



CODEN [USA]: IAJPBB

ISSN : 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

SJIF Impact Factor: 7.187

<https://doi.org/10.5281/zenodo.23217957>Available online at: <http://www.iajps.com>

Research Article

**STUDY OF SECONDARY METABOLITES IN CITRUS
MEDICA EXTRACT USING UV AND CHROMATOGRAPHIC
TECHNIQUES****Kirti Ahirwar*, Mr. Ankit Diwan, Dr. Kuldeep Ganju**
Sagar Institute of Pharmacy and Technology (SIPTec), Bhopal (M.P.)**Abstract:**

The present study was undertaken to evaluate different extraction techniques for the preparation and phytochemical characterization of the hydroalcoholic extract of *Citrus medica*. Maceration, ultrasonic-assisted extraction (UAE), and microwave-assisted extraction (MAE) were employed, and the obtained extracts were evaluated for percentage yield, preliminary phytochemical constituents, thin-layer chromatography (TLC), total phenolic content, total flavonoid content, quercetin content, and antioxidant activity. The MAE method produced the highest extract yield of 15.04%, compared with 13.08% for maceration and 9.38% for UAE. Phytochemical screening revealed the presence of several bioactive constituents, particularly flavonoids, phenols, proteins, and saponins. TLC analysis using rutin as a marker showed an R_f value of 0.48 for the extracts. Among the extraction methods, the MAE extract exhibited the highest total phenolic content (7.884 ± 1.87 mg GAE/100 mg extract) and total flavonoid content (5.972 ± 1.36 mg QE/100 mg extract). HPLC analysis confirmed the presence of quercetin in the MAE extract with a retention time of 2.300 min and an assay value of 0.085%. The antioxidant activity of the selected extract was concentration dependent. In the DPPH assay, the extract showed an IC_{50} value of 88.31 μ g/ml, while the IC_{50} of ascorbic acid was 24.15 μ g/ml. In the hydrogen peroxide scavenging assay, the extract showed an IC_{50} value of 75.32 μ g/ml compared with 25.83 μ g/ml for ascorbic acid. The findings indicate that microwave-assisted extraction was the most effective method among those investigated for obtaining a phenolic- and flavonoid-rich hydroalcoholic extract of *Citrus medica*, which demonstrated appreciable antioxidant potential.

Keywords: *Citrus medica*, hydroalcoholic extract, microwave-assisted extraction, phytochemical screening, flavonoids, phenolic content, quercetin, antioxidant activity, DPPH, hydrogen peroxide.

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Please cite this article in press **Kirti Ahirwar et al., Study Of Secondary Metabolites In Citrus Medica Extract Using Uv And Chromatographic Techniques., Indo Am. J. P. Sci, 2026; 13(10).**

INTRODUCTION:

Citrus medica L., commonly known as citron, belongs to the family Rutaceae and is an important medicinal and aromatic plant. Different parts of the plant have traditionally been used for their nutritional and therapeutic properties. *Citrus medica* is reported to possess considerable nutritional and phytochemical importance, making it a promising source of biologically active compounds (Chhikara et al., 2018).

The plant is reported to contain diverse secondary metabolites, including flavonoids, phenolic compounds, alkaloids, glycosides, terpenoids, tannins, saponins, and other bioactive constituents. These phytochemicals are associated with various biological activities, particularly antioxidant, antimicrobial, anti-inflammatory, and protective effects (Nwozo et al., 2023).

Secondary metabolites present in medicinal plants play an important role in determining their pharmacological potential. Phenolic compounds and flavonoids are particularly important because of their ability to scavenge free radicals and reduce oxidative stress. Their presence is therefore frequently considered an important indicator of the therapeutic and antioxidant potential of medicinal plant extracts (Hussein and El-Anssary, 2019).

Compounds such as quercetin and rutin are important flavonoid constituents that can serve as chemical markers for the standardization and quality assessment of plant extracts. Therefore, identification and quantitative estimation of these constituents are important for establishing the phytochemical profile of *Citrus medica* and supporting the quality evaluation of its extracts (Benedetto et al., 2023).

