berberine (98.84 %) was computed from the regression equation. Benchmark limits were set rationally as check points for the two bioactive markers along with the fingerprint profile of the formulation as testimonials for its effectiveness.



(I) **(II)**

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

2. EXPERIMENTAL

2.1 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.

2.2 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 Salcocheck were obtained from the QA/QC department of AYURVET LTD, Baddi.

2.3 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. Each TLC plate was developed to a height of about 9.0 cm, under laboratory conditions with a mobile phase of Chloroform: Methanol: Water (6:3.5:0.5, v/v/v) for fingerprint profile generation. Chloroform: Methanol (8.5:1.5, v/v) and Propan-1-ol: Water: Formic acid (9:0.9:0.1, v/v/v) for the quantification of Catechin and Berberine, respectively. Developed plates were dried in a stream of air and fingerprint profile generated by observation and scanning the TLC using Camag TLC Scanner 3 at 254 & 366 nm, spraying the TLC with dilute sulphuric acid and heating at 105°C for 5 minutes. Quantitative determination of spots corresponding to **I** and **II** were done by Camag TLC Scanner 3 at 285 & 366 nm respectively with a slit size of 6 × 0.3 mm.

2.4 Preparation of sample & standard solutions

2.4.1 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.

2.4.2 Preparation of sample solutions

Weighed accurately around 5 g of Salcochek 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.

3. RESULTS & DISCUSSION

A broiler trial was conducted for 38 days at Veterinary College and Research Institute, Namakkal, Tamilnadu, India, to assess the efficacy of the polyherbal product Salcochek on gut health and reducing the impact of *Clostridium perfringens* induced enteritis using 120 birds. Body weight of birds, feed intake, feed conversion efficiency, litter moisture, nutrient digestibility (dry matter, crude protein & crude fibre), dressing percentage, intestinal length, blood parameters (Hb, total proteins, albumin, globulins, uric acid, serum sodium, potassium, chloride levels), villi height and crypt depth in the duodenum region and HI titre against Ranikhet disease (RD) did not vary significantly

between treatment groups. However, the feed cost per kg live weight was relatively less in polyherbal supplemented groups when compared to the un-supplemented groups. Moisture excretion through excreta and colonization of beneficial bacteria such as *Lactobacilli* spp. & *Bifidobacterium* spp in the intestine and reduction in *Clostridium* spp. load were comparatively better in polyherbal supplemented groups suggesting that the polyherbal product is having certain beneficial effects in improving gut heath and immunity(5).

An exercise was carried out to establish the phyto equivalence by generating the fingerprint profile of the clinically efficacious product under study. As the two herbs *Acacia catechu* with hepatoprotective action & *Berberis aristata* with endotoxin damage neutralizing property are among the main active ingredients in polyherbal formulation, which is a combination of herbs viz. *Plantago ovata, Acacia catechu, Aegle marmelos, Punicia granatum Holarrhena antidysenterica, Berberis aristata & Woodfordia fruticosa* quantifying them with their respective bioactive markers and setting the limits along with fingerprint profile will help us in setting the phyto equivalence, hence, ensuring batch to batch consistency of the product for its efficacy.

Fingerprint profile of the efficacious batch was generated and the new HPTLC methods were developed to quantify Catechin & Berberine, thus establishing the phyto equivalence of clinically efficacious formulation. This exercise established the profile of chemical constituents present in the specific mobile phase and cut off limits for its bioactive phyto constituents below which optimum efficacy will not be observed, hence ensuring batch to batch consistency in terms of chemical profiling & pharmacological effect. The mobile phase used was optimized to resolve the chemical constituents for fingerprint profile generation (Figure 2 a-f). For the quantification, different combinations of mobile phases were used to resolve Catechin (Figure 3 b; Rf 0.19) and Berberine (Figure 4 b; Rf 0.33) from other phytoconstituents in the formulation. Finally, Chloroform: Methanol: Water (6:3.5:0.5, v/v/v), Chloroform: Methanol (8.5:1.5v/v) and Propan-1-ol: Water: Formic acid (9:0.9:0.1, v/v/v) were chosen as mobile phases for fingerprint profile generation and for quantification.

