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High-performance Thin Layer Chromatographic quantification of hesperidin and naringenin from *Citrus sinensis* peel extract

Research Article

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Abstract

Orange peel is a member of *Citrus sinensis*, the Rutaceae family, and is widely used for its nutritional properties. Orange peels are the waste part of the fruit and contain various therapeutic molecules. Hesperidin and naringenin are two flavonoids present in orange peel. The present study depicted the quantification of high-performance thin layer chromatography of orange peel extract using hesperidin and naringenin. The HPTLC densitometric analysis of the ethanolic extract of orange peel was carried out using a CAMAG HPTLC system, Linomat 5 applicator, and Vision CAT software. The results were obtained in the form of chromatograms scanned at 254 & 366 nm. The Rf values of hesperidin were 0.5 with mobile phase Ethyl acetate: Methanol: Water (15:04:01) and 25.93 nanograms in 1 mg of orange peel extract. Naringenin was quantified at 254 & 366 nm with Rf value 0.6 & mobile phase used was Toluene: Ethyl acetate: Formic acid (12:8:1.6v/v/v), and 14.92 nanograms of naringenin were found in 1 mg of orange peel extract.

Keywords: Orange peel extract, HPTLC, Hesperidin, Naringenin, Densitometry.

Introduction

Oranges are currently the most produced fruit in the world. Tropical and subtropical regions are home to the sweet orange, *Citrus sinensis*, a citrus fruit that belongs to the Rutaceae family. Today, the world produces around 110 million tons of citrus fruit per year (1). Orange juice will be made from about 8.3% (2 million tons) of the 24 million tons of oranges that are anticipated to be produced globally in 2016–17 (2). However, as orange peels comprise around 44% of the fruit body, they will produce a large number of by-products. Since these orange peels are typically thrown away as waste, there may be major disposal issues (3). There are numerous techniques to prepare orange peels for use in cosmetics, medications, and food (4). Orange peel extracts are high in physiologically active substances, including phenolic acids and flavonoids, which have antioxidant, anti-inflammatory, anti-atherosclerosis and anti-carcinogenic potential, and they are also a major source of dietary fiber (5). Orange peel is a common waste product that contains both soluble fibers like pectin and insoluble fibers, including lignin, cellulose, hemicellulose (6). Orange peels also contain bioactive chemicals and essential oils with antioxidant and antibacterial potentials, such as α- α-pinene, β-pinene, farnesene, limonene, γ-terpinene, myrcene and α-terpinolene. Orange peels contains compounds like essential oils and pectin that have antibacterial activity and can be used to prolong the shelf life of some food products. Numerous extraction methods are employed to extract natural chemicals from orange peels (7). Flavonoids and phenolic acids are among the many phenolic chemicals found in citrus fruit peels and seeds, out of both flavonoids are more prevalent in the peel than in the seeds (8). Flavonoids are naturally occurring compounds with different phenolic structures that are found in plants. They are oxidized by radicals to form a less reactive and more stable radical. They include flavonols (such as kaempferol, quercetin, fisetin and myricetin), flavonones (such as flavanone, hesperidin, and naringenin), flavones (such as flavone, luteolin and apigenin), and additional classes (9). Certain flavonoids can directly scavenge superoxides, but other flavonoids can scavenge peroxynitrite, a highly reactive radical generated from oxygen. Epicatechin and rutin are also powerful radical scavengers. Owing to its inhibition of the xanthine oxidase enzyme, rutin may possess scavenging capabilities (10). Numerous flavonoids, such as apigenin, catechin, quercetin, naringenin, rutin, venoruton, and, have been demonstrated to possess hepatoprotective qualities (11). Hesperidin is a flavanone glycoside found in several citrus fruits. Among its many pharmacological activities are antioxidant, anti-inflammatory, neuroprotective, anticancer, and antiallergy (12). According to Wei et al., treating asthmatic mice decreased blood levels of IgE as well as BALF levels of eosinophils, neutrophils, macrophages, IL-4, IL-5, and IL-13. This data suggests that hesperidin helps to cure allergic asthma by suppressing the Th2 response and reducing