The efficiency of extraction depends on several factors, including the extraction technique, solvent composition, temperature, extraction time, and nature of the plant matrix. Conventional maceration is simple, economical, and widely used for extraction of plant constituents; however, it generally requires prolonged extraction time and may provide comparatively lower extraction efficiency (Shrivastav et al., 2025).

Modern techniques such as ultrasonic-assisted extraction (UAE) and microwave-assisted extraction (MAE) can improve the recovery of phytoconstituents by enhancing solvent penetration and disrupting plant tissues. Microwave-assisted extraction can provide rapid heating and improved mass transfer, thereby facilitating the release of bioactive compounds from plant materials. Comparative evaluation of these techniques can therefore help in selecting an appropriate method for obtaining a phytochemically rich extract (López-Salazar et al., 2023).

Chromatographic techniques are valuable tools for the separation, identification, and estimation of secondary metabolites. Thin-layer chromatography (TLC) provides a rapid and economical method for preliminary identification of marker compounds, whereas high-performance liquid chromatography (HPLC) offers greater sensitivity, selectivity, and accuracy for the qualitative and quantitative analysis of phytoconstituents (Parys et al., 2022).

Ultraviolet-visible (UV-Vis) spectrophotometric methods are also widely employed for the estimation of total phenolic and flavonoid contents and for evaluating the antioxidant activity of plant extracts. Spectrophotometric approaches provide relatively simple and reproducible methods for preliminary quantitative assessment of biologically active compounds in herbal materials (Butnariu and Coradini, 2012).

In view of the diverse phytochemical composition and potential therapeutic value of *Citrus medica*, systematic investigation of its secondary metabolites is necessary (Chhikara et al., 2018). The present study was therefore designed to prepare hydroalcoholic extracts of *Citrus medica* using maceration, ultrasonic-assisted extraction (UAE), and microwave-assisted extraction (MAE) and to comparatively evaluate their extractive yield and phytochemical constituents. The selected extract was further investigated using UV and chromatographic techniques for estimation of total phenolic and flavonoid contents and identification and quantification of marker compounds such as rutin and quercetin. Antioxidant activity was also evaluated using DPPH and hydrogen peroxide scavenging assays to establish the biological relevance of the identified phytoconstituents.

MATERIAL AND METHODS:**Material**

The chemicals and reagents used in the study were procured from standard pharmaceutical and analytical-grade suppliers. Major chemicals included potassium mercuric iodide, iodine, potassium iodide, potassium bismuth iodide, picric acid, sodium nitroprusside, sodium hydroxide, pyridine, ferric chloride, gelatin, lead acetate, nitric acid, copper acetate, sodium chloride, methanol, ethanol, chloroform, Folin-Ciocalteu reagent, and Fehling's solution. All chemicals were of analytical grade and obtained from reputed suppliers such as Thomas Baker, Loba Chemie, S. D. Fine Chem. Ltd., Qualigens Fine Chemicals, and Central Drug House, Mumbai/New Delhi.

Methods**Extraction of plant materials by different methods**

50 gram of dried leaves of *Citrus medica* were coarsely powdered and subjected to extraction by different methods.

Maceration process

Leaves of *Citrus medica* were extracted with hydroalcoholic solvent (Ethanol: Water; 70:30) using maceration process (48hrs) (Handa *et al.*, 2008). The extract was evaporated above their boiling points. Finally the percentage yields were calculated of the dried extract.

Ultrasonic Assisted Extraction

Fifty grams of dried leaves of *Citrus medica* were accurately weighed and transferred into a 100 mL stoppered bottle containing 80 mL of hydroalcoholic extraction solvent (ethanol: water, 70:30 v/v). The mixture was subjected to ultrasonic-assisted extraction in an ultrasonic bath for 40 minutes at room temperature (25°C) to facilitate efficient extraction of bioactive coloring constituents. After completion of extraction, the obtained extract was filtered through Whatman No. 42 filter paper to remove insoluble materials. The filtered extract was then stored under refrigerated conditions until further analysis was carried out (Briones-Labarca *et al.*, 2015).

