ORIGINAL ARTICLE

Cytomorphological studies and HPTLC fingerprinting in different plant parts of three wild morphotypes of *Datura metel* L. "Thorn Apple" from North India

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Datura metel L. (Solanaceae) with its trade name "Thorn Apple" is a herb that exists in tropical, warm temperate regions of the old world, throughout the hills of India, up to an altitude of 8000 ft. The objective of the present study is to investigate the morphological and phytochemical variabilities in the wild taxa of *D. metel*. On the basis of 17 phenotypic traits three morphotypes I, II and III were identified, and highly significant variations were seen among them. The three morphotypes of the species, with quite distinct flower colours, that is, purple, yellow and white, had the same diploid chromosome number n=12. The pharmacological activities of *D. metel* are mainly attributed to the presence of two tropane alkaloids, namely hyoscyamine and scopolamine. For the systematic quantification of hyoscyamine and scopolamine in different plant parts such as, leaves, roots and seeds of these three wild morphotypes (I, II and III) of *D. metel*, the high performance thin layer chromatography (HPTLC) technique was employed. Better resolution was achieved by using chloroform:acetone:diethyl amine (50: 40: 10 v/v/v) as a mobile phase. Quantitative, densitometric evaluation of the plate was performed in the absorbance/reflectance mode at 530 nm. The average recovery of hyoscyamine and scopolamine was 97.4 and 96.8%, respectively, showing the excellent reproducibility of the method. The calibration curves were linear in the range of 1000–4000 ng for hyoscyamine and 500-2000 ng for scopolamine, respectively. The method was simple, precise, specific, sensitive, accurate and could be used for routine analysis as well as quality control of raw materials and herbal formulations. The present study has established a link between cytomorphological variations and chemical characterization for the first time and was also helpful in discovering the best genotype with richer active constituents for future herbal formulations.

Key words: Cytotypes, *datura metel* L, morphotypes, high performance thin layer chromatography (HPTLC), hyoscyamine, scopolamine

INTRODUCTION

"Thorn Apple" *Datura metel* L. (Solanaceae) is a sub-glabrous shrubby herb that exists throughout the world. It is probably a native of America, but it has long been introduced and naturalized in Asia. It is found in tropical, warm temperate regions of the old world, throughout the hills of India, up to an altitude of 8000 ft. and is common in the north-western Himalayas. It is an annual, erect herb and may grow up to 1m in height. In general, the leaves are alternate, simple, triangular ovate to ovate-lanceolate and unequal at the base, with entire margins. Flowers vary in colour, with an elongated calyx tube, which is green in colour. Fruits are capsules, covered with spines. Flowering and fruiting commonly occurs in the months of April to October.[1,2] The *D. metel* plant has been well known for its use in traditional Chinese and Indian systems of medicine for centuries as a narcotic, anodyne and antispasmodic,[3] and a lot of botanical pharmacognostic studies are also known. The seeds are considered to have a strong aphrodisiac effect. According to Ayurveda, the seeds are acrid, bitter, tonic, febrifuge, anthelmintic, alexiteric, emetic and useful in leucoderma, skin disorders, ulcers, bronchitis, jaundice, piles and diabetes.[4] The pharmacological activities of *D. metel* are mainly attributed to the presence of tropane alkaloids.[5] Two main tropane alkaloids, namely, hyoscyamine and scopolamine, the main active constituents of *D. metel*, are responsible for anticolinergic and central nervous system (CNS) properties.[6-10] Industrially, these natural substances are exclusively produced by plants and are in high demand.[11] Scopolamine (also called hyoscine) is an antimuscarinic agent used as an analgesic and smooth muscle relaxant. It is also an antispasmodic agent with antinauseant properties, extensively used in the treatment of motion sickness and in pre-operative medication.[12-14]

Germplasm of *D. metel* was collected from different regions of Punjab and Himachal Pradesh. The Indian genetic resources, wild as well cultivated, showed a

