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Original Article

Comprehensive Study on the Pharmacognostical, Physico-Phytochemical, and Analytical Properties of *Citrus limon* (L.) Burm. F. Fruit

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ABSTRACT

Background: Pharmacognosy is a scientific and systematic study of natural products helps in identification of raw material through morphological and chemical properties. *Citrus limon* (L.) Burm. f. is a tree, and its fruits are used for nutritional and therapeutic values in Indian traditional system of medicine, Ayurveda, and other like African and Romanian traditional systems. *Citrus limon* has a wide use but its pharmacognostical, physicochemical, phytochemical profile is not published till date. Thus, to develop a preliminary pharmacognostical, physicochemical, and phytochemical profile of *Citrus limon* current research has been carried out. **Materials and Methods:** *Citrus limon* fruits were collected from its natural habitat and authenticated at CSIR-National Institute of Science Communication and Policy Research (CSIR-NIScPR). Pharmacognostical, physicochemical analysis was done as per Ayurvedic Pharmacopeia of India. **Results:** The macroscopic analysis suggested that fruit of *Citrus limon* was ovoid; the microscopic analysis revealed the presence of prismatic calcium oxalate crystals; in the physicochemical parameters loss on drying was found 11.90% and pH 2.98. The phytochemical analysis revealed presence of proteins, alkaloids and flavonoids. The fourier transform infrared spectroscopy showed presence of alcohol, alkenes, and aromatic compounds. Fingerprinting analysis of *C. limon* through high performance thin layer chromatography showed presence of various phytoconstituents in different concentrations through different peaks and R f values and also sample showed presence of 2.36% limonene. **Conclusion:** The present study will serve as a quality control parameter of *Citrus limon* fruit. The study will be helpful in easy identification, authentication, and detection of adulteration in marketed samples.

Keywords: Calcium oxalate, Ethnomedicine, Limonene, Phytochemical, Quality control, Traditional medicine.

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INTRODUCTION

Plant-based medicines are widely used by most of the population for health (Nasim, *et al*., 2022). In Ayurveda, various plant species are described for therapeutic effect reliant on the genuineness of raw material. The raw material of plants are derived from various geographical sources; hence, the correct identification of raw material is essential (Shrestha *et al*., 2018). Pharmacognosy is a scientific and systematic study of natural products may be of animal, mineral, metal or of plant origin (Khan *et al*., 2020). It includes the study of morphological and chemical properties (Orhan I E, 2014) which helps in identification.

Citrus limon (L.) Burm. f. is a tree with yellow edible fruits of rutaceae family. It is used in Ayurveda to manage various diseases like *Kasa* (cough), *Vibandha* (constipation) etc. (Chunekar, 2015). The fruit juice has nutritional value along with vitamin C. Apart from Ayurveda traditional medicine, the juice is used in other traditional systems like in African traditional system, it is used to treat hypertension, cough, cold and irregular menstrual cycles. In Romanian traditional system, it is used to treat scurvy, sore throats, fever, and chest pain (Klimek-Szczykutowicz, M *et al*., 2020). The published literature revealed that peel and flesh of *Citrus limon* (*C. limon*) of Tunisia was evaluated for its phytochemical and physicochemical analysis (Makni *et al*., 2018). *C. limon* has been also evaluated for its cytotoxic, anxiolytic, and anti-spasmodic etc. activities in various studies (Dosoky & Setzer, 2018). However, *C. limon* fruit of Indian origin is not evaluated for its pharmacognostical, physicochemical, phytochemical,

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and analytical properties yet thus, the present study has been undertaken as it has therapeutic as well as nutritional values.

MATERIALS AND METHODS

Collection and authentication

Collection of mature fruits of *C. limon* was done from local market of Amritsar, Punjab. The sample was identified, authenticated, and the voucher specimen was placed in Raw Materials Herbarium and Museum (RHMD), CSIR-NIScPR, New Delhi.

Drying and grinding of *C. limon* fruits

The fresh mature *C. limon* fruits were taken, washed with tap water, and converted into small pieces with the help of knife, then dried in shade. After proper drying, the pieces of *C. limon* grinded to make fine powder with the help of mixer grinder.

Pharmacognostic evaluation

The morphological characteristics of *C. limon* fruit parts, including color, shape, size, taste, and texture, were assessed. The freshly collected fruits underwent for macroscopic observation. Anatomical study of samples was done with the help of stained hand/microtome sections which were observed under trinocular microscope after mounting. Staining was done with diluted aqueous safranin and fast green and after thoroughly washing sections was mounted in 40% glycerin. For examining powder characteristics, 5g of fine fruit powder was taken in chloral hydrate solution on a slide, warmed on a hot-plate for a short time, and then it was covered with a cover slip and observed under trinocular microscope. The microscopic images were thoroughly examined and the anatomical characteristics were noted.

