

Evaluation Techniques Employed for Herbal Drugs: Special emphasized on Phytochemical Evaluation of Polyherbal Formulation

Minakshi Verma^{1*}, Rajana Sharma, Amita Tilak, Sudhir Singh Gangwar,

Department of pharmacy, G. S. V. M. Medical College Kanpur

Received 01 Dec 2015; Accepted 30 Dec 2015

ABSTRACT

Herbs are produced in two main ways: collection from wild plants from their natural habitats and cultivation of herbs that are grown that the plant collected is the one that is desired and having uniform quality attributes while in wild-crafted herbs there is a chance that the wrong herb has been picked, which could lead to serious consequences. So herbal drugs or its standardized extracts or pure active compound needs analytical techniques to confirm its identity, quality, purity, potency, safety and efficacy of the plant. Evaluation of herbal drug is an important tool in the formulation of high quality herbal products. Quality of herb depends upon many factors like cultivation, collection, drying, storage, processing for market etc. Now a day's substitution and adulteration of herb is very common due to scarcity of drug and its high price prevailing in the market. Owing to medicinal properties attributed to an herb, it is necessary to maintain its quality and purity in the commercial market. A present overview covering various tools like morphological, microscopical, physical, chemical and biological employed for evaluation of herbal drugs including the recent techniques employed for phytochemical evaluation of polyherbal formulation.

INTRODUCTION:

A system to ensure that every single medicinal plant, part of a plant or an extract, an isolate or an enriched portion or a product thereof, being sold has the correct substances in the correct amount and will induce its therapeutic effect, is known as standardization. Medicinal plants, being an important aspect of various traditional systems of medicine, have been used therapeutically around the world. From Ayurveda to Chinese traditional medicine, from Unani to Tibetan Medicine and from Amazonian to African Medicine, all systems of traditional medicine, although based on different theoretical and cultural models, integrate phytotherapy into their doctrine [1-3]. In high-income countries, however, the widespread use of phytotherapy declined at the end of the first part of the twentieth century, due to the development and production of synthetic medicines. During the past few decades, however, phytotherapy has started to be increasingly used even in industrialized countries. In low and middle-income countries, phytotherapy never stopped being important, often representing the only therapeutic system preferred by certain people; in the Indian sub-continent about 70% of the population (WHO) extensively use traditional and alternative medicines for health care [2-4]. The growing use of botanicals by the public has initiated evaluation of

the health claims of these agents and steps are being taken to develop standards for their safety, efficacy and quality. In addition, the WHO has developed a series of technical guidelines and documents relating to the safety and quality assurance of medicinal plants and herbal materials as a minimum requirement [1-5].

The increasing interest in the use of plant based formulations is leading to a fast growing market for Ayurvedic, nutraceutical and polyherbal formulations. The development of stable polyherbal formulations is a challenging task because of the large number of varied chemical compounds present in the different medicinal plants. Hence, the entire herbal drug or herbal drug preparation is regarded as active drug substance, regardless of whether constituents with defined therapeutic activity are known. This difficulty has been acknowledged in the draft of the Strategic Plan for Regional Traditional Medicine of the World Health Organization [2-4].

The chemical incompatibility leads to changes in the chemical nature, solubility, absorption and therapeutic response of these drugs. Therefore, during the formulation of new drugs or the reformulation of existing products, the interaction study between active markers of various plant extracts and commonly used excipients should be carried out thoroughly. However, no

*Corresponding author: Minakshi Verma

universally accepted protocol is available for evaluating the compatibility of drugs with different excipients. Assessment of possible compatibility between active component and different excipients along with the evaluation of stability are crucial part of a normal study prior to the final formulation [6-8].

In compatibility studies, temperature variation is one of the most important parameter to induce rapid chemical and physical alternation in formulations, which is determined by quantification of the active constituents over the time. Unlike a single chemical entity that forms the basis of conventional medicine, traditional Ayurvedic medicine views the poly herbal preparations as they induce combined therapeutic activity this creates a challenge in the development of a stable poly herbal formulation [7-9].

In this present review article covering various tool like morphological, microscopical, physical, chemical and biological employed for evaluation of herbal drugs including the recent techniques employed for phytochemical evaluation of poly herbal formulation.

