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Comparative Pharmacognostical Studies of Blue and White Flower Varieties of *Clitoria ternatea l.*

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Abstract

Objective: To establish the quality control parameters for two varieties of Clitoria ternatea among which one is medicinally important whereas second is ornamental.

Method: The pharmacognostic studies were focused on macroscopy, qualitative and quantitative microscopy, physicochemical parameters, quantitative estimation of phenolics, flavonoid and also the TLC profiling with two phenolics (caffeic and ferulic acid) and two terpenoid markers (lupeol and β-sitostrol).

Results: The distinguishing macroscopical feature is white and blue colour of corolla while the microscopy showed more starch grains in root; variations in pericyclic fibers, xylem vessels and pith in stem and quantitative parameters of leaf. Some variations were also observed in physicochemical parameters. However, TLC fingerprint profiles of both the varieties showed similarities in the presence of lupeol, β-sitostrol, caffeic acid and ferulic acid. However, characteristic additional bands e.g. blue fluorescent band at Rf 0.38 under UV 366 nm and greyish blue band at Rf 0.58 under visible light after derivatization were observed only in white variety.

Conclusion: Present study provided the scientific data for the proper identification and establishment of standards for the two varieties of C. ternatea and blue variety may be used as substitute of white variety with less therapeutic activity.

Introduction

Clitoria ternatea L. belongs to Family: Fabaceae commonly known as 'Butterfly pea' a perennial twining herb, found throughout India in tropical areas. Traditionally it is recommended for the treatment of snakebite, scorpion sting, chronic bronchitis, indigestion, constipation, fever, arthritis, eye ailments, sore throat, skin diseases, rheumatism, syphilis, eye and ear-diseases in India [1-5]; mental problems, epilepsy, insanity, for muscular strength and complexion tonics [6]; as a remedy for hemicranias and in swollen joints [7-8] beside this it is a good source of forage legumes in India [9]. Ethno botanically it is used in various urinary troubles like infection, burning sensation in urinary tract, lack of urination, frequent urination [10] and also reported for purification after surgical removal of tumor [11]. C. ternatea has two varieties, i.e., white flowered and blue flowered. The white-flowered one is found to be therapeutically more active and has been accepted as 'Shankhpushpi' by most of the south Indian vaidyas [12]. 'Shankhpushpi' consists of conch or 'Shankha' shaped flowers, categorized under Ayurvedic Medhya Rasayana drugs that claim as brain tonic and have memory and intelligence enhancing properties. The blue-flowered variety is generally mixed with white-flowered one. Various groups of phytochemicals have been reported from white variety of *C. ternatea* like phenolics- kaempferol, quercetin, myricetin and their glycosides [13,14]. The fatty acid content of *C. ternatea* includes palmitic, stearic, oleic, linoleic, and linolenic acids [15-18]. Lactones aparajitin and clitorin were also reported from leaves [19-21]. The leaf mucilage contains anhydro-galacatan, anhydro pentosan and methyl pentosan and an alkaloid [22]. The anthocyanins and delphinidin glucoside present in blue flowered variety [23]. Pharmacologically it is reported for improved cognitive abilities, learning and memory, neuronal degenerative disorders [24-27]; nootropic and anticonvulsant activities [28]; antimicrobial and insecticidal [29-30]; antipyretic, analgesic, anti-inflammatory [31-32]; antioxidant, hepatoprotective, antidiabetic, [33] and platelet aggregation inhibitory [34] activities. The detailed pharmacognostic study of two varieties of *C. ternatea* viz. white and blue have not been reported so far. Therefore, an attempt has been made to standardize the two varieties through macro-microscopical, physico and phytochemical parameters.

Materials and Method

Collection and authentication of plant material

The selected blue and white variety of *Clitoria ternatea* were collected from Lucknow, India in April 2012, their herbarium specimens were prepared as per standard herbarium procedure [35] and deposited in the herbarium of CSIR-National Botanical Research Institute, Lucknow wide voucher specimen number LWG-002 and LWG-32 respectively.