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Received: 12-09-2008; **Accepted:** 02-01-2009; **DOI:** 10.4103/0973-8258.49373

number of morphological and phytochemical variabilities, which remain largely undocumented. Thus the systematic cytomorphological and chemical characterization of *D. metel* is of great significance, for future programmes on quality enhancement of hyoscyamine and scopolamine for herbal formulations. Different analytical methods and techniques have been applied for the analysis of these tropane alkaloids, including spectrophotometry, thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), HPTLC, high-speed counter current chromatography (HSCCC), gas chromatography (GC), liquid chromatography-ultraviolet-mass spectrometry (LC-UV-MS) and liquid chromatography-nuclear magnetic resonance (LC-NMR).[15-22] However, there is no HPTLC report on the simultaneous quantification of hyoscyamine and scopolamine in different plant parts such as the leaves, roots and seeds of these three wild morphotypes of *D. metel*. In most of the herbal preparations, the medicinal plants have been utilized without taking into account their different variants. At present, it is a well known fact that many medicinal plants have a number of cytological variabilities at the same ploidy level and a number of morphological variabilities with different chemotypes, which differ in their contents of active principles. In spite of the use of synthetic drugs, now the trend is going towards herbal drugs because they are safe. Hence, keeping this view, there is a need to document the complete morphological, cytological and chemical characterization of *D. metel*, so that one of the better morphotypes of this plant species can be used for further herbal formulations. Although many studies have been performed separately by a number of workers, there is a need for a combined approach to identify these three wild morphotypes by using cytomophological and phytochemical parameters.

Thus, in continuation of our attempts on the chromatographic determination of secondary metabolites in medicinal plants,[23-27] the present study is aimed at identifying three wild morphotypes of *D. metel*, linked to phenotypic variabilities with chemical relatedness, by using the HPTLC fingerprinting method, based on two important chemical markers such as hyoscyamine and scopolamine, and also to find the best genotype with richer active constituents for future herbal formulations.

MATERIALS AND METHODS

Plant Materials

Samples of *D. metel* were collected from different areas of Punjab: Hoshiarpur, Patiala and Kangra of Himachal Pradesh in the months from July to November, 2006. They were authenticated by the Department of Botany, Punjabi University, Patiala (Punjab) India.

Morphometric Studies

Data of average morphological characters of 10 plants were recorded for each morphotype. On the basis of 17 phenotypic traits, these three morphotypes (I, II and III) showed highly significant variations from each other.

Cytological Studies

Floral buds were collected from the study areas in Carnoy's fixative for 24 hours and preserved in 70% alcohol at 4^0C for further use. For meiotic studies, a smear was made using the standard acetocarmine technique to analyse cytological variability. Pollen analysis was made by mounting pollens from mature flowers in 50% glycerol-acetocarmine and by taking well-filled pollen grains with stained nuclei as apparently viable. The photomicrographs of the chromosome count were made from permanent and freshly prepared slides, with the help of Leica Qwin Digital Imaging System.

Chemical Characterization and Validation of HPTLC Densitometry Method

Chemicals

All the chemicals, including solvents, were of analytical grade from E. Merck, India. The HPTLC plates Si $60F_{254}$ (20 cm × 10 cm) were purchased from E. Merck (Darmstadt, Germany). The standards hyoscyamine and scopolamine were purchased from Sigma (New Delhi, India).

Preparation of Sample and Standard Solutions

The air dried (1.0 gm) parts of each of the different plant parts (leaves, roots and seeds) of all the three morphotypes of *D. metel* were extracted thrice, with 20 mL of methanol, for 45 minutes, in a sonicator. After mixing they were filtered and vacuum dried at 45°C. The dried extracts were re-dissolved in 2 mL of methanol and samples of the varying concentrations (5 µL of seeds, 10 µL of roots and 20 µL of leaves) were spotted for quantification. Standard solutions of hyoscyamine and scopolamine (2.5 mg/ 10 mL) were prepared in methanol, and different amounts of hyoscyamine and scopolamine were loaded onto a TLC plate to get the calibration curve.

