Image processing and Ultra-Violet and Visible reflectance spectroscopy combined with chemometrics for discrimination as well as authentication powder and extract with anti- diabetic polyherbal formulation.

Jayanta Kumar Maji[*], Shikha Sharma[**], V.J.Shukla[***]
[*]Ph.D scholar (Ayu.), [**]M-pharm (Ayu.) [***] Head of the department Pharmaceutical Chemistry,
I.P.G.T & R.A, PGT-SFC, Gujarat Ayurved University, Jamnagar, Gujarat-361008, India.
E-mail: jkmaji67@gmail.com.
Mob.no-9537130948

Abstract: _ The proposed anti-diabetic polyherbal formulation "Diabetogen" is composed of Indian Kino (Ht.wd.), Indian Liac (Lf.), Ram's horn (Lf.), Fenugreek (Sd.), black berry (Sd.) and heart-leaved moonseed (St.) is categorized antioxidant rich medicament and has been clinically used in the Indian subcontinent to various permutation and combination. To establish a real time identification system in favor of non –destructive ultra-violet visible (UV-VIS) spectroscopy & preliminary image processing. The multivariate chemometrics technique principal component analysis (PCA), hierarchical cluster analysis (HCA) use and allow an overall evaluation of the significant difference between groups and discriminate the polyherbal powder and extract. The authenticated individual herbal, polyherbal pulverized powder and the dry extracts were both shifted through eighty mesh. The samples were subjected to UV-Vis diffuse reflectance spectral detection at the interval of 1 nm. The macroscopic image powders of herbal plants planning for plant identification, was carried out by L*A*B color based image segmentation. Plant powders macroscopic image shown distinct L*A*B color based segmentation for identifying sample. Samples discriminated by first-order derivative preprocessed reflectance spectra on favor of various transitions marker bands. Discrimination of the two classes of remedy was also able in natural grouping by PCA and HCA technique. An analytical method which is rapid, simple and accurate for discriminating two forms of polyherbal formulation using (UV-VIS) diffuse reflectance spectroscopy combined with well-known chemometrics method was developed along with powder image processing.

KeyWords: Chemometrics, Discrimination, Diabetogen, Image processing, UV-VIS reflectance Spectroscopy.

1. Introduction:

Herbal medicine (HM) and their preparations have been used widely for hundreds of years all over the world. Among the characteristics of herbal medicine preparations, is that they are all presented either as single herbs or as a combination of several herbs in composite formulae. Quality control protocols for herbal drugs have constituted much concern and at present it is a real challenge, especially with the development of herbal remedy of herbal formulation. A fingerprint can be demarcated as a characteristic profile reflecting the compound chemical composition when the sample analyzed and can be achieved by spectroscopic, chromatographic or RAPD technique. Fingerprinting profile have to be highlighted by the fundamental designation of 'integrity' and 'fuzziness' (sameness and difference) so as to chemically exemplify the HM has been investigated Welsh.[1] It is recommended that with the help of the chemical fingerprints , the authentication and identification of herbal medicine can be conducted accurately, 'integrity', even if the amount concentrations of characteristic constituents are not exactly same for different samples, hence 'fuzziness'.[2] Therefore, HM extracts should be considered holistically, and the model of using only one or two marker components should not be considered for HM products. However, in any polyherbal powder and its extract, there are hundreds of unknown components, many of them present only in trace amounts. Consequently, to obtain reliable fingerprints that represent pharmacologically active and chemically characteristic components is not an easy or trivial task. [3] Recently increased quality requirements for herbal preparations raise new tasks to the science of pharmacy. Modern requirements are linked to better support of industrial production of traditional based multi-ingredient Phyto-preparations. In recent times the field of Chemometrics and multivariate analysis encompasses the interdisciplinary sciences of mathematics, analysis and chemistry to create results that are precise and specific, which is very essential in the field of medicine and has been widely implemented in the quality control herbal medicine. [4] On the other hand, many pharmaceutical companies have demand the automatic identification of powders from herbal plants. Due to lack of specialists, identification work is very tribal task. Taking

macroscopic image of powders, applying image processing task is successively accurately classified.[5] The objective of this study was to determine non-destructive diffused UV-VIS reflectance on the electromagnetic spectrum ranges from (200-780nm) to authenticate & discriminate herbal & polyherbal natural medicament ; analyze highly absorbing Chromophores, conjugation (electronic transition) on favor of dried, pulverized fine particles & plant dry extract of respective contents which are stem of *Tinospora cordifolia* (Willd), Seed of *Syzygium cumini* (Skeels), Heart wood of *Pterocarpus marsupium* R, Leaf of *Azadirachta indica* A.Juss, Seed of *Trigonella-foenum-graecum* L, Leaf of *Marsdenia sylvestre* R.Br; to distinguish as well as recognize two class of formulation ,to develop qualitative discrimination method in combination chemometrics method and also applying image segmentation tool to create L*A*B space powder image signature. In this way develop a significant relevant library to get monitoring antidiabetic formulation along with Zero –Defect quality control norm.