The fingerprint profile was generated by observation and scanning the TLC using Camag TLC Scanner 3 at 254 & 366 nm, spraying the TLC with dilute sulphuric acid and heating at 105°C for 5 minutes (Figure 2 a-f).

For quantitative determination the chromatograms were scanned at 285 & 366 nm for the two bio actives and the identities of the bands in the sample extracts were confirmed by comparing their Rf values and their absorption spectra with those obtained from reference standards (Figures 3 & 4, a-c).

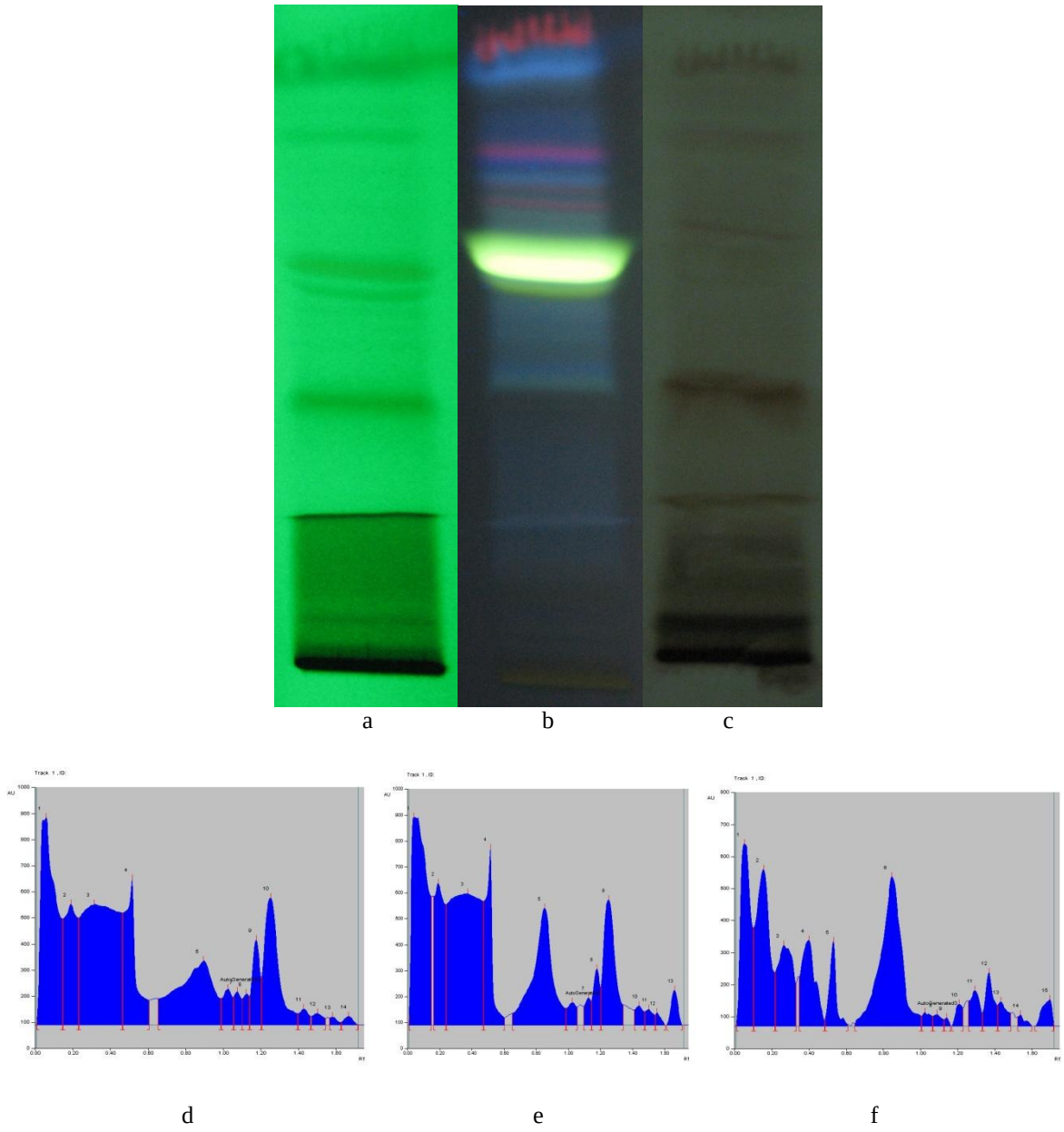


Figure 2: Fingerprint profile and chromatograms of Salcochek (a,d) at 254 nm, (b,e) at 366 nm, (c,f) after spraying with dilute sulphuric acid.

