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# Purification of Eriocitrin (Eriodictyol 7-O Rutinoside) from Orange (Citrus Sinuses) Peel by Using Column Chromatography and Its Cytotoxic Activity against Breast Cancer Cell Line

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**ABSTRACT:** A huge effort has been made to create a safe and cost-effective breast cancer treatment. An objective of the present study is to purify Eriocitrin a flavanone from Orange peel by using Column Chromatography Technique and to check its Cytotoxic activity against Breast Cancer cells. The MTT system is a means of measuring the activity of living cells via mitochondrial dehydrogenases. The MTT method yields reproducible results. The key component is (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) or MTT. Orange peel was shadow dried for 7-10 days and defatted using nHexane, then the residue was extracted using Ethyl Acetate at RT overnight. The extract was air dried and suspended in 5ml of ethyl acetate. A silica gel column of size 300mm X10mm was packed and the 5ml of ethyl acetate extract was loaded onto the column and eluted by using 30 ml ethyl acetate, each 5ml fractions. All these fractions were collected and checked separately for Eriocitrin content by Reverse Phase HPLC analysis. Results showed that concentration of Eriocitrin in orange peel powder was found to be 37.68 µg/ml and purity was found 90 %. Eriocitrin shown strong cytotoxic effect proven by IC50 value of 16.7 µg/ml with 80.2% inhibition

**KEYWORDS:** Eriocitrin, Column Chromatography, RP-HPLC Analysis, UV absorption at 280nms, MCF-7 Cells, MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) from Sigma, 96 well plate, 570nms.

### I. INTRODUCTION

Orange tree is mostly cultivated and rarely found in the forests. Oranges form a rich source of vitamin C, flavonoids, phenolic compounds and pectin's. Flavonoids are water soluble polyphenolic molecules containing 15 carbon atoms [3,4]. The main flavonoids found in citrus species are hesperidin, narirutin, naringin and eriocitrin. Just one orange provides 116% of the daily requirement for vitamin C. Vitamin C is the primary water- soluble antioxidant, which prevents free radical generation in the body and damage to the tissues in the aqueous environment both inside and outside the cells [5,6]. These characteristics place them among the most attractive natural substances available to enrich the current therapy options because they also help support detoxification of potentially tissue-damaging molecules. [7]. Flavonoids have been found to scavenge free radicals that can damage macromolecules, including DNA, interfere with biotransformation enzymes and efflux transporters, possibly preventing the activation of pro carcinogenic chemicals and promoting their excretion from the body and regulate proliferation, DNA repair or activation of pathways leading to apoptosis (programmed cell death) in case of irreversible DNA damage and inhibit tumor invasion and angiogenesis. Hence flavonoids have been shown to reveal cytotoxic activity towards various human cancer cells with little or no effect on normal cells and this fact has stimulated large interest in developing of potential flavonoid-based chemotherapeutics for anticancer treatment [8]. Traditionally, the in vitro determinations of toxic effects of unknown compounds have been performed by counting viable cells after staining with a vital dye. The key component is 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide or MTT, is a water soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells.

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Vol. 7, Issue 10, October 2018

This water insoluble formazan can be solubilized using DMSO, acidified isopropanol or other solvents (Pure propanol or ethanol). The resulting purple solution is spectrophotometrically measured. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material [9].

## II. REVIEW OF LITERATURE

Several phytochemicals of medicinal interest have been found throughout the Citrus plant including the leaf, flower, fruit, seeds, and peel. Leaf and flowers contains the essential or volatile oil 0.2% to 0.4%. It also contains monoterpene hydrocarbons, alcohols, and flavonoid-glycosides. The following constituents are also found in the flowers: synephrine, 5,8-epidioxyergosta-6,22-dien-3  $\beta$ -ol, adenosine, asparagine, tyrosine, valine, isoleucine, alanine,  $\beta$ -sitosterol, and  $\beta$ -daucosterol. The fruit contains octopamine, synephrine, and tyramine. Synephrine is the main chemical constituent in the fruit (0.1% to 0.35%) Oranges as excellent source of vitamin C, contain powerful natural antioxidant, folate, dietary fiber and other bioactive components like carotenoids and flavonoids that prevent cancer and degenerative diseases. Consumption of foods rich in vitamin C improves body immunity against infectious agents and scavenging harmful pro-inflammatory free radicals from the blood. Sweet orange contains a variety of phytochemicals like hesperidin and naringenin. Naringenin has a bioactive effect on human health as antioxidant, free radical scavenger, anti-inflammatory, and immune system modulator (1). Cancer is a very complex disease that occurs from accumulation of multiple genetic and epigenetic changes in individuals who carry different genetic backgrounds and have suffered from distinct carcinogen exposures. These changes affect various pathways which are necessary for normal biological activities and gene networks are driving these pathways in disorder in the center. Breast tumors bear all the hallmarks of multiple-gene alterations where the sequence of events is poorly understood. The genome of breast cancer cells is in general unstable and is mostly classified as chromosome instability (CIN). The understanding of CIN in breast tumors is rather poor but can be explained in a minority of tumors, i.e. in carriers of BRCA1 and BRCA2 mutations due to an improper DNA repair mechanism. Alterations at some chromosome regions are frequently detected in tumors with an unstable genome, while alterations at other chromosomes seem to be unrelated to genetic instability (2)