2. Material & methods

2.1 Chemical

Analytical grade methanol for solvent extraction was obtained from Merck (Darmstadt, Germany).

2.2 Sample preparation

A total of six fresh identified samples, were collected between March and May of 2013, and identified by, V.P.Prasad, Scientist-'C', Botanical Survey of India (Ref. No. CNH/51/2013/GAU/1085 dated: 24/9/2013). Voucher specimens of all samples are kept at the Pharmacognosy Department, Faculty of Pharmacy, and Gujarat Ayurveda University (Table 1). All samples were air dried in the shade and ground into powder, then passed through an 80-mesh stainless steel sieve. The sieved powders were stored in tightly closed containers away from light. Equal amounts of the powdered six individual samples were mixed (Diabetogen) to prepare polyherbal formulation. Each sample (10 g) was soxhalated for 12 h in 100mL of methanol for UV analysis and samples were filtered, transferred to china crucible, dried under temperature controlled water bath.

Table-1. Origin, codes and source of polyherbal samples included in that study

Sample set	Name	Voucher specimen	Origin	Code	Source
Identified samples	*Tinospora cordifolia*	JKM01-GAU	Wild	TCP	W.B. (Kalyani)
	Syzygium cumini	JKM02-GAU	Wild	SCP	W.B. (Jalpaiguri)
	Azadirachta indica	JKM03-GAU	Wild	AIP	W.B. (Kalyani)
	Trigonela foenum –graecum	JKM04-GAU	Cultivated	TFGP	W.B.(Medinipur)
	Gymnema sylvestre	JKM05-GAU	Cultivated	GSP	W.B. (Kolkata)
	Pterocarpus mersupium	JKM06-GAU	Wild	PMP	Odhisha (Baruipada)
	Diabetogen (Polyherbal powder)	GAU1	Powdered	MIXP	Gujarat (Jamnagar)
Extracted samples	Heart leaved moon seed	GAU2	Extracted (Powdered form)	TCE	Gujarat(Jamnagar)
	Black berry	GAU3	Extracted (Powdered form)	SCE	Gujarat (Jamnagar)
	Indian Liac	GAU4	Extracted (Powdered form)	AIE	Gujarat (Jamnagar)
	Fenugreek	GAU5	Extracted (Powdered form)	TFGE	Gujarat (Jamnagar)
	Ram's horn	GAU6	Extracted (Powdered form)	GSE	Gujarat (Jamnagar)
	Indian Kino tree	GAU7	Extracted (Powdered form)	PME	Gujarat (Jamnagar)
	Diabetogen (Polyherbal extract)	GAU8	Extracted (Powdered form)	MIXE	Gujarat (Jamnagar)

2.3 Image processing:

A lab colour space is a colour component space with dimension L for lightness and a and b for the colour-comonent dimensions, based on nonlinearity compressed CIE XYZ colour space coordinates. "Lab" colour space is to create a space which can be computed via simple formulas from the XYZ space, but is more perceptually uniform than XYZ. Perceptually uniform means that a change of the same amount in a colour value should produce a change of about the same visual impotance.When storing colours in limited precision values, this can reproduction of tones. Both Lab spaces are relative to the white point of the XYZ data they were

converted from lab values do not define absolute colours unless the white point is also specified.[6] Our goal is to identify different colours in image by analyzing the L*a*b colour space. The image was acquired using the Image Acquisition Toolbox by Matlab 2016.

