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Original Article

High-Performance Thin-Layer Chromatography (HPTLC) Fingerprinting, Bioactive Composition, and Antioxidant Activity of *Citrus sinensis* Peel Extract

Moinuddin Vakil [1], Nishat Fatima Shaikh [1], Rehana Anjum Shah [2]

1. *Department of Botany, Khairul Islam Higher Education Society's Maharashtra College of Arts, Science and Commerce, Mumbai, IND*
2. *Department of Botany, Rizvi Education Society's Rizvi College of Arts, Science & Commerce, Mumbai, IND*

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Abstract

Citrus sinensis (Malta) fruit peels, often regarded as waste, represent a cost-effective and eco-friendly source of bioactive compounds. The present investigation focused on phytochemical screening, High-Performance Thin-Layer Chromatography (HPTLC) fingerprinting, citric acid quantification, and antioxidant evaluation of *C. sinensis* peel extract. Preliminary phytochemical analysis confirmed the presence of alkaloids, flavonoids, phenols, saponins, and terpenoids in the 70% ethanolic peel extract. Quantitative estimation revealed 0.5% citric acid content in the peel. HPTLC fingerprinting was performed for absolute hexane, acetone, and 70% ethanolic extracts of *C. sinensis* peel. Among the tested extracts, the acetone extract showed the most promising results, exhibiting greater compound separation compared to the others. Furthermore, the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay demonstrated a concentration-dependent activity of the extract, ranging from 40.97% inhibition at 200 µg to a maximum of 87.38% at 1,000 µg, establishing it as a potent natural antioxidant. These findings highlight the potential of Malta peel as a sustainable source for incorporation into food, pharmaceutical, and cosmetic formulations, thereby improving product safety, shelf life, and consumer acceptability while reducing reliance on synthetic preservatives with possible toxic effects.

Categories: Food security and sustainability, Food biochemistry, Food nutrition

Keywords: citrus sinensis, antioxidant, citric acid, hptlc, phytochemical

Introduction

Citrus sinensis (L.) Osbeck, also known as Malta, belongs to the family Rutaceae and is widely distributed across tropical and subtropical regions. In India, *C. sinensis* is widely cultivated in the hilly regions of Uttarakhand and represents one of the most economically important citrus fruits [1]. Beyond its consumption as a fresh fruit, *C. sinensis* is also utilized for its medicinal properties in several countries [2]. Citrus peels constitute approximately 44% of the total fruit mass [3], and large quantities are routinely discarded as agro-industrial waste. However, these by-products represent a valuable reservoir of bioactive compounds with significant potential for applications in health-related and industrial sectors [4]. Citrus peels are particularly rich in phenolic compounds, flavonoids, carotenoids, essential oils, steroids, terpenoids, and alkane groups [5-7]. In addition, they contain high levels of antioxidant compounds, including phenolics, ascorbic acid, essential oils, and carotenoids, which play a crucial role in mitigating oxidative cellular damage [8]. These phytochemicals exhibit diverse biological activities, including antimicrobial, anti-inflammatory, anticancer, and cardioprotective properties [7]. Numerous studies have demonstrated that *C. sinensis* peel extracts possess strong antioxidant activity,

suggesting their protective role against oxidative stress and associated disorders [7]. Consequently, antioxidants derived from citrus peels have gained considerable attention for the development of nutraceuticals and therapeutic formulations [9]. High-performance thin-layer chromatography (HPTLC) is an advanced form of thin-layer chromatography that offers improved resolution, higher sensitivity, better reproducibility, and the capability for precise quantitative analysis through automated sample application and densitometric scanning, whereas conventional TLC is primarily used for qualitative or preliminary separation with limited accuracy and reproducibility [10]. This refined chromatographic method is particularly adept at the rapid separation and analysis of complex herbal extracts, minimizing the need for extensive sample clean-up due to its optimized design [11,12]. The instrumental sophistication of HPTLC provides excellent separation capabilities for a wide array of compounds, making it suitable for analysing substances from dietary supplements to traditional medicines [13]. HPTLC is also effective in evaluating different extraction methods, including conventional and ultrasound-assisted extraction, and can quantify compounds like hesperidin, which often show higher concentrations with ultrasound-assisted extraction [14]. Moreover, HPTLC is characterized by its capacity for simultaneous analysis of numerous samples, offering considerable time and cost efficiencies compared to other chromatographic techniques [15,16]. This parallel processing capability not only enhances throughput but also ensures consistent analytical conditions across all samples, thereby improving the overall precision and accuracy of the results [17]. Despite these advantages, there is a recognized need for expanded HPTLC-effect-directed analysis to better delineate specific compounds driving bioactivities and for more systematic application for standardization of *C. sinensis* peel extracts [18,19].

