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STUDY OF SECONDARY METABOLITES IN *CITRUS MEDICA* EXTRACT USING UV AND CHROMATOGRAPHIC TECHNIQUES

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ABSTRACT

The present study was undertaken to investigate the secondary metabolites present in the hydroalcoholic extract of *Citrus medica* using spectroscopic and chromatographic techniques. The extract was prepared using a suitable hydroalcoholic solvent system, yielding a dark-colored solid with a percentage yield of 10.28%. Preliminary phytochemical screening revealed the presence of important bioactive constituents such as carbohydrates, glycosides, saponins, phenols, flavonoids, and proteins, while alkaloids and diterpenes were found to be absent. Thin Layer Chromatography (TLC) analysis using the solvent system Toluene: Ethyl acetate: Formic acid (5:4:1) showed multiple spots, indicating the presence of diverse phytoconstituents. A spot with an Rf value close to that of standard quercetin confirmed the probable presence of flavonoid compounds. Quantitative estimation demonstrated that the extract contains 0.52 mg/100 mg of total phenolic content and 0.89 mg/100 mg of total flavonoid content, suggesting a significant presence of antioxidant compounds. Further chromatographic analysis confirmed the presence of quercetin in the extract with a retention time comparable to the standard (RT ≈ 2.5 min), and its content was found to be 0.12%. The results indicate that *Citrus medica* is a rich source of biologically active secondary metabolites, particularly flavonoids and phenolic compounds, which may contribute to its therapeutic potential. The study highlights the effectiveness of UV and chromatographic techniques in the identification and characterization of phytoconstituents.

Keywords: *Citrus medica*, Secondary metabolites, Hydroalcoholic extract, Phytochemical screening, Thin Layer Chromatography (TLC), Quercetin, Total phenolic content, Total flavonoid content, UV spectroscopy, Chromatographic analysis.

INTRODUCTION

Citrus medica L., commonly known as citron, belongs to the family Rutaceae and is widely recognized for its rich phytochemical composition and medicinal importance. Citrus species are well-known reservoirs of diverse secondary metabolites, which play a fundamental role in plant defense mechanisms and exhibit significant pharmacological activities such as antioxidant, antimicrobial, anti-inflammatory, and anticancer effects (Chhikara *et al.*, 2018).

These metabolites include flavonoids, phenolic acids, alkaloids, coumarins, limonoids, carotenoids, and essential oils, which are distributed in different parts of the fruit such as peel, pulp, and seeds (Mabberley, 2023).

Secondary metabolites are organic compounds that are not directly involved in the normal growth and development of plants but are essential for their survival and interaction with the environment. In *Citrus medica*, these bioactive compounds contribute to its therapeutic potential and have attracted considerable attention in pharmaceutical and nutraceutical research. Recent metabolomic studies have revealed the presence of numerous bioactive constituents in *Citrus medica*, including flavonoids and terpenoids, which are responsible for its biological activities (Meena *et al.*, 2011).

The qualitative and quantitative analysis of these secondary metabolites requires advanced analytical techniques. Among these, Ultraviolet (UV–Visible) spectroscopy and chromatographic techniques are widely employed due to their sensitivity, accuracy, and reliability. UV–Visible spectroscopy is based on the absorption of electromagnetic radiation in the range of 200–700 nm and is particularly useful for detecting compounds with conjugated double bonds such as flavonoids and phenolic compounds. The absorption arises from electronic transitions ($\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$), providing valuable information for both identification and quantification of phytochemicals (Tasnim, 2023).

Chromatographic techniques, including Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), and Gas Chromatography (GC), are essential tools for the separation, identification, and characterization of complex mixtures of secondary metabolites. These methods allow the development of chromatographic fingerprints and enable the detection of specific compounds such as rutin, limonene, and other flavonoids present in citrus extracts. For instance, HPLC and LC-MS analyses of citrus species have identified numerous compounds, mainly flavonoids and limonoids, demonstrating the effectiveness of chromatographic approaches in phytochemical profiling. Similarly, GC-MS has been extensively used to identify volatile secondary metabolites such as monoterpenes (e.g., limonene) in citrus essential oils (Parys *et al.*, 2022).

