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Original Article

# NEAR-INFRARED SPECTROSCOPY: A POTENTIAL NEW MEAN OF ASSESSING MULTICOMPONENT POLYHERBAL FORMULATION ON WAY BEFORE AND AFTER EXTRACTION

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**ABSTRACT**

**Objective:** To establish a real-time sustainable identification system of polyherbal powder and extract in favor of near-infrared-spectroscopy (NIR) on applying the chemometrics technique principal component analysis (PCA) and hierarchical cluster analysis (HCA) based the fundamental attribution of integrity (similarities) and fuzziness (differences).

**Methods:** The authenticated individual, polyherbal pulverized powders and the soxhleted dry extracts containing heart-leaved moonseed, Indian Kino, Indian Liac, Ram's horn, Fenugreek, blackberry were sifted through eighty meshes. The samples were subjected to NIR spectral detection from 750 to 2500 nm at the interval of 1 nm. The multivariate data were analyzed with the help of the Unscrambler and Matlab software. The powder microscopy of polyherbal powder for identification was carried out by following routine procedure.

**Results:** Original NIR spectra of polyherbal medicament powder and extract showed characteristic hydroxyl hump peaking at about 2200 nm by the naked eye. Two classes remedy discriminated through the first order derivative transformation and natural grouping by PCA, HCA in context relative intensity of the NIR sensitive registered generic CH, OH and NH functional groups.

**Conclusion:** Chemometric method PCA and HCA is established as a reference library which significantly influences real-time quality monitoring of uncontrolled natural variation plant kingdom. NIR analysis is useful because a sample may be rapidly tested without destroying its integrity.

**Keywords:** Chemometrics, Multivariate, PCA, Polyherbal, Discrimination


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## INTRODUCTION

This is a part of a long-term research effort aimed at establishing a sustainable identification system in unavoidable cultivated and wild harvesting raw materials of plant origin in India. This paper presents a detailed study on the multivariate fingerprinting comparison of both herbal powders and extracts in favor of nondestructive method i.e. near infrared spectroscopy. The objective of the study was to determine diffused reflectance on the electromagnetic spectrum ranging from 700-2500 nm (12821-4000 $cm^{-1}$) of authenticated herbal and polyherbal samples; analyze highly absorbing functional group (alkyl, phenyls, amines, thiols, hydroxyls, acids, esters etc.) in favor of dried pulverized fine particles and plant extracts of respective contents, which are stem of *Tinospora cordifolia* (Willd), seed of *Syzigium cumini* (Skeels), heartwood of *Pterocarpus mersupium* R, Leaf of *Azadirachta indica* A. Juss, seed of *Trigonella foenum-graecum* L, leaf of *Marsdenia sylvestre* R. Br; determine distinguishing character to compute principal component analysis after preprocessing of the raw diffuse reflection data using mathematical transformation; establish library which significantly influence real-time quality monitoring and discuss easy unambiguous identifying tool to qualify products from uncontrolled natural variation plant kingdom.

The upcoming trend, NIR spectroscopy has got wide acceptance within the pharmaceutical industry for raw material testing, intermediate product, finished product quality control and process monitoring [1, 2]. The major advantages of NIR spectroscopy is due to a direct result other than any analytical technique. The NIR region is unique due to forbidden transition, highly transmitting window to radiation compare to allowed transition responsible neighbouring region such as UV, VIS, mid-IR. Many bands arising from overtone and combination mode overlap each other, due to fermi resonance appear in the region. The radiation can penetrate into powder materials like herbal powder, dry extract and the diffusely reflected or transmitted radiation will provide a vast amount of spectral information about the sample [3]. Unfortunately, the overlap of these overtone and combination bands strongly decreases the specificity of NIR spectroscopy which was the main reasons to neglect for using conservative spectroscopists such a long time. But the availability of chemometric evaluation procedures for qualitative discrimination and quantitative determination is new opportunity to overcome data evaluation.

On the other way, the natural product has complex matrices that may contribute to their benefit of use. It also fulfils all the regulatory demands as any other " chemically " raw materials and finished product materials 21 CFR 11 guidelines, food and pharma GMP guidelines, USP chapter 1119 [4]. With NIR spectroscopy, the pharmaceutical industry will move one step closer to "Zero defect" quality control. Here the spectra taken from a sample are representative of the sample as a whole. Since the drug is used as a whole there is no need for extensive sample preparation. NIR analysis is useful because a sample may be rapidly tested without destroying its integrity. Rubinovitz studied a variety of botanicals including chickweed, echinacea, golden seal, echinea root, goldenseal herb, golden seal root, horsetail and St. John's wort. The clustering algorithm permitted the reliable use of subtle differences in the powder spectra to identify each type of powder and to accurately distinguish it from other powders [5]. Applying multivariate chemometric cluster analysis easily separated materials of raw burdock root powder and concentrated burdock root extract [6]. Basic information exists in literature that indicates the frequencies (Wavelength) of bond absorption occurring for the generic functional group as well as those functionalities most important to the NIR analyst. In the light of the above background, the present study was designed and undertaken to discriminate the individual 'drug' both power and 'extract' form along with polyherbal formulation in favor of multivariate discrimination chemometric analysis.

