Cytotoxic Effect of flavonoids extracted from *Conocarpus erectus* leaves on SKG cell

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Abstract

The present study was conducted to investigate the cytotoxic activity flavonoids extracted from *Conocarpus erectus* L. leaves using ethyl acetate and chloroform, the results of the extracted flavonoids were detected by HPLC. The evaluation of its cytotoxic effect on cancerous SKG cell line Human Esophageal Cancer and non-cancerous REF (rat embryonic fibroblast) using MTT assay. HPLC analysis of both extracts showed 6 out of 10 flavonoids matched the standard peaks which were (Catechin, Rutin, Myrecetin, Quercetin, Apigenine and Kaempferol). The cytotoxicity of the ethyl acetate extract was greater than that of the chloroform extract against the SKG cell line, and the cytotoxic effect of both extracts increased with increasing concentration. At 200 µl/ml the cytotoxicity of the ethyl acetate and chloroform extracts were 71% and 64.6%, respectively, while the cytotoxicity of the extracts on REF cells was 10.5% and 11.2%. This implies that *C.erectus* extract has little negative effects on normal cells and is thus a safe and promising anticancer drug candidate.

Keywords: Cytotoxicity, *Conocarpus erectus*, Esophageal Cancer, SKG

1. Introduction

Medicinal plant has different concentration of secondary metabolites compounds such as alkaloids, tannins, flavonoids, and phenolic compounds, that affect the physiological actions of the human body which enhance their medicinal value and heal diseases or lower their symptoms, they were globally used as traditional medicine. (Azam *et al*., 2019). Plant secondary metabolites are compounds that results from metabolic reactions, they are not responsible for plant growth and development. They defend plants against environmental threats, like pathogens and herbivores and abiotic stressors (Mawalagedera *et al.*, 2019). Flavonoids have different functions in plants, plant microbe interactions developmental regulation, and photoprotection. Flavonoids decrease cancer cell proliferation and invasiveness via modulating ROS scavenging enzyme activities, participating in cell cycle arrest, inducing apoptosis and autophagy, and suppressing cancer cell proliferation and invasiveness (Kopustinskiene *et al*., 2020). (*Conocarpus erectus* L.) is often a 1–10 m tall, thick, multi-trunked shrub. They feature two salt glands at the base of each leaf, are dark green and lustrous on top, and lighter with fine, silky hairs below. It can survive in arid and semi-dry places because it grows quickly and has the capacity to withstand saline in the soil and drought. In tropical and subtropical places across the globe, vegetation similar to Iraq grows on shorelines. (Imran *et al.*, 2019) . The extract of *C. erectus* from different parts (leaves, stems, fruits, and flowers) showed high antioxidant, hepatoprotective and anticancer activity due to the presence of phenolic compounds. It is an attempt to review the pharmacognostic characteristics, traditional uses, phytochemistry and biological activities of the plant. (Khan *et al.*, 2015). Esophageal cancer is a major cause of cancer-related mortality and has shown a sharp increase in incidence rates that have increased by more than 6-fold globally but vary widely by region. It is a malignancy that

has developed in the esophagus, the food conduit that connects the neck to the stomach. A hoarse voice, swollen lymph nodes around the collarbone, weight loss, trouble swallowing, discomfort while swallowing, a dry cough, and maybe coughing up or vomiting blood are common symptoms (Jemal *et al*., 2011). The purpose of this study is to determine if the flavonoids extracts of *C.erectus* leaves have any cytotoxic effects against the malignant human cell line (SKG) and against the normal cells shown in REF (rat embryonic fibroblast).

2. Material and Method

2.1. Collecting and Preparation of Conocarpus erectus leaves

The leaves of *C.erectus* were collected from 5th to 10th December 2022 from local gardens of Al-Jadriah / Baghdad and classified by herbarium of biology department of Baghdad university. The whole leaves samples of *C.erectus* were washed with tap water twice and dried under shade at room temperature for 2 weeks with heater. The dried leave samples (280 g) were grounded by hands into coarse pieces. The leave powder samples were packed in sealed plastic bags until extraction (Imran *et al*., 2019).