2.4 Different channel in lab colour space:

Transform the RGB colour space as a imput image and the template image to lab color space. Different LAB represent the lightness of the color (L^*= 0, yield black and L^*= 100 Indicates diffuse white; specular white may be higher), its position between red/magenta and green (a^*, negative values indicate green while positive values indicate magenta) and its position between yellow and blue (b^*, negative values indicate blue and positive values indicate yellow) coordinate ranges from 0 to 100. The possible range of a^* and b^* coordinates is independent of the color space that is convering from, when it projected in two dimensions X and Y come from RGB the red/green and yellow/blue opponent channels are computed as differences of lightness transformation of cone response. The non-linear relations for L*, a* and b* are intended to mimic the nonlinear response of the eye. Furthermore uniform changes of components in the L*a*b colour space aim to correspond to uniform changes in perceived color. The L*a*b* color space includes all perceivable colours which means to identify different colours in image by analyzing the L*a*b* colour space. [7]

2.5 UV-VIS reflectance spectroscopy measurement

Samples were analyzed using a bench top Perkin Elmer Lambda 19 UV-VIS-NIR spectrophotometer system in range between 200-780 nm in diffused reflectance mode with 1 nm sampling interval and 2.0 nm slit width. The materials powders & dry extract (passed through 80#) both were placed in a closed rotating sample lead sulphide cell cup with scan speed 240 nm/min. The contact probe was placed against 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 obtained reflectance reading of all samples was converted into a data matrix by using Microsoft Excel 2007 with the wavelength as variables represented by columns and the corresponding spectral reflectance measurements of different samples represented by rows. Instrumentation selected standard for the wavelength and absorption calibration validation traceable to NIST. The UV-VIS - NIR spectroscopy study was done NABL accreditated laboratory Sciart at Anand, Gujarat.

2.6 UV-VIS spectral data pre-treatment

Spectral data pre-treatment is an important step before subjecting the UV-VIS reflectance spectra data for multivariate analysis. Here spectral raw reflectance data are converted as absorbance scale applying the well-known equation, Absorbance = log (1/ Reflectance). It is necessary to perform this step in order to minimize the effect of light scattering, baseline variation, noise, etc, in all UV-VIS absorbance spectra of the representative samples.[8] In this study data from three pretreatment methods, namely standard normal variate (SNV), and first and second order derivative spectra, were compared.

2.7 Multivariate analysis

Multivariate methods of data analysis applied to data of chemical interest employing formal logic via mathematical and statistical sense**.** The search for natural groupings among the samples is a preliminary way to study data sets. The spectroscopic parameters were subjected to exploratory data analysis, performed by applying principal component analysis (PCA) using Unscrambler®9.7 (CAMO SA, 108 Oslo, Norway) and hierarchical cluster analysis (HCA) using Hierarchical Clustering in Matlab, 2015 version.

3. Result and Discussion

3.1 Image processing L*A*B scale:

Image segmentation is a set of segments that collectively cover the entire image and set of contours extracted from the raw image. Each of the pixels in a region is similar with respect to some characteristic or computed property such as color, intensity and texture. The most important attributes of the L*a*b* model is the device where raw powder of individual drug and preprocessed image is tabulated in Fig-1 and Fig-2.In that way the real time color documentation was stored as a database of the diabetogen formulation .

