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Original Article

DISCRIMINANT ANALYSIS OF PURIFICATION ON TURMERIC (*CURCUMA LONGA* LINN) SAMPLES BASED ON PHYTO-PHARMACOGNOSTICAL AND MULTIVARIATE CHEMOMETRIC TECHNIQUE

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ABSTRACT

Objective: To establish an acceptable identification system of various purification effects in context classical based different media on turmeric samples and relates its altering pattern in favor of phyto-pharmacognostical, image processing and multivariate chemometric analysis.

Methods: Authenticated turmeric samples purified through different processes by using different media such as cow's urine, panchapllava (five different plants tender leaves), the inflorescence of alambusha (*Sphaeranthus indicus*, Linn) decoction, water and buttermilk. Resultant samples dried, pulverized and undertaken powder microscopy, image processing, physicochemical and chromatographic fingerprinting (HPTLC). The multivariate chemometric analysis, principal component analysis (PCA) analyzed with help of Unscrambler and image processing in Matlab software.

Results: The addition of characters of medias drug with turmeric powders like the crystal of gomutra, pollen grain and starch grain of Alambusha, epidermis, fibre, the crystal of panchapllava. Identify different perceivable colors in variously processed turmeric by analyzing the Lab color space through the Image segmentation. PC_1 and PC_2 explained (90 + 9) % total variance in score plot of respective purify turmeric samples shown clear grouping in relation to the physicochemical constant. Quantification of curcumin in various treated turmeric samples displayed variation due to additive effect in high-performance thin layer chromatographic profile.

Conclusion: This study proved that purification in ayurveda not only refers to the elimination of toxins and unwanted particles but also the transformation in the properties in the primary substance rendering it safe as well as many desired qualities are imbibed in it.

Keywords: Curcumin, Discrimination, HPTLC, Image Processing, Powder-Microscopy, Purification, Turmeric

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INTRODUCTION

Turmeric (*Curcuma longa* Linn) belongs to the family Zingiberaceae, is also referred to the Ayurvedic system of medicine [1]. It has good therapeutic effects like antioxidant, anti-inflammatory, antitumoral, anti-fertile, antidiabetic, antibacterial, antimicrobial [2,3]. Ayurveda advocates purification procedures (Shodhana) for poisonous substances to render it safe and effective for therapeutics. Ayurveda classics also mentioned non-poisonous substance like turmeric internal administration after proper purification [4]. Applying various media on turmeric purification is sometimes the bottle-neck problem to pick up best reasonable and reliable media. Previously comparative physicochemical profile of Gunja seeds was processed through water and nimbu swarasa [5] are successfully done. Effect of sodhan on (processing) on kupeelu (*Strychnos nuxvomica* Linn.) with special reference to strychnine and brucine content is also explored [6].

However non-poisonous drug turmeric also recommended passing through specific purification process in the traditional system. Recently image processing technique has been applied increasingly for food quality evaluation. Generally speaking, color images are captured by a color camera and saved in the three-dimensional RGB (red, green and blue) color space. Color space transformation is a powerful tool for color feature extraction L*a*b space [7]. Ayurveda has more attached to holistic belief drug act as a whole [8] for supporting intact quality control of herbal medicine. As per chemical point of view adding materials with purified drugs is nothing but impurities (reductionist approach) but Ayurvedic argument therapeutically point of view may prove useful in potentiating therapeutic effect (synergistic effect) and neutralize the toxic effect (antagonistic approach). In another context fingerprinting profile have to be highlighted the fundamental designation of 'integrity' and 'fuzziness' (similarity and dissimilarity) [9]. In that circumstances the present study designed with minimum distortion of ayurvedic logic, applying the multivariate chemometric technique (principal component analysis) to visualize the physicochemical pattern of respective media treated turmeric samples. As a part of a long-term research effort aimed at inaugurating a workable identification system in few media treated turmeric samples. The paper presents a detailed study on the phyto-pharmacognostical comparison of examined turmeric samples in favor powder microscopical, image processing, and chromatographical fingerprinting. The objective of the study was to determine physicochemical, pharmacognostic and chromatographical pattern of previously authentic sodhan turmeric medicament, analyse preliminary image processing in favor of dried pulverized fine powder of respective various media treated turmeric samples, which are cow's urine, panchapllava (Five different plants tender leaves), inflorescence of alambusha (*Sphaeranthus indicus*, Linn) decoction, and water and buttermilk.; determine distinguishing feature microscopically and compute principal component analysis in physicochemical data; establish the library which significantly influences real-time quality monitoring and discuss qualitative discrimination identification tool to select best purification media of turmeric samples along with curcumin quantification. The present study revealed the impact of sodhan on turmeric through various media.

