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Original Article

Comparative Pharmacognostic Evaluation of Leaves of *Citrus sinensis* Var. *Jaffa* and *Citrus paradisi* var. *Redblush*

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ABSTRACT

Background: The genus *Citrus* (family: Rutaceae), a native to South East Asia and China, comprises aromatic shrubs and trees which are highly acknowledged by folk medicine of various tropical tribes and cultures and also by modern science for the treatment of various ailments. *Citrus sinensis* var. *jaffa* and *Citrus paradisi* var. *redblush* are two medicinally important members of this genus. However, the first step in utilization of a medicinal plant is authentication which involves pharmacognostic examination of the plant material. Systematic pharmacognostic studies for leaves of these *Citrus* species have not yet been done. **Objective:** To study and compare pharmacognostic profile of leaves of *Citrus sinensis* var. *jaffa* and *Citrus paradisi* var. *redblush*. **Methods:** Leaves were subjected to detailed macroscopic, microscopic (qualitative and quantitative), physiochemical, fluorescence and preliminary phytochemical analysis as per standard pharmacopoeial procedures and WHO guidelines. **Results:** Macroscopic examination showed that leaves of the two species can be differentiated based on nature of petiole, size and shape. Diagnostic microscopic features including the size of the epidermal cells, the type of stomata, stomatal index and location of secretory cavities help to distinguish the two species. Results for physiochemical and fluorescence analysis were recorded which will serve as reference standards. Preliminary phytochemical screening showed the presence of alkaloids, flavonoids, tannins, saponins, steroids, volatile oils, proteins and sugars. **Conclusion:** Pharmacognostic standards for leaves have been generated for the first time. These may prove useful to establish identity, quality and purity of these medicinally important *Citrus* species.
Key words: *Citrus*, Fluorescence, Leaf, Microscopic, Physiochemical, Phytochemical.

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INTRODUCTION

Citrus, a premier genus of family Rutaceae (sub-family: Aurantioideae), comprises 16 species of evergreen aromatic shrubs and small trees, native to the Indo-Malaysian region, South-East Asia and China. There are also numerous cross-fertile varieties and cultivars. *Citrus* species are cultivated throughout the tropical and temperate regions globally for their fruit.[1] There has been an increased interest in the study of *Citrus* species in the past few years because of the presence of secondary metabolites in different parts of the plant. *Citrus* fruit, juice and peel oil are renowned for their health benefits and have been widely employed in herbal medicine and aromatherapy. However the lesser explored leaves are equally promising in terms of therapeutic potential.[2] *Citrus spp.* leaves contain a wide range of phytoconstiuents, including the phenolic flavonoid compounds (flavonones, flavones flavonols and polymethoxyflavones), anthocyanin, psoralens, coumarins and tannins as well as essential oils, carotenoids and limonoids.[3,4,5] Two *Citrus* species representing different horticultural groups, *Citrus sinensis* (Linn.) var. *jaffa* (from the group 'Oranges') and *Citrus paradisi* (Macf.) var. *redblush* (from the group 'Pummelos') were selected for the study.

Citrus sinensis (Linn.) var. *jaffa*: is native to north-east India and China.[6] The leaves are used as a folk remedy for insomnia, neurological disorders, diabetes, malaria, cardioprotective, skin diseases, nausea, cough, inflammation, sores, ulcers, scorpion stings and to facilitate digestion of food.[7,8,9,10] Leaf extracts have shown antioxidant, antifungal, anti-*Helicobacter pylori* and larvicidal activity against the dengue vector and negative ionotropic activities in experimental studies.[11,12,13,14,15] The leaf oil has insecticidal activity against larvae of *Culex pipiens molestus* mosquito.[16]

Citrus paradisi (Macf.) var. *redblush*: which is native to the Island of Barbados in the West Indies, is now cultivated in all tropical and subtropical regions for fruit.[17] Leaves are used as a folk remedy for gout, arthritis, swellings, ulcers, insomnia, infections, cuts and wounds.[18,19,20,21] Leaf extracts have shown antioxidant, antitumor, cytotoxic and antibacterial activities.[22,23,24]