2.2. Chloroform Extraction

The dried powder samples of *C.erectus* (100 g) were Macerated with D.W. (900 ml) and HCL (10 ml) in a beaker using glass stirrer to mix it and let it sit overnight to break the glyosidic bond of flavonoid aglycon, then cover the beaker with foil. After 24 hr. The sample was put in water bath at 60 ℃ for 30 minutes and left to be cooled then filtered twice. The filtered liquid (650 ml) and the used leaves weighted (110 g). The crude extracts or filtrate (150 ml) that has the aglycon part which is the active component of flavonoids were transferred into aspiratory funnel and finally extracted by organic solvent chloroform (150 ml) with different polarity respectively. The funnel was shaken very well while the lid was open then left until the two layers were separated (Hossain *et al.*, 2011)

2.3. Ethyl acetate extraction

The extraction was performed in accordance with Dong *et al.*, (2011). The dried plant leaves (100 g) were added to 3 L of D.W. for 40 minutes at 80 °C. The extract was filtered using 110 nm filter paper, and the supernatant was extracted three times with ethyl acetate, each time using 1.5 L of ethyl acetate. To separate flavonoids, the organic phases were re-extracted three times using 1.5 L of aqueous citric acid solution. The citric acid solution had a concentration of 10 mg/L. The aqueous extract was evaporated, and the resultant solid were weighted using the equation:% flavonoids (w/w) = (weight of flavonoids extract/weight of plant sample) x 100.

2.3.1. Maintenance cell culture preparation

This research made use of the rat embryonic fibroblasts (REF) and the human esophageal cancer cell line (SKG). They were kept in RPMI-1640 that had 10% fetal bovine serum, 100 units/mL of penicillin, and 100 µg/mL of streptomycin added as supplements. Trypsin-EDTA was used to passage the cells, and they were reseeded at 80% confluence twice a week and cultured at 37 °C. (Sulaiman *et al*., 2018).

2.3.2. Cell growth preparation and cytotoxicity assay

To determine the cytotoxicity effect of *C.erectus* L**.** flavonoids consequently on Human Esophageal Cancer cell line. The MTT cell viability assay was done using 96-well plates. Cell lines were seeded at 1×10^4 cells/well. After24hrs. of a confluent monolayer was achieved, cells were treated with tested compounds at different concentration. Cell viability was measured after 72 hrs. The cell viability was assessed by removing the medium, adding 28 µ L of an MTT solution containing 2 µ g/mL, and incubating the cells for 2.5 h at 37 °C. The residual crystals in the wells were

solubilized after the MTT solution was removed by adding 130 µL of DMSO (Dimethyl Sulphoxide), which was then incubated for 15 minutes at 37 °C while being shaken (Al-Shammari *et al.,* 2016). The experiment was carried out in triplicate, and the absorbency was measured using a microplate reader at 492 nm (the test wavelength). The following equation was used to compute the percentage of cytotoxicity, or the rate at which cell growth was inhibited.:

$$cytotoxicity = \frac{A - B}{A}\ x\ 100$$

Where A and B are the optical densities of the control and the test, respectively.
200µ L of cell suspensions were planted at a density of 1x104 cells mL-1 in 96-well micro-titration plates and cultured for 48 hours at 37°C in order to observe the morphology of the cells under an inverted microscope. After the medium had been withdrawn and flavonoid extract had been added, the plates had been stained with 50 µL of crystal violet and incubated at 37 °C for 15 min. Finally, the stain had been removed by gently washing with tap water. The cells were examined using an inverted microscope at 100x magnification, and photographs were taken with a digital camera. (Jabir *et al.,* 2019).

3. Results and discussion

3.1 HPLC analysis

The identification of flavonoids found in chloroform and ethyl acetate extract of *C.erectus* leaves were analyzed using HPLC.

Table (1) Standard flavonoids and their retention time and concentration

RT	Name of standard	Concentration (ppm)
3.938	Ascorbic acid	1 ppm
4.470	Gallic acid	1 ppm
5.106	Catechin	0.5 ppm
5.485	Caffeic acid	1 ppm
5.926	Rutin	1 ppm
6.147	Coumarin	1 ppm
7.144	Myrecetin	0.1 ppm
7.767	Quercetine	0.2 ppm
9.177	Apigenine	0.1 ppm
9.741	Kaempferol	0.5 ppm

A typical HPLC chromatogram of the standard mixture recorded at 220 nm is presented in Table-1 as shown in the chromatogram, all investigated compounds had response at 220 nm. where they were successfully separated. The result presented in Figure-2 and 3 showed 10 peaks for different flavonoids in both extracts. By comparing their retention times with those of the authentic standards Figure-1 only 6 of them matched the standard peaks which were (Catechin, Rutin, Myrecetin, Quercetin, Apigenine and Kaempferol) with retention times as followed respectively (5.106, 5.926 , 7.144, 7.767, 9.177, 9.741).