This study aimed to conduct preliminary phytochemical screening and comprehensive phytochemical profiling of *C. sinensis* peel. Extracts were prepared using solvents of varying polarity, specifically acetone, hexane, and 70% ethanol, and subsequently analyzed by HPTLC. This multi-solvent approach maximizes the recovery of both lipophilic and hydrophilic molecules, thereby providing a more complete representation of the plant's bioactive profile [20]. The antioxidant potential of these extracts was then evaluated using the DPPH radical scavenging assay. Through the integration of these approaches, this research endeavors to address existing knowledge gaps and underscore the significant 'waste-to-wealth' potential of citrus by-products for their valorization in industrial and therapeutic applications.

Materials And Methods

The citrus fruits (Malta) were purchased from Jogeshwari Fruit Market, Mumbai, Maharashtra, India, and identified as Linnaeus Osbeck variety of the *C. sinensis* in the Department of Botany, Maharashtra College of Arts Science and Commerce. The fruit peels were removed with the help of knife and washed with distilled water, chopped into bits, and dried in an oven for 72 hours [2]. The dried peels were powdered using mortar and pestle to obtain fine powder, and the powdered sample was further ground using a commercial electrical blender for further use (Figure *1*).

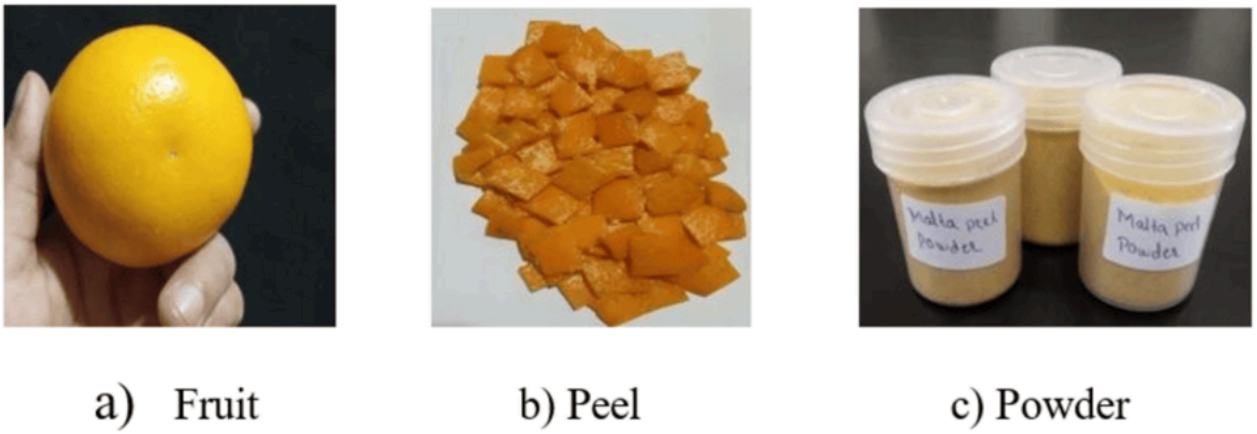

FIGURE 1: Citrus sinensis (L. Osbeck)

Preliminary phytochemical test

Preparation of Extracts

The citrus peel powder was soaked in 70% hydroethanolic solvent for 72 hours in an airtight container with occasional stirring [21]. The extract was filtered through Whatman filter paper No. 1 and concentrated by using a rotary evaporator at low pressure. The concentrated extract was stored at 4°C till further use. The extract was subjected to phytochemical screening, as described by Harbone [22] and Trease and Evans [23]. For the screening, a stock solution of the extracts at a concentration of 1 mg/ml was prepared.

• Test for alkaloids (Dragendroff's Reagent Test): 1 ml of the sample extract was treated with a few drops of Dragendorff's reagent. An orange-brown precipitate indicates the presence of alkaloids.

• Test for flavonoid (Alkaline Reagent Test): 1 ml of NaOH was added to 3 ml of the sample extract. A yellow coloration indicates a positive test for flavonoids.

• Test for saponins (Froth Test): 2 ml of the sample extract was added to 5 ml of distilled water, and the solution was shaken vigorously for 30 seconds; stable, persistent frothing indicates the presence of saponins.

• Test for tannins (Ferric Chloride Test): 2 ml of the sample extract was treated with 1 ml of ferric chloride ($FeCl_3$); the formation of a blue-black or greenish-black precipitate indicates the presence of tannins.