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inflammation (13). Citrus fruits, such as oranges and grapefruit, and tomatoes are the main sources of naringenin (4′,5,7-trihydroxyflavonone). It comes in a variety of conjugated forms, mostly as aglycone glycosylated, neohesperidoside, which is derived from phenylalanine, the aromatic amino acid (14). Each of these forms has altered pharmacokinetic properties, including absorption, distribution, metabolism, and elimination. Grapefruits are the most common source of naringenin, where it is present as "naringin," an inactive glycone form. Naringin, a 4′,5,7-trihydroxyflavonone 7-rhamnoglucoside, (15). The most active aglycone form. Naringenin is reported to enter the bloodstream fast due to its easy absorption by the gastrointestinal tract. Ever since its rapid absorption, Naringenin is the most pharmacologically effective form of naringin (16). Two intermediates, naringenin and rhamnose, are produced shortly from naringin after consumption by the intestinal bacterial naringinase enzyme (17).

Materials and Methods

Collection and authentication

The orange peels were collected from the local market and authenticated by Dr. Nitin Dongarwar, Department of Botany, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur

Extraction

The orange peels were collected and shade dried for 5 days. The dried peels were powdered using a mechanical grinder into coarse powder (18). As per the literature and our study revealed that the maceration is the best process for extraction of phytochemicals. The 500 g of coarse powdered material was placed in a glass jar and macerated for 3-5 days using ethanol (3 volumes). The extract was filtered and concentrated using a rotary evaporator with 50-60˚C temperature (19)

Phytochemical Study

The phytochemical screening of the orange peel extract was performed and shown the presence of flavonoids, phenolics, tannins, and alkaloids (20)

Test for flavonoids

Shinoda test: A little piece of magnesium, 1.5 mL of 50% methanol solution, and 4 mL of extract solution were heated. When 5–6 drops of concentrated HCl were added, flavonoids showed a crimson hue (21).

Dil. NH_3 test: 5 mL of diluted NH3 solution in extract was taken, and concentrated H_2SO_4 was added. Flavonoids were suggested when a yellow-colored precipitation appeared (22).

Test for polyphenol

Ferric Chloride test: When 3 to 4 drops of 10% $FeCl_3$ were added to the diluted extract, gallo tannins became blue while catechol tannins caused the solution to become green (23).

Test for Tannins

Lead acetate Test: 10 mg of plant extract was taken and 0.5 ml of 1 % lead acetate solution was added and the formation of precipitate indicates the presence of tannins (23).

Test for alkaloids

Meyer's test: 1ml of Meyer's reagent was added to 2 ml of extract. Presence of pale yellow precipitate confirms the presence of alkaloids in the OPEE (24).

Dragendorff's reagent test: 2ml of extract were heated using 2% H_2SO_4. A little amount of Dragendorff's reagent was added. The presence of alkaloids was revealed by an orange-red precipitate (25).

HPTLC Instrument Specifications

Instrumentation

A CAMAG HPTLC system equipped with LINOMAT 5 applicator fitted with a 100 µl syringe with CAMAG TLC scanner and vision CAT software was used (26).

Solvents and Chemicals

Standard hesperidin & naringenin was purchased from Sigma-Aldrich, St. Louis, MO, USA. All the HPLC grade solvents were used for HPTLC analysis, and all the chemicals of analytical grade were used for the above study.

Sample preparation

Dried orange peel extract 10 mg was dissolved in 10 mL of methanol and then filtered.

Standard preparation

1 mg hesperidin dissolved in 10 ml of methanol.1 mg of naringenin dissolved in 10 ml of methanol. Both the standard solutions were sonicated using a sonicator, used for an HPTLC quantification study.

