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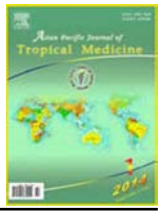

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Development of quality control parameters for the standardization of *Limonia acidissima* L. leaf and stem

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ABSTRACT

Objective: To evaluate the pharmacognostic characters of *Limonia acidissima* L. (*L. acidissima*) leaf and stem, an important traditional medicinal plant.

Methods: The present study provides pharmacognostic, physicochemical and phytochemical details of leaf and stem of *L. acidissima*. Micro and macroscopic characters were analyzed. WHO recommended parameters were followed in the entire study.

Results: The macroscopic study showed that the leaf was alternate, imparipinnately compound leaf with entire margin, long petiole, apex obtuse and base decurrent, with surface appearance and txture glabrous. The inflorescence was lateral and terminal panicles. The microscopic study of leaf revealed the presence of actinocytic stomata, multicellular trichome, prismatic calcium oxalate crystals, vascular bundles, *etc.* The powder microscopy also revealed prism like calcium oxalate crystals, multicellular trichome and actinocytic stomata. Physiochemical analysis of dried leaf powder showed total ash, water soluble ash and acid insoluble ash as 9.33%, 1.83% and 1.16% w/w respectively. Preliminary phytochemical screening revealed the presence of maximum amount of flavonoids and tannins. The main microscopic characteristic of stem was 2-3 layers of phellem, phellogen 2-3 layered followed by 7-8 layered phelloderm. Among other microscopic components were presence of xylem parenchyma, xylem vessels, xylem fibres and tracheids. The powder microscopy also revealed presence of annular, spiral vessel, prism crystals and multicellular trichome. Physiochemical analysis of dried stem powder showed total ash, water soluble ash and acid insoluble ash as 3.16%, 0.66% and 0.66% w/w respectively. Preliminary phytochemical screening showed the presence of maximum amount of only flavonoids.

Conclusions: Various pharmacognostical characters observed in this study will help in botanical identification and standardization of leaf and stem of *L. acidissima* and will also help in quality control and formulation development.

1. Introduction

Plants with medicinal properties have been known for thousands of years and have been used as traditional medicines by the people to treat diseases. Due to many side effects of drugs of medical science and their high cost, the traditional medicines are being used all over the world. Botanically derived medicines have played a major role in human society throughout history and prehistory[1]. The WHO estimates that about 80% of the population living in developing countries relies almost exclusively on traditional medicine for their primary healthcare needs. In spite of great advances of modern scientific medicine, traditional medicine is still the primary form of treating diseases of majority of people in developing countries including India; even among those to whom western medicine is available, the number of people using one form or another of complementary of alternative medicine is rapidly increasing worldwide. In such a situation, correct identification and quality assurance of the starting materials is an essential prerequisite to ensure reproducible quality of herbal medicine which will contribute to its safety and efficacy[2]. Pharmacognostic study is the preliminary step in the standardization of crude drugs. The detailed pharmacognostic evaluation gives valuable information regarding the morphology, microscopic and physical characteristics of the crude drugs[3].

Limonia acidissima L. (*L. acidissima*) belongs to the family Rutaceae. It is distributed throughout India. In Sanskrit, it is known as 'Kapitthah' and in Gujarati it is known as 'Kotha'. There are many synonyms of this

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plant like *Feroniam elephantum*, *Feronia limonia* and *Schinus limonia*[4]. The decoction of the leaves is used in the treatment of constipation, vomiting, cardiotonic and diuretic[5]. The leaves contain coumarin, triterpinoids and steroids[6]. The fruits are used for tumors, asthma, wounds, cardiac debility and hepatitis[7]. The leaves are reported to possess hepatoprotective activity[8]. *L. acidissima* is an important medicinal plant with various uses of its different parts for treating different illnesses. The different parts of the plant are reported to show wound healing, antioxidant activities[9], analgesic activity[10], antidiabetic activity[11], antiproliferative effect[12], antioxidant activity[13], antimicrobial and cytotoxic activity[14], phytochemical and antimicrobial activity[15], pharmacognostical and physicochemical evaluation of stem bark[16], *etc.*

In the present study, an attempt has been made to lay down some standardization parameters for *L. acidissima* leaf and stem. Hence, the objectives of the study were to evaluate various pharmacognostic parameters like macroscopic and microscopic characters, phytochemical and physicochemical characterization including fluorescence study.