Physicochemical evaluation

The dried fruit powder of *C. limon* was used for quantitative determination of physicochemical values including total ash, acid insoluble ash, moisture content (loss on drying), alcohol and water-soluble extractive as per Ayurvedic Pharmacopoeia of India (API) methods (Ministry of Health and Family Welfare [MoHFW], 2011).

Phytochemical investigation

Analytical grade chemicals were used in the experiment. HPLC-grade methanol, hydrochloric acid and ferric chloride were purchased from qualigen chemicals (Mumbai, India). Dragendorff's reagent & Millon's reagent were purchased from CDH (GIDC, Gujarat), sodium hydroxide was purchased from Merck (Mumbai, India).

5 g fine dried fruit powder was mixed in 30 mL methanol and 30 mL distilled water separately, and they were soaked for 6 hr on a rotatory shaker. After proper shaking of samples, they were kept for 18 hr to complete 24 hr, maceration procedure. After that, they were filtered through 1 no. Whatman filter paper to prepare methanolic and aqueous extracts respectively.

The preliminary phytochemical investigation of methanolic and aqueous extracts was performed according to standard protocol for the determination of proteins, alkaloids, flavonoids, tannins, and saponins (Kokate *et al.*, 2015).

Analytical study

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis was done through PerkinElmer spectrum by using Universal Attenuated Total Reflectance (UATR) technique and spectra were taken in 4000-400 cm^{-1} range. The interpretation of peaks was done by IR spectrum table.

High Performance Thin Layer Chromatography (HPTLC)

Preparation of test solution

1 g sample was mixed in 10 mL n-hexane and kept for 24 hr maceration. Next day after 20 min. sonication it was filtered through Whatman filter paper no.1. This solution or extract was used for chromatographic analysis. The stationary phase was used HPTLC aluminium precoated Silica gel 60 F_{254} (Merck KGaA) plate of 10×10 cm with 0.2 mm thickness. A mobile phase of n-hexane: ethyl acetate (8:2 v/v) was emloyed. Camag Linomat 5 auto sampler is used for sampling.

The sample solution of 8 µL and 10 µL volumes was applied to the HPTLC plate using an 8 mm band width, maintaining a distance of 11.40 cm between the bands.

Derivatization: Anisaldehyde-sulphuric acid sprayed, and plate was heated at 100°C for 3 min.

Visualization: Visualization is performed at 254 nm, 366 nm and 540 nm wavelengths.

HPTLC with standard limonene

The volatile oil of *C. limon* peel was extracted with the help of Clevenger's apparatus. n-hexane: ethyl acetate (8:2 v/v) was used as mobile phase. Limonene used as standard was procured from Sigma-Aldrich.

Sample preparation: 500 µL volatile oil dissolved in 1 mL n-hexane (v/v).

Standard preparation: 500 µL of standard limonene dissolved in 1 mL n-hexane (v/v).

RESULTS

Authentication

The *C. limon* fruit authentication no. is NIScPR/RHMD/Consult/2022/4292-92-1 (Annexure 1).

Pharmacognostic evaluation

Macroscopic evaluation

C. limon fruits (3-5) were investigated from bottom to tip for its size, shape, color, etc., and the macroscopic features are noted below in Figure 1 and Table 1.

Microscopic evaluation

Transverse Section of C. limon

Transverse Section (TS) of fruit part of *C. limon* showing circular to oval shaped outline. Bright yellowish smooth outer epicarp followed by hypodermis, outer mesocarp region embedded with large volatile oil glands, the inner portion being occupied by 10-11 or more segments of endocarp and reaching up to the central axis. TS of the pericarp shows a layer of epicarp, consisting of polygonal-shaped cells covered with single layer of cuticle, followed by hypodermis, spongy parenchymatous celled zone of mesocarp embedded with oval shaped lysigenous oil glands. Thick-walled lignified cells are located near the oil cavity walls. Prismatic crystals of calcium oxalate are present in the mesocarp region. Transverse Section of seed part of *C. limon* showing oval in outline shows cotyledon embedded with a row of vascular bundles and filled with aleurone grains. Testa shows radially elongated sclerenchymatous cells of epidermis with mucilaginous outer walls followed by a thin collapsed cells of perisperm, and endosperm is a narrow band of 3 to 4 rows of cells, embedded with aleurone grains; its innermost cells being collapsed. The cotyledon shows upper epidermis layer enclosing the cotyledonary cells embedded with small aleurone grains (Figure 2).