Chromatographic Conditions

A 10 x 20.0 cm pre-coated silica gel 254 HPTLC plate (E. MERCK) was used for the HPTLC densitometric analysis. No plate modification or pre-washing. A CAMAG Linomat applicator with a 100 µl syringe was used to apply the sample solution on the plate in the form of a band. Samples were applied to the TLC plates as 8 mm bands using a Camag Automatic TLC Sampler 4 (ATS4) sample applicator (Switzerland) fitted with a Camag microlitre syringe. A constant application rate of 150 nL/s was used. Linear ascending development of the plates to a distance of 70 mm was performed with ethyl acetate-methanol-water 15:4:1 (%, v/v) previously saturated for 20 min and the hesperidin standard and sample-loaded plates were developed in an automated development chamber. The procedure repeated for naringenin using Toluene: Ethyl acetate: Formic acid (12:8:1.6 v/v/v) mobile phase previously saturated for 20 min and the naringenin standard and sample-loaded plates were developed in an automated development chamber with distance of 70 mm and the application rate was 150 nL/s.

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Results and Discussion

The *Citrus sinensis* L. peels were subjected to ethanolic extraction by using maceration and the extract was evaporated to dryness by rotary evaporator, and the extract were concentrated and tested for different phytoconstituents, by phytochemical study as shown in Table 1.

Table 1: Phytochemical study of Orange peel ethanolic extract

Sr. No	Phytochemical Test	Observation
1	Flavonoids (Shinoda test)	Positive
2	Tannins (Lead acetate Test)	Positive
3	Phenolics (Ferric Chloride test)	Positive
4	Alkaloids (Dragendorff test)	Positive

The polyphenolic compounds were found in the extract based on the above qualitative phytochemical study. The extract was assessed using HPTLC and fingerprinting using naringenin and hesperidin as standards. The outcomes are shown in Tables 2 and 3.The chromatograms were obtained upon scanning at UV at 254 & 366 nm. Figures 1, 2 & 3 depicted for hesperidin and Figures 4, 5 & 6 depicted for naringenin, respectively. The Rf values, area percentage, and peak height of both hesperidin and naringenin were calculated.

Table 2: Tracks of hesperidin as a standard & Orange-peel ethanolic extract

Track no	Ref/Sample ID	X (mm)	Y (mm).	Appl. Volume	Rf	Conc.
1	Hesp	20.0	40.7	1.0 µl	0.527	0.1 mg/ml
2	Hesp	31.4	40.8	2.0 µl	0.529	0.1 mg/ml
3	Hesp	42.8	40.1	3.0 µl	0.518	0.1 mg/ml
4	Hesp	54.2	40.4	4.0 µl	0.523	0.1 mg/ml
5	Hesp	65.6	40.3	5.0 µl	0.521	0.1 mg/ml
6	Hesp	77.0	40.1	6.0 µl	0.518	0.1 mg/ml
7	Hesp	88.4	40.1	7.0 µl	0.518	0.1 mg/ml
8	Hesp	99.8	40.2	8.0 µl	0.519	0.1 mg/ml
9	OPE	111.2	40.3	2.0 µl	0.521	1mg/ml
10	OPE	122.6	39.8	4.0 µl	0.513	1mg/ml
11	OPE	134.0	40.1	6.0 µl	0.518	1mg/ml
12	OPE	145.4	40.4	8.0 µl	0.523	1mg/ml
13	OPE	156.8	40.3	10.0 µl	0.521	1mg/ml
14	OPE	168.2	40.0	12.0 µl	0.516	1mg/ml
15	OPE	179.6	39.7	14.0 µl	0.511	1mg/ml