Macro-microscopical studies

The macroscopy of two varieties of *C. ternatea* was described with the help of Flora [36]. Plant materials were dried at 40 ± 2°C for 4-5 days in a hot air oven. The samples were stored at 25 ± 2°C in air-tight containers and grounded to form fine powder when required and filtered through sieve of 345 micron pore size. Qualitative were done by hand cut sectioning in transverse planes best sections were picked out for mounting after the staining and dehydration were completed, quantitative microscopy for stomatal number, somatal index and palisade ratio were done by slide preparation after clearing

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with chloral hydrate solution. Observation and photography was made under the microscope (Olympus CX3) and compatible software (Magnus Pro image analysis software) in different magnification (10X, 40X). Fluorescence analysis (in Ultraviolet fluorescent analysis cabinet, Sonar) and powder studies were done according to the standard methods [37-39].

Physicochemical and phytochemical studies

Total ash, acid-insoluble ash, alcohol soluble extractive, water soluble extractive and residual moisture content were calculated as per pharmacopoeial methods [40]. The preliminary phytochemical screening for the presence of steroid, triterpenoids, flavonoids, alkaloids, carbohydtrate, glycosides, tannins and saponins etc. was done according to Evans [39] and estimation major phytoconstituents according to following described methods.

Estimation of total sugar: Total amount of sugar present in the drug was calculated based on the Montgomery method [41] using a spectrophotometer (Thermo electronic, Double Beam UV-vis Spectrophotometer). 10% homogenate of the plant tissue in 80% ethanol was prepared and centrifuged at 2000 rpm for 15 minutes. The supernatant obtained was made upto known volume (10 ml or depending expected conc. of sugar). 0.1 ml aliquote was taken and 0.1 ml of 80% phenol and 5 ml conc. H_2SO_4 were added. Cooled and then the absorbance at 490 nm were noted. D-Glucose was taken as positive control. Standard curve was made by plotting a graph between optical density (OD) and concentrations of different dilutions (0.01, 0.02, 0.03, 0.04 and 0.05mg/ml) of standard D-Glucose. The percentage of sugar was calculated using formula. % sugar = Con. At UV x Ext value x 100/1000.

Estimation of total starch: Total amount of starch present in the drug was calculated based on the Montgomery method [41] using the spectrophotometer (Thermo electronic, Double Beam UV-vis Spectrophotometer). 10% homogenate of the plant tissue in 80% ethanol was prepared and centrifuged at 2000 rpm for 15 minutes. Added 4 ml of distilled water to the residue heated on a water bath for 15 minutes and macerated with the help of glass rod. To each of the samples, added 3 ml 52% perchloric acid and centrifuged at 2000 rpm for 15 minutes. The supernatant thus obtained was made upto known volume (generally upto 10 ml). 0.1 ml aliquot was taken, 0.1 ml of 80% phenol and 5 ml conc. H_2SO_4 were added to it. Cooled and then noted the absorbance at 490 nm. The percentage of starch was calculated using formula. % sugar = Con. At UV x Ext value x 100/1000.

Estimation of total phenolic content (TPC): The amount of total phenolics present in the drug was calculated according to the Bray and Thrope [42]. A stock solution of 1 mg/ml methanolic plant extract was prepared. 0.5 ml stock solution was taken in the test tube and added 10ml distil water and 1.5 ml folin reagent, kept for 5 minutes then added 4 ml 20% Na_2CO_3 made the volume upto 25 ml with distil water, and kept for 30 minute. The OD (optical density) was taken at 765 nm using the spectrophotometer (Thermo electronic, Double Beam UV-vis Spectrophotometer). Gallic acid of different dilutions (0.2, 0.4, 0.6, 0.8 µg/ml) was used as standard. TPC was calculated in percent by the following formula: TPC = conc. In 1 ml x Ext. value x 100/1000.

Estimation of total flavonoids content (TFC): The amount of total flavonoid present in the drug was calculated according to the Woisky and Salatino [43]. A stock solution of 1 mg/ml methanolic plant extract was prepared. 0.5 ml stock solution was taken in a test tube and added 0.5 ml 2% methanolic $AlCl_3$ and volume made upto 5

ml with methanol. Yellow colour indicated the presence of flavonoid. Read the optical density (OD) at 420 nm using the spectrophotometer (Thermo electronic, Double Beam UV-vis Spectrophotometer). Quercetin solution used as standard in serial dilutions of 4, 8, 12, 16, 20 µg/ml. TFC was calculated in percent by the given formula: TFC = conc. In 1 ml x Ext. value x 100/1000.