Microwave-assisted extraction

Microwave-assisted extraction was employed for the extraction of phytochemicals from dried leaves of *Citrus medica*. The dried leaf material was mixed with a hydroalcoholic solvent system consisting of ethanol and water (70:30 v/v) and subjected to microwave treatment at varying power levels ranging from 230 to 840 W for different exposure times (10–110 seconds) (Pinela *et al.*, 2016).

Determination of percentage yield

The extraction yield is evaluate of the solvent's efficiency to extracts bioactive components from the selected natural plant samples and it was defined as quantity of plant extracts recovered in mass after solvent extraction compared with the initial quantity of plant samples. After extraction, yield of the plant extracts obtained were calculated in grams and then converted it into percentage (Arwande *et al.*, 2018). The percentage yields of each extract were calculated by using following formula:

$$\text{Percentage yield} = \frac{\text{Weight of extract}}{\text{Weight of powder drug taken}} \times 100$$

Qualitative evaluation

Medicinal plants are resources of traditional medicines and many of the modern medicines are produced indirectly from plants. Phytochemical constituents are of two type primary bioactive constituents (Chlorophyll, proteins, amino acids,

sugar etc.) and secondary bioactive constituents include (Alkaloids, terpenoids, phenols, flavonoids etc.). Phytochemical tests were done as per the methods given (Talukdar and Chaudhary, 2010).

Quantitative studies of bioactive constituents

Estimation of total phenol content

The total phenolic content of the extract was determined using the modified Folin–Ciocalteu method (Javanmardi *et al.*, 2003). A stock solution of gallic acid was prepared by dissolving 10 mg of gallic acid in 10 mL of methanol, and different concentrations (5–25 µg/mL) were prepared for calibration. For sample preparation, 10 mg of dried extract was dissolved in 10 mL of methanol and filtered. An aliquot of 2 mL of the extract solution (1 mg/mL) was used for total phenol estimation. Subsequently, 2 mL of the extract or standard solution was mixed with 1 mL of Folin–Ciocalteu reagent diluted previously with distilled water (1:10, v/v) and 1 mL of sodium carbonate solution (7.5 g/L). The reaction mixture was vortexed for 15 seconds and allowed to stand for 10 minutes to facilitate color development. The absorbance of the developed color was measured at 765 nm using a UV–Visible spectrophotometer.

Estimation of total flavonoids content

The total flavonoid content of the extract was determined by the aluminium chloride colorimetric method (Khan *et al.*, 2018). A standard stock solution was prepared by dissolving 10 mg of quercetin in 10 mL of methanol, and different concentrations (5–25 µg/mL) were subsequently prepared for calibration. For sample analysis, 10 mg of dried extract was dissolved in 10 mL of methanol and filtered. An aliquot of 3 mL of the extract solution (1 mg/mL) was used for flavonoid estimation. To this, 1 mL of 2% aluminium chloride (AlCl₃) solution was added and the reaction mixture was allowed to stand at room temperature for 15 minutes for color development. The absorbance was then measured at 420 nm using a UV–Visible spectrophotometer to determine the total flavonoid content.

Identification of marker compound (Rutin) by HPLC

Rutin was kindly provided by Scan Research Laboratories, Bhopal (India). Methanol and acetonitrile were of HPLC grade and purchased from Merck Ltd, New Delhi, India. Water used was of HPLC grade water from Merck Ltd, New Delhi, India. A thermospectronic model of Labindia 3000 + UV/VIS Spectrophotometer with 1cm. matched quartz cells was used for determination of λ_{max} . The HPLC system (Waters) consisted of a pump, a U.V. Visible detector, a Thermo C₁₈ (250 X 4.6 mm, 5µm) column, a Data Ace software. The chromatographic analysis was performed at

ambient temperature on a RP-C18 analytical column with a mobile phase composed of methanol: water (50:50 v/v) pH 2.8 (adjusted with phosphoric acid) and was isocratically eluted at a flow rate of 1 mL min⁻¹. A small sample volume of 20 µL was used for each sample run, being injected into the HPLC system. The chromatogram was monitored with UV detection at a wavelength of 254 nm (Kuntić et al., 2007).