## MATERIALS AND METHODS

### Plant materials and chemicals

Leaves of *Meshashringi* (*Marsedenia sylvestre* (Retz.) P. I. Forst; Asclepiadaceous) and *Nimba* (*Azadirachta indica* A. Juss. Meliaceae),

the stem of *Guduchi* (*Tinospora cordifolia* (Willd.) Miers; Menispermaceae), Heartwood of *Beejaka* (*Pterocarpusmarsupium* Roxb; Fabaceae), seed of *Jambu* (*Syzygiumcumini* (L.) Skeels; Myrtaceae) and *Methika* (*Trigonellafoenum-graecum L.*; Fabaceae) were collected from West-Bengal (22 °26'21''N-88 °23'45''E) and Odisha (21.49 °N-86.93 °E) in the month of July 2013. Then the plant herbariums were authenticated by a botanical survey of India, Kolkata (Voucher specimens No. CNH/51/2013/1085). A specimen of each drug has also been submitted to pharmacognosy laboratory for further references. Drugs were dried properly by shade drying and stored in an air-tight container. Then the required amount of each sample was pulverized by mechanical mixer grinder and sieved through 80#. Powder of above mentioned six ingredients were mixed in equal proportion to prepare a polyherbal formulation. Then powders of all individual plants and polyherbal formulation were subjected for pharmacognostical and NIR spectroscopic study.

**Pharmacognostical study**

Powder of all six individual ingredient and polyherbal formulation were studied under a microscope for proper identification by standard operating procedure [7].

**Extraction study**

All the individual powdered drugs and the formulation were extracted with methanol by using soxhlet apparatus. The extracts were dried, passed through 80 meshes (#) and used NIR spectroscopy. The concentration of trace heavy metals such as lead, cadmium, arsenic and mercury in formulations were analyzed by a double-beam Systonic UV-VIS spectrophotometric (2201) with help of 'cyanidin' as a chromogenic reagent [8]. All chemicals used in the study and for extraction were of analytical grade.

**Near infrared spectroscopy**

Samples were analyzed using a bench top Perkin Elmer Lambda 19 UV-VIS-NIR spectrophotometer system in the range between 750-25000 nm in diffused reflectance mode. The polyherbal powders and extract both were placed in a closed rotating sample lead sulfide cell cup with scan speed 240 nm/min. The contact probe was placed against a block surface, and spectral data were collected. At each position, the exposure time was twenty-five seconds. The spread between the spectra of each material is characteristic of reflectance spectra of powder and dry extract. The Particle size of these spectra is represented by the numerical values corresponding to the reflectance of the material at each wavelength (one-nanometer interval). Instrumentation selected standard for the wavelength and absorption calibration, validation traceable to NIST [9]. The NIR spectroscopy study was done NABL accreditated laboratory sciart at Anand, Gujarat.

**Data analysis**

Data were manipulated into two forms: 1) Data were exported as 2D ASCII files at all NIR wavelength region at 1 nm interval to a corresponding reflectance value to build a matrix for powder and extract sample (matrix contain 1750 × 7), i.e., wavelength is one direction and samples in other direction; 2) Data were exported as ASCII files to reduced reflectance value of whole wavelength (a single profile by both powder and extract sample giving a matrix with 280 points). All data operations (preprocessing, 1st derivative) were performed using MATLAB R2008 (Math works) on a computer intel Pentium 4 processor containing 500 MB RAM and running Microsoft windows seven.

**Data preprocessing**

Raw reflectance is converted to absorbance using the function Absorbance =-log (10)* Reflectance, commonly referred to as log (1/R). Raw data were submitted to chemometric analysis with first order derivative preprocessing for multivariate distribution.

**Principal component analysis (PCA) and hierarchical clustering analysis (HCA)**

Chemometric is an interdisciplinary field that involves multivariate statistics, mathematically modelling, statistical principle and other logic-based methods in the field of chemistry and in particular analytical chemistry [10]. Rapid technological advances, particularly in the area of computerized instruments for analytical chemistry have enabled the extraction of the maximum of chemical information from analytical data [11]. Qualitative spectroscopic analysis depends on comparing spectra of the specimens to be identified with spectra of 'known' or 'standards'. Basically, a chemometric assisted powerful algorithm (PCA, Cluster, discrimination) allows accurate identification which be made by small absorbance differences of various samples. Simultaneously the enormous signal and noise ratios that the instrument provide in that circumstances a computer is needed to separate information that cannot be always be detected with an unaided analytes eye. Qualitative discrimination analysis is one of the tools which can frequently use some algorithms, to match spectra across the entire spectral region, while others use relatively few individually selected wavelength from the spectrum, with an auxiliary algorithm that optimises the selection [12]. PCA represent (real phenomena) is the effective rank of the matrix. Principal component analysis refers to a method of data analysis for building linear, multivariate model of the complex data set. Developed using orthogonal basis vector (Eigenvector), usually called $PC_S$. The principal components model the statistically significant variation in the data set as well as the random measurement error. One of the significant goals of PCA is to estimate the principal components associated with noise, thereby reducing the dimensionality of complex problems and minimising the effects of measurement error. In PCA a set of P correlated variables is transformed to a smaller set of a uncorrelated hypothetical construct called Principal component. Empirical mathematical model: [13]

$A = T_K \partial$ [$T_K$ is the n × k matrix of Principal component score, $V_K$ is the m× k matrix of eigenvectors, loading, ∈ = residual.