High Performance Thin Layer Chromatography

A Camag HPTLC system equipped with an automatic TLC sampler ATS_4, TLC scanner 3 and integrated software winCATS version 1.2.3, was used for the analysis. HPTLC was performed on a pre-coated silica gel HPTLC $60F_{254}$ (20 cm × 10 cm) plate of 200 µm-layer thickness, for the quantification of hyoscyamine and scopolamine in *D. metel*. The samples and standards were applied on the plate as 8 mm wide bands with a constant application rate of 150 nL s^{-1}, with an automatic TLC sampler (ATS_4) under a flow of N_2 gas, 15 mm from the bottom, 15 mm from the side, and the space between two spots was 6 mm of the plate.

Detection and Estimation of Hyoscyamine and Scopolamine

The linear ascending development was carried out in a Camag twin through chamber (20 cm × 10 cm), which was pre-saturated with a 25-mL mobile phase, with chloroform: acetone: diethyl amine (50: 40: 10 v/v/v), for 30 minutes, at room temperature (25°C ± 2°C) and 50 ± 5% relative humidity. The length of the chromatogram run was up to 90 mm. Subsequent to the development; the TLC plate was dried in a current of air, with the help of an air dryer, in a wooden chamber with adequate ventilation. The dried plate was dipped into freshly prepared Dragendorff's reagent. Quantitative evaluation of the plate was performed in the absorption-reflection mode at 530 nm, using a slit width 6 × 0.3 mm, with data resolution 100 mm $step^{-1}$ and scanning speed 20 mm s^{-1}, and the baseline correction was used. The source of radiation utilized was a tungsten lamp emitting continuous visible spectra of 370 and 700 nm. Determination of hyoscyamine and scopolamine in different extracts was performed by the external standard method, using pure hyoscyamine and scopolamine as standards. Each sample was carried out in triplicate.

Calibration Curve and Linearity

Stock solutions of hyoscyamine and scopolamine were prepared in methanol and different amounts, (4, 8, 12 and 16 µL for hyoscyamine) and (2, 4, 6 and 8 µL for scopolamine), were loaded onto a TLC plate, using ATS 4 for preparing four-point calibration curves. The calibration curves were performed by analysis of working standard solutions of hyoscyamine and scopolamine with at least four different concentrations ranging between 1,000-4,000 ng for hyoscyamine and 500-2,000 ng for scopolamine. Each concentration was spotted and measured in triplicate. The regression equation and correlation curves for hyoscyamine were, Y = 2893.107 + 5638.031X and 0.99973 and for scopolamine were, Y = -1583.434 + 3.934X and 0.99956.

Selectivity and Accuracy

Each compound was separated with baseline return. For accuracy, the pre-analysed samples, 125 and 250 ng each of hyoscyamine and scopolamine were added and the mixture was analysed by the proposed method. The experiment was conducted in triplicate to check the recovery and accuracy of the system. The results are summarized in Table 1, showing the accuracy (expressed as recovery) of the method as mean values and the % CV values of hyoscyamine and scopolamine.

Limits of Detection and Quantification

In order to estimate the limit of detection (LOD) and limit of quantification (LOQ), blank methanol was spotted six times following similar conditions and methodology as mentioned above. The signal-to-noise ratio was determined as 3:1 and 10:1 for LOD and LOQ, respectively. The limits of detection for hyoscyamine and scopolamine were 60 ng and 25 ng, respectively, and the limits of quantification were 1000 ng for hyoscyamine and 500 ng for scopolamine.

Precision

System precision was performed by spotting six samples of the same concentration, each from the stock solutions of hyoscyamine (1000 ng) and scopolamine (500 ng), on the silica gel $60F_{254}$ plate, and analysing them with the proposed method. Six different samples of the same concentration were spotted on a plate and analysed by the proposed method to determine the variation arising from the method itself. The results are summarized in Table 1.