Preparation of standard stock solution

10mg of rutin was weighed accurately and transferred to a 10ml volumetric flask, and the volume was adjusted to the mark with the methanol to give a stock solution of 1000ppm.

Preparation of working standard solution

From stock solutions of rutin 1 ml was taken and diluted up to 10 ml. from this solution 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 ml solutions were transferred to 10ml volumetric flasks and make up the volume up to 10 ml with mobile phase, gives standard drug solution of 5, 10, 15, 20, 25, 30 µg/ ml concentration.

Analysis of extract

10 mg extract was taken in 10 ml volumetric flask and dilute upto the mark with Methanol; resultant solution was filtered through Whatmann filter paper and finally volume made up to mark with same solvent to obtain concentration of 1000 µg/ml. The resulting solution was again filtered using 0.45 µm membrane filter and then sonicated for 10 min.

In-vitro antioxidant activity of extract (Microwave-Assisted Extraction) of *Citrus medica*

DPPH method

Total free radical scavenging capacity of extract from *Citrus medica* estimated according to the previously reported method with slight modification (Parkhe and Jain, 2018). Solution of DPPH (6 mg in 100ml methanol) was prepared and stored in dark place. Different concentration of standard and test (10-100 µg/ml) was prepared. 1.5 ml of DPPH and 1.5 ml of each standard and test was taken in separate test tube; absorbance of this solution was taken immediately at 517nm. 1.5 ml of DPPH and 1.5 ml of the methanol was taken as control absorbance at 517nm. The percentage inhibition of free radical DPPH was calculated from the following equation:

$$\% \text{ inhibition} = \frac{[(\text{absorbance of control} - \text{absorbance of sample}) / \text{absorbance of control}] \times 100\%}{}$$

Hydrogen peroxide method

In-vitro antioxidant activity of extracts of *Citrus medica* using hydrogen peroxide was performed as Czocho and Widensk (2002) proposed. Added 2ml hydrogen peroxide (43 mol) and 1.0 ml extract

solution (20-100 µg/ml) accompanied by 2.4 ml 0.1 M phosphate buffer (pH 7.4). The resulting solution was maintained for 10 minutes and the absorbance at 230 nm was recorded. Without adding hydrogen peroxide, blank was ready and control was prepared without sample. It was used as a conventional compound with ascorbic acid. Free radical hydrogen peroxide scavenging activity (percent) has been calculated.

$$\% \text{ inhibition} = \frac{[(\text{absorbance of control} - \text{absorbance of sample}) / \text{absorbance of control}] \times 100\%}{}$$

RESULTS AND DISCUSSION:

The physical characteristics of the hydroalcoholic extracts of *Citrus medica* obtained by different extraction techniques are presented in Table 1. All extracts were black in colour. The microwave-assisted extraction (MAE) method produced the highest extract weight (7.52 g) and percentage yield (15.04%), followed by maceration (6.54 g; 13.08%) and ultrasonic-assisted extraction (UAE) (4.69 g; 9.38%). The higher yield obtained with MAE may be attributed to rapid heating and improved disruption of plant tissues, which facilitates the release of soluble phytoconstituents into the extraction solvent.

Preliminary phytochemical screening of the macerated hydroalcoholic extract showed the presence of flavonoids, phenols, proteins and saponins, while alkaloids, glycosides, diterpenes, carbohydrates, tannins and sterols were absent (Table 2). The UAE extract showed a broader phytochemical profile, with positive reactions for flavonoids, glycosides, diterpenes, phenols, proteins, carbohydrates, saponins, tannins and sterols. Hager's test for alkaloids was positive, whereas Wagner's test was negative (Table 3). The MAE extract demonstrated positive reactions for flavonoids, glycosides, diterpenes, phenols, proteins, carbohydrates, saponins and sterols. Alkaloids were negative by Hager's test but positive by Wagner's test, while tannins were absent (Table 4). These variations may be related to differences in extraction efficiency, temperature and exposure of phytoconstituents during the respective extraction processes.