• Test for steroids (Sulphuric Acid Test): Five drops of concentrated H_2SO_4 were added to 1 ml of the sample extract. A red coloration indicates the presence of steroids.

• Test for terpenoids (Salkowski Test): 2 ml of chloroform and 3 ml of concentrated H_2SO_4 were carefully added to 5 ml of the sample extract to form a layer. A reddish-brown color at the interface indicates the presence of terpenoids.

• Test for phenol (Ferric Chloride Test): 5 ml of the sample extract was boiled with 5 ml of 70% ethanol in a water bath for 5 minutes and then filtered through Whatman No. 1 filter paper. After cooling, five drops of 5% ferric chloride were added and mixed. The appearance of a green precipitate indicates the presence of phenols.

• Test for cardiac glycosides (Keller Kiliani Test): 5 ml of the sample extract was treated with 2 ml of glacial acetic acid and 1 drop of ferric chloride solution. Then, 1 ml of concentrated H_2SO_4 was added. A brown ring at the interface indicates the presence of a deoxysugar characteristic of cardenolides.

Estimation of Citric Acid by Titrimetric Method

Citric acid, NaOH, and phenolphthalein of analytical grade were purchased from S.D. Fine-Chem Ltd., India. Citric acid was estimated using a titrimetric method, with 0.1 M NaOH serving as the titrant. A working standard of citric acid anhydrous aqueous solution (1 mg/ml) was prepared, and 10 ml of this solution was pipetted into an Erlenmeyer flask. A few drops of phenolphthalein indicator were then added. Upon titration with 0.1 M NaOH, a faint pink color appeared. Similarly, the Malta peel aqueous solution was titrated against 0.1 M NaOH. To obtain consistent burette readings, the titration was carried out three times for each sample.

• Balanced equation for citric acid and sodium hydroxide titration:

$$3\,\mathrm{NaOH\,(aq)} + \mathrm{C_3H_5O(COOH)_3\,(aq)} \rightarrow \mathrm{Na_3C_3H_5O(COO)_3\,(aq)} + 3\,\mathrm{H_2O}$$

• Calculation of grams and percentage of citric acid:

$$N_1 \times V_1 = N_2 \times V_2$$

$$N_1 = \mathrm{Normality\ of\ NaOH} = \mathrm{Molarity\ of\ NaOH\ (0.1)} \times \mathrm{Oxidation\ number\ (1)}$$

$$N_2 = \frac{N_1 \times V_1}{V_2}$$

$$\mathrm{Molarity\ of\ citric\ acid} = \frac{\mathrm{Normality\ of\ citric\ acid}}{\mathrm{Oxidation\ number\ (3)}}$$

$$\mathrm{Grams\ of\ citric\ acid} = \mathrm{Number\ of\ moles} \times \mathrm{Molecular\ weight}$$

$$\%\ \mathrm{of\ citric\ acid} = \frac{\mathrm{Normality\ of\ citric\ acid} \times \mathrm{Equivalent\ weight\ of\ citric\ acid} \times 100}{\mathrm{Volume\ of\ sample}}$$

where,

N_1 = Normality of NaOH

V_1 = Average volume of NaOH used from the burette

N_2 = Normality of citric acid (unknown)

V_2 = Whole volume of citric acid sample in the Erlenmeyer flask (ml)

Molecular weight of citric acid = 192.124 g/mol

Equivalent weight of citric acid = 64.04 g/mol

HPTLC Fingerprinting

A CAMAG HPTLC system equipped with a LINOMAT 5 applicator, TLC Scanner 4, TLC Visualizer 2, and WINCATS-4 software (CAMAG, Muttenz, Switzerland) was used to analyze the HPTLC fingerprinting of *C. sinensis* (Malta) peel. For fingerprinting, three different solvents, namely acetone, hexane, and 70% ethanol, were used for the preparation of Malta peel extracts, and extraction was carried out using bath-type sonication. One gram of Malta peel powder was dissolved separately in 10 ml of 70% ethanol, 10 ml of hexane, and 10 ml of acetone. The extracts were centrifuged at ,3000 rpm for 5 minutes, and the supernatant obtained from each solution was used as the test sample. The samples were applied onto aluminum plates pre-coated with silica gel 60 F254 (Merck, Germany). Volumes of 2 µl and 5 µl of each extract were applied onto 10 × 10 cm plates as bands of 8 mm width using a CAMAG Linomat 5 sample applicator. The plates were developed in a TLC twin-trough developing chamber (after saturation with solvent vapor for 20 minutes) using toluene:chloroform:ethanol (4:4:1, v/v) as the mobile phase. The developed plates were sprayed with anisaldehyde sulfuric acid reagent (ASR) and then dried using hot air to evaporate the solvents. The plates were placed in a TLC Visualizer 2 chamber, and images were captured under RT White-Developed (Rem TransVis), R 254 (UV 254 nm), and R 366

(UV 366 nm). The plates were then fixed on the scanner stage (CAMAG TLC Scanner 4, winCATS version 3.2.2), and scanning was performed at UV 254 nm and 366 nm before and after derivatization with ASR. The HPTLC fingerprint of *C. sinensis* peel was recorded using a computer-aided printing machine, and the peak display, peak table, and densitogram were noted.

