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RESEARCH ARTICLE

Micrometric Study and Random Amplified Polymorphic Deoxyribonucleic Acid Analysis of *Wattakaka volubilis* (Linn. f.) Stapf. Leaves

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ABSTRACT

The leaves of Jīvantī are considered to be the best among the vegetables in the classical texts of Ayurveda. In some parts of India, *Wattakaka volubilis* (Linn. f.) Stapf. is used in the name of Jīvantī for treatment of pyoderma, fever, cough, cold, rheumatic pain, diabetes, eye diseases, snake bite etc. The present study deals with the micrometric evaluation and molecular characterization of *Wattakaka volubilis* (Linn. f.) Stapf. leaves by random amplified polymorphic deoxyribonucleic acid (RAPD) markers by following standard parameters. Morphological study showed that leaves are 6.5–14 × 4.5–11 cm, in size, broadly ovate or suborbicular with reticulate venation. Transverse section of petiole measures about 2.7 µm, showing single-layered epidermis with multicellular, warty trichomes. Cortex contains chloroplast and several prismatic and rosette crystals. Section through midrib measures about 3.4 × 3.2 µm, having centrally located bicollateral vascular bundle supported by ground tissue. Powder microscopy showed the presence of multicellular and glandular trichomes, paracytic stomata, rosette crystals of calcium oxalate, oil globules and chlorophyll pigments. In RAPD analysis, all the 10 primers showed good amplification. The unique bright and light bands obtained in polymerase chain reaction amplification along with quantitative pharmacognostical characters can be considered as a measure for authentication and standardization of the plant.

Keywords: Jīvantī, Pharmacognosy, Random amplified polymorphic deoxyribonucleic acid analysis, *Wattakaka volubilis* (Linn. f.) Stapf.

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INTRODUCTION

Jīvantī is one of the important medicinal herbs having immense medicinal excellence described in almost all the classical texts of Āyurveda. It is considered to be the best among vegetables.[1] The plant has been reported for its use as an ingredient in 223 compound formulations in the Āyurvedic classics.[2] Though *Leptadenia reticulata* Wight & Arn. is accepted as a genuine botanical source,[3] various herbs are used under the name of Jīvantī in different parts of the country, namely *Wattakaka volubilis* (Linn. f.) Stapf., *Holostemma annulare* Roxb. of Asclepiadaceae family, *Dendrobium normale* Fale (Orchidaceae) etc.[4]

The *Wattakaka volubilis* (Linn. f.) Stapf. is a large, stout, twining shrub, distributed throughout the hotter parts of India, up to 1,500 m in the Himalayas, China, Taiwan, Sri Lanka, Malaysia, etc.[5] Leaves measure up to 15 × 11 cm and are broadly ovate, acute, or shortly acuminate at apex, rounded or cordate at base; the petioles are up to 6 cm long. Cymes are shorter than or as long as the leaves. Corolla is 1.5 cm in diameter; lobes are triangular. Follicles are up to 10 × 3 cm, tomentose when young, afterward glabrous.[6]

The plant has been considered as the botanical equivalent of the classical Āyurvedic drug Jīvantī, which is highlighted as the best among vegetable drugs in different Āyurvedic texts.[7] Traditionally, tender leaves have been eaten in curries and used in the treatment of pyoderma and fevers in children.[8] The plant has been frequently used as a remedy for cough, fever, severe cold, rheumatic pain, diabetes, eye diseases, snake bite etc.[9,10]

The general approaches to herb identification and standardization are dependent on morphological,[11] anatomical,[12] chemical,[13] and molecular[14] techniques. Literature review of *Wattakaka volubilis* (Linn. f.) Stapf. revealed that though certain pharmacognostical studies of the leaf have been reported,[15,16] details regarding its quantitative microscopy and deoxyribonucleic acid (DNA) molecular characterization are still lacking. Recently, the random amplified polymorphic DNA (RAPD) method has been used for the estimation of genetic diversity of medicinal plants and certain plants have been reported for their molecular characterization through RAPD markers.[17]

Hence, the present investigation was undertaken to establish quantitative pharmacognostical parameters and DNA molecular characterization of *Wattakaka volubilis* (Linn. f.) Stapf. by RAPD markers.