TLC analysis was performed for identification of the marker phytoconstituent in *Citrus medica* extracts. Rutin was used as the marker compound, and the solvent system consisting of methanol:glacial acetic acid:formic acid:water (3:0.9:0.9:0.5) produced an R_f value of 0.48 for all three extraction methods (Table 5). A distinct yellow fluorescent spot was observed in the maceration extract, a well-resolved compact spot was observed in the UAE extract, and an intense fluorescent spot was observed in the MAE extract. The consistent R_f value with the rutin standard supported the presence of rutin in the extracts.

Quantitative estimation of phenolic and flavonoid compounds revealed differences among the extraction methods (Table 6). The MAE extract showed the highest total phenolic content (7.884 ± 1.87 mg GAE/100 mg extract) and total flavonoid content (5.972 ± 1.36 mg QE/100 mg extract), followed by UAE and maceration. The enhanced recovery of phenolic and flavonoid constituents by MAE may be associated with efficient microwave energy transfer and improved penetration of the extraction solvent into the plant matrix.

The chromatographic analysis further supported the presence of the selected phytoconstituent. The chromatogram of standard rutin is shown in Figure 1, while the chromatogram of the hydroalcoholic extract obtained by MAE is presented in Figure 2. Quantitative HPLC estimation of quercetin showed a retention time of 2.300 min for both the standard and MAE extract. The extract exhibited a quercetin content of 0.085%, indicating the presence of quercetin in the selected extract (Table 7).

The antioxidant potential of the MAE hydroalcoholic extract was evaluated using the DPPH free-radical scavenging assay and compared with ascorbic acid (Table 8). The percentage inhibition increased progressively with increasing

concentration. The extract showed 13.96%, 21.07%, 32.43%, 39.56%, 43.87% and 51.71% inhibition at concentrations of 10, 20, 40, 60, 80 and 100 µg/ml, respectively. Ascorbic acid showed comparatively higher inhibition at all tested concentrations. The IC₅₀ value of the extract was 88.31 µg/ml compared with 24.15 µg/ml for ascorbic acid, demonstrating concentration-dependent antioxidant activity of the *Citrus medica* extract.

The hydrogen peroxide scavenging activity also demonstrated concentration-dependent inhibition (Table 9). The MAE extract exhibited 21.65%, 32.57%, 39.91%, 55.06% and 61.78% inhibition at concentrations of 20, 40, 60, 80 and 100 µg/ml, respectively. Ascorbic acid produced higher inhibition, ranging from 46.38% to 92.17%. The IC₅₀ value of the extract was 75.32 µg/ml, whereas that of ascorbic acid was 25.83 µg/ml. The antioxidant activity may be associated with the phenolic and flavonoid constituents identified in the extract. Overall, among the three extraction techniques, microwave-assisted extraction produced the highest extract yield and the highest phenolic and flavonoid contents, and therefore the MAE extract was selected for further investigation.

Table 1: Physical characteristics of hydroalcoholic extracts of *Citrus medica*

Methods	Colour	Weight of extract	% Yield
Maceration process	Black	6.54	13.08
Ultrasonic Assisted Extraction	Black	4.69	9.38
Microwave-assisted extraction	Black	7.52	15.04

Table 2: Phytochemical tests of extract of *Citrus medica* (Maceration process)

S. No.	Constituents	Hydroalcoholic extract
1.	Alkaloids Hager's test: Wagner's test:	-ve -ve
2.	Glycosides Conc. H ₂ SO ₄ test:	-ve
3.	Flavonoids Lead acetate test: Alkaline reagent test:	+ve +ve
4.	Diterpenes Copper acetate test:	-ve
5.	Phenol Ferric chloride test:	+ve
6.	Proteins Xanthoproteic test:	+ve
7.	Carbohydrate Fehling's test: Benedict's test:	-ve -ve
8.	Saponins Froth test:	+ve
9.	Tannins Gelatin test:	-ve
10.	Sterols Salkowski test	-ve