Hierarchical clustering is the visualization tool as a supervised approach (classification approach). It is used usually based on object wise similarities or distances. One of the most valid arguments is that most clustering methods are heuristic in nature with solid statistical foundation. Dendrogram plotting can handle visualize more than three independent variables using a hierarchical family tree like structure. The algorithm performs n-1 steps where n is the sample size. In all the steps it calculates the Euclidean distance of the two closet samples to a new one with, for example, single coordinate, complete coordinate (single linkage, complete linkage algorithom). After n-1 steps, a dendrogram can be drawn to visualize hierarchically in which samples are most probably comparable even considering many independent variables. This tool is very useful if classification using PCA along a sample set needs to consider more than three factors. [14] Each distance is generally converted value

$s_{ik} = 1-d_{ik}/d_{max}$ [$s_{ik}$ is the measure of similarity between samples i and k, $d_{ik}$ is the Euclidean distance between samples i and k, $d_{max}$ is the distance between the two most dissimilar distance] In this work, PCA and HCA were carried out for the 14 samples employing as multivariable whole wavelength and result are shown as score, loading plots and dendrogram without data preprocessing of the method. The PCA were performed using the algorithm Unscrambler Camo student version and HCA included in the statistical toolbox of matlab.

**RESULTS AND DISCUSSION**

**Pharmacognostical analysis**

Detail finding of organoleptic characters of all individual powder and polyherbal formulation are depicted in table 1. Detail powder microscopical characters of all individual ingredients along with polyherbal formulation are given in table 2. Microscopic characters are a border pitted vessel, tannin content (fig. 1, A, D) and warty trichome, acicular crystal (fig. 1, E, F) seen in beejaka (*Pterocarpus marsupium* Roxb) and Nimba (*Azadirachta indicum* A. Juss) respectively. In the same way powder microscopic characters are starch grain, brown content (fig. 2, A, D) and cluster crystal, anomocytic stomata (fig. 2, E, F) seen Guduchi (*Tinospora cordifolia* Miers) and meshasringi (*Marsdenia sylvestre* P. I. Forst) simultaneously. Remaining two ingredients powder microscopy

characters are aleuronic grain, the epidermis of testa (fig. 3, A, D) and spiral vessel, brown content (fig. 3, E, G) seen methika (*Trigonela foenum graecum* L) and jambu (*Syzygiumcumini* L.). Finally, polyherbal formulation encompasses starch grain for guduchi, anomocytic stomata for meshasringi, a fragment of the epidermal cell as methika, scleride of jambu, a fragment of fiber passing through medullary ray's beejaka, annular vessel are seen in photomicroscopy in 10X. On the basis of the results obtained in the present study, the ingredients of the polyherbal formulation are pure without adulterant and authentic material.

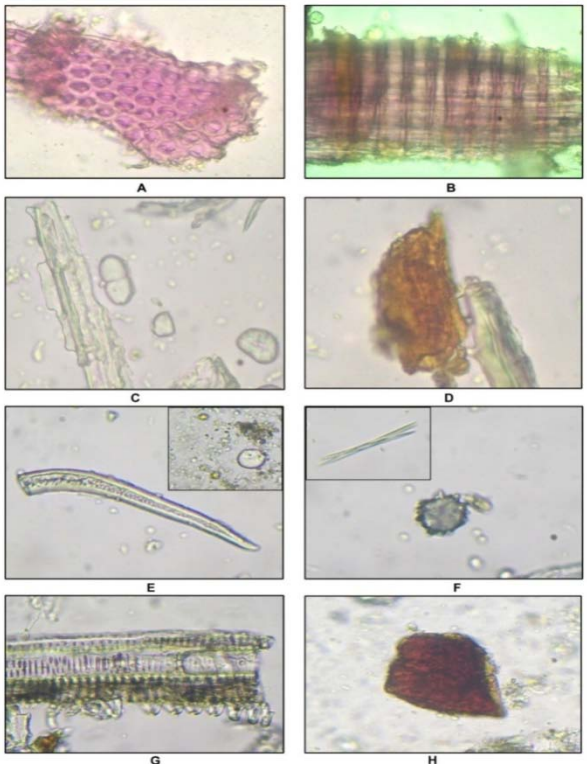


**Fig. 1: A. Fragment of border pitted vessel of beejaka (Ht. wd). B. Fiber passing through the medullary rays of Beejaka (Ht. wd). C. Simple and compound starch grain with hilum of Beejaka (Ht. wd). D. Tanin content of Beejaka (Ht. wd). E. Warty trichome of neem (Lf.). F. Acicular crystal and cluster crystal of neem (Lf.). G. Fragment of spiral and annular vessel of neem (Lf.). H. Tanin content of neem (Lf.)**

