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UV-Vis scanning coupled with PCA as an alternative method for phytochemical screening of natural products – *Costus Igneus* leaf metabolites

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Abstract
Utilization of medicinal plants to discover new drugs and to invent nutraceuticals and functional foods is the current trend for natural product researchers and food technologists. Availability of cost effective and reliable methods to identify the functional groups and bioactive molecules from natural products are scarce. In this study the patterns of absorbance at different wavelengths by the bioactive compounds were detected using principal component analysis of the absorbance data of extracts of *Costus igneus* plant leaf. Thin layer chromatography method and qualitative phytochemical screening chemicals methods were done simultaneously. Principal component analysis depicted significant correlation ($p<0.5$) between wavelengths 410nm-670nm, 330nm-350nm-370nm-410nm and 210nm-290nm. Principal component analysis and consequent literature search indicated the presence of flavonoids, catechins, chlorophyll a and b and antioxidant polyphenolic compounds. These results correlates with qualitative phytochemical screening and thin layer chromatography. UV-Vis scanning coupled with principal component analysis is a cheap and reliable method for preliminary screening of natural products for bioactive flavonoids and chlorophylls.

Keywords: UV-Visible spectrum, phytochemical screening, flavonoids, principal component analysis

1. Introduction
Medicinal plants have been traditionally utilized as food and medicine across different cultures in different regions. Almost all the regions are known to have versatile plant based remedies for acute and chronic ailments. The well advanced orthodox plants based remedies were vastly found in and around the Indian subcontinent including India, Sri Lanka, Pakistan, Nepal and Bangladesh. India is one of the seventeen mega-diverse countries and houses four global biodiversity hotspots. A total land area of 2.4% secures 7-8% of recorded species biota based on species rarity and endemism (Dar *et al*., 2012) [2]. Rich plant diversity in India accounts for 11% of world plant diversity with over 45,000 plant species of which 7500 are medicinal plant species (Khandekar and Srivastava, 2014) [13]. Their importance in the health care and socio-cultural needs of communities is well known (Dikshit and Kala, 2014) [5]. Current trend in utilization of medicinal plants takes the forms of drug-discovery (Dias *et al*., 2012, Newman and Cragg, 2012) [4, 20], chemical biology (Kumagai *et al*., 2012, Hicks and Raikhel, 2012) [16, 9], agrochemicals (Dayan *et al*., 2012) [3] and food preservatives (Rukayadi *et al*., 2013) [22].
The renowned importance of medicinal plants are derived from their rich and diverse phytochemical composition. Plant secondary metabolites of medicinal importance are grouped into alkaloids, tannins, flavonoids, saponins, terpenoids, lactones, glucosides, anthocyanosides, anthroquinones and polyphenolos (Singh *et al.*, 2003) [25]. On the pursuit of novel drug discovery for diabetes mellitus, various indigenous and invasive plants have been phytochemical checked in the past. Recent additions to this list are *Hygrophilaauriculata* (Rastogi *et al*., 1995) [21], *Cucumis sativus* (Saidu *et al*., 2014) [23], *Cola nitida* (Dorothy *et al*., 2014) [6], *Vernonia glaberrima (Asteraceae)* (Abdullahi *et al*., 2015) [1] and *Vernonia amygdalina (bitter leaf)* (Johnson *et al*., 2015) [10]. For our purpose to validate the UV-Vis scanning coupled with principal component analysis (PCA) as an effective method for phytochemical screening we used extracts of *Costus igneus* aka Insulin plant leaves.
Costus igneus is a perenial shrub plant which grows in western ghats of India is known for its curative properties against various ailments (Urooj, 2008). The plant has been studied for its anti-inflammatory activity (Krishnan *et al*., 2014) [14], antidiabetic properties such as Insulin mimicking property (Joshi *et al*., 2013) and plasma glucose lowering property (Kalailingam *et al*., 2011) [12], antibacterial activity (Sulakshana *et al*., 2013) [26], antifungal activity

(Saraswathi *et al*., 2010) [24], hypolipidemic properties (Krishnan *et al*., 2011) [15] and anti-tumor properties (Sun *et al*., 2007) [27]. This study compares the effectiveness of UV-Visible scanning coupled with principal component analysis as an alternative method to TLC and qualitative phytochemical screening for the identification of metabolites from *C.igneus* plant leaves.