+ ve – Present, - ve – Absent

Table 3: Phytochemical tests of extract of *Citrus medica* (Ultrasonic Assisted Extraction)

S. No.	Constituents	Hydroalcoholic extract
1.	Alkaloids Hager's test: Wagner's test:	+ve -ve
2.	Glycosides Conc. H ₂ SO ₄ test:	+ve
3.	Flavonoids Lead acetate test: Alkaline reagent test:	+ve +ve
4.	Diterpenes Copper acetate test:	+ve
5.	Phenol Ferric chloride test:	+ve
6.	Proteins Xanthoproteic test:	+ve
7.	Carbohydrate Fehling's test: Benedict's test:	+ve +ve
8.	Saponins Froth test:	+ve
9.	Tannins Gelatin test:	+ve
10.	Sterols Salkowski test	+ve

+ ve – Present, - ve – Absent

Table 4: Phytochemical tests of extract of *Citrus medica* (Microwave-Assisted Extraction)

S. No.	Constituents	Hydroalcoholic extract
1.	Alkaloids Hager's test: Wagner's test:	-ve +ve
2.	Glycosides Conc. H ₂ SO ₄ test:	+ve
3.	Flavonoids Lead acetate test: Alkaline reagent test:	+ve +ve
4.	Diterpenes Copper acetate test:	+ve
5.	Phenol Ferric chloride test:	+ve
6.	Proteins Xanthoproteic test:	+ve
7.	Carbohydrate Fehling's test: Benedict's test:	+ve +ve
8.	Saponins Froth test:	+ve
9.	Tannins Gelatin test:	-ve
10.	Sterols Salkowski test	+ve

