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PHARMACOGNOSTIC INVESTIGATION, ISOLATION AND EVALUTION OF DIOSGENIN FROM *COSTUS PICTUS* D.DON

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Keywords:

Costus pictus, Insulin plant, Costaceae, Microscopical evaluations

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ABSTRACT

In the last few years, there has been an exponential growth in the field of herbal medicine and gaining popularity both in developing and developed countries because of their natural origin, useful medicinal activities and less side effects. The secondary metabolites such as alkaloids, tannins, glycosides, carbohydrates, starch, volatile oils/fixed oils, proteins, flavonoids, saponins, steroids are biocompatible, synthesized and semi synthesized, easily available because of their lack of toxicity, low cost, irritant nature they are utilized in the pharmaceutical formulations. Present study was focusing on the pharmacognostic investigations (Morphological and Microscopical characteristics) by utilizing the leaf powdered drug and isolation of diosgenin from herbal leaf powdered drug"*Costus pictus*"D.Don for evaluations. Characterized for various parameters such as Color, odour, pH, physical appearance, solubility, etc.Leaf powdered drug has been reported to have the many medicinal activities. The physical, chemical and microscopical test shows presence and confirmation of alkaloids, glycosides, saponin, steroids, and volatile oils. Study of isolated diosgenin shows, it is yellowish green waxy in nature, odourless, soluble in organic solvents. By analytical investigations like IR spectra and HPTLC, fluorescence analysis was evaluated and the obtained results shows that isolated diosgenin and marketed diosgenin has same results and acceptable for pharmaceutical formulation.

1. INTRODUCTION

Costus pictus D. Don is commonly known as spiral ginger, belonging to the family Costaceae. It is a magical cure of diabetes. Its leaf helps to build up insulin the human body. So, it is commonly known as Insulin plant .Insulin plant was grown in America and is becoming popular in India because of its medicinal value. It is now accepted and used widely as an Ayurvedic medicinal herb. Leaf is traditionally used as antidiabetic, Antioxidant, Antibacterial, Anti-cancer and diuretic. It was reported that fresh leaves contains 18 chemical compounds were identified by using GC-MS. From the chromatogram, it was evident that the major component in the ether fraction is bis (2'- ethyl hexyl)-1, 2-benzene dicarboxylate (59.04%). The major component in the acid fraction are haxadecanoic acid (44.53%) and 4, 8, 12, 16- tetra methyl hepta decan 4-olide (27.86%). Trace elemental analysis showed that the leaves of *C. pictus* contains appreciable amounts of the elements K, Ca, Cr, Mn, Cu, and Zn. Steam distillation of leaves of *C. pictus* D. Don yielded clear and yellowish essential oils. The major constituents were Dodecanoic acid, Hexadecanoic acid, 1, 1-diethoxy Ethane, cis-3-Hexenol, 2-ethoxy Butane, 2-Pentanol, Tetradecane, β-Ionone, α-Ionone, n-Nonadecane, Farnesyl acetone identified 20. In spite of the numerous medicinal uses attributed to this plant, limited report available on its pharmacognostical information. These standards are of utmost importance not only in finding out genuity, but also in detection of adulterants in marketed drug. The objective of the present study was to establish various Pharmacognostic standards, important microscopical feature (Midrib and Lamina) and to evaluate preliminary phytochemical and physicochemical analysis of *Costus pictus* can facilitate identification and assist in preparation of monograph of plant [1, 2, 3, 4, 5, 13].

1.1 Saponins:

Saponins are a diverse group of compounds widely distributed in the plant kingdom, which are characterized by their structure containing a triterpene or steroid aglycone and one or more sugar chains. Consumer demand for natural products coupled with their physicochemical (surfactant) properties and mounting evidence on their biological activity (such as anticancer and anticholesterol activity) has led to the emergence of saponins as commercially significant compounds with expanding applications in food,cosmetics, and pharmaceutical sectors. Saponins, are glycosides widely distributed in the plant kingdom, and include a diverse group of compounds characterized by their steroidal or triterpenoid aglycone structure with one or more

sugar chains. Their structural diversity is reflected in their physicochemical and biological properties, which are exploited in a number of traditional (as soaps, fish poison, and molluscicides) and industrial applications (Price et al., 1987; Oakenfull, 1981; Fenwick et al., 1991; Hostettmann and Marston, 1995; Oakenfull and Sidhu, 1989). Research has establishedsaponins as active components in herbal medicines (Liu, 2002; Alice et al., 1991) [17].