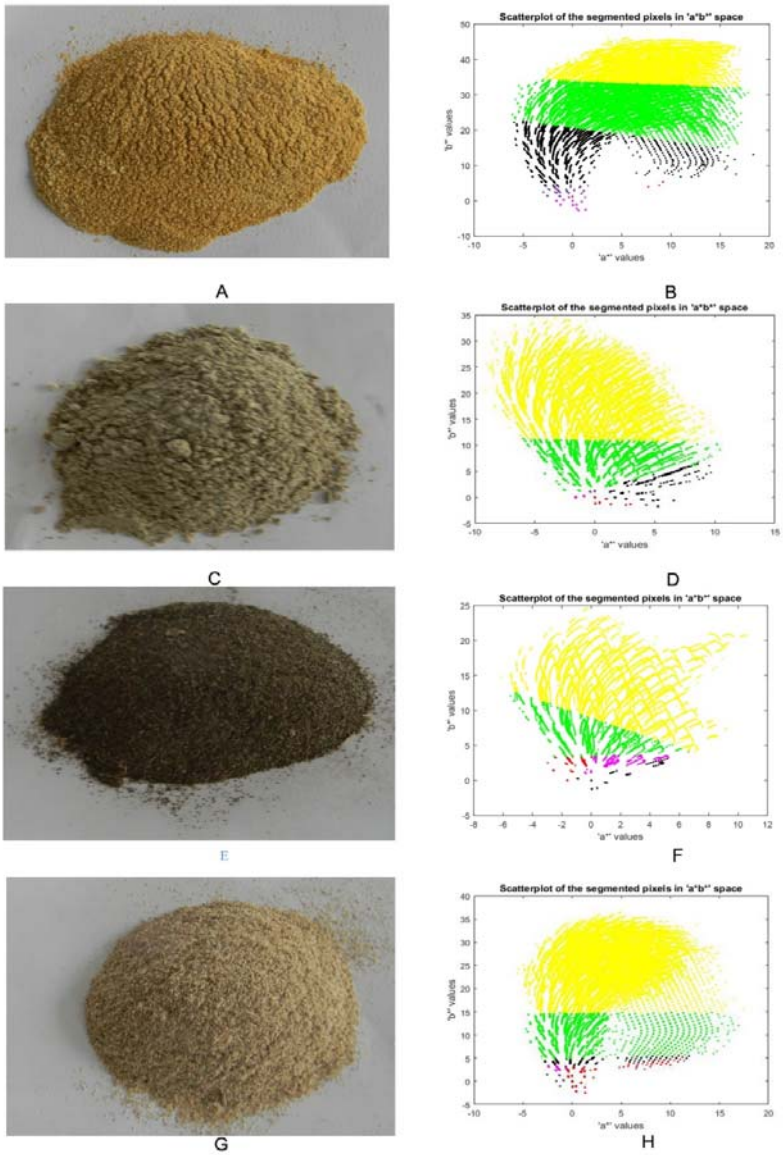


Fig-1. Representative different image of Fenugreek, Heart-leaved moon seed, Indian liac and Indian kino using image acquisition tool box (1) A,C,E,G raw powder images and (2) B,D,F,H as L*a*b colour space converted images.

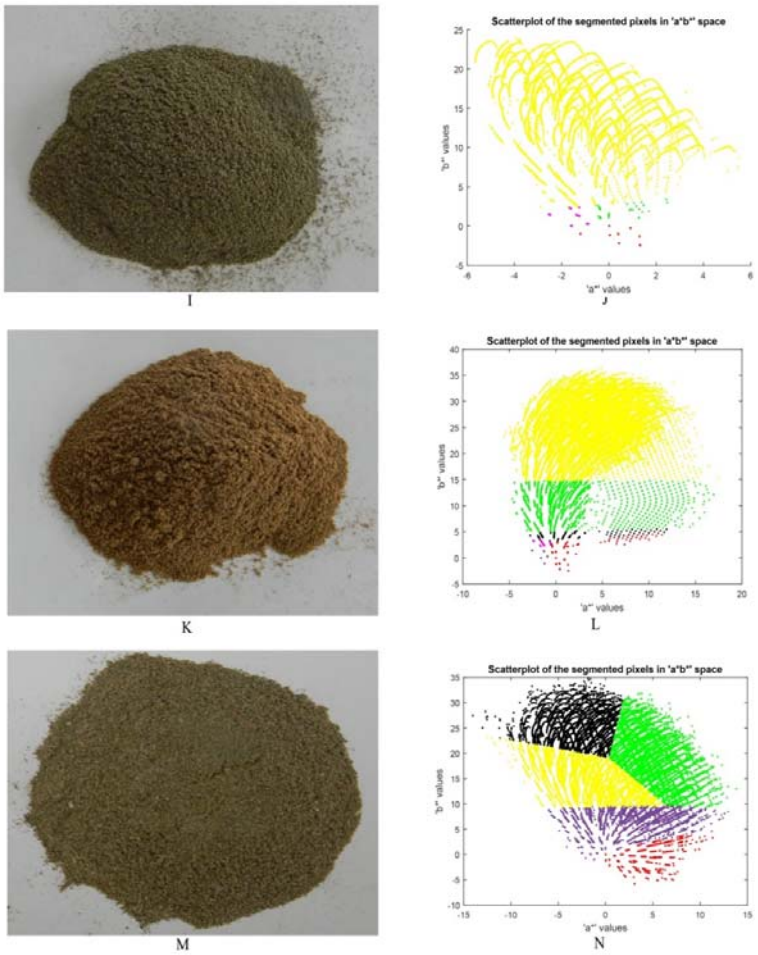


Fig-2. Representative different image of Ram's horn, Black berry and Diabetogen using image acquisition tool box (1) I,K,M raw powder images and (2) J, L, N as L*a*b colour space converted images.