2. Materials and methods

L. acidissima L. leaf and stem were collected from Rajkot, Gujarat, India in August, 2013. The plant was compared with voucher specimen (voucher specimen number PSN426) deposited by Dr. Nagar PS at the Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India. The plant parts were washed thoroughly with tap water, shade dried, homogenized to fine powder and stored in air tight bottles.

2.1. Macroscopic study

For morphological observations, fresh leaf and stem were collected from Rajkot, Gujarat, India in August, 2012. The macromorphological features of the leaf and stem were observed under magnifying lens[17].

2.2. Phytochemical analysis

2.2.1. Qualitative phytochemical analysis

The crude powder of leaf and stem was subjected to qualitative phytochemical analysis[18,19]. The phytochemicals analyzed were alkaloids, flavonoids, tannins, phlobatanins, triterpenes, steroids, saponinis and cardiac glycosides.

2.2.2. Fluorescence analysis

Fluorescence study of leaf and stem powder was performed as per reported standard procedures[20]. A small quantity of the stem powder was placed on grease free clean microscopic slid and 1-2 drops of freshly prepared reagent solution was added, mixed by gentle tilting of the slide and waited for a few minutes. Then the slide was placed inside the UV chamber and observed in visible light, short (254 nm) and long (365 nm) UV radiations. The colour observed by application of different reagents in different radiations was recorded.

2.2.3. Physicochemical analysis

The physicochemical analysis of the crude powder *L. acidissima* leaf and stem was carried out as per WHO guidelines[21]. The parameters analysed were loss on drying, total ash, water soluble ash, acid insoluble ash, petroleum ether soluble extractive, ethyl acetate soluble extractive, acetone soluble extractive and water soluble extractive.

3. Results

3.1. Pharmacognostic study

3.1.1. Organoleptic and macroscopic characteristics of leaf

The organoleptic features of *L. acidissima* leaf is given in Table 1 and Figure 1. The macroscopic study showed that the leaf was alternate, imparipinnately compound leaf with entire margin, long petiole, apex obtuse and base decurrent, with surface appearance and texture glabrous. The inflorescence was lateral and terminal panicles.

Table 1

Organoleptic features of *L. acidissima* leaf.

Parameters	Observations
Colour	Dark green
Lamina	Obovate
Composition of lamina	Imparipinnate
Dimensions	2.5-8×5.5 cm
Leaf	Compound
Margin	Entire
Apex	Obtuse
Base	Decurrent
Surface appearance and texture	Glabrous
Venation	Pinnate
Inflorescence	Lateral and terminal panicles

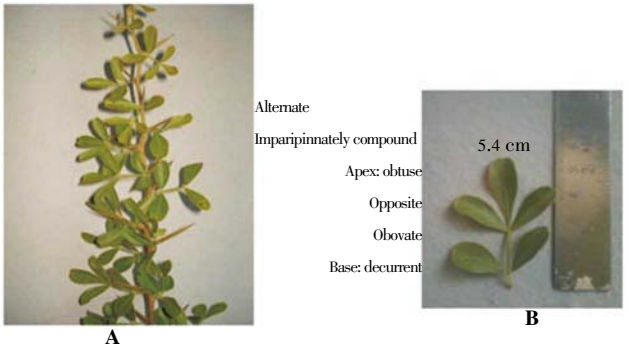

Figure 1. Macroscopic characteristics of *L. acidissima* leaf.

3.1.2. Microscopic characteristics of leaf

The microscopic characters of leaf are shown in Figure 2. Lamina of the leaf was dorsiventral type. The upper and lower epidermis consisted of small polygonal parenchymatous cells which appeared wavy in outline and after epidermis, chlorenchyma cells were present (Figure 3). The chlorenchyma cells were further seen as upper palisade and lower spongy tissue. The palisade with two layers of regular, long, columnar cells, beneath which, three to four layered spongy tissues was present. The cortex consisted of 6 to 8 layers of parenchymatous cells in the midrib region. The vascular bundle was ovoid in shape around the vascular bundle layer of sclerenchyma cell. Multicellular trichomes were present on the surface. Stomata were actinocytic (Figure 3).