MATERIALS AND METHODS

Plant material

Mother rhizomes of Turmeric (*Curcuma longa* Linn.) were collected from Sasoi Botanical Garden, Sasoi village, Gujarat (Latitude 22°18'30.06"N, Longitude 69°58'55.11"E) in the month of December 2016, fresh leaves *Panchapallava* (five different plant's Tender leaves) *Trichoanthes dioica*, *Azadirachta indica*, *Syzygium cumini*, *Mangifera indica* were collected from Gujarat Ayurved University and Kapita*, (Feronia limonia)* from Bhavnagar. Alambusa, *Sphaeranthus indicus* Linn. dried head of flowers was purchased from Ayu medicines, Jamnagar and authenticated (Voucher number Phm-6219/17-18) by H. O. D. of Pharmacognosy Harisha CR. A specimen of each drug has been submitted to Pharmacognosy laboratory for further references.

Chemical

All chemicals used in the study and for extraction were of analytical grade. Curcumin was purchased from Alfa Aesar (95% purity) from turmeric rhizome (Batch number- B21573, lot: 10189524) England.

Standard operating procedure of turmeric sodhan (test sample)

Sample (TT)

Turmeric mother rhizomes were treated with butter-milk (takra) by dipping (nimajjana) them in takra for 10 days [10], where takra [11] was prepared with 1/4th amount of water), sample (TW): turmeric mother rhizomes treated with water (by boiling (*Swedana*) of turmeric mother rhizome in water for 3 h.) [12] sample (TGM): turmeric mother rhizome boiling in water for 3 hrs. then followed by steaming with fresh cow's urine for 15 min. sample (TZ): turmeric mother rhizome boiling in fresh gomutra (cow's urine) (1h) followed by boiling in panchapallava (decoction*)* kwatha (1h), alambusa kwatha (1h) and finally steaming in fresh gomutra (15 minutes)[13, 14], sample (TR): raw turmeric mother rhizome without any treatment. These all coded samples dried in sunlight, subsequently pulverized and passed through a sieve (#60) and packed in the well-closed container.

Pharamacognostical study (Powder microscopy)

Slides prepared with help of water, chloral hydrate as a clearing agent, stained with $FeCl_3$ Iodine etc, to detect chemicals like tannin, starch, lignin etc. Microphotographs were taken under a carl-zeiss trinocular microscope attached with camera [15]. Powders of all samples (turmeric) was studied under a microscope for proper identification by the standard operating procedure given by Quality standard of Indian medicinal plants [16].

Image processing

A lab color space is a color component space with dimension L for lightness a* and b*, based on nonlinearity compressed CIE XYZ color space coordinates. The original images of respective samples captured in RGB color space. Only $a^*$and b^* component of L. a. b were used for color feature extraction make the system more illumination independent. The image was acquired using the Image Acquisition Toolbox by Matlab 2016. In brief L. a. b represent the lightness of the color (L*= 0, yield black and L*= 100 indicates diffuse white, * negative values indicate green while positive values indicate magenta, b* negative values indicate blue and positive values indicate yellow). The nonlinear relations for L*, a* and b* are intended to mimic the nonlinear response of the eye.

HPTLC study

Test samples phytochemical screening

Preliminary phytochemical studies were carried out using 5 g powder material of raw and shodhita samples of Turmeric by Maceration procedure [17] with HPLC grade alcohol. The HPTLC separation was performed on pre coated aluminium HPTLC plates of 0.2 mm layer thickness with silica gel $60F_{254}$ using toluene-chloroform-methanol (5:4:1, v/v/v) as a mobile phase [18]. Densitometric analysis was performed at 430 nm.

This system was found to have the compact spot of curcumin at R_f value of 0.34. Band wise application, 6 tracks, band length 8 mm, track distance 11.4 mm, distance from left edge 20 mm, distance from lower edge 8 mm, application volume 2.5 µL. A stock solution of curcumin (500 µg/ml) was prepared in methanol. Dilutions were made in methanol as C0=blank (methanol), C1=30, C2=40, C3=50, C4=100, C5=200 (µg/ml) and were spotted on HPTLC plate. Turmeric methanolic extracts (raw and purified) with curcumin (standard) and the plate was observed under visible light, UV254 nm (short wavelength) and UV366 nm (long wavelength).

Physicochemical constants

Physicochemical constants, such as pH [19], the percentage of total ash (TA) [20], acid-insoluble ash (AIA) [21], water (WSE) [22] and alcohol (MSE) [15] soluble extractives were calculated as per the Ayurvedic pharmacopoeia of India.