MATERIALS AND METHODS

Collection and Preservation of the Sample

The plant, *Wattakaka volubilis* (Linn. f.) Stapf., was collected from its natural habitat, Rakakhatia forest area of Jamnagar, Gujarat, India, in November 2015, after being authenticated by the local taxonomist with the help of botanical flora.[18] A herbarium specimen was deposited in the pharmacognosy laboratory of the Institute for Postgraduate Teaching and Research in Ayurveda, Jamnagar, India, for future references vide Ref No. PHM 6202/21/11/2016 (Fig. 1A).

Morphological Study

The morphological study includes size, shape, apex, margin, venation, base, petiole, surface and color of leaves of *Wattakaka volubilis* (Linn. f.) Stapf.

Microscopical Study

Detailed microscopic characters were studied by taking free hand, thin transverse sections of fresh plant parts. Sections were stained with phloroglucinol and hydrochloric acid to notice the lignified elements like fibers, vessels etc.[19] Microphotographs of the section were taken with the help of a Canon digital camera attached to a Quasmo microscope. Powder characters were studied as per the guidelines of Ayurvedic Pharmacopoeia of India.[20]

Quantitative Microscopy

Quantitative microscopy was carried out to determine epidermal cell number, stomatal number, stomatal index, size of the stomata, etc.[21]

Powder Microscopy

Dried leaf powder was studied by following standard procedures.[22] The microphotographs were taken by using Carl Zeiss trinocular microscope.

Histochemical Test

To confirm the presence and absence of the chemical constituents, the test drug was subjected to various tests. The histochemical tests were carried out according to the standard guidelines of practical pharmacognosy.[23]

Molecular Characterization (DNA Fingerprints)

Fresh leaves of *Wattakaka volubilis* (Linn. f.) Stapf. were used for molecular characterization and DNA fingerprints, by standard and the most convenient RAPD method. The RAPD reaction was performed by following standard procedures at Junagadh Agricultural University, Junagadh, Gujarat, India. For DNA fingerprinting through RAPD markers, DNA was extracted using the Doyle and Doyle[24] method with minor modifications. The DNA quantification was done by using a Picodrop spectrophotometer and DNA sample was diluted by using TE buffer up to 50 ng/µL. The quality of four samples of DNA was checked by 0.8% agarose gel electrophoresis. The RAPD-polymerase chain reaction (PCR) was carried out in Veriti ABI thermal cycler. The resolved amplified products were visualized by illumination under ultraviolet light in a Gel document system. List of RAPD primers used for the analysis of plant DNA sample is given in Table 1.

RESULTS AND DISCUSSION

Morphological Study

Leaves are simple, alternate, petiolate, measure about 6.5–14 × 4.5–11 cm, broadly ovate or suborbicular, acuminate, glabrous, more or less softly pubescent, reticulate venation with a few small glands just above the petiole (Figs 1B and C).

Microscopic Study

Transverse section (TS) of petiole: The TS of the petiole is almost circular in shape with two notchs at one side (Fig. 2A). The TS of petiole measures about 2.7 µm (Fig. 2B). Epidermis consists of single-layered small, thin-walled cells covered with thin cuticle. Epidermis is interrupted by some simple, multicellular, and warty trichomes (Fig. 2C). About three to four layers of circular-to-oval collenchymatous cells were observed beneath the epidermis (Fig. 2D). Inner to the collenchyma, cortex region is observed, which is made up of thin-walled circular-to-oval parenchymatous cells with large