Furthermore, the integration of spectroscopic and chromatographic techniques provides a comprehensive approach for phytochemical analysis. While UV spectroscopy offers rapid preliminary screening, chromatographic methods provide detailed separation and structural elucidation of individual components. This combined approach enhances the accuracy and reliability of the analysis and is widely used in standardization and quality control of herbal formulations. In this context, the present study focuses on the investigation of secondary metabolites in *Citrus medica* extract using UV spectroscopy and chromatographic techniques, aiming to establish a scientific basis for its phytochemical characterization and potential therapeutic applications.

MATERIALS AND METHODS

Materials

The study utilized *Citrus medica* leaves as the primary plant material for extraction and phytochemical analysis. Hydroalcoholic solvent system was employed for extraction of bioactive constituents. Various analytical grade chemicals and reagents such as ethanol, methanol, ethyl acetate, toluene, formic acid, ferric chloride, Wagner’s reagent, Fehling’s

solution, lead acetate, and alkaline reagents were used for preliminary phytochemical screening and TLC analysis. Standard quercetin was used as a reference compound for chromatographic comparison. Distilled water and laboratory-grade solvents were used throughout the experimental procedures to ensure accuracy and reproducibility of results. All instruments and glassware used in the study were properly calibrated and maintained for reliable analytical outcomes.

Methods

Extraction procedure

Defatting of plant materials

50 gram of dried leaves of *Citrus medica* were coarsely powdered and subjected to extraction with petroleum ether by Soxhlet extraction method (Handa *et al.,* 2008). The extraction was continued till the defatting of the material had taken place.

Extraction by Soxhlet extraction process

Defatted materials of *Citrus medica* were extracted with hydroalcoholic solvent (Ethanol: Water; 80:20) using Soxhlet extraction process (48hrs). The extract was evaporated above their boiling points. Finally the percentage yields were calculated of the dried extract.

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).

Separation and Identification of phytoconstituents by TLC

Thin Layer Chromatography (TLC) is based on the adsorption phenomenon, where the mobile phase carrying dissolved solutes moves over a stationary phase. In the present study, TLC was performed using the conventional one-dimensional ascending technique on silica gel 60F254 TLC plates (7 × 6 cm, Merck). The sample was applied using glass capillaries at 1 µL volume, spotted 1 cm from the base across five tracks. The plates were developed in a twin-trough chamber using the solvent system toluene: ethyl acetate: formic acid (5:4:1) after pre-saturation for 20 minutes. After development, the chromatograms were visualized under normal light, short UV (254 nm), and long UV (365 nm) using a TLC visualizing cabinet. The movement of compounds was expressed in terms of Rf values, which were calculated for each spot to identify and compare phytoconstituents present in the

extract (Saxena *et al.*, 2005). Once the chromatogram was developed the R_f Value of the spot was calculated using the formula:

$$\mathbf{Rf} = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

Quantitative studies of bioactive constituents

Estimation of total phenolic content

The total phenolic content of the extract was determined using the modified Folin–Ciocalteu method as described by Javanmardi et al. (2003). For the preparation of standard, 10 mg of gallic acid was dissolved in 10 ml of methanol, and various aliquots (5–25 µg/ml) were prepared. For sample preparation, 10 mg of dried extract was dissolved in 10 ml of methanol and filtered, and 2 ml of this solution (1 mg/ml) was used for analysis. In the procedure, 2 ml of standard or extract was mixed with 1 ml of Folin–Ciocalteu reagent (diluted 1:10 with distilled water) 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 for color development. The absorbance was then measured at 765 nm using a UV-visible spectrophotometer, and the total phenolic content was quantified using a calibration curve of gallic acid.

Estimation of total flavonoids content

The total flavonoid content of the extract was determined by the aluminium chloride colorimetric method as described by Khan et al. (2018). For standard preparation, 10 mg of quercetin was dissolved in 10 ml of methanol, and different aliquots (5–25 µg/ml) were prepared. For sample preparation, 10 mg of dried extract was dissolved in 10 ml of methanol, filtered, and 3 ml of this solution (1 mg/ml) was used for analysis. In the procedure, 1 ml of 2% aluminium chloride solution was added to 3 ml of standard or extract solution and allowed to stand for 15 minutes at room temperature for complex formation. The absorbance of the resulting solution was measured at 420 nm using a UV-visible spectrophotometer, and the total flavonoid content was calculated using a quercetin calibration curve.