Data analysis

Physicochemical data were manipulated into two forms: 1) Data were exported as 2D ASCII files at all physicochemical constant value (pH, WSE, MSE, TA, AIA) of five samples matrix contain (5 × 5), i. e., physicochemical constant are one direction and samples in other direction; 2) Data were exported as ASCII files to reduce in a single profile by five coded samples giving a matrix with 25 points). All data operations (PCA) were performed using Unscrambler and on a computer Intel Pentium 4 processor containing 500 MB RAM and running Microsoft Windows seven.

RESULTS AND DISCUSSION

Pharmacognostical analysis

Photomicrographs (a) and (b) at 40X showed powder characters of raw Turmeric and different characters of purified samples. TR (raw) showed general characters of Turmeric powder (fig. 1.01 to fig. 1.16), TT showed almost similar except fibre attached with starch gain (fig. 2.4). TW also showed similar characters except for the decrease in the size of oleoresin. TGM showed the character of media cow's urine as Calcium oxalate crystal (fig. 4.5) and fibre attached with calcium oxalate crystal (fig. 4.6). Characters of media can be seen in TZ also, fig. 5.07 showed the character of cow's urine, fig. 5.08 and fig. 5.09 showed characters of alambusha and fig. 5.10 to 5.16 showed characters of C decoction.

It seems, Shodhana act as synergic and antagonist due decrease in the characters of Turmeric and addition of the characters of media. Shape and size variation can be seen in the starch grain and oleoresin after the process that directly shows the effect of media and process. Scalariform vessel of TGM and annular vessel of TZ got burst due to boiling (heat treatment). Media characters incorporated with the components of Turmeric in TGM as the crystal of cow's urine attached with the fibre and same in TZ with the characters of Alambusha and Panchapallava shows the addition of extra chemical material those may alter the therapeutic efficacy also.