RESULTS

Morphological Variations

The data on the average morphological characters of 10 plants were recorded for each morphotype on the basis of 17 phenotypic traits. These three morphotypes showed highly significant variations from each other [Table 2]. However, the colour of flowers (purple, yellow and white) remained the most important morphological character, on the basis of which all the three morphotypes could be differentiated [Figure 1a-l]. Morphotype-I had a dark violet stem and purple flowers with simple corolla tubes, morphotype-II was characterized by a green stem and simple white infundibuliform flowers and morphtype-III had a green stem and yellow flowers with two to three corolla tubes. According to one research report the varieties of *D. metel* have been distinguished on the basis of stem colour, flower colour and the number of corolla tubes.[28] In a few reports, detailed examinations of leaves, stomatal

Table 1: Method validation parameters for the quantification of hyoscyamine and scopolamine by the HPTLC method

Compound	Accuracy				Precision		LOD (ng)	LOQ (ng)
	Amount present (ng)	Amount added (ng)	Amount found (ng)	Mean recovery (%)	Method precision (% CV) (n = 6)	System precision (% CV) (n = 6)		
Hyoscyamine	168.60	125	287.20	97.8	2.43	1.33	60	1000
		250	405.70	96.9				
Scopolamine	66.65	125	182.31	95.1	2.94	1.68	25	500
		250	311.43	98.4				

