

Phytochemical Analysis and Effect of Various Parameters on the Extraction Yield of Flavonoids from the *Citrus medica L* Peel Extract

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Abstract: *Citrus medica L*, widely known as Citron belongs to family Rutaceae. Its fruit peel is considered as an important source of dietary phenolics and flavonoids. The objective of this work is to perform the Qualitative phytochemical analysis for identifying the various compounds present in the peel extract and to study the effect of various parameters on the extraction yield of flavonoids (quercetin, rutin, and kaempferol). For the extraction of flavonoids, the optimum results were observed for the effect of different organic solvents such as: methanol, ethanol, ethylacetate and water, with various concentrations (20% - 100%, v/v), temperatures (40°C-60°C), extraction times (1-5 hrs), extraction pH (2-6) and material ratio (1:10-1:50). Results showed that maximum amount of quercetin, rutin and kaempferol of concentrations 21.4µg/ml, 46.8µg/ml and 12.3µg/ml were obtained at optimum conditions of 80% methanol at pH-5 of 50°C temperature and 1:30 % of material ratio for 4hrs extraction time.

Keywords: Flavonoids (Quercetin, Rutin, and Kaempferol), *Citrus medica L* (Citron fruit), Methanol.

I. INTRODUCTION

Polyphenols constitute one of the most common and widespread groups of substances in flowering plants, occurring in all vegetative organs, as well as in flowers and fruits. These molecules are involved in many physiological processes such as cell growth, root formation, seed germination and fruit ripening. Moreover, these compounds are considered as secondary metabolites involved in the chemical defense of plants against predators, pathogens, environmental stresses and in plant-plant interferences. Phenolic compounds represent a unique and a functional place, composed of bioactive products, present in plant-derived foods and beverages and included in the formulations of well-marketed cosmetic and pharmaceutical products. Furthermore, polyphenols exhibit various biological activities such as anticancer, antioxidant, antimicrobial and anti-inflammatory activities. Therefore, in recent years, the determination of phenolic compounds concentrations in fruits, vegetables [2] and some aromatic and medicinal plants has been of increasing interest in the scientific community. In order to recover bioactive compounds from plant raw materials, extraction is widely used and it constitutes the first important step [3]. Different solvents and techniques are used for the extraction of polyphenols from there are several experimental factors that can influence the rate of extraction and the quality of extracted bioactive phenolic compounds. These factors include extraction method, Solvent type used and solvent concentration [4], temperature, pH of extraction [5], extraction time [6], and solvent-to-sample ratio [7]. Different plants may require different extraction conditions to achieve the optimum recovery of phenolic compounds [8], [9]. The family Rutaceae includes aromatic and medicinal plants, which are used in traditional medicine. *Citrus medica L* is commonly known as citron, grown in Assam, central India

and Western Ghats of India [10]. It is more commonly present in the Mediterranean region [11], central and southern parts of America. In ancient times, the citron was used mainly for medical purposes: to combat sea sickness, pulmonary troubles, intestinal ailments, and other disorders. The peel of *Citrus* fruits is a rich source of flavanones and many polymethoxylated flavones which are very rare in other plants. Quercetin and hesperidin have many biological activities such as antioxidant, antimutagenic effect, analgesic, anti-inflammatory etc. [12].

However, to the best of our knowledge, optimizing the extraction of phenolic compounds from *Citrus medica L* peel extract using different extraction conditions has not been reported yet. Hence, the purpose of the current study was to investigate the effects of different extracting conditions (organic solvent type, concentration of organic solvent, temperature, pH, material ratio and time) on the extraction of phenolic compounds; flavonoids (quercetin, rutin, and kaempferol) from *Citrus medica L* peel extract.

II. MATERIALS AND METHODS

2.1 Collection and processing of plant material:

The fresh fruits of *Citrus medica L* were collected in the month of October 2010 from its natural habitat at Visakhapatnam region, Andhra Pradesh, India. Fresh plant material was washed under running tapwater and cleaned with a dry cloth, Peeled off the skin carefully with the help of knife. The peels were divided into 2 parts, i.e. albedo (white colour) and flavedo (green colour). Separated peel was air dried under the shade up to 48 hours. By using the kitchen blender the plant material was homogenized to a fine powder and stored in airtight bottles.