Thin layer chromatographic (TLC) finger printing

For TLC studies, powdered plant materials were placed in appropriately sized volumetric flasks. 25 mL methanol was added to 4 g of powder of each plant, shaken on shaker for 2 hrs, kept at rest overnight. The methanolic extracts were filtered through Whatmann No. 1 filter paper. The procedure was repeated thrice with methanol (25 mL) at room temperature (25°C ± 2°C). The extracts were concentrated under reduced pressure at a temperature of 45 ± 2°C. Accurately weighed 10 mg of the extract was dissolved in 1 mL methanol, and filtered through a 0.45 µm filter membrane, the filtrate was used as sample solution. 1 mg each of ferulic acid, caffeic acid, β-sitostrol and lupeol were dissolved in 10 mL methanol to get 0.1 mg/ mL solution of standard markers. TLC was performed on 20 x 10 cm silica TLC aluminium sheet, coated with 0.2 mm layer of silica gel containing UV 254 fluorescent indicator (S.D. Fine Chemicals, India). Samples (20 µL) and standards (10 µL) were applied to the plates by means of a Camag (Switzerland) Linomat 5 sample applicator. The plates were developed to a distance of 8 cm from the lower edge of the plate with 20 mL toluene-ethyl acetate-formic acid (8.5:1.5:0.1 v/v/v) as mobile phase, in a Camag twin-trough chamber, previously saturated with mobile phase vapor for 30 min at 25 ± 2°C. After removal from the chamber, plates were completely dried in air at room temperature (25 ± 2°C) and documented under UV 254 nm and UV 366 nm. The plates were dipped in anisaldehyde sulphuric acid reagent, dried and heated at 110 ± 2°C for 5 min and documented under visible light after derivatization.

Results

Macro and microscopy

Both varieties of Clitoria are morphologically very similar except white and blue colour of petals. Stem: 20-45 cm, slender, terate, downy, splintery fibrous, surface smooth, internode 6-13 cm, taste bitter; Leaf: leaflets 4, opposite imperipinnate, ovate or oblong, obtuse, subcoriaceous 2-5 cm in length and 1-2 cm in width, apex macronate, surface hairy; Stipules: minute, linear; Flowers: solitary, axillary, bracteoles large, obtuse; Calyx: 1-1.5 cm, teeth lanceolate nearly as long as the tube; Corrolla: 3-5 cm long; Pod: 6-8 cm, flat, sparingly hairy, 6-10 seeded (Figure 1). Comparative qualitative, quantitative microscopy, powder (Table 1; Figures 2 and 3) and fluorescent analysis details of two studied variety of *C. ternatea* are given in Table 2.

Physicochemical and phytochemical studies

The comparative results of physicochemical parameters viz. water extractive; alcoholic extractive, total ash, acid insoluble ash, residual moisture content, total sugar, starch; TPC and TFC have been represented in Figure 4. The results of preliminary phytochemical screening are presented in Table 3.

TLC finger printing

TLC fingerprint profile of white and blue variety of *C. ternatea* showed common as well as differentiating bands. The similar bands at Rf 0.12, 0.14, 0.19, 0.30 (under UV 366 nm) at Rf 0.12, 0.14, 0.19, 0.25, 0.30, 0.54 (under UV 254 nm) and at Rf 0.30, 0.48, 0.62 and 0.75

Figure 1: Plants of two *Clitoria ternatea* varieties. A: white variety, B: blue variety.

(under visible light after derivatization) were observed in both the varieties. However, the characteristic blue fluorescent band at Rf 0.38 under UV 366 nm and greyish blue band at Rf 0.58 after derivatization under visible light were observed only in white variety (Figure 5). The corresponding bands of four chemical markers viz. caffeic acid, ferulic acid, β-sitosterol and lupeol were represented at Rf 0.14, 0.30, 0.48, 0.62, respectively (Figure 5).