Powder microscopy of *C. limon*

Brownish yellow colored powder shows cells from mesocarp, fragment of seed coat, spiral vessels, prismatic calcium oxalate crystals up to 20µ, fibre up to 175 µ in length with a narrow lumen, thick-walled lignified cells, tannin cell, aleurone grain from cotyledons, oil globule from pericarp, and elongated sclereids from seed coat (Figure 3).

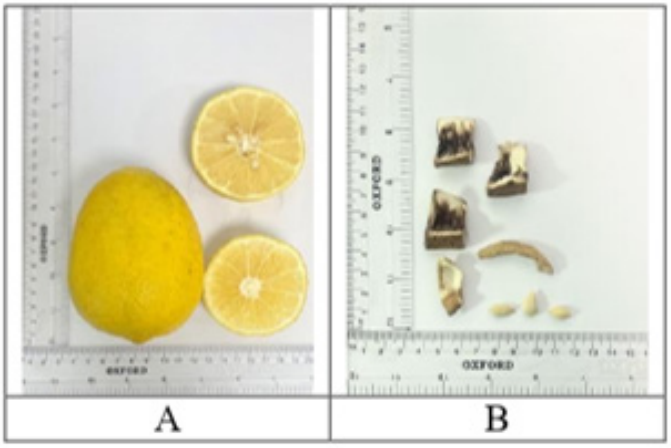

Figure 1 (A-B): Macroscopic evaluation of *C. limon*; A: fresh sample; B: dry sample.

Physicochemical evaluation

The physicochemical profile of *C. limon* fruit powder has been performed, and the results are summarized in Table 2.

Phytochemical Investigation

Qualitative phytochemical screening of two types of extracts (methanolic and aqueous) of *C. limon* fruit have been performed, and the results are presented in Table 3.

Fourier Transform Infrared Spectroscopy (FTIR)

C. limon fruit powder was investigated in IR spectrum ranging 4000-400 cm^{-1}, and the results after interpretation summarized in Table 4.

High Performance Thin Layer Chromatography (HPTLC)

The HPTLC analysis of *C. limon* revealed the presence of various unknown phytochemicals through chromatograms obtained by scanning of sample at 254, 366 and 540 nm (Figure 4). The peak tables with R_f values are depicted in the Table 5.

Limonene was used as marker compound and R_f was observed at 0.61. The R_f values of all seven tracks of samples and limonene are mentioned in Table 6. The results showed presence of 2.36% of limonene in *C. limon*. The chromatographic scanning images and detailed calibration plot is mentioned in Figures 5 and 6.

DISCUSSION

The present research study has been undertaken to establish preliminary quality control parameters including pharmacognosy, physicochemical, phytochemical, and analytical parameters of *C. limon* because these parameters are integral part of standardization and indicators of the safety and efficacy of herbal drugs (Alam F & Us Saqib QN, 2015a). In the present study the macroscopic

Table 1: Macroscopic characteristics of *C. limon*.

Parameters	Observations
Length	7-13 cm
Width	5-9 cm
Shape	Ovoid
Nipple	Present
Surface	Irregular wart rind
Colour	Greenish yellow
Fragrance	Citrus
Pulp	Pale yellow
No. of seeds	30-40 in approx. 11 locules
Taste	Sour