3.2 UV-VIS Reflectance spectroscopy

Medicinal plants are a complex mixture of chemicals, so their UV-VIS reflectance spectra will show overlapping of some characteristic absorption bands maxima (Chromophores, functional group, conjugation, double bond extended conjugation etc.) in the table-2. [9]. On the other hand, this study reflected the excitation of Sigma and Pi electron in sense of UV-VIS sensitive functional group as holistic molecular socialism in favor of understanding of the interaction of the electromagnetic radiation. The UV-VIS reflectance spectra of respective samples Indian Kino (Ht.wd.), Indian Liac (Lf.), Ram's horn (Lf.), Fenugreek (Sd.), black berry (Sd.) and heart-leaved moon seed (St.) are shown in Fig-3(A) under the same experimental conditions, the UV-VIS reflectance spectra of each plant along with polyherbal formulation. The characteristics peaks in the UV-VIS spectra of the two type's class antidiabetic formulation showed moderate similarity. As can be seen in Fig.3 (B), the characteristic peaks in the UV-VIS of the respective samples as well as polyherbal powder and extract appeared at 280-300 nm corresponds to CHO absorption ,at 252 nm to anthracene (180,204,255nm) are assigned to aromatic skeletal band transition. And also relative intensity differences some chromophore containing short UV sensitive (CHO, ONO, absorption) and visible region both powder and extract in reflectance concern. Comparison of the UV-VIS spectra of both classes of medicament showed slight differences because they are from the same materials that may have similar components.

Table-2.Tentative list of UV-VIS transition guide sensitive compound alignment of registered wavelength [9, 12]

Chromophores	Transition	Structure	λ(nm)
Aldehyde	n-π*, π-π*	CHO	280-300
Amine	π-π*	NH2	145
Bromide	n-□*	Br	208
Carbonyl	n-π*	C=O	195,270-281
Disulfide	n-□*	S-S	194, 255
Ester	n-π*	COOR	205
Ether	n-π*	-O-	185
Nitrile	n-π*	-ONO	220-300
Oxime	n-□*	-NOH	190
Thio ether	n-π*,n-□*	-S	194
alkyne	π-π*,n-π*	-C=C,(C=C)2,(C=C)3	190,210-230, 260
Carbonyle	π-π*, n-π*	-C=C-C=O	210-250
Benzene	π-π*,n-π*	Aromatic ring	184,204,255
Diphenyl	π-π*	Link Aromatic	246
Anthracene	π-π*,n-π*	fused aromatic ring	252

3.3 Combination of UV-VIS reflectance spectra and chemometrics method for discrimination of polyherbal diabetogen powder & extract

To confirm the results obtained from the visual inspection of UV-VIS spectra for discrimination of polyherbal powder & extract antidiabetic formulation, a combination of UV-VIS spectra and chemometrics method was used. Chemometrics is widely used to analyze a huge amount of data such as in UV-VIS reflectance spectra with an aim to resume information contained in the data matrix by reducing dimension of the data, and finding the similarities or dissimilarities between observations and variable. The Pre –treatment of UV-VIS reflectance spectra is a standard procedure before using the spectra in chemometric analysis. SNV and first and second order derivative spectra were applied and compared. SNV mechanisms mainly to correct differences in baseline offset and path length due to differences in particle size distributions in UV-VIS reflectance spectra of powdered samples [8].In SNV transform the mean of each spectrum is subtracted and the length is normalized to 1. The first and second order derivative accustomed to eliminate the baseline offset and for the heightening of small spectral feature. [9]. In this work, pre-treatment of the UV-VIS reflectance spectra using gave the best result for optimum group's separation for the two class of antidiabetic polyherbal formulation. In this case, SNV was selected to normalize the UV-VIS spectra before subjecting the spectra to PCA and HCA. Here 1^{ST} order derivative was selected for pre-processing the UV-VIS spectra before subjecting PCA other than SNV & 2^{nd} order derivative for better discrimination. Enhancement of the spectral resolution and amplification of small difference in the ordinary spectra could be obtained using 1^{st} derivative derivative spectra; some overlapping bands could be resolved. Fig. 3 (C) shows the first order derivative spectra of the samples in the region 550-570nm, 600-650 nm and it is seen that there are relative intensity differences in the spectra features. As illustrated in Fig 3 (C), it was clearly observed the typical positive and negative peak. A reasonable corollary is that a close similarity of molecular structure may lead to a similarity in the allowed energy levels.