Image analysis

Color and texture are critical factors in human visual perception. Segmentation approaches use both factor to the homogenous region for segmentation. 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. L*a*b* model is developed to measure color differences consistently with the perceived color differences [fig. c]. In this way, this technique is fulfilled automatic identification system with-out help of lack of subject specialist. In this way, identify different colors in the image by analyzing the image color space to build reference database for various turmeric purificatory environment.

HPTLC quantification

In the HPTLC chromatogram, the VIS spectrum of standard curcumin various concentration level (R_f -0.34) at 430 nm [fig. d], and the calibration curve of curcumin were established in the range of (30-200µg/ml) spot versus average area of the peak [fig. e].

Curcumin estimation by regression method

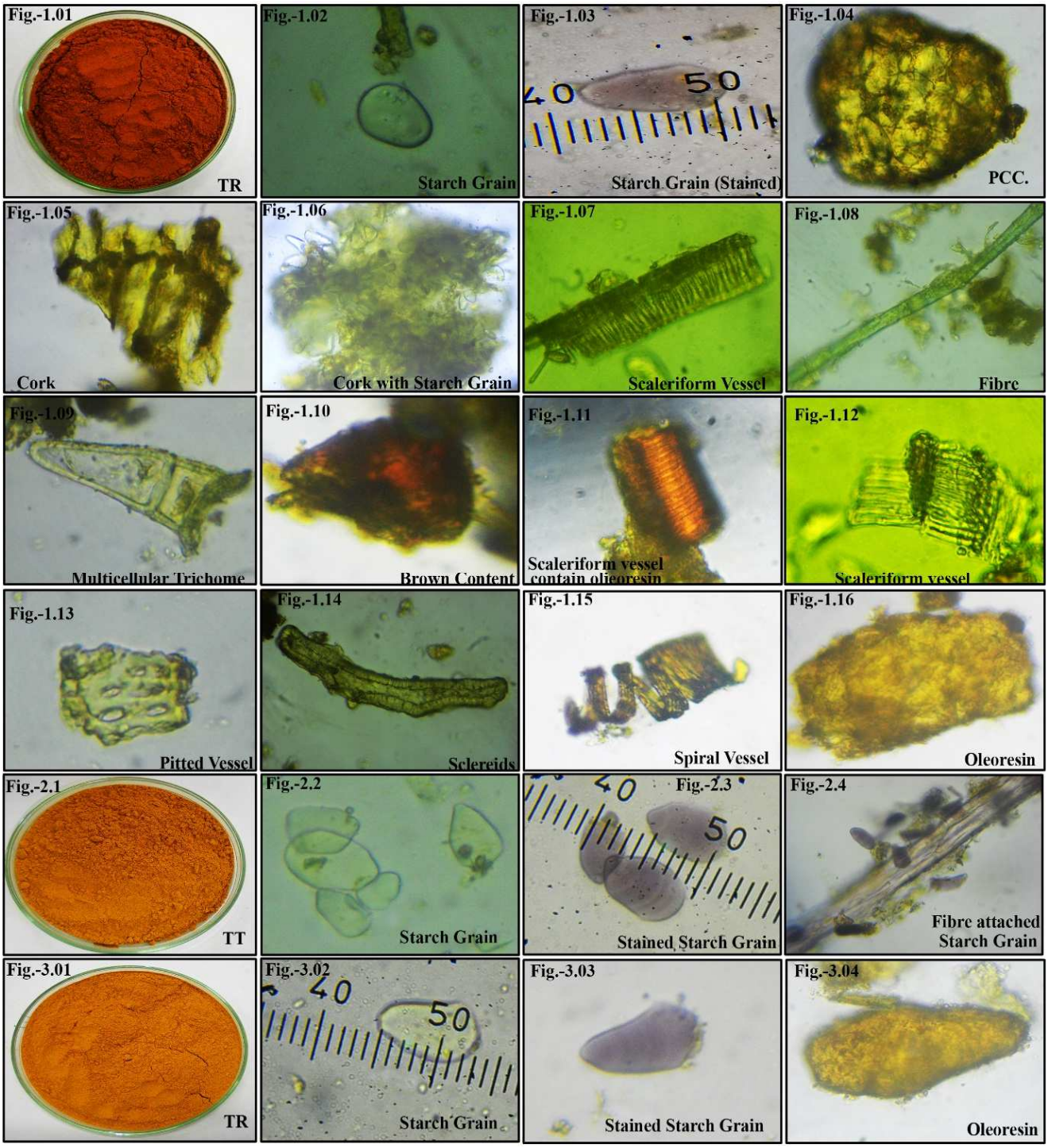
In statistical modeling, regression analysis is a statistical process for estimating the relationships among variables. It includes many techniques for modeling and analyzing several variables when the focus is on the relationship between a dependent variable and one or more independent variables (or 'predictors'). More specifically, regression analysis helps one understand how the typical value of the dependent variable (or 'criterion variable') changes when any one of the independent variables is varied, while the other independent variables are held fixed [23]. The regression equation from the Calibration curve is used for estimation of curcumin (fig. f) $y = 40.899x + 470.22$ with R^2 =