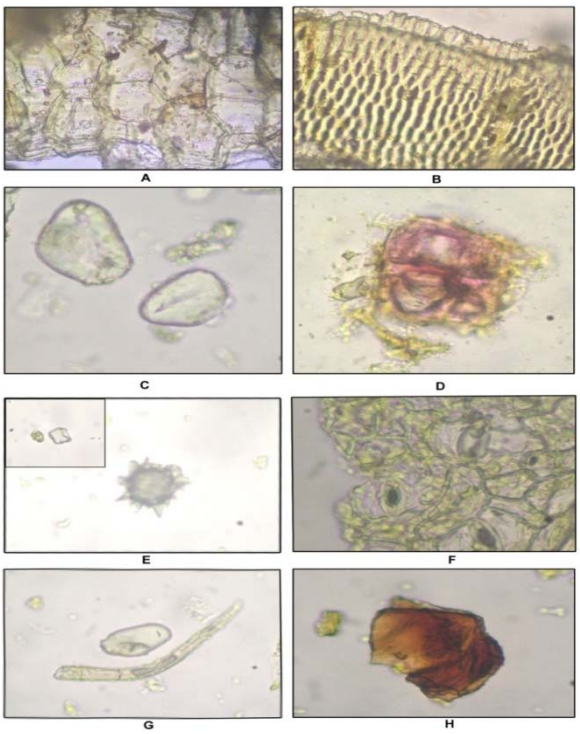


**Fig. 2: A. Parenchyma cell with a starch grain of guduchi (St.). B. Vessel elements of guduchi (St.). C. Starch grain with hilum of guduchi (St.). D. Brown content of guduchi (St.). E. Cluster crystal of meshasringi (Lf.). F. Anomocytic stomata of meshasringi (Lf.). G. Fragment of warty trichome of meshasrinigi (Lf.). H. Brown content of meshasringi (Lf.)**

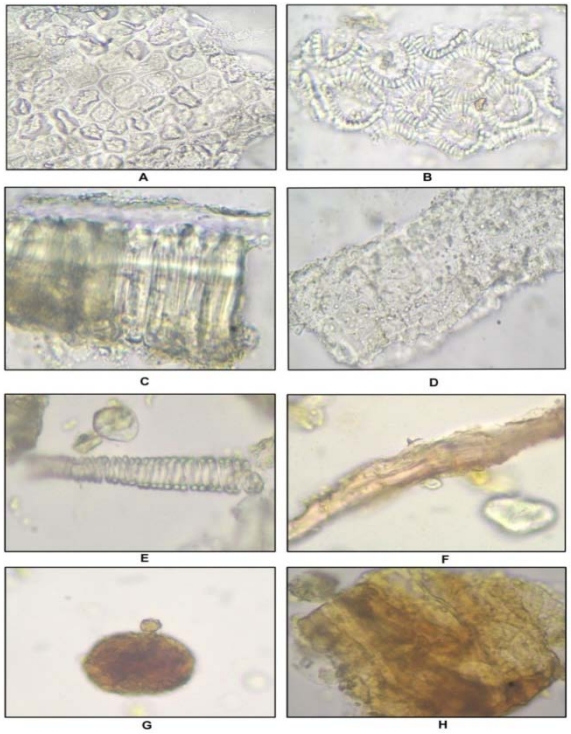


**Fig. 3: A. Thick-walled endosperm cell containing aleurone grain of methika (Sd.). B. Sub-epidermis of the testa in a surface view of methika (Sd.). C. Fragment of the columnar cell of Methika (Sd.). D. The epidermis of the testa in a surface view of methika (Sd.). E. Spiral vessel with a starch grain of Jambu (Sd.). F. Scleride of jambu (Sd.). G. Brown content of jambu (Sd.). H. Tanin content of jambu (Sd.)**

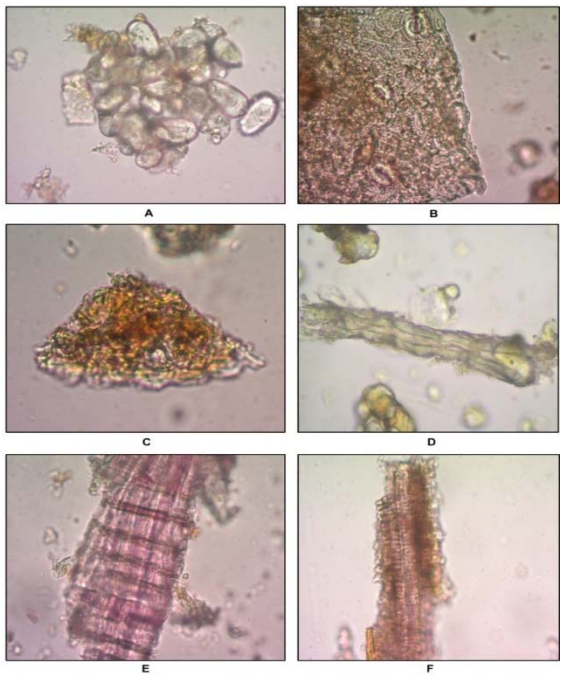


**Fig. 4: A. Starch grain of guduchi (St.). B. anomocytic stomata of meshasringi (Lf.). C. Fragment of epicarp cell methika (Sd.). D. scleride of jambu (Sd.) E. fragment of fiber passing through medullary rays in beejaka (Ht. wd.). F. annular vessel of neem (Lf.)**