2.2 Qualitative analysis

Preliminary phytochemical Analysis of the crude peel powder of the fruit collected was determined as follows:

Test for reducing sugars : (200 mg plant material in 10 ml distilled water, filtered); a 2ml of filtrate + 2ml of Fehling's solution and boiled for 10min, thick brick red precipitate indicated the presence of reducing sugars.

Test for aminoacids: (200 mg plant material in 10 ml distilled water, filtered); a 2ml of filtrate + 3 drops of 5% v/v of Ninhydrin reagent and boiled, blue coloration indicate the presences of amino acids.

Test for proteins: A 2ml of filtrate + 1ml of 4% w/v NaOH and few drops of 1% w/v copper sulphate solution were added and a blue color was observed indicate the presence of proteins.

Test for Steroids: A 2ml filtrate + 1ml of chloroform. Then 3-4 drops of acetic anhydride and 3 drops of concentrated sulphuric acid added by sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence indicate the presence of steroids.

Test for Flavonoids: (200 mg plant material in 10 ml ethanol, filtered); A 2 ml filtrate + conc. HCl + magnesium ribbon pink tomato red color indicated the presence of flavonoid.

Test for terpenoids (Liebermann- Burchard reaction): (200 mg plant material in 10 ml chloroform, filtered); A 2 ml filtrate + 2 ml acetic anhydride + conc. H₂SO₄. Blue-green ring indicated the presence of terpenoids.

Test for Cardiac glycosides (Keller-Kiliani test): A 2 ml filtrate + 1 ml glacial acetic acid + FeCl₃ + conc. H₂SO₄; green blue color indicated the presence of cardiac glycosides.

Test for Fattyacids: A 0.5ml of filtrate + 5 ml of ether, allow it for evaporation on filter paper and dried the filter paper, appearance of transparence on filter paper indicated the presence of fatty acids.

Test for Alkaloids: (200 mg plant material in 10 ml methanol, filtered); A 2ml filtrate + 1% HCl + steam, 1 ml filtrate + 6 drops of Dragendroff reagent, orange precipitate indicated the presence of respective alkaloids.

Test for Tannins: (200 mg plant material in 10 ml distilled water, filtered); A 2ml filtrate + 2ml FeCl₃, blueblack precipitate indicated the presence of Tannins.

Test for Saponins (frothing test): A 0.5ml filtrate + 5 ml distilled water); frothing persistence indicated presence of saponins.

Test for Phenols: A 2ml filtrate + 1ml of alcohol and then few drops of neutral ferric chloride solution was added, blue colour formation indicated the presence of phenols.

2.3 Extract preparation:

Two grams of peel powder *Citrus medica peel* were extracted using 50 ml of extraction solvent with different concentrations, introduced in conical flask of capacity 100 ml sealed with parafilm and wrapped with aluminum foil to prevent solvent loss and exposure to light and the mixture was stirred at 150 rpm for a particular duration. After completing the extraction process, the extract was filtered using Whatman No. 1 filter paper in

order to obtain a clear crude extract solution. To determine the optimal conditions for phenolic and flavonoids extraction from *Citrus medica L* peel, the extraction conditions were set according to the experimental design described below:

- **Organic solvents type:** Three kinds of organic solvents (methanol, ethanol and acetone) were selected. Distilled water was tested as control.
- **Solvent concentrations:** Five different concentrations (20%, 40%, 60%, 80% and 100% v/v) of solvent were prepared in distilled water.
- **Extraction time:** Five different time intervals (1, 2, 3, 4 and 5 hours) for the extraction were chosen.
- **Extraction temperatures:** Four different temperatures (30°C, 40°C, 50°C and 60°C) were used to find the optimum temperature for the extraction of flavonoids and phenolics
- **Extraction pH:** Five different pH (2, 3, 4, 5 and 6) were selected to find the optimum pH for extraction.
- **Material ratio (w/v):** Different amounts of sample (Peel powder of *Citrusmedica L* to solvent ratio) viz. 1:10, 1:20, 1:30, 1:40 and 1:50 were used to find out the maximum concentrations of quercetin, rutin and kaempferol.

