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DNA Based Authentication of *Solanum melongena* var. *insanum* (V. Elabatu) Roots in Herbal Medicine Market to Circumvent the Use of Noxious Adulterant, *Solanum melongena* (V. Eggplant) Roots

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RUNNING TITLE: DNA based authentication of *Elabatu* roots

ABSTRACT

Elabatu roots are an important constituent of indigenous medicine which has frequently been adulterated with eggplant (brinjal) roots which is widely cultivated as a vegetable crop in Sri Lanka. Eggplant has no marked therapeutic value in Ayurvedic medicine and has been considered to have a high allergenic potential. Yet no studies have been conducted to date to investigate the illegal adulterations for Elabatu with eggplant roots. Therefore, the aim of the present study was to optimize a protocol to extract DNA from dried roots and to develop a DNA based method for accurate identification of Elabatu from eggplant cultivars. Further an attempt was also made to confirm the general observation that morphological traits cannot be used to differentiate the Elabatu from commonly grown eggplant cultivars in Sri Lanka. An efficient method of dried-root grinding for DNA extraction and a DNA extraction procedure were developed for Elabatu roots. The SSR markers used were monomorphic across eggplant cultivars and Elabatu, thus they cannot be used to find adulterations to Elabatu with eggplant. The ISSR marker was highly polymorphic thus cannot be used to differentiate Elabatu from the adulterants. DNA barcoding with matK primers showed that it is possible to discriminate Elabatu from eggplant referring to matK region DNA sequences. The morphological parameters cannot be used to discriminate Elabatu from other eggplant cultivars.

Keywords: DNA Based Authentication of herbal medicince, *Solanum melongena* var. *insanum*, adulteration, *Solanum melongena*

1.Introduction

Solanum melongena var. *insanum* (Prain) known as *Elabatu* is an important constituent of indigenous medicine in Sri Lanka and also in South and East Asia. The estimates of the World Health Organization (WHO) show that 80% of world population use indigenous medicine for their primary health care needs. *Ayurveda* is a traditional system of medicine native to Indian subcontinent. Its' main objective is to counteract the imbalance of three essential elements; *vata, pitta* and *kapha* known as *Tridoshas* from which the body originates and helps to regularize the normal function of the human body [1]. The traditional system of *Ayurveda* uses the root of *Bruhathi* (*Solanum torvum*), which is one of the ingredients of *Dashamoola* (ten roots) [2]. Even though, there are some controversies regarding the usage of *Bruhathi* in Sri Lanka, the indigenous *Ayurvedic* herbal practitioners are using *Elabatu* (*Solanum melongena* var. *insanum*) in place of *Bruhathi*. The *Elabatu* root is a rare single drug component yet it is frequently used in multi component herbal formulations. *Elabatu* is popularly being used in ailments as bronchitis, asthma, rhinitis, oedema, dysentery, dysuria and depressants of nervous system [3]. *Dashamoolarista, Vyaghriharitaki, Chavanaprashavelaha, Kalyanaka ghrta* and *Eranda saptaka qwata* are few formulations that use *Elabatu* root as one of their ingredients.

The popularity for natural products has increased the demand for herbals in the market. Which has resulted in adulteration by other plant species or cultivars that are morphologically and phytochemically indistinguishable. The roots of *S. melongena* var. *insanum (Elabatu)* are frequently been adulterated with *Solanum melongena* L. (eggplant or brinjal) which is widely cultivated as a vegetable crop in every corner of Sri Lanka (Pers. comm.). Although species such as *S. indicum, S. nigrum, S. violaceum, S. torvum* and *S. xanthocarpum* are being used in different alternative and complementary systems of medicine, eggplant is not a significant drug or an ingredient in *Ayurveda*. *S. melongena* has an allergenic potential and the consumption is prohibited in certain disease conditions such as respiratory diseases [4]. A species belonging to a genus containing medicinal benefits does not mean that all other species in that genus are equally medicinally important. Therefore, the correct identification and the quality assurance of herbal medicine is an essential prerequisite that contributes to its' safety and efficacy. Hence the standardization of herbals is a current need.

The morphological characteristics of dried plant parts of genuine and adulterants are more or less similar, thereby presenting difficulty in discrimination. The chemical variability within plant materials hinders the confirmation of its' botanical identity as it may vary based on the stage of development, grower to grower, crop to crop or even at storage and during harvest and post-harvest processing. These limitations in chemical and morphological markers in authentication generate the need for new methods for quality control. DNA based authentication methods such as DNA fingerprinting and DNA barcoding are in progress in many research studies all over the world.

DNA based techniques have been widely used for authentication of plant species of medicinal importance. Such DNA fingerprinting methods are widely used for differentiation of large number of medicinal species from their close relatives or adulterants viz, *Coptis* spp. [5], *Panax* spp. [6,7,8], *Echinacea* spp. [9], turmeric [10]; *Dendrobium officinale* [11]; *Typhonium* spp. [12]; *Tinospora cordifolia* [13]; *Glycirrhiza glabra* [14]; *Piper nigrum* [15] and *Cuscuta reflexa* [16]. These DNA based methods are reliable since the genetic composition is unique for each species and is not affected by age, physiology or with environmental factors. Unlike the other analytical techniques, DNA can be extracted from fresh or dried organic tissue of botanical material. Hence the physical form of the sample for the assessment does not restrict the detection. Thus, DNA based molecular techniques have a great utility in herbal drug analysis and can be widely used for authentication of plant species of medicinal importance [17].

