ORIGINAL ARTICLE

A rapid and simple scheme for the standardization of polyherbal drugs

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In America and Europe, fast growing awareness and confidence has been shown by consumers for alternative medicine. The Indian system of medicine comprises of Ayurved, Unani and Siddha. In these systems maximum drugs are made up of polyherbal materials. The Indian System of Medicine is a pioneer in the use of herbominiral material as medicines. The World Health Organization (WHO) in 1999, had given a detail protocol for the standardization of herbal drugs comprising of a single content, but very little literature is available for the standardization of polyherbal drugs. We have developed a rapid and simple scheme for the standardization and authentication of a polyherbal drug comprising of many substances. Madhunashini is a polyherbal drug, which is a complex mixture of different herbal and mineral substances. We have undertaken the task of developing a novel scheme for a sensitive, specific and accurate standardization of Divya Madhunashini. The present scheme could also be applicable for the standardization of other polyherbal drugs, for their consistency, potency and efficacy. A packet of 120 tablets of Madhunashini, 500 mg, has been taken from the Divya pharmacy, Haridwar outlet; batch No.DPO-12 Exp. 9/2009. Madhunashini is a mixture of 23 poly herbal materials. We have categorized all 23 herbominiral materials into four categories, alkaloid content, glycoside content, bitter content and tannin content. Our result indicates that the extraction of polyherbal drugs in different solvents, in a particular sequence, yields all the four categories of active constituents, which are further identified by high performance thin layer chromatography, whereas, material extracted with one solvent has not exhibited any clear Rf values and always appears in the form of a tail.

Key words: Ayurvedic formulation, polyherbal drugs, standardization

INTRODUCTION

The quality and therapeutic efficacy of herbal drugs is dependant on the active constituents which are present in the plant cell. Of the newly approved drugs reported between 1983 and 1994, drugs of natural origin predominated (78%) in the antibacterial area, while 61% of the 31 anticancer drugs approved in the same period were either natural products, nature-derived products or compounds modelled on natural product parents or "leads". In addition, 50% of the best selling pharmaceuticals in 1991 were either natural products or their derivatives.[1] Madhunashini is an Ayurvedic Proprietary Drug. It is a combination of 23 polyherbal materials, and controls and manages Diabetes mellitus effectively.[2] Chemical and instrumental analysis is routinely used for analysing single herbal ingredient drugs for the purpose of standardization.[3] A single herbal drug extract was standardized on the basis of its active principles. We reviewed a lot of literature for the standardization of polyherbal drugs, but there very few chemical or analytical methods available for polyherbal drug standardization.[4] We developed a novel scheme for the standardization of the finished Ayurvedic product, made up of more then one polyherbal material, by broadly classifying it into four categories of active constituents, as a group, which was responsible for the pharmacological activity of the herbominiral material. The pharmacological property of the herbominiral material was due to the stimulation or depression of one or more physiological systems of the body and its action was due to constituents known as specific active principles.

Most of the bulk of the biomass, irrespective of whether it is of plants or microbes, exists as a fairly inert, insoluble and often polymeric material, such as cellulose of plants or fungi and the microbial cell wall.[5] The first step of the extraction is therefore to release and solubilise the smaller secondary metabolites in the matrix, resulting in the initial extract. In liquid extractions the choice of extraction of a solvent or solvents provides the first and most obvious means of sample preparation.[6] Initial extraction with low-polarity solvents yields the more lipophilic components, while alcohols isolate a broader spectrum of polar compounds from the material. In addition to the choice of extraction of a solvent, there are also different approaches to the actual extraction procedure. The simplest method of extraction, however, needs no extraction medium. Mechanical

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pressing has been traditionally applied to the extraction of oils from oilseeds.[7] This process may be combined with some form of pretreatment such as cleaning, dehulling, crushing or flaking before the extraction, but in general, the only equipment needed is a hydraulic press. Despite the simple operating principle, there are several operating parameters that need to be controlled in order to obtain a sufficient extraction rate and yield. The most important parameters affecting the yield of the extraction procedure are the moisture content of the material and temperature.[8] Standardization of the Triphala mixture of *Emblica officinalis, Terminalia chebula* and *T. belerica* in equal proportions has been reported by the HPLC method by using the RP18 column with an acidic mobile phase. A complete extraction of phenolic compounds was also studied, which enabled the efficient separation of total phenol compounds, that is, gallic acid, tannic acid, syringic acid and epicatechin along with ascorbic acid, within a 20 minute analysis. Validation of the method was also performed in order to demonstrate its selectivity, linearity, precision, accuracy and robustness.[9] We proposed the scheme for the standardization of Madhunashini, which will give answers for almost all the requirements for polyherbal medicine standardization.