1.2 Diosgenin Chemistry:

Steroids form an important group of compounds based on the fundamental saturated tetra cyclic hydrocarbon: 1, 2- cyclopentanoperhydrophenanthrene (sterane or gonane).According to their chemical structure, the wide array of steroid molecules may be divided into several groups as Sterols, Brassinosteroids and Sapogenins. All these compounds have basic structural skeleton or nucleus of four fused rings of 17-carbon atoms but they differ in chemical groups or side chain attached to the basic skeleton and double bond at specific position in the nucleus (Asolkar and Chadha., 1979). Saponins are categorized according to number of sugar chains in their structure. Bidesmosidic saponins have two sugar chains with one attached through an ether linkage at C-26 are furastanol saponins.The nature of the aglycone and the functional groups on the aglycone backbone and number and nature of the sugars can vary greatly resulting in a very diverse group of compounds (Price et al., 1987; Hostettmann and Marston, 1995) Diosgenin has spiroketal side chain attached at positions 16 and 17 of the sterane and has a double bond at 5-6. It has a hydroxyl group at 3rd position; hydroxyl groups are mostly found combined with sugars, making the compounds water soluble and highly saponaceous. It is a steroidal sapogenin that is isolated from plants and is structurally similar to cholesterol.Diosgenin is obtained entirely from natural sources (Yams of dioscorea spp.) since the synthetic product is not an attractive proposition commercially [17].

1.3 Plant Description

1.3.1 Botanical Name – *Costus Pictus* D.Don Family- Costaceae

Synonym: Spiral ginger

1.3.2 Chemical constituents

A Steroidal Sapogenin-Quercetin, Diosgenin, Protein, Iron, Antioxidant components-ascorbic acid, α-tocopherol, β-carotene, terpinoids, steroids, flavonoids, carbohydrates, triterpenoids, alkaloids, tannins,Minerals-Calcium, potassium, Zinc.

1.3.3 Medicinal uses

- Used as natural source of diosgenin which is steroidal sapogenin used for synthesis of sex hormones-cortisone and oral contraceptive.
- Consumption of fresh leaf of costus pictus maintains blood glucose in diabetic patients.
- Juice of Rhizome is utilized for cooling and relief from headache.
- Bruised leaves are applied in fever decoction of stem is used in fever and dysentery.
- It used as Antidiabetic,Antioxidant,Antibacterial,Anticancer
- The rhizomes have anti-fertility, anabolic properties.
- It also used as cardiotonic, hydrochloretic, diuretic and CNS depressant.

2. MATERIAL AND METHODS

2.1Chemicals

Formalin, acetic acid, ethyl alcohol, choral hydrate, phloroglucinol, hydrochloric acid, n-hexane and all other chemicals used in this study were of analytical grade. Standard diosgenin was procured from the Durvesh Labchem Pvt Ltd, Dist.Palghar, Mumbai.

2.2Plant collection and authentication

The leaves of the *Costus pictus* selected for present study was collected from Dr.Gour, Rock Garden, G Amsapuram, Dist.Theni, and TamilNadu, India during the month of August 2015(Figure 1).The plant specimen was identified and authenticated as '*Costus pictus'*(Costaceae)-BSI/WRC/Cert./2016/635 by Dr.Priyanka Ingle, Scientist, Botanical Survey of India, Pune, Maharashtra, India.

2.3 Isolation of Diosgenin

The diosgenin of plant from the Costaceae family was collected from *Costus pictus*.Leaves of the plant were collected, washed n kept for sundried for 24 hrs.After that dried leaves was grind for obtained fine and coarse powdered of leaf drug(500gms).Pigments and chlorophyll was removed by treating powdered drug with pet ether and chloroform. Obtained material was dried.500gms fine ground was weighed and mixed with a solution of sodium acetate (20mg) in 1 litre water.The mixture was allowed to stand for 24 hrs and then hydrolyzed with 5% Hydrochloric acid(2 litres) for about 14 hrs. After 14 hrs the hydrolysate mass was filtered and repeatedly washed with water until the it free from acid. The residue was dried and extracted with hexane in a soxhlet apparatus for 4 hrs.Concentration of hexane extract gaved crude diosgenin as slightly yellow solid. Solid was crystallized by adding 95% Ethanol to colorless needles (diosgenin) [14].

