Anti-Tumor Activity of *Phoenix dactylifera L.* Pit Extracts against *Hela* and $L_{20}B$ cell lines

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Abstract
Date palm has antioxidant activity which is able to control cancer cells. This study was designed to assess the anti-tumor activity of aqueous and ethanolic extracts of Phoenix dactylifera L. pits extracts against (Hela) and ($L_{20}B$) cancer cell lines. HPLC technique detected that both extracts contained several phenolic compounds (Caffeic acid, Ferulic acid, p-coumaric acid, Chlorogenic acid, Sinapic acid, Genstic acid, p-hydroxy benzoic acid, Gallic acid and Protocatechuic acid). The percentage of growth inhibition (PGI) of four extracts concentrations (6.5, 12.5, 25 and 50 µg/ml) was in vitro assessed with using two cancer cell lines.

The results revealed that the four concentrations of the two plant extracts had anti-tumor activity in a concentration-dependent manner and the ethanolic extract gave best results of PGI and IC_{50} than aqueous extract in both cell lines. As a conclusion in accordance to the results, the ethanolic extract of Phoenix dactylifera L. pits showed obvious anti-tumor activity in both cell lines. This activity appeared to be particularly significant in both treated cell lines.

Keywords: Date Palm pits, Anti-Tumor Activity, Human cell line, mice cell line.

Introduction

Date palm (*Phoenix dactylifera L.*) is considered as one of the most important source of food for humans in many regions[11]. Its contain sugars reaching to 88% in some varieties[7], also rich in vitamins and mineral salts and different chemical compounds such as Calcium (Ca), Cadmium (Cd), Zinc (Zn), Potassium (K), Saturated and Unsaturated fatty acids. The pits powder is also used in some traditional medicines and has been tested for human used for health purposes[12]

Cancer is the abnormal cellular growth that usually invades and destroys normal cells. As a result of imbalance in the body these cells are born and so various types of cancer treatments were used to correct the imbalance. In spite of many cancer researches, the nature of cancer does not understand exactly[13]. Thus, all over the world cancer kills about 3500 M people[9]. More than 270,000 people in USA die because of cancer[4]. In Iraq, breast cancer considered to be the most dominant type of all cancers representing around 43% with a mortality rate of 23%[8].

Cancer therapy depends on surgery radiotherapy, chemotherapy, or combinations of them. New and /or complementary treatments methods are needed as a result of significant death rate in response to cancer and because of the serious side effects of radiation therapy and chemotherapy. Because of the need for effective chemotherapy and in line with recent discoveries in cell biology to cure cancer without serious side effects, recent researchers are doing their best[19].

Considered as a major treatment, chemotherapy is used for the control of advanced stages of cancer cells yet shows serious side effects on normal cells and tissues[19,27].

Since the beginning of civilizations, the plant has been used to treat many diseases for both humans and animals. Without causing toxicity, they may maintain the vitality and health of people and also cure diseases including cancer[6]. About 50% of all modern drugs in medicinal use are from natural products which have the ability to treat tumor cells[24].

Phenolic compounds in plants have a major role in control cancer cells[17], because some synthetic antioxidants have the ability to enhance development of cancer in mice and the increased interested of peoples in natural foods products. Plant phenolic compounds and other natural antioxidants are extremely desirable[2].

Material and Methods

Collection of the Plant: The date palm pits were freshly collected from Iraqi local strains Barhi and Khastawi of date palm in year 2017.

Preparation of aqueous and ethanolic extracts of *Phoenix dactylifera L.* pits for anti-tumor activity: Pits of *Phoenix dactylifera L.* were ground into fine powder using grinding machine[3].

Aqueous extract: About 50 g of the powdered pits was mixed with 250 ml double distilled water. Then mixture was left in a shaker incubator at 37°C for 24 hrs, then filtered by a filter paper (Whatmann no. 1). Using rotary evaporator, the filtrate was concentrated at 40°C until dryness and the extract residue was weighed and kept until used[28].

Ethanolic extract: About 50 g of the powdered pit was extracted with 250 ml of 70% ethanol in flask and put on

magnetic stirrer for 72 hrs. at room temperature, then filtered through filter paper (Whatmann no.1). Using a rotary evaporator, the solution was evaporated to dryness at 40°C and the extract residue was weighed and kept until used[16].