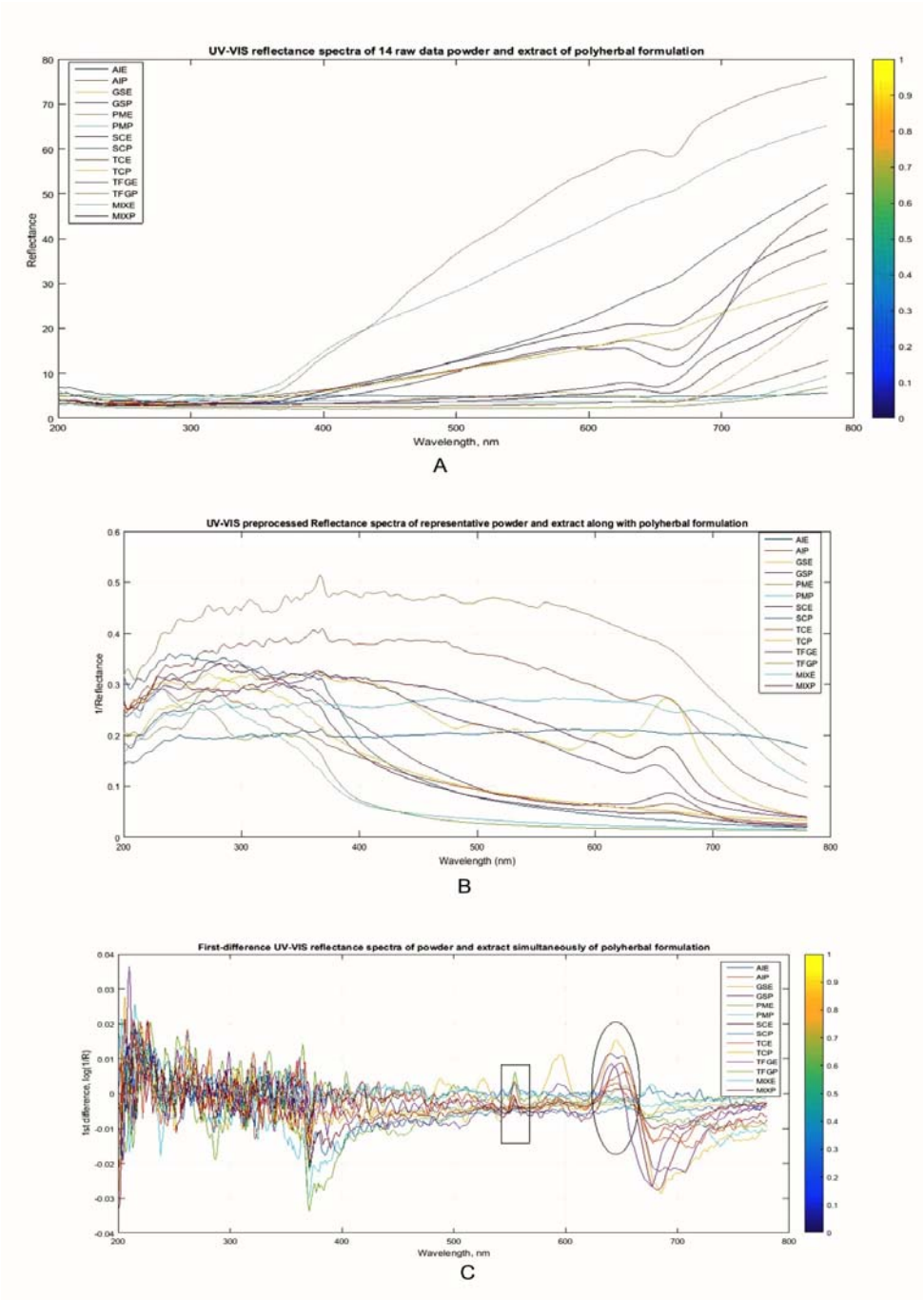
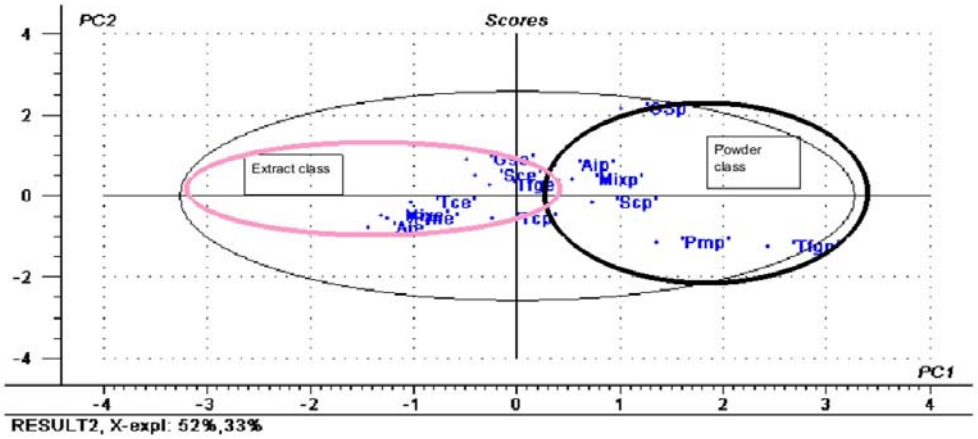