## III. MATERIALS AND METHOD

Citrus sinuses were purchased from super market, Silica gel column (300mm x 10mm), Standards: Eriocitrin Standard (Sigma) stock solution (1mg/mL) was prepared in HPLC grade Methanol. Working standard solution: Test concentrations 25, 50 and 100  $\mu$ g/mL were prepared from stock solution. Samples: Ethyl acetate fraction (10mg/mL) was prepared from stock and Used for HPLC analysis. Instrument used was from Shimadzu LC-Prominence 20AT, Column: C18 column 250 mm x 4.6 mm, 5 $\mu$ M particle size. GC-MS (Thermo scientific GC Trace 1310 Equipped with Thermo Scientific MS TSQ 8000). MTT powder (the solution was prepared and is filtered through a 0.2  $\mu$  m filter and stored at 2–8  $^{\circ}$ C for frequent use or frozen for extended periods), DMSO, CO<sub>2</sub> Incubator, Tecan Plate reader, Centrifuge.

## IV. SAMPLE PREPARATION

The orange peel was shadow dried for 7-10 days. 500g of powdered orange peel was weighed and extracted with 2500ml of nhexane for 4 hours at RT in a magnetic stirrer maintained at 2000rpm. The extract was filtered using 0.4 $\mu$ M filter and the residue was air dried at RT. The residue was extracted by using 2500ml of Ethyl acetate overnight (16 hours) at RT in a magnetic stirrer at 2000 RPM. The extract was evaporated to dryness and reconstituted in 5ml of Ethyl Acetate [10].

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Vol. 7, Issue 10, October 2018

## V. COLUMN PURIFICATION

Five ml of the concentrated Ethyl Acetate extract was loaded onto the silica gel column (300mm x 10mm). The column was washed with 125 ml of hexane and 5ml fractions were collected [11]. Elution was done using 30ml of ethyl acetate and 5ml fractions were collected. Then the 5ml fractions were checked separately for Eriocitrin content by using reverse phase HPLC [12].

## VI. HPLC ANALYSIS

Ethyl acetate fraction (10mg/mL) was prepared from stock and Used for HPLC analysis by using a C18 reverse phase column (RP-18, 250mm× 4.6mm, 5μM) with a linear gradient. The solvent system contained mobile phase A- 0.1% formic acid in acetonitrile (20%) and mobile phase B- 0.1% formic acid in HPLC water (80%). The injection volume was 10 μL and the flow rate was 1mL per min and the absorbance was monitored at 280 nm.

## VII. MTT ASSAY

MCF-7 (NCCS, Pune) cells were collected when they reach about 70-80% confluence. Cells were centrifuged at 1000rpm and supernatant was discarded. To a 96 well plate 50,000 cells per well were seeded and incubated for 24 hours at 37°C, 5 % CO<sub>2</sub> incubator. Next day test items were prepared as 10 mM stocks by adding directly in to phosphate buffered saline, PBS and the working stock of 2X (2000, 600, 200, 60, 20, 6, 2 and 0.6 μg/ml) concentration was prepared in complete DMEM supplemented with 10% FBS. 100μL volume of 2X stock was transferred to respective wells to maintain the final concentration range of 1000, 300, 100, 30 and 10μM and the plate was further incubated for 48 hours in the CO<sub>2</sub> incubator. MTT solution was prepared at 5mg/ml concentration in PBS and after 48 hours incubation 50μl was pipetted into each well to achieve a final concentration of 1mg/mL. The plate was further incubated for 3 hours in incubator and the medium was carefully decanted. The formazan crystals were air dried in dark place for 30min at room temperature and dissolved in 100μL DMSO and the plates were mildly mixed at room temperature for 10 min and the OD was measured using Synergy HT microplate reader at 570nm.

