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EXTRACTION, MODELLING AND PURIFICATION OF FLAVONOIDS FROM *CITRUS MEDICA* PEEL

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Abstract

Soxhlet extraction technique is widely employed for the extraction and separation of chemical constituents in the medicinal plants. *Citrus medica* L commonly called as Citron belongs to family Rutaceae, is a slow-growing shrub. It is mainly cultivated for the production of edible fruits which are sour in taste like lime and lemon and the main content of a citron fruit is the thick rind, which is very adherent to the segments. From the phytochemical analysis the peel extract is rich source of phenols, flavonoids and alkaloids. The objective of present work is to develop a modelling equation for quercetin, rutin and kaempferol and the crude extract obtained by soxhlet extraction was further purified by solvent-solvent extraction and Column chromatography. Extraction was carried out by 80% methanol as a solvent Soxhlet extractor. Soxhlet extraction with methanol was carried out with varying time intervals, to evaluate modelling equation. The proposed modelling equation was $E_s = 0.0849(t) + 7.0286$ for Quercetin and $E_s = 0.0912(t) + 25.971$ for rutin, and $E_s = 0.0267(t) + 7.3714$ for Kaempferol. High yield was obtained for 180min of Soxhlet extraction with 80% methanol. Yield of quercetin, rutin and kaempferol after solvent-solvent extraction and column chromatography was 22.6µg/ml, 43.7µg/ml and 10.8µg/ml respectively. The proposed model showed good agreement with the experimental data.

Keywords: *Citrus medica* L; Quercetin; Rutin; Kaempferol; Soxhlet Extraction; Solvent-Solvent Extraction; Column Chromatography.

Introduction

Citrus medica Linn., commonly known as a Citron in English and *bijapura* in Ayurvedic literature is a shrub or small tree. This plant is found apparently wild in Kumaon, Pachamarhi, Sikkim, Khasia Hills, Garo hills, Chittagong, Upper Yunzalin valley, the Western Ghats and Satpura range in Central India. Various parts of citron are widely used as medicine. In ancient times, the citron was used mainly 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.. These compounds, not only play an important physiological and ecological role, but are also of commercial interest because of their multitude of applications in the food and pharmaceutical industries. Quercetin, Naringin and hesperidin have many biological activities such as antioxidant, antimutagenic effect, analgesic, anti-inflammatory etc. Citrus cultivation is probably one of the most important commercial and industrial agricultural activities of the world. Soxhlet extractor (Ahmad, 2009) is a liquid- solvent extraction and it is the most common method for separating bioactive components from their natural resources. It is employed for the extraction of compounds with limited solubility in a solvent, and the impurity is insoluble in that solvent The

advantages of this method over other extraction methods are as follows: (i) the sample is repeatedly brought into contact with the fresh portions of the solvent, thereby helping to displace the transfer equilibrium, (ii) the temperature of the system remains relatively high due to the heat applied to the distillation flask, (iii) sample throughput can be increased by simultaneous extraction in parallel, (iv) it has the ability to extract more sample mass and it is non-matrix dependent. However, for toxicological reason, drug and medicine producers are required to minimize the number and amount of solvents employed in pharmaceutical processes (Beatriz and Luis, 2005). The presence of a solvent in the extract may also affect the kinetics of crystallisation and the crystal morphology of the product. In order to optimize the utilisation of solvent in the extraction of bioactive components (Bhuiyan and Begum, 2009) from natural resources, an estimation of the extract yield obtained is necessary. The objectives of this work were to develop a mathematical model to quantitatively describe the extraction phenomena of Quercetin, Rutin and Kaempferol from *Citrus medica* peel and purification of extract obtained after soxhlet extraction by using liquid-liquid extraction and column chromatography to get maximum yield of flavonoids.

Materials and Methods

Collection and Processing of Plant Material

The fresh unripen fruits of citron were collected from a local market, near Visakhapatnam, Andhra Pradesh, India. 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 form. The powder was placed in small plastic bags and stored at 4°C until further use.

Soxhlet extraction

Prior to the solvent extraction study, 5 grams of dried powder of *Citrus medica* peel was placed in a cellulose thimble. An amount 250 ml of solvent was used for the extraction using a standard soxhlet method for 10,800 seconds in a soxhlet extraction system. The standard soxhlet extraction method (Darshan *et al.*, 2014) was conducted using methanol at different extraction times to verify the mathematical model proposed in this work. The extracts were then filtered and methanol was evaporated on vacuum rotary evaporator. The crude extracts were then analysed for their Quercetin, Rutin and Kaempferol content using UV-visible spectrophotometer (Kalpesh Panara *et al.*, 2012).