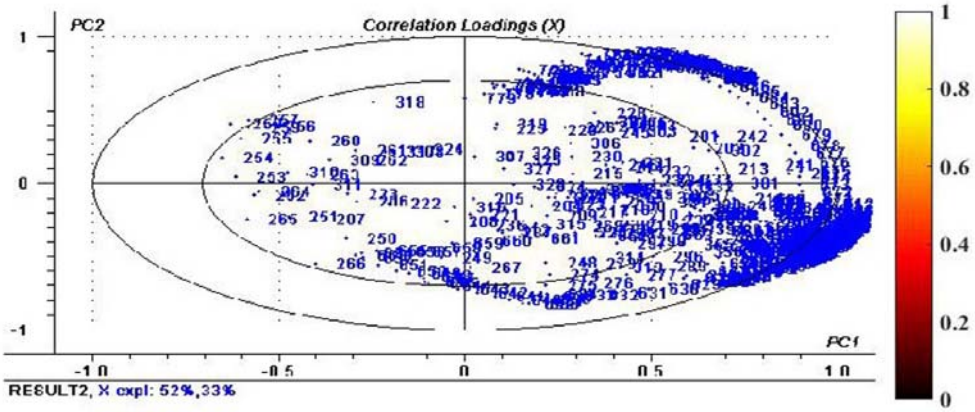
Fig 3. Raw (A), Preprocessed (B) and First derivative(C) UV-VIS reflectance spectra of polyherbal powder & extract.

3.4 Principal Component Analysis

PCA is well-known unsupervised pattern recognition. The main objective of the PCA is to reduce the dimension of the data and extract the information in order to find a combination of variables for describing major trends in their values and emphasize the similarities and differences between samples on a score plot a data set. PCA will transform the original variables into new uncorrelated variables called principal components (PC_s) that maximize the explained variance in the data on each successive component under the constraint of being orthogonal to the previous PC_S. [10] In this study, PCA was employed to discriminate the samples according to the two class of medicament based on the UV-VIS spectra in the region of 200-800nm. This region was selected because it is complex and full of information with various electronic transition attributed to the chemical components in all samples. The full 14 objects x 450 variables data matrices were submitted to PCA. The score plot in Fig.3 showed that the powder samples were grouped together in the (GSp, Scp, AIp, Mixp) upper quadrant of the score plot, though remaining samples (Tcp, Pmp, Tfgp) appear below the horizontal line of score plot.



A



B

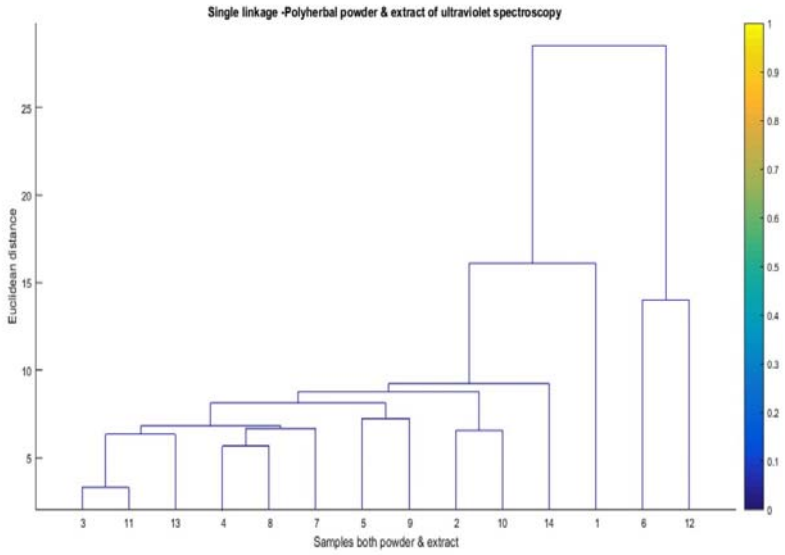
Fig-4. Score (A) and loading (B) plot of both powder & extract classes of samples based on its UV-VIS reflectance data showing the distribution of samples. The ellipse represents the Hotelling T2 with 95% confidence in score plot.