**Pharmacognostical alignment of NIR generic functional group**

The structure of cellulose and lignin are known tentative band assignments with respect of its structure alignment of Pharmacognostic finding [Table-3]. The vascular portions of leaves and stems are encapsulated by a material called lignin which contained some ingredients of Dt. During some success calibration study for the major fibrous component in favor of plant, substances were reported [15]. The most important factor that affects the behavior of solid samples a) chemical composition b) physical texture coarse and smooth [table 1]. Bulk density may interfere of packing characteristic in the sample cells which affect the diffuse reflectance from the surface. Another way powder characteristic–processing (drying, grinding) method, temperature, colour are grossly reflectance of light energies from the surface and can introduce bias basically low wavelength. The individual colour of the respective powder is one of the factor reflectances of resultant mixture profile [Table-1]. Chemical composition affects the sample, a

composition factor, the presence of high cellulosic constituents generates long narrow particles, which pack differently than materials lower in fibre. On the other way starch and cellulose, are two very similar monomers, glucose based repeat unit. Glucose unit in starch is connected by alpha linkage and cellulose by beta linkage. Powder characterizations of the resultant mixture with its ingredient are found (fig. 1-C, 2-C, 3-E, 4-A) along with its individual component. Polyherbal commodities are very complicated substances, with oil, protein, moisture, brown component in various degrees and in substances with very big differences in physical nature and functionality. In the alignment of powder characteristic, the energy quanta absorbed are bond specific but are also affected by the chemical matrix and environmental factors such as the type of functional group neighbouring molecules and hydrogen bond indirectly of the polyherbal formulation [16].

**Table 1: Different organoleptic parameters of individual plant powder and polyherbal powder**

| Drug name | English name | Organoleptic characters | | | |
|---|---|---|---|---|---|
| | | Texture | Colour | Taste | Odour |
| *Nimba* (*Azadirachta indica*) | Indian liac (lf.) | Coarse | Green | Bitter | Characteristic odour (leafy) |
| *Meshashringi* (*Marsedeniasylvestrie*) | Ram's horn (lf.) | Coarse | yellowish brown on adaxial and dark green on abaxial side | Bitter (paralyzing the sense of taste for new hours particularly for sweet substances). | pleasant aromatic odour |
| *Beejaka* (*Pterocarpus marsupium*) | Indian Kino (Ht. wd) | Coarse | dark reddish brown | astringent | nill |
| *Jambu* (*Syzygium cumini*) | Black berry (Sd.)/Black plum | Smooth | dark violet to brownish | astringent | agreeable and aromatic |
| *Guduchi* (*Tinospora coordifolia*) | Heart leaved moon seed (St.) | Smooth | Creamish brown | bitter | Odorless |
| *Methika* (*Trigonella foenum-graecum*) | Fenugreek (Sd.) | Smooth | yellowish brown | mucilaginous bitter | spicy |
| Polyherbal mixer(powdered pills) | Diabetogen | Coarse | Greyish yellow | Bitter | Spicy |

**Table 2: Different macroscopic and microscopic characters of individual plant powder and polyherbal powder**

| Individual drug and polyherbal powder | Microscopic powder character |
|---|---|
| *Nimba*(*Azadirachta indica*) | Warty trichome, acicular crystal, prismatic crystal, rhomboidal crystal, cluster crystal, tannin content, fragment of spiral and annular vessel |
| *Meshashringi*(*Marsedeniasylvestrie*) | Anomocytic-stomata, fragment of warty trichome, cluster crystal, Tracheidalfibre, starch grain, brown content, fragment of spiral vessel |
| *Beejaka*(*Pterocarpus marsupium*) | Fragment of border pitted vessel, fibre passing through the medullary rays, simple and compound starch grain with hilum, prismatic crystal of calcium oxalate, Tannin content |
| *Jambu*(*Syzygium cumini*) | Simple starch grain with and without hilum, scleride, spiral vessel, tannin content, testa in surface view, |
| *Guduchi*(*Tinospora coordifolia*) | Starch grain, parenchyma cell with starch grain, crystal fiber, vessel elements, prismatic crystal, brown content, surface view of cork, parenchyma cells with oil globules, fragment of collenchyma cells |
| *Methika*(*Trigonella foenum-graecum*) | Fragment of bordered pitted vessel,thick-walled endosperm cell containing aleurone grain, a fragment of the columnar cell, cluster crystal, sub-epidermis of the testa in surface view, the epidermis of the testa in surface view with full of aleurone grain. |
| Polyherbal mixer (powdered pills) | Acicular crystal, annular vessel, bordered pitted, cluster crystal, collenchyma cell, a fragment of cork cell, a fragment of epicarp cell, a fragment of an epidermal cell, a fragment of fiber passing through medullary rays, a group of the stone cell, starch grain, normocytic stomata, warty trichome etc. |

**Table 3: A common NIR "Generic functional group" absorption wavelength demonstrated by the literature in favors pharmacognostic alignment**

| Type of material (Fibrous component) | Tentative band assignment | Wavelength (nm) | Comment | Reference source |
|---|---|---|---|---|
| Cellulose | O-H str. first overtone, | 1490 | The unbranched polymer of glucopyranose unit | Shenk (17) |
| | C-H str. first overtone | 1780 | linked by carbon (β,1-4) bond. | Shenk (18) |
| | O-H str. and second over C-O str. | 1820 | | |
| | C-H str. and C-H deform | 2335 | | |
| | C-$H_2$ sym. str. and =$CH_2$ deform | 2347 | | |
| | $CH_2$ deform the second overtone | 2352 | | |
| | CH str./C-C str. comb. | 2488 | | |
| Lignin | C-H str. second overtone | 1170 | Phenyl propanoid residue | Coleman(19) |
| | O-H str. first overtone, | 1410 | | |
| | C-H str. combination; | 1417 | | |
| | O-H Str. first overtone, | 1420 | | |
| | C-H str. combination | 1440 | | |
| | C-H str. first overtone | 1685 | | |