Evaluation Techniques Employed for Herbal Drugs

Morphology and Microscopy

Morphology [3]

Macroscopic characters of the medicinal plant material are based on shape, size, colour, surface characteristics, texture, fracture and appearance of the cut surface.

Microscopy

Anatomical studies [3, 4]

Free hand sections of the specified parts can be boiled with chloral hydrate to remove all the colouring matter and then carefully stained with phluoroglucinol and HCl (1:1).

Powder analysis [5]

The dried and powdered plant materials which passed through sieve no. 60 can be used for powder analysis. The macroscopic characters of the powders are observed first, following which, the powders are examined microscopically by mounting in chloral hydrate solution, iodine solution and by staining with fluoroglucinol: HCl.

Physical/physicochemical standardization [3-7]

Total ash

About 2 g of the powdered drug should accurately weigh in a tarred silica crucible. The powdered drug can be spread as a thin layer at the bottom of the crucible. The

crucible incinerated at a temperature not exceeding 450°C until free from carbon. The crucible is cooled and weighed. The procedure would be repeated till a constant weight is observed. The percentage of the total ash is calculated with reference to the air-dried drug.

Acid insoluble ash

The ash obtained as described in the determination of total ash is boiled with 25 mL of hydrochloric acid for 5 min. The insoluble ash is collected on an ash-less filter paper and washed with hot water. The insoluble ash is transferred into a tarred silica crucible, ignited, cooled and weighed. The procedure is repeated till a constant weight is observed. The percentage of acid insoluble ash is calculated with reference to the air-dried drug.

Water soluble ash

The ash obtained as described in the determination of total ash is boiled for 5 min with 25 mL of hot water. The insoluble matter is collected on an ash-less filter paper and washed with hot water. The insoluble ash is transferred into a tarred silica crucible and ignited at a temperature not exceeding 450°C. The procedure is repeated until a constant weight is observed. The weight of the insoluble matter is subtracted from the weight of the total ash. The difference in weight is considered as water-soluble ash. The percentage of water soluble ash is calculated with reference to air-dried drug.

Extractive values [8-11]

Ethanol soluble extractive

5 g of previously weighed air-dried drug is taken in a stopper flask to which 100 mL of 95% ethanol is added. It is shaken continuously for 4 h on a magnetic stirrer. Then it is filtered rapidly taking precautions against loss of the solvent. 25 mL of this filtrate is evaporated to dryness in a tarred flat-bottomed Petri dish, dried at 105°C and weighed. The percentage of ethanol soluble extractive is calculated with reference to the air-dried drug.

Water soluble extractive

5 g of previously weighed air-dried drug is taken in a stopper flask to which 100 mL of chloroform water is added. It is shaken continuously for 4 h on a magnetic stirrer. Then it is filtered rapidly taking precautions against loss of the solvent. 25 mL of this filtrate is evaporated to dryness in a tarred flat-bottomed Petri dish, dried at 105°C and weighed. The percentage of water-soluble extractive is calculated with reference to the air-dried drug.

Ether soluble extractive

5 g of previously weighed air-dried drug is taken in a Stoppard flask and 100 mL of ether is added to it. It is shaken continuously for 4 h on a magnetic stirrer. Then it is filtered rapidly taking precautions against loss of the solvent. 25 mL of filtrate is evaporated to dryness in a tarred flat-bottomed Petridis, dried at 105 °C and weighed. The percentage of ether-soluble extractive is calculated with reference to air-dried drug.

Foreign organic matter [3]

The sample (100-500 g) is spread on a white tile or a glass plate uniformly to form a thin layer without overlapping. The sample is inspected with the unaided eye or by means of a lens (5x or above). The foreign organic matter is separated manually. After complete separation, the matter is weighed and percentage w/w present in the sample is determined as described in WHO guidelines.

Moisture content by Loss on drying [3]

About 2-5g of accurately weighed drug is dried at 100-105°C for 5 h, and then weighed again. Percentage is calculated with reference to the initial weight.

Volatile content [3]

Volatile content of the plant material is determined using Clavenger's apparatus as described in WHO guidelines.

