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RESEARCH ARTICLE

Standardization of an anti stress poly herbal formulation Stresroak liquid with respect to its antioxidant phytoconstituents

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Abstract

Stress evokes harmful responses that interferes with the general health, productivity and result in immunosuppressant. Exposure of birds to stress is an inevitable event in poultry husbandry, when the threshold level of stress is crossed it results in distress to birds. Supplementation of antistressor products can alleviate adverse effect of various stressors in poultry. Polyherbal formulations have plant-based pharmacological agents which may exert synergistic, potentiative, agonistic or antagonistic actions by virtue of its diverse active principles within themselves. These pharmacological principles work together in a dynamic way to produce maximum therapeutic efficacy with minimum side effects. Standardization is an important aspect for maintaining and assessing the quality and safety of the polyherbal formulation as these are combinations of more than one herb.

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1. Introduction

Phytopharmaceuticals could only be considered as a rational drug if these are standardized and their pharmaceutical quality is approved. Standardization based on a single or small number of chemical markers or classes of compounds serve mainly to promote quality control and batch-to-batch consistency in terms of efficacy. Methods of standardization should take into consideration all aspects that contribute to the quality and pharmacological efficacy of the herbal drugs.

Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease (1) Stress evokes harmful responses that interferes with the general health, productivity and result in immunosuppression (2).

Exposure of birds to stress is an inevitable event in poultry husbandry, when the threshold level of stress is crossed it 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. Supplementation of antistressor products can alleviate adverse effect of various stressors in poultry.

Stresroak liquid, a proprietary polyherbal formulation of AYURVET, is a blend of extracts of medicinal plants viz. Phyllanthus emblica, Withania somnifera, Mangifera indica, Ocimum sanctum and Shilajit. This combination has excellent antistress properties and immunomodulatory activity (3–12) resulting in increased adaptive response in birds (poultry) and non-specific modulation of the immune system. The major active components of the formulation, for example gallic acid, hydrolyzable tannins (13), mangiferin (14), withanolide-A (15), eugenol, ursolic acid (16), dibenzo α- pyrones and fulvic acid (17), contribute to the anti-mutagenic, anti-

cancer, anti-oxidant, immunomodulatory activity of the product (18-23) These compounds and their quantification have been reported in various parts of the plants (24,25).
Standardization of the product with respect to the bioactive phyto constituents was taken up to ensure the batch to batch consistency in efficacy. New HPTLC & HPLC methods were developed for the quantification of two main ingredients of the formulation i.e Phyllanthus emblica with Gallic acid (Fig.1, I) & Mangifera indica with Mangiferin having antioxidant activity (26,27, Fig.1, II) The analytical methods were validated for linearity, accuracy, and precision in accordance with the statistical method of validation given in ICHQ2R1 (28). The average recovery of Gallic acid (99.47 %) and Mangiferin (98.38 %) was computed.

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. HPLC was performed with WATERS, USA having binary pump 515 with PDA 2996 detector. The data was acquired on the Empower 2.0 controlling software. Separation was obtained on Phenomenex luna C18 column (250 mm x 4.6 mm, 5 µm).

2.2 Reagents and materials

Chemicals and reagents used were of analytical reagent grade. Ethyl acetate, formic acid, acetonitrile and water were purchased from Rankem, whereas, acetic acid and ortho phosphoric acid were purchased from SD Fine chemicals. Gallic acid was purchased from HIMEDIA & Mangiferin was isolated in house and characterized by different spectroscopic methods before use. TLC plates were purchased from Merck (Darmstadt, Germany). Controlled samples of Stresroak liquid were obtained from the QA/QC department of AYURVET LTD, Baddi.

2.3 Chromatographic conditions

HPTLC 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 8.0 cm, under laboratory conditions with a mobile phase of Toluene : Ethyl acetate : Formic acid : Methanol (3.0:3.0:0.8:0.4, v/v/v/v) for quantification. Quantitative determination of spots corresponding to Fig. 1 (**I)** was done by Camag TLC Scanner 3 at 260 nm with a slit size of 6 × 0.3 mm.