DPPH Antioxidant Analysis

The DPPH radical scavenging activity of Malta (*C. sinensis*) peel extract was determined using the method described by Brand-Williams [24], with slight modifications. Briefly, five different concentrations of the extract (200, 400, 600, 800, and 1,000 µg/ml) were prepared in 70% ethanol. The same concentrations were prepared for ascorbic acid, which served as the standard antioxidant.

For each concentration, 3 ml of the sample solution was mixed with 0.5 ml of 0.01 mM DPPH solution in ethanol. The mixtures were vortexed briefly and incubated in the dark at room temperature for 30 minutes to allow the reaction to occur. A blank solution containing ethanol was used to set the baseline. The negative control consisted of 2 ml of DPPH solution and 1 ml of ethanol, while the positive control included ascorbic acid at the corresponding concentrations.

After incubation, the absorbance of each solution was measured at 517 nm using a UV-Vis spectrophotometer (Systronics India Limited). All measurements were performed in triplicate. The DPPH radical scavenging activity (%) was calculated using the following formula:

$$\text{Radical Scavenging Activity } (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

where A_{control} is the absorbance of the negative control and A_{sample} is the absorbance of the test sample (extract or standard).

Results And Discussion

Phytochemical screening and citric acid content

The comprehensive phytochemical analysis of the 70% ethanolic extract from *C. sinensis* peel revealed a rich presence of several secondary metabolites, such as alkaloids, flavonoids, terpenoids, saponins, and phenols, which are commonly associated with various therapeutic properties [25,26]. Based on the phytochemical profile and existing literature, flavonoids and phenolic compounds are considered to have the most significant functional role in *C. sinensis* peel extract, primarily due to their well-documented antioxidant activity and associated therapeutic potential. Conversely, the absence of steroids, cardiac glycosides, and tannins further delineates the specific chemical profile of this extract, distinguishing it from other Citrus varieties, where these compounds might be present (Figure *2* and Table *1*) [25]. This distinct phytochemical fingerprint aligns with previous research on *C. sinensis* peels, which often highlights a prevalence of flavonoids and organic acids [25,27]. These findings are significant as they indicate the potential health benefits of *C. sinensis* peel, consistent with observations that various *C. sinensis* fruits contain diverse phytochemicals such as coumarin glycosides, flavonoids, and volatile oils that contribute to anti-inflammatory, antibacterial, and anti-diabetic activities [28]. The quantitative analysis further indicated that the 70% ethanolic extract of *C. sinensis* peel, at a concentration of 1 mg/ml, contained 0.49 mg/ml of citric acid, which translates to 5.12% of the total sample (Figure *3*). This high concentration of citric acid is notable, given its established roles as a natural antioxidant, chelating agent, and acidulant in various industrial and pharmaceutical applications [28]. This substantial citric acid content also underscores the extract's potential for contributing to the overall acidic profile and preservative qualities, which are critical in food science and pharmaceutical formulations. Furthermore, the presence of these diverse phytochemicals and a substantial citric acid content suggests a synergistic effect that could amplify the extract's bioactivity for further pharmacological investigations [25,29]. The presence of such a diverse array of phytochemicals, particularly the high concentration of

flavonoids and terpenoids, corroborates findings in other studies indicating the significant pharmacological potential of *C. sinensis* peels [25]. This aligns with the understanding that *C. sinensis* is a rich source of secondary metabolites, including flavonoids and other beneficial compounds, that contribute to its observed pharmacological activities [30].