3.1.3. Powder study of leaf

The crude powder of leaf was dark green in colour while

stem was light brown in colour with characteristic odour and astringent taste. The powdered leaf of *L. acidissima* under microscopic investigation showed prism like crystals, multicellular trichomes, epidermal cells and actinocytic stomata (Figure 4).

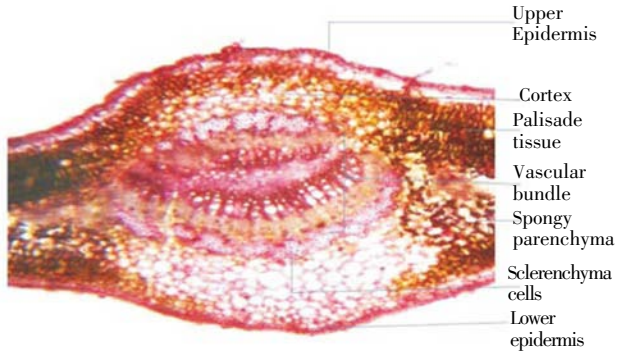

Figure 2. Photomicrographs of microscopic characteristics of *L.* acidissima leaf.

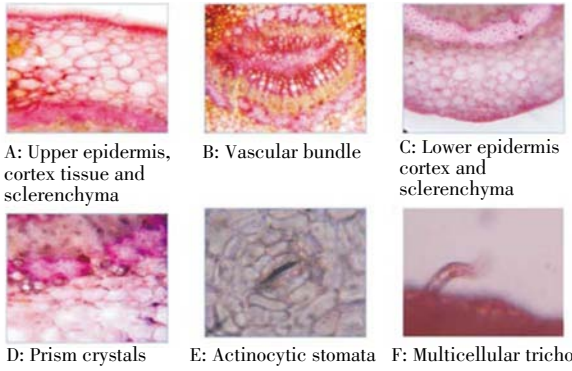

Figure 3. Photomicrographs of macroscopic characteristics of *L. acidissima* leaf.

Figure 4. Photomicrographs of macroscopic characteristics of powder of *L. acidissima* leaf.

3.2. Microscopic characteristics of stem

The microscopical study of stem showed very large pith and vascular bundles arranged in a ring (Figure 5). The stem was thick hollow, continuous cylinder of xylem and phloem. Around the vascular bundle patches of sclerenchyma cells were present. Layer of cuticle was present around 2–3 layers of cork (phellem), composed of rectangular, thick walled cells and filled with reddish brown content. Phellogen was 2–3 layered having polygonal, tangentially elongated, thin walled, parenchymatous cells whereas phelloderm (secondary cortex) was 7–8 layered having oval to polygonal, tangentially elongated, thin walled parenchymatous cells. In the xylem region the xylems were guarded by bi seriate lignified medullary rays. Xylem tissues consisted of xylem vessels, xylem parenchyma, xylem tracheids and xylem fibers. Xylem vessels were larger; these are mainly responsible for conduction of water (Figure 6). The powder microscopy of the stem showed the presence of annular spiral vessel, prism crystals, multi cellular trichome (Figure 7).

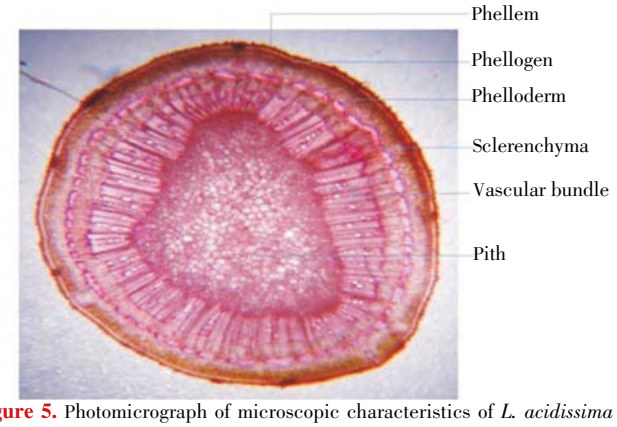

Figure 5. Photomicrograph of microscopic characteristics of *L. acidissima* stem.