Material which is Standardized by its Alkaloid Content
As a class character the alkaloid was precipitated from the solution by salts of heavy metals and iodine solution.[10]

Material which is Standardized by its Glycoside Content
Glycosides are the combination of sugars with other organic structures found in the vegetable kingdom. Large glycosides do not combine with acids to form salts.[11] When warmed with mineral acid the molecules of glycoside hydrolyse and split the sugar or sugars in the molecules of the residue into glycone or genin.

Material which is Standardied by its Bitter Content
Bitters are nonpoisonous substances that occur widely in medicinal plants.

Material which is Standardized by its Tannin Contents
Tannins are nonnitrogenous plant constituents characterized by their astringent action upon the muscular membrane.

MATERIALS AND METHODS

A packet of 120 tablets of Madhunashini 500 mg has been taken from batch No. DPO12 Exp. 9/2009. Madhunashini is a mixture of 23 polyherbal materials.

Madhunashini is a Mixture of the Following 23 Polyherbal Materials
Tinospora cordifol,
Salacia oblonga
Azadirachta indica (Bitters)
Swertia chirayita
Holarrhena antidysenterica
Gymnema sylvestre
Withania somnifera
Tribulus terrestris
Terminalia chebula
Terminalia bellirica
Emblica officinalis
Aegle marmelos
Curcuma zedoaria
Ficus bengalensis
Adhatoda vasica
Curcuma longa
Acacia arabica
Strychnos nux-vomica
Centratherum anthelminicum
Picrorhiza kurroa
Syzygium cumini
Trigonella foenum-graecum
Asphalatum (Miniral)

Steps for Standardization of Herbal Medicine
Step 1. Extraction and identification of the active therapeutic constituent from the finished medicine.
Step 2. Extraction, identification and quantification of pesticides from the finished medicine.
Step 3. Extraction, identification and quantification of heavy metals from the finished medicine.
Step 4. Microbiological load test of the finished medicine.

Step 1. Extraction and authentication of active therapeutic constituent from finished medicine
Isolation of total glycoside contents: We weighed 10 gm of the Madhunashini sample powder into a 100 ml volumetric flask and made it acidic with dilute Hydrochloric Acid (HCL) (5%). Then we took the sample in separating funnel with 50 ml of chloroform, and the chloroform layer was taken out and evaporated on a water bath.The residue was now ready for glycoside and phenolic compounds.

Isolation total alkaloids content: We weighed 10 gm of the Madhunashini sample powder in a conical flask and added 50 ml of distilled water and 25 ml of 1 N sulphuric acid to it. It was further stirred on a magnetic stirrer for 15 minutes and filtered through a Whatman No. 1 filter paper. The filtrate was transferred into a separating funnel and extracted thrice, each with 50 ml of chloroform. The chloroform layers were separated and discarded. Ten percent of ammonia solution was added, to maintain a pH of 7-8. It was extracted thrice, each with 50 ml of chloroform. The combined chloroform layers were passed over anhydrous sodium sulphite. The combined chloroform layers were concentrated to dryness on a rotary evaporator,

under vacuum. The residue was dissolved in 10 ml × twice the ratio of methanol : chloroform (50 : 50), transferred into a rotary flask and evaporated to dryness under vacuum. Now the residue was ready for total alkaloid content.

Isolation of total tannin content: Extract powder of 400 mg was weighed accurately in a 100 ml volumetric flask. Fifty millilitres of hot water was added to it, pH was above 7, the temperature was maintained at above 49°C and it was shaken well. The aqueous fraction contained the tannin content.