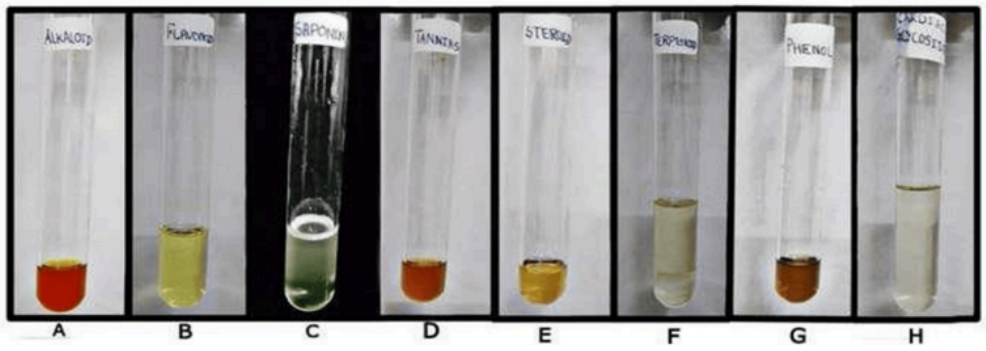

FIGURE 2: Malta peel 70% ethanolic extract phytochemical test: (A) alkaloids, (B) flavonoids, (C) saponins, (D) tannins, (E) steroids, (F) terpenoids, (G) phenol, and (H) cardiac glycoside

Sr. No.	Phytochemicals	Citrus sinensis peel extract
1	Alkaloids	+
2	Flavonoids	+
3	Saponins	+
4	Tannins	-
5	Steroids	-
6	Phenols	+
7	Terpenoids	+
8	Cardiac Glycoside	-

TABLE 1: Qualitative phytochemical composition of 70% ethanolic extract of Citrus sinensis (Malta) peel

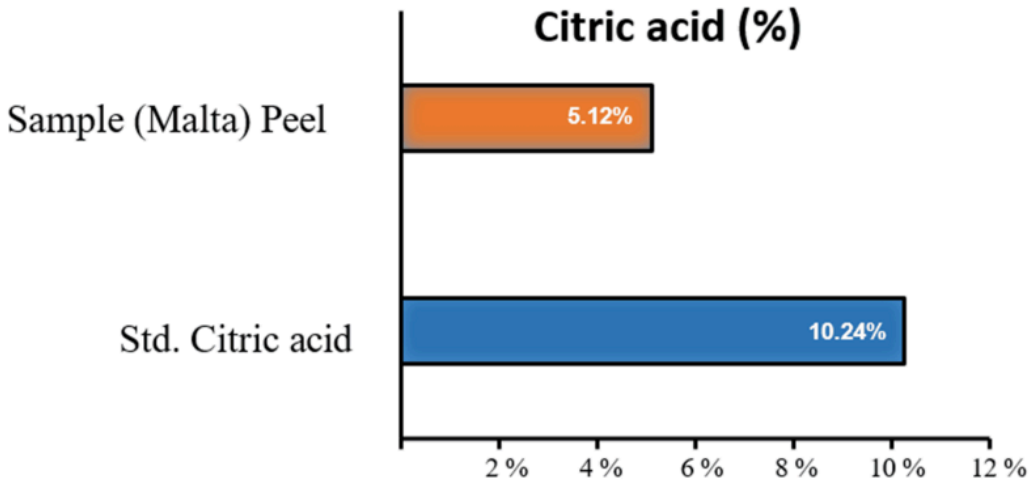

FIGURE 3: Determination of citric acid by titration method expressed in percentage

HPTLC fingerprinting analysis

The current HPTLC findings for *C. sinensis* peel extracts demonstrate significant differences in phytochemical profiles depending on the extraction solvent, supporting previous research that highlighted the variability of secondary metabolite recovery based on solvent polarity [18]. Specifically, the enhanced separation observed with acetone extraction, yielding seven distinct bands, aligns with the principle that solvent polarity plays a crucial role in the efficient extraction of a broader spectrum of compounds from plant matrices (Figure *4*) [31]. This superior performance of acetone is likely attributed to its intermediate polarity, which facilitates the dissolution of a wider range of compounds compared to the more non-polar hexane or the moderately polar 70% ethanol [19]. Conversely, the limited number of bands observed in the hexane extract suggests its selectivity towards highly apolar compounds, while the 70% ethanol extract, despite its moderate polarity, may not have been as effective in solubilizing the full complexity of compounds present in the peel, potentially due to the specific dielectric constant and hydrogen bonding capabilities of the solvent mixture [16].