Phytochemical evaluation [10-16]

Preparation of extracts

The plant/material is either extracted successively with different organic solvents from lowest polarity to highest polarity, or exhaustively extracted with ethanol and then fractionated successively with solvents of lowest polarity to highest polarity.

Procedure

A. The dried coarsely powdered plant/ material (50 g) is successively in increasing order of polarity extracted with n-hexane, chloroform, ethyl acetate, n-butanol and methanol by the hot extraction process using a soxhlet apparatus. After completion of each extraction process (4 h) the solvent is removed by distillation and the extracts are concentrated in vacuo.

The marc left after each extraction process, is dried so that it is completely free from the solvent.

B. Plant material (3 kg) is exhaustively extracted with ethanol (95%) in a soxhlet apparatus for 6 h. After extraction the solvent is distilled from the extract under pressure, till syrupy consistency after which it is evaporated to dryness under pressure. The solvent free

extract (50 g) is suspended in sufficient water. The suspension is fractionated with various solvents of ascending polarity (n-hexane, chloroform, ethyl acetate, n-butanol and methanol). Each fraction is then separated and distilled to remove solvent and concentrated in vacuo

Physico-chemical evaluation of the fractions

Determination of solubility [9]

100 mg each of various fractions (n-hexane, chloroform, ethyl acetate, n-butanol) obtained from the ethanolic extract of whole plant parts are accurately weighed, dissolved in 10 mL volume of various solvents and solubility is observed.

Fluorescence analysis [10, 11]

The hexane, chloroform, ethyl acetate, butanol fractions and total ethanol extracts are observed under daylight and under UV [254 nm] and the color is recorded. The test material is further treated with different reagents namely, 1N Hydrochloric acid, 1N sodium hydroxide (aqueous), ferric chloride, 1N nitric acid, ammonia, iodine, 1N sodium hydroxide (alcoholic), picric acid and 1N Sulphuric acid, and then observed for any color change in daylight as well as under UV [254 nm].The change in colour is recorded

Preliminary phytochemical screening [7]

Each of the extracts/ fractions is subjected to various phytochemical tests to detect the presence of various phytoconstituents such as carbohydrates (Molisch's, & Fehling's tests), glycosides (Borntrager's & Modified Borntrager's test), saponins (Foam test), flavonoids (Shinoda's test), alkaloids (Mayer's, Dragendroff's, Wagner's & Hager's reagent tests), sterols (Libermann Burchard test), fixed oils (Spot & Saponification test), tannins and phenols (Ferric chloride, Lead acetate & Aqueous bromine solution), proteins and amino acids (Biuret test, Ninhydrin test).

Estimation of total phenolic content [12, 13-18]

CHEMICALS: DPPH, Folin ciocalteu reagent, ferrous sulphate, ascorbic acid, gallic acid, ferric chloride, trichloroacetic acid, sodium carbonate, sodium nitroprusside, NED, sulphonillic acid, quercitin, dextrose, sodium citrate, citric acid, sodium chloride, thiobarbutaric acid, potassium ferricyanide, ammonium molybdate, sulphuric acid, potassium acetate, TBA, deoxy ribose, EDTA, PMS, NBT, NADH are of analytical grade and are obtained from SRL, Rankem and Merck.

PREPARATION OF THE EXTRACTS

Poly herbal formulation: The poly herbal formulation is extracted with different solvents viz water, diethylether, methanol, acetone, chloroform, and hexane. Approximately 1.5g of polyherbal formulation is dissolved in each solvent in different tubes and kept overnight at 40°C, after that the solvents with extract are filtered and the filtrates are stored at 40°C for further uses.

PHYTOCHEMICAL ANALYSIS ESTIMATION [15-20] OF TOTAL POLYPHENOLIC CONTENT

PRINCIPLE:

This is based on the principle that poly phenol reacts with folin ciocalteau's reagent gives blue colored chromogen in alkali medium, which can be measured at 760nm and the concentration of poly phenol in extract are calculated by using standard curve prepare with gallic acid.