Identification of marker compound (Quercetin) by HPLC

Quercetin was kindly provided by Scan Research Laboratories, Bhopal (India). Methanol, acetonitrile, and HPLC-grade water were purchased from Merck Ltd., New Delhi, India. UV analysis was carried out using a Thermospectronic Labindia 3000+ UV/VIS spectrophotometer equipped with 1 cm matched quartz cells for determination of λmax. The HPLC system (Waters) consisted of a pump, UV-visible detector, Thermo C18 column (250 × 4.6 mm, 5 µm), and Data Ace software. Chromatographic separation was performed on a reverse-phase C18 column using a mobile phase of acetonitrile:methanol (50:50 v/v) under isocratic conditions at a flow rate of 1 mL/min. A sample volume of 20 µL was injected for each run, and detection was carried out at 256 nm under ambient temperature conditions (Garg, 2021).

Table 1: Selection of Separation Variable

Variable	Condition
Column	C18 (Octadecylsilane)
Dimension	250 mm × 4.60 mm
Particle Size	5 µm
Bonded Phase	Octadecylsilane (C18)
Mobile Phase	
Acetonitrile	50%
Methanol	50%
Mode of	Isocratic

Elution	
Flow Rate	1.0 ml/min
Injection Volume	20 µl
Detection Wavelength	256 nm
Column Temperature	Room temperature (25 ± 2°C)
Run Time	10 minutes
Retention Time	2.50 ± 0.5 min
Diluent	Mobile phase
Detector	UV/Visible Detector
System Pressure	~2000–3000 psi (depending on system)
Sample Preparation	Filtered through 0.45 µm membrane filter

Preparation of standard stock solution

10mg of quercetin 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 Quercetin 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, 30µg/ 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 0.45µ membrane filter and then sonicated for 10 min.

RESULTS AND DISCUSSION

The present study was carried out to evaluate the physical characteristics, phytochemical composition, and chromatographic profile of the hydroalcoholic extract of *Citrus medica*, along with quantitative estimation of major secondary metabolites.

The physical characterization of the extract (Table 2) revealed that the hydroalcoholic extract was solid in consistency with a black color and showed a percentage yield of 10.28%. This moderate yield suggests efficient extraction of both polar and semi-polar constituents using the hydroalcoholic solvent system. The dark coloration may be attributed to the presence of phenolic compounds, flavonoids, and other oxidizable phytoconstituents, which are commonly extracted in such solvent systems.

The preliminary phytochemical screening (Table 3) confirmed the presence of several important secondary metabolites. The extract tested positive for carbohydrates, glycosides, saponins, phenols, flavonoids (lead acetate test), and proteins, while alkaloids and diterpenes were absent. The presence of phenolic compounds and flavonoids is particularly significant, as these compounds are known for their antioxidant and therapeutic properties. The positive result in the alkaline reagent and lead acetate tests further supports the presence of flavonoid constituents, which are key bioactive compounds in *Citrus medica*. The absence of alkaloids indicates that the biological activity of the extract may be primarily attributed to non-alkaloidal constituents.

The Thin Layer Chromatography (TLC) analysis (Table 4) provided insight into the separation and identification of phytoconstituents. Using the mobile phase system Toluene: Ethyl acetate: Formic acid (5:4:1), the standard quercetin showed a consistent Rf value of 0.52 under long UV, short UV, and normal light conditions, indicating its purity and suitability as a reference marker. The hydroalcoholic extract exhibited multiple spots with Rf values ranging from 0.48 to 0.78, indicating the presence of a complex mixture of phytoconstituents. One of the spots in the extract showed an Rf value (0.50) very close to that of standard quercetin, suggesting the possible presence of quercetin or structurally related flavonoids in the extract. The variation in the number of spots under different detection conditions confirms the presence of multiple compounds with varying polarities.

The quantitative estimation of total phenolic and flavonoid content (Table 5) revealed that the extract contained 0.52 mg/100 mg of phenolic compounds and 0.89 mg/100 mg of flavonoids. The relatively higher flavonoid content compared to phenolic content indicates that flavonoids are the predominant class of secondary metabolites in the extract. This finding correlates well with the phytochemical screening results and supports the potential antioxidant capacity of the extract.