Discussion

Therapeutic efficacy of medicinal plants depends upon the quality and quantity of chemical constituents which is starts with wrong identification of plant material [44]. There are several evidences of unraveling this problem by pharmacognostic studies of medicinal plants even present time [45-46]. Morphological, microscpical, phytochemical and physicochemical analysis are major pharmcognostical parameters for above incongruity [44]. Microscopical method of valuing medicinal plants is based on the examination of mounts of the thin sections of them under a compound microscope. Every plant possess a characteristic histology in respect to its organs and diagnostic features of these are ascertained through the study of the tissue and their arrangement, cell walls and cell contents, when properly mounted in stains, reagent or mounting media. The microscopical features of two studied varieties

of *C. ternatea* (Table 1) clearly showed some identifying anatomical structures like more starch grains in transverse section of root; broad patches of pericyclic fibers, more vessels with broad lumen and broad pith region in the stem of white variety (Figure 2 and 3). Further, the quantitative leaf microscopy showed slight variation in stomatal number, somatal index and palisade ratio (Table 1). Fluorescence analysis also an important parameter for quality control point of view, because some phytochemicals showed fluorescence in different UV range after reacting with different reagents [44]. Two studied varieties of *C. ternatea* showed some significant variation in fluorescence analysis (Table 2). White and blue flower varieties showed different intensity of florescence with acetic acid (5%) that indicates variation in the quality and quantity of phyto-constituents in two varieties. Physicochemical and phytochemical studies showed that white variety have high ash value but low acid insoluble ash depicted comparatively high level of carbonates, phosphates and low level of silicates and silica content in white variety (Figure 4). Extractive value of the crude drug also useful parameter for the evaluation and standardization and gives the idea of nature of the chemical components soluble in particular solvent. High value of water and alcohol extractive percentage in white *C. ternatea* variety showed high concentration of polar compounds. These parameters are useful in determining authenticity and purity of drug and also these values are important quantitative standards [37-42].

Further, the preliminary phytochemical screening showed presence of steroid, flavonoids, alkaloid, carbohydrate, glycosides, tannins and saponins in both variety but white variety gave better results for flavonoid, alkaloid, carbohydtare and glycosides while blue variety for saponins and tannins (Table 3). These secondary metabolites have infatuated different pharmacological effects and liable for various pharmacological activities of *C. ternatea* varieties. Sugar and starch contents were also more in white variety showed its high rate of photosynthesis and high level of glucosides bounded chemical groups; this is a useful standardization parameter [47]. The characteristic bands showed in TLC profiling (Figure 5) are significant point of quality standards. The results showed that the marker components viz. caffeic acid, ferulic acid, β-sitosterol and lupeol were present in both the varieties and hence, these may be substituted to each other. Polyphenols have neuroprotective effects in various pathological states of the nervous system as they are able to modulate and control oxidative stress and reactive oxygen species production, metal accumulation, inflammation, apoptosis, and mitochondrial dysfunction, all mechanisms which contribute to the etiology of neurodegenerative diseases [48]. caffeic acid and ferulic acid are polyphenolic compounds and present in both the varieties of *C. ternatea*. Further, several reports indicated that caffeic acid and ferulic acid also showed neuroprotective effect [49-53]. Similarly, terpenoids viz. β-sitosterol and lupeol, available in both the verities reported for their activity against neuroblastoma, glioblastima and various neurodegenarative disorders [54-56]. These reports validated the traditional claim of *C. ternatea*. However, presence of high flavonoid, phenolics, sugar and starch content in the white variety indicated its more therapeutic values. Further pharmacological investigations are required for therapeutic activities of *C. ternatea*.

Conclusion

Quality of herbal drug in term of chemical constituents and their efficacy necessitates the need of quality control studies of raw drug materials using pharmacognostical standardization. World health organization (WHO) has also created awareness towards validation of