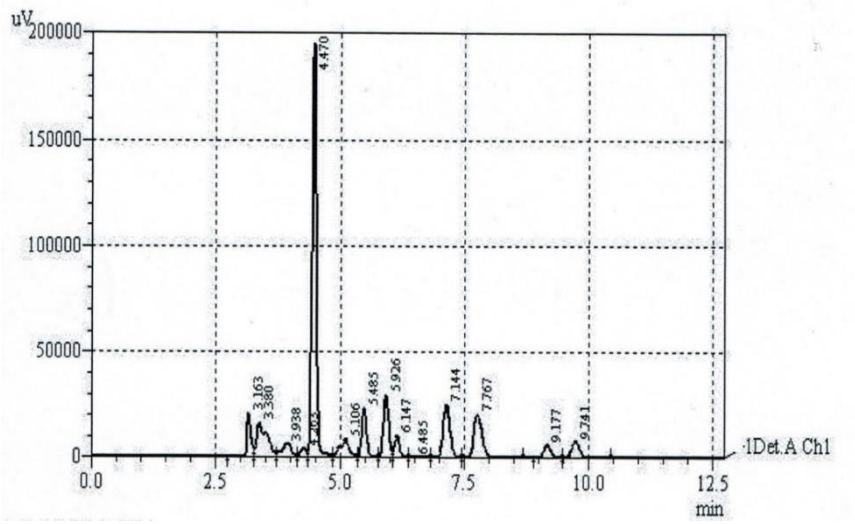

Figure (1) flavonoids standard curve analyzed by HPLC

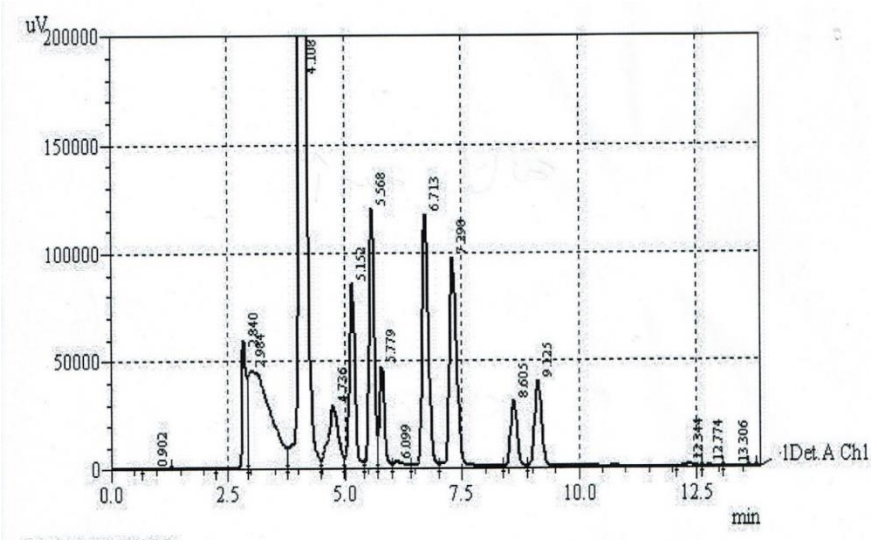

Figure (2) Chloroform extract of flavonoids curve analyzed by HPLC

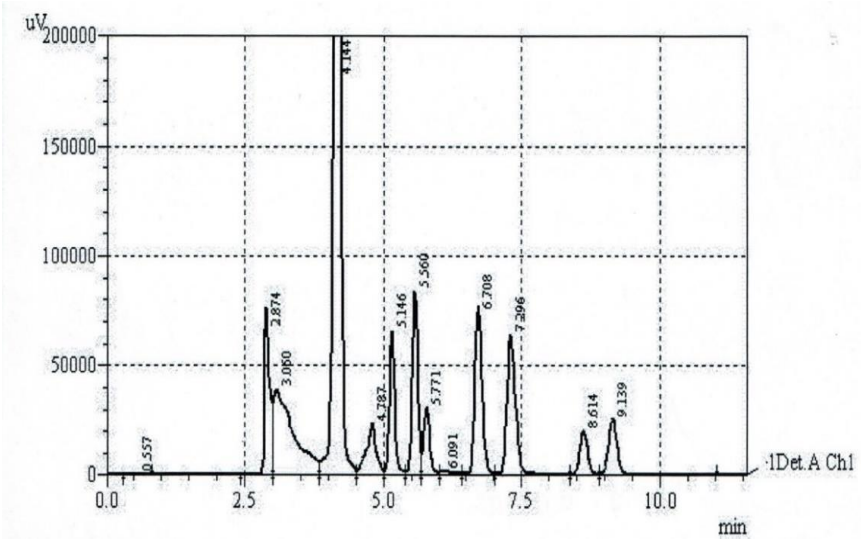

Figure (3) Chloroform extract of flavonoids curve analyzed by HPLC

3.2 Cytotoxicity evaluation

3.2.1 Ethyl acetate extract

Table-2 show that the cytotoxicity assay of flavonoids (ethyl acetate extract) against SKG cell line reached to 71% at 200 µg/mL it was also increasing with the increasing of the concentrations mentioned above as follows (5, 10.3, 22.3, 37, 51.6) % respectively.

Table-3 show the cytotoxic effect of *Conocarpus erectus* leaves flavonoids (ethyl acetate extract) on Rat Embryo Fibroblast (REF), which is represent normal cells and consider as control, for the same suggested concentrations (6.25, 12.5, 25, 50, 100, 200) µg/mL was reached to (2.6, 2.9, 4.1, 7.1, 9.1, 10.5) % respectively.

Table (2) The cytotoxicity of flavonoids in *C.erectus* leaves (ethyl acetate extract) against SKG cell line.

Concentration µg/mL	6.25	12.5	25	50	100	200
	3	11	19	33	55	66
Cytotoxicity %	5	8	22	41	49	71
	7	12	26	37	51	76
Mean	5	10.3	22.3	37	51.6	71

Table (3) The cytotoxicity of flavonoids in *C. erectus* leaves (ethyl acetate extract) against REF.

Concentration µg/mL	6.25	12.5	25	50	100	200
	1	1.8	3.5	8	11.4	8.5
Cytotoxicity %	3	3	5	7	8	13
	2.2	4	4	6.3	7.9	10
Mean	2.06	2.9	4.1	7.1	9.1	10.5

3.2.2. Chloroform extract

Results in Table-4 show the cytotoxic effect of flavonoids extracted from *Conocarpus erectus* leaves using chloroform on SKG cell line, we can see an increase in cell killing percentages (5, 11, 25, 34, 52, 69) % when concentrations of flavonoids were increased (6.25, 12.5, 25, 50, 100, 200) µg/mL respectively. When comparing the cytotoxicity effect of flavonoids on cell lines under study with normal cell lines representing in REF we found in the Table-5 decreasing in the percentage of killing despite the slight increase reached to (1.7, 2.8, 4.3, 6.2, 8.4, 11.2) % respectively.