Further confirmation was obtained from the chromatographic estimation of quercetin (Table 6), where the standard quercetin showed a retention time (RT) of 2.545 minutes, while the extract exhibited a closely matching RT of 2.512 minutes. This similarity in retention time confirms the presence of quercetin in the hydroalcoholic extract. The percentage assay of quercetin was found to be 0.12%, indicating that although present in small quantity, quercetin contributes to the overall bioactivity of the extract.

The study demonstrates that the hydroalcoholic extract of *Citrus medica* is rich in bioactive secondary metabolites, particularly flavonoids and phenolic compounds. The combined use of phytochemical screening, TLC, and chromatographic analysis proved to be effective in identifying and characterizing these constituents. The presence of quercetin, along with other phytochemicals, supports the potential use of *Citrus medica* in pharmaceutical and nutraceutical applications, especially in formulations targeting antioxidant and therapeutic benefits.

Table 2: Physical characteristics of extract

Extract	Consistency	Weight of extract	Colour	% Yield
Hydroalcoholic	Solid	5.14	Black	10.28

Table 3: Phytochemical tests of extract of *Citrus medica*

S. No.	Bioactive constituents	Hydroalcoholic extract
1.	**Alkaloids** Wagner's Test	 -ve
2.	**Carbohydrates** Fehling's Test	 +ve
3.	**Glycosides** Legal's Test	 +ve
4.	**Saponins** Froth Test	 +ve
5.	**Phenols** Ferric Chloride Test	 +ve
6.	**Flavonoids** Alkaline Reagent Test Lead acetate Test	 -ve +ve
7.	**Proteins** Xanthoproteic Test	 +ve
8.	**Diterpenes** Copper acetate Test	 -ve

+ ve – Present, - ve – Absent

Table 4: Identification of phytoconstituents by TLC of *Citrus medica*

Hydroalcoholic extract of *Citrus medica*		
S. No.	**Mobile phase** Toluene: Ethyl acetate: Formic acid (5:4:1)	***Rf* value**
1.	**(Quercetin)** Dis. travel by mobile phase= 6cm No. of spot at long UV= 1 No. of spot at short UV = 1 No. of spot at normal light= 1	 Long- 0.52 Short- 0.52 Normal- 0.52
2.	**(Hydroalcoholic extract)** Dis. travel by mobile phase= 6cm No. of spot at long UV = 5 No. of spot at short UV = 1 No. of spot at normal light= 1	 Long- 0.48, 0.50, 0.62, 0.72, 0.78 Short- 0.50 Normal- 0.50, 0.54, 0.82
	Spot Sequence	
	Quercetin	1st
	Hydroalcoholic extract	2nd

Table 5: Estimation of total phenol and flavonoids content

S. No.	Total phenol content	Total flavonoids content
1.	0.52 mg/100mg	0.89 mg/100mg

Table 6: Quantitative estimation of Quercetin in extract

S. No.	Standard/Extract	RT	% Assay
1.	Quercetin	2.545	-
2.	Hydroalcoholic extract	2.512	0.12%

CONCLUSION

The present study on the hydroalcoholic extract of *Citrus medica* demonstrated the presence of a wide range of bioactive secondary metabolites, including phenols, flavonoids, glycosides, saponins, carbohydrates, and proteins. The preliminary phytochemical screening confirmed that flavonoids and phenolic compounds are the major constituents contributing to the phytochemical profile of the plant extract. Thin Layer Chromatography analysis revealed multiple phytoconstituents with distinct Rf values, and the presence of a spot corresponding to quercetin suggested the existence of flavonoid derivatives in the extract. Quantitative estimation further confirmed significant levels of total phenolic and flavonoid content, indicating strong antioxidant potential. Chromatographic analysis also confirmed the presence of quercetin with comparable retention time to the standard, validating its occurrence in the extract. The study concludes that *Citrus medica* is a rich source of biologically active compounds, particularly flavonoids and phenolics, which may be responsible for its therapeutic potential. The combined use of UV spectroscopy, TLC, and HPLC proved to be effective for the identification, separation, and quantification of secondary metabolites, supporting the standardization and future pharmacological exploration of this plant.

DECLARATION OF INTEREST

The authors declare no conflicts of interests. The authors alone are responsible for the content and writing of this article.

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