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CYTOTOXIC EFFECT OF FLAVONOIDS EXTRACTED FROM *CONOCARPUS ERECTUS* LEAVES ON HELA CELL AND REF

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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 were detected by HPLC. The evaluation of its cytotoxic effect on cancerous Hela cell line for breast 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 extract ethyl acetate showed higher cytotoxicity against Hela cell line than chloroform and the cytotoxic effect of both extracts increased with the increasing of their concentration, the cytotoxicity of both extract were at 200 µL 81.6 %. and 73.6 % respectively and for REF were 10.5 % and 11.2 % at 200 µL. This implicit that *Conocarpus erectus* extract has minimum side effect on normal cells thus may be considered safe and potential candidate as anticancer agent.

Key words : Cytotoxicity, *Conocarpus erectus*, breast cancer, L., Hela, inhibition rate.

Introduction

Medicinal Plants

Medicinal plant has different concentration of chemical components 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. considered phenolic compound a subgroup of secondary metaboiltes, flavonoid structure includes a C6-C3-C6 carbon framework. Depending on the position of the linkage of the aromatic ring to the benzopyran (chromano) moiety, this group of natural products may be divided into three classes: Flavonoids (2-phenylbenzopyrans), Isoflavonoids (3-benzopyrans) and Neoflavonoids (4-benzopyrans) (Lei *et al.,* 2019) (*Conocarpus erectus* L.) is usually a dense multiple-trunked shrub, 1–10 m tall. They are dark green and shiny on top, and paler with fine silky hairs underneath, and have two salt glands at the base of each leaf. (Stevens *et al*. 2001) grows rapidly, Has the ability to resist drought and soil salinity so it can endure dry and semi dry areas. Like Iraq grows on shorelines in tropical and subtropical regions around the world (Imran *et al.,* 2019) is one of the major causes of death in the world, it starts with the deformation of a natural cell caused by genetic mutations in DNA. It reproduces asexually in an abnormal way by ignoring signals related to regulation of cell's growth around it and obtains invasion characteristics and causes changes in surrounded tissues. Anticancer medicines prepared from plants are tested by cytotoxic compounds and screened by their raw extracts. To attain natural products with higher effectiveness and less side effects. Medicinal herbs are important for cancer treatment because they have multiple chemical compounds that are active material against cancer. Breast cancer is a heterogeneous disease composed of a growing number of recognized biological subtypes. The prognostic and etiologic importance of this diversity is complicated

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by many factors, including the observation that differences in clinical outcomes often correlate with race. Age-adjusted mortality. Biological differences among breast cancers may reflect genetic influences, differences in lifestyle, or nutritional or environmental exposures. (Carey *et al.,* 2006). The aim of this research is to investigate the cytotoxic activity of the crude ethyl acetate and chloroform extract of *C. erectus* leaves against cancerous human cell line (Hela) and against normal cell represented in REF (rat embryonic fibroblast). In fact, it should also be mentioned that only limited studies were done on cytotoxic effect of *C. erectus* leaves extract on cancer cell lines and it was worth noting that ethyl acetate and chloroform extracts were not used previously with these cell lines Hela cell line.

Materials and Methods

Collecting plant material

The leaves of *C.erectus* were collected from 5th to 10th December 2018 from local gardens of Al-Jadriah / Baghdad and classified by herbarium of biology department (Imran *et al.,* 2019).

Preparation of *Conocarpus erectus* leaves

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).

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 aglycone, then cover the beaker with foil. After 24 hr. The sample was put in water bath at 60 °C for 30 minutes and left to be cooled then filtered twice. The filtered liquid (650 ml) the used leaves weighted (110 g). The crude extracts or filtrate (150 ml) that has the aglycone part which is the active component of flavonoids were transferred into a separatory funnel and finally extracted by organic solvent chloroform (150 ml) with different polarity respectively. Shake very well then open the lid and left on

Ethyl acetate extraction

The extraction was carried out according to Dong *et al.,* (2011). The dried plant leaves (100 g) was added into 3 L of D.W. at 80°C for 40 min. The extract was filtered through 110 nm filter paper, and then the supernatant was extracted with ethyl acetate three times using 1.5 L of ethyl acetate in each extraction. The organic phases were re-extracted with 1.5 L of aqueous citric acid solution three times to separate flavonoids. The concentration of citric acid solution was 10 mg/L Aqueous extract was evaporated and the resulting solid was weighted according to the following equation:

% flavonoids (w/w) = (weight of flavonoids extract/ weight of plant sample) × 100.

Maintenance cell culture preparation

Human breast cell line (Hela) and rat embryonic fibroblasts (REF) were used in this study. They were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were passaged using Trypsin-EDTA reseeded at 80% confluence twice a week, and incubated at 37°C (Sulaiman, *et al*., 2018).