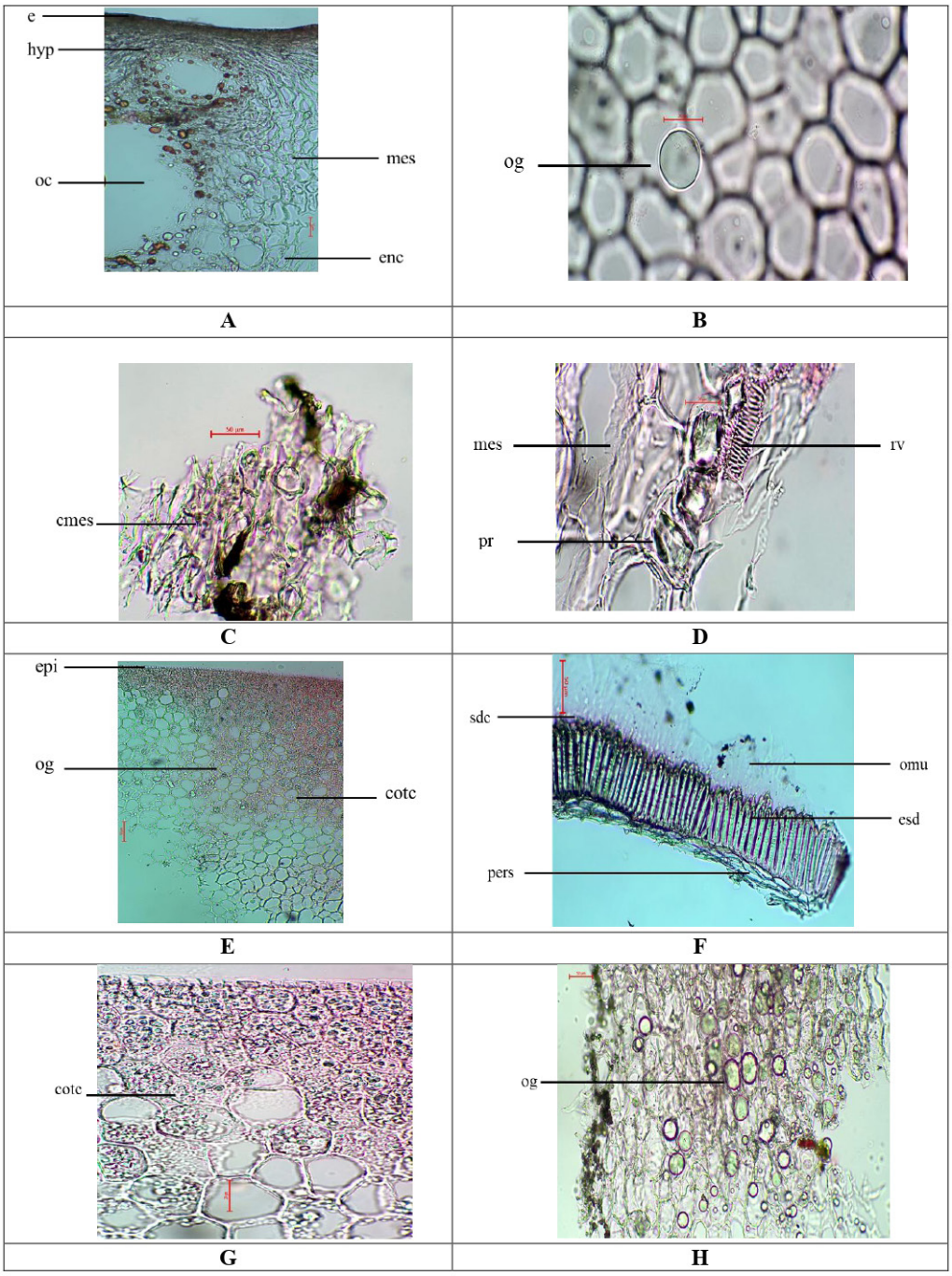

Figure 2 (A-H): Transverse Section of *C. limon*; A: T.S of pericarp, 10x view B: Oil globules, 40x view, C: Cells from mesocarp, 10x view, D: Reticulate vessels and prismatic crystals, 40x view, E: T.S of cotyledon, 10x view, F: T.S of seed coat, 10x view, G: T.S of cotyledon-enlarged view, 40x view, H: Oil filled thin walled parenchyma from cotyledon, 10x view. e: epidermis; hyp: hypodermis; oc: oil cavity; mes: mesocarp; enc: endocarp; og: oil globule; cmes: cells of mesocarp; rv: reticulate vessels; pr: prismatic crystal; epi: epicarp; cotc: cotyledonary cells; sdc: seed coat; omu: outer mucilaginous wall; esd: epidermal cells of seed; pers: perisperm.

study reveals presence of nipple and irregular wart rind in fresh sample of *C. limon* which are key identifying features.

The anatomical features such as vascular bundles, fibers, and cortex of a plant provide the key identifying features and have a crucial role in quality control and standardization (Majid *et al.*, 2021a). The TS of *C. limon* showed presence of oval-shaped lysigenous oil glands and prismatic crystals of calcium oxalate in the mesocarp region. TS of seed showed presence of aleurone grain.

The powder microscopy also provides structural components of a powdered raw plant material (Majid N *et al.*, 2021b). In the present study spiral vessels, prismatic calcium oxalate crystals, fibre with narrow lumen, thick-walled lignified cells, tannin cell, aleurone grain were seen in powder microscopic study

indicating the presence of all fruit parts and seeds in the powder. The macroscopic and microscopic studies are important tool for detection of adulteration (Ragesh *et al.*, 2016). Phytochemical classes of phytoconstituents or secondary metabolites are the ones responsible for the pharmacological activity (Alam & Us Saqib, 2015b). In the present study preliminary phytochemical screening was done to find out groups of phytochemicals in methanolic and aqueous extracts. The present study showed the presence of protein, flavonoids, alkaloids and tannins in methanolic extract. The aqueous extract showed only the presence of flavonoids.