From the optical densities percentage growth was calculated using the following formula:

$$\text{Percentage growth} = 100 \times [(T - T_0) / (C - T_0)]$$

Where,

T is optical density of test,

C is the optical density of untreated control,

T<sub>0</sub> is the optical density at time zero (at the time of  
compound addition).

From the percentage growth, a dose response curve was generated and IC<sub>50</sub> value was interpolated from the growth curve.

## VIII. RESULTS AND DISCUSSION

### Quantification:

The retention time of samples were matched with that of each standard and concentration of respective standard were calculated using the formula.

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Eriocitrin ((Microgram/mL) = Sample Area / Standard Area x Standard Concentration Injected x Dilution Factor.

### HPLC Analysis:

HPLC conditions were carried out for separation of Eriocitrin. HPLC chromatograms of standards and ethylacetate fraction are shown in Figure 2-6. Eriocitrin content in the unknown fractions of orange peel were determined using the regression equation  $Y=5.201X + 12.73$ , derived from standard Eriocitrin response (Table 1), Where Y = Area of peak (mV.S), X= Concentration of Eriocitrin in  $\mu\text{g/mL}$ .

$$X = \frac{12.73 - 208.743}{5.201} = 37.68$$

$$X = 37.68 \mu\text{g/mL.}$$

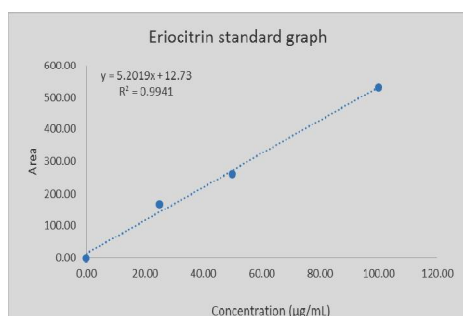
### MTT Assay

The results showed that purified Eriocitrin shown strong cytotoxic effect. 80.2% inhibition was found with IC50 value of 16.7 $\mu\text{g/ml}$  concentration.

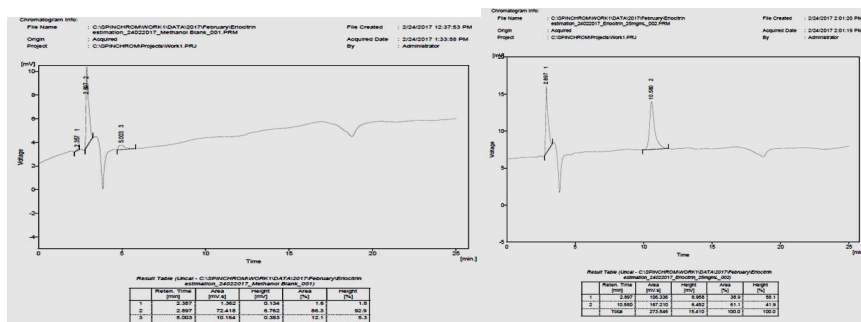
## IX. TABLES AND CHROMATOGRAMS

Sl no	Standard concentration ( $\mu\text{g/mL}$ )	Retention time (min)	Area of Eriocitrin (mV.s)	Chromatogram references
2	Solvent Blank	-	0	Fig.2
3	25	10.580	167.210	Fig.3
4	50	10.590	261.636	Fig.4
5	100	10.650	532.406	Fig.5

Table 1



(1)



(2)

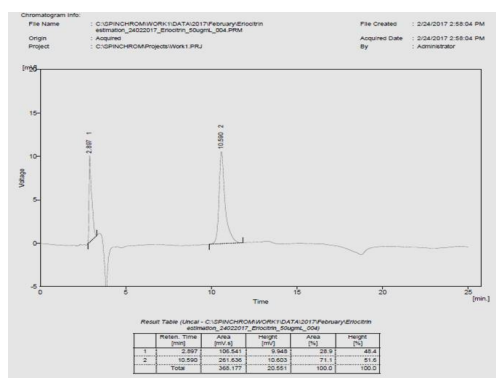
(3)

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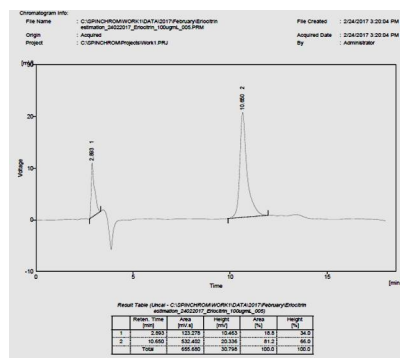
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Vol. 7, Issue 10, October 2018