Parts	White flower variety	Blue flower variety
Root	Circular in outline, outer most cork 5-7 layered; cortical region is well developed with more starch grains; phloem region is narrow, and xylem region is comparatively well developed.	Almost similar in anatomical features except very less starch deposition in cortical region, xylem comparatively less developed.
Stem	TS almost circular with wavy outline, outermost layer of cuticle bearing both simple and glandular trichomes; cortical region is parenchymatous, 4-5 layered followed by endodermis; pericyclic fibers are in broad patches; phloem region is comparatively narrow and xylem region broad and comprises of more broad lumen vessels; pith parenchymatous and comparatively broad.	Similar to white variety except, narrow patches of pericyclic fibers, narrow xylem region and vessels with comparatively narrow lumen.
Leaf	TS of leaf dorsiventral with collateral, centrally located meristele; trichomes present, similar as in stem; epidermal cells wavy in surface view, cuticle striated, stomata subcoriaceous, rubeaceous present on both side, calcium oxalate crystals are prismatic in shape and present along the veins. Stomatal number ranges 290-400 on lower epidermis and stomatal index 17.25-18.90 while on the upper epidermis stomatal number ranges 150-250 and stomatal index ranges 14.5-16.8. Palisade ratio ranges from 4-6.	Similar as in white variety. Stomatal number ranges 250-350 on lower epidermis and stomatal index 15.50-1675 while on the upper epidermis stomatal number ranges 200-250 and stomatal index ranges 12.25-14.45. Palisade ratio ranges from 5-7.
Powder	Cork cells, glandular and simple trichomes, vessels with scalariform and reticulate thickenings, fibres, patches of pericyclic fibres and prismatic crystals of calcium oxalate are observed.	Similar as in white variety

Table 1: Comparative microscopical detail of two varieties of *Clitoria ternatea.*

Reagents	Under visible light		Under UV 254		Under UV 366	
	White	Blue	White	Blue	White	Blue
FeCl$_3$ (5%)	G	YG	DG	G	G	G
I$_2$ (5%)	DG	BLG	G	LG	DG	YG
NaOH	G	LG	G	LG	YG	Y
NH$_3$	BKG	BKG	DB	B	YG	Y
HNO$_3$	LB	RB	RO	LO	DB	B
CH3COOH	B	B	FG	FY	RO	O

Abbreviations: Green (G), Dark green (DG), Blackish green (BKG), Light brown (LB), Brown (B), Yellowish green (YG), Bluish green (BLG), Light green (LG), Reddish brown (RB), Dark green (DG), Dark brown (DB), Reddish orange (RO), Light orange (LO), Florescent green (FG), Florescent yellow (FY), Yellow (Y), Orange (O).

Table 2: Comparative fluorescence analysis details of two varieties of *Clitoria ternatea.*
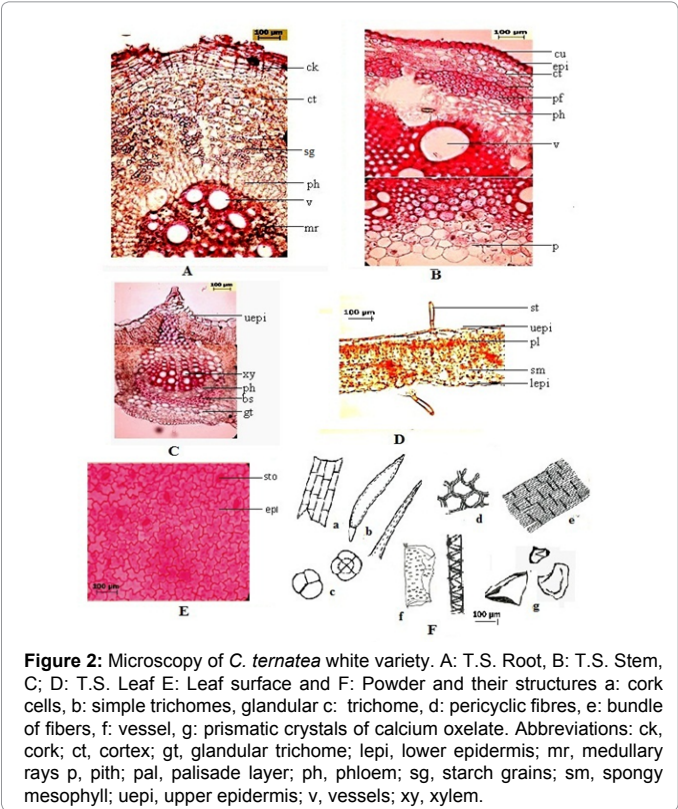