Isolation of total bitter content: Ten grammes of dry powder was accurately weighed in a round bottom flask. One hundred millilitres of methanol, of analytical grade, was added and refluxed for one hour. It was extracted with further 50, 50, 25, 25 ml of methanol AR grade till the extract became colourless. The combined methanol extract was evaporated on a rotary evaporator, under vacuum, to a thick paste, which was dissolved in 40 ml of hot water. The aqueous extract was transferred into a 250 ml separating funnel. It was extracted repeatedly with 50, 50, 50, 25, 25 ml of ethyl acetate till the ethyl acetate extract became colourless. The combined ethyl acetate extract was concentrated on a rotary evaporator, under vacuum, to dryness. The residue was dissolved in methanol in a 50 ml rotary flask and evaporated to dryness under vacuum.

IHPTLC details for the identification of extracted material as their active chemical constituents

Sample solution: Extracted material of 1 gm of all four groups was dissolved in 5 ml of methanol and 2 ul was spotted on a coated silica gel 60 F254 TLC plate using a micropipette.

Development system: We tried many solvent systems, but ultimately we got the best result with Mercuric Chloride : Ethyl Acetate (90 : 10)

Stationery phase: We also tried four stationery phases, but ultimately we got the best result with a precoated silica gel 60 F254 TLC plate of 0.2 mm thickness and plates of micro crystalline cellulose for sugar contents.

Scanning: Absorption/refection mode from 200 to 800 nm.

Spectrum: 200 to 400 nm

Step 2. Extraction, identification and quantification of pesticides from the finished medicine

Extraction of common pesticide from material: Ten grammes of the sample was taken in an R.B. flask and added sodium sulphide with 100 ml n-Hexane. It was refluxed for 1 hour. The filtrate was taken in a separating funnel and extracted with 50 ml and 25 ml of acetonitrile. The acetonitrile layer was mixed with 500 ml DM water with 2.5 ml saturated sodium sulphide and again shaken in a separating funnel with an N-Hexane layer and evaporated on a water bath. Now this residue was ready for the analysis of organochloro, organophosphate and carbamate pesticides.[12]

Step 3. Extraction, identification and quantification of heavy metals from the finished medicine

Extraction of heavy metals from material: A sample of 10 gm was taken in a silica crucible and heated to remove the moisture. It was then put in a muffle furnace at 600°C, for 3 hours, to remove the organic material. After the crucible had cooled down the color of material was observed to see if it gave a color, it contained the copper and zinc. Next the residue was boiled with 10 ml of dilute HCL and filtered. This filtrate contained the metal-like arsenic, mercury, lead, cadmium and zinc.[13]

Materials:

1. Copper strip, 5 × 10 mm, or a spiral of the copper wire wound tightly around a glass rod 10 times
2. Nitric acid, 2.5 N

Procedure for the test of heavy metals: Wash the copper strip with 2.5 N nitric acid, then rinse with 95% ethanol and dry. Place 20 ml of residue dissolved or suspended in 20.0 ml of water, into a small Erlenmeyer flask. Add 4.0 ml of concentrated HCL. Add the freshly washed copper strip to the flask. Heat the solution for about 1 hour. Next, remove the copper and examine it.

Step 4. Microbiological load test in finished medicine

One gramme of sample was taken and poured onto specific media and incubated in an appropriate condition for microbial testing, according to Bergey's manual.[14]

Materials:

1. Soyabean Casein Digest Broth Medium (SCDM)
2. Soyabean Casein Digest Agar (SCDA)
3. Cetrimide Agar Medium

RESULTS

Identification of the extracted material for the presence of active chemical constituents by HPTLC:

RF values: The Rf values of each class of active chemical constituents was different and specific [Figures 1 and 2]. We have identified specific peaks and their respective spectra [Figure 3] for each group of active plant constituents.

The results of HPTLC are as follows. AU is Absorbance Unit and Rf is Retention Factor of the separated components.