There is a good level of traditional and experimental evidence to support various claims about the therapeutic potential of these *Citrus* species. However, scientific comparative pharmacognostic studies of the leaves of these two species have not been reported to date. The WHO has recommended that a specific set of standards for medicinal plant products should be generated using modern techniques and standard procedures for authentication of the plant material.[25] Hence, the present study attempted to establish pharmacognostic profiles of the leaves of *Citrus sinensis* var. *jaffa* and *Citrus paradisi* var. *redblush* to ensure identity, quality and purity of the plant material.

MATERIALS AND METHODS

Chemicals

Phloroglucinol, glycerine, hydrochloric acid, potassium hydroxide and all other chemicals used in the study were of analytical grade.

Plant material

The leaves of *C. sinensis* var. *jaffa* and *C. paradisi* var. *redblush* were collected from Regional Fruit Research Station, Abohar, which is affiliated to Punjab Agriculture University, Ludhiana. Collection occured in the month of November 2014 and were authenticated by Dr. Anil Kumar, Assistant Horticulturist, Regional Fruit Research Station, Abohar, Punjab.

Macroscopic and microscopic evaluation

Leaves were subjected to detailed macroscopic examination.[26] For microscopy evalution, fresh leaves were fixed in FAA (formalin: glacial acetic acid: alcohol 5:5:90). Ventral and paradermal sections stained with phloroglucinol-HCl and mounted in glycerin were studied at 100x, 400x and 1000x magnification using a compound microscope. Photographs were taken with a Nikon DS-L1-5M camera. Leaf surface constants were determined as per standard procedures.[26,27,28]

Physiochemical evaluation

The physiochemical parameters of moisture content, volatile oil content, ash values and extractive values were determined as per standard pharmacopoeial procedures and WHO Guidelines.[25,29]

Fluorescence analysis

Dried powdered leaves were treated with chloroform, methanol, petroleum ether, ethyl acetate, sulphuric acid, nitric acid, hydrochloric acid, acetone, sodium hydroxide etc and observed for colour reactions under visible and ultra violet light.[27,30]

Preliminary phytochemical screening

Various leaf extracts (petroleum ether, chloroform, methanol and aqueous) were prepared by successive Soxhlet extraction and subjected to qualitative phytochemical screening to check for the presence or absence of different classes of phytoconstituents.[31] Percentage yields and physical parameters of all the leaf extracts were recorded.

RESULTS AND DISCUSSIONS

Macroscopic characteristics

According to World Health Organization, the macroscopic and microscopic description of a medicinal plant is the first step towards establishing its identity and should be carried out before any other tests are undertaken.[25] Therefore, various macroscopic features of the leaves were recorded (Table 1) and the diagnostic feature that can help differentiate given two species was found to be a narrowly winged petiole in *C. sinensis,* whilst a broadly winged petiole having obovate wings was seen in *C. paradisi* (Figure 1a and 1b). Also, the leaves of *C. paradisi* were longer and broader than leaves of *C. sinensis*.

Microscopic characteristics

Examination of ventral sections and paradermal sections of leaves revealed following histoanatomical characteristics:

Upper epidermis

The upper epidermis of both the leaves was composed of three to four layers of polygonal cells having straight anticlinal walls covered with thick layer of cuticle. However, the epidermal cells of *C. sinensis* are larger (20.5-36.0μ long, 10.6-17.6μ wide) than the epidermal cells of *C. paradisi* (13.6-30.7μ long, 6.5-17.1μ wide) as shown in Figure 2a and 2b. Prismatic Ca-oxalate crystals (upto 15μ long) can be seen throughout the upper epidermis (Figure 3a and 3b). In both the species, stomata and trichomes are absent.