2. Materials and Methodos

Chemicals and reagents

All solvents for extraction and TLC were purchased from Sigma-Aldrich Co. LLC. Sulfuric acid(98%), acetic anhydride, ferric chloride, lead acetate, ammonium solution, potassium hydroxide, hydrochloric acid (37%), glacial acetic acid, acetyl chloride, zinc chloride, iodine, potassium iodide, picric acid and sodium hydroxide were purchased from Sigma-Aldrich Co. LLC.

2.1 Sample preparation

Costus igneus plant leaves were collected from the plants which were purchased from Herbal Garden, PJTSAU, Hyderabad. Green full-grown leaves were shade dried for five days until they reach a moisture content below 5% (dw). Plant leaves were fine ground (60 mesh size) using a commercial grinder. The grinder jar loaded with dried leaves was refrigerated for half an hour before grinding every batch. The powdered leaves were stored in a cool dry place until they were used for extraction.

2.2 Extraction of phytochemicals from powdered leaves

Phytochemical constituents of powdered *Costus igneus* leaves were extracted in solvents with different polarity. 0.5 g of Leaf powder was soaked in 10 ml solvents (acetone, acetone/water (1:1, v/v), acetone/water (4:1, v/v), acetone/water/acetic acid (7:2.9.5:0.05, v/v/v), ethanol/water (7:3, v/v), methanol/water (7:3, v/v), deionized water, ethanol, methanol, hexane, chloroform, petroleum ether and diethyl ether). The mixture was taken into 15 ml polypropylene centrifuge tubes and shaken at 300 rpm at ambient temperature in an orbital shaker for 3 h. The centrifuge tubes were left to stand for 12 h in the dark overnight and centrifuged at 3000 rpm for 15 min. Supernatants were carefully collected into new tubes. Residues were re-extracted with 5 ml of the respective solvents. First and second extracts were pooled in a sample bottle and stored at 4° C in the dark.

2.3 UV-Vis Spectroscopic measurement of optical density

Extracts were scanned for their absorbance against solvent blank at wave lengths ranging from 200nm to 700nm by Thermo Scientific UV 100 UV-Vis spectrophotometer. Peak pick option was used for identifying peaks in the spectrum. All the peak absorbance for respective wavelengths were recorded and tabulated in Microsoft Excel Application.

2.4 Thin Layer Chromatography profile of phytochemicals

Mobile phases were prepared for each extract as given below.

Table 1: Mobile Phases for the TLC

Extract	Mobile Phases
Hexane	Methanol/Chloroform (9:1, v/v)
Chloroform	Methanol/Chloroform (9:1, v/v)
Ethyl acetate	Methanol/Ethyl acetate/ Hexane/Acetic acid (2:7:1:0.5 v/v)
Methanol	Ethyl acetate/Water/Methanol (6:3:1 v/v)

TLC plate with silica gel 60 F254 TLC (Sigma-Aldrich), 7X6 cm was cut with scissors. Plate markings were made with soft pencil. A sample volume of 1 µl was spotted by using a glass capillary at a distance of 1 cm from the solvent end. After pre-saturation of the TLC chamber with mobile phase for 20 minutes the plates were introduced in to the TLC chamber. Iodine chamber was used to detect the bands. The distance travelled by solvent front and the distance from the band to penal line was measured using a standard length scale. Retention factor (R_f) for each extract was calculated as:

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}} \quad (1)$$

2.5 Qualitative Phytochemical Screening

Solvent extracts were subjected to standard methods of qualitative phytochemical screening (Harborne, 1998) [8]. The tests were carried out for alkaloids, phytosterols and terpenoids, saponins, triterpenes, flavonoids, anthroquinones, tannins, glycocides and anthocyanocides.

2.6 Phytosterols and terpenoids

Salkowski Test: 5.0ml extract was shaken with 2.0ml of chloroform in a test tube and 2.0ml of concentrated sulfuric acid was added along the sides of the test tube. A reddish brown coloration of the interface was considered to be the observation for positive results (presence of terpenoid).

Liebermann-Burchard test: 5.0ml of extract was shaken with 2.0ml of chloroform in a test tube and two drops of acetic anhydride was added. The test tube was boiled in a water bath and rapidl cooled in iced water. 2.0 ml of concentrated sulfuric acid was added along the walls of the test tube. Formation of a brown ring at the junction of two layers and turning the upper layer to green was considered as the presence of steroids while formation of deep red color was considered as the presence of triterpenoids.

Tshugajeu's test method: Chloroform extract was filtered, an excess of acetyl chloride and a pinch of zinc chloride was added to the filtrates and heated in water. Appearance of eosin red color was considered as an indication of triterpenes.