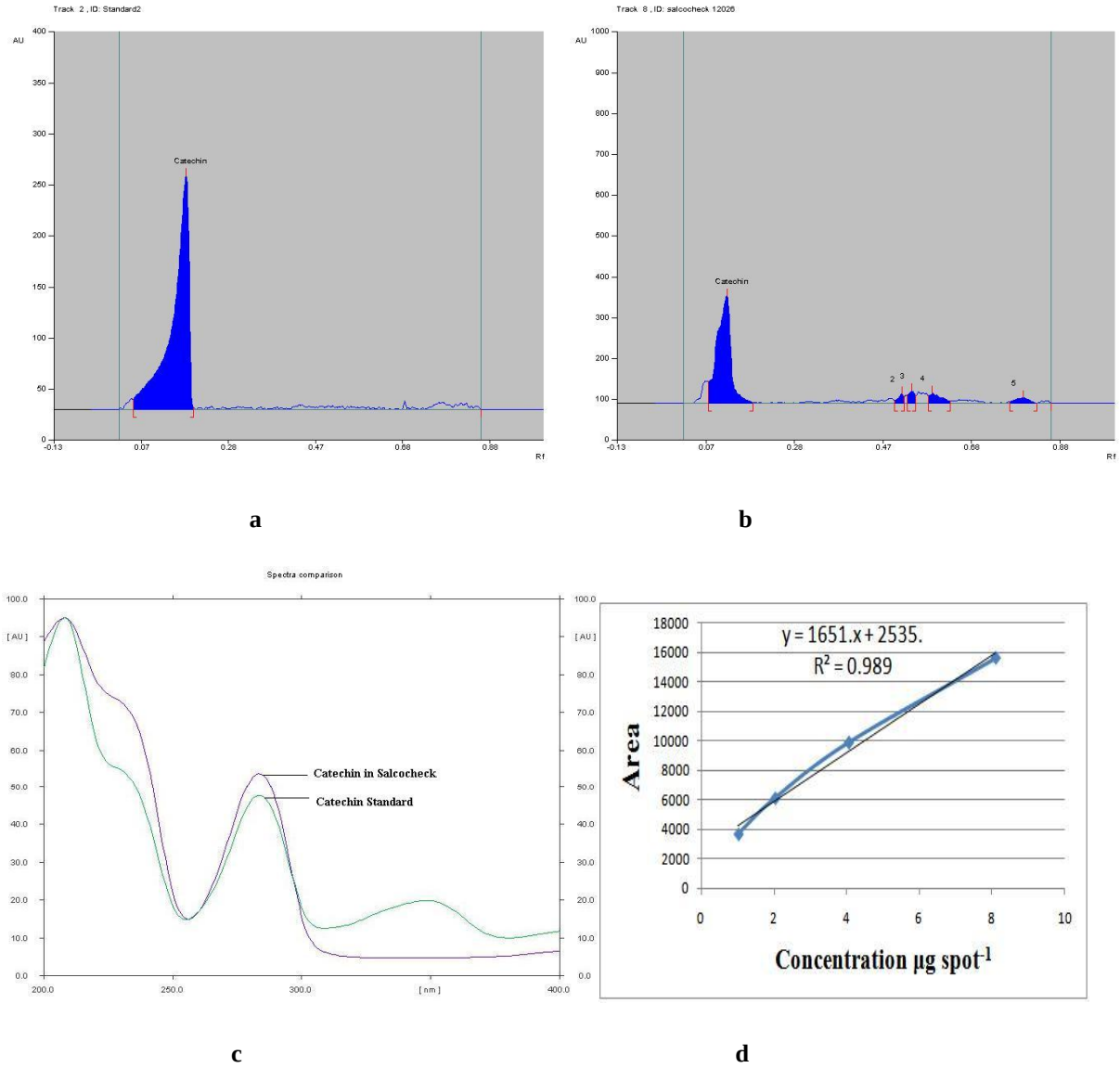


Figure 3: Chromatograms showing the resolution of marker compound in the formulation Salcochek. (a) Chromatogram of the marker compound Catechin (I). (b) Chromatogram of the formulation Salcochek. (c) Overlay of spectra of Catechin standard with its counterpart in formulation. (d) Calibration plot for Catechin standard.