Figure no 1-Habit of Costus *pictus*

Figure no 2- Plant of *Costus pictus*

Figure no 3- Isolation of Diosgenin

Table 1: Comparative account of the three species of *Costus*

Parameter	***C.pictus***	***C.speciosus***	***C.igneus***
Habit	Perennial herb	Perennial herb	Perennial herb
Leaves	Narrow with wavy Edges	Large, pubescent and dark green	Large, smooth, dark green with purple undersides
Flower	Yellow with reddish Stripes	White ,crepe paper	Orange in colour
Bracts	Large, greenish	Large, reddish	Large, greenish
Seed	Minute, black with white fleshy aril	Minute, black with white fleshy aril	Minute, black with white fleshy aril

Table 2: Medicinal importance of different parts of *Costus* species

Plant part used	**Phyto-Constituent**	**Activity**
Whole plant	Diosgenin	Astringent, aphrodisiac, purgative, anthelmintic, depurative and expectorant.
Roots	Diosgenin, sitosterol, dioscin, gracillin, cycloartanol, Cycloartenol and cycloalaudenol.	Antibacterial, antifungal, tonic, expectorant and stimulant.
Rhizomes	Diosgenin, dioscin, gracillin and Beta-sitosterol.	Antispasmodic,antidiabetic, anti-inflammatory,antivermin, antiarthritic, cardiotonic, Hydrochloretic, diuretic and CNS depressant.
Leaves	Diosgenin	Fever, dysentery, diabetes, eye and ear infections, diarrhorea and mental disorders.

2.4 Macroscopic analysis

Macroscopic observation of the plant was done.

Color-Greenish yellow in colour.

Odour- Sweet odour was evaluated by smell of powder, characteristic taste and odour.

Shape-Fresh leaves are simple and looking spirally arranged, oblong lanceolate being dark green. Above and lighter green, narrowly elliptic with length 10 to 25cm and width 2.5 to 6cms.

2.4.1 Microscopic analysis

Transverse section midrib region of fresh leaf pieces were cut and fixed in FAA and then dehydrated by employing graded series of ethyl alcohol and tertiary butyl alcohol 23. Sections were taken using microtome. The sections were then stained with toludine blue as per standard procedure 24. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. The sections were also stained with saffranin and fast-green and iodine wherever necessary.

