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Research Article

BIO STANDARDIZATION OF AN ANTISTRESS POLYHERBAL FORMULATION, STRESROAK LIQUID

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ABSTRACT

Objective: The study was designed to biostandardize the formulation with respect to the free radical scavenging antioxidant potential of the formulation which in turn will substantiate its antistress property. Standardization of this potential will add value in ensuring the batch to batch consistency in terms of biological activity.

Method: The antioxidant activity of the product on the basis of the scavenging of free radical 2, 2- diphenyl-2-picrylhydrazyl (DPPH) was determined. Ascorbic acid was used as standard; the difference in absorbance between the test and the control was calculated and expressed as % scavenging of DPPH radical. The radical scavenger activity is calculated in terms of Ascorbic acid equivalent antioxidant capacity (AEAC). The IC50 was evaluated from concentration vs. inhibition graphical curve plot.

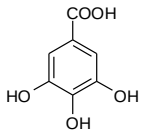
Results: The experiments were performed in triplicate on 5 different batches and Antioxidant activity of each concentration was determined. Six different concentrations i.e. 10, 20, 50, 100, 250 & 500 ppm of the samples were used each time. Mean value of each concentration of individual batches was taken into account for statistical calculation for 5 different batches with their standard deviations. The formulation exhibited the IC 50 value of 128.85 µg/ml.

Conclusion: The product under investigation is having excellent antistress and immunomodulatory properties resulting in increased adaptive response in poultry and non-specific modulation of the immune system. The free radical scavenging potential of the formulation ameliorates its antistress capability. Standardization of this potential will add value in ensuring the batch to batch consistency in terms of biological efficacy.

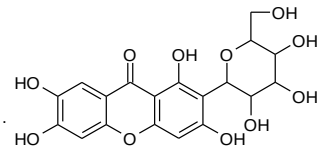
Keywords: Stresroak, Antistress, DPPH, Gallic acid, Ascorbic acid, Free radical scavenging

INTRODUCTION

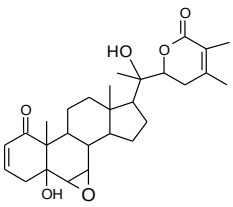
The herbal medicines have stood the test of time for their safety, efficacy, cultural acceptability and lesser side effects. As commercialization of this medicine has happened, assurance of safety, quality and efficacy of medicinal plants and herbal products has become an important issue. The need of the hour is to evolve a systematic approach and to develop well designed methodologies for the standardization of herbal raw materials and herbal formulations.



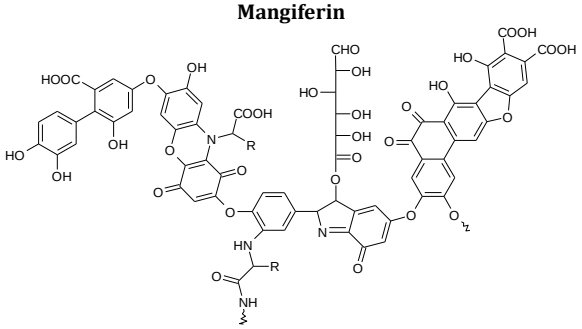
Gallic acid



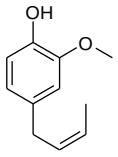
Mangiferin



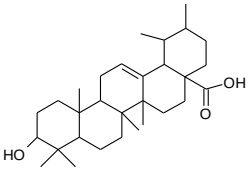
Withanolide – A



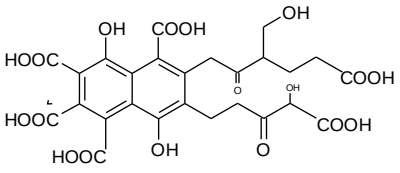
Humic acid



Eugenol



Ursolic acid



Fulvic acid

Fig. 1: The main constituents of the herbs *Phyllanthus emblica*, *Mangifera indica*, *Withania somnifera, Ocimum sanctum* & Shilajit

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 to attain the desire therapeutic effect [1]. Methods of standardization should take into consideration all aspects that contribute to the quality and pharmacological efficacy of the herbal drugs. The fingerprint profiles serve as guideline to the phytochemical profile of the drug in ensuring the quality, while quantification of the marker compound/s would serve as an additional parameter in assessing the quality of the sample. Standardization of herbal formulations for their biological activity will ensure the batch to batch consistency for desired therapeutic effect.

Living cells may generate free radicals and other reactive oxygen species by-products as a results of physiological and biochemical processes. 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. [2]

Stress evokes harmful responses that interferes with the general health, productivity and result in immunosuppression [3]. 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. The main goals of dietary antioxidants are to control oxidative stress in humans and animals and to improve poultry meat and their products. Today there is an increasing trend among consumers to seek natural ingredients for their nutrition, pressuring the food industry to use antioxidants of plant origin in poultry diets [4].