HPLC was performed with WATERS, USA having binary pump 515 with PDA 2996 detector. The data was acquired on the Empower 2.0 controlling software. Separation was obtained on Phenomenex luna C18 column (250 mm x 4.6 mm, 5 µm). The mobile phase was filtered through 0.45 µm Millipore filter and degassed. To optimize the RP-HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for Mangiferin [Fig. 1, **II**] was obtained with a mobile phase Ace tonitrile : Water : Ortho phosphoric acid :: 30 : 69: 0.1 % at a flow rate of 1.2 ml/min to get better reproducibility and repeatability. Selecting 258 nm as the detection wavelength resulted in an acceptable responses and enabled the detection of compound under investigation.

2.4 Preparation of sample & standard solutions

2.4.1 Preparation of standard solutions

Stock solutions (~ 0.1 mg/mL) of standards (marker compounds) **I** and **II** were prepared in methanol/aqueous methanol (6:4), different concentrations were spotted/injected in order to prepare the calibration graphs and quantification of bioactives.

2.4.2 Preparation of sample solution for quantification of Gallic acid:

Sonicated 5.0 g of Stresroak liquid in a 100 ml volumetric flask with 50 ml of water for 15 minutes, adjusted the volume to the mark with same solvent. Filtered the solution through 0.45 µ filter before spot application.

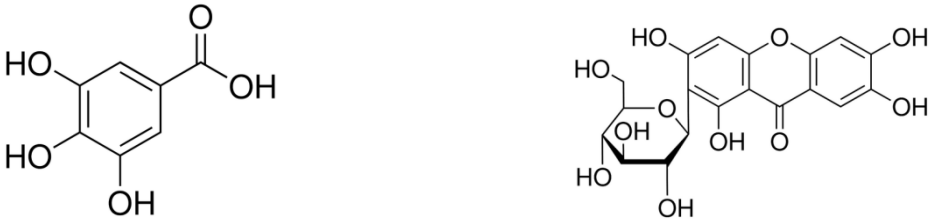
2.4.3 Preparation of sample solution for quantification of Mangiferin:

Sonicated 10.0 g of Stresroak liquid in a 100 ml volumetric flask with 75 ml of aqueous methanol (60:40) for 15 minutes, adjusted the volume to the mark with same solvent. Filtered the solution through 0.45 µ filter before injecting into HPLC.