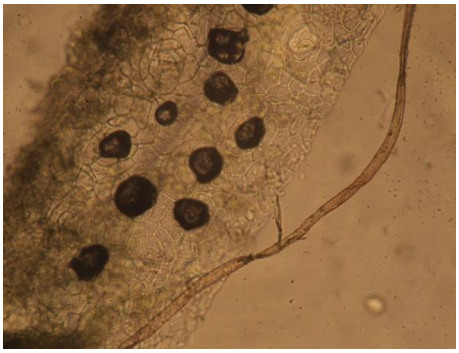


Figure no 4-Mesophyll tissue

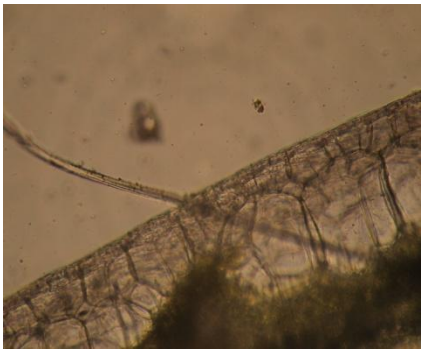


Figure no 5- Trichome

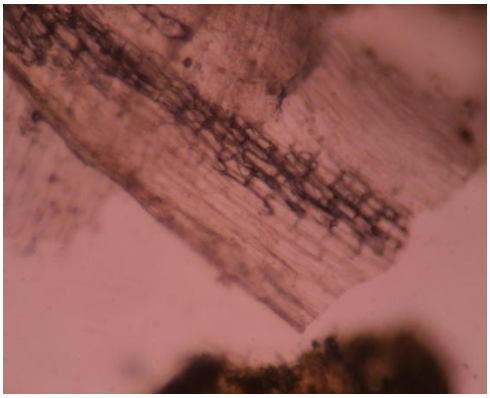


Figure no 6- Venation pattern of lamina

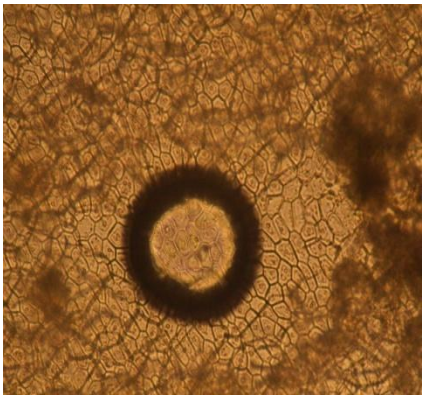


Figure no 7- Vascular bundles

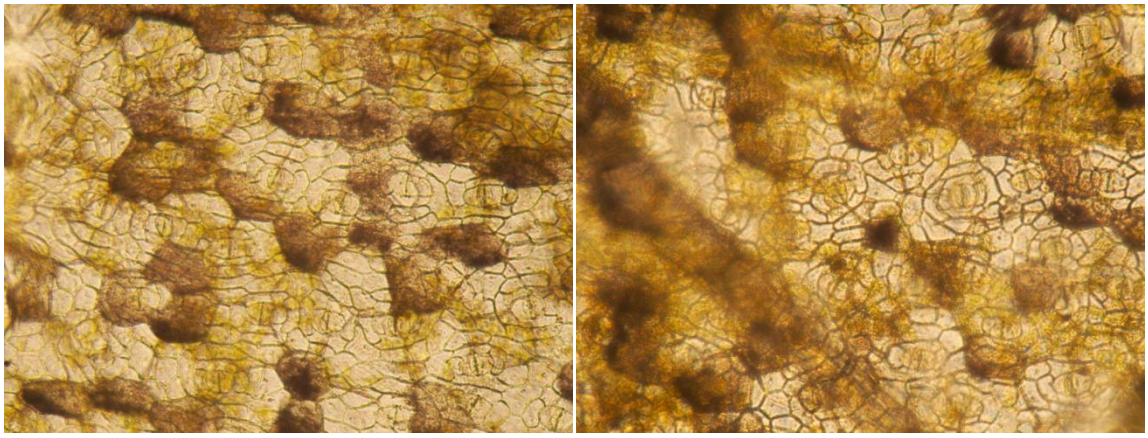


Figure no 8-Paradermal section showing stomata on the Abaxial Epidermis

2.4.2 Leaf constants

Quantitative analytical microscopy is useful for the measurement of cell contents of the crude drug and thus helps in their identification, characterization and standardization. Leaf constants such as vein islet numbers, vein terminal number, stomatal number, stomatal index and palisade ratio were determined[1,16]

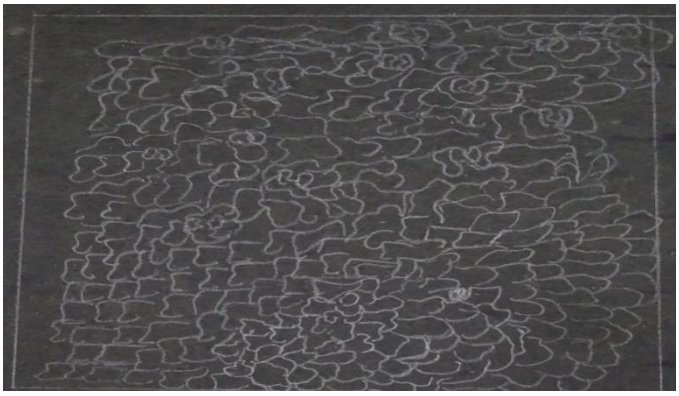


Figure no 9-Leaf constants

Table 3: Quantitative analytical microscopical parameters of the *Costus pictus*

Sr.No	Parameters*	Value obtained
1.	Stomatal number(lower epidermis)	13±0.27
2.	Stomatal index(lower epidermis)	10.37±0.23
3.	Vein islet number	4.0±0.057
4.	Vein termination number	3.8±0.088

*mean of 6 readings ± SEM

2.4.3 Physicochemical analysis

Total ash, acid insoluble ash, water soluble ash, loss on drying, extractive values, foaming index, swelling index and moisture content were performed as per standard procedure.

I. Loss on drying (LOD)

It is an gravimetric method to determine the moisture content in a powder. Weight of empty porcelain dish was taken and noted as W1. 1.5g of powder was weighed in porcelain dish and weight was noted as W2.Porcelain dish containing 105 g powder was kept in an oven at 100^0c until two consecutive readings of weight differ by more than 0.5 mg.[9] There was no significant difference between three consecutive weight in gm taken at one hour interval. As per limit it should not be more than 0.5 mg, values obtained are within the limits.

II. Total Ash value

It is used to determine quality and purity of crude and to establish the identity of it, used to determine foreign inorganic matter present as an impurity. Weight of empty crucible was noted as X. 2gms of powder was weighed in crucible dish and weight was noted as Y. Powdered was kept for incineration at 450^oc until all carbon is burnt off. After that cooled in a desiccators and total ash were calculated.