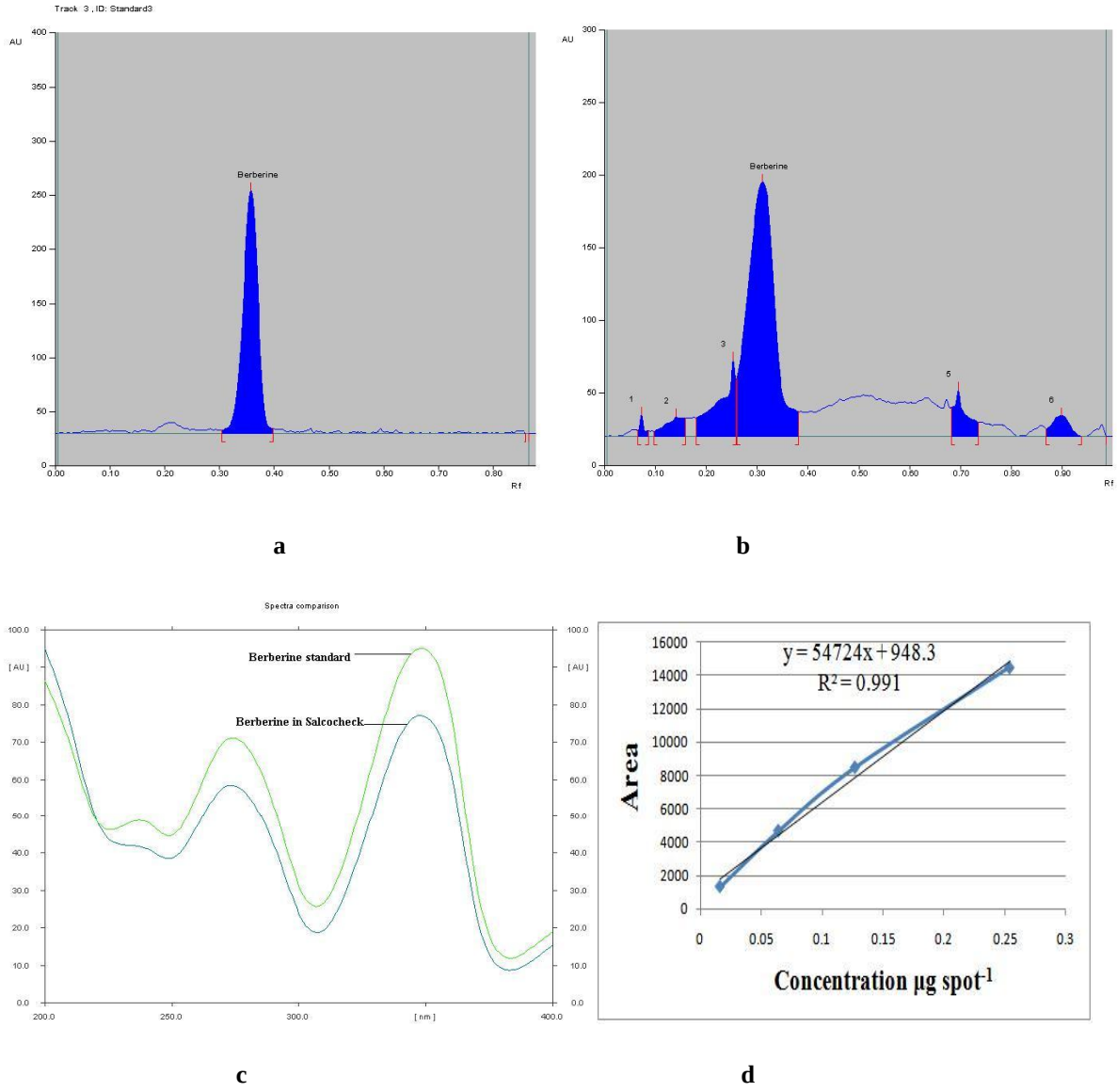


Figure 4: Chromatograms showing the resolution of marker compound in the formulation Salcochek. (a) Chromatogram of the marker compound Berberine (II). (b) Chromatogram of the formulation Salcochek. (c) Overlay of spectra of Berberine standard with its counterpart in formulation. (d) Calibration plot for Berberine standard.

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

Parameters	Catechin	Berberine
Concentration range [μg spot^{-1}]	1.01 – 8.11	0.01 – 0.25
Regression equation	y = 1651x + 2535	y = 54724x + 948.3
Correlation Coefficient (r2)	0.989	0.991
Amount of marker compound in Salcochek [%] (w/w)[a]	1.19 ± 0.02	0.13 ± 0.01
Method precision (Repeatability) – RSD %	0.92	0.87
Intermediate precision (Reproducibility) - RSD [%]		
Intraday 1	0.89	0.79
Interday 3	0.81	0.90
LOD	0.22 μg spot^{-1}	1.0 ng spot^{-1}
LOQ	0.74 μg spot^{-1}	3.25 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}]	11.9	11.9	11.9	1.3	1.3	1.3
Concentration added [mg g^{-1}]	0	6.02	12.05	0	0.70	1.35
Total concentration [mg g^{-1}]	11.9	17.92	23.95	1.3	2.00	2.65
Concentration found [mg g^{-1}]	12.0	17.99	23.86	1.25	1.97	2.7
RSD [%] (n=7)	0.33	0.43	0.60	0.30	0.3	0.19
Recovery [%]	100.84	100.39	99.62	96.15	98.5	101.88
Mean recovery [%]	100.28			98.84		

4. METHOD VALIDATION

4.1 Calibration curve (Linearity)

The method was validated in accordance with the statistical method of validation given in ICHQ2R1 (5). Two independent calibration equations were obtained. Linear regression analysis was used to calculate the slope, intercept, and coefficient of determination/regression coefficient (r2) for each calibration plot. 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 2d and 3d). Evaluation was on the basis of peak area.

4.2 Accuracy (% Recovery)

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. Catechin & Berberine standards were added to the formulation at two different concentrations, extraction and analysis was performed as described in preparation of sample solution. Recovery was calculated for each standard at each concentration. The results obtained are listed in Table 2.