It is well reported that plant proteins have the potential to reduce the risk of developing metabolic syndrome, cancer, and useful in diabetes and weight control (Ahnen *et al.*, 2019). Natural polyphenol substance groups are flavonoids having anti-oxidant and other pharmacological activities like antimicrobial and anti-inflammatory (Shen *et al.*, 2022). Alkaloids are useful

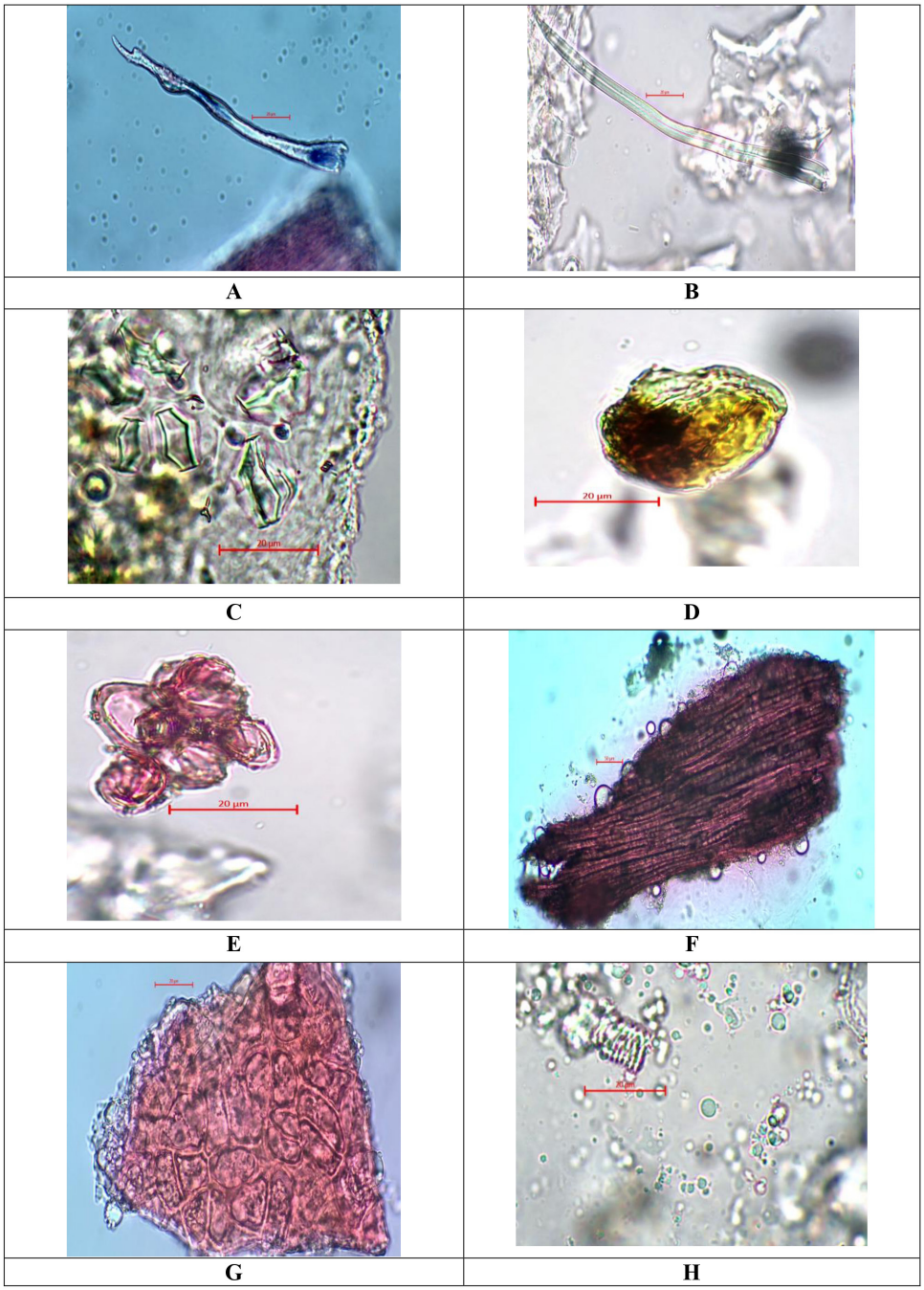

Figure 3 (A-H): Powder microscopy of *C. limon*; A: fibre, 40x view, B: fibre, 40x view, C: Prismatic calcium oxalate crystals, 40x view, D: Tannin cell, 40x view, E: Thick walled lignified cells, safranin stained 40x view, F: Elongated sclereids from seed coat, Safranin stained, 10x view, G: Cells from seed coat, Safranin stained, 40x view, H: Oil globules and spiral vessels, 40x view.

in human physiology and are used to improve body mass and composition (Sellami *et al.*, 2018). Tannins are class of polyphenols useful for anti-inflammatory, antimicrobial, antioxidant, and anticancer properties (Maugeri *et al.*, 2022).