Table 4: Standardization parameters of leaves of Costus pictus

Sr.No	Parameters*	Values* expressed as
1.	Moisture content	12.37±0.032
	Ash value	
	Total ash	15.24±0.140
2.	Acid insoluble ash	3.15±0.080
	Water soluble ash	9.60±0.110
3.	Foreign organic matter	0.06±0.080
4.	Extractive values	
	Petroleum ether	25.74±0.150
	Chloroform	7.19±0.300
	Ethyl acetate	8.60±0.030
	Ethanol	5.10±0.110
	Methanol	16.24±0.040
	Water	11.12±0.600
	Benzene	5.76±0.080
5.	Foaming index	Less than 100
6.	Swelling index	Expressed as ml
	Initial volume	3.2±0.10
	Final volume	7.6±0.140

2.4.4 Chemical tests

I. Tests for Carbohydrates:

a) Molisch‘s test: The sample powder solution was treated with few drops of alcoholic α-napthol. Add 0.2 ml of concentrated H2SO4 slowly through the sides of the test tube, Purple to violet color ring appears at the junction.

b) Benedict‘s test: The sample powder solution was treated with few drops of Benedict‘s reagent (alkaline solution containing cupric citrate complex) followed by boiling on water bath and reddish brown precipitate was observed to check reducing sugars are present or not.

c) Barfoed‘s test: (General test for monosaccharide) The test tube containing 1ml reagent and 1 ml of sample powder solution heated in a beaker of boiling water. Precipitate of red cuprous oxide was observed to check monosaccharides are present or not. Disaccharides on prolonged heating (about 10min) may also cause reduction, owing to partial hydrolysis into monosaccharide.

II. Tests for steroids and triterpenoids:

a) Salkowski test: Sample was treated with 2 ml chloroform and 2 ml conc. H2SO4.After sometime, chloroform layer appeared red in the lower layer and acid layer shows greenish yellow fluorescence.

III. Tests for reducing sugar:

a) Fehling‘s test: Mix 1 ml of Fehling‘s solution A and B and heat on a water bath for 2 minutes. Add sample powder. Heat on water bath for 5- 10 min. First yellow, then red ppt observed.

IV. Test for anthraquinone glycosides:

a) Borntrager‘s test: Sample powder treated with dil.H2SO4, boiled and filtered. To cold filtrate, equal volume of benzene or chloroform was added. The organic solvent Separated and ammonia added. Ammonical layer turns pink or red.

b) Modified Borntrager‘s test:To sample powder add 5 ml 5% FeCl3 and 5ml diluted HCl added, boiled and filtered. To cold filtrate, benzene or chloroform was added. Shake well. Organic solvent separated and equal volume of dilute ammonia added to it. Ammonical layer showed pinkish red color.

V. Test for coumarin glycosides:

a) Powder when made alkaline, shows blue or green fluorescence.

b) Take moistened dry powder in test tube. Cover test tube with filter paper soaked in dilute NaOH. Keep in water bath. After sometime expose filter paper to UV light it showed yellow-green fluorescence.

VI. Test for alkaloids:

a) Mayer‘s test (Potassium mercuric iodide solution): 2-3 ml powder solution, add few drops of Mayer‘s reagent, creamy white precipitate is produced.

b) Dragendroff‘s test (Potassium bismuth iodide solution): 2-3 ml powder solution, add few drops of Dragendroff‘s reagent, reddish brown precipitate is produced.

c) Wagner‘s test (Solution of Iodine in Potassium Iodide): 2-3 ml of powder sample solution; add few drops of Wagner‘s reagent, reddish brown precipitate is produced. d) Hager‘s Test (Saturated solution of Picric acid): 2-3 ml of powder sample solution, add few drops of Hager‘s reagent, yellow precipitate is produced.

VII. Tests for tannins compounds:

a) Lead acetate solution: Powder with lead acetate gives white precipitate.

b) Bromine water: decoloration of bromine water.

c) Dilute iodine solution: transient red colour.

VII. Tests for phenolic compounds:

a) Ferric chloride test: Powder gives blue-green color with few drops of Fecl3.

b)Dilute Potassium permanganate solution: decoloration.

VIII. Tests for flavonoids:

a) Shinoda test (Magnesium Hydrochloride reduction test): Powder was treated with 5 ml of 95% ethanol, few fragments of magnesium turnings and concentrated hydrochloric acid drop wise so pink or red color appears after few minutes.

b) Sulfuric acid test: To the powder add water and few drops of Sulfuric acid; formation of an intense deep yellow solution.Chalcones and aurones gives red or red bluish solutions. Flavanes give orange to red colours[16].