Table 1: List of RAPD primers used for the analysis of plant DNA sample

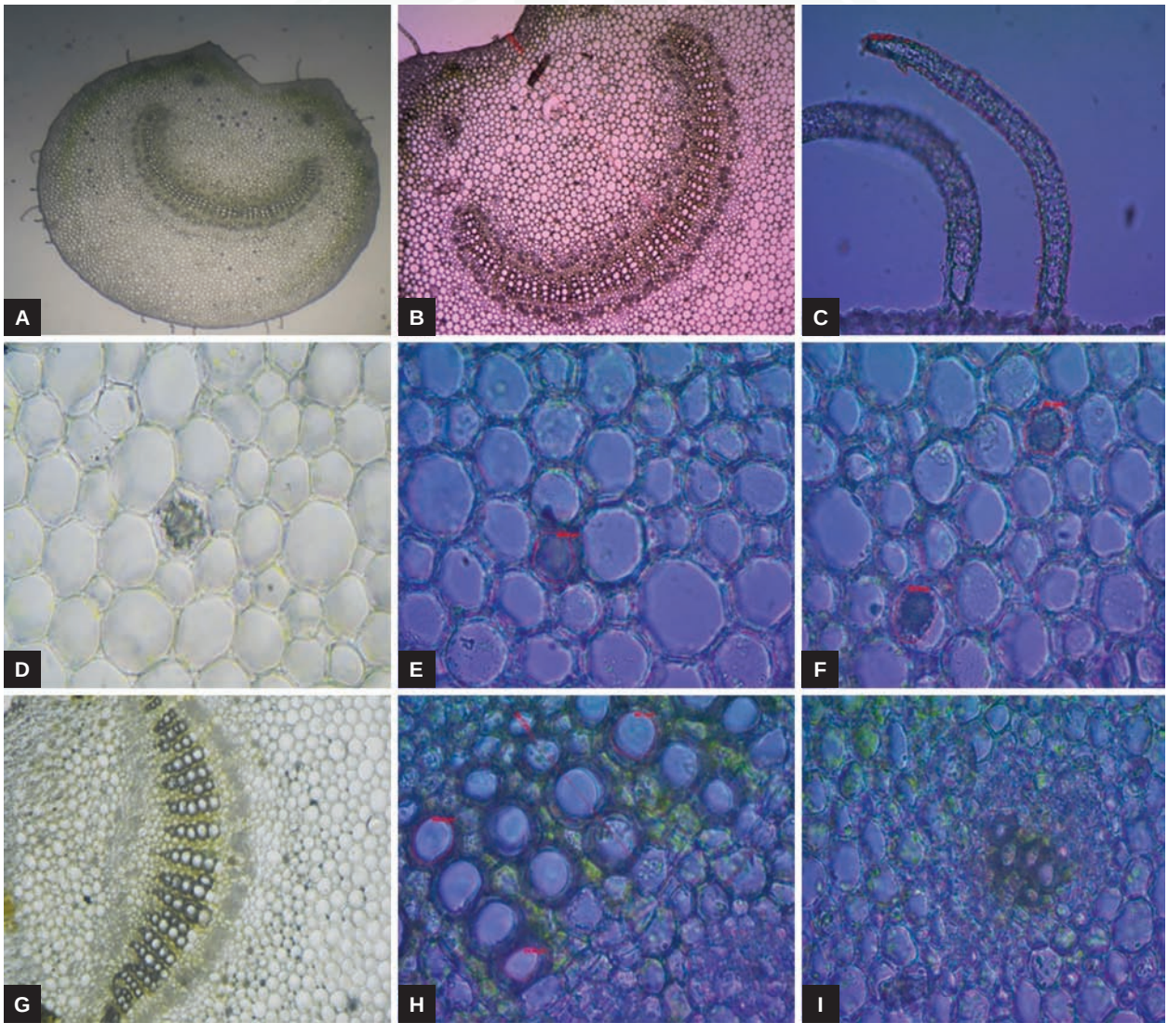
Sl. no	*Primer*	*Sequence 5′–3′*	*Tm (°C)*
1	OPM-01	GTTGGTGGCT	25.0
2	OPM-03	GGGGGATGAG	27.0
3	OPM-06	CTGGGCAACT	25.0
4	OPN-02	ACCAGGGGCA	27.0
5	OPN-08	ACCTCAGCTC	25.0
6	OPO-05	CCCAGTCACT	25.0
7	OPO-06	CCACGGGAAG	27.0
8	OPO-07	CAGCACTGAC	25.0
9	OPO-08	CCTCCAGTGT	25.0
10	OPO-09	TCCCACGCAA	25.0

Figs 1A to C: Morphology of *Wattakaka volubilis* (Linn. f.) Stapf.: (A) Herbarium; (B) plant morphology; and (C) leaf