Modelling of Extraction Using Soxhlet Extractor

In order to describe the Quercetin, Rutin and Kaempferol transfer from the peel powder to the bulk of the solvent, the following hypotheses were used: (i) every powder particle is symmetrical, (ii) the mass transfer coefficient is constant, (iii) the solvent in the extractor is perfectly mixed, while the transfer resistance in the liquid phase is negligible and the flavonoids (Khoddami *et al.*, 2013) concentration in the solvent depends only on time, (iv) the transfer of the flavonoids is a diffusion phenomenon and independent of time, (5) at the interface, the concentration of flavonoids in the solution between the internal liquid (in pores) and external to particles are equal. The final form of the equation obtained from this modelling is:

$$E_s = A(t) + B$$

(Where A & B are equation constants, E_s = yield extract ($\mu\text{g/ml}$ of flavonoids) and t = extraction time (min)).

Purification of Flavonoids

Crude extract obtained after the Soxhlet extraction, was purified by two different methods: solvent-solvent extraction and column chromatography.

1) Solvent – Solvent Extraction:

Solvent – Solvent extraction was done with extract (i.e., obtained from Soxhlet extractor) and hexane of different

proportions by varying extract: hexane proportion in the ratio of 1:0.5 to 1:2.0 used as solvent. Extraction was done in separating funnel for 1hr to 2hrs and then the two phases: raffinate and extract phases were separated. The sample is collected from both the phases for the estimation of flavonoids: quercetin, rutin and Kaempferol concentrations and also the partition coefficient of quercetin was estimated.

Partition coefficient

$$= \frac{\text{Amount of component present in extract}}{\text{Amount of component present in raffinate}}$$

2) Column Chromatography

Extract obtained from the soxhlet extractor contain many other components along with flavonoids and phenols. So the impurities are to be removed to get purified flavonoids by using column chromatography technique. In this method, 200 micron particle size silica gel was used as stationary phase. Before starting the experiment first insert a piece of cotton into the column towards outlet. Fix the column to the clamp tightly. Pour the sea sand of 1cm bed in the column. Add silica gel powder in the column up to 10cm length from the neck of the column. Run the solvent methanol in the column up to the bed was entirely wet. Add excess solvent on the top of the silica gel bed. Gently tap the column with hand or soft materials. After tapping gentle pressure can be applied. Before loading the sample in the column, little silica gel was added to the sample. Pour the 20ml of sample along the side walls of the column. Add sand on the top of the sample. After collecting the samples for every 5 minutes from the column, take 1ml of sample from each test tube and quantitatively determine the flavonoids content by using spectrophotometer (Pawar N.P and Salunkhe V. R 2013).

Results and Discussion

Soxhlet Extraction

Flavonoids were extracted from the *Citrus medica* peel with 80% methanol in the Soxhlet extractor for 210min. Non-polar solvents shows high yield of extraction when compared to polar solvents. Non- polar solvents were almost used for the extraction of flavonoids. Methanol was found to be the best solvent for the extraction (Santana *et al.*, 2009) of flavonoids. The highest amounts of flavonoids were extracted from 80% of methanol. The final form of proposed model equation for Quercetin was $E_s = 0.0849(t) + 7.0286$, and for rutin was $E_s = 0.0912(t) + 25.971$, and for Kaempferol was $E_s = 0.0267(t) + 7.3714$. Results for the extraction of flavonoids with 80% methanol were tabulated in Table 1 and Fig. 1, 2 and 3. From the obtained data it was observed that maximum extraction of flavonoids: Quercetin, Rutin and Kaempferol has occurred for 180min, further continuing the extraction for 210 min and then it shows slower depletion in flavonoids concentration.

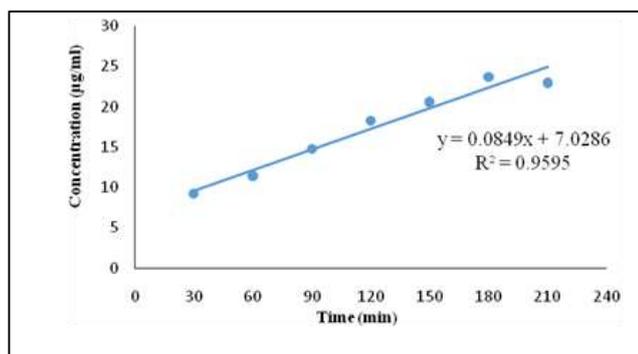


Fig 1: Effect of Extraction yield with Extraction time of quercetin.