Figure 2: Microscopy of *C. ternatea* white variety. A: T.S. Root, B: T.S. Stem, C; D: T.S. Leaf E: Leaf surface and F: Powder and their structures a: cork cells, b: simple trichomes, glandular c: trichome, d: pericyclic fibres, e: bundle of fibers, f: vessel, g: prismatic crystals of calcium oxelate. Abbreviations: ck, cork; ct, cortex; gt, glandular trichome; lepi, lower epidermis; mr, medullary rays p, pith; pal, palisade layer; ph, phloem; sg, starch grains; sm, spongy mesophyll; uepi, upper epidermis; v, vessels; xy, xylem.
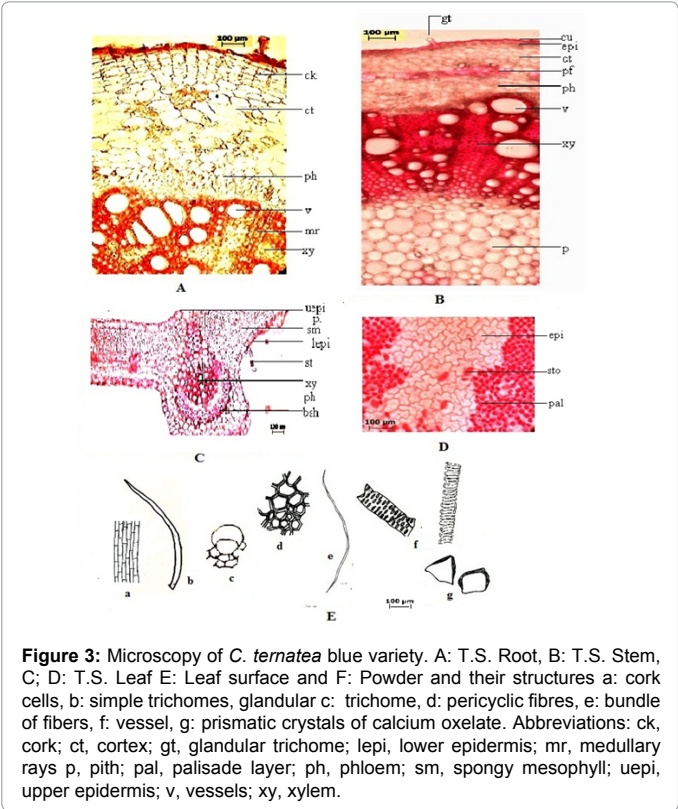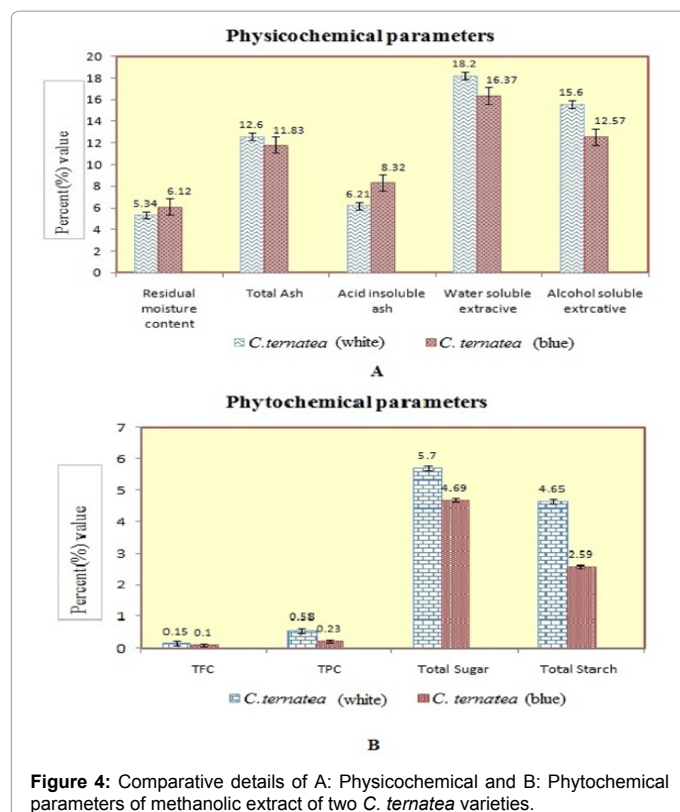