**Multivariate analysis**

**NIR fingerprints of powder and extract**

In this study, NIR spectrum has shown characteristic "fingerprints" for individual powder and extract along with polyherbal formulation [fig. 5; A and B] cover up a targeted functional group of organic compound [Table-4]. The multivariate distribution used as an indirect measure of plant chemistry NIR sensitive mainly C=H, O=H, N=H. Solid form powders and extracts were subjected by reflectance mode to generate the discriminating criteria of the different class materials in the expression source of variation like particle size, density and NIR sensitive stretching and bending primary bond with combination and overtone vibration mode. Particularly NIR spectra of the resultant materials are applied preprocessing (the first order derivative) technique to eliminate the baseline offset for improving the resolution of overlapping bands. The first derivative of the spectra (fig. 5; C and D) highlights the key differences between the respected samples. A significant amount of spectral variability observed on relative intensity of the respective extract and powder on the favor of various generic functional group at approximately 1143,1160,1170, 1195, 1360, 1395, 1415, 1417, 1420, 1440, 1446, 1450, 1620, 1685, 1695, 1705, 1860, 1900, 1908,1920 nm which is depicted on[table 5]. On the other hand, the remaining frequencies shown more or less same response pattern in the context of a functional group (alkyl, phenyl, amines, thiols, hydroxyl, acids) [table 4]. In this way, herbal preparation be characterized in its completeness in favor of respective functional groups. In powders solids, and various discontinuous samples, these discontinuities at the boundaries of the particles in the powder are beneficial rather than detrimental since they allow spectral measurements to be taken using diffuse reflection rather than transmission. From a theoretical point of view NIR is basically separated cumulative form band in favor of electronic and vibrational transition in the context of d-d transition, charge transfer (CT transition), and pi-pi transition or large conjugated system, OH and NH stretching bands of monomeric and polymeric species. Actually, the various band described to free, and terminal OH and NH groups of the polymeric species may be differentiated in the NIR region.

Larger anharmonicity, band ascribed to the first overtone of OH and NH stretching modes of monomeric species are enhanced compared with the corresponding bands arising from poly component species. The spectrum also contains all of the information due to light interaction with the sample as well as instrumental artefacts [20]. Since NIR spectra are typically composed of broad overlapping ill-defined absorption bands containing chemical and physical information of all sample components.

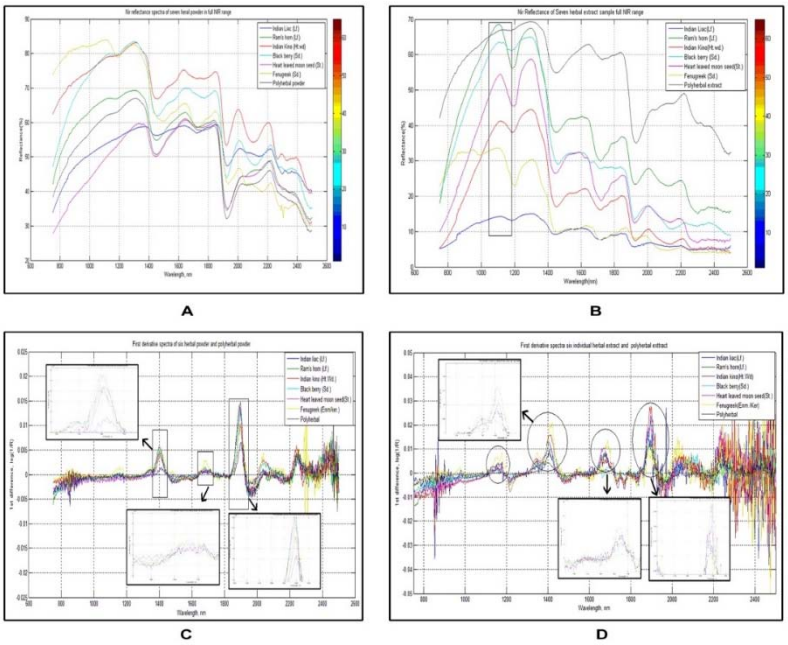


**Fig. 5: Raw reflectance spectra of various herbal materials in powder (A) and extract (B). First derivative spectra of various materials in Powder (C) and extract (D)**

**Table 4: Major analytical bands and relative peak positions for prominent real-infrared absorption region respective functional group on combination and overtone mode [21]**

| Highly absorbing functional group | Overtone mode (NIR region) nm | Combination mode(NIR region)nm |
|---|---|---|
| Alkyl | C-H 1st overtone-(1650-1750)<br>C-H 2nd overtone(1100-1150)<br>C-H 4th overtone (700-750) | C-H (1350-1450)<br>C-H+C. H (2200-2300)<br>C-H+C. C (2300-2400). |
| Phenyl (Aromatic) | 1143 2nd overtone,<br>CH stretch 1st overtone | 1417,1446–CH, 1492 Aromatic NH combination |
| Amines | N-H 1st overtone(1450-1500),<br>N-H 2nd overtone(1000-1050)<br>N-H 3rd overtone(800-850) | N-H combination (2050-2600) |
| Thiols | S-H 1st overtone (1740) | |
| Hydroxyl | O-H 1st overtone-(1400-1450)<br>O-H 2nd overtone(900-950)<br>O-H third overtone<br>(700-750) | O-H combination (1900-2000) |
| Acids (Acetamide) | C==O (1920) stretch second overtone | 1980,Asym N––H stretch/amide IIb combination,<br>N––H/Amide IIb or 2050,CONH N––H/Amide IIIb or combination |