Physicochemical parameter play important role in standardization and remain constant for a plant thus, they are helpful in detection of adulteration (Alam & Us Saqib, 2015c). The moisture content in physicochemical analysis is determined by Loss on drying (LOD), the ash value indicates the carbonate, oxalate, and silicate impurities, contamination with earthy material indicated by acid insoluble ash and the pH values suggests the acidic or basic nature of raw material (Khan *et al.*, 2020). The physicochemical analysis of *C. limon* is mentioned in Quality Standards of Indian Medicinal Plants published by Indian Council of Medical Research (ICMR) and the current research results are comparable with it (Tandon N, 2016).

The FTIR analysis is indicative of possible functional groups with structural information (Song *et al.*, 2020). The FTIR of *C. limon* suggested the presence of alcohol, alkenes, aromatic compounds, aldehydes, ketones, esters etc. functional groups (Figure 7).

Table 2: Physicochemical profile of *C. limon*.

Parameters	Results
pH value (10% w/v)	2.98
Loss on drying at 105 ^{0}C (w/w)%	11.90
Total ash (w/w)%	3.76
Acid insoluble ash (w/w)%	0.15
Alcohol soluble extractive (w/v)%	33.72
Water soluble extractive (w/v)%	18.08

Table 3: Phytochemical screening of methanolic and aqueous extracts of *C. limon*.

Phytochemical group	Methanolic Extract	Aqueous Extract
Proteins	++	-
Tannins	++	-
Flavonoids	++	+
Alkaloids	+	-
Saponins	-	-

* +=Present, -=Absent.

Table 4: Functional group present in *C. limon* FTIR analysis.

Peak wavelength	Appearance	Group	Compound class
3341.49	Broad	O-H stretch	Alcohol
3008.70	Medium, Weak	=C-H stretch, C-H stretch	Alkenes, Aromatic Compounds
2923.07	Sharp	C-H stretch	Alkanes
2853.29	Sharp	C-H stretch, C-H stretch	Alkanes, Aldehydes
1738.19	Sharp	C=O stretch, C=O stretch, C=O stretch	Aldehydes, Ketones, Esters
1629.26	Broad	C=C stretch	Alkenes
1365.49	Sharp	NO2 stretch	Nitro Compounds
1217.14	Weak	C-F stretch	Alkyl & Aryl Halides

Table 5: Peaks at 254, 366, 540 nm with R_f of track 1 and 2.

Track No.	R_f at 254 nm	R_f at 366 nm	R_f at 540 nm
1	0.03, 0.08, 0.18, 0.51	0.02, 0.08, 0.18, 0.28, 0.51, 0.56, 0.62	0.20, 0.27, 0.40, 0.53, 0.66, 0.78
2	0.03, 0.08, 0.18, 0.50, 0.77	0.03, 0.08, 0.19, 0.28, 0.56	0.19, 0.27, 0.40, 0.52, 0.65, 0.78

Table 6: Peaks at 366 nm with R_f of *C. limon* and standard Limonene all tracks.

Track No.	Sample	R_f	Area
1	*C. limon*	0.61	0.001
2		0.60	0.003
3		0.60	0.009
4	Limonene	0.61	0.0007
5		0.59	0.001
6		0.60	0.003
7		0.60	0.006