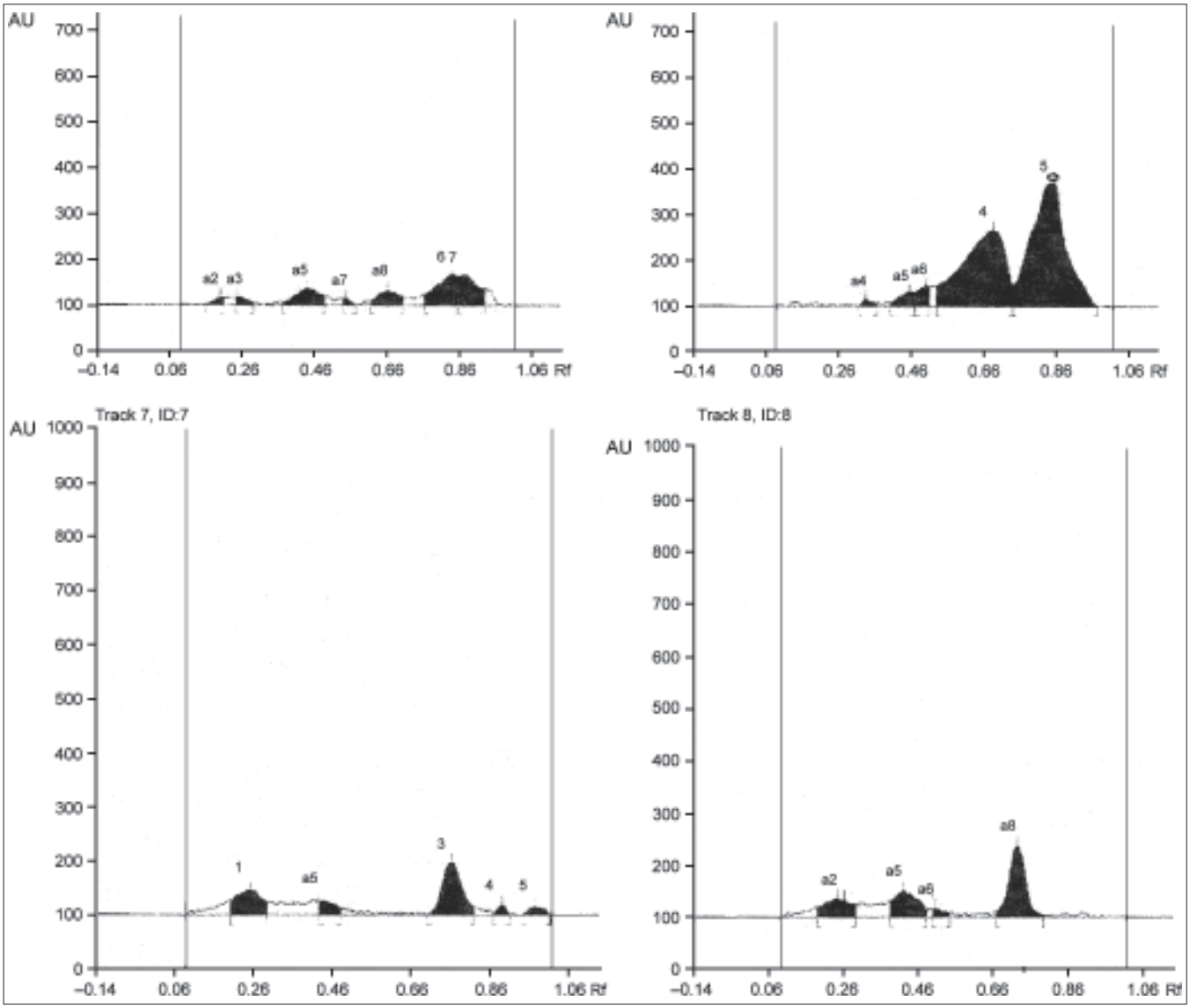

Figure 1: An Rf value of each class of active chemical constituents is different and specific