The DNA fingerprinting methods like Randomly Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Sequence Characterized Amplified Region (SCAR) and DNA barcoding are widely used for differentiation of large number of medicinal species from their close relatives or adulterants, but very few are reported with SSR (Simple Sequence Repeats) markers [18,19]. Microsatellites or SSR represent an ideal class of genetic markers in plant genome analysis, as they are evenly distributed throughout the genome [18]. They identify alleles with high reliability, reproducibility, sensitivity and accuracy making it superior from other genetic markers, providing the full confidence to the consumer at the stage of confirmation of genetic identity. DNA barcoding is also a very powerful tool to identify species accurately [20].

Therefore, objectives of the present study were to check the suitability of morphological traits to differentiate the *Elabatu* from commonly grown eggplant cultivars in Sri Lanka, establish a protocol to extract DNA from dried roots and use DNA fingerprinting and DNA barcoding for accurate discrimination of *S. melongena* var. *insanum* (*Elabatu*) from *S. melongena* L. (Eggplant) cultivars.

2.MATERIALS AND METHODS

Selection of *Elabatu* and possible adulterant eggplant cultivars

Nine cultivars of eggplant (the possible adulterants) and *Elabatu* were selected and authenticated seeds of these cultivars were purchased from Horticultural Crop Research and Development Institute (HORDI), Kandy and from Agricultural Stores at *Polgasovita* and *Piliyandala*, Sri Lanka (Table 1). The ten cultivars were grown in five replicates at the Greenhouse of Department of Molecular Biology and Biotechnology, Faculty of Science, University of Peradeniya, Sri Lanka in 2013.

Table 1 : Details of the selected eggplant and *Elabatu* cultivars

No	Cultivar/Regional /Commercial Name	Botanical Name	Source
1	*Padagoda*	*S. melongena*	Department of Agriculture, Sri Lanka
2	*Fullness*		
3	*Amanda*		Private Seed Company
4	*Amali*		
5	*Praveena*		
6	*Lenaeri*		
7	*Raveena*		
8	*SM164*		
9	*Kermit* (Hybrid)*		Imported seeds
10	*Elabatu*	*S. melongena* var *insanum*	Private Seed Company

*Hybrid cultivar showing the in-between traits of eggplant and *Elabatu*. Fruits are very large.

Growing / establishing plants in the nurseries

In establishing nurseries, 1:2 ratio of compost and soil mixture was filled into clay pots (height: 6 cm, diameter: 21 cm), each cultivar was sown separately on a different pot and labeled. The soil was sprayed with a fungicide (Dithane M-45) 0.2% (w/v) solution. The pots were then placed in mild sunlight and covered with a net. Irrigation was done twice a day, in the morning and in the evening. Seedlings bearing two to four leaves were selected and transplanted into plastic containers (20 liters of volume) filled with soil: compost mixture in 6:1 ratio. Each container was provided with fertilizer; Urea 3.75 g, Triple super phosphate 16.75 g and Murate of potash 4.25 g. Five replicates of each cultivar were prepared adding up to a total of 55 replicates. It was laid as a Completely Randomized Design (CRD) in the Greenhouse. Irrigation was continued twice daily, in the morning and in the evening. Tender leaves from all 55 samples were collected into autoclaved and labeled falcon tubes and stored at -80 ^{o}C till DNA extraction. When plants were three months old, a plant from each cultivar was randomly selected and pulled out without disturbing the root system. They were washed thoroughly. Roots were then air dried for seven days. Then they were labeled and sealed in air tight bags and were stored till DNA extraction.

Morphological characterization

Three out of five plants grown for each accession were chosen randomly for recording the morphological data, corresponding to quantitative and qualitative traits (Table 2). The quantitative data were measured on metric scale and qualitative data were recorded on an arbitrary scale.

Table 2 : Morphological descriptors used in characterization

Descriptor	Morphological characters
Plant	Height
	Breath
Leaf	Shape
	Arrangement
Leaf	Type
	Blade length/width (cm)
	Presence of thorns
	Presence of hair
Flower	Diameter (cm)
	Flower length/width(cm)
	Number of petals
	Petal color
	Presence of hair on flower
	Number of sepals
	Color of sepals
	Length of sepals(cm)
	Width of sepals(cm)
	Presence of thorns on sepals
	Number of stamen
	Color of stamen
	Length of stamen(cm)
	Presence of hair on stamen
	Length of filament(cm)
	Length of anther(cm)
	Width of anther(cm)
	Color of style
	Length of style(cm)
	Color of stigma
	Width of stigma(cm)
	Number of carples/locules?
	Height/Width of ovary(cm)
Fruit	Fruit color
	Fruit shape
	Fruit diameter (cm) upper/lower
	Height /Width of fruit(cm)
	Color of the seeds
	Shape of seeds
	Seed height/width(cm)

Molecular characterization

Collection of plant materials for DNA extraction

Tender leaves and dry roots were obtained from the grown plants belonged to 10 cultivars. The '*Elabatu*' (*Solanum melongena* var. *insanum*) roots (could be reliable or adulterant) were purchased from five local herbal drug stores in Colombo and Kandy, Sri Lanka (Table 3).