(4)

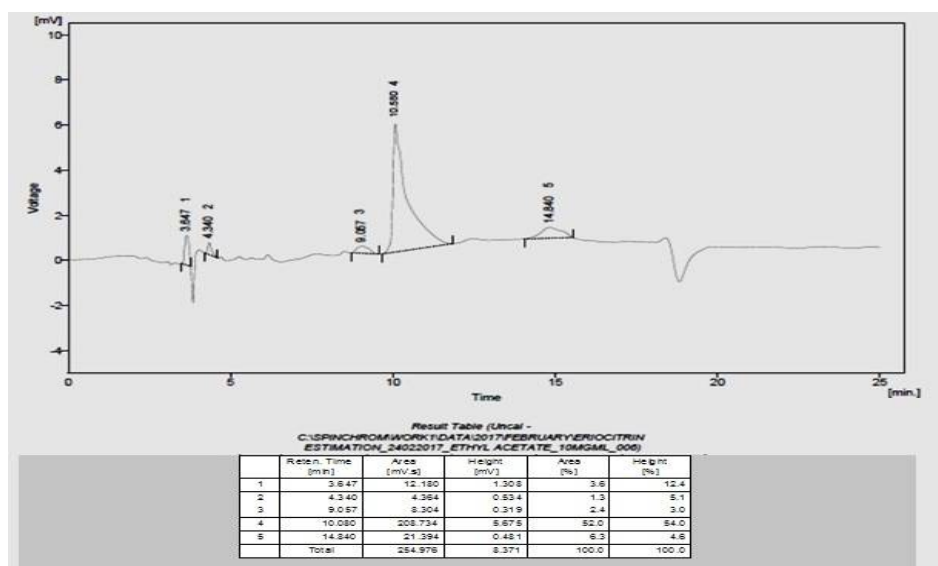


(5)

Eriocitrin content in the unknown fractions of orange peel were determined using the regression equation  $Y = 5.201x + 12.73$ , derived from standard eriocitrin response (Table 1 and Fig 1) and Figures 2 is HPLC chromatogram of blank(Methanol), Figure 3, 4 and 5 are chromatogram of standard with 25, 50 and 100 µg/mL of concentration.

Sl. No.	Sample (concentration 10 mg/mL)	R.T (min)	Area of (mV.s)	concentration (µg/mL)	Chromatogram references
1	Ethyl acetate fractions	10.08	208.734	37.68	Fig.6

Table 2



(6)

Table 2 and Fig 6 shows the Eriocitrin content in the eluted fraction of orange peel powder

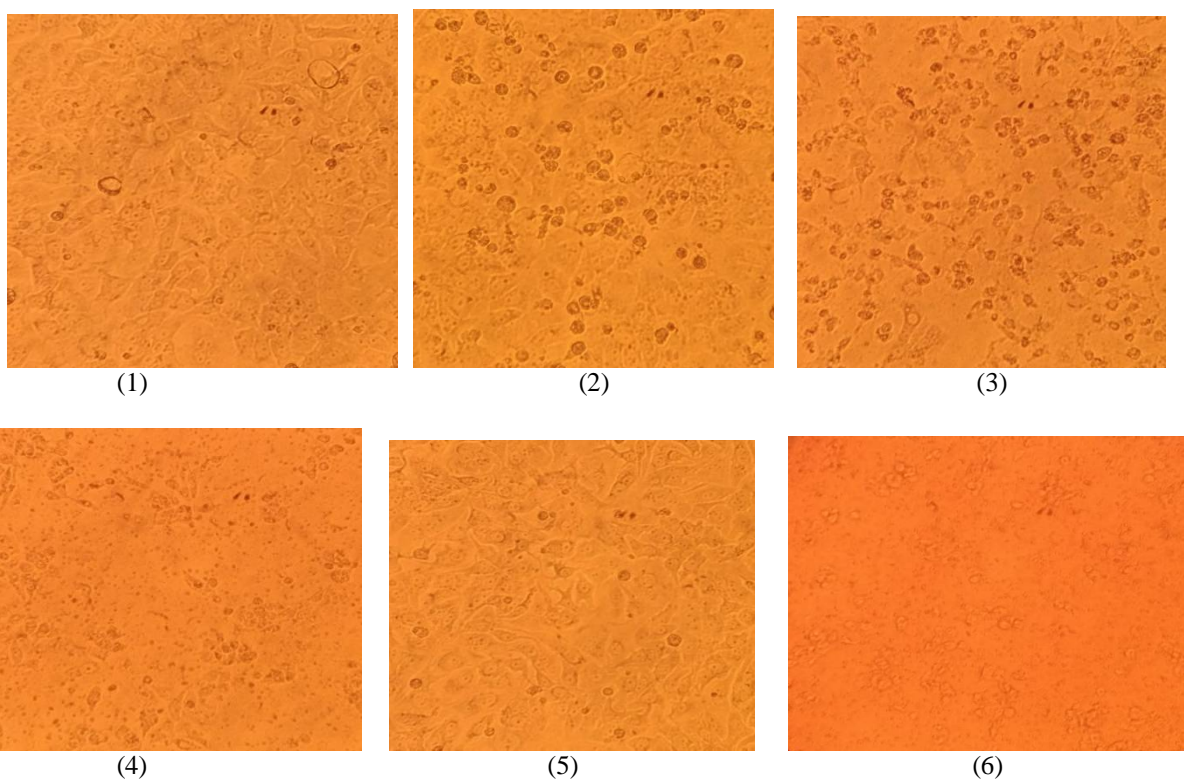
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Vol. 7, Issue 10, October 2018