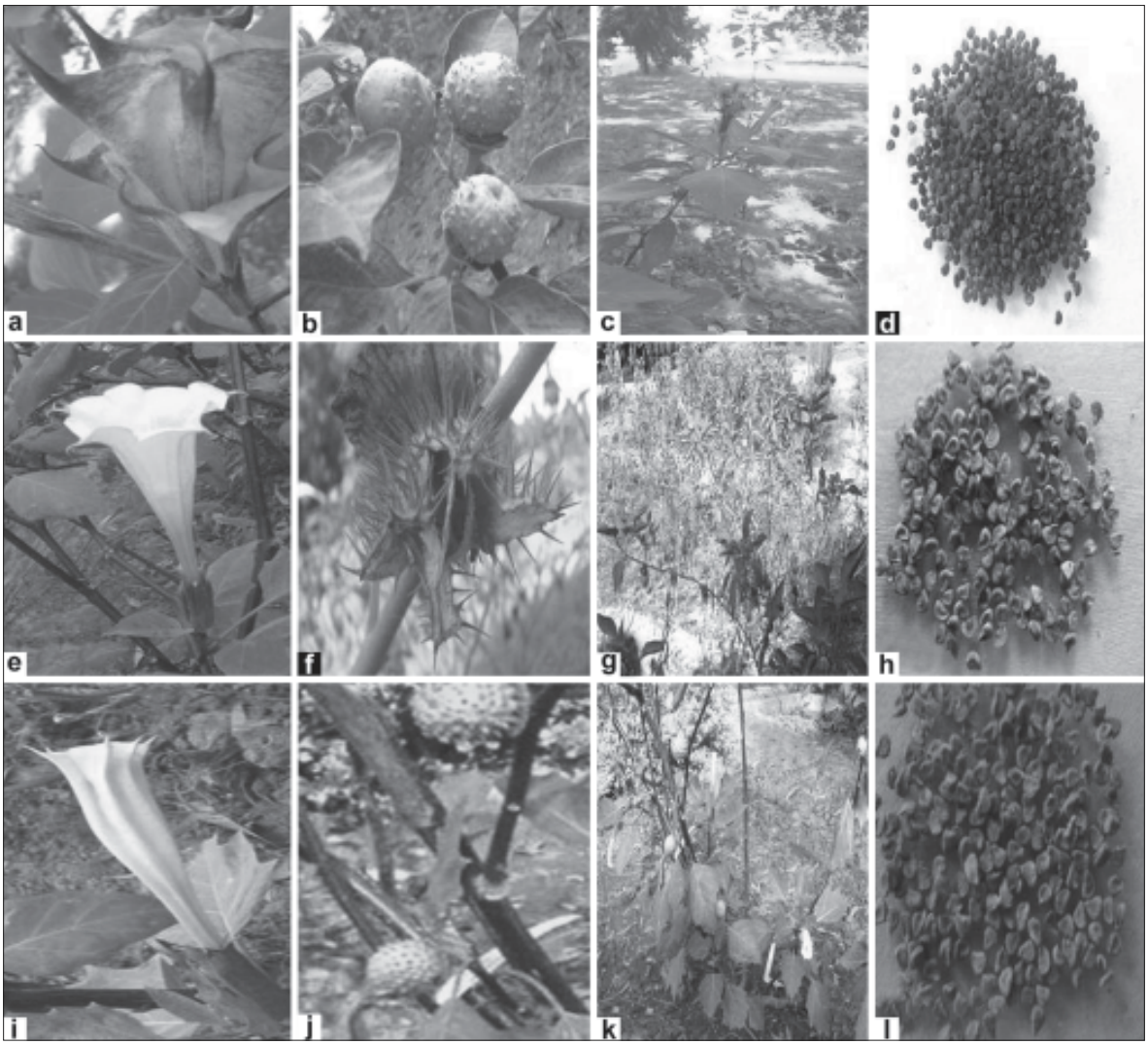

Figure 1: a-l: Datura metel: (a) Purple flower (Morphotype I); (b) Fruits with small and few spines (Morphotype I); (c) Whole plant (Morphotype I); (d) Black seeds (Morphotype I); (e) White Flower (Morphotype II); (f) Fruits with long and numerous spines (Morphotype II); (g) Whole plant (Morphotype II); (h) Dark yellow seeds (Morphotype II); (i) Yellow flower (Morphotype III); (j) Fruits with small and numerous Spines (Morphotype III); (k) Whole plant (Morphotype III); (l) Light yellow seeds (Morphotype III)

Table 2: Average morphological characters of 10 plants were recorded for each morphotype of *D. metel* on the basis of 17 phenotypic traits

Characteristics	Morphotype-I	Morphotype-II	Morphotype-III
Plant height	124.3 cm	105.2 cm	132.0 cm
Leaf colour	Dark green	Greenish	Greenish
Leaf size (tenth leaf from the base)	17.4 x 8.3 cm	12.5 x 6.5 cm	22.5 x 1.2 cm
No. of leaves/branch	23.8	22.7	17.3
No. of branches/plant	24.2	27.3	35.1
Length of fifth internode from base	9.5 cm	6.2 cm	7.3 cm
Flower colour	Purple	White	Yellow
No. of flowers/plant	7.4	10.5	8.3
Colour of fruit	Pale with green spines	Green with purple spines	Pale with green spines
Size of fruit with calyx cap	3.5 x 1.6 cm	2.2 x 1.3 cm	4.2 x 2.5 cm
Size of fruit without calyx cap	1.1 x 0.4 cm	0.7 x 0.6 cm	1.3 x 0.9 cm
Spines on fruit	Small and few	Long and numerous	Small and numerous
No. of seeds/capsule	43.2	35.3	47.0
Colour of seeds	Black	Dark yellow	Light yellow
Primary root length	24.23 cm	13.25 cm	28.11 cm
Root diameter (2 cm below base)	1.67 cm	1.34 cm	2.05 cm
Root colour	Light yellow	Dark yellow	Light yellow

numbers and seeds of various *Datura* species have already been performed.[29,30]

Cytological Studies

The extensive cytological study was carried out for each germplasm of *D. metel*. Cytologically all these three morphotypes showed equal distribution at the anaphase, with normal meiosis having diploid chromosome numbers 24 (n=12). However, the pollen grains of the three morphotypes showed variability in their size and shape [Figure 2a-i]. Intraspecific polyploidy within the species is well reported from India and from other countries.[31,32] Previously, a detailed study on the cytogenetics of one of the genus, *D. fastuosa* L., had also been performed.[33] The reduction division in haploid, diploid, triploid and tetraploid *Daturas*,[34] and geographical distribution of chromosomal types in *D. metel* have already been studied.[35]