Table 3 : Details of the purchased '*Elabatu*' root samples from local herbal drug stores

No	Sample*
S1	'*Soma*'- Maharagama
S2	'*Seth Medura*- Maharagama
S3	'*Hela Osu*- Piliyandala
S4	'*Shafira*'- Gelioya
S5	'*Mihira*'- Peradeniya

* Herbal medical stores; Colombo and Sri Lanka

Extraction of genomic DNA

DNA was extracted from fresh tender leaves using the KCl method. Dried hard root materials were brought into fine powder by using two methods, (i) grinding with liquid nitrogen and (ii). filing with steel files followed by grinding with liquid nitrogen prior to DNA extraction. The commercially available Promega DNA Extraction Kit (USA) and modified CTAB method were used to extract genomic DNA from the dried root samples.

Isolation of genomic DNA from dried roots

In addition to the roots from the cultivars listed in Table 1, '*Elabatu*' roots were purchased (Table 3) and used in DNA extraction to check the reliability of commercially available '*Elabatu*' roots in Sri Lanka.

PCR amplification

Species specific Simple Sequence Repeat (SSR) and Inter Simple Sequence Repeat (ISSR) marker were (Table 4) selected for the PCR amplification PCR was carried out in 15 µl reaction mixture containing 50 ng of template DNA, 0.1 µl of forward and reverse primers each and 1× Go Taq® Green Master Mix and nuclease free water to balance the final reaction volume. The amplification was performed in a 96 well Thermal Cycler (PTC Thermocycler).

A touch down PCR protocol was applied to the SSR markers (*emg01G23, emi02E03* and *emd24G09*) consisting of 94 °C/1min denaturation, 10 cycles of 94 °C/30 s, 65 °C/60 s, decreasing by 1 °C per cycle and 72 °C/60 °C, followed by 30 cycles of 94 °C/30 s, 55 °C/60 s, 72 °C/60 °C and final step of 5 minutes at 72 °C was added to allow the complete extension of all amplified fragments. For the marker *issr09*, the cycles were programmed as follows; initial step of 5 min at 94°C, followed by 40 cycles of 30 s at 94 °C, 45 s at 52 °C and 2 min at 72 °C and a final 6 min extension at 72°C.

Table 4 : Specific SSR and ISSR markers used in the study

Marker	Primer	Sequence (5'→3')	Reference
emg01G23	emg01G23/U	TTCGCCTATTTTATTAGGCTCCCC	
	emg01G23/L	CAAGATTTGTGAATTTGGGAGGTG	
emi02E03	emi02E03/U	CTTGAATGTTACCAACTTGGCGTG	[21]
	emi02E03/L	CCAGCATCATCAAATTCTTCTCCA	
emd24G09	emd24G09/U	GTTCTGACAGAGTATGGGCGAGGT	
	emd24G09/L	AATTTCCTCATTACCCCCAATTCC	
issr09		CTCCTCCTCCTCCTCCTC	[22]

DNA barcoding

PCR amplification for DNA barcoding was carried out using *matK* primer pair (5'CGATCTATTCATTCAATATTTC3' and 5'TCTAGCACACGAAAGTCGAAGT3') [23]. For each species 15 ìl total reaction volumes were prepared with 7.5 ìl of PCR master mix (Promega, USA), 5 ìl of nuclease free water, 0.5 ìl from each forward and reverse primers and 60 ng of extracted DNA. PCR protocol for each primer comprised of initial denaturation at 94 °C for 5 mins followed by 35 cycles of 94 °C for 30 sec, 48 °C for 2 mins, 72 °C for 2 mins for extension and finally 72 °C for 10 mins for the final extension. The amplified samples were verified on an agarose gel and sequenced using ABI 3500 Genetic Analyzer.

Data analysis

The morphological data were analyzed using ANOVA and the mean separation procedure DUNNETT in the Statistical Package 9.1 (NC, USA). A cluster analysis was performed for morphological data of *Elabatu* and eggplant cultivars by having McQuitty Linkage, Euclidean distance using MINITAB 14 software package. DNA banding patterns for SSR and ISSR markers were compared among *Elabatu* and other eggplant cultivars to establish individual cultivar identities. The DNA barcoding sequences of *Elabatu* and eggplants were aligned using the package ClustalW 2.1.

3.Results discussion

Morphological characterization

Plant height and leaf parameters

At maturity, mean plant height of *Elabatu* (28.32 cm) was significantly different from the mean height of four eggplant cultivars but not significantly different from the mean height of other five eggplant cultivars ($P<0.05$). The mean leaf diameter and plant height were compared among *Elabatu* and other eggplant cultivars. Apparently *Elabatu* had smaller leaves (8.61 cm of diameter) compared to that of many eggplant cultivars however, leaf diameter of *Raveena* (9.20 cm), *SM164* (7.44 cm) and *Kermit* (7.38 cm) were smaller leaves ($P<0.05$) compared to *Elabatu* (Table 5). Therefore, the plant height at maturity or mean leaf diameter are not good morphological parameters to differentiate *Elabatu* from the eggplant cultivars.