2.7 Alkaloi ds

Extract was shaken with 10.0ml dilute hydrochloric acid and filtered. The filtrate was separately treated with Dragendorff's, Mayer's and Wagner's Reagent to test for the presence of alkaloids.

Dragendorff's test: To one portion of the filtrate, 1.0ml of Dragendorff's reagent (Potassium bismuth iodide solution) was added; an orange red precipitate was considered as the presence of alkaloids.

Mayer's test: To one portion of filtrate, 1.0ml of Mayer's reagent (Potassium mercuric iodide solution) was added. Formation of cream colored precipitate was considered as the presence of alkaloids.

Wagner's test: 2g of potassium iodide and 1.27g iodine were dissolved in 5ml distilled water and the solution was diluted to 100.0 ml with distilled water. Few drops of this solution was added to the filtrate. A brown colored precipitate was considered as the presence of alkaloids.

2.8 Tannins

Tannins present in the extract was identified by ferric chloride test, gelatin test and match stick test for catechin.

Ferric Chloride test: A few drops of 5% ferric chloride was

added to the extract. Subsequent black or blue-green coloration or precipitate was considered as the presence of tannins

Gelatin test: The extract was treated with 5.0 ml of 1% gelatin solution containing sodium chloride and observation was made. Precipitation of gelatin was considered as the presence of tannins.

Match stick test for catechin: Match stick wood was soaked in the extract for 1min and left to dry. Then the matchstick was wetted with concentrated HCl and warmed near a flame. Care was taken not to put the matchstick on fire. Appearance of pink or red stain on the wood was considered as the presence of catechins.

2.9 Flavonoids

Tests for flavonoids were done by alkaline reagent test, lead acetate test, ferric chloride test.

Alkaline reagent test: Extract was treated with 5 ml of 1% sodium hydroxide solution and observation was made. Golden yellow or green coluor solution was be considered as the presence of alkaloids.

Lead acetate Test: Extract was treated with 5 ml of 3% lead acetate solution. Formation of yellow precipitates confirms the presence of alkaloids.

Ferric chloride Test: Extract was treated with 1% ferric chloride solution. Appearence of green colour was considered as the presence of alkaloids.

2.10 Glycosides

Extract was hydrolyzed with dilute diluted hydrochloric acid and then subjected to test for glycosides

Anthraquinone glycoside (Borntrager's test): 1.0ml of 5% hydrochloric acid was added to 1.0ml of extract. The mixture was boiled in a water bath and then filtered. Filtrate was then shaken with equal volume of chloroform and kept to stand for 5 min. Then lower layer of chloroform was shaken with half of its volume of dilute ammonia. The formation of rose pink to red color of the ammoniacal layer was considered as the indication of anthraquinone glycosides.

Alkaline reagent test: Extract was treated with 5.0 ml of diluted sodium hydroxide and formation of yellow color solution was considered as the positive results.

Modified Brontrager's Reagent test: Extract was treated with dilute, ferric chloride solution immersed into the boiling water for 5 minutes, cooled and shaken with equal volume of benzene and then resultant solution was treated with dilute ammonium hydroxide.

Cardiac glycoside (Keller-Kiliani test): Extract was shaken with 5.0ml of distilled water. To this, 2.0ml of glacial acetic acid containing was added. 1.0ml concentrated sulfuric acid was added along the side of the test tube. The formation of brown ring at the interface was considered a positive indication for cardiac glycoside and a violet ring may appear below the brown ring.

2.11 Saponins

1.0ml of extract was shaken with 10.0ml of distilled water in a test tube. The formation of frothing, which persists on warming in a water bath for 5 min, indicated the presence of saponins.

2.12 Anthocyanosides

1ml of extract was taken in a test tube and treated with 5ml dilute hydrochloric acid. Pale pink color solution was considered as the confirmation for the presence of anthocyanosides.

2.13 Statistical analysis

Pricipal component analysis (PCA) of the spectroscopic data was carried out using XLSTAT-2016 software. Heat map diagram was constructed using ClustVis online tool (Metsalu *et al.,* 2015) [19].

3. Results and Discussions

Bioactive phytochemicals in plants are of different molecular make-up that they are differently soluble in solvents with different polarity, hydrophilic and hydrophobic nature. The results of this study shows that the UV-Vis spectrum scanning of extracts prepared with different solvents with varying polarity will produce an array of absorbance spectra. This array stores values of absorbance at different wavelengths for corresponding solvent. This array is specific for a given plant material and the solvents utilized. Further factor reduction of the data through PCA and cluster analysis arranges the absorbance peaks according to the peak location and absorbance values. Peaks locations or the peak wavelengths are specific for different bioactive compound groups and the peak height depends on the concentration of the compound.