2.4 Estimation of Quercetin:

Aluminum chloride method was used for quercetin determination[14]. In this method Quercetin was determined by using a standard graph For this purpose, the calibration curve of quercetin was prepared by taking 1ml of standard or extract solution of different concentrations (2, 4, 6, 8,10 µg/ml) into 10ml test tubes, containing 4ml of distilled water. 0.3ml of 5%NaNO₂ was added to the test tube. After 5min, 0.3ml 10%AlCl₃ was added to the mixture. At the 6th min add 2ml of 1M NaOH was added and volume made up to 10ml with distilled water. The absorbance was noted at 510nm using UV-Visible spectrophotometer.

2.5 Estimation of rutin:

Rutin concentration was determined by preparing a standard graph from working stock solution of rutin which is prepared by dissolving 50mg of rutin in 50ml of methanol (1000 µg/ml) [15]. A quantity of *Citrus medica* powder 50mg was transferred to 50 mL volumetric flask and dissolved in methanol and final volume was made up with methanol.

The sample solution was then filtered through Whatman filter paper No.1. From the above solution 0.373ml of solution was taken and diluted to 10 mL with methanol to get final concentration. The concentration of rutin was determined by taking absorbance value at 359nm using UV-Visible spectrophotometer.

2.6 Estimation of kaempferol:

Kaempferol concentration was determined by preparing a standard graph from working stock solution of kaempferol which is prepared by dissolving 5mg of kaempferol in 5ml of methanol (1000 µg/ml) [16]. The working stock solution of kaempferol (10 µg/ml) was prepared by diluting the stock solution.

A quantity of *Citrus medica* extract 50mg was transferred to 50 mL volumetric flask and dissolved in methanol and final volume was made up with methanol. The sample solution was then filtered through Whatman filter paper No.1. From the above solution 0.1ml of solution was taken and diluted to 10 mL with methanol to get final concentration. The concentration of rutin was determined by taking absorbance value at 265nm using UV-Visible spectrophotometer.

III. RESULTS AND DISCUSSION

3.1 Qualitative analysis

Preliminary phytochemical analysis revealed the presence of primary metabolites like carbohydrates, proteins and fattyacids, and secondary metabolites like steroids, alkaloids,phenols, tannins and flavonoids etc. were present in trace amounts in the peels. At the same time, the phytochemical constituents like Cardiac glycosides, saponins and terpenoids are absent, and the results were shown in the table 1:

Phytochemical tests	Peel extract	Inference
Fehling's test	+	Carbohydrates present
Ninhydrin test	+	Aminoacids present
Biuret test	+	Proteins present
Test for steroids	+	Steroids present
Keller-Kiliani test	-	Cardiac glycosides absent
Test for alkaloids	+	Alkaloids present
Test for saponins	-	Saponins absent
Test for phenols	+	Phenols present
Test for fatty acids	+	Fatty acids present
Test for flavonoids	+	Flavonoids present
Test for terpenoids	-	Terpenoids absent
Test for tannins	+	Tannins present

Constituents present = +, Constituents absent = -
Table 1: Preliminary phytochemical screening of *Citrus medica* peel extract

3.2 Optimization results for the Extraction of Flavonoids: Effect of Different Solvents on Extraction of Quercetin, Rutin and Kaempferol:

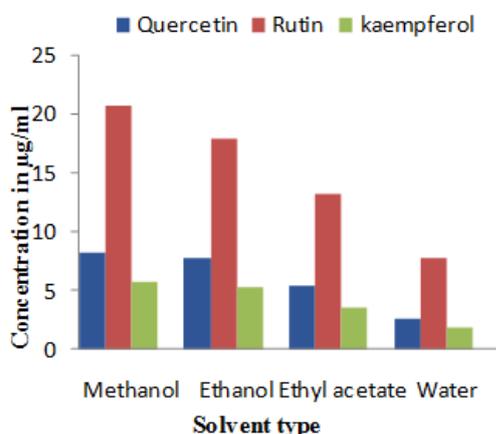


Fig 1: Effect of Different Solvents on Extraction of Quercetin, Rutin and kaempferol

The choice of extraction solvents is important for maximum extraction of phytoconstituents. Different organic solvents such as methanol, ethanol, ethylacetate and water were used to extract the optimum yield. Methanol gave maximum yield of Quercetin, Rutin, and Kaempferol of concentrations 8.3µg/ml, 20.61µg/ml, and 5.7µg/ml from *Citrus medica* peel powder. The results were shown in the Figure 1.

