ejpmr, 2015,2(4), 779-787

SJIF Impact Factor 2.026

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 3294-3211

EJPMR

STANDARDIZATION OF PHYTOTHERAPEUTIC CONSTITUENTS IN AYUCEE PREMIX

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Article Received on 25/06/2015 **Article Revised on 16/07/2015** **Article Accepted on 07/07/2015**

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ABSTRACT

Lack of proper standard parameters for herbal preparation and several instances of substandard herbs, adulterated herbs have come into existence and thus it becomes imperative to standardize the polyherbal formulation to ensure the batch to batch consistency in quality of product and its efficacy. As pharmacological properties of an herbal formulation depend on phytochemical constituents present therein, development of authentic analytical methods which can reliably profile the phytochemical quantification to maintain reproducible efficacy and safety of phytopharmaceutical is paramount responsibility of herbal drug industry. Standardization of the product Ayucee premix with respect to its phytotherapeutic constituents was undertaken and new validated HPTLC methods were developed for the quantification of two of its bioactive phytoconstituents.

KEY WORDS: HPTLC, Gallic acid, Ursolic acid, Ayucee premix.

1. INTRODUCTION

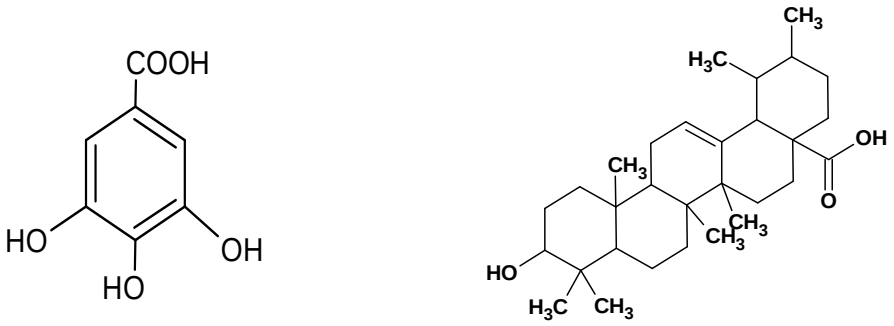
Modernizing the ancient art of herbal medicine bequeathed from generations entails addressing two interrelated issues i.e. efficacy and safety prior to their acceptance and use worldwide. WHO stresses the importance of the qualitative and quantitative methods for quantification of the biomarkers and/ or chemical markers and the fingerprint profiles. Lack of proper standard parameters for herbal preparation and several instances of substandard herbs, adulterated herbs have come into existence and thus it becomes imperative to

standardize the polyherbal formulation to ensure the batch to batch consistency in quality of product and its efficacy.

Exposure of birds to stress is an inevitable event in poultry husbandry, when the threshold level of stress is crossed results in distress to birds. Most of today's problems in poultry are caused by combinations of factors such as management, stress, nutrition, overcrowding, poor ventilation, high intensity of light, immunosuppressant and exposure to disease agents. Stress evokes harmful responses that interferes with the general health, productivity and result in immunosuppression.[1] Supplementation of antistressor products can alleviate adverse effect of various stressors in poultry.[2]

Ayucee premix (proprietary polyherbal formulation of AYURVET) a natural Vitamin C and bioflavonoids containing product owes its beneficial properties of reduction in mortality and losses due to oxidative stress, proper growth of poultry and maintaining immunity and livability to the constituent herbs *Phyllanthus emblica, Ocimum sanctum, Terminalia chebula* and *Withania somenifera*.[3] All of the herbs are well known for their antistress activity. It is well known that constituents Ursolic acid & Gallic acid which are the bioactive constituents of *Ocimum sanctum*, *Phyllanthus emblica* & *Terminalia chebula* posses strong free radical scavenging and antioxidant activities. As pharmacological properties of an herbal formulation depend on phytochemical constituents present therein, development of authentic analytical methods which can reliably profile the phytochemical quantification to maintain reproducible efficacy and safety of phytopharmaceutical is paramount responsibility of herbal drug industry. To meet new thrust of inquisitiveness, standardization of the product with respect to its phytotherapeutic constituents was undertaken and new validated HPTLC methods were developed for the quantification of two above mentioned phytoconstituents. The analytical methods were validated for linearity, accuracy, and precision in accordance with the statistical method of validation given in ICHQ2R1.[4] The average recovery of Gallic acid (99.96 %) and Ursolic acid (99.41 %) was computed.

The method is simple, precise, specific, accurate and has the potential for routine quality control of the formulation.