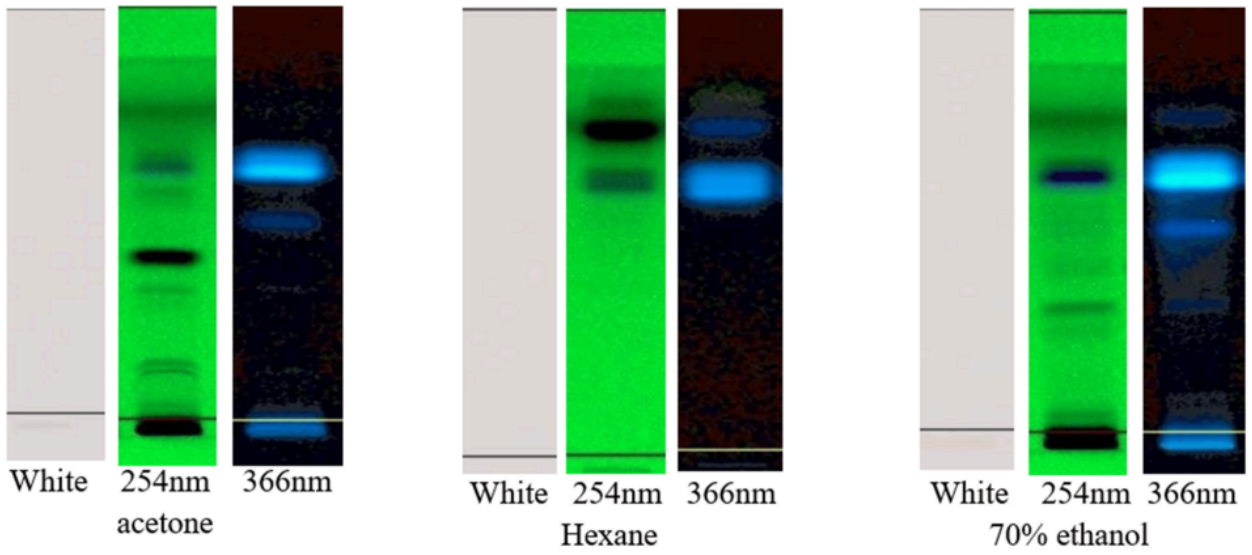

FIGURE 4: HPTLC fingerprinting of Malta peel extracts (acetone, hexane, and 70% ethanol) after derivatization

HPTLC, High-Performance Thin-Layer Chromatography

This is further supported by densitometric analysis, which quantitatively affirmed the superior compound resolution achieved with acetone, indicating a richer phytochemical repertoire accessible by this solvent (Figures *5*-*7* and Table *2*) [32]. The identification of multiple bands under various illumination conditions (white light, 254 nm, and 366 nm) both before and after derivatization further underscores the chemical diversity within these extracts, suggesting the presence of compounds with varying chromophoric and fluorophoric properties [32,33]. These results underscore the utility of HPTLC as a sensitive and cost-effective technique for profiling complex plant extracts, enabling the identification and quality control of distinct phytochemicals [34]. The comprehensive HPTLC analysis, particularly with techniques like GMD-DAD-HPTLC, offers a robust platform for the preliminary characterization of complex natural product mixtures, allowing for the separation of over 50 compounds on a single HPTLC plate and laying the groundwork for further effect-directed analysis [19]. This initial fingerprinting serves as a vital preliminary step in identifying potential bioactive fractions, thereby guiding subsequent targeted isolation and characterization studies to elucidate the specific compounds responsible for observed biological activities [32,35,36]. The ability to detect a broad range of pharmaceutically active compounds across varying polarities using diode-array detection, coupled with the visualization of separated zones through contour plots, significantly enhances the utility of HPTLC for comprehensive sample overview [32]. Furthermore, the distinct banding patterns observed, particularly with fluorescence under 366 nm, imply the presence of compounds such as flavonoids and other phenolic compounds known for their autofluorescent properties, warranting further investigation into their specific identities and concentrations. The application of HPTLC for separating various phytochemicals, such as alkaloids, flavonoids, and saponins, further emphasizes its versatility in profiling diverse plant extracts [37]. This precise and reproducible method provides a robust platform for the standardization of herbal medicines, ensuring consistent quality and efficacy of botanical products [38]. Moreover, the distinct HPTLC fingerprints obtained for each extract serve as valuable tools for quality control, authentication, and the detection of adulteration in *C. sinensis*-derived products by establishing unique chromatographic profiles [39,40]. Such detailed fingerprinting is crucial for the pharmaceutical industry, enabling the rapid screening and assessment of plant extracts in relation to their total bioactivity profile, particularly given the complexity of herbal drugs whose effects are often attributed to synergistic interactions of multiple metabolites [41,42]. This approach is particularly valuable for identifying representative principle components of herbal samples, fulfilling fundamental requirements for ethnobotanical applications and enabling the selection of compounds with targeted biological activities for further characterization [43,44].