Hesp - Hesperidin, OPEE - Orange peel ethanolic extract

Figure 1: Calibration curve of Hesperidin standard

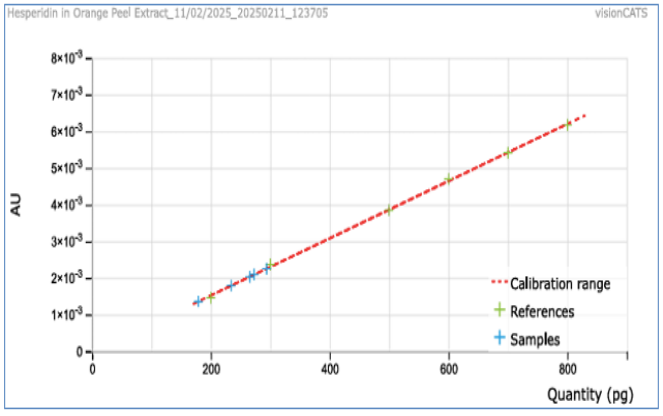
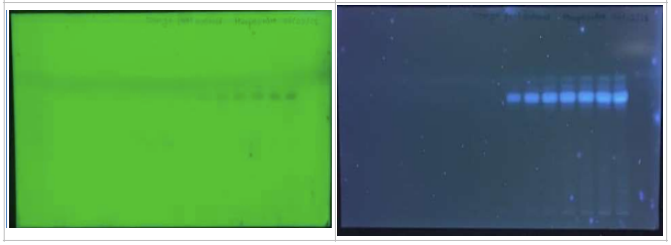


Figure 2: Scanned image of Hesperidin and OPEE at 254 nm

Figure 3: Scanned image of Hesperidin and OPEE 366 nm



After performing the phytochemical study revealed that the polyphenolic compounds are abundantly present, and the same extract was studied using High-performance thin layer chromatography (HPTLC), and the extract was fingerprinted with the use of naringenin and hesperidin as standards, and the results are displayed in Table 2 & 3 respectively. The chromatograms were obtained upon scanning at UV at 254 & 366 nm. Figure 7 shows the chromatographic overlay of hesperidin standard & OPEE while figure 8 shows the UV overlay spectra of OPEE extract at 254 nm. Figure 9 displays the chromatographic overlay of naringenin & OPEE while figure 10 shows the UV overlay spectra of naringenin and OPEE at 254 nm.

Table 3: Tracks of naringenin & orange peel ethanolic extract (OPEE)

Track no	Ref/Samp.ID	X (mm)	Y (mm)	Appl. Volume	Rf	Conc.
1	Naring	20.0	45.9	1.0 µl	0.611	0.1 mg/ml
2	Naring	31.4	44.9	2.0 µl	0.595	0.1 mg/ml
3	Naring	42.8	45.4	3.0 µl	0.603	0.1 mg/ml
4	Naring	54.2	45.4	4.0 µl	0.603	0.1 mg/ml
5	Naring	65.6	45.0	5.0 µl	0.597	0.1 mg/ml
6	Naring	77.0	44.8	6.0 µl	0.594	0.1 mg/ml
7	Naring	88.4	44.7	7.0 µl	0.592	0.1 mg/ml
8	Naring	99.8	44.7	8.0 µl	0.592	0.1 mg/ml
9	OPEE	111.2	45.2	2.0 µl	0.600	1mg/ml
10	OPEE	122.6	45.9	4.0 µl	0.611	1mg/ml
11	OPEE	134.0	45.2	6.0 µl	0.600	1mg/ml
12	OPEE	145.4	45.3	8.0 µl	0.602	1mg/ml
13	OPEE	156.8	42.8	10.0 µl	0.561	1mg/ml
14	OPEE	168.2	44.6	12.0 µl	0.590	1mg/ml
15	OPEE	179.6	44.6	14.0 µl	0.590	1mg/ml