3. RESULTS & DISCUSSION

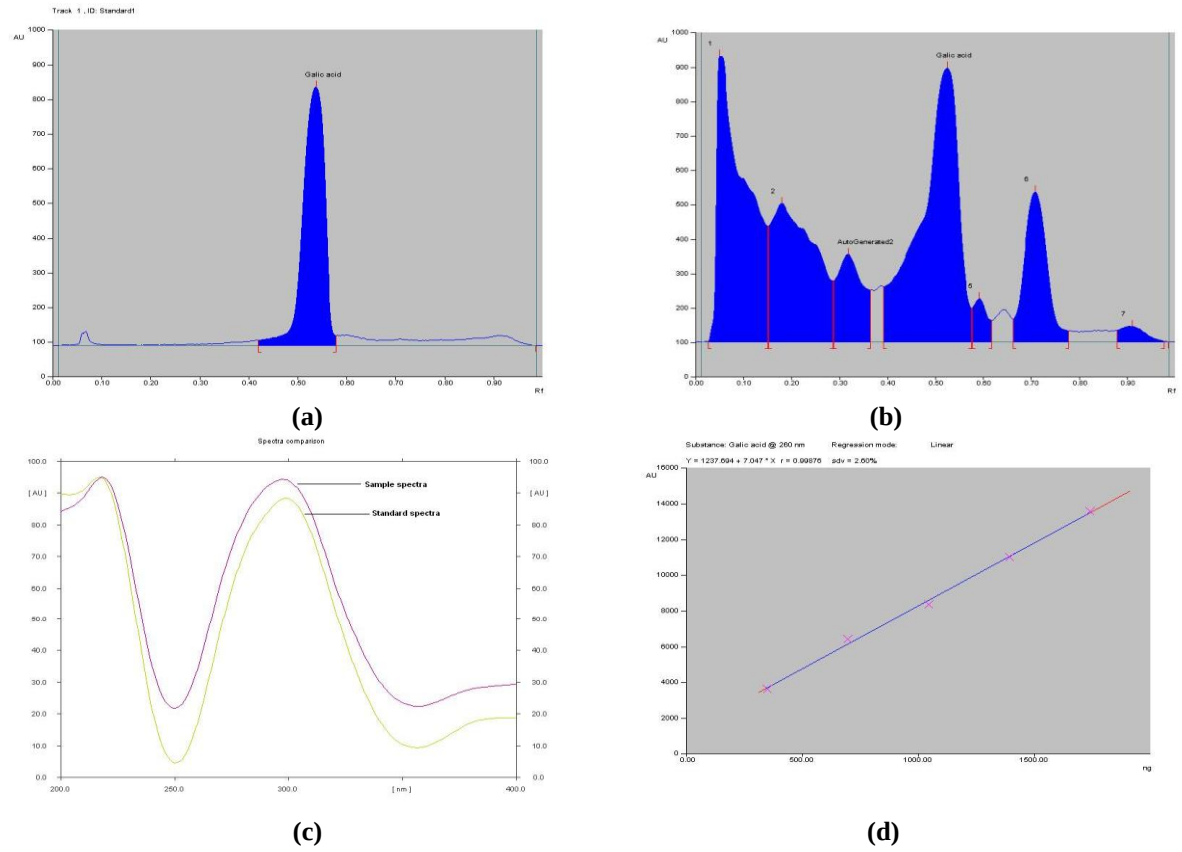
Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Phenols, a major group of antioxidant phytochemicals, have profound importance due to their biological and free radical scavenging activities. It has already been exhibited that polyphenolic compounds are responsible for radical scavenging activity, due to the ease of their hydrogen atom donation to active free radical (29). The content of total polyphenols in Stresroak liquid expressed as mg of gallic acid equivalent per gram of formulation was found to be 2.4 mg/g . The antioxidant potential of the formulation under study was found to give IC 50 value (to scavenge 50% of DPPH free radicals) of 128.85 µg/ml. This ensured that formulation would exhibit minimum 96.85 ± 2.4 % DPPH free radical scavenging potential at 500 ppm (30).
The main pharmaco active ingredients of the blend are Phyllanthus emblica, Withania somnifera, Mangifera indica, Ocimum sanctum and Shilajit. As the two herbs Phyllanthus emblica, & Mangifera indica with strong antioxidant activity [28] are among the main active ingredients in polyherbal formulation, quantifying them with their bioactive markers Gallic acid & Mangiferin with antioxidant activity potential will ensure its antioxidant efficacy hence the quality of the product.

HPTLC & HPLC methods were developed to quantify Gallic acid & Mangiferin. For quantitative determination the chromatograms were scanned at 260 & 258 nm for the two bio actives respectively and the identities of the bands in the sample extracts were confirmed by comparing their Rf /RT values and their absorption spectra with those obtained from reference standards (Figures 2:a-c & 3: a-c, e-f).