Mesophyll (Figure 4a, 4b, 5a and 5b)

This region shows typical dicot leaf-like characteristics in both the species. There are two to three layers of cylindrical, tightly packed palisade parenchyma cells. Palisade cells containing Ca-oxalate crystals which protrude into upper epidermis and secretory cavities (upto 75μ in diameter) containing volatile oil can be seen in this region. The rest of the mesophyll is composed of approximately eight-layer thick spongy parenchyma

Table 1: Macroscopic features of leaves.

Feature	*C. sinensis* var. *jaffa*	*C. paradisi* var. *redblush*
Colour	US: bright and dark green, LS: light green	US: bright and dark green LS: light green
Odour	Sharp and pleasant similar to fruit	Citrus like, dull as compared to C. sinensis
Taste	Bitter and astringent	Bitter and astringent
Size	8 to 13 cm × 2 to 5 cm	10 to 16 cm × 3 to 8 cm
Shape	Ovate	Ovate- obovate
Margin	Crenate	Crenate
Apex	Acute	Acute to obtuse
Base	Obtuse, symmetric	Obtuse, symmetric
Petiole	Narrowly winged petiole	Broadly winged petiole having obovate wings
Spines	Axillary spines	Axillary spines
Surface	Glabrous, oil dotted	Glabrous, oil dotted
Texture	Leathery	Leathery
Venation	Pinnate reticulate	Pinnate reticulate
Type	Simple	Simple

US: upper surface, LS: lower surface

Figure 1: (a). Leaves of C. sinensis var. jaffa, (b).Leaves of *C. paradisi* var. redblush, column width.

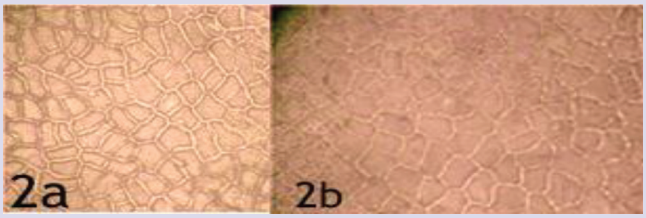

Figure 2: (a). Upper epidermis of C. sinensis var. jaffa in surface view, (b).Upper epidermis of *C.paradisi* var. redblush in surface view, column width.

where cells vary in shape and size and have large intercellular spaces. Cells near the lower epidermis are nearly spherical and tightly packed.

Vascular system

The mid-rib shows a large vascular bundle comprising a crescentic bundle on the abaxial side and a smaller arc on the adaxial side with two xylem and phloem rings with the phloem outermost. The xylem and phloem elements of upper bundle have inversed distribution so that the xylem of the two bundles lies opposite to each other, but separated by a narrow zone of parenchyma. The lower bundle is more developed and is protected by a discontinuous sclerenchymatous sheath (Figure 4a and 4b). Small vascular bundles surrounded by pericyclic fibres can be seen in the lamina portion (Figure 5a and 5b).

Lower epidermis

Cells in this region resemble the cells of upper epidermis in shape and size and are covered with a thick layer of cuticle. Both the species are hypostomatic as abundant shrunken paracytic type of stomata are present on lower epidermis (Figure 6a and 6b). The stomata are more numerous in the case of *C. paradisi* leaf. Some earlier studies on the epidermal morphology of *C. paradisi* and *C. sinensis* have reported the presence of anomocytic stomata.[32,33] whilst some researchers have reported the presence of paracytic stomata in both species.[34,35,36] Trichomes are absent. Cells containing prismatic Ca-oxalate crystals can also be seen. However, secretory cavities adjoining the lower epidermis were found only in *C. paradisi.* This is of diagnostic importance (Figure 4a and 4b). Leaf surface constants for both the species were determined and compared which help to identify and differentiate closely allied species Table 2.