**Table 5: Selected wavelength region where after 1st order difference of samples is discriminated [22]**

| Wavelength (nm) | Bond vibration | Structural assignment |
|---|---|---|
| 1143 | C-H second overtone | Aromatic |
| 1160 | C=O stretch fourth overtone | C=O |
| 1170 | C-H second overtone | . HC=CH |
| 1195 | C-H second overtone | . $CH_3$ |
| 1360 | C-H combination | . $CH_2$ |
| 1395 | C-H combination | . ROH,OIL |
| 1415 | C-H combination | . $CH_2$ |
| 1417 | C-H combination | Aromatic |
| 1420 | O-H first overtone | ArOH |
| 1440 | C-H combination | . $CH_2$ |
| 1446 | C-H combination | Aromatic |
| 1450 | O-H strech first overtone | Starch |
| 1620 | C-H strech first overtone | Aromatic |
| 1685 | C-H strech first overtone | Aromatic |
| 1695 | C-H strech first overtone | . $CH_3$ |
| 1705 | C-H strech first overtone | . $CH_3$ |
| 1860 | C-Clstrech sixth overtone | C-Cl |
| 1900 | C=O stretch fourth overtone | -COOH |
| 1908 | O-H strech first overtone | P-OH |
| 1920 | C=O stretch second overtone | -CONH |

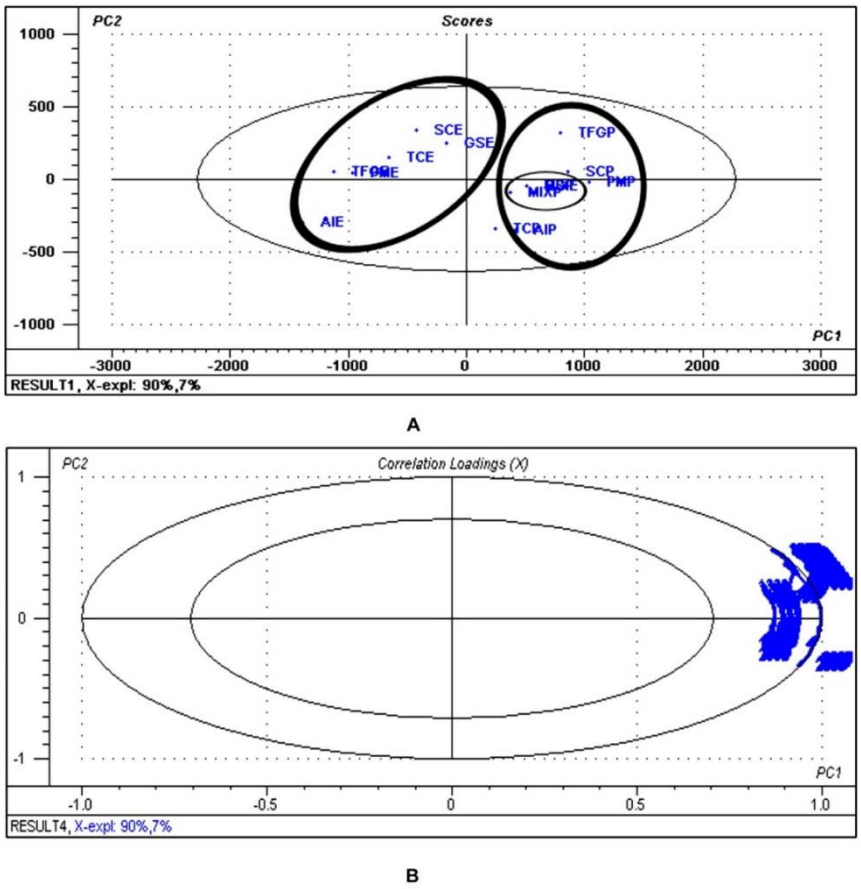


**Fig. 6: Score (A) and Loading (B) plot (PC1 vs. PC2) of PCA results obtained from whole NIR reflectance spectra of various powder (AI-Nimba, SC-Jambu, GS-Meshasringi, TC-Guduchi, TFG-Methika, PM-Beejak,Mix-Polyherbal),E-Extract,P-Powder. The ellipse represents the Hotelling T2 with 95% confidence in score plot**

**Principal component analysis**

The experimental data was examined by PCA to visualize the response pattern in the feature space of principal components ($PC_S$). The measured parameter using the whole wavelength was displayed in the loading plot (fig. 5, B), while each point corresponded to a various powder and extract sample in the score plot (fig. 5, A); Two $PC_S$ explaining the cumulative 97 (%) of the data variance, were chosen based on the eigenvalues (>2). $PC_1$ and $PC_2$ accounted for 90 and 7 (%) total variance respectively.