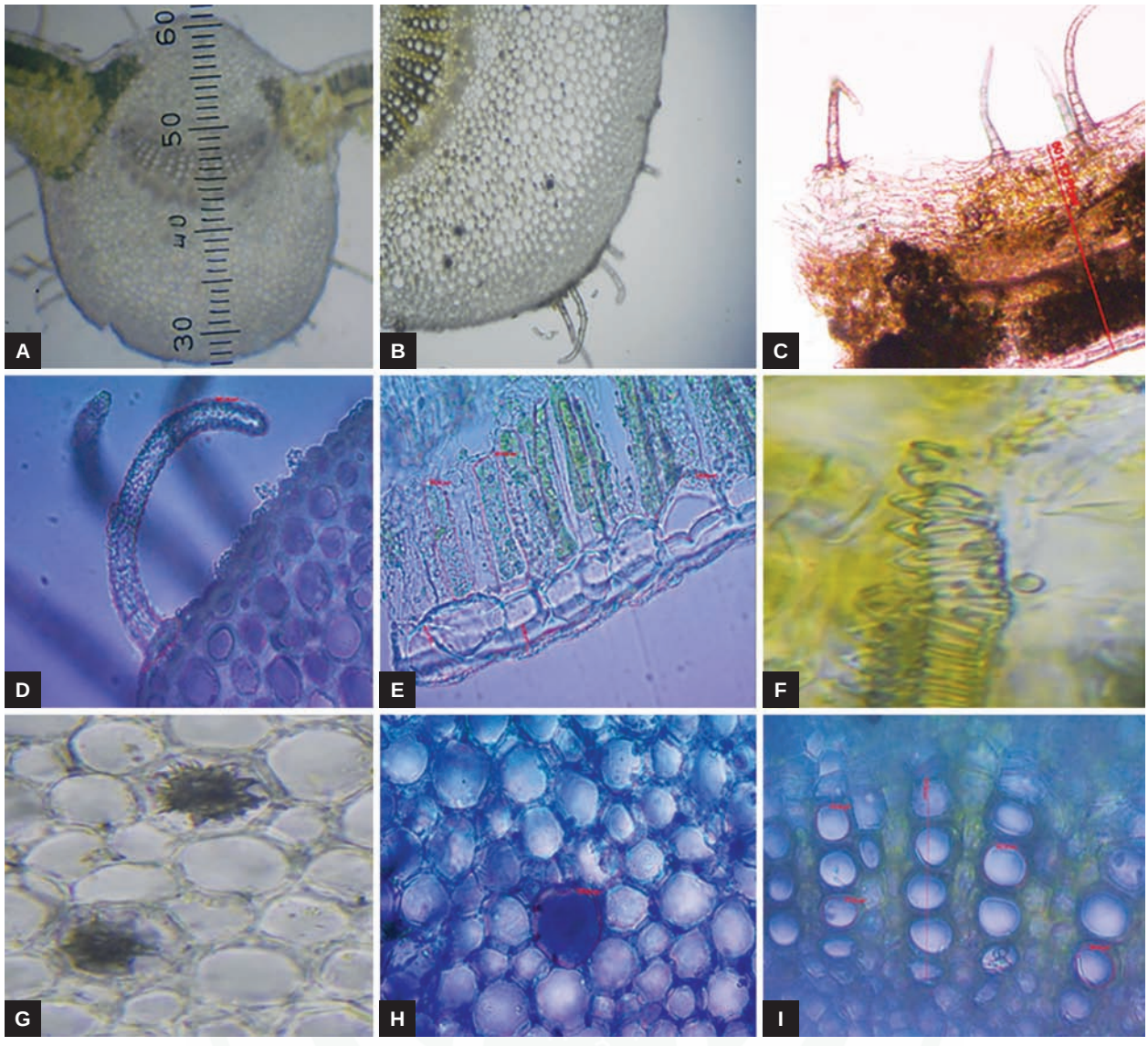
Intercellular spaces. These parenchymatous cells contain chloroplast, lactiferous cells, several prismatic and rosette crystals (Figs 2E to G). The vascular bundle is bicollateral, protoxylem facing toward center and metaxylem toward the epidermis (Figs 2H and I).