0.9987. *x*-axis simply represents concentration while the *y*-axis represents the area of retained curcumin in the calibration curve. Area of the maximum R_f 0.34 in all the samples Track were noted down. These areas were placed as "y" in above regression equation and arrange it.

$$x = \frac{(y - 470.22)}{40.899}$$

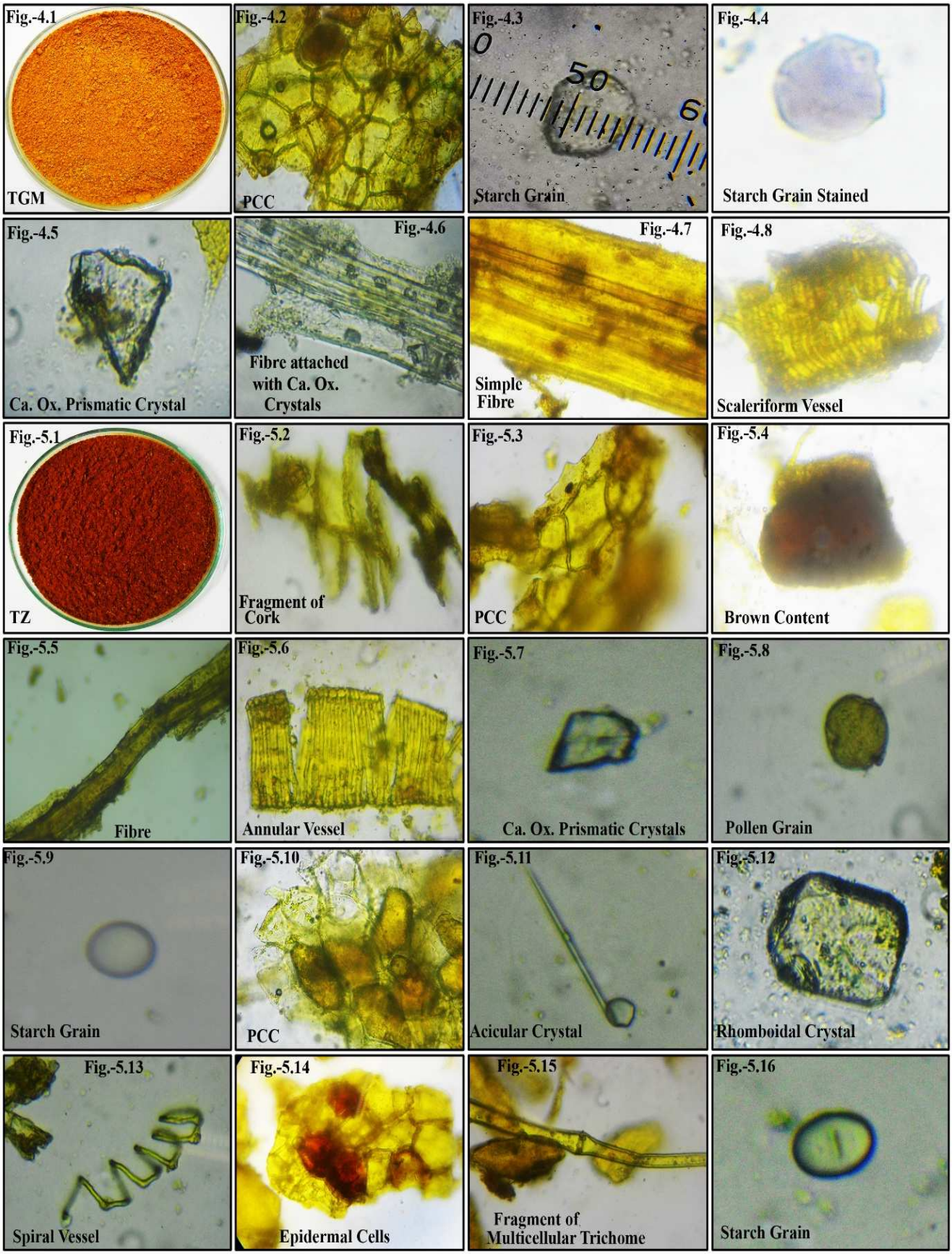
The amount of curcumin in the raw and purified samples was computed from the calibration curves, which suggests that the highest reduction of curcumin was found to be takra treated sample. It might be due to the fact that prolonged contact of the turmeric rhizome with takra (acidic medium) not only helped to extracted out some quantity curcumin [(1,7–bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione–a yellow pigment] [24] but also converted di-hydroxy curcumin, and so on.