Physiochemical evaluation and fluorescence analysis

Unlike taxonomic identification, pharmacognostic study also includes parameters which help in identifying adulteration in dry powder forms. Therefore, physiochemical parameters of powdered leaves were also determined. The percentage of active chemical constituents in the crude drugs is mentioned on an air-dried basis. Therefore, the moisture content of leaves was determined. Aromatic drugs are standardized based on volatile oil content. Hence, the volatile oil content of leaves was also determined. The physiochemical evaluation revealed higher volatile oil content for the leaves of *C. sinensis* whilst extractive values were found to be higher for *C. paradisi* (Table 3). Ash values were determined. These values represent inorganic salts or silica naturally occurring in the crude drug, or adhering to it, or deliberately added to it as a form of adulteration. The extractive values were determined and are primarily useful for the determination of exhausted or adulterated drug.[37] Fluorescence analysis helps to identify the drug in the powder form, as fluorescence is shown by various phytoconstituents in the range of visible and UV light when treated with different reagents (Table 4).[31]

Preliminary phytochemical screening

The yields of leaf extracts were recorded as extracts obtained by exhausting plant materials with specific solvents are indicative of approximate measures of their chemical constituents extracted with those solvents from a specific amount of air-dried plant material. This parameter is employed for materials for which as, yet no suitable chemical or biological assay exists.[37] Physical parameters and percentage yields of various leaf extracts are summarized in Table 5. Preliminary phytochemical screening of different leaf extracts of both the plants showed the presence of alkaloids, flavonoids, tannins, saponins, steroids, volatile oil, proteins and sugars (Table 6). Results of preliminary phytochemical screening will be useful in finding out the genuity of the drug.

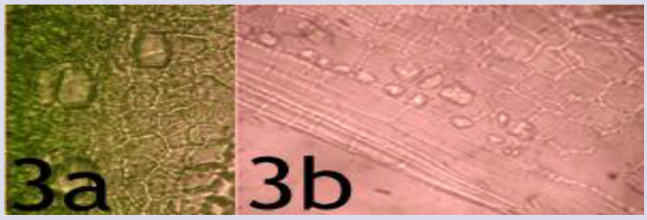

Figure 3: (a). Ca oxalate crystals of C. sinensis var. jaffa in surface view, (b).Ca oxalate crystals of *C. paradisi* var. redblush in surface view, column width.

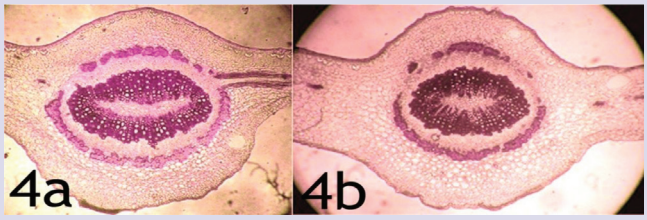

Figure 4: (a). Ventral section of C. sinensis var. jaffa leaf through midrib, (b).Ventral section of *C. paradisi* var. redblush leaf through midrib, column width.

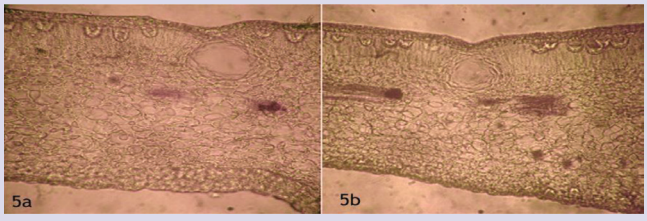

Figure 5:(a). Ventral section of C. sinensis var. jaffa leaf through lamina,(b).Ventral section of *C. paradisi* var. redblush leaf through lamina, column width.

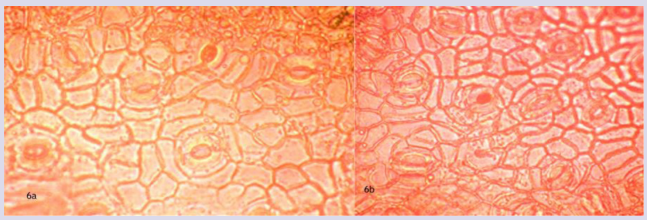

Figure 6: (a). Paracytic stomata of C. sinensis var. jaffa,(b).Paracytic stomata of *C. paradisi* var. redblush, column width.