- **Folin ciocalteau's (FC) reagent (1:1 v/v)**
- **Sodium carbonate 10%**
- **Gallic acid solution in water 1mg/ml**

PROCEDURE: The total polyphenolic content is determined colorimetrically using folin ciocalteau's method for extracts. Aliquot (0-5µl) of gallic acid is taken in the tubes. Volume of all the tubes is made up to 1ml with distilled water. The extracted also diluted accordingly; 1ml of FC reagent and 2ml of 10% sodium carbonate is added to each of the tubes. After 30 mins absorbance is read at 760 nm against a blank. Concentration of poly phenol in poly herbal formulation extracts is calculated using standard curve and expressed as % concentration.

DETERMINATION OF PHENOLICS [3-8, 16-24] AND SAPONINS BY TLC

PRINCIPLE

The test sample is applied as a spot on the pre coated silica G plate and then placed in a reservoir of mobile phase that allowed passing over the plate. By simple capillary action the mobile phase moved rapidly across the layer.

REAGENTS REQUIERD

TLC plate, sprayer,

- **capillary tubes,**
- **Solvent system and sample.**

PROCEDURE: Phenolics are separated with acetic acid: chloroform (45:5) and the saponin is developed with chloroform: methanol: water (13:7:2) and detected with 10% Sulphuric acid. The Rf values of the spots are calculated as the ratio of the distance travelled by the solute to that by the solvent front.

$$R_f = \frac{\text{distance travelled by compound (solute)}}{\text{distance travelled bu solvent}}$$

ESTIMATION OF TOTAL PROTEINS

The protein content is determined by Lowry's method, taking BSA as the standard (0.075g) 50µl of poly herbal formulation extract are taken and the volume is made up to 1ml using distilled water, this is followed by addition of 5ml of Lowry's reagent.

The reaction mixture is allowed to stand for 10minuts at room temperature, 0.5ml of 1:1 dilution of folin ciocalteau's reagent added and allowed to stand 30 minutes at room temperature. And absorbance is measured at 670 nm. The total protein content is calculated using standard BSA calibration curve.

ESTIMATION OF TOTAL CARBOHYDRATES

The total carbohydrate content is determined by phenol-Sulphuric acid method, taking glucose as standard (100 mg).100µl of poly herbal formulation extract are taken and the volume is made up to 1ml using distilled water, this is followed by addition of 1ml of 5% phenol and 5ml of 96% Sulphuric acid. The reaction mixture is allowed to stand for 20 minutes at 25-30°C and the absorbance is read at 490 nm. The total carbohydrate content is calculated by using standard glucose calibration curve.

ESTIMATION OF TOTAL FLAVONOID

The aluminum chloride colorimetric method is modified from the procedure reported by Woisky and Salatino. Quercetin (0-100µg) is used to make the calibration curve. 0.1 ml of polyherbal formulation extract is taken and the volume is made up to 1ml using methanol, this is followed by additional of 1ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water. The reaction mixture is allowed to stand for 30 minutes at room temperature and then the absorption is measured at 415nm. The total flavonoid content is calculated using standard curve.

QUALITATIVE TEST

HAGER'S TEST: Alkaloids give yellow colour precipitate with Hager's reagent (saturated solution of picric acid).

TERPENOIDS: 5ml of each polyherbal formulation extract is mixed in 2ml of chloroform; 3ml of concentrated H2SO4 is then added to form a layer. A reddish-brown precipitate colouration at the interface formed indicated the presence of terpenoids.

STEROIDS: 1ml of the poly herbal formulation extracts is dissolved in a few drops of acetic acid. It is gently warmed and cooled under the tap water and a drop of concentrated Sulphuric acid is added along the sides of the test tube. Appearance of green color indicates the presents of steroids.

Total phenolic content is estimated by Folin–Ciocalteu colorimetric method using gallic acid as a standard phenolic compound.

a. Reagents –

1) Folin–Ciocalteu reagent (0.2 N)

2) Saturated sodium carbonate (75 g/l)

b. Procedure - 100 µl (two replicates) of the samples (1 mg/mL) are mixed with 900 µl of distilled water and 5 mL of 0.2 N Folin–Ciocalteu reagents. After 5 min, 4 mL of saturated sodium carbonate (75 g/l) are added. The absorbance of the resulting blue-coloured solution is measured at 765 nm after incubation at 30°C for 1.5 h with intermittent shaking. Quantitative measurements are performed, based on a standard calibration curve (10, 20, 40, 80, 160 and 320 µg/mL of gallic acid in 95% methanol). The total phenolic content is calculated as gallic acid equivalents (GAE) in milligrams per gram of dry material by the following formula

$$T = \frac{C \cdot V}{M}$$

Where T = total phenolic compounds, mg/g plant extract, in GAE;

C = concentration of gallic acid established from calibration curve, mg/mL;

V = the volume of extract, mL;

M = the weight of ethanolic plant extract, gram.