Table 5: The comparison of mean leaf size and mean plant height at maturity stage

Cultivar	Mean leaf diameter (cm)	Plant height (cm)
Elabatu	**8.61^{a}**	**28.32^{a}**
Padagoda	20.76^{b}	17.42^{f}
Fullness	18.35^{c}	38.55^{b}
Amanda	17.48^{d}	29.60^{a}
Amali	13.82^{e}	28.78^{a}
Praveena	13.77^{f}	30.86^{c}
Lenaeri	11.99^{g}	27.72^{a}
Raveena	9.20^{a}	28.88^{a}
SM164	7.44^{h}	30.20^{d}
Kermit	7.38^{i}	23.38^{e}

Means denoted by same letters are not significantly different at $P< 0.05$ (Sample size=15 per cultivar)

Floral parameters

The mean values of the floral parameters, flower; diameter, length, width, length of filament, width of anther, length of style, width of stigma, height of ovary were compared among *Elabatu* and other eggplant cultivars (Table 6). However the mean floral parameters were not significantly different to differentiate *Elabatu* from other eggplant cultivars *($P<0.05$)*.

The flower diameter of *Elabatu* (3.38 cm) had no significant size difference when compared with other eggplant cultivars, SM164 (3.19 cm), *Praveena* (3.25 cm) and *Fullness* (3.68 cm). The mean flower lengths of *Padagoda* (1.62 cm), and *Lenaeri* (1.60cm), were similar to mean value of *Elabatu* (1.64 cm). The flower width was also not a good parameter to differentiate *Elabatu* from other *batu* cultivars, as *Fullness* (1.36 cm) *Amali* (1.24 cm), *Kermit* (1.24 cm) and *Raveena* (1.23 cm) had a similar mean flower width when compared with *Elabatu* (1.33 cm). The lowest sepal length (0.64 cm) was noted in *Elabatu*. Apparently *Amanda* (0.74cm), *Praveena* (0.75 cm) and SM164 (0.73 cm) had a smaller sepal length which was not significantly different from *Elabatu*. In addition *Padagoda* (0.26 cm) and *Amali* (0.26 cm) had a similar sepal width when compared to *Elabatu* (0.25 cm; $P<0.05$; Table 6).

Table 6 : The comparison of mean flower; diameter, length, width, sepal length and sepal width

Cultivar	Flower diameter (cm)	Flower length(cm)	Flower width(cm)	Sepal Length (cm)	Sepal Width (cm)
Elabatu	**3.38^{a}**	**1.64^{a}**	**1.33^{a}**	**0.64^{a}**	**0.25^{a}**
Padagoda	4.17^{b}	1.62^{a}	1.00^{d}	0.93^{g}	0.26^{a}
Fullness	3.68^{a}	1.90^{b}	1.36^{a}	1.13^{c}	0.31^{a}
Amanda	3.96^{d}	1.84^{a}	1.42^{a}	0.74^{a}	0.17^{e}
Amali	2.96^{a}	1.74^{a}	1.24^{a}	1.06^{f}	0.26^{a}
Praveena	3.25^{a}	1.45^{a}	1.06^{c}	0.75^{a}	0.16^{d}
Lenaeri	3.66^{a}	1.60^{a}	1.09^{b}	1.10^{e}	0.15^{c}
Raveena	4.30^{c}	2.07^{c}	1.23^{a}	1.24^{b}	0.18^{a}
SM164	3.19^{a}	1.57^{a}	1.10^{a}	0.73^{a}	0.10^{b}
Kermit	3.59^{a}	1.93^{d}	1.24^{a}	1.11^{d}	0.15^{c}

The stamen length of *Elabatu* (1.34 cm) was not significantly different from that of eggplant cultivars, *Padagoda, Amanda, Amali, Praveena* and *Kermit*. The mean length of the filament of anther for *Elabatu* was 0.51 cm and it was not significantly different from that of eggplant cultivars *Fullness, Amali, Praveena, Sum164* and *Kermit*. Similarly, the floral parameters for *Elabatu*, mean length of anther: 0.80 cm, width of anther: 0.18 cm, length of style: 0.69 cm, width of stigma: 0.01 cm, height of ovary: 0.42 cm and width of ovary: 0.36 cm was not significantly different from at least few cultivars of eggplants *($P<0.05$)*. In summary none of these floral parameters were significantly different enough to discriminate *Elabatu* from other eggplant cultivars grown in Sri Lanka (Table 7).