Table (4) The cytotoxicity of flavonoids in *C.erectus* leaves (chloroform extract) against SKG cell line.

Concentration µg/mL	6.25	12.5	25	50	100	200
	4	9	18	33	51	61
Cytotoxicity %	3	10	17	40	44	64
	5	11	25	34	52	69
mean	4	10	20	35.6	49	64.6

Table (5) The cytotoxicity of flavonoids in *C.erectus* leaves (chloroform extract) against REF.

Concentration µg/mL	6.25	12.5	25	50	100	200
	1.2	3	4	5.6	8.1	9.1
Cytotoxicity %	3.	2	5.2	6.2	7.4	13
	1	3	3.7	7	9.20	11.5
mean	1.7	2.8	4.3	6.2	8.4	11.2

3.3 Statistical analysis

SKG with Chloroform and Ethyl acetate, results of the statistical analysis shown in Table-6. The results of the statistical analysis in the above table showed that there were significant differences or effects between the extracts (chloroform and ethyl acetate) and SKG cell line by relying on (*P*-value) and its statistic (chi-square) as well as the values of coefficient of determination or interpretation (R^2), where the results showed that there are significant differences at the significance level (0.05) that's due to *P*-value is less than (0.05).

Table (6) The statistical analysis.

		Chi-Square	*P* -Value	R^2
SKG Cell	**Chloroform**	**20.66**	**0.001**	**0.98**
	Ethyl acetate	**13.831**	**0.031**	**0.95**

Then we accept the null hypothesis (H_0) that says there are differences or effects between the cell line and two extracts and refuse substitution hypothesis (H_1) that says there are no differences, as the results of the SKG cell line with the extract chloroform reached a ratio (33.4 %) at (6.25) concentration while at a concentration (200 µl/ml) the ratio is (66.5 %), and with ethyl acetate extract the ratio is (17.2 %) at (6.25 µl/ml) con and (82.5%) at (200 µl/ml) concentration.