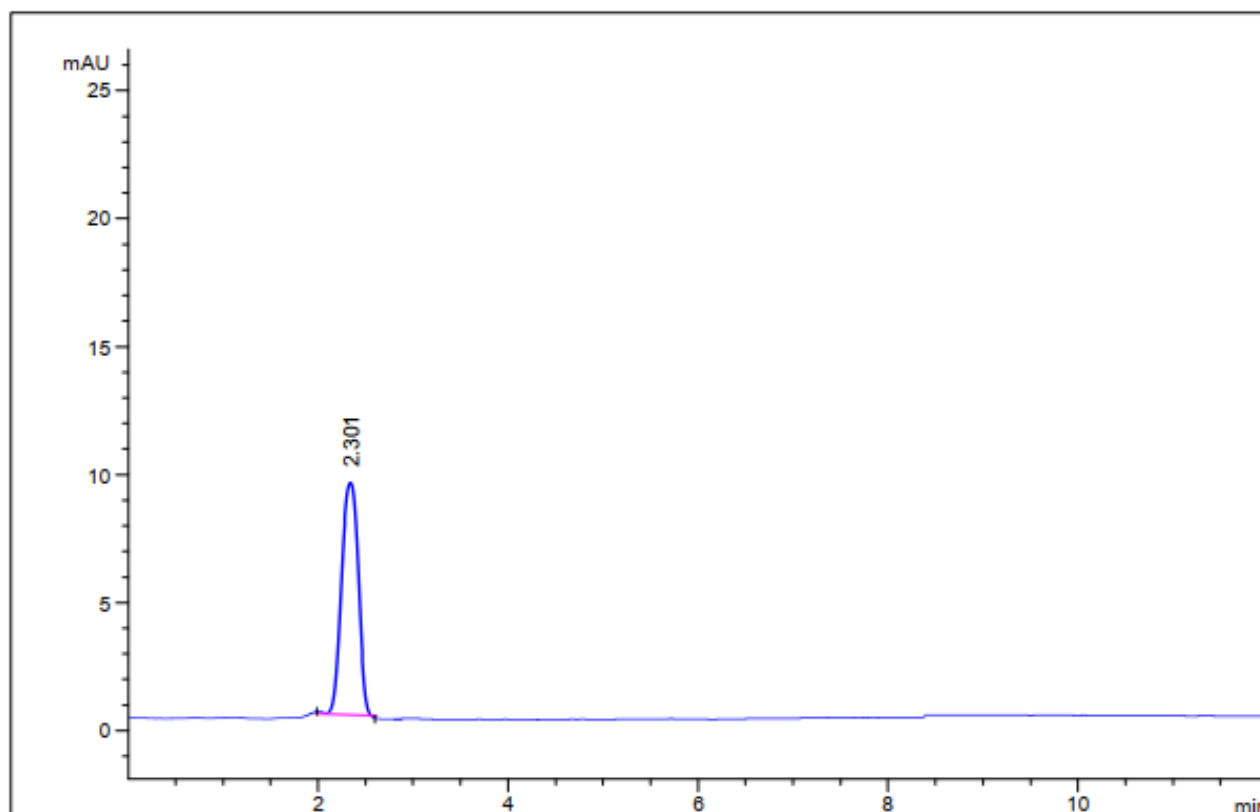
+ ve – Present, - ve – Absent

Table 5: Identification of phytoconstituents by TLC of *Citrus medica*

S. No.	Extraction Method	Plant Material	Solvent System Used for TLC	Marker Compound	Rf Value	Observation
1	Maceration Process	<i>Citrus medica</i>	Methanol : Glacial Acetic Acid : Formic Acid : Water (3 : 0.9 : 0.9 : 0.5)	Rutin	0.48	Distinct yellow fluorescent spot observed
2	Ultrasonic Assisted Extraction	<i>Citrus medica</i>	Methanol : Glacial Acetic Acid : Formic Acid : Water (3 : 0.9 : 0.9 : 0.5)	Rutin	0.48	Well-resolved compact spot observed
3	Microwave-Assisted Extraction	<i>Citrus medica</i>	Methanol : Glacial Acetic Acid : Formic Acid : Water (3 : 0.9 : 0.9 : 0.5)	Rutin	0.48	Intense fluorescent spot indicating presence of rutin

Table 6: Estimation of total phenol and flavonoids content

S. No.	Methods	Total Phenol Content (mg GAE/100mg extract)	Total Flavonoid Content (mg QE/100mg extract)
1	Maceration Process	4.826 ± 1.24	3.218 ± 0.96
2	Ultrasonic Assisted Extraction	6.145 ± 1.52	4.536 ± 1.14
3	Microwave-Assisted Extraction	7.884 ± 1.87	5.972 ± 1.36