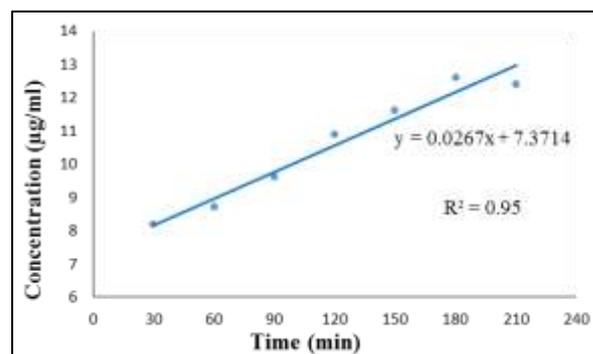


Fig 3: Effect of Extraction yield with Extraction time of Kaempferol

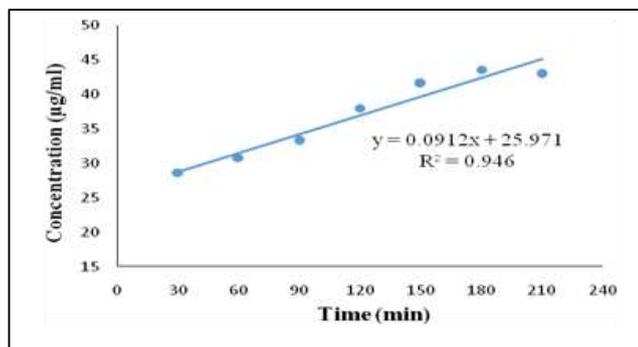


Fig 2: Effect of Extraction yield with Extraction time of Rutin.

Solvent-Solvent Extraction

Methanolic peel extract of *Citrus medica* obtained from Soxhlet extractor was purified by solvent – solvent extraction method, using hexane as a solvent. Purification was done with different hexane proportion ranging from 0.5 to 2 times of extract. 1: 0.5, 1:1, 1: 1.5, and 1:2 of extract: hexane mixture was taken in four different conical flasks and allowed to shake continuously in rotary shaker for 2hrs and then separated by using separating funnel. After 1hr, among the different ratios of extract and hexane, 1:0.5 has showed optimum purification Tab 2 and the partition coefficient was found to be 4.07.

Table 1: Extraction of flavonoids with respective to time in soxhlet extractor

Time(min)	Concentration of Quercetin (µg/ml)	Concentration of Rutin (µg/ml)	Concentration of kaempferol (µg/ml)
30	9.2	28.6	8.2
60	11.4	30.8	8.7
90	14.7	33.2	9.6
120	18.2	37.9	10.9
150	20.5	41.5	11.6
180	23.6	43.7	12.7
210	22.9	42.9	12.6

Table 2: Purification of flavonoids with different ratios of n-hexane

Extract : hexane	Extraction time: 1 hr			Extraction time: 2 hrs		
	Quercetin (µg/ml)	Rutin (µg/ml)	Kaempferol (µg/ml)	Quercetin (µg/ml)	Rutin (µg/ml)	Kaempferol (µg/ml)
1 : 0.5	25.7	48.2	12.9	22.5	46.4	11.7
1 : 1	23.2	45.7	11.4	20.4	43.9	10.7
1 : 1.5	20.8	41.2	10.7	18.2	38.2	9.4
1 : 2.0	19.7	39.5	10.2	17.6	36.5	8.9

Column Chromatography

Extract obtained after soxhlet extraction was subjected to column chromatography. 50ml of the extract was taken in to the column for purification and finally 38 ml of purified extract was collected. The flavonoids concentration was enhanced after the column chromatography when compared to that of the before. The values of Quercetin, Rutin, Kaempferol before the purification were 20.3µg/ml, 38.4µg/ml and 9.6µg/ml, after column chromatography their concentrations have been increased to 22.6µg/ml, 43.7µg/ml and 10.8µg/ml respectively. Mainly rutin is purified more during chromatography because of presence of larger amounts in the extract.

Conclusion

Methanol was the best solvent for extraction of flavonoids from the peel extract of *Citrus medica*. 80% methanol shows highest yield of flavonoids. The final proposed model equation was $E_s = 0.0849(t) + 7.0286$ for Quercetin and $E_s = 0.0912(t) + 25.971$ for rutin, and $E_s = 0.0267(t) + 7.3714$ for Kaempferol. Among the two purification methods, solvent-solvent extraction was concluded as the best purification process, since it shows the highest yield of flavonoids compared to column chromatography. From the experimental observation the proposed model shows the best match.

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