(A) **(B)**

Fig 1. Structure of Gallic acid (A) and Ursolic acid (B)

2. MATERIAL AND METHODS

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. 20 × 10 cm aluminum 60F254 TLC plates (E-Merck-Germany) with stationary phase silica gel and layer thickness 0.2 mm were used for the resolution of chemical constituents.

2.2 Reagents and materials

Chemicals and reagents used were of analytical reagent grade. Toluene, Ethyl acetate, Formic acid, Chloroform, Methanol and Water were purchased from RANKEM. Gallic acid and Ursolic acid were isolated in our lab and structures were established by interpreting the 1H, 13C and 2D NMR spectra. Controlled samples of Ayucee premix 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) on pre-activated (110°C) silica gel 60 F254 TLC plates under a flow of nitrogen gas 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. Toluene : Ethyl acetate : Formic acid : Water (3:3:0.8:0.4 v/v) and Chloroform: Methanol (95:05, v/v) were the mobile phase developed for the resolution of and quantification of Gallic acid & Ursolic acid, respectively. Quantitative determination of spots corresponding to **I** and **II** were done by Camag TLC Scanner 3 at 280 & 520 nm using deuterium & tungsten lamp respectively with a slit size of 6 × 0.3 mm.

2.4 Preparation of sample and standard solutions

Preparation of standard solutions

Stock solutions (0.1 mg/ml) of standards (marker compounds) A and B were prepared in methanol, different concentrations were spotted on TLC in order to prepare the calibration graphs and quantification of bioactives.

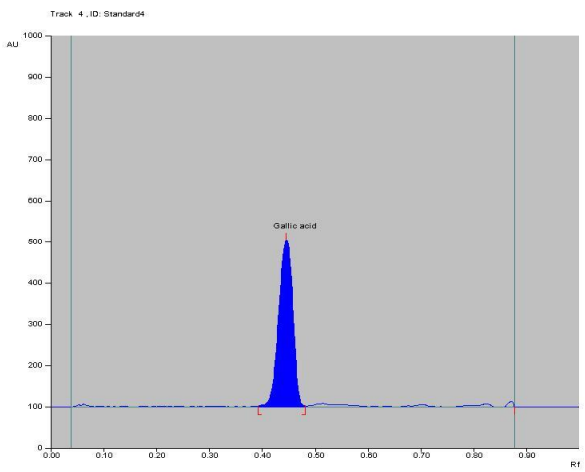
Preparation of sample solution

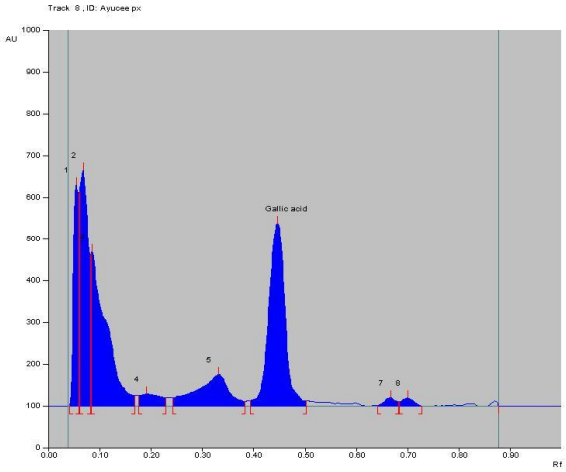
Weighed accurately around 5 g of Ayucal premix and transferred to a 100 ml round bottom flask. Added 50 ml of methanol, refluxed for 1 hour, repeated the process two more times. Filtered, concentrated and made up the volume to 100ml. Filtered the solution through 0.45µ syringe filter before application of spots on TLC.

3. RESULTS AND DISCUSSION

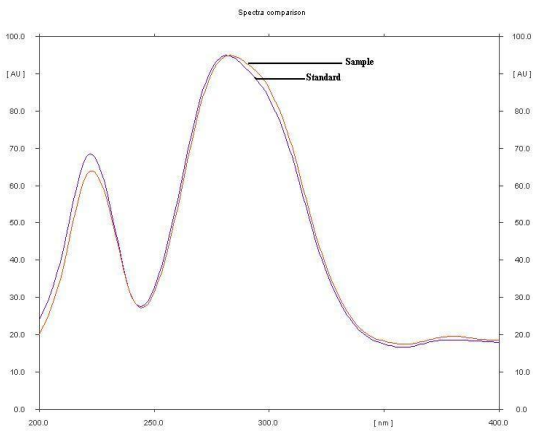
Ocimum sanctum, *Phyllanthus emblica* & *Terminalia chebula* are well known for their antistress activity and their constituents Ursolic acid & Gallic acid posses strong free radical scavenging and antioxidant and free radical scavenging activities. Standardization of these phytotherapeutic constituents will ensure the batch to batch consistency in efficacy of the product on commercial scale.