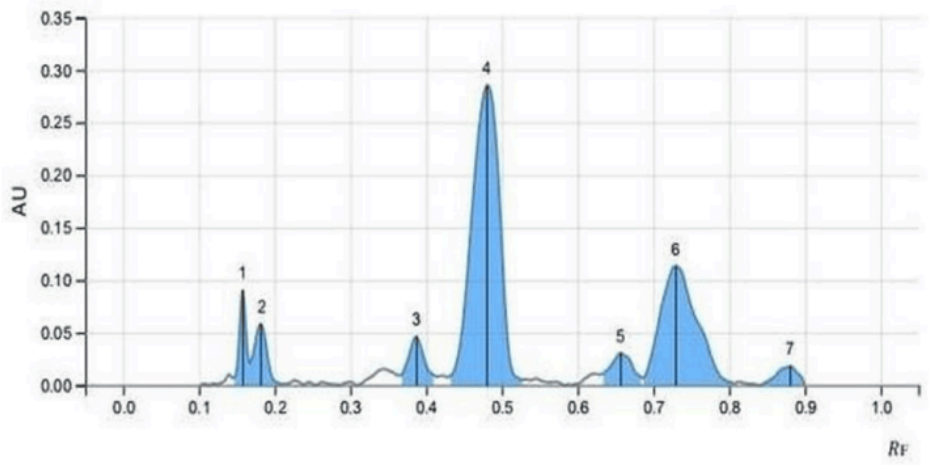

FIGURE 5: HPTLC chromatogram with peak Rf value recorded in densitogram of C. sinensis absolute acetone extract scanned at 254 nm

HPTLC, High-Performance Thin-Layer Chromatography

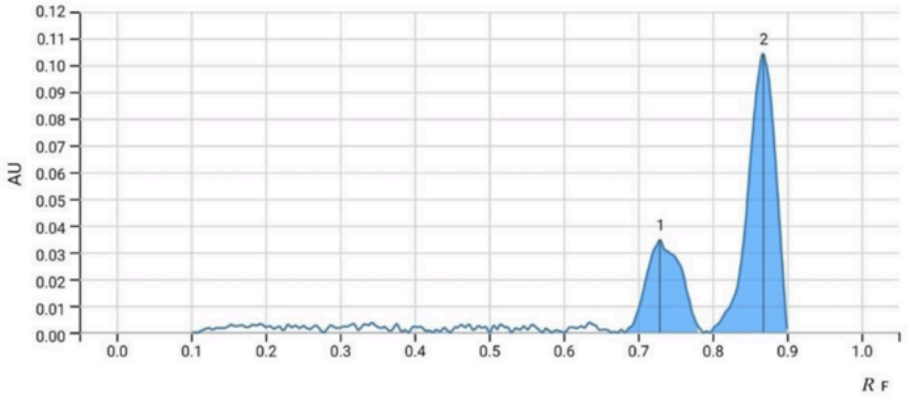

FIGURE 6: HPTLC chromatogram with peak Rf value recorded in densitogram of C. sinensis absolute hexane extract scanned at 254 nm

HPTLC, High-Performance Thin-Layer Chromatography

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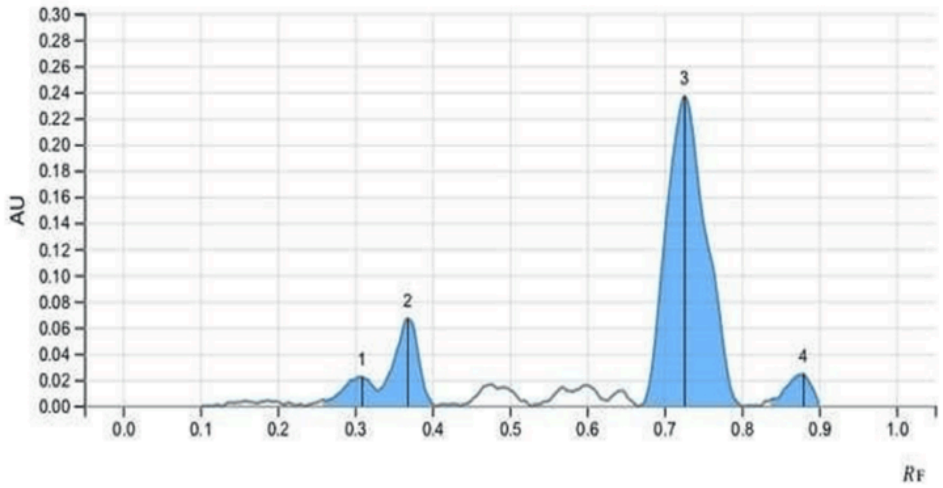