Cell growth preparation and cytotoxicity assay

To determine the cytotoxicity effect of *C. erectus* L**.** flavonoids consequently on breast 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. of treated cells by removing the medium, adding 28 µL of 2 µg/mL solution of MTT and incubating the cells for 2.5 h at 37 °C. After removing the MTT solution, the crystals remaining in the wells were solubilized by the addition of 130 µL of DMSO (Dimethyl Sulphoxide) followed by 37 °C incubation for 15 min with shaking (Al-Shammari *et al*., 2016). The absorbency was determined on a micro plate reader at 492 nm (test wavelength); the assay was performed in triplicate. The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated as the following equation:

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

Where A and B are the optical density of control and the optical density of test.

For visualize the shape of cells under inverted microscope, 200 µL of cell suspensions were seeded in 96-well micro-titration plates at density $1x10^4$ cells mL^{-1} and incubated for 48 hrs at 37°C. Then the medium removed, and flavonoid extract was added after 24hr, the plates were stained with 50 µL with Crystal violet and incubated at 37°C for 15 min, the stain was washed gently with tap water until the dye was removed. The cells were observed under inverted microscope at 100x magnification microscope filed and photographed with digital camera (Jabir *et al.,* 2019).

Results and discussion

HPLC analysis

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

Table 3-1: 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 3-1 as shown in the chromatogram, all investigated compounds had response at 220 nm. where they were successfully separated. The result presented in Fig. 3-2 and 3-3 showed 10 peaks for different flavonoids in both extracts. By comparing their retention times with those of the authentic standards fig. 3-1 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).

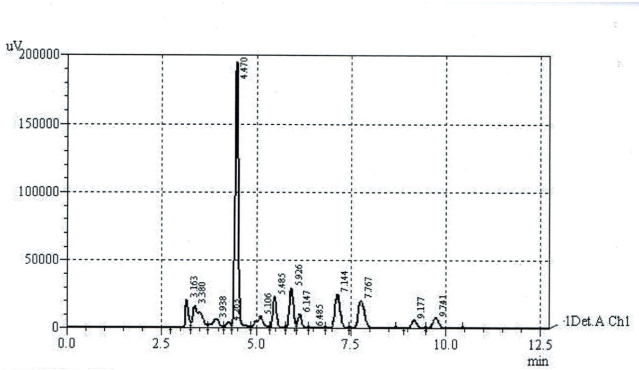

Fig. 1-2: Flavonoids standard curve analyzed by HPLC.

Cytotoxicity evaluation

Ethyl acetate extract

Table 1 shows that the cytotoxic effect of flavonoids (ethyl acetate extract) was elevating with the increasing of the interactions which reached to 81.5% at 200 mg/ml of extract concentration. On the other hand, the results obtained from cytotoxicity assay of flavonoids (ethyl acetate extract) against normal cell represented in REF was 10.5% at 200 mg/ml. (Table 2).

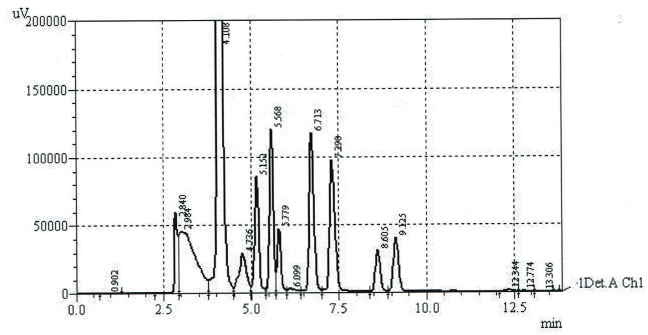

Fig. 1-2: Chloroform extract of flavonoids curve analyzed by HPLC.

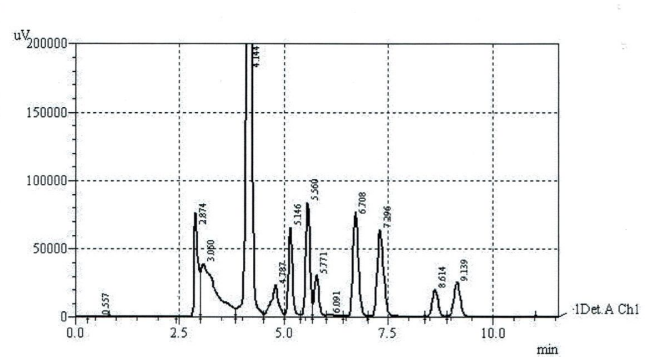

Fig. 1-2: Chloroform extract of flavonoids curve analyzed by HPLC.

Chloroform extract

Results in table 3 that the cytotoxic effect of flavonoids (chloroform extract) was elevating with the increasing of the interactions which reached to 73.6% at 200 mg/ml of extract concentration. On the other hand, the results obtained from cytotoxicity assay of flavonoids (chloroform extract) against normal cell represented in REF was 11.2% at 200 mg/ml. (Table 2).