On the other hand the score plot in Fig.4(A) showed the extract samples were naturally grouped together in the (GSe, Tfge and Sce) upper left quadrant of the score plot through remaining Tce, MIXe, AIe & Pme) below the horizontal line of score plot. In that way the both class of material are sucessivefully discriminated. From the loading plot in Fig.4 (B), it appeared the correlation for the variables to the principal components. Center position variable was less informative due to consider average data set. Variables (253-262nm); (309-312nm); (318-324nm) closely correlated and show similarities among the samples.Anticorrelated with variable (630-638nm) show dissimilarity. Above variable mainly indicative of conjugated or aromatic system of absorption bands of both powders and dry extracts presented are due to the presence of different Chromophores intensity, usually exemplified in the presence of aromatic components as phenolic and flavonoids as well as various conjugated system.[11]

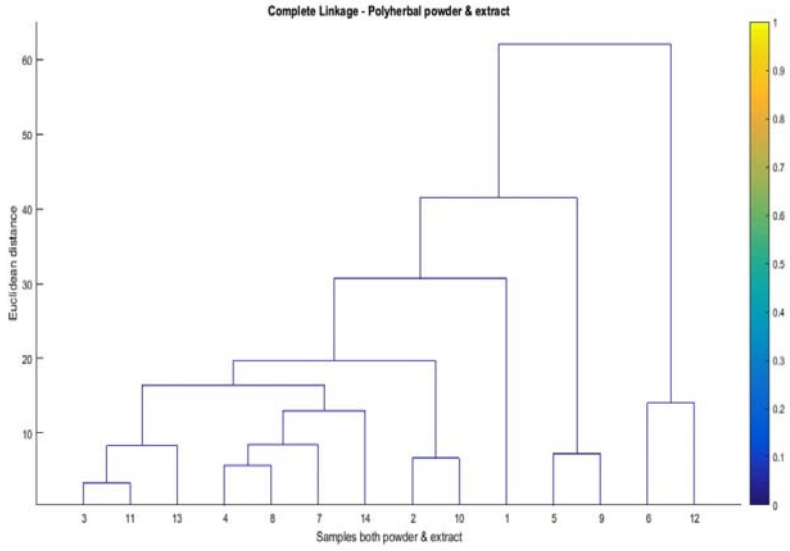
3.5 HCA analysis

Hierarchical cluster analysis (HCA) was performed to authenticity of two classes of material samples on the basis of the similarities of UV-VIS spectral reflectance. Fig-5 (A) Single linkage favors the formation of large linear cluster instead of the usual spherical & elliptical clusters.Fig-5(B) complete linkage favors the small

spherical cluster. Both hierarchical trees showed of the similarity chemical profile of the two classes of materials. Finding suggest resembles of grouping as found by PCA calculation in respect to 3, 11, 13 extract as well as the 4, 8 powder samples. On other way both methods generate the same number of cluster. All samples were aggregated into two clusters with the similarity level of 95 %.On the other way two multivariate methods are complement each other clustering offer formal classification while the visualization gives clear with PCA.



A



B

Fig-5. Dendogram of Diabetogen formulation using single linkage (A) and complete linkage (B) with Euclidean distance similarity measure .3(GSE), 11(TFGE), 13(Dt.P); 4(GSP),8(SCP),7(SCE),14(Dt.E); 2(AIP), 10(TCP): 1(AIE);5(PME),9 (TCE);6(PMP) ,12(TFGP).

4. Conclusion

The chemo (bio) analyze of two class of diabetogen formulation (powder & extract) was successfully assessed by using a typical secondary metabolic platform involving spectroscopic technique (UV-VIS) chemometrics. The analytical approach described showed to be suitable pharmaceutical industries regarding the usage and quality control process of that raw materials. The two class of materials evaluate to their potential usage in

pharmaceutical industry, taking into indirectly secondary metabolite constituent's e,g. conjugated property. Such comprehensive analytical approach can conceive an intuitive resolution to evaluate natural products from holistic perspective. Identification of raw powder its colour, texture etc and L*a*b preprocessed image is help to build a database of respective formulation by image processing. Discrimination of polyherbal powder and extract was achieved by UV-VIS reflectance spectral analysis in combination with PCA, HCA and visual analysis of the raw and 1st order derivative UV-VIS reflectance spectra. In that way chemometrics tools are superbly useful in monitoring of changes formulation make up and equally be exploited to monitor metabolic divergence or unexpected chemical signature of the two class of formulation.

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