FIGURE 7: HPTLC chromatogram with peak Rf value recorded in densitogram of C. sinensis 70% ethanol extract scanned at 254 nm

HPTLC, High-Performance Thin-Layer Chromatography

Samples/Tracks	Peak	Rf	Area
Acetone	1	0.158	0.00096
	2	0.182	0.00113
	3	0.387	0.00118
	4	0.481	0.01208
	5	0.656	0.00105
	6	0.729	0.00639
	7	0.881	0.00059
Hexane	1	0.727	0.00792
	2	0.866	0.00840
70% Ethanol	1	0.308	0.00101
	2	0.368	0.00244
	3	0.726	0.01411
	4	0.879	0.00092

TABLE 2: Rf values and peak areas corresponding to acetone, hexane, and 70% acetone extracts of Citrus peels

Antioxidant activity (DPPH radical scavenging activity)

DPPH radical scavenging activity analysis revealed that the antioxidant potential of *C. sinensis* peel increases in a dose-dependent manner. These findings support previous research indicating that the antioxidant potential of *C. sinensis* peel extracts is directly correlated with increasing concentrations, largely attributable to their rich phytochemical composition, particularly flavonoids and phenolics [28]. The observed dose-dependent enhancement in antioxidant activity, reaching 87.38% DPPH radical scavenging at 1,000 µg, suggests a strong correlation between extract concentration and free radical neutralization (Table *3*) [2]. This efficacy approaches that of the standard (88.56% at 200 µg), indicating significant antioxidant potential within the *C. sinensis* peel 70% ethanolic extracts [29]. Such robust radical scavenging capacity is likely attributable to the rich phytochemical profile of *C. sinensis* peel, which often includes potent flavonoids like hesperidin and nobiletin, known for their antioxidant properties [18]. Previous research supports that methanolic extracts of citrus peels have exhibited varying degree of antioxidant potentials [45]. Furthermore, the selection of ethanol as an extraction solvent is crucial, as it has been shown to yield significant amounts of extract compared to other solvents, thereby maximizing the recovery of these bioactive compounds [46]. This substantial

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antioxidant activity positions *C. sinensis* peel extracts as promising natural sources for therapeutic applications, particularly given their capability to donate hydrogen atoms to stable organic free radicals like DPPH, thereby reducing them to a non-radical form [29,47].

Concentration	Ascorbic acid OD at 517 nm	Sample OD at 517 nm	% of RSA ascorbic acid	% of RSA sample
200 µg	0.223	1.151	88.56%	40.97%
400 µg	0.219	1.062	88.76%	45.53%
600 µg	0.214	0.942	89.02%	51.69%
800 µg	0.212	0.707	89.12%	63.74%
1,000 µg	0.210	0.246	89.23%	87.38%

TABLE 3: DPPH radical scavenging activity of 70% ethanolic extract of Malta peel was evaluated at 517 nm, with the control (DPPH) absorbance value recorded as 1.950

OD, Optical Density; RSA, Radical Scavenging Activity

Conclusions

The peel of *C. sinensis* is a rich source of natural antioxidants, including citric acid, phenols, alkaloids, terpenoids, and flavonoids, making it a valuable resource for applications in the food and cosmetic industries, owing to its notable antioxidant and anti-ageing properties. HPTLC fingerprinting of the peel extracts demonstrated that acetone was the most effective solvent for phytochemical extraction, further substantiating the high concentration of bioactive compounds within the peel. The prominent presence of citric acid, in particular, underscores its potential role as a natural food preservative and antioxidant, capable of mitigating oxidative damage through the scavenging of reactive oxygen species. Consequently, *C. sinensis* peel, which is frequently discarded as agro-industrial waste, represents a promising source of natural preservatives and antioxidants. Its valorization not only offers a sustainable solution to environmental waste management issues but also contributes to reducing reliance on synthetic chemicals, thereby fostering the adoption of natural components across various industries vital to human life.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

Concept and design: Moinuddin Vakil, Rehana Anjum Shah

Acquisition, analysis, or interpretation of data: Moinuddin Vakil, Nishat Fatima Shaikh

Drafting of the manuscript: Moinuddin Vakil

Critical review of the manuscript for important intellectual content: Moinuddin Vakil, Nishat Fatima Shaikh, Rehana Anjum Shah

Supervision: Moinuddin Vakil

Disclosures

Plant subjects: All authors have confirmed that this study did not involve any herbarium specimen. **Human subjects:** All authors have confirmed that this study did not involve human participants or tissue. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

Data Availability Statements

All data generated or analyzed during this study are included in this published article and/or its appendices.

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