4.3 Precision

4.3.1 Method precision (Repeatability)

The precision of the instrument was checked by repeated scanning of the same spot of Catechin & Berberine without changing the position of the plate for the HPTLC methods. The precision of the instrument was checked by repeated scanning of the same spot (n = 7) of Carechin (1.0 µg/spot) and Berberine (250 ng/spot) without changing the position of the plate for the HPTLC method.

4.3.2 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).

4.4 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-Visible 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 2c and 3c), which indicated respective peak purity.

4.5 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 220 ng $spot^{-1}$ and 1.0 ng $spot^{-1}$ respectively, whereas, the LOQ was 740.0 ng $spot^{-1}$ and 3.25 ng $spot^{-1}$, respectively.

Meticulously examining the finger print & amount of Catechin & Berberine in clinically efficacious batches we were in a position to establish the phytoequivalence which ensures the batch to batch consistency in efficacy of the product on commercial scale.

5. CONCLUSION

To ensure the batch to batch consistency in efficacy the fingerprint profile of the clinically efficacious batch was generated and the product was standardized to establish the phyto equivalence. New HPTLC methods were developed for the quantification of two main ingredients of the formulation i.e. *Acacia catechu* with the active tannin catechin and Berberis *aristata* with bioactive alkaloid berberine reported to exhibit hepatoprotective activity. Benchmark limits were set rationally as check points for the two bioactive markers in the formulation as testimonials for its effectiveness.

6. ACKNOWLEDGEMENT

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