The curcumin aromatic ring systems are consisted polyphenol are connected by two α, β unsaturated carbonyl group which is good Michael acceptor and undergo nucleophilic addition [25] along with its hydrophobic nature, the water treated methanolic extract is shown high-level curcumin.



Note: PCC stands for parenchymatous cells

Fig. a: Photomicrograph of powder pharmacognostical characters observed at 40x lens



Note: Ca. Ox. stands for calcium oxalate crystal and PCC stands for Parenchymatous cells.

Fig. b: Photomicrograph of powder pharmacognostical characters observed at 40x lens

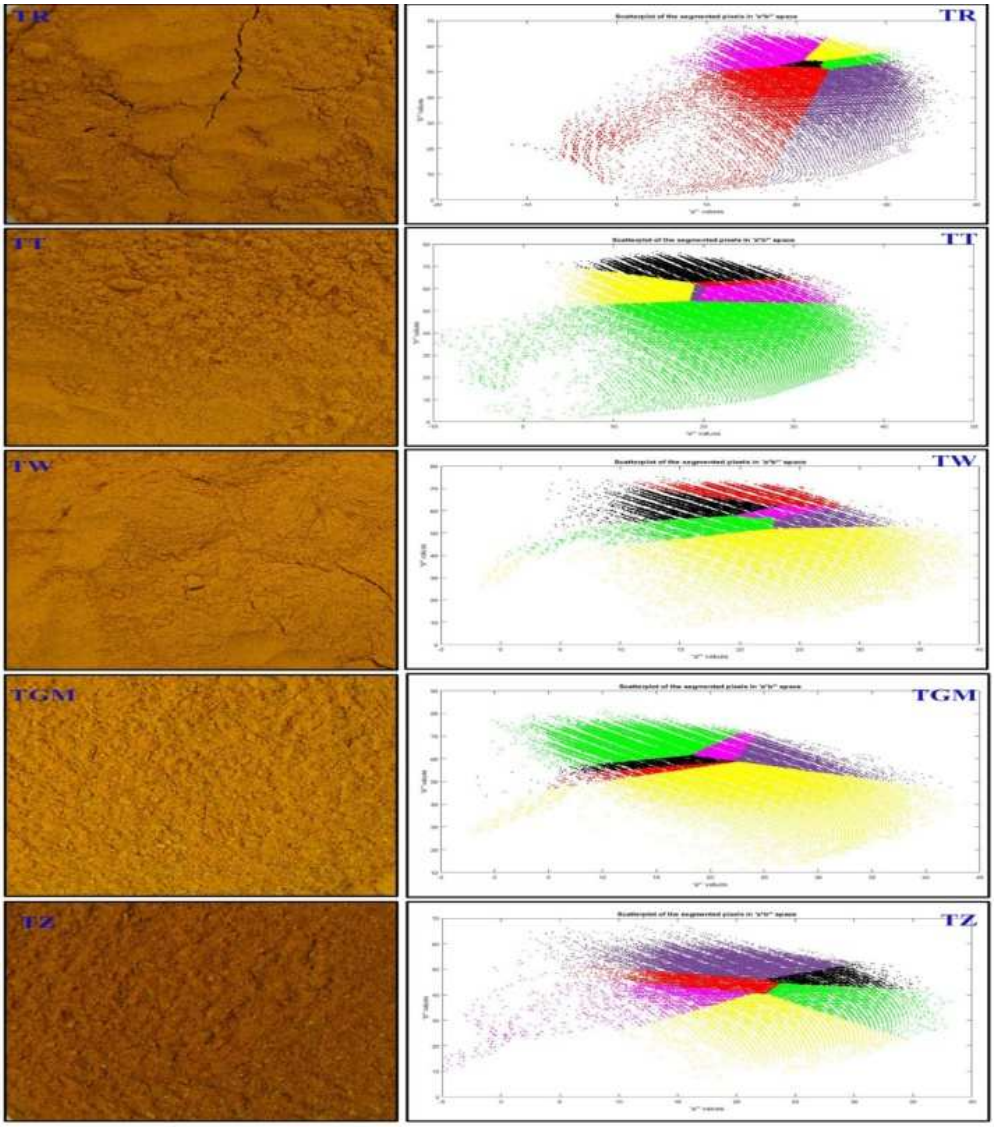


Fig. c: Representative different image of coded samples raw image and color space converted image

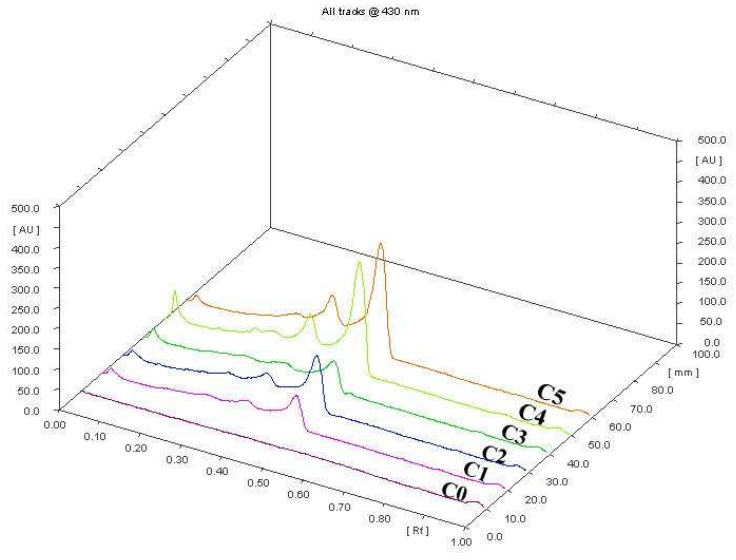


Fig. d: 3D densitogram of curcumin standard with the blank, Calibration curve was described by the regression equation, y = 40.899x + 470.22 with R^2 = 0.9987