Chemical Characterization

The compositions of the mobile phase for the development of the chromatographic method was optimized for better resolution of hyoscyamine and scopolamine, by testing different solvent mixtures of varying polarities. The best results, with well resolved, symmetrical and reproducible peaks of analytes, were obtained using chloroform: acetone: diethyl amine (50: 40: 10, *v/v/v*). Densitometric scanning of all the tracks showed peaks corresponding to hyoscyamine and scopolamine with R_f values of 0.56 and 0.76, respectively. Different parts (leaves, roots and seeds) of the three morphotypes (*D. metel* with purple, yellow and white

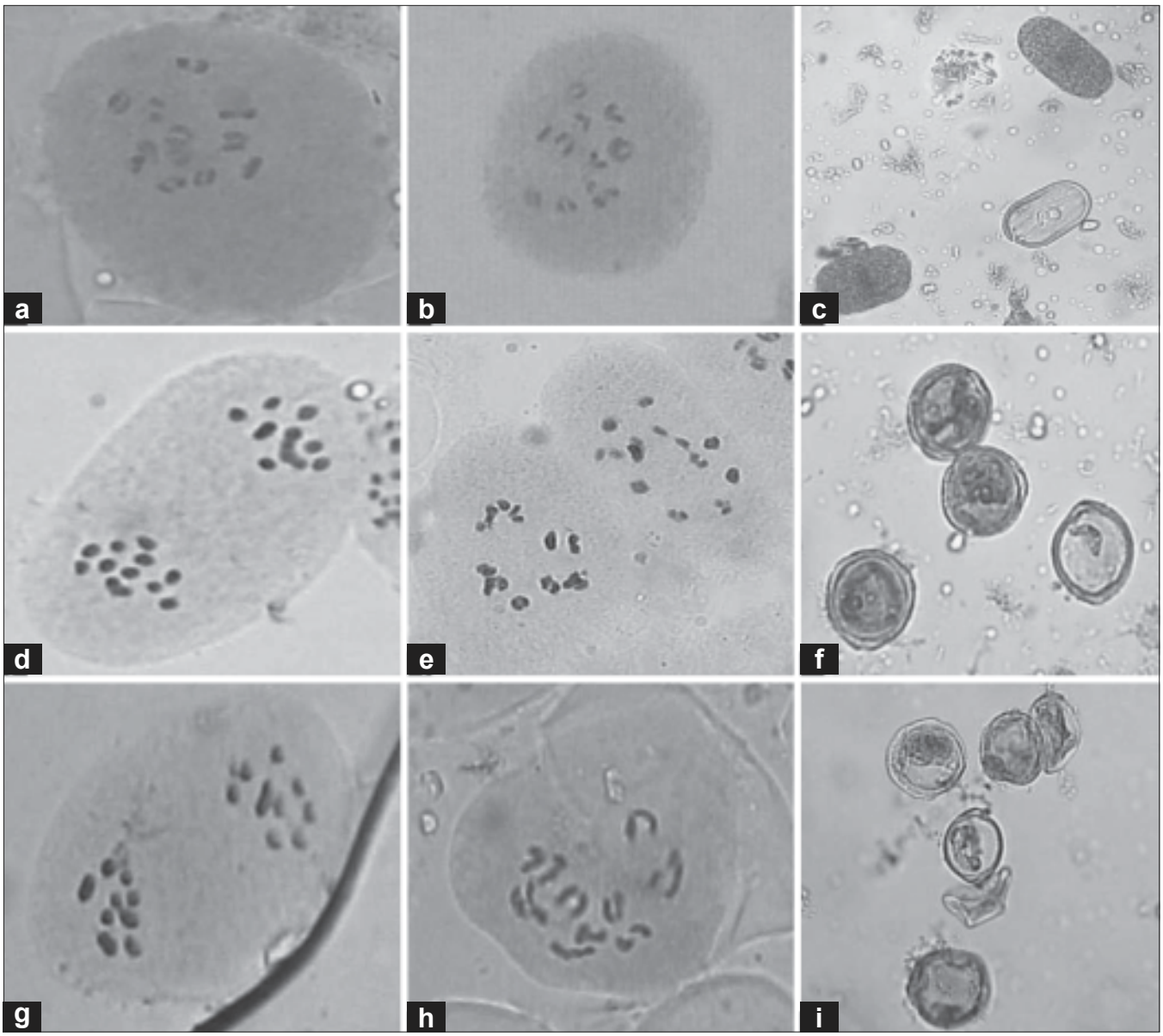

Figure 2: a-i: *Datura metel*: (a) Diakinesis - I (n=12) (Morphotype I); (b) Metaphase (n=12) (Morphotype I); (c) Eliptical and fertile spores (Morphotype I); (d) Anaphase- I (n=12) (Morphotype II); (e) Metaphase (n=12) (Morphotype II); (f) Round and fertile spores (Morphotype II); (g) Anaphase- I (n=12) (Morphotype III); (h) Metaphase-I (n=12) (Morphotype-III); (i) Round and sterile spores (Morphotype III)

flowers) were evaluated for the presence of hyoscyamine and scopolamine [Figure 3]. The identities of hyoscyamine and scopolamine, in the methanolic extract of *D. metel*, were determined by the comparison of R_f values and UV spectral characteristics of the peaks, for standards of hyoscyamine and scopolamine. The calibration curves were linear in the range of 1000-4000 ng for hyoscyamine and 500-2000 ng for scopolamine, respectively. Peak purity tests of hyoscyamine and scopolamine were also conducted by comparing UV visible spectra of hyoscyamine and scopolamine in the *D. metel* standard and sample track [Figure 4a-b]. Higher contents of hyoscyamine and scopolamine were observed in seeds of purple (585.40 ng/μL) and yellow (153.92 ng/μL) flower morphotypes, respectively, whereas, the lowest amount of both analytes was found in the leaves and parts of the yellow (51.40 ng/μL hyoscyamine, 42.41 ng/μL scopolamine) and white (56.15 ng/μL hyoscyamine, 41.36 ng/μL scopolamine) flower morphotypes of *D. metel.* The highest levels of hyoscyamine and scopolamine were observed in the seed of *D. metel*. In the present study, the higher contents of both alkaloids (hyoscyamine and scopolamine) were detected in the seeds followed by the root and leaf parts of all the three morphotypes, with an exceptionally high amount of scopolamine (107.85 ng/μL) in the leaves of the purple flower morphotype [Table 3].

PFL-I (Purple Flower Leaf of Morphotype-I); PFR-I (Purple Flower Root of Morphotype-I); PFS-I (Purple Flower Seed of Morphotype-I); WFL-II (White Flower Leaf of Morphotype-II); WFR-II (White Flower Root of Morphotype-II); WFS-II (White Flower Seed of Morphotype-II); YFL-III (Yellow Flower Leaf of Morphotype-III); YFR-III (Yellow Flower Root of Morphotype-III); YFS-III (Yellow Flower Seed of Morphotype-III)

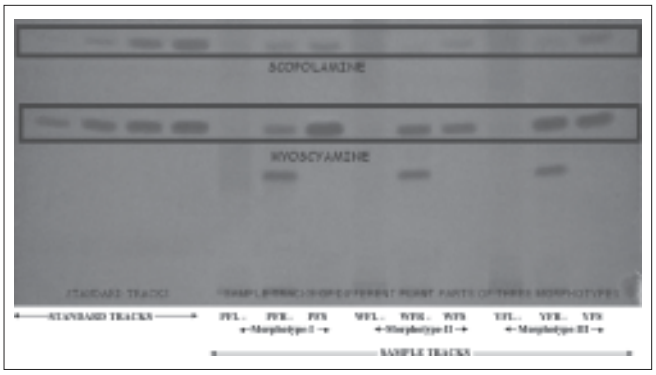

Figure 3: V.: HPTLC fingerprinting of different plant parts of three morphotypes of *Datura metel* at X = 530, showing best resolution of hyoscyamine and scopolamine by using mobile phase chloroform: acetone: diethylamine (50 : 40 : 10 v/v/v)

Table 3: Hyoscyamine and scopolamine content (ng/μL^{-1}) found in different plant parts in three morphotypes of *Datura metel* by the HPTLC method (*n* = 3)