UV-Vis absorbance of plant material extracts enables the identification of flavonoids and other polyphenolic compounds in extracts prepared using different media. Flavonoids have an absorption maximum around 240–290 nm, which is affected by the conjugation of ring structure and its substitution pattern. Other flavonoids have an extra absorbance maximum at around 300–550 nm, which is detected in flavonoids having conjugated rings. This absorption maximum is at around 460–560 nm for anthocyanins and at 310–370 nm for flavones and flavonols (Kumar and Pandey, 2013) [17]. Highest peaks for solvents were at around 300nm to 390 which also indicated the presence of conjugated flavonoids, flavones and flavonols (Kumar and pandey, 2013) [17]. Flavonoids have both polar and non-polar compounds (Xiao *et al.,* 2011 and Ferreira and Pinho, 2012) [30, 7]. More polar flavonoid glycocides are easily extracted with mixed solvents or polar solvents including acetone, acetone/water (1:1, v/v), acetone/water (4:1, v/v), acetone/water/acetic acid (7:2.9.5:0.05, v/v/v), ethanol/water (7:3, v/v), methanol/water (7:3, v/v), deionized water, ethanol and methanol (Ferreira and Pinho, 2012) [7]. Hence the presence of polar flavonoids is shown by the higher absorbance at 200-400 nm in polar solvents. Prominent peaks at 210nm and 290nm indicated the presence of flavonols and negative correlation of absorbance at 290 indicated the presence of catechins in the extracts (Kumar and Pandey, 2013) [17].

The characteristic absorbance spectra for chlorophyll a and b was observed. Chlorophyl a and chlorophyl b compounds are highly soluble in non-polar solvents like pet ether and chloroform. The absorbance peaks at 270 and 390 and 410 and 690 had a significant correlation ($p<0.01$) with each other hence classified together in hierarchical tree diagram. This indicated the presence of chlorophyll a and b in subsistence amounts in non-polar solvents.

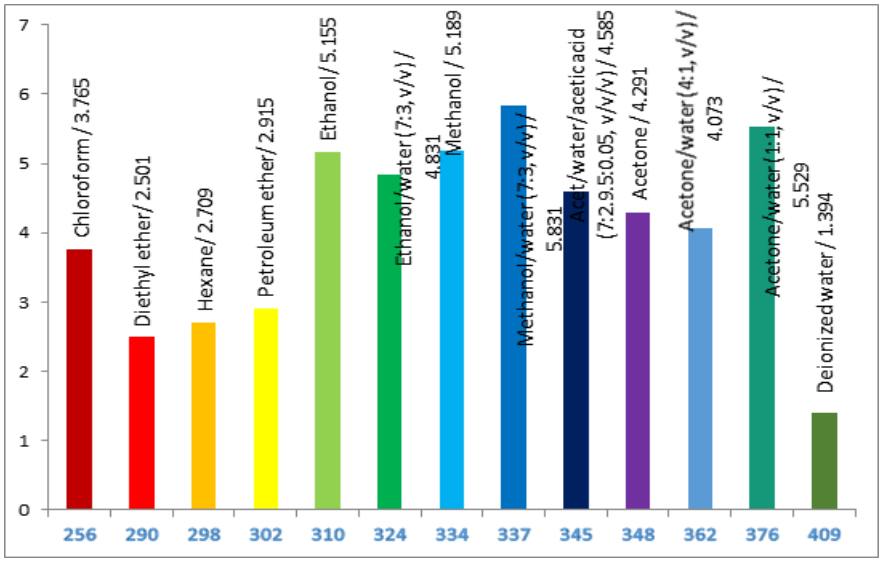

Fig 1: Absorbance maximum of different solvent extracts of *Costus igneus* leaf

Among all the solvents used in this experiment methanol/water (7:3) shows highest absorbance at 337nm followed by acetone/water (1:1) at and absolute methanol (Figure 1.0). methanol/ water (7:3) extract registerd the highest abosrbance of 5.831 at 310nm. Chloroform extract shows most number of peaks with 64 followed by hexane and diethyl ether each sharing 54 peaks.

Major peak wavelengths were tabulated in table 2 as an array. The table gives an overview of the peak wavelengths obtained from different solvent extracts of *Costus igneus* leaf.