Figure 3: Microscopy of *C. ternatea* blue variety. A: T.S. Root, B: T.S. Stem, C; D: T.S. Leaf E: Leaf surface and F: Powder and their structures a: cork cells, b: simple trichomes, glandular c: trichome, d: pericyclic fibres, e: bundle of fibers, f: vessel, g: prismatic crystals of calcium oxelate. Abbreviations: ck, cork; ct, cortex; gt, glandular trichome; lepi, lower epidermis; mr, medullary rays p, pith; pal, palisade layer; ph, phloem; sm, spongy mesophyll; uepi, upper epidermis; v, vessels; xy, xylem.

Figure 4: Comparative details of A: Physicochemical and B: Phytochemical parameters of methanolic extract of two *C. ternatea* varieties.

Figure 5: TLC profile of methanolic extract of two *C. ternatea* varieties. 1, white; 2, blue; R1, Lupeol; R2, β-sitosterol; R3, caffeic acid and R4, Ferulic acid. A: visualization under UV 254nm, B: visualization under UV 366nm, C: visualization under visible light after derivatization.

Phytochemicals	White variety	Blue variety
Steroid	+	+
Triterpenoids	+	+
Flavonoids	++	+
Alkaloids	++	+
Carbohydrate	++	+
Glycosides	++	+
Tannins	+	++
Saponins	+	++

Table 3: Results of phytochemical screening of methanolic extract of two varieties of *Clitoria ternatea*.

plant based drug to maintain the quality, safety and efficacy. The macro-microscopic characterization is an important parameter for proper authentication of crude drug even in powdered form. However, the physico-chemical values are useful to ascertain the identity, purity and strength of both the varieties of *C. ternatea*. As *C. ternatea* white variety being a nerve tonic in traditional systems of medicine, has a potential to develop neuroprotective drug. The blue variety may be a substitute of white variety. In addition, the parameters which are reported here can be considered as a distinctive enough to identify and decided the authenticity of the more medicinally valuable variety of *C. ternatea* in herbal industries and also helpful as reference for researchers.

Acknowledgement

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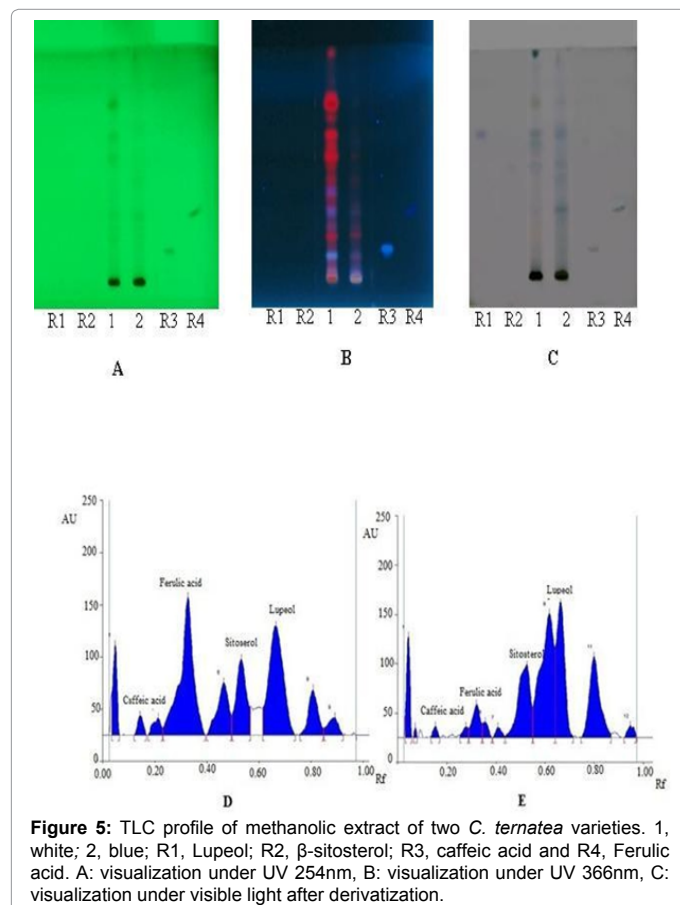
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