REF with Chloroform and Ethyl acetate, results of the statistical analysis shown in Table-7. The results of the statistical analysis in the above table showed that there were no significant differences or effects between the extracts (chloroform and ethyl acetate) and REF cell line by relying on (*P*-value) and its statistic (chi-square) as well as the values of coefficient of determination or interpretation (R^2), where the results showed that there are no significant differences at the significance level (0.05) that's due to *P*-value is more than (0.05).

Table (7): The statistical analysis.

		Chi-Square	*P*-Value	R^2
REF Cell	Chloroform	14.027	0.071	0.90
	Ethyl acetate	12.727	0.064	0.85

Then we refuse the null hypothesis (H_0) that says there are differences or effects between the cell line and two extracts and accept the substitution hypothesis (H_1) that says there are no differences, as the results of the REF cell line with the extract chloroform reached a ratio (16.7 .4 %) at (6.25) concentration while at the concentration (200 µl/ml) the ratio is (83.3 %), and with ethyl acetate extract the ratio is (16.4 %) at (6.25 µl/ml) con and (83.6%) at (200 µl/ml) concentration. It should be mentioned that only limited studies were conducted on the cytotoxic effect of *C.erectus* leaves extract on cancer cell lines and it is worth noting that this is the first study on *C.erectus* plant growing in Iraq concerned about using flavonoids as anticancer on SKG and REF cell lines. Anticancer effects of polyphenols depend on several factors: Their chemical structure and concentration, and also on the type of cancer. Malignant cells from different tissues reveal somewhat different sensitivity toward flavonoids (Ren *et al*., 2003)

The results of this study proved that flavonoids and their derivatives extracted from leaves of *C.erectus* by ethyl acetate have anticancer specifications, as the cytotoxicity effect reached to 71% on (SKG) compared to normal cells (REF) (10.5%) at 200 mg/ml while the cytotoxicity of flavonoids extracted by chloroform reached to 11.2 % on the cell lines mentioned above respectively at 200 µl/ml.

Our results were in agreement with studies achieved by Abdel-Hameed *et al*., (2012); and Bashir *et al.*, (2015). They mentioned that flavonoids extracted by ethyl acetate and *n*-butanol from different parts of *C. erectus* have anticancer properties towards Hep G2 and MCF7 cell lines.

Conclusions

From the above results we conclude that HPLC analysis showed six flavonoids (Catechin, Rutin, Myrecetin, Quercetin, Apigenine and Kaempferol) matched with the standard peaks in both extracts. Flavonoids extracted from *C.erectus* leaves for (Ethyl acetate) showed cytotoxic activity on Human Esophageal Cancer cell line (SKG) reach to 71.6% at concentration 200 mg/ml compared with a minimum cytotoxicity on normal rat embryonic fibroblast cell line (REF) reached to 10.2% at the same concentration and for chloroform extract 64.6% at 200 µl/ml of extract concentration and was 11.2% in REF at 200 mg/ml.

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التأثير السام لخلايا للفلافونيدات المستخرجة من أوراق نبات الكاربس على خلايا (SKG) و (REF)

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الخلاصة

أجريت الدراسة الحالية للتحقق من فعالية مركبات الفلافونويد السامة للخلايا المستخلصة من أوراق نبات الكاربس. باستخدام أسيتات الإيثيل والكلوروفورم ، وقد تم الكشف عن النتائج بواسطة جهاز (HPLC). تم تقييم تأثيره السام للخلايا على خط خلايا السرطانية (SKG) سرطان المريء البشر و (REF)غير السرطاني الخلايا الليفية الجنينية للجرذان باستخدام مقياس (MTT). أظهر تحليل(HPLC) لكلا المستخلصين أن 6 من أصل 10مركبات فلافونويد تطابق القمم القياسية وهي (كاتشين، روتين، ميريستين، كيرسيتين، أبجينين وكايمبفيرول). كانت السمية الخلوية لمستخلص أسيتات الإيثيل أكبر من مستخلص الكلوروفورم ضد خط خلايا (SKG)، وزاد التأثير السام للخلايا لكلا المستخلصين مع زيادة التركيز. عند 200 ميكرولتر، كانت السمية الخلوية لمستخلصات أسيتات الإيثيل والكلوروفورم 71٪ و 64.6٪ على التوالي ، بينما كانت السمية الخلوية لمستخلصات REF 10.5٪ و 11.2٪ . هذا يعني أن مستخلص نبات الكاربس له آثار سلبية قليلة على الخلايا الطبيعية، وبالتالي فهو دواء مرشح آمن وواعد مضاد للسرطان.