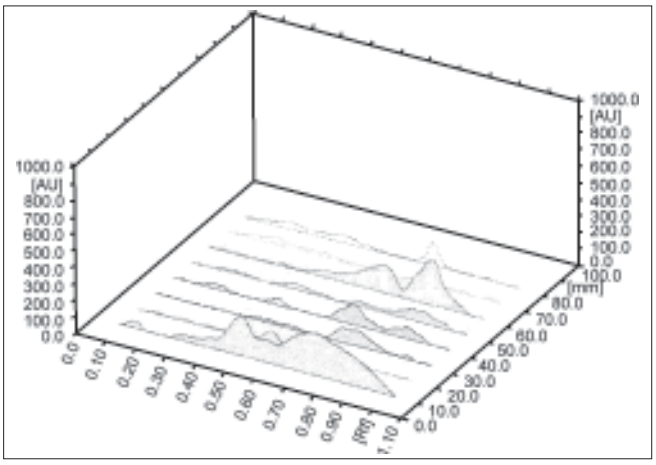

Figure 2: 3D representation of active constituents from track 1 to 8

1. Materials which are standardized with their alkaloid contents Rf 0.66 and 0.86, Area 66%
2. Material which is standardized with its glycoside contents Rf 0.26, Area 46%
3. Material which is standardized with its total bitter content Rf 0.46, Area 50%
4. Material which is standardized with its Tannin contents Rf 0.96, Area 10%

Spectrum: Spectra were taken from 200 to 400 mm and comparison has been done with the known reference standards of HPTLC Data. Spectra comparison indicates the presence of four major active constituents in the finished Madhunashini drug.

Detection of Pesticides from the Finished Medicine

Detection of organochloro, organophosphate and carbamate pesticides: - The detection of pesticides has been done by swift and speedy colour test methods. We have divided the pesticides into three broad categories for speedy analysis