Table 2: Leaf surface constants.

Parameter	Value per sq mm (25 Fields)	
	C. sinensis* var. *jaffa	***C. paradisi* var. *redblush***
Stomatal Number	142-**180**-231	378-**410**-432
Stomatal Index	12.7-**17.2**-19.2	16.3-**22.9**-26.5
Palisade Ratio	2.5-**4.5**-7.0	4.0-**5.0**-7.0
Vein-Islet Number	10.0-**13.5**-16.5	8.0-**9.5**-14.0
Veinlet Termination Number	21-**24**-27	20-**22**-25

Table 3: Physiochemical parameters of powdered leaves.

Parameter	Mean (%w/w On dry weight basis, n=3)	
	C. sinensis* var. *jaffa	***C. paradisi* var. *redblush***
Foreign matter	0.46	0.55
Loss on Drying	4.20	3.60
Volatile oil content	1.00	0.50
Total ash	10.30	11.80
Acid insoluble ash	1.00	1.30
Ethanol soluble extractive	22.38	24.65
Water soluble extractive	29.14	31.82

Table 4: Fluorescence analysis of powdered leaves.

Reagent	*C. sinensis* **var.** *jaffa*			*C. paradisi* **var.** *redblush*		
	Visible	*Short UV*	*Long UV*	*Visible*	*Short UV*	*Long UV*
Petroleum Ether	Green	Pale Green	Dark Green	Green	Pale Green	Black
Chloroform	Bright Green	Fluorescent Green	Red	Green	Fluorescent Green	Red
Methanol	Green	Pale Green	Red	Green	Pale Green	Red
Acetone	Pale Green	Green	Brown	Pale Green	Green	Orange
1% Glacial Acetic acid	Green	Pale Green	Brown	Green	Pale Green	Brown
50% Sulphuric acid	Dark Brown	Pale Brown	Black	Dark Brown	Pale Brown	Black
50% Hydrochloric acid	Orange	Pale Green	Dark Green	Orange	Pale Green	Green-Black
50% Nitric acid	Orange	Green	Green	Orange	Green	Green
10% Sodium hydroxide	Brown	Green	Dark Green	Brown	Green	Dark Green
As such powder	Green	Dark Green	Brownish green	Green	Dark Green	Brownish green

Where short UV=254nm, long UV=365nm

Table 5: Physical parameters of leaf extracts.

Leaf Extract	***C. sinensis* var. *jaffa***		***C. paradisi* var. *redblush***	
	Yield (%w/w)	**Colour**	**Yield (%w/w)**	**Colour**
Petroleum Ether	1.89	Teak Brown	3.17	Teak Brown
Chloroform	4.88	Chestnut Brown	5.93	Chestnut Brown
Methanol	16.69	Mustard Brown	16.78	Golden Brown
Aqueous	18.47	Pompeian Red	24.59	Pompeian Red

Table 6: Phytochemical screening of leaf extracts.

Phytoconstituent	*C. sinensis* **var.** *jaffa*				*C. paradisi* **var.** *redblush*			
Test/Reagent	*PE*	*CH*	*ME*	*AQ*	*PE*	*CH*	*ME*	*AQ*
Alkaloids								
Mayer's Test	–	–	+	–	–	–	+	–
Dragendorff's Test	–	–	+	–	–	–	+	–
Wagner's Test	–	–	+	–	–	–	+	–
Hager's Test	–	–	+	–	–	–	+	–
Carbohydrates								
Molisch Test	–	–	+	+	–	–	+	+
Fehling's Test	–	–	+	+	–	–	+	+
Benedict's Test	–	–	+	+	–	–	+	+
Barfoed's Test	–	–	+	+	–	–	+	+
Selvinoff's Test	–	–	–	+	–	–	+	+
Phloroglucinol Test	–	–	–	–	–	–	–	–
Cobalt Chloride Test	–	–	+	+	–	–	+	+
Iodine Test	–	–	–	–	–	–	–	–
Coumarins								
Fluorescence Test	–	–	+	–	–	–	–	–
Fats and Fixed Oils								
Tincture of Alkanna	+	+	–	–	+	–	–	–
Flavonoids								
Shinoda Test	–	–	+	–	–	–	+	–
Lead Acetate Test	–	–	+	–	–	–	+	–
Sodium Hydroxide Test	–	–	+	–	–	–	+	–
Proteins								
Millon's Test	–	–	+	–	–	–	–	–
Biuret Test	–	–	–	–	–	–	–	–
Phenolic Compounds andTannins								
Ferric Chloride Test	–	–	+	+	–	–	+	+
Lead Acetate Test	–	–	+	+	–	–	+	+
Saponins								
Foam Test	–	–	+	–	–	–	–	–
Steroids								
Salkowski Test	–	+	–	–	+	+	–	–
Triterpenoids								
Liebermann-Burchard's Test	–	+	+	–	–	+	+	–