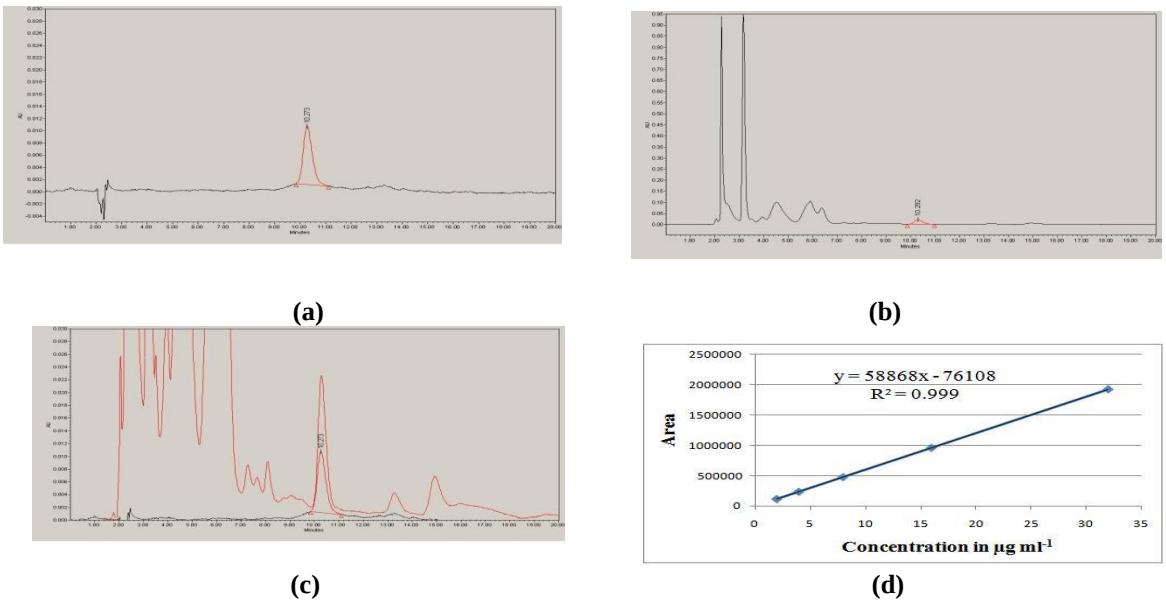
(I) **(II)**

Figure 1. Structure of Gallic acid (I) & Mangiferin (II)

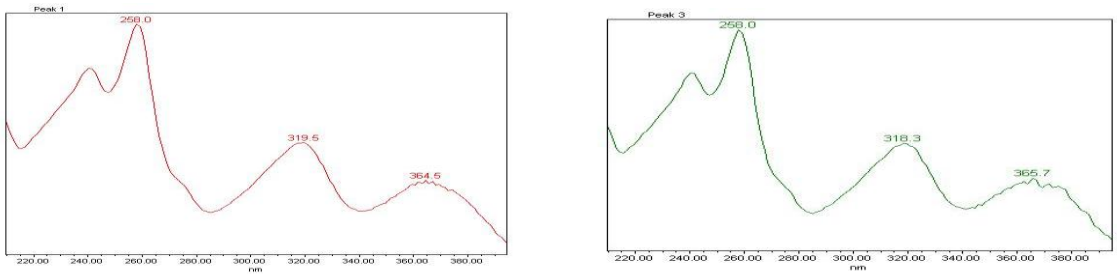


(a) **(b)** **(c)** **(d)**

Figure 2: Chromatograms showing the resolution of marker compound in the formulation Stresroak liquid. (a) Chromatogram of the marker compound Gallic acid (**I**). (b) Chromatogram of the formulation Stresroak liquid. (c) Overlay of spectra of Gallic acid standard with its counterpart in formulation. (d) Calibration plot for Gallic acid standard.



(a) **(b)** **(c)** **(d)**



(e) **(f)**

Figure 3: Chromatograms showing the resolution of marker compound in the formulation Stresroak liquid. (a) Chromatogram of the marker compound Mangiferin **(II)**. (b) Chromatogram of the formulation Stresroak liquid. (c) Overlay of chromatograms of Mangiferin standard with its counterpart in formulation. (d) Calibration plot for Mangiferin standard. (e) Chromatogram of spectra of Mangiferin standard. (f) Chromatogram of spectra of Mangiferin in formulation Stresroak liquid.