Polyherbal formulations have plant-based pharmacological agents which may exert synergistic, potentiative, agonistic 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 [5]

Stresroak, 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 [6–15] 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 [16], mangiferin [17], withanolide-A [18], eugenol, ursolic acid [19], dibenzo α- pyrones and fulvic acid [20], contribute to the anti-mutagenic, anti-cancer, anti-oxidant, immunomodulatory activity of the product [21-26] These compounds and their quantification have been reported in various parts of the plants [27,28]. It's proven pharmacological efficacy lead us to standardize it with respect to it's free radical scavenging / antioxidant activity, hence ensuring the batch to batch consistency for desired antisterss activity .

MATERIAL AND METHODS

Materials

Chemicals and reagents used were of analytical reagent grade. Ascorbic acid & DPPH were of Sigma Aldrich make while absolute ethanol was purchased from Merck. Controlled samples of Stresroak liquid were obtained from the QA/QC department of AYURVET LTD, Baddi, Himachal Pradesh, India.

Methodology for estimation of total antioxidant activity

The antioxidant activity of the Stresroak liquid on the basis of the scavenging activity of the stable 2, 2- diphenyl-2-picrylhydrazyl (DPPH) free radical was determined according to the method described by Brand-Williams et.al. [29] with slight modification. The different concentrations of the formulation were prepared in ethanol. The test tubes were incubated for 30 min at room temperature and the absorbance was measured at 517nm. Lower the absorbance of the reaction mixture indicates higher free radical scavenging activity. Ascorbic acid was used as a standard and the concentrations prepared were same as of the test solutions. The different in absorbance between the test and the control (DPPH in ethanol) was calculated and expressed as % scavenging of DPPH radical. The IC50 value of the formulation was calculated from graphical plot. The free radical scavenger activity is calculated in terms of Ascorbic acid equivalent antioxidant capacity (AEAC) by using the following formula:

$$\mathbf{AEAC} = \frac{(\mathbf{A}\ \text{control} - \mathbf{A}\ \text{sample})}{(\mathbf{A}\ \text{control} - \mathbf{A}\ \text{ascorbic acid})}\ \mathbf{X}\ \frac{\text{Concentration of ascorbic acid (mg/ml)}}{\text{Concentration of sample (mg/ml)}}\ \mathbf{X}\ 100$$

The following methodology was used to estimate the antioxidant activity (hydrogen donating ability) of extract in the presence of DPPH stable radical.

Absorbance (**A** control): Mix one milliliter of 0.3mM DPPH ethanol solution and 2.5 ml of ethanol, allow to react them at room temperature. After 30 minutes measure the absorbance at 517nm against a blank of ethanol

Absorbance (**A** ascorbic acid): Mix one milliliter of 0.3mM DPPH ethanol solution and 2.5 ml of ascorbic acid solution, allow to react them at room temperature. After 30 minutes measure the absorbance at 517nm against a blank prepared by mixing ethanol (1.0 ml) and ascorbic acid solution (2.5 ml).

Absorbance (**A** sample): Mix one milliliter of 0.3mM DPPH ethanol solution and 2.5 ml of sample solution, allow to react them at room temperature. After 30 minutes, measure the absorbance at 517nm against a blank prepared by mixing ethanol (1.0 ml) and sample solution (2.5 ml).

Methodology for estimation of total phenolic contents:

In order to ensure that every batch of the formulation has uniformity and consistency of the biological effects exhibited, determination of total phenolic content was carried out using the Folin-Ciocalteu method adapted from Singleton et.al. [30] and the gallic acid was used as the standard phenolic compound. 1 ml of the formulation was added to a mixture of 2.5 ml of 10 % Folin-Ciocalteu reagent and 2 ml of 7.5 % Na_2CO_3. After incubation at 45°C for 30 minutes the absorbance was measured at 765 nm. A linear dose response curve was generated using absorbance reading of gallic acid. The content of total polyphenols in the formulation is expressed as mg of gallic acid equivalent per gram of formulation.

C = A/B, where C is expressed as mg GAE/g weight of formulation. A is the equivalent concentration of gallic acid established from the calibration curve (mg) and B is the weight of formulation.

Table 1: Total Phenolics and DPPH free radical scavenging potential of Stresroak liquid

Concentration(ppm)	GAE (mg/g)	(%)[a] DPPH free radical scavenging
10	-	4.25 ± 2.7
20	-	8.44 ± 2.4
50	-	21.06 ± 1.4
100	-	40.50 ± 4.1
250	-	84.05 ± 7.7
500	2.4	96.85 ± 2.4