Table 5: Results of chemical tests

Sr.No	TEST	OBSERVATION	RESULT
1.	Carbohydrates- a) Molisch's test b) Benedict's test c) Barfoed's test	Violet ring at junction Green color appears Red precipitate	Carbohydrates present Carbohydrates present. Carbohydrates present.
2.	Steroids and Triterpenoids- Salkowski test	Acid layer shows green fluorescence	Steroids present
3.	Reducing sugar- Fehlings test	Red precipitate	Reducing sugar present
4.	Anthaquinone glycosides-	Ammonical layer do not show pink color	Anthraquinone glycosides absent
5.	Alkaloids- a)Mayer's reagent b)Dragendroff's reagent c)Wagner's reagent d)Hager's reagent	Precipitate observed. Orange color observed. Reddish brown color observed Yellow precipitate observed	Alkaloids present Alkaloids present Alkaloids present Alkaloids present
6.	Tannins- a)Lead acetate solution b)Bromine water c)Dilute iodine solution	White precipitate not observed. No decoloration. No transient red color observed	Tannins absent Tannins absent Tannins absent
7.	Phenolic compound- a)Ferric chloride solution b)Dilute Potassium permanganate solution	Blue green color observed decoloration observed	Phenolic compound present Phenolic compound present
8.	Flavonoids- a)Shinoda test b)Sulphuric acid test	Orange,pink,red,purple color observed Orange color observed	Flavonoids present Flavonoids present

3. ANALYTICAL EVALUATIONS

I. Melting point Determination:

- **Melting point of Standard diosgenin:205^0c-212^0c**
- **Melting point of Isolated diosgenin:208^0c**

II. pH Determination:

pH was determined using litmus paper. pH of dissolved powder sample in hot water was found to be slightly acidic in nature.

III. Solubility:

10 mg of powder was taken and dissolved in 5 ml of various solvent like cold water hot water, benzene, di-methyl –sulphoxide, pet ether, ethanol, Ethyl acetate, chloroform, N-hexane Sonicated and observed visually to check solubility, Powder sample was found to be dissolved in hot water, Ethyl acetate and chloroform and slightly soluble in cold water and 0.1 N Hydrochloric acid.

IV. Infra-red (IR) spectroscopy:

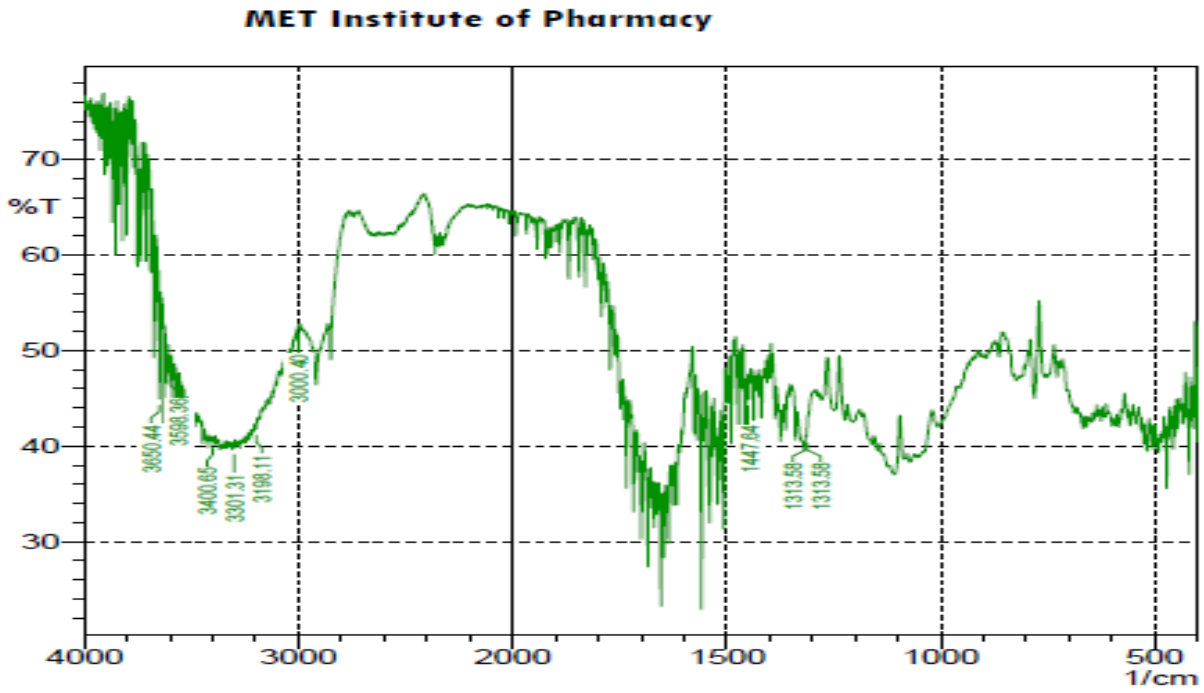


Figure no 10- IR spectrum of Isolated diosgenin

Table 6: Functional group with wave numbers

Reported wave number (cm^{-1})	Observed wave number (cm^{-1})	Functional group
1000-1400	1313.58	C-H bend
1400-2000	1447.64	C-H stretch
3400-3200	3400.65	N-H stretch
3600-3650	3650.44	O-H stretch