Effect of Different Solvent percentages on Extraction of Quercetin, Rutin and Kaempferol:

Mixtures of methanol with different proportions of water have shown to be more effective in extracting flavonoids. The dried peel powder of *Citrus medica* was extracted with various percentages of the methanol (20%, 40%, 60%, 80%, and 100%) were checked for the optimum extraction yield of flavonoids, and the results showed that the maximum rate of Quercetin, Rutin and Kaempferol concentrations of 11.9 µg/ml, 29.92 µg/ml and 8.24 µg/ml were observed at 80% of methanol respectively, and the results were shown in Figure 2.

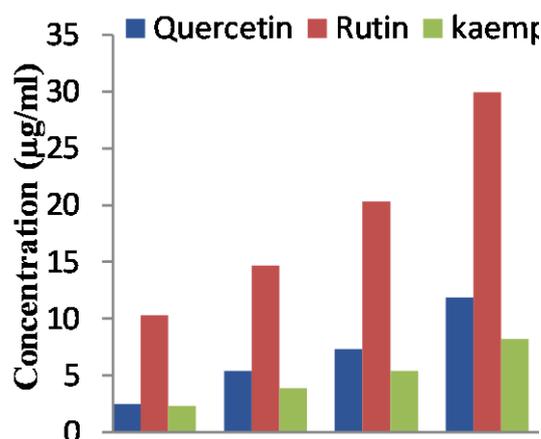


Fig 2: Effect of Solvents percentages on Extraction of Quercetin, Rutin and kaempferol

Effect of Soaking time on extraction of Quercetin, Rutin and Kaempferol:

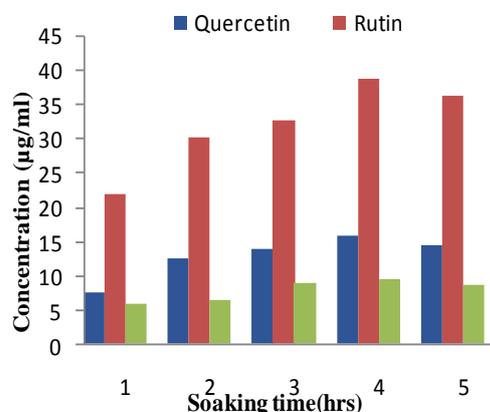


Fig 3: Effect of Soaking time on Extraction of Flavonoids

Soaking time represents another key parameter in optimizing the phenolic compounds extraction. The powder was soaked in

under proper conditions at different time intervals viz., 1, 2, 3, 4 and 5hrs to investigate the influence on extraction of flavonoids. It was observed that, extraction time of 4hrs was the optimum condition to get maximum concentration of Quercetin, Rutin and Kaempferol of concentration 15.82 $\mu\text{g/ml}$, 38.7 $\mu\text{g/ml}$ and 9.43 $\mu\text{g/ml}$. The results were shown in figure 3.

Effect of Extraction temperature on extraction of Quercetin, Rutin and Kaempferol:

The effectiveness of extraction process of flavonoids is largely regulated by different experimental parameters particularly by the extraction temperature. An increase of temperature is mainly due to an increase of the diffusion rate and solubility of the extracted substances. Extraction at different temperatures such as 30 $^{\circ}\text{C}$, 40 $^{\circ}\text{C}$, 50 $^{\circ}\text{C}$ and 60 $^{\circ}\text{C}$ were used, and the results showed that maximum concentration of Quercetin, Rutin and Kaempferol of 20.1 $\mu\text{g/ml}$, 44.2 $\mu\text{g/ml}$ and 11.7 $\mu\text{g/ml}$ observed at 50 $^{\circ}\text{C}$ incubation temperature. The results were shown in figure 4.