Midrib

The TS of the leaf (lamina) passing through the midrib is strongly convex in outline (Fig. 3A). Upper and lower epidermis consist of single-layered, thin-walled, barrel-shaped cells covered with cuticle (Fig. 3B). The TS



Figs 2A to I: The TS of petiole of *Wattakaka volubilis* (Linn. f.) Stapf.: (A) TS of petiole; (B) petiole measurement; (C) warty trichomes; (D) rosette crystal; (E) lactiferous cell; (F) cluster crystals; (G) xylem and phloem; (H) xylem fibers; and (I) vascular bundle



Figs 3A to I: The TS of midrib of *Wattakaka volubilis* (Linn. f.) Stapf.: (A) TS of midrib; (B) epidermis and hypodermis; (C) lamina measurement; (D) warty trichomes; (E) epidermis and palisade; (F) spiral vessels; (G) rosette crystal; (H) lactiferous cells; and (I) xylem measurement

of lamina through midrib measures about 3.4 × 3.2 µm (Fig. 3C). Some of the epidermal cells possess multicellular, glandular and warty trichomes (Fig. 3D). Below the upper epidermis, one to two layers of palisade parenchyma cells with chlorophyll pigments (Fig. 3E) and oil globules, spiral vessels (Fig. 3F), prismatic and rosette crystal of calcium oxalate (Fig. 3G) and lactiferous cells (Fig. 3H) are observed. Section through midrib shows centrally located bicollateral vascular bundle, supported by ground tissue (Fig. 3I). On the lower side of the transverse section, one to three layers of collenchymatous cells are present.

Powder Microscopy

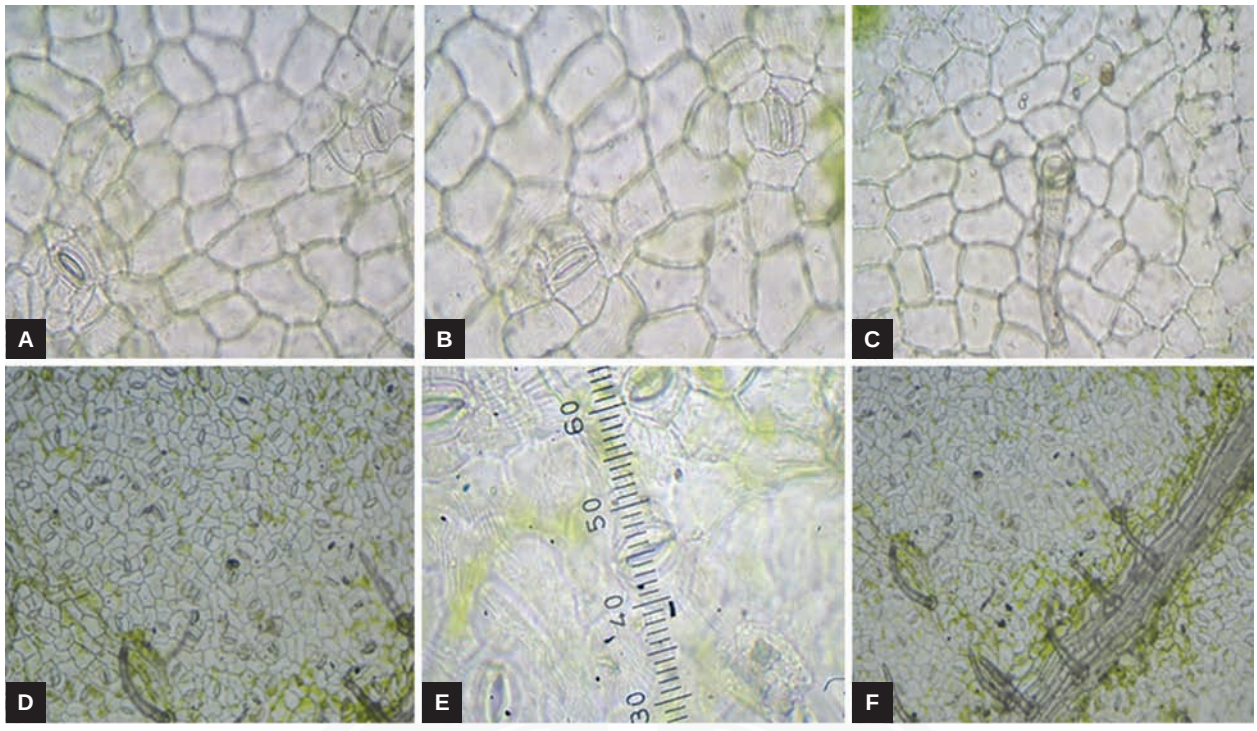
Organoleptic characters showed the presence of greenish color with leafy odor and bitter taste. Diagnostic characters of dried powder showed multicellular and glandular trichomes, paracytic stomata, rosette crystals of calcium oxalate, oil globules and chlorophyll pigments.