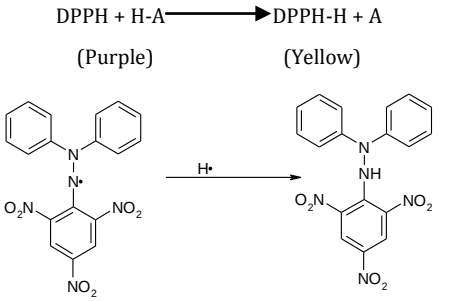
[a] = Mean ± SD, n=5

RESULT AND DISCUSSION

Stresroak is a renowned polyherbal formulation for poultry. It is used as an antistress treatment, immunomodulator, adaptogenic, and performance enhancer. The main pharmaco active ingredients of the blend are *Phyllanthus emblica, Withania somnifera, Mangifera indica, Ocimum sanctum* and Shilajit. A unique HPTLC method has already been developed at our premises for standardization and quantification of gallic acid, mangiferin, and withanolide-A in solid dosage form of the product. The average content of these markers in different batches of the formulation was found to be 0.654, 0.627, and 0.325% (w/w), respectively. The method was validated for linearity, accuracy, and precision in accordance with the statistical method of validation given in ICHQ2R1 [31]. The phytochemical profile is of special significance since it has a direct bearing on the activity of the herbal drugs, the batch to batch consistency in pharmacological efficacy was ensured by the standardization of formulation with respect to its antioxidant activity.

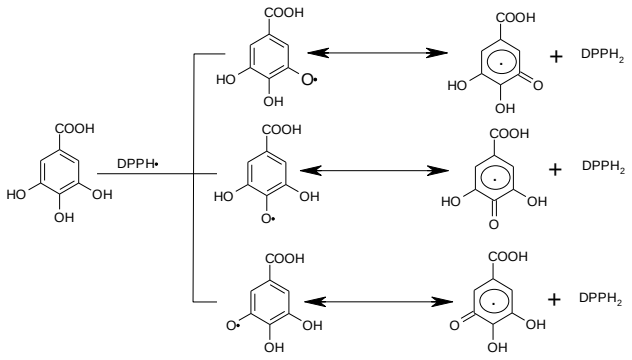
Phenols, a major group of antioxidant phytochemicals, have profound importance due to their biological and free radical scavenging activities. It has already been exhibited that polyphenolic compounds are responsible for radical scavenging activity, due to the ease of their hydrogen atom donation to active free radical [32]. The content of total polyphenols in the formulation is expressed as mg of gallic acid equivalent per gram of formulation and was found to be 2.4 mg/g (Table 1).

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples [33]. DPPH is a stable nitrogen-centered free radical the color of which changes from violet to yellow by either the process of hydrogen- or electron- donation (Scheme 1). Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers [34]. It was found that the radical-scavenging activity increased with increasing concentration. Usually, higher total phenol and flavonoid contents lead to better DPPH-scavenging activity [35 – 36].



Scheme 1: Reaction of DDPH free radical to DPPH non radical

A possible free radical scavenging mechanism by the Gallic acid (Scheme 2), the main active ingredient of the herb *Phyllanthus emblica* is being postulated to understand the antioxidant potential of the formulation in part.



Scheme 2: Free radical scavenging by Gallic acid, a possible mechanism

The experiments were performed in triplicate on 5 different batches and mean values of Antioxidant activity (Table 1) of each concentration was determined. Ascorbic acid was used as a standard to convert the inhibition capability of formulation to the Ascorbic acid equivalent. The antioxidant activity of the formulation is expressed as IC50 which is the concentration of the sample required to scavenge 50% of DPPH free radicals. Six different concentrations i.e. 10, 20, 50, 100, 250 & 500 ppm of the samples were used each time. Mean value of each concentration of individual batches was taken into account for statistical calculation for 5 different batches with their standard deviations. The IC50 value was evaluated from concentration vs. inhibition graphical curve plot [37]. The formulation exhibited the IC 50 value of 128.85 µg/ml (Fig. 2). This exercise for biostandardization shall ensure that formulation should exhibit minimum 96.85 ± 2.4 % DPPH free radical scavenging potential at 500 ppm to ensure batch to batch consistency in term of its biological activity.

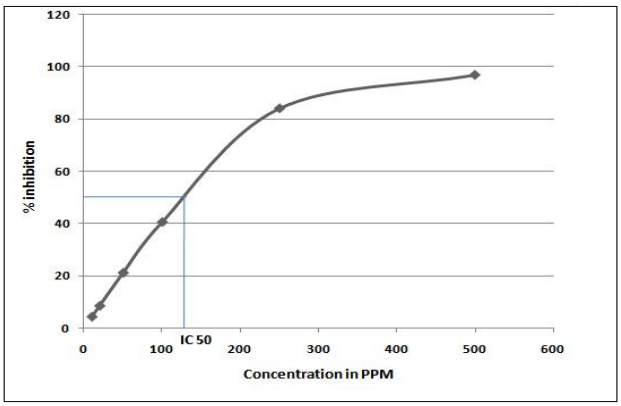


Fig. 2: Graphical representation of free radical scavenging activity of Stresroak liquid and its IC50 value

CONCLUSION

The product under investigation is a blend of extracts of medicinal plants viz. *Phyllanthus emblica, Withania somnifera, Mangifera indica, Ocimum sanctum and* Shilajit, having excellent antistress and immunomodulatory properties resulting in increased adaptive response in birds (poultry) and non-specific modulation of the immune system. The free radical scavenging potential of the formulation ameliorates its antistress capability. Standardization of this potential will add value in ensuring the batch to batch consistency in terms of biological efficacy.

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