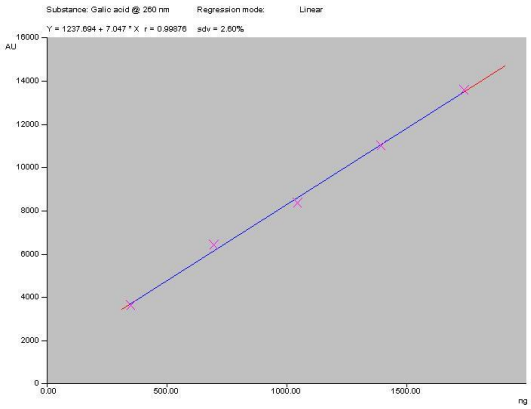
Mobile phase development and its optimization was carried out to resolve the chemical constituents which ultimately helped in quantitative estimation of two major phytoactives major contributors for the efficacy of Ayucee premix. Toluene : Ethyl acetate : Formic acid : Water (3:3:0.8:0.4 v/v) and Chloroform: Methanol (95:05, v/v) were the mobile phase finalized for the resolution of and quantification of Gallic acid & Ursolic acid , respectively. Quantitative determination of spots corresponding were done by Camag TLC Scanner 3 at 280 & 520 nm using deuterium & tungsten lamp respectively with a slit size of 6 × 0.3 mm and the identities of the bands in the sample extracts were confirmed by comparing their Rf values and overlaying their absorption spectra with those obtained from reference standards (Figures 2 & 3, a-c).