Table 2: UV-VIS Characterization of solvent exrtacts of *Costus igneus*

Solvent	Peak λ (nm)
Deionized water	220 236 249 274 291 409 530 667
Acetone	212 238 243 269 287 305 331 348 529 669
Acetone/water (1:1, v/v)	220 242 269 289 316 325 355 376 384
Acetone/water (4:1, v/v)	206 221 250 280 284 310 328 348 362
Acetone/water/acetic acid (7:2.9.5:0.05, v/v/v)	202 239 253 266 297 320 324 345 362
Ethanol/water (7:3, v/v)	216 236 244 265 298 320 324 356 373
Methanol/water (7:3, v/v)	207 236 243 263 294 319 337 345 361
Ethanol	210 239 247 277 310 331 341 532 666
Methanol	208 239 245 266 291 306 334 366 529 665
Hexane	211 238 257 279 298 410 472 670
Chloroform	208 224 256 266 298 305 353 413 535 670
Petroleum ether	202 239 241 271 298 302 371 410 471 531 669
Diethyl ether	220 240 252 277 290 409 530 667

Factor reduction from PCA and consequent clustering analysis resulted in groups and relates the data in heat map diagram (HMD). More than 80% positive correlation was detected among 330nm, 350nm, 370nm and 410nm. This indicated the presence of extractable compounds which showed characteristic peak absorbance at all four wavelengths. Xu and Howard (2012) [31] and Harborne (1998) [8] described that these peaks indicated the presence of antioxidant phenolic compounds such as, flavones and flavonols.

HMD explicated the relationship in absorbance among absorbance peak wave lengths and depicts characteristic groups based on the absorbance for specific wavelengths. Peaks at 210nm, 530nm, 290nm, 310nm and 610nm are in one group, 230-270nm and 330-390nm are in another and 410nm and 670nm are in the last group.

Observation of groups and absorbance data showed prominent peaks around 210nm to 290nm. This ensures the presence of flavonols (Harborne, 1998) [8]. At 290nm, strong negative correlation between polar and non-polar solvents was observed (red and blue colours in HMD). This demonstrated the presence of catechins and catechin derivatives (Zhang *et al*., 2013) [32]. The higher positive correlation among group three (410nm and 670nm) by non-polar solvents (AC, HX, PE) indicated the presence of chlorophylls a and b in the extracts (Lichtenthaler and Buschmann, 2001) [18].

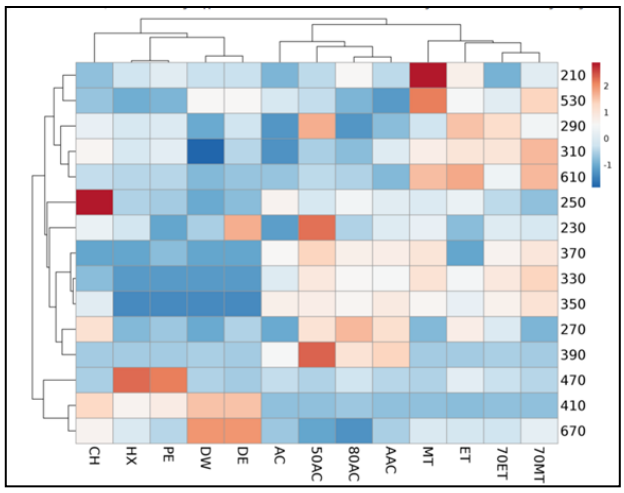

Fig 2: HMD and HCA for absorbance of solvents

TLC results (Table 3) confirmed the presence of saponins, flavonoids, tannins and alkaloids in *C. igneus* leaf extracts.

Table 3: TLC of *C. igneus* leaves ethanol extract in mobile phase petether: benzene: methanol (16:3:2).

Band number	Rf value	Spraying agent	Colour of band	Phytochemical detected
1	0.10	Con. HCl	Dark brown	Saponin
2	0.53	Dil. Ammonia	Yellow	Flavonoid
3	0.85	$FeCl_3$	Intense red	Tannin
4	0.90	Dragendorf's reagent	Orange	Alkaloid

Qualitative phytochemical screening results confirmed the presence of steroids, triterpenes, alkaloids, tannins, flavonoids, glycosides and saponins (Thiruchenduan *et al.*, 2016) [28].

4. Conclusion

The results showed that the metabolites present in *C.igneus* plant leaves can be identified using UV-Visible spectrum coupled with principal component analysis as an alternative to TLC and qualitative phytochemical screening methods in identifying flavonoids, catechins and chlorophyll a and b from natural sources. It is suggested that this method should be validated using standards and different solvents.

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