	Chi - Square		*P* -Value	R^2
Hela Cell	Chloroform	12.419	0.000	0.97
	Ethyl acetate	33.694	0.000	0.87

Statistical analysis

The results of the statistical analysis in the above table showed that there were significant differences between the extracts (chloroform and ethyl acetate) with the Hela cell line by relying on the value of (*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)

Then we accept the null hypothesis (H_0) that says

Table 1: The cytotoxicity of flavonoids in *C. erectus* leaves (ethyl acetate extract) against Hela cell line.

Concentration µg/mL	6.25 µg/mL	12.5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
Cytotoxicity %	8.000	13.000	27.00	45.00	63.00	81
	7.000	16.000	31.00	50.00	67.00	77
	11.000	17.000	34.00	49.00	66.00	87
Mean	8.6	15.3	30.6	49	65.3	81.6

Table 2: The cytotoxicity of flavonoids in *C. erectus* leaves (ethyl acetate extract) against REF.

Concentration µg/mL	6.25 µg/mL	12.5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
Cytotoxicity %	1.000	1.800	3.50	8.00	11.40	8.5
	3.000	3.000	5.00	7.00	8.00	13.0
	2.200	4.000	4.00	6.30	7.90	10.0
Mean	2.06	2.9	4.1	7.1	9.1	10.5

Table 1: The cytotoxicity of flavonoids in *C. erectus* leaves (chloroform extract) against Hela cell line.

Concentration µg/mL	6.25 µg/mL	12.5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
Cytotoxicity %	7.000	14.000	24.00	41.00	55.00	77
	6.000	13.000	30.00	37.00	61.00	73
	9.000	15.000	27.00	44.00	57.00	71
Mean	7.3	14	27	40.6	57.6	73.6

Table 2: The cytotoxicity of flavonoids in *C. erectus* leaves (chloroform extract) against REF.

Concentration µg/mL	6.25 µg/mL	12.5 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
Cytotoxicity %	1.200	3.000	4.00	5.60	8.10	9.1
	3.000	2.500	5.20	6.20	7.40	13.0
	1.000	3.000	3.70	7.00	9.20	11.5
Mean	1.7	2.8	4.3	6.2	8.4	11.2

there are differences or effects between the two cell lines and two extracts and refuse substitution hypothesis (H_1) that says there are no differences, as the results of the Hela cell line with the extract chloroform reached a ratio (16.7 %) at (6.25) concentration while at concentration (200) at ratio (83.3 %), while with ethyl acetate extract the ratio is (16.9 %) at (6.25) con and (83.1%) at (200) concentration.

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

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)

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 concentration (200) the ratio is (83.3%), and with ethyl acetate extract the ratio is (16.4%) at (6.25) con and (83.6%) at (200) concentration.

Phenolic compounds brought attention into medicine field for having anticancer properties (Selassie *et al.*, 2005). Two other studies Özçelik *et al.*, (2011) and Sak, (2014) that focused on flavonoids specifically and found having cytotoxic effect. Bashir *et al.*, (2015) found that the extract of *C. erectus* from different parts (leaves, stems, fruits and flowers)

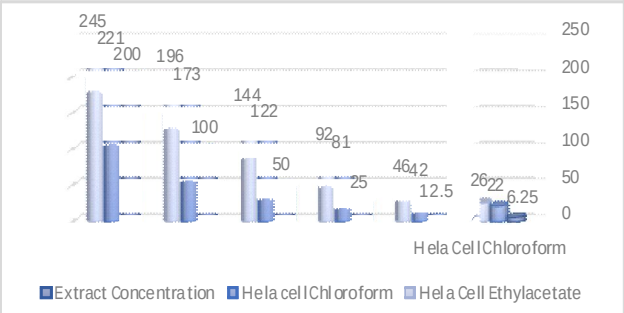

Fig. 1-1: Cytotoxic effect of *Conocarpus erectus* L. ethyl acetate and chloroform extract (µg/ml) on Hela cell line at 72 hours incubation period.

had different phenolic compounds including flavonoids detected by HPLC showed high showed high antioxidant hepatoprotective and anticancer activity. Regarding to *Conocapus* plant a study done by Nascimento *et al.*,

(2016). Showed acute toxicity of aqueous *C. erectus* leaves extract with the presence of flavonoids. On the other hand the research accomplished by Abdel-Hameed *et al.,* (2012) who found that *C. erectus* leaves essential oil is cytotoxic when measured by MTT assay.

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 breast cancer cell line (Hela) reach to 81.6% 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 73.6% at 200 mg/ml of extract concentration and was 11.2% in REF at 200 mg/ml.

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