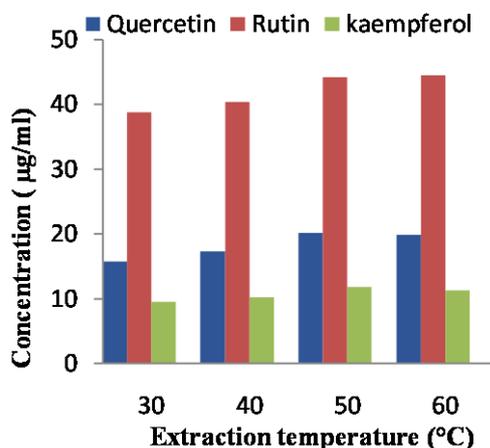


Fig 4: Effect of Extraction temperature on Extraction of Flavonoids

Effect of pH on Extraction of Quercetin, Rutin and Kaempferol:

To determine the effects of various pH values on extraction yield of flavonoids from the peel of *Citrus medica* fruit. The extraction yield of components was observed at different pH values namely 3, 4, 5, 6, 7, and 8.

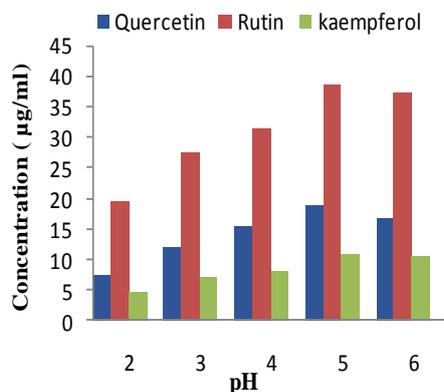


Fig 5: Effect of pH on Extraction of Flavonoids

The results showed the maximum concentrations of Quercetin, Rutin, and Kaempferol of 18.72 $\mu\text{g/ml}$, 38.5 $\mu\text{g/ml}$ and 10.8 $\mu\text{g/ml}$ was obtained at pH 5 using methanol as solvent. The results of maximum rate of extraction of phytoconstituents were shown in the figure.5

Effect of Material ratio on extraction of Quercetin, Rutin and Kaempferol:

Different amounts of sample powder to solvent ratio of 1:10, 1:20, 1:30, 1:40, and 1:50 were used to find out the maximum concentrations of quercetin, rutin and kaempferol and the results were shown in the figure 6. The results showed that the higher concentrations of Quercetin, Rutin, and Kaempferol of 21.4 $\mu\text{g/ml}$, 46.8 $\mu\text{g/ml}$ and 12.3 $\mu\text{g/ml}$ was obtained at 1:30 materials ratio. Further increase in the material ratio leads to a gradual decrease in the flavonoids content revealing a saturated condition.

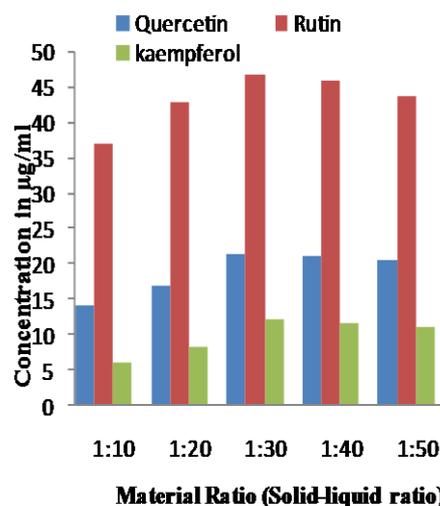


Fig 6: Effect of Material ratio on Extraction of Flavonoids

IV. CONCLUSION

Experiments were performed for the Qualitative phytochemical analysis and optimization of physico-chemical parameters for the extraction of Quercetin, Rutin and Kaempferol from *Citrus medica* peel powder. The parameters like effects of different solvents, solvent percentages, extraction time, extraction temperature, extraction pH and material ratio for the extraction of Quercetin, Rutin and Kaempferol and their concentrations were observed from the experimental work. The highest concentration of Quercetin, Rutin and Kaempferol for optimized conditions were 21.4 $\mu\text{g/ml}$, 46.8 $\mu\text{g/ml}$ and 12.3 $\mu\text{g/ml}$.

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