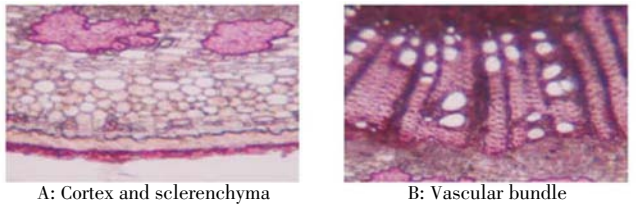

Figure 6. Photomicrograph of microscopic characteristics of *L. acidissima* stem.

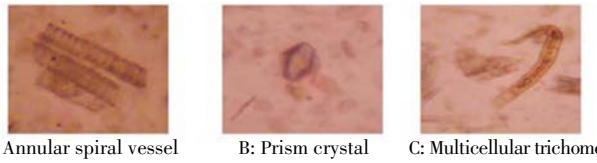

Figure 7. Photomicrograph of microscopic characteristics of powdred *L. acidissima* stem.

3.3. Phytochemical analysis

The results of qualitative phytochemical analysis of the crude powder of *L.* acidissima leaf and stem are shown in Table 2. The leaf had maximum amount of flavonoids and tannins while stem had only flavonoids. Some amount of cardiac glycosides, triterpenes and steroids were present and saponins were completely absent in both leaf and stem.

Table 2

Qualitative phytochemical analysis of *L. acidissima* leaf and stem.

Phytochemicals	Reagents	Leaf	Stem
Alkaloids	Drangroff	+	+
	Mayer	–	–
	Wagner	+	+
Flavonoids		+++	+++
Tannins		+++	–
Cardiac glycosides		+	+
Triterpenes		+	+
Steroids		+	+
Saponins		–	–

3.4. Fluorescence analysis

The fluorescence character of powdered drug plays a vital role in the determination of quality and purity of the drug material. The fluorescence characteristics of leaf and stem powder of *L.* acidissima are summarized in Tables 3 and 4. Some constituents show fluorescence in the visible range in daylight. The UV light produces fluorescence in many natural products which do not visibly fluoresce in daylight. If substance themselves are not fluorescent, they may often be converted into fluorescent derivatives or decomposition products by applying different reagents.

Table 3

Fluorescence analysis of *L. acidissima* leaf powder.

Treatment	Visible light	Under UV light	
		Short wavelength (254 nm)	Long wavelength (365) nm
Powder+1 mol/L NaOH (aq)	Light brown	Black	Green
Powder+1 mol/L NaOH (alc)	Green	Black	Dark green
Powder+Ammonia	Dark green	Black	Dark green
Powder +Picric acid	Green	Black	Black
Powder +Petroleum ether	Green	Black	Light green
Powder+50% HCl	Dark brown	Black	Dark green
Powder+50% H_2SO_4	Brown	Black	Black
Powder+Ethyl acetate	Light green	Black	Orange
Powder+Ethyl alcohol	Green	Black	Orange
Powder+Methanol	Green	Black	Red

Table 4

Fluorescence analysis of *L. acidissima* stem powder.

Treatment	Visible light	Under UV light	
		Short wavelength (254 nm)	Long wavelength (365 nm)
Powder+1 mol/L NaOH (aq)	Dark brown	Black	Dark green
Powder+1 mol/L NaOH (alc)	Brown	Black	Dark green
Powder+Ammonia	Brown	Black	Green
Powder+Picric acid	Yellow	Black	Black
Powder+Petroleum ether	Light brown	Black	Light green
Powder+50% HCl	Brown	Black	Dark green
Powder+50% H_2SO_4	Brown	Black	Dark green
Powder+Ethyl acetate	Light brown	Black	Green
Powder+Ethyl alcohol	Brown	Black	Green
Powder+Methanol	Light brown	Black	Green

3.5. Physicochemical study

The physiochemical characterization of *L. acidissima* leaf and stem are shown in Table 5. The moisture content of leaf and stem was 9.00% and 6.50% respectively. The ash value was determined by three different forms *viz.*, total ash, water soluble ash and acid insoluble ash. The total ash in leaf was 9.33%, while water soluble ash and acid insoluble ash was 1.83 and 1.16 respectively. The total ash in stem was 3.16% while both water soluble ash and acid insoluble ash was 0.66%. The extractive values of leaf and stem are shown in Table 5. The maximum extractive value was found in methanol solvent and minimum was in petroleum ether solvent in both leaf and stem of *L. acidissima*.