Sample	Average hyoscyamine	%CV	Average scopolamine	%CV
PFL-I	99.25	3.22	107.85	1.32
WFL-II	56.15	2.34	41.36	1.87
YFL-III	51.40	2.43	42.41	2.46
PFR-I	168.60	2.67	66.65	2.32
WFR-II	230.10	2.02	55.69	3.33
YFR-III	288.50	1.86	79.93	3.42
PFS-I	585.40	1.56	116.55	1.47
WFS-II	351.0	2.86	132.10	2.85
YFS-III	206.20	1.97	153.92	1.87

DISCUSSION

High performance thin layer chromatography densitometry is a rapid, reproducible, accurate and selective alternative to HPLC for the separation of hyoscyamine and scopolamine in *D. metel.* From the study conducted, it can be concluded that all three morphotypes (I, II and III) differ in their morphological and chemical characteristics. The advantage of TLC is the high sample throughput, which results from the small amount of sample preparation required and the simultaneous quantification of several samples. The presence of morphological differences coupled with differences in the chemical characterization indicate that these three morphotypes are quite distinct and there taxonomical status needs reconsideration on the basis of experimental studies including crossing and meiotic behaviour, to check for the genomic homology. However, a controversy still remains over the taxonomy of this species.

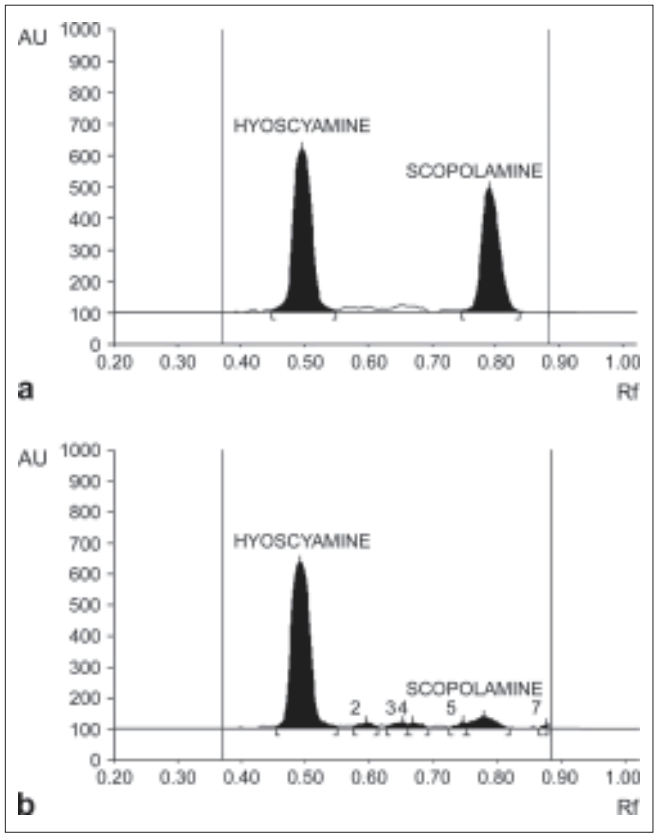

Figure 4: HPTLC chromatogram of (a) standards hyoscyamine and scopolamine (b) resolution of hyoscyamine and scopolamine in *Datura metel* in one of the sample

Do all these morphotypes belong to the same species *D. metel* or to some other one? Whether to keep these three morphotypes at the same level or to raise them to higher levels, such as, varieties, sub-species or even different species. In view of the above observations, it may be suggested that a critical re-examination be performed, with the help of further biochemical parameters, with respect to its morphotypes, to solve the taxonomical controversy of *D. metel*.

ACKNOWLEDGMENTS

The authors are grateful to Dr. P.S. Ahuja, Director, IHBT (CSIR), Palampur (H.P) for providing the necessary facilities, and part of this study is also supported by SAP-II DRS (UGC), Department of Botany, Punjabi University, Patiala (Punjab).

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Source of Support: SAP-II DRS (UGC),
Conflict of Interest: None declared.