## X. MTT ASSAY PICTURES



Picture 1 is control and Picture 2 to 6 shows the inhibition effect of the test item concentration ranging from at 10, 30, 100, 300 and 1000 ug/ml respectively. As the concentration is increasing Inhibition of growth in MCF-7 cell line is observed due to cytotoxic effect of Purified Eriocitrin.

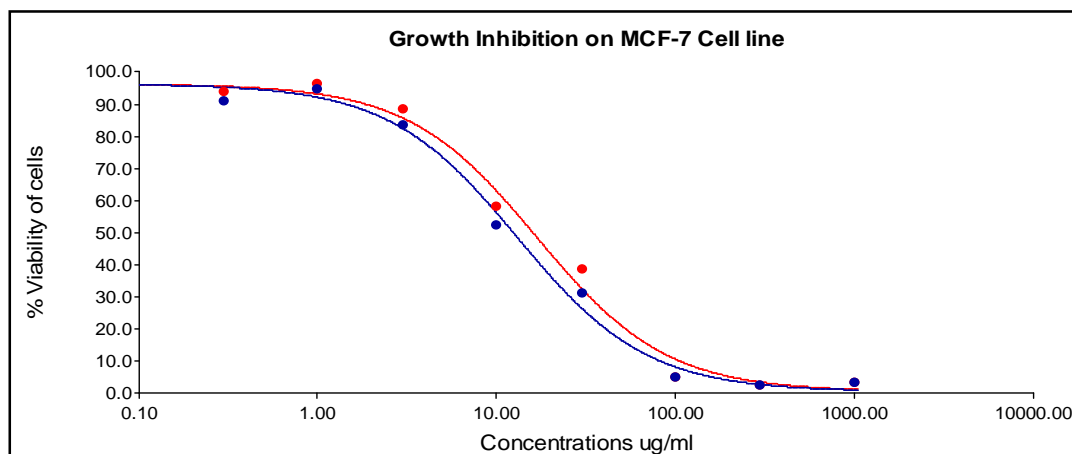


Figure 7 compare the Growth Inhibition effect of standard and Purified Eriocitrin on MCF-7 cells.

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Curve Name	Well ID	Curve Formula	A	B	C (IC50)	D	R2
Test	MCF-7	$Y = \frac{(A-D)}{1+(X/C)^B} + D$	97.2	1.19	16.7	0.0588	0.991
STD	MCF-7	$Y = \frac{(A-D)}{1+(X/C)^B} + D$	95.2	1.22	13.4	1.17	0.993

Table 3 shows IC50 value of Standard and test sample i.e., Eluted Fraction of Purified Eriocitrin

## XI. CONCLUSION

The above results showed that the concentration of Eriocitrin in orange peel powder was found to be 37.68 µg/ml with 90 % purity. Eriocitrin shown strong cytotoxic effect proven by IC50 value of 16.7 µg/ml with 80.2% inhibition. The above process is simple, efficient and economical for the purification of eriocitrin from orange peel. The effective utilization of Eriocitrin in orange peel should be utilized to formulate effective anti-breast cancer drug for the benefit of society.

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