Estimation of total flavonoid content [14]

The total flavonoid content is determined by the following methods-

a. Reagent – 1) Aluminium trichloride (2%)

b. Procedure - 5 mL of 2% aluminium trichloride is mixed with the same volume of sample (1 mg/mL). Absorbance at 415 nm is taken after 10 min against a blank sample consisting of 5 mL of sample solution and 5 mL of methanol without aluminium trichloride. The total flavonoid content is determined using a standard curve of quercetin at 1–64 µg/mL. The average of three readings is expressed as quercetin equivalents (QE) on a dry weight (DW) basis.

Estimation of tannin content [15].

Tannin content of the samples is determined by the following methods-

a. Principle [16, 17]

Phosphomolybdic-phosphotungstic acid (Folin-Denis reagent) is reduced to a blue colour complex of tungsten and molybdenum oxide in alkaline solution by phenols. The intensity of the blue colour produced is measured using spectrophotometer at 760 nm. Tannic acid is used as a standard to calculate the concentration of phenols in the sample and is expressed as tannic acid equivalent.

b. Reagents – 1) Folin-Denis reagent.

2) Saturated sodium carbonate solution. (35 g in 100 mL water)

c. Extract solution 10 mg of extract is dissolved in 10 mL of methanol.

d. Procedure

1 mL (0 - 1000 µg/mL in distilled water) of the standard tannic acid solution is pipette into 10-mL standard volumetric flasks containing 7.5 mL of water. 0.5 mL of Folin-Denis reagent and 1.0 mL of sodium carbonate solution is added and diluted to the mark with water. The solution is mixed well and the absorbance is determined at 760 nm after 30 min. Absorbance is plotted against concentration of tannic acid. Determination of samples is carried in the same manner as standard, where 1 mL of each fraction (1 mg/mL) is taken instead of standard and total tannin content is expressed in mg tannic acid equivalent (TAE)/g dry weight (DW) of fraction. The blank consisted of all the reagents without the sample.

Heavy metal analysis

Determination of heavy metal content [3]

Heavy metal content for crude drugs is determined by using the Atomic Absorption Spectroscopy as per the recommendations of WHO.

Microbiological evaluation

Determination of micro-organisms [8, 9]

Presence of micro-organisms are detected as per WHO guidelines.

Total Aerobic Bacterial Count:

1 g of sample (crude drug powder) is weighed in a sterile test-tube. It is dissolved / suspended in 9 mL of buffered sodium chloride-peptone solution pH 7.0 (PB). Then the contents are vortexed to form a uniform suspension. Dilutions are made upto 10⁻³ in PB. 1 mL of each dilution is taken in a sterile petri plate and to it, 18 mL of liquefied soyabean casein digest medium (TSA) is added at a temperature not exceeding 45°C. The contents are mixed and the plate is allowed to set. These plates are then incubated at 28°C for 5 days. The plates are examined daily for the bacterial count. The numbers of colonies formed are counted and the results are expressed in terms of cfu/g or cfu/mL.

Total Yeast and Mould Count

Sample (crude drug powder, 1 g) is weighed in a sterile test-tube. It is dissolved and suspended in 9 mL of PB. The content is vortexed to form a uniform suspension.

Dilutions are made upto 10⁻³ in PB. To 1 mL of each dilution taken in a sterile petri plate, 18 mL of liquefied Sabouraud's agar (SAB) is added at a temperature not exceeding 45°C. The contents are mixed and the plate is allowed to set. This is followed by incubation at 23°C for 5 days and daily examination for the count. Numbers of colonies formed are counted and the results are expressed as cfu/g or cfu/mL.

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