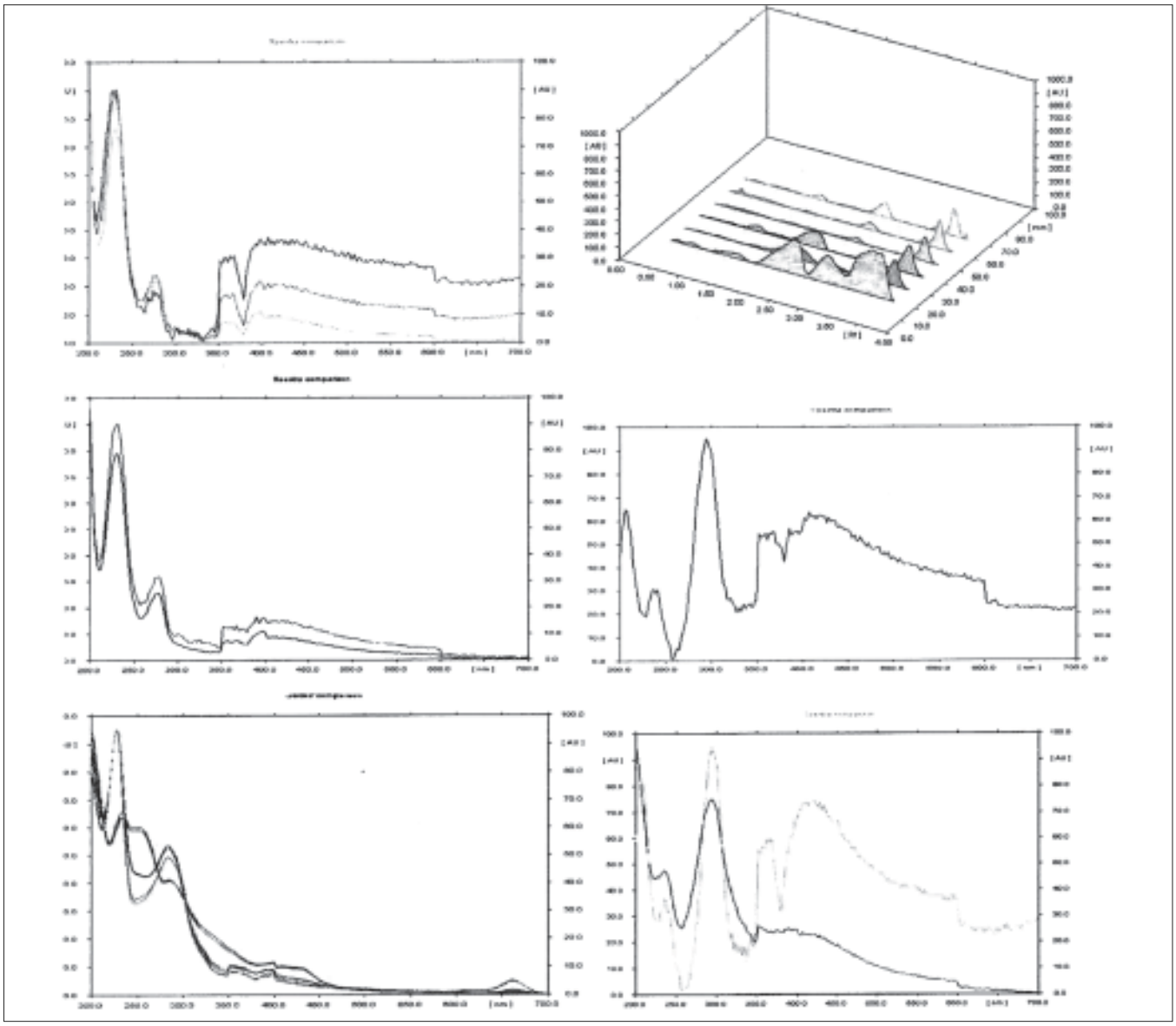

Figure 3: Peaks and their respective spectra of each group of active plant constituents

by a group of specific colour tests, and the sensitivity of the colour test is (0.05 ppm).

Result of the Colour test for the Detection of Pesticides

Organochloro colour test

1. Hydroquinone of 0.5% in concentrated sulphuric acid - no colour (Dichloropropone absent)
2. Residue + Isopropyl alcohol- no colour (Dichloropropone absent)

Organophospho colour test

Residue of 1 ml in 5 ml ethanol + Potassium Hydroxide - no colour (Phosphate absent)

Carbamate colour test

Residue of 1 ml in ml ethanol + 1 drop Furfural + 1 drop of HCL - no colour (Amide group absent)

TLC Procedure for Pesticides

Sample solution: Residue in methanol

Development system: We have tried many solvent systems, but ultimately we got the best result with a common solvent system, for the detection of three classes of pesticides.

Benzene: Methanol (60 : 40)

Stationery Phase: We used a precoated silica gel 60 F254 TLC plate of 0.2 mm thickness.

Detection: By UV Absorption Range from 200 to 300 nm

TLC results of pesticides: Our TLC results corroborate our

findings of the absence of pesticide by a colour test of all the three broad groups of pesticides in the Madhunashini sample, whereas, the reference samples (Accu Standards, USA) exhibit the characteristic spot for the organochloro, organophosphate and carbamate class of pesticides.

Detection of Heavy Metals by Colour Test

Calculation: A silver deposit indicates the presence of mercury, a dark deposit indicates the presence of bismuth, and a dull black deposition indicates the the presence of arsenic. We have estimated the arsenic contents in the material by comparing the deposit with those obtained from a solution of known arsenic content processed in the same manner, (Dull black arsenic deposition, if it is as little as 0.010 mg of As). A fair estimate of the quantity present may be obtained by comparing the deposit of the unknown with that obtained from the reference solution. This test is sensitive to 0.010 mg

Interfering substances: Parallel analysis of all reagents is necessary to rule out a false positive result due to the reagent.

Result of heavy metals by colour test: Parallel analysis of all reagents with the control sample exhibits the following observations in the Madhunashini samples.

Arsenic < 1 ppm
Heavy metals < 8 ppm

Results for the Test of Microbial Load

TAMC (Total Aerobic Microbial count): 670 cfu/gm
TYMC (Total Yeast and Mould count): 83 cfu/gm
(a) Test for *E. coli.* Absent
(b) Test for *S. aureus.* Absent
(c) Test for *P. aeruginosa*.
(d) Test for *Salmonella.* Absent