Table 7 : The comparison of floral parameters

Cultivar	Length of stamen (cm)	Length of filament (cm)	Length of anther(cm)	Width of anther (cm)	Length of style(cm)	Width of stigma(cm)	Height of ovary(cm)	Width of ovary(cm)
Elabatu	**1.34^{a}**	**0.51^{a}**	**0.83^{a}**	**0.18^{a}**	**0.69^{a}**	**0.01^{a}**	**0.42^{a}**	**0.36^{a}**
Padagoda	1.29^{a}	0.39^{e}	0.86^{a}	0.18^{a}	1.10^{i}	0.12^{a}	0.53^{e}	0.45^{a}
Fullness	1.52^{d}	0.55^{a}	0.96^{a}	0.20^{a}	1.15^{h}	0.13^{a}	0.42^{a}	0.45^{a}
Amanda	1.49^{a}	0.73^{c}	0.76^{a}	0.18^{a}	1.20^{g}	0.10^{a}	0.57^{c}	0.49^{e}
Amali	1.38^{a}	0.54^{a}	0.82^{a}	0.18^{a}	1.20^{f}	0.15^{b}	0.44^{a}	0.38^{a}
Praveena	1.41^{a}	0.54^{a}	0.87^{a}	0.18^{a}	0.45^{e}	0.08^{a}	0.19^{b}	0.21^{b}
Lenaeri	1.57^{b}	0.71^{d}	0.86^{a}	0.18^{a}	1.34^{d}	0.14^{a}	0.50^{d}	0.57^{d}
Raveena	1.56^{c}	0.74^{b}	0.82^{a}	0.18^{a}	1.20^{c}	0.10^{a}	0.59^{a}	0.48^{a}
SM164	1.10^{e}	0.45^{a}	0.65^{b}	0.18^{a}	1.79^{b}	0.11^{a}	0.50^{a}	0.44^{a}
Kermit	1.43^{a}	0.50^{a}	0.78^{a}	0.18^{a}	1.10^{a}	0.12^{a}	0.56^{d}	0.60^{c}

Means denoted by same letters are not significantly at different at $P<0.05$ (Sample size=15 per cultivar)

Fruit parameters

The mean fruit parameters for the fruit; height, width, and upper and lower diameters were compared among *Elabatu* and other eggplant cultivars at their maturity stage (Table 8). The mean fruit height of *Elabatu* (2.93 cm) was significantly different from that of all eggplant cultivars except the cultivar Kermit (2.31 cm). The mean fruit width (2.53 cm), the mean diameter of the upper side of the fruit (7.58 cm) and mean diameter of the lower side of the fruit (7.69) cm were not significantly different from other eggplant cultivars except the cultivar *Fullness* which was having the values of 5.39 cm, 17.63 cm and 17.65 cm respectively ($P<0.05$). Therefore, the fruit measurements were not significantly different between *Elabatu* and other eggplant types to discriminate *Elabatu* specifically.

Table 8 : The comparison of mean fruit; height, width, the upper and the lower diameter of *Elabatu* and eggplant cultivars

Cultivar	Fruit height (cm)	Fruit width (cm)	Diameter-upper (cm)	Diameter lower(cm)
Elabatu	2.93^{a}	2.53^{a}	7.58^{a}	7.69^{a}
Padagoda	7.00^{c}	2.24^{a}	14.26^{a}	7.38^{a}
Fullness	5.43^{c}	5.39^{b}	17.63^{b}	17.65^{b}
Amanda	5.62^{d}	2.81^{a}	6.98^{a}	7.25^{a}
Amali	7.37^{c}	3.36^{a}	6.71^{a}	7.00^{a}
Praveena	9.28^{f}	2.65^{a}	6.91^{a}	8.56^{a}
Lenaeri	16.98^{g}	3.17^{a}	7.78^{a}	9.67^{a}
Raveena	9.82^{h}	2.83^{a}	8.18^{a}	8.39^{a}
SM164	6.87^{i}	2.55^{a}	7.16^{a}	8.26^{a}
Kermit	2.31^{a}	2.43^{a}	8.57^{a}	8.59^{a}

Means denoted by same letters are not significantly at different at $P<0.05$(Sample size=15 per cultivar)

Seed parameters

The mean seed height and width were compared between *Elabatu* and other eggplant cultivars (Table 9). There was no apparent significant size difference of seed length or width to distinguish *Elabatu* from other eggplant cultivars. Seed parameters proved to be inefficient in differentiating other cultivars from *Elabatu* ($P< 0.05$).

Table 9 : The comparison of mean seed height and seed width

Cultivar	Seed length (cm)	Seed width (cm)
Elabatu	0.16^a	0.12^a
Padagoda	0.15^a	0.09^a
Fullness	0.17^a	0.13^a
Amanda	0.15^a	0.10^a
Amali	0.17^a	0.09^a
Praveena	0.18^a	0.11^a
Lenaeri	0.18^a	0.12^a
Raveena	0.16^a	0.10^a
SM164	0.18^a	0.08^a
Kermit	0.16^a	0.15^a

Means denoted by same letters are not significantly different at $P<0.05$ (Sample size=15 per cultivar)

According to the dendrogram based on the cluster analysis of morphometric data, *Elabatu* cannot be differentiated from other possible adulterants using flower, fruit or plant quantitative parameters (Figure 1). Thus morphological data proved to be not suitable in differentiating *Elabatu* from its' possible adulterants.