V.Thin layer chromatography

Sample preparation:1) Prepared hydro alcoholic extract of *costus pictus* leaf (control)

2) Prepared hydro alcoholic extract of *costus pictus* leaf (standard)

Various mobile phases were tried in different ratios, but good result was obtained by using the mobile phase: Toluene: Ethyl acetate: Methanol (5:3:2) TLC plate was observed under UV cabinet absorbance at 254nm & 366nm. Rf value was calculated.

Rf value for standard=1.052 Rf value of control=1.048

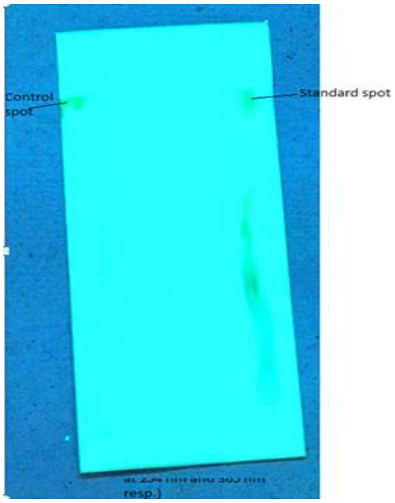


Figure no 11-Chromatogram of standard and control hydro alcoholic extract at 254nm and 366nm

VI. High performance liquid chromatography analysis

TITLE: HPTLC FINGERPRINTING OF RAW MATERIAL

NAME OF SAMPLE: COSTUS PICTUS

Instrument: CAMAG Linomat 5 "Linomat5-210175" S/N 210175 (1.00.13)

Scanner: CAMAG TLC scanner 4

Standard preparation: 10mg of Standard diosgenin and isolated diosgenin individually was dissolved in 10ml of methanol each.

Table 7: Sample Application: CAMAG Linomat 5

Track Number	Name of Samples	Applied volume	Vial
1.	Control costus pictus	5µl	1.
2.	Standard costus pictus	5µl	2.
3.	Diosgenin	8µl	3.
4.	Isolated diosgenin	8µl	4.

Chromatography:

Solvent system: Toluene: Ethyl acetate: Glacial acetic acid: Formic acid (4:2:2:1.4)

Stationary phase: 7× 10 cm TLC silica gel 60 F_{254} (Merk)

Chamber type: 10×10 –cm twin-trough chamber.

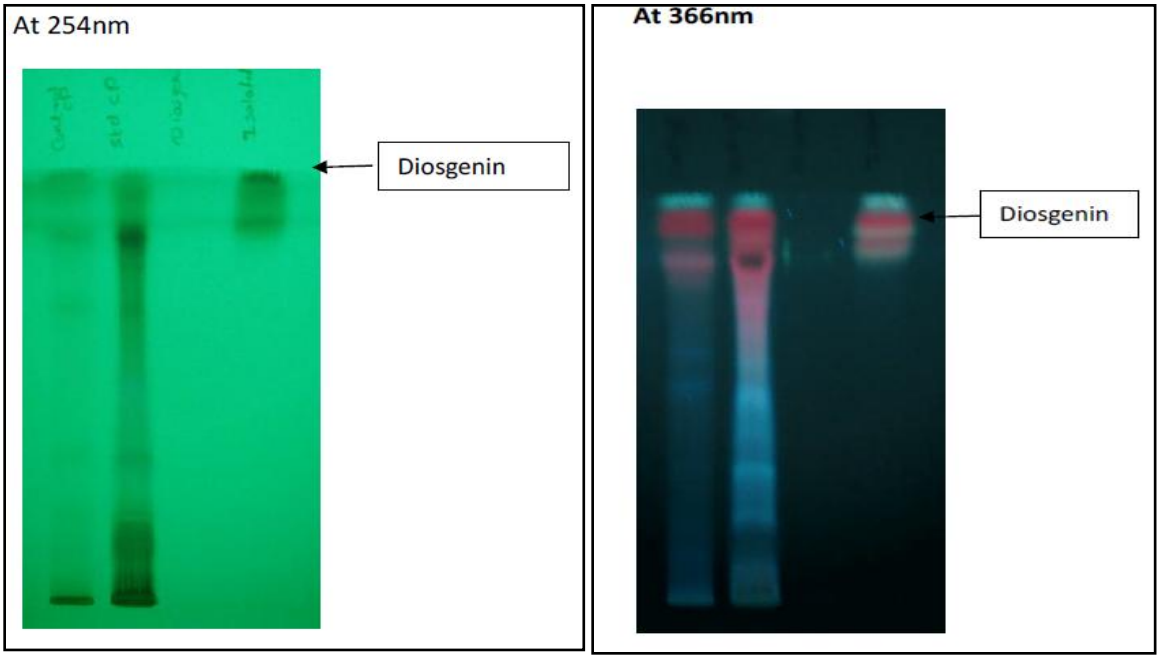
Saturation time: 20 minutes **Developing distance:** 80mm from lower edge of plate.