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

Parameters	**Gallic acid**(μg spot^{-1})	**Mangiferin (**ppm)
Concentration range	0.5 – 2.0	2.0 – 32.0
Regression equation	y = 7.047x + 1237.69	y = 58868x - 76108
Correlation Coefficient (r2)	0.998	0.999
Amount of marker compound in Stresroak liquid [%] (w/w)[a]	0.60 ± 0.05	0.0085 ± 0.0005
Method precision (Repeatability) – RSD %	0.95	0.92
Intermediate precision (Reproducibility) - RSD %		
Intraday 1	0.99	0.96
Interday 3	0.91	0.93
LOD	0.02 μg spot^{-1}	1.0 ppm
LOQ	0.06 μg spot^{-1}	3.0 ppm

y = peak area response

x = amount of marker compound

a = Mean ± SD, n=6

Table 2: Results from determination of recovery.

Parameter	Gallic acid			Mangiferin		
Initial concentration in formulation [mg g^{-1}]	6.0	6.0	6.0	0.085	0.085	0.085
Concentration added [mg g^{-1}]	0	3.0	6.0	0	0.20	0.40
Total concentration [mg g^{-1}]	6.0	9.0	12.0	0.085	0.285	0.485
Concentration found [mg g^{-1}]	6.02	8.91	11.89	0.082	0.283	0.482
RSD [%] (n=6)	0.58	0.75	0.60	0.50	0.65	0.90
Recovery [%]	100.33	99.00	99.08	96.47	99.29	99.38
Mean recovery [%]		99.47			98.38	

4. METHOD VALIDATION

Validation parameters: The method was validated according to ICH guideline for linearity, precision, accuracy, selectivity, limit of detection and limit of quantification.

4.1 Linearity

The method was validated in accordance with the statistical method of validation given in ICHQ2R1 (30). 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). Evaluation was on the basis of peak area.

a. Calibration: The marker compound in the formulation was quantified using a calibration curve established with five dilutions of the standard at concentrations ranging from 0.5 – 2.0 µg $spot^{-1}$ for the Gallic acid and 2 – 32 ppm for Mangiferin standard compound. The corresponding peak area in formulation was plotted against the concentrations of the standard injected. Peak identification was achieved by comparison of both the Rf/RT and UV absorption spectrum with those obtained for standard.

b. Linearity: Linear regression analysis was used to calculate the slope, intercept, and coefficient of determination/regression coefficient (r2) for calibration plot. Linearity was determined by using five concentrations of the standard solution. The calibration curve was obtained by plotting the area versus the concentrations of the standard solution. Response was linear in the concentration ranges investigated (Fig. 2 d, 3 d, Table 1).

c. **Range:** Range is the interval between upper and lower concentration (amount) of analyte in sample for which it has been demonstrated that the analytical method has suitable level of precision, accuracy and linearity. The linear response was observed at 260 nm / 258 nm over a range of 0.5 – 5.0 µg $spot^{-1}$ for the Gallic acid and 2 – 32 ppm for Mangiferin standard compound (Fig. 2 d, 3 d, Table 1).

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 & Mangiferin 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

Three different concentrations of marker compound solution in triplicates were injected 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. The low %RSD values of Intraday and Interday for marker compound reveals that the proposed method is precise (Table 1).

4.4 Selectivity

The selectivity of the respective method was determined by comparing the retention factor/retention time 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 & Mangiferin peaks separately were, therefore, not masked by any peak of other compound present in the formulation (Figures 2c and 3 e & f), which indicated respective peak purity.

4.5 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. The LOD for Gallic acid & Mangiferin was 0.02 μg $spot^{-1}$ and 1.0 ppm respectively, whereas, the LOQ was 60.0 ng $spot^{-1}$ and 3.0 ppm , respectively.

Quantification of the amount of Gallic acid & Mangiferin in clinically efficacious batches hence ensures batch to batch reproducibility and consistency in efficacy of the product on commercial scale.

5. CONCLUSION

The biostandardization exercise of product ensured the formulation to exhibit minimum 96.85 ± 2.4 % DPPH free radical scavenging potential at 500 ppm. Quantification of the amount of Gallic acid & Mangiferin in clinically efficacious batches vis a vis standardizing the two herbs Phyllanthus emblica, & Mangifera indica with antioxidant activity potential ensures batch to batch reproducibility and consistency in efficacy of the product on commercial scale.

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