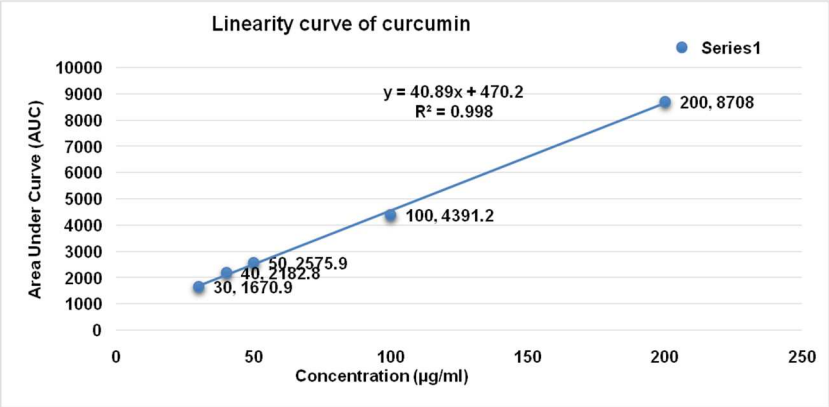


Fig. e: Calibration curve curcumin at 430 nm

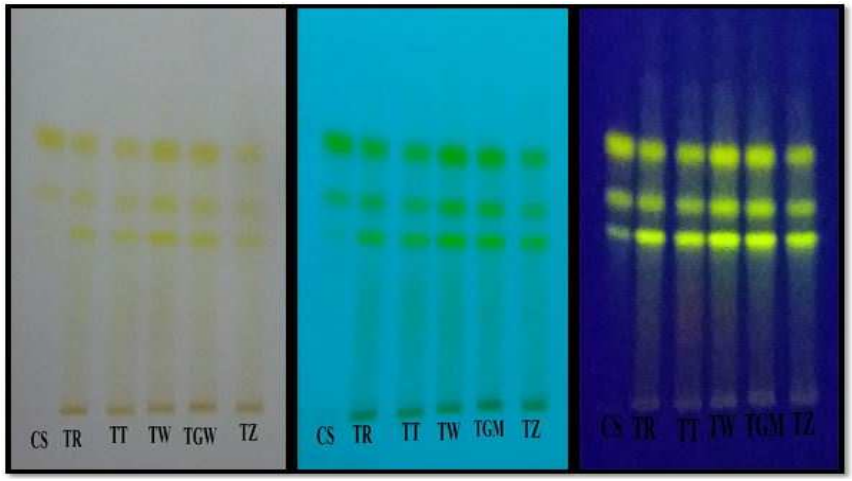


Fig. f: Developed plate under daylight, UV 254 nm, 366 nm with standard, different purified samples

Table 1: Curcumin present in different treated turmeric powders

Samples	Conc^{n}. Prepared (mg/ml)	CD (µg)	% of C. in extract (mg/100 mg)	M. E. Value (%)	Powder of drug (gm)	M. Extract (gm)	EC. in powder drug (µg)
TR	10.1	57.2576	0.5669	10.639	5.142	0.547	3100.98
TT	10.3	56.9079	0.5525	8.446	5.131	0.433	2392.34
TW	10.9	76.8302	0.7048	8.877	5.172	0.459	3235.32
TGM	10.6	61.9371	0.5843	10.442	5.146	0.537	3137.75
TZ	10.1	49.1474	0.4866	9.411	5.021	0.472	2296.78

Note: Conc^{n}.= Concentration, CD = Curcumin Determined, C.= Curcumin, M. E. = methanolic extractive, M. =Methanolic, EC = estimated curcumin

PCA with physicho-chemical analysis

Table 2: Physicochemical constant result expressed as % w/w except pH (n=5; ±SD)

Sam.	pH	WSE	MSE	TA	AIA
TR	4.982	20.648	10.627	8.054	0.492
	±0.008	±0.326	±0.109	±0.149	±0.022
TT	4.964	15.797	8.458	7.994	0.716
	±0.005	±0.147	±0.049	±0.072	±0.042
TW	5.022	14.416	8.886	7.493	0.611
	±0.010	±0.092	±0.043	±0.195	±0.022
TGM	5.452	13.277	10.410	7.378	0.623
	±0.016	±0.171	±0.052	±0.235	±0.037
TZ	5.46	16.450	9.509	7.678	0.784
	±0.023	±0.056	±0.302	±0.178	±0.033

Note: n stands for number of experiments and ±SD stands for Standard deviation

The results of physicochemical properties of the five coded samples are depicted in table 2. Principal component analysis (PCA) was executed to deliver the physicochemical (thermal and solubility categorical variable) data in a reduced dimension, covering the maximum amount information present in the data in order to show the possible trends in their values and emphasize the similarities and differences between samples on a score plot. The score plot in (fig. g) indicated cow urine treated turmeric (TGM) was grouped together in the upper left quadrant of the score plot. The samples (TT, TW, TZ) cluster indicating their similarity, and TGM, TR dissimilarity due to the position of the respective coordinate. From the loading plot in (fig. h) it appeared that the pH and WSE were the physicochemical parameters contributing to the grouping of turmeric samples and that these attributes corresponded to the PC1 which explained about 90% of the total variance. It should be noted that TGM and TZ samples are differentiated from other samples by their higher pH and water soluble extractive value (WSE) as well as lower total ash (TA) content.