Table 5

Physiochemical parameters of *L. acidissima* leaf and stem.

Parameters	% Value (w/w) leaf	% Value (w/w) stem
Loss on drying	9.00	6.50
Total ash	9.33	3.16
Water soluble ash	1.83	0.66
Acid insoluble ash	1.16	0.66
Petroleum ether soluble extractive value	1.14	0.62
Ethyl acetate soluble extractive value	2.58	1.30
Acetone soluble extractive value	2.63	1.12
Methanol soluble extractive value	13.94	5.47
Aqueous soluble extractive value	19.64	4.47

4. Discussion

Establishing standards is an integral part of establishing the correct identity and quality of a crude drug. The microscopic characters, the physiochemical studies and fluorescence analysis can be used for the quality control of the crude drug and these are prime stem for this evaluation[22]; these are prime steps for pharmacognostic study. *L. acidissima* is traditionally used to treat many ailments and illness hence it is imperative to standardize it for use as a drug. According to WHO, the macroscopic and microscopic description of a medicinal plant is the first step towards establishing its identity and purity and should be carried out before any tests are undertaken. Morphological and microscopic studies are reliable, simple and cheapest in establishing the identity of source materials[23].

L. acidissima is an important medicinal plant with many traditional uses, hence it becomes necessary to standardize it for using as a drug. The salient diagnostic characteristics of the leaf were actinocytic stomata, multicellular trichome, prismatic calcium oxalate crystals and vascular bundles while the salient characteristics of the stem were 2-3 layers of phellem , phellogen 2-3 layered followed by 7-8 layered phelloderm, xylem parenchyma, xylem vessels, xylem fibres and tracheids. The phytochemical analysis revealed considerable amount of flavonoids and tannins in leaf while stem showed only the presence of tannins. Saponins were completely absent and other phytoconstituents were present in trace amounts in both leaf and stem powder. The leaf and stem showed less moisture content; it was only 9.00% and 6.50% respectively hence it would not encourage the growth of microorganisms. The ash values were determined by three different forms *viz.* total ash, acid-insoluble ash and water soluble ash. The total ash includes physiological ash and non physiological ash. The physiological ash is derived from the plant tissue itself and non physiological ash is the residue of the extraneous matter adhering to the plant surfact such as metallic salits and/or silica. Low amount of total ash, acid insoluble ash and water soluble ash indicates that the inorganic matter and non-physiological matter is less in leaf and stem. The physiocochemical parameters like ash values, moisture content ensures the purity of the drug and helps in

preventing adulteration and substitution.

The extractive values are useful to evaluate the chemical constituents present in the crude drug and also help in estimation of specific constituents soluble in a particular solvent[2]. Thus, preliminary analysis of phytochemical and physicochemical analysis ensures purity, identity and quality of the drug and also gives an idea about the phytoconstituents present for further analysis[24]. Fluorescence study of the leaf powder helps in the qualitative evaluation that can be used as a reference data for the identification of adulterations. The fluorescent analysis under the visible light and UV light by treatment of different chemical reagents showed different colours. This is attributed to the UV light which produces fluorescence in many natural products that do not visibly fluoresce in daylight. Thus fluorescence is used for qualitative assessment of crude drug[25]. Pharmaognotic studies on different plants like *Polyalthia longifolia*[26], *Cissus quadrangularis*[27], *Woodfordia fruticosa*[28], *Psidium guajava*[29], *Ficus racemosa*[30] and *Heterophragma quadriloculare*[31] are also reported.

In conclusion, it can be stated that the results of the present work lays down the standard parameters which can be useful for checking the authenticity of this important useful medicinal plant, can ensure in maintaining the quality of crude drug and can be also useful for the preparation of a monograph.

Conflict of interest statement

We declare that we have no conflict of interest.

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