**Figure 1: Chromatogram of standard Rutin**

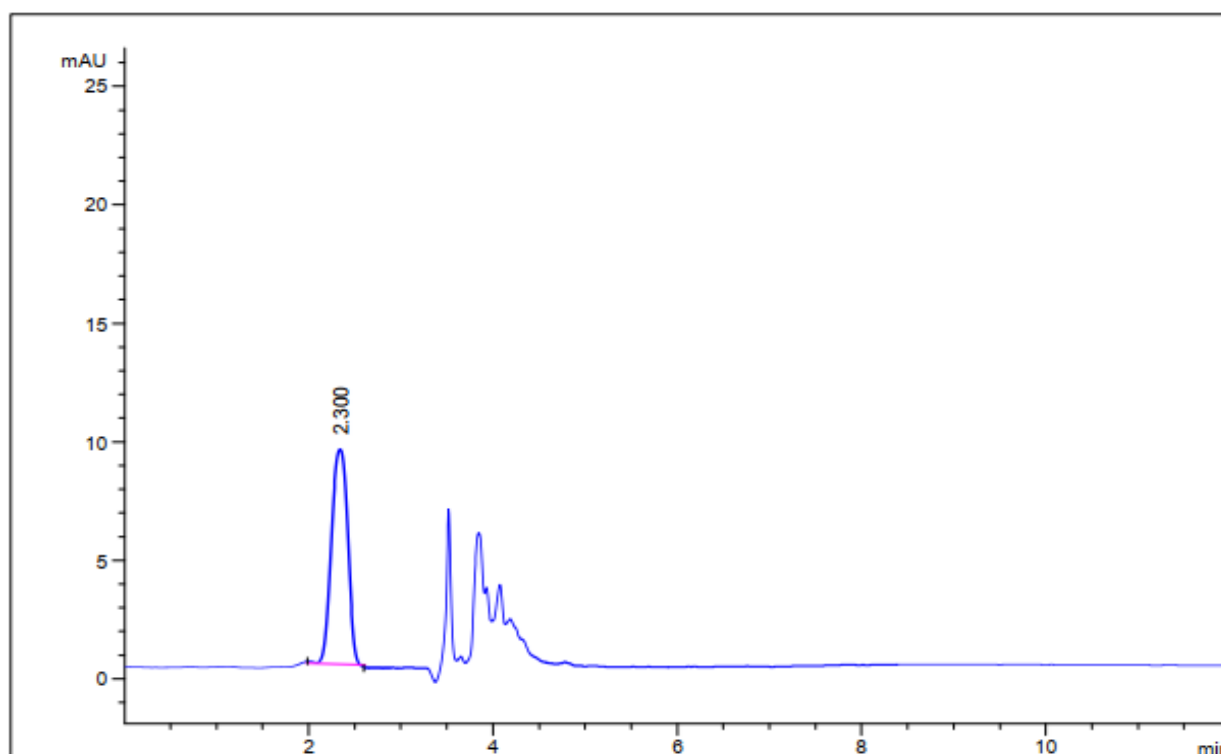


Figure 2: Chromatogram of hydroalcoholic extract by Microwave-Assisted Extraction of *Citrus medica*

Table 7: Quantitative estimation of Quercetin in extract

S. No.	Standard/Extract	RT	% Assay
1.	Quercetin	2.300	-
2.	Hydroalcoholic extract (Microwave-Assisted Extraction)	2.300	0.085%

Table 8: % Inhibition of ascorbic acid and hydroalcoholic extract (Microwave-Assisted Extraction) of *Citrus medica* using DPPH method

S. No.	Concentration (µg/ml)	% Inhibition	
		Ascorbic acid	Hydroalcoholic extract
1	10	42.75	13.96
2	20	48.96	21.07
3	40	57.81	32.43
4	60	65.27	39.56
5	80	72.05	43.87
6	100	83.11	51.71
IC ₅₀ value		24.15	88.31

Table 9: % Inhibition of ascorbic acid and Hydroalcoholic extract (Microwave-Assisted Extraction) of *Citrus medica* using hydrogen peroxide method

S. No.	Concentration (µg/ml)	% Inhibition	
		Ascorbic acid	Hydroalcoholic extract
1	20	46.38	21.65
2	40	60.72	32.57
3	60	65.96	39.91
4	80	76.84	55.06
5	100	92.17	61.78
IC ₅₀ value		25.83	75.32

CONCLUSION:

The present study demonstrated that the extraction method significantly influenced the yield and phytochemical composition of the hydroalcoholic extract of *Citrus medica*. Among the investigated methods, microwave-assisted extraction produced the highest extract yield (15.04%) and showed the highest total phenolic content (7.884 ± 1.87 mg GAE/100 mg extract) and total flavonoid content (5.972 ± 1.36 mg QE/100 mg extract). TLC analysis confirmed the presence of rutin, while HPLC analysis demonstrated the presence of quercetin (0.085%) in the MAE extract. The extract also exhibited concentration-dependent antioxidant activity in both DPPH and hydrogen peroxide scavenging assays. Microwave-assisted extraction was found to be the most efficient extraction technique for obtaining a phytochemically rich *Citrus medica* hydroalcoholic extract with appreciable antioxidant potential. Further studies are recommended to isolate individual active constituents and establish their pharmacological efficacy and safety.

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