As indicated in the PC loading plot (fig. 5, B), all the variables (75%) located in the positive side pc1 or pc2 axis. On this way, PCA of the NIR spectra from the powder and extract (individual and mixture) samples showed a clear grouping. It is interesting to note that individual powder drug of the polyherbal formulation appears to

cluster more closely with the extract of the polyherbal medicament with Indian Liac (Lf.),Ram's horn (Lf.),Indian Kino (Ht. wd), Blackberry (Sd.), heart-leaved moonseed (St.), Fenugreek (Sd.).

The first derivative of the spectra (fig. 5, Cand D) highlights the key differences between the powder and extract along with polyherbal formulation. A key feature of this graph is a feature at approximately C-H stretching in the context of the aromatic structural assignment in first, 2nd overtone and combination mode at 1143, 1417,1446,1620 and 1685 nm in form of relative intensity differences of experimental samples. During Phytochemical screening of extract level, the aromatic ring contains flavonoid, carbohydrate, steroids gave positive response respectively Shinoda, Molish and Salkowski test. On examining the log (1/R) graph without derivative, it is clear that the samples contain a discrete peak at this position no overlapping happened here. This indicates that discrete differences in polyherbal composition are important when assessing polyherbal mixer in both powder and extract form.

However, a good discrimination between individual powder and extract along with polyherbal form could very easily be obtained when using only the first component (PC1) from PCA analysis [fig. 6A]. The primary and secondary metabolite metabolites sensitive as a functional group allowing this discrimination were clearly [table 5]. This is the indication that the indirectly primary and secondary metabolite content of two class of material is quite similar in some absorbance frequency region. The loading plot was shown a correlation of the variables (wavelength) to the principal component [fig. 6B]. The wavelength (800-1000 nm), (2000-2500 nm) values were far from the centre of the loading plot and close to each other, suggesting a strong correlation between them on favor of the generic functional group. In that way differentiation between samples was possible by visual inspection of the spectra; it was accomplished by multivariate data analysis method more easy. This was in agreement with the result obtained from NIR diffuse reflectance spectra of the respective materials. A negative correlation observed at selective (1100-1200 nm), (1300-1400 nm), (1600-1700 nm) wavelength regions.

**Hierarchical cluster analysis (HCA)**

Hierarchical cluster analysis (HCA) was performed to the authenticity of two classes sample on the basis of the similarities of NIR spectral reflectance. The result was obtained are shown as a dendrogram [fig.-7]. Euclidean distance of the complete linkage favors the small spherical two clusters. Sample 8 9,11,12,13 and samples 1, 5and 3, 4 give cluster effect which are closely together following PCA analysis. All samples were aggregated into respective clusters with the similarity level of 95%. On the other way, two multivariate methods are complemented each other clustering offer former classification while the visualization gives clear in PCA.

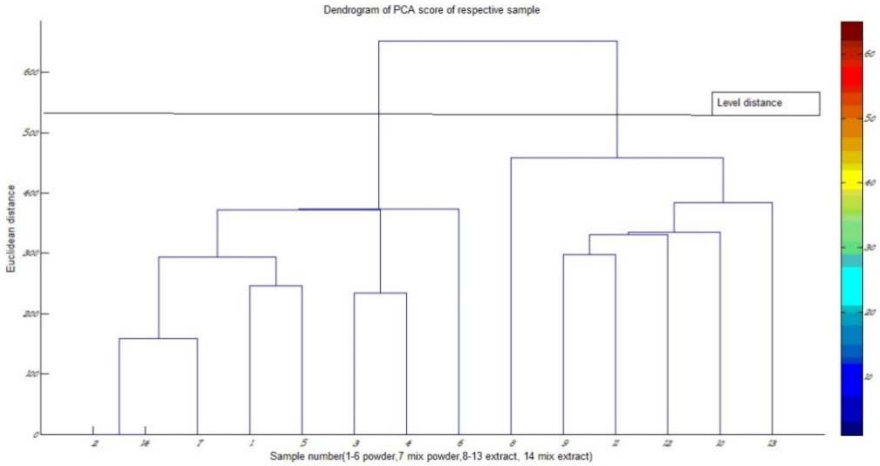


**Fig. 7: Dendrogram form of whole class material of complete linkage, HCA on of NIR spectra**

## CONCLUSION

Near infrared spectroscopy after being analysed the samples could still be used for another purpose, avoid toxic, corrosive, expensive chemicals and also concomitant avoidance of the need to dispose of the chemicals and big boost of new technology. The quality control of natural products such as primary metabolite like carbohydrate, protein, fat and secondary plant metabolite alkaloids, phenol, flavonoids and other biological samples is one of the most challenging tasks. Vibration spectroscopy NIR has proven to be a nondestructive and fast method requiring both minimal sample preparation and minimum amount of analyte as compared to traditional chromatographic techniques like HPLC, HPTLC and GC. NIR is a cumulative effect of the chemical bonds of a specific wavelength. Applying diffuse reflectance mode generates the discriminating criteria of the different materials due to the face of the extraneous sources of variation in the spectra. The first derivative spectra highlight the discriminating feature in context relative intensity differences of respective samples in favor of some registered generic functional group schedule wavelength. PCA and HCA were performed on the choice pretreatment process resulting in suitable exploratory methods to find similarities among powder and extract of individual and mixture with regards to structural properties of registered generic functional group contained from these and which resulted responsible of their NIR spectroscopic behavior.

## CONFLICT OF INTERESTS

Declared none

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