DISCUSSION

Herbal medicines are prepared from materials of plant origin which are prone to contamination, deterioration and variation in composition. Batch-to-batch variations start right from the collection of raw material itself. So the need arises to evaluate the finished polyherbal drug for the presence of bioactive constituents. The chemical marker for herbal standardization means establishing a characteristic chemical pattern for the plant material or its cut or fraction or extract. A biomarker on the other hand is a group of chemical compounds which, in addition to being unique for that plant material, also correlates with the biological efficacy.[15] The conventional method of standardization is based on the fingerprinting of each ingredient active constituent. This process is time consuming and not viable at the manufacturing level. To overcome this situation a swift scheme is very much needed for the standardization of the polyherbal drug. We proposed the scheme for the standardization of a mixture of herbal drugs on the basis of the extraction of a group of active compounds present commonly in more then one herb. Triphala is an antioxidant-rich herbal formulation containing fruits of *Emblica officinalis, Terminalia chebula* and *T. belerica* in equal proportions. Triphala is known to have anti-mutagenic effects of the polyphenolic present in all three plant species, that is, *Emblica officinalis, Terminalia chebula* and *T. belerica*. In our scheme we classify the herbal ingredients into a broad group of active compounds, by which the detection of active constituents in a finished drug is easy, fast and has commercial viability, in place of standardizing each ingredient active compound.[16] Therefore, the speedy standardization of polyherbal material is essential for maintaining the quality of herbal drugs. We have used major class compounds as markers in the plant material for the fingerprinting of the mixture of herbal material by HPTLC. Each extract of Madhunashini exhibits the characteristic spot at different Rfs.

Our findings have corroborated with various studies on the extraction of herbal material. In this article we have suggested the scheme of extraction of a complex mixture with various solvents according to the polarity of the material. A proper extraction scheme for the extraction of a desired class of compounds is essential and necessary for the accurate standardization of the therapeutic ingredients in the polyherbal material. Our results suggest that the extraction of polyherbal material with a single solvent results in an incomplete extraction and the extracted material never shows the clear spot on the TLC plate and no clear Rf values can be achieved for batch-to-batch standardizations. We also demonstrate the fact that the existing method of standardization of finished herbominiral material, which is based on direct sampling of the material on to the stationery phase is insufficient.

In most cases, direct sampling of a polyherbal material on TLC plates yield vague results, whereas, the proper extraction of various constituents of a sample exhibits the clear spot and specific Rf of the separated material. In [Figure 4] the comparison of the HPTLC fingerprint of the Madhunashini material by different extraction schemes demonstrate that sample 1, which is directly applied on to the stationery phase exhibits no clear spot, but sample 2, which is extracted with different solvents exhibits clear and separate spots with specific Rf values. Furthermore, we have identified the specific peaks of highest concentration for each category, that is, the material which is standardized with its alkaloid content, material which is standardized with its glycoside content, material which is standardized

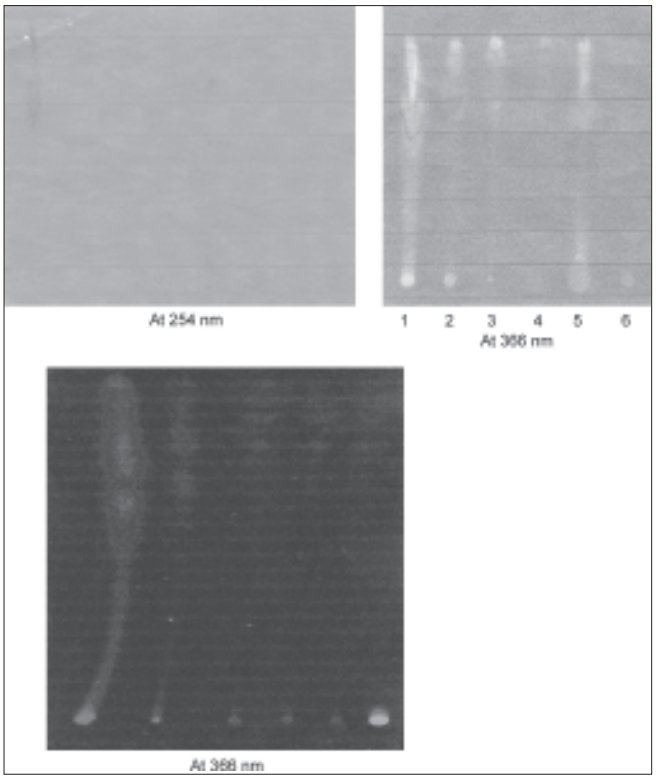

Figure 4: Comparison of HPTLC fingerprints of Madhunasni material

with its total bitter content, and material which is standardized with its Tannin content. The corresponding peaks were found to be identical with the corresponding spectra and it was further confirmed by superimposing the UV spectra of tannin, alkaloid, glycoside and bitter contents.

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