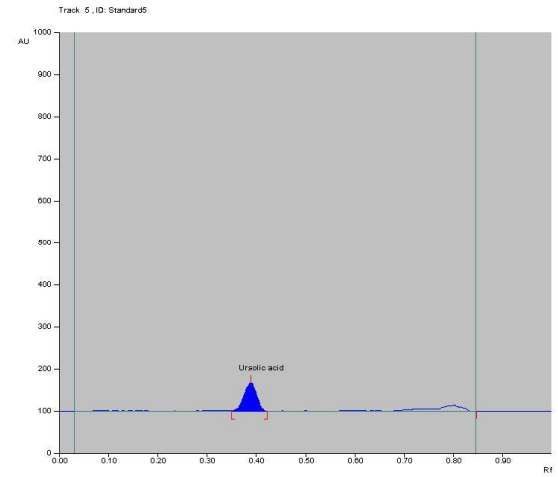
(a) **(b)**

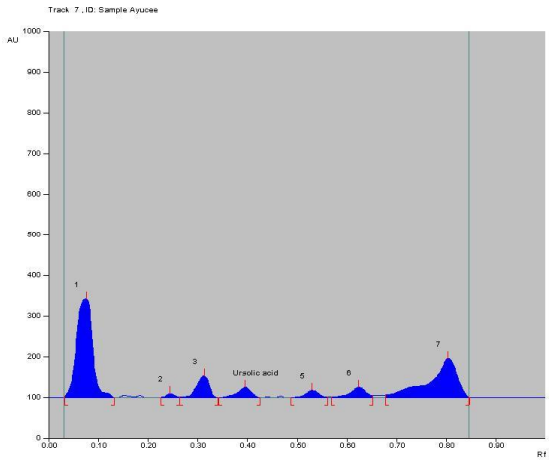




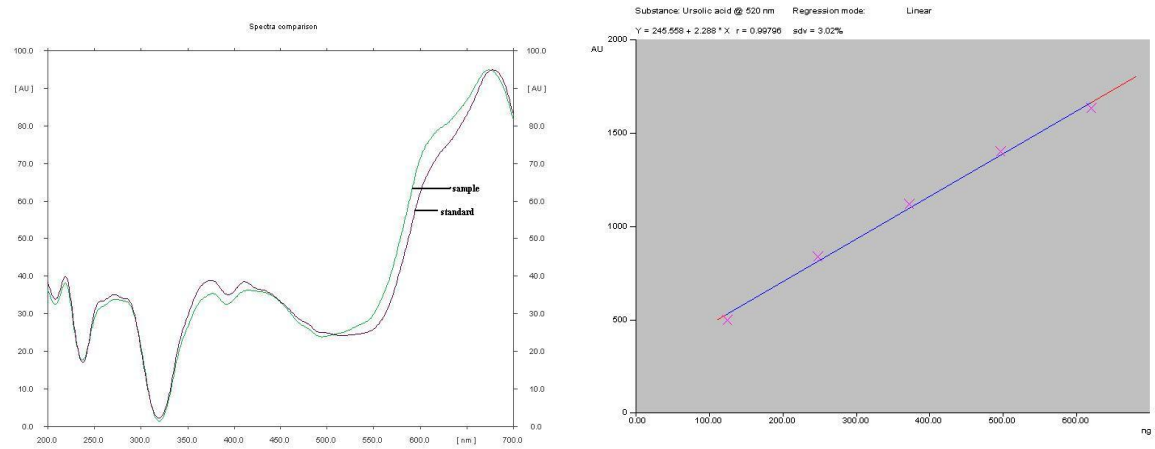
(c) **(d)**

Fig 2: Chromatograms showing the resolution of marker compound in the formulation Ayucee premix. (a) Chromatogram of the marker compound Gallic acid (A). (b) Chromatogram of the formulation Ayucee premix. (c) Overlay of spectra of Gallic acid standard with its counterpart in formulation. (d) Calibration plot for Gallic acid standard.





(a) **(b)**



(c) **(d)**

Fig. 3: **Chromatograms showing the resolution of marker compound in the formulation Ayucee premix. (a) Chromatogram of the marker compound Ursolic acid (B). (b) Chromatogram of the formulation Ayucee premix. (c) Overlay of spectra of Ursolic acid standard with its counterpart in formulation. (d) Calibration plot for Ursolic acid standard.**

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

Parameters	**Gallic acid**	**Ursolic acid**
Concentration range [µg/ng spot^{-1}]	0.5 – 2.5 µg/spot	124-620 ng/spot
Regression equation	y = 7.047x + 1237.69	y = 2.288 x + 245.56
Correlation Coefficient (r2)	0.998	0.998
Amount of marker compound in Ayucee premix [%w/w] [a]	1.58 %w/w	0.035 %w/w
Method precision (Repeatability) – RSD %	0.95	0.91
Intermediate precision (Reproducibility) - RSD [%] Intraday 1 Interday 3	 0.99 0.91	 0.90 0.94
LOD	0.02 µg spot^{-1}	0.093 µg spot^{-1}
LOQ	0.06 µg spot^{-1}	0.279 µg spot^{-1}

y = peak area response

x = amount of marker compound

[a] *= Mean, n=8*

Table 2: Results from determination of recovery.

Parameter	**Gallic acid**			**Ursolic acid**		
Initial concentration in formulation [mg g-1]	15.8	15.8	15.8	0.35	0.35	0.35
Concentration added [mg g-1]	0	8.0	16.0	0	0.20	0.5
Total concentration [mg g-1]	15.8	23.8	31.8	0.35	0.55	0.85
Concentration found [mg g-1]	15.9	23.7	31.7	0.34	0.556	0.85
RSD [%] (n=7)	0.99	1.05	1.02	0.98	1.01	0.97
Recovery [%]	100.63	99.57	99.68	97.14	101.09	100.00
Mean recovery [%]	99.96			99.41		

4. METHOD VALIDATION

4.1 Calibration curve (Linearity)

The method was validated in accordance with the statistical method of validation given in ICHQ2R1.[4] 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. Response was linear in the concentration ranges investigated (Table 1; Figures 2d and 3d). Quantification 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. Gallic acid & Ursolic acid 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 Gallic acid & Ursolic acid 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 Gallic acid (75 ng/spot) and Ursolic acid (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 Gallic acid & Ursolic acid 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-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 Gallic acid & Ursolic acid 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 Gallic acid & Ursolic acid 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 Gallic acid & Ursolic acid was 20 ng spot^{-1} and 93.0 ng spot^{-1} respectively, whereas, the LOQ was 60.0 ng spot^{-1} and 279.0 ng spot^{-1}, respectively.

Quantification of Gallic acid & Ursolic acid in clinically efficacious batches helped in standardization of the product with respect to its phytotherapeutic constituents. The newly developed method ensures the batch to batch consistency in efficacy of the product on commercial scale.

5. CONCLUSION

It has become imperative to standardize the polyherbal formulation to ensure the batch to batch consistency in quality of product and its efficacy. New HPTLC methods were developed for the fine resolution of various phyto constituents of the product. Standardization of phototherapeutic constituents eg. Gallic acid & Ursolic acid possessing strong free radical scavenging and antioxidant activities helped and will help in ensuring the batch to batch consistency in quality & efficacy of the product on commercial scale.

6. ACKNOWLEDGEMENT

We thank Mr. Mohanji Saxena, Managing Director, AYURVET Limited, for providing necessary facilities, help and guidance.

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