Surface Study and Quantitative Microscopy of Epidermis

Stomata were rarely distributed in upper epidermis. Some of the trichomes and cicatrix are also observed (Figs 4A to C). The lower epidermis consisted of numerous stomata of the paracytic and anisocytic type. Some of the trichomes and cicatrix were also observed (Figs 4D to F). The results of quantitative microscopy of *Wattakaka volubilis* (Linn. f.) Stapf. leaves are presented in Table 2.

Histochemical Test

To confirm the presence and absence of the chemical constituents, the material was subjected to various tests.



Figs 4A to F: Surface study of *Wattakaka volubilis* (Linn. f.) Stapf.: (A) Upper epidermis; (B) stomata in upper epidermis; (C) cicatrix in upper epidermis; (D) lower epidermis; (E) stomata in lower epidermis; and (F) cicatrix in lower epidermis

Table 2: Quantitative microscopy of leaf of *Wattakaka volubilis* (Linn. f.) Stapf

Sl. no	*Parameter*	*Micrometeric values*
1	Size of stomata (Length × width)	0.8 × 0.6 µm
2	Laticeferous cavity (surface measurement)	498.83 µm^2
3	Xylem measurement from proto to metaxylem	106.11 µm
4	Xylem surface measurement	391.71 µm^2
5	Rosette crystals	568. 12 µm^2
6	Warty trichome	3845. 65 µm^2
7	Palisade cell measurement	1080. 48 µm^2
8	Epidermal cell measurement	212. 64 µm^2
9	Cuticle layer measures	18.55 µm
10	Stomatal index	3
11	Palisade ratio	2.5

Table 3: Histochemical tests of *Wattakaka volubilis* (Linn. f.) Stapf. leaf

Sl. no.	*Reagents*	*Observation*	*Characteristics*	*Results*
1	Phloroglucinol + conc. HCl	Red	Lignified cells	++
2	Iodine	Blue	Starch	++
3	Phloroglucinol + conc. HCl	Dissolved	Calcium oxalate crystals	++
4	FeCl3 solution	Dark blue to black	Tannin cells	++
5	Sudan III	Red	Oil globules	++

"++": Present

The results of histo-chemical test shows that, lignified cells, starch, calcium oxalate crystals, tannin and oil globules are present in the leaf (Table 3).

RAPD Analysis

The RAPD has frequently been used for the detection of genetic variability in plants. The advantages of this method are its rapidity, simplicity and lack of need for any prior genetic information about the plant. The RAPD patterns are consistent irrespective of the plant source or age.[25,26]

In RAPD analysis of *Wattakaka volubilis* (Linn. f.) Stapf., all the 10 primers showed good amplification. In primer 1, range of band size was observed at 300 and 800 bp; in primer 2, range of band size was observed at 500 to 1500 bp; in primer 3, single band size was observed 500 bp; in primer 4, band size was observed from 250 to 1,000 bp; in primer 5, band size was observed at 700 and 800 bp; in primer 6, single band size was observed at 400 bp; in primer 7, range of band size was observed from 300 to l,500 bp; in primer 8, band size was observed at 500 and 700 bp; in primer 9, range of band size was observed at 600, 700 and 900 bp; in primer 10, range of band size was observed at 300 to 1500 bp (Fig. 5). The unique bright and light bands obtained in PCR amplification are indicative of the genuinity of the plant. These bands are unique for the particular species. The observed bright and light bands in RAPD analysis make identification and characterization of genotype very easy. These characters are especially advantageous for the identification of any herbal drugs because of little DNA existing in the dried material (Fig. 5).