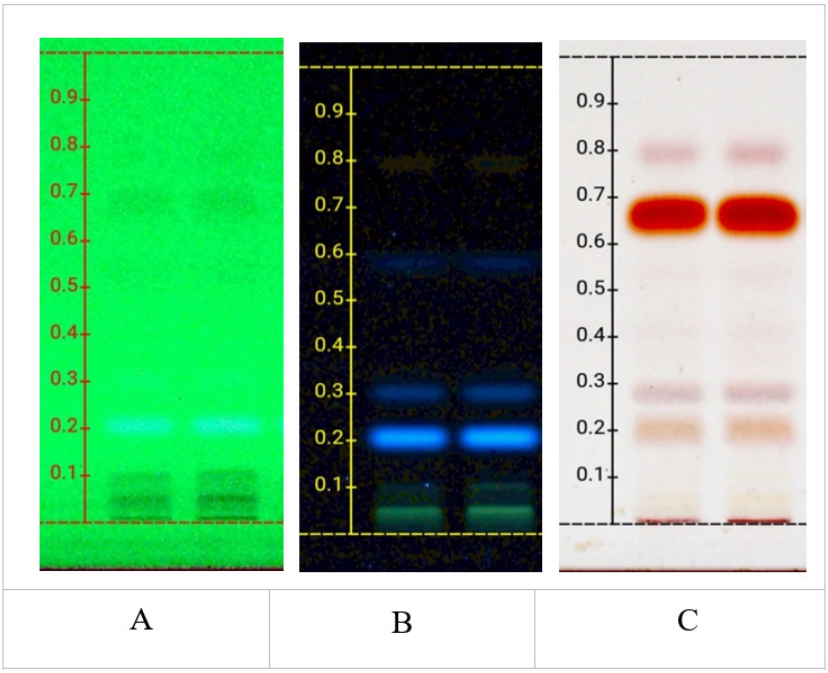

Figure 4: HPTLC chromatograms of *C. limon* visualized under A UV 254 nm, B UV 366 nm, C HPTLC visualizer 540 nm.

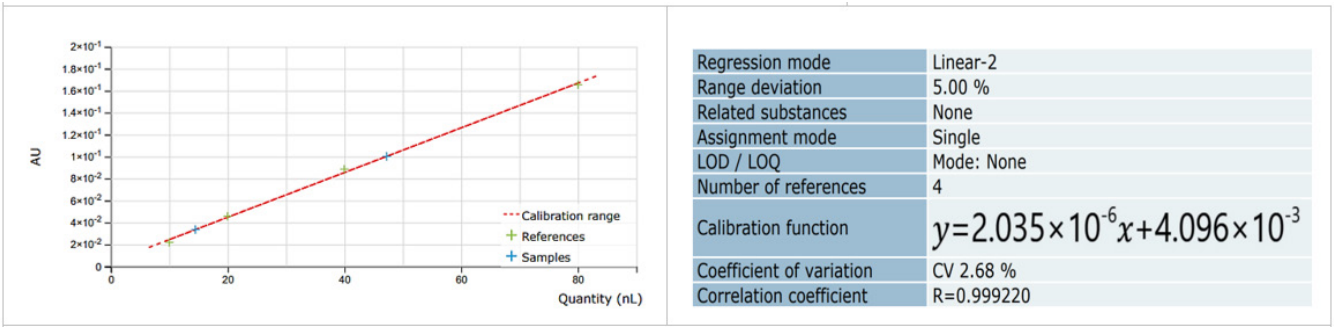

Figure 5: Callibration curve of standard limonene and *C. limon*.

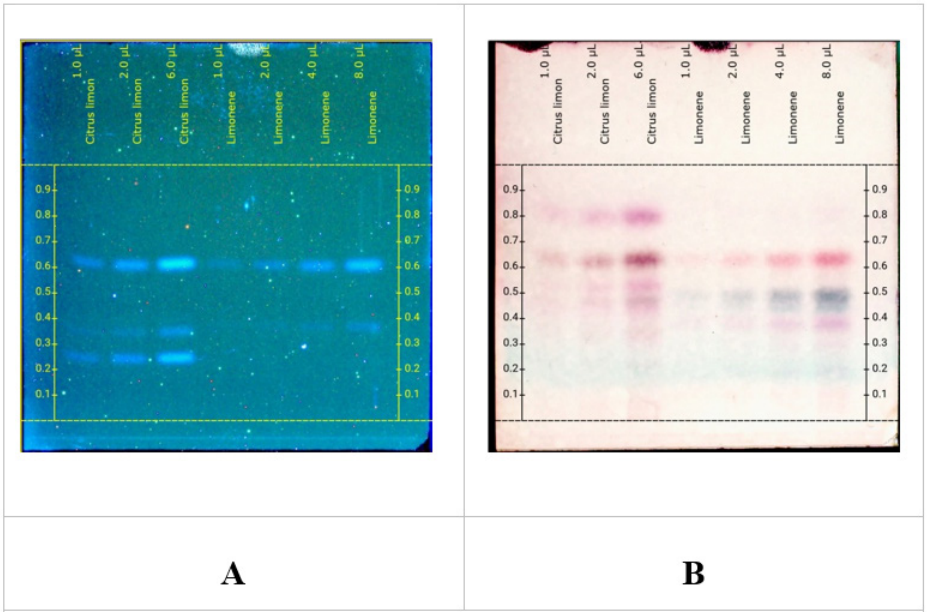

Figure 6: HPTLC chromatograms of *C. limon* visualized under A UV 366 nm, B HPTLC visualizer 540 nm.