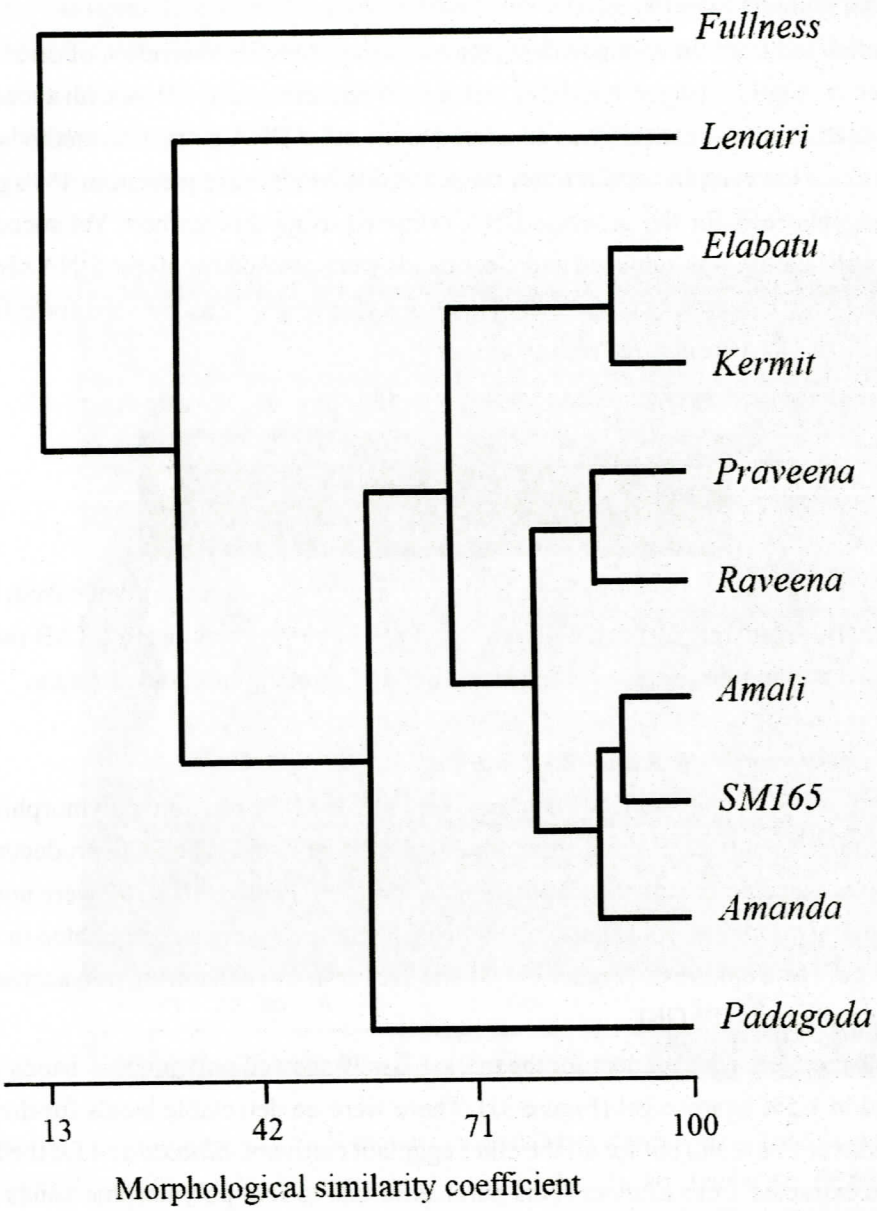

Figure 1 : Morphological similarity dendrogram for *Elabatu* and eggplant cultivars

Molecular characterization

DNA extraction

DNA extracted from the root powder (obtained using steel file aberration of dried roots followed by liquid nitrogen grinding) yielded the highest quality DNA with successful results in PCR amplification when compared to the other DNA extraction methods from dried roots. However, in certain times no detectable bands were present in 1% agarose gel electrophoresis for the genomic DNA extracted using this method. Yet successive PCR amplification was achieved and clear bands were showed for all the DNA samples extracted from dried roots (Figure 2). Therefore, this method can be used for efficient extraction of DNA from dried root samples

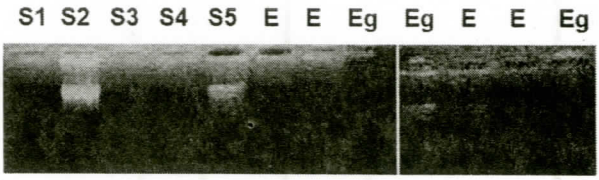

Figure 2 : Agarose gel electrophoresis of genomic DNA samples extracted from dried *Elabatu* (E), eggplant (Eg) and *Elabatu*, S1→S5 (Table 3) roots using CTAB method with sawdust samples prepared using steel files and grinding in liquid nitrogen.

Banding patterns for SSR and ISSR markers

Initially three SSR and one ISSR markers were screened for possible polymorphism to detect and differentiate *Elabatu* from other eggplant cultivars. The PCR products with primers for selected SSR markers, *emg01G23*, *emi02E03* and *emd24G09* were not able to produce any polymorphic bands when the PCR products were size separated in 1.5% agarose gel electrophoresis (Figure 3H, 3I and 3K) or in 6% denaturing polyacrylamide gel electrophoresis (PAGE).

But PCR products with primers for the marker *issr09* showed polymorphic bands when resolved in 1.5% agarose gel (Figure 3J). There were no detectable bands for the egg-plant cultivar *Praveena* but for all the other eggplant cultivars, *Elabatu* and for the DNA samples extracted from *Elabatu* roots purchased, one to five polymorphic bands were detected. However, the bands generated were highly polymorphic making all 16 DNA samples different from each other. This could be due to the very high level of intra species genetic diversity observed in eggplant and *Elabatu* species so that *issr09* maker was highly polymorphic to differentiate *Elabatu* in general from other eggplant cultivars.