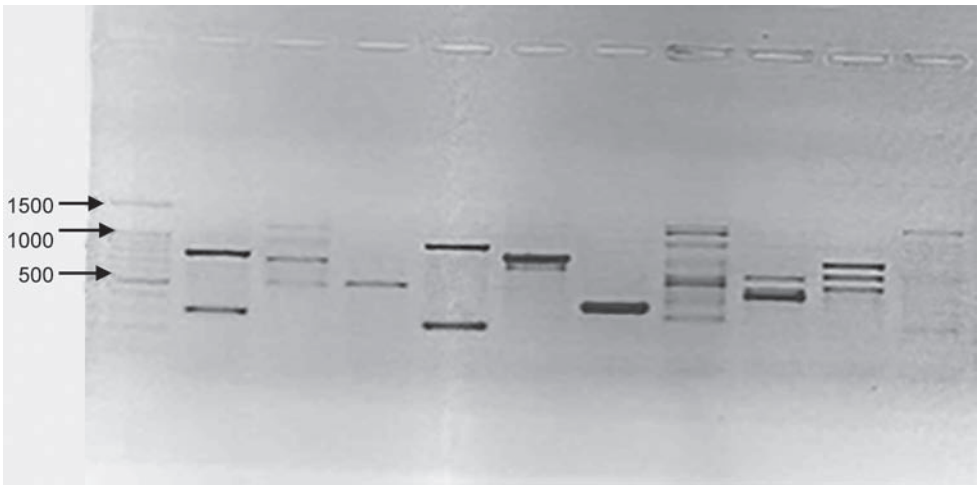


Fig. 5: RAPD analysis. *Wattakaka volubilis* (Linn. f.) Stapf. DNA sample with 10 RAPD primers

CONCLUSION

The standardization of a crude drug is an integral part for establishing its correct botanical identity. The observed macroscopic and microscopic characters of the leaf of *Wattakaka volubilis* (Linn. f.) Stapf., reported for the first time, can serve as diagnostic parameters. Observed RAPD marker can be used to differentiate genuine as well as adulterated samples.

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हिन्दी सारांश

वट्टाकाका वोल्युबिलिस के पत्र का माईक्रोमेट्रिक् अध्ययन एवं आर. ए. पी. डी. विश्लेषण

राघवेन्द्र नाईक,[1] सी आर हरीशा,[2] रबिनारायण आचार्य[3]

आयुर्वेद ग्रन्थो में जीवन्ती के पत्र को शाकद्रव्यों मे श्रेष्ठ माना गया है। वट्टाकाका वोल्युबिलिस को जीवन्ती का एक वानस्पतिक स्रोत माना जाता है और उसका उपयोग ज्वर, कास, प्रतिश्याय, सन्धिशूल, मधुमेह, अक्षिरोग, सर्पदंश इत्यादि व्याधियों मे किया जाता है। प्रस्तुत अध्ययन मे वट्टाकाका वोल्युबिलिस के पत्र का माईक्रोमेट्रिक् अध्ययन एवं आर. ए. पी. डी विश्लेषण किया गया। वर्तमान अध्ययन मे पत्र की आकृति उपगोलाकार और प्रमाण ६.५–१४ × ४.५–११ से.मी. पाया गया। पर्णवृन्त का अनुप्रस्थ छेद का प्रमाण २.७ माईक्रो मीटर और उसमे एक लेयर का एपीडर्मिस, मल्टीसेल्युलार ट्राईकोम, क्लोरोप्लास्ट, प्रिस्मेटिक एवं रोसेट क्रिस्टल पाये गये। मिडरिब के अनुप्रस्थ छेदका प्रमाण ३.४ × ३.२ माईक्रो मीटर और उसमे मध्य स्थित वेस्क्युलार बंडल पाया गया। पाऊडर माईक्रोस्कोपि मे बहुकोषकीय ट्राईकोम, पेरासाईटिक स्टोमाटा, क्लोरोफिल पिग्मेंट, प्रिस्मेटिक एवं रोसेट क्रिस्टल पाये गये। आर. ए. पी. डी विश्लेषण मे सभी १० प्राईमरों ने उत्तम परिवर्धन दिखाया। प्रस्तुत अध्ययन मे पाये गये मानकों का उपयोग वट्टाकाका वोल्युबिलिस के पहचान और मानकीकरण के लिए उपयोग किया जा सकता है।