Naring – Naringenin, OPEE – Orange peel ethanolic extract

Figure 4: Calibration curve for Naringenin Standard

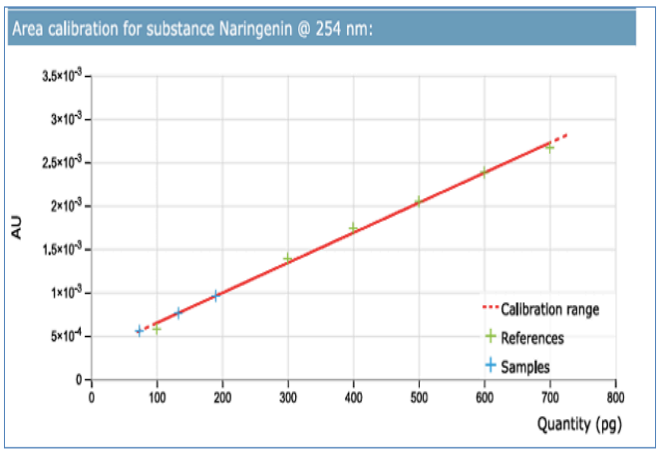


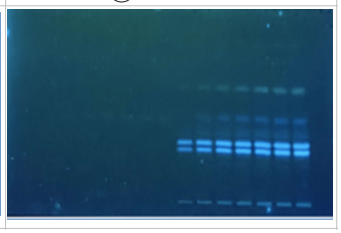
Figure 5: Scanned image of naringenin and OPEE @ 254 nm	Figure 6: Scanned image of naringenin and OPEE @ 366 nm
	

Table 4: Calibration curve parameters of Hesperidin Standard

Parameters	Values
Regression mode	Linear-2
Range deviation	5.00%
Related substances	None
Assignment mode	Single
Number of references	6
Calibration function	$y=3.463\times10^{-6}x+3.002\times10^{-4}$
Coefficient of variation	2.61%
Correlation coefficient	R=0.997633

Table 5: Calibration curve parameters of Naringenin Standard

Parameters	Values
Regression mode	Linear-2
Range deviation	5.00%
Related substances	None
Assignment mode	Single
Number of references	6
Calibration function	$y=7.803\times10^{-6}x$
Coefficient of variation	1.17%
Correlation coefficient	R=0.999601

Figure 7: Overlay Chromatograms of hesperidin in OPEE	Figure 8: UV Overlay Spectra of hesperidin in OPEE at 254 nm

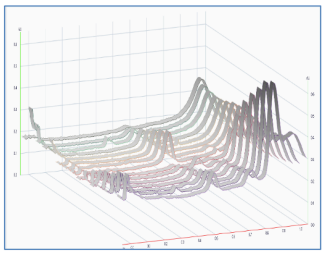
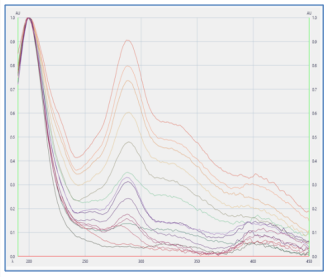
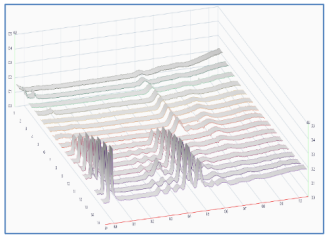
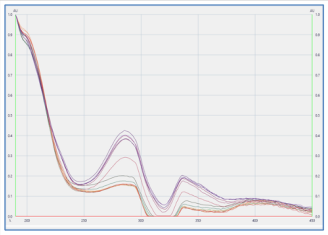

	

Figure 9: Overlay chromatograms of Naringinin in OPEE.	**Figure 10: UV Overlay Spectra of Naringinin in OPEE at 254 nm.**
	

Conclusion

Orange fruit is a waste part of the orange fruit contains different chemical constituents, which are responsible for various therapeutic potential. Hesperidin is a major flavonoid present in orange peel. In the present study, the HPTLC analysis of orange peel extract was performed and the densitometric analysis of the ethanolic extract of orange peel was carried out using a CAMAG HPTLC system and Vision CAT software. The results were observed in the form of chromatograms scanned at 254 & 366 nm. The Rf values of hesperidin was found to be 0.5 with mobile phase Ethyl acetate: Methanol: Water (15:04:01). 25.93 nanograms of hesperidin was found in 1 mg of orange peel extract. Naringenin was quantified at 254 & 366 nm with Rf value 0.6 & mobile phase used was Toluene: Ethyl acetate: Formic acid (12:8:1.6v/v/v). The hesperidin and naringenin was done 14.92 nanograms of naringenin was found in 1 mg of orange peel extract.

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