The two *Elabatu* samples used in the study shared some DNA banding patterns but the five root samples purchased from the market were having different banding patterns to the authenticated *Elabatu* samples. This may imply the adulteration of *Elabatu* roots with roots of any other *Solanum* species or any other species with similar roots. However, our results indicated that the repeatability of *issr09* marker was questionable as some of the low intensity bands disappeared in independent runs

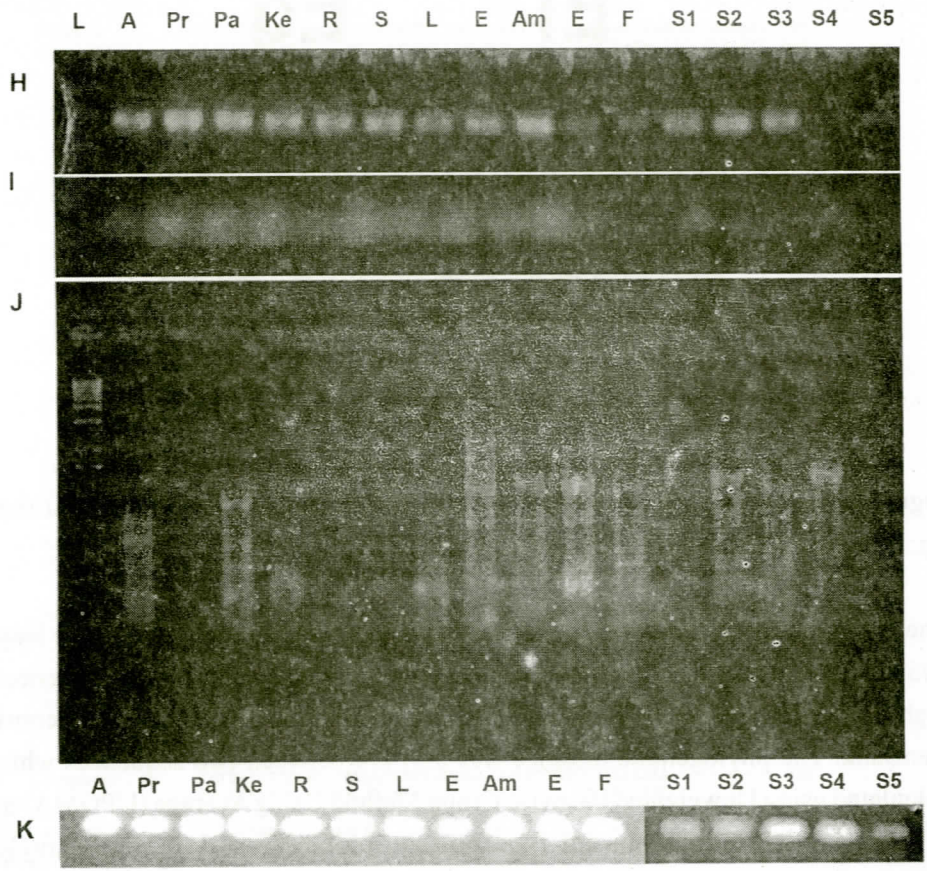

Figure 3 : Electrophoresis of PCR products with the markers A: *emg01G23*, B: *emi02E03*, C: *ISSR09* and D: *emd24G09* markers. L: 123 bp ladder, A: *Amali*, Pr: *Praveena*, Pa: *Padagoda*, Ke: *Kermit*, R: *Raveena*, S: *SM164*, L: *Lenairi*, E: *Elabatu* (included twice), Am: *Amanda*, F: *Fullness*, S1àS5 dried *Elabatu* roots purchased (Table 3)

DNA barcoding

As none of the SSR or ISSR markers tested were able to differentiate *Elabatu* from other eggplant cultivars, DNA barcoding was employed with one standard DNA barcoding primer pair *matK*. The PCR with *matK* primers *Elabatu* and eggplant genomic DNA templates yielded the expected band nearly 850 bp (Figure 4).

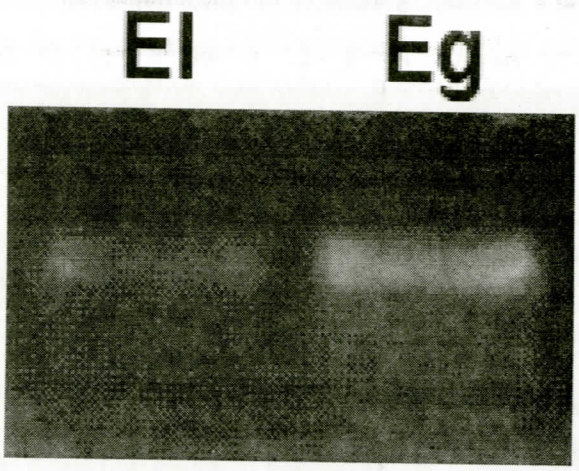

Figure 4 : The amplified bands for *matK* primers on an agarose gel for El: *Elabatu* and Eg: Eggplant DNA samples

The *matK* sequences obtained for *Elabatu* and eggplant samples were in the lengths of 905 bp and 869 bp respectively. The BLAST search revealed that these sequences were highly correlated (80%) with the *matK* sequences of related *Solanum* spp. deposited in GenBank. The phylogenetic distance was 0.31494 between two sequences which was calculated using Unweighted Pairwise Group Method Using Average (UPGMA) method (Figure 5). There were in-del type polymorphisms and 180 single nucleotide polymorphisms (SNPs) between the two sequences. The relative sequence difference was 5%. It is quite evident from the two sequences that two species are different for *matK* barcoding sequences and can be used to differentiate *Elabatu* roots from the eggplant roots. However, repeated sequencing is required to set up the *matK* sequences as the *Elabatu* and eggplant barcodes for routine testing requirements to check adulterations.