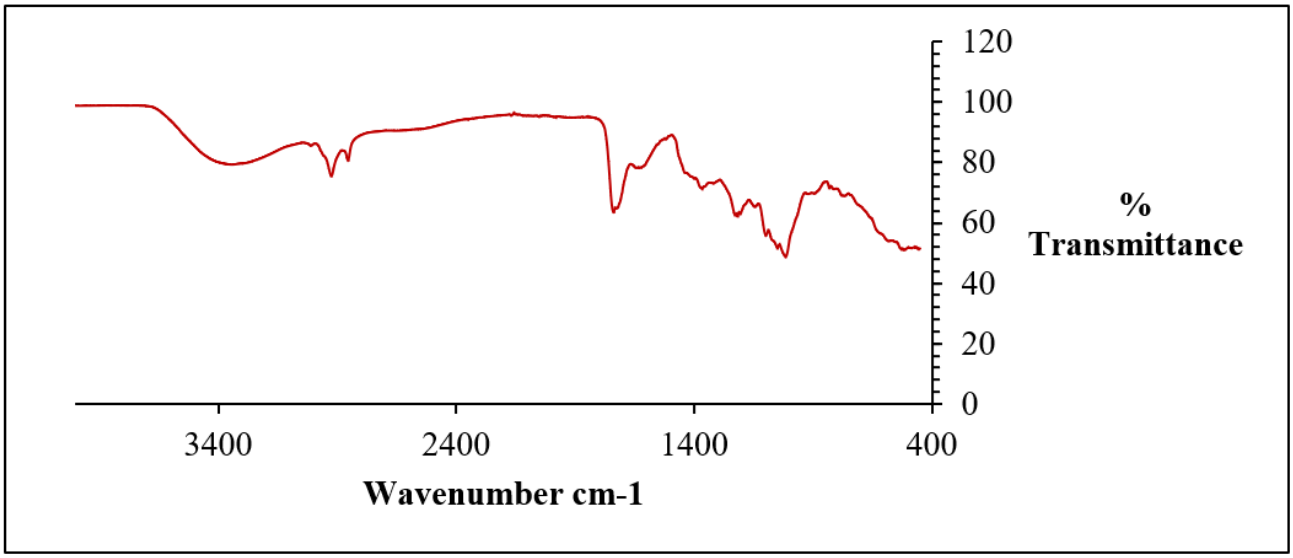

Figure 7: Peaks in FTIR analysis of *C. limon* powder with respect to wavenumber and % transmittance.

The preliminary chromatographic fingerprinting is also done by HPTLC which is the advanced version of TLC, rapid, most acceptable, and authoritative analytical tool used for phytochemical and biochemical analysis (Bhargava A *et al.*, 2021). In the present study the n-hexane extract of *C. limon* showed presence of various phytoconstituents in different concentrations (Figure 4, Table 5). The chromatogram of 10 µL scanned at 254 nm showed 5 peaks on track 2, while the chromatogram of 10 µL scanned at 366 nm showed 5 peaks on track 2 (Table 5). After derivatization the chromatogram of 10 µL was scanned at 540 nm and 6 peaks were present on track 2 (Table 5). The R_f values showed in Table 5 can be used for further evaluation and identification of unknown compounds. However, limonene is quantified in the study which was found 2.36% in peel oil. Limonene is reported having various pharmacological activities including neuroprotective (Eddin LB *et al.*, 2021), anti-inflammatory (Kathem SH *et al.*, 2024), anti-microbial (Han Y *et al.*, 2019), cardio protective (Al Saffar RM *et al.*, 2022) etc.

CONCLUSION

The fruits of *C. limon* are used as a food for nutritional value and for medicinal purpose as well. Thus, the quality control parameters of the *C. limon* fruits are very essential. The present study will serve as a quality control parameter of *C. limon* fruit. The study will be helpful in easy identification, authentication and detection of adulteration in marketed samples.

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ABBREVIATIONS

CSIR-NIScPR: CSIR-National Institute of Science Communication and Policy Research; ***C. limon*:** *Citrus limon*; **RHMD:** Raw Materials Herbarium and Museum; **API:** Ayurvedic Pharmacopoeia of India; **FTIR:** Fourier Transform Infrared Spectroscopy; **UATR:** Universal Attenuated Total Reflectance; **HPTLC:** High Performance Thin Layer Chromatography; **TS:** Transverse Section; **LOD:** Loss on drying; **ICMR:** Indian Council of Medical Research.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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