Drying: 5min hair dryer.

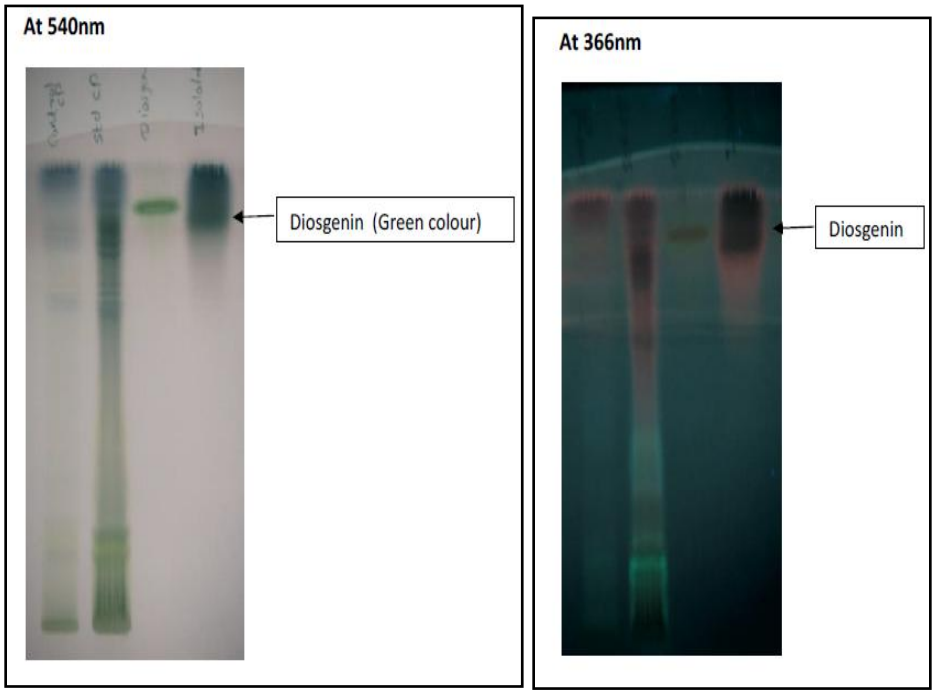
Derivatization: The plate was immersed into Anisaldehyde reagent for 1s then heated at 110˚C for 5 minutes.

Images of chromatogram:

Before Derivatization:



After derivatization:



Scanning: Table 8 :Results

Wavelength	RF value	Sample
Before derivatization		
At 254 nm		
254nm	0.88	Control costus pictus
254nm	0.88	Standard costus pictus
254nm	0.90	Diosgenin
254nm	0.92	Isolated diosgenin
At 366nm		
366nm	0.90	Standard costus pictus
366nm	0.92	Isolated diosgenin
After Derivatization:		
At 540nm	0.88	Control costus pictus
540nm	0.88	Standard costus pictus
540nm	0.92	Diosgenin
540nm	0.89	Isolated diosgenin
At 366nm	0.91	Control costus pictus
366nm	0.90	Diosgenin

3. RESULT AND DISCUSSION

The pharmacognostical studies of leaves of *Costus pictus* D. Don gave the valuable information regarding the morphology of crude drugs. They can be useful for the authentication of this plant among all species of *Costus*. The microscopic character, leaf constants, quantitative analysis and physico-chemical parameters studied are useful for setting standards for crude drug and to judge the adulteration and purity of this drug. Chemical test and analytical evaluations clarified the present of components and its value quantitatively. By analytical investigation like IRspectra and chromatographic analysis diosgenin was evaluated. Since the parameters are constant and any change in these values are indicative of substitution and adulteration of the plant materials.

4. CONCLUSION

It can be concluded that the Costus pictus (control) and costus pictus (standard) shows the presence of similar secondary metabolites by chemical tests, microscopical, analytical evaluations Quantitative analytical microscopy is useful for the measurement of cell contents of the crude drug and thus helps in their identification, characterization and standardization and provides useful information about crude drug.

5. ACKNOWLEDGEMENT

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