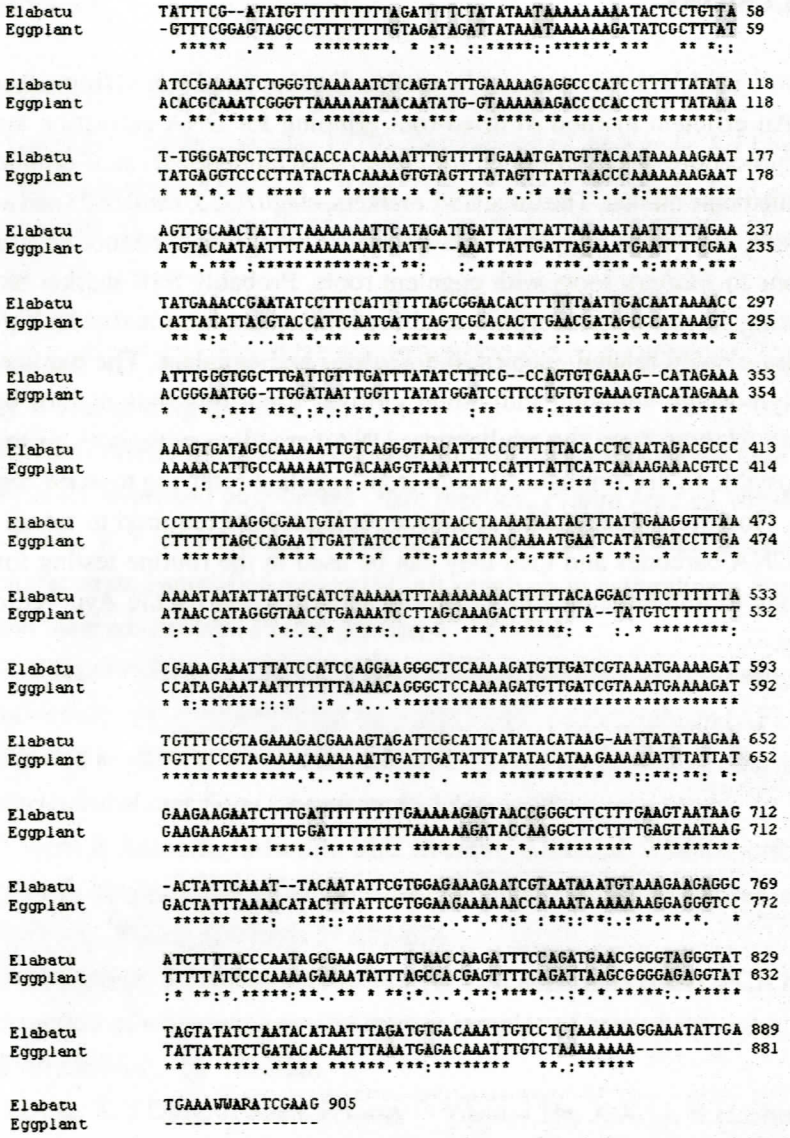

Figure 5 : Sequence alignment of *matK* homologues sequences for *Elabatu* and *Eggplant*. The alignment was performed using the ClustalW 2.1 multiple sequence alignment programme **(http://www.ebi.ac.uk/Tools/services/web/toolresult.ebi?jobId=clustalw2).** The branch length of phylogenetic tree is 0.31494 between two sequences calculated using Unweighted Pairwise Group Method Using Average (UPGMA) method.

4. CONCLUSIONS

The morphological parameters cannot be used to discriminate *Elabatu* from other eggplant cultivars. An efficient method of dried-root grinding for DNA extraction and a DNA extraction procedure were developed for DNA based authentication of *Elabatu* roots in the herbal medicine market. The three SSR markers, *emg01G23*, *emi02E03* and *emd24G09* are monomorphic across eggplant cultivars and *Elabatu* thus, cannot be used to find adulterations to *Elabatu* roots with eggplant roots. Probably SSR marker based DNA fingerprinting is too monomorphic to discriminate taxonomically ambiguous and evolutionary closely related species like *Elabatu* and eggplant. The marker *issr09* is highly polymorphic showing individual cultivar identities thus cannot be used to differentiate *Elabatu* from the adulterants. DNA barcoding with *matK* primers shows that it is possible to discriminate *Elabatu* from eggplant referring to *matK* region DNA sequences. However, independent sequence replicates are required to set up the *matK* sequence DNA barcodes and then they can be used in the routine testing for possible adulterations to authenticate *Elabatu* roots as demanded by the Ayurvedic medical community.

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