PE: petroleum ether, CH: chloroform, ME: methanol, AQ: aqueous

CONCLUSION

In the present study, an attempt has been made to establish pharmacognostic profile of leaves of *C. sinensis* var. *jaffa* and *C. paradisi* var. *redblush* which may prove useful to ensure identity, quality and purity of these medicinally important plants for future use.

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ABBREVIATIOSN USED

WHO: World Health Organization; **PE:** Petroleum ether; **Ch:** Chloroform; **ME:** Methanol; **AQ:** Aqueous.

CONFLICT OF INTEREST

There is no conflict of interest.

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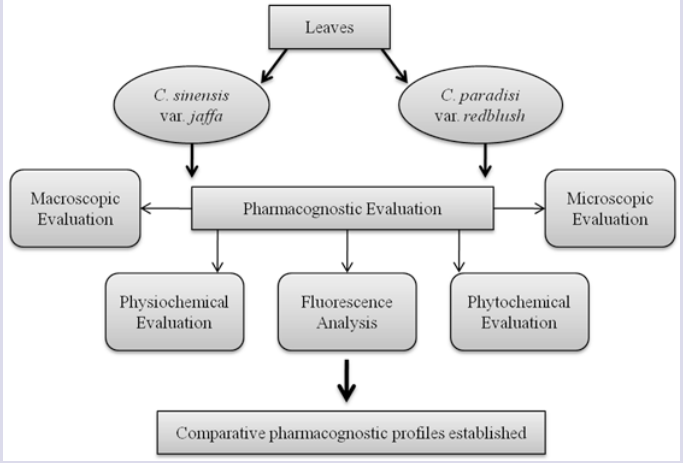
PICTORIAL ABSTRACT



SUMMARY

- The leaves of Citrus sinensis var. *jaffa* and *Citrus paradisi* var. redblush are used in traditional medicinal systems of various regions for treatment of a wide range of ailments. Systematic pharmacognostic studies for leaves of these *Citrus species* have not yet been done. In the present study, an attempt has been made to establish pharmacognostic profile of leaves of *C. sinensis* var. *jaffa* and *C. paradisi* var. *redblush* which may prove useful to ensure identity, quality and purity of these medicinally important plants for future use. Leaves were subjected to detailed macroscopic, microscopic (qualitative and quantitative), physiochemical, fluorescence and preliminary phytochemical analysis as per standard pharmacopoeial procedures and WHO guidelines. Macroscopic examination showed that leaves of the two species can be differentiated on the basis of nature of petiole, size and shape. Diagnostic microscopic features including the size of the epidermal cells, the type of stomata, stomatal index and location of secretory cavities help to distinguish the two species. Results for physiochemical and fluorescence analysis were recorded which will serve as reference standards. Preliminary phytochemical screening showed the presence of alkaloids, flavonoids, tannins, saponins, steroids, volatile oils, proteins and sugars. Pharmacognostic standards for leaves have been generated for the first time. These may prove useful to establish identity, quality and purity of these medicinally important *Citrus species*.