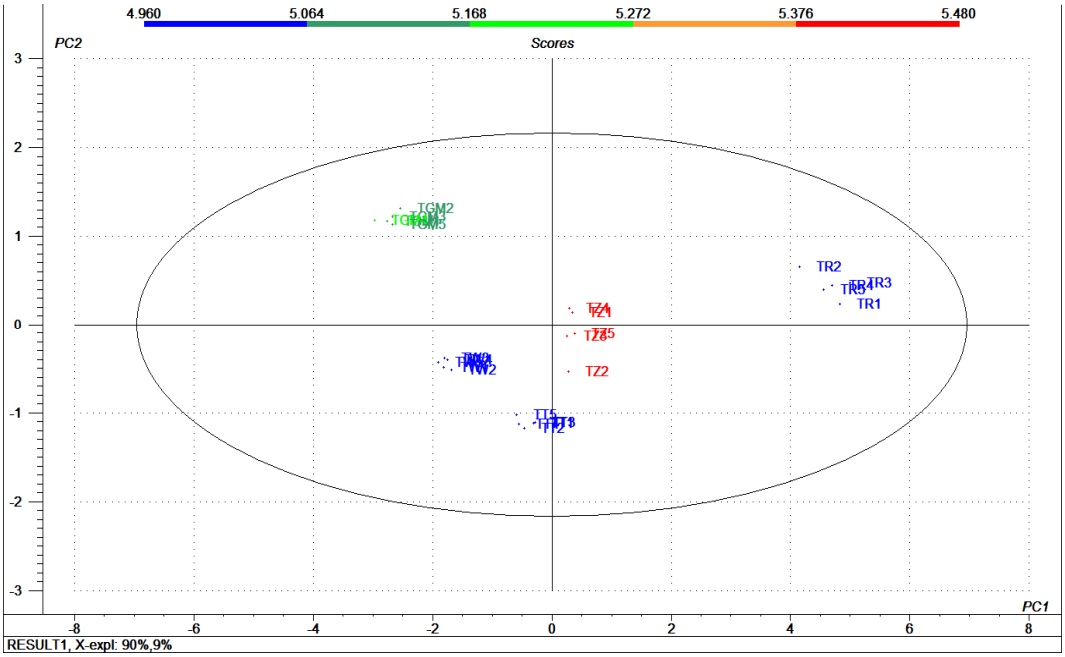


Fig. g: PCA score plot of various coded samples based on its physicochemical data showing the distribution pattern, the ellipse represents the Hotelling T2 with 95 % confidence in score plot

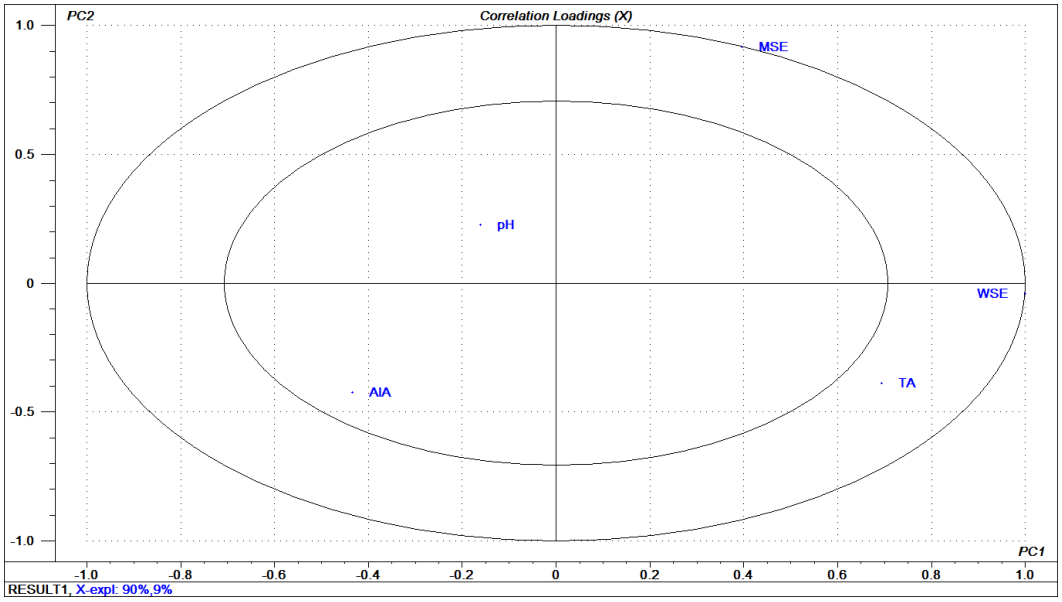


Fig. h: Loading plot showing the parameters contributing to the grouping of the samples

CONCLUSION

This study proved the discrimination pattern of various purified turmeric samples with help of phyto-pharmacognostical, image processing, multivariate chemometric technique (PCA) to develop an unorthodox identification system in favor of reference unique database. It also established the various media (purification system) give additive, reductive effect on turmeric samples in context HPTLC –fingerprinting. Addition of characters of medias drug with turmeric powders like the crystal of cow's urine, pollen grain and starch grain

of Alambusha, epidermal cells, fiber, the crystal of panchapllava. Identify different perceivable colors in variously processed turmeric by analyzing the Lab color space as an image signature. PC_1 and PC_2 explained (90 + 9) % total variance in score plot of respective purify turmeric samples shown clear grouping in relation to the physicochemical constant. It should be noted that the sample TGM are differentiated from other samples by their higher pH value and water soluble extractive value. HPTLC chromatographic fingerprinting reveals that samples treated water contained maximum amount of curcumin with help of standard curve.

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CONFLICTS OF INTERESTS

Declared none

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