Detection of some phenolic compounds of date palm aqueous pits extract:

- Estimation of phenols date palm pit by HPLC: The main compound was separated on FLC (Fast Liquid Chromatography) column under the optimum condition.
- Column: phenomenex C-18, 3µm particle size (100x 4.6 mm I.D) column.
- Mobile phase: Linear gradient of solvent A 0.1 % trifluoro acetic acid (TFA acid) in deionized water: solvent B was acetonitrile gradient program from 0% B to 100 % B for 10 minutes.
- Flow rate 1.3 ml/min.
- Detection: UV at 280 nm.
- Calculation:
  Concentration of sample µg/ml = (sample area / standard area) x conc. of standard x dilution Factor.
- Extraction: 10 mg of the extraction phenolic compounds dissolved in 10 ml methanol were exposed to ultra – sonication (Branson sonifier, USA) at 60 % duty cycles for 25 min. at 25 ºC and centrifugation (7500 rpm for 15 min.), then to remove pigments prior to evaporation under vacuum (Buchi Rotary evaporator Re Type) the clear supernatant of sample was subjected to charcoal treatment. Re-suspend the dried samples in 1.0 ml methanol by vortex, the mixture was passed through 2.5 µm disposable filter and stored at 4ºC for further analysis, then according to the optimum separation condition, 20 µl of the sample was injected into HPLC system[5,20].

Assessment of Cytotoxic Activity (*in vitro*): The *in vitro* anti-tumor activity of agrimony aqueous and ethanol extracts was estimated at the Biotechnology Research Center (Al-Nahrain University). In this study, the cytotoxic activity of the two pits extracts was determined against two tumor cell lines (Human cell line (HELA) and ($L_{20}B$) mice cell line)[15]. The percentage of growth inhibition was calculated according to an equation presented by Phuangsab et al.[23]

$$\text{Growth inhibition (\%)} = \left(\frac{\text{Control Absorbance - Treated Absorbance}}{\text{Control Absorbance}} \right) \times 100$$

Growth Inhibition Assessment in Hela and $L_{20}B$ Cell Lines: The laboratory assessment of growth inhibition was carried out according to a method that was adopted by Abdul-Majeed[1]. The method included the following steps:

1. The cells (Hela and $L_{20}B$) were supplemented as monolayer attached cells in Falcon culture flasks (75 cm) containing RPMI-1640 medium. The cells were washed with PBS and then 1 ml of trypsin –versine solution was added with a gentle shaking until the cells were detached from the flask surface. Such manipulation was carried out with aid of phase contrast inverted microscope. Then, the contents of the flask were removed to another flask and incubated at 37ºC for 15 minutes (sub-culture).

2. The cells were counted and their number was adjusted to 1 x 10^6 cell/ml. At the same time, viability was assessed using a dye-exclusion test (trypan blue stain) and it was always greater than 96%.

3. The cells were seeded in the wells of 96-well tissue culture plate, which was carried out by pipetting 200 µl of the cell suspension into each well in a cell number of 10^4 cells/ well and the plate was incubated overnight at 37ºC.

4. The day after, the wells were examined to inspect the cell growth, then four concentrations 6.5, 12.5, 25 and 50 µg/ml were added to each well in a triplicate manner.

5. After the end of each incubation period, 20 µl of MTT solution was added to each well. The plate was re-incubated at 37ºC for 2-4 hrs.

6. After incubation, DMSO was added to dissolve the MTT crystals.

7. The wells were read under the ELISA reader at wave length of 570 nm and the absorbance was recorded.

Statistical Methods: The values of the resulted parameters were given in terms of mean and the differences between means were assessed by analysis of variance test, using the computer program SPSS (using T-test). The difference was considered significant when the probability value was equal or less than 0.05.

Results and Discussion

Result estimated by HPLC methods showed that palm pits extracts contain a significant amount of some important phenolic compounds with higher concentrations for Caffeic acid and Ferulic acid respectively (table 2). Both tested strains showed similar results in aqueous and ethanolic extracts.

Both tested cell lines show similar results of PGI in aqueous and ethanolic extracts. Results in table 3 and figure 1 showed that ethanolic extract has the best cytotoxic activity with highest PGI at the higher concentration used (83.8 % at 50 µg/ml) than that obtained by aqueous extract (70 % at 50 µg/ml).

Nevertheless, the calculated IC_{50} values showed no significant differences between both different extracts against HeLa cells and slightly lower value for the aqueous extract.

Cytotoxic effects of date palm pits extracts (aqueous and ethanol) on $L_{20}B$ cell line: The results in table 4 and figure

2 showed that ethanolic extract has the best anti-tumor activity with highest PGI at the higher concentration used (85.7 % at 50 µg/ml) than that obtained by aqueous extract (77.1 % at 50 µg/ml). Nevertheless, the calculated IC_{50} value was significantly different and had the lower value for the aqueous extract.

Plants are sources of different compounds. They contain and can produce a variety of chemical substances that have different biological actions with a special reference to their medicinal importance[6,21]. Therefore, they are employed by herbalists of different cultures, anciently and recently to provide remedy to peoples of their sicknesses[10].

Since cancer is a major cause of death, every day tumor treated research is conducted worldwide. These studies often involve the properties and the effects of biologically active compounds on tumor cells and they often originate from plants[25]. There is an urgent need to examine reliable and inexhaustible sources of natural compounds. In addition, it is important to understand the mechanisms of anti-tumor activity for future application in cancer treatment[14].

Figure 1: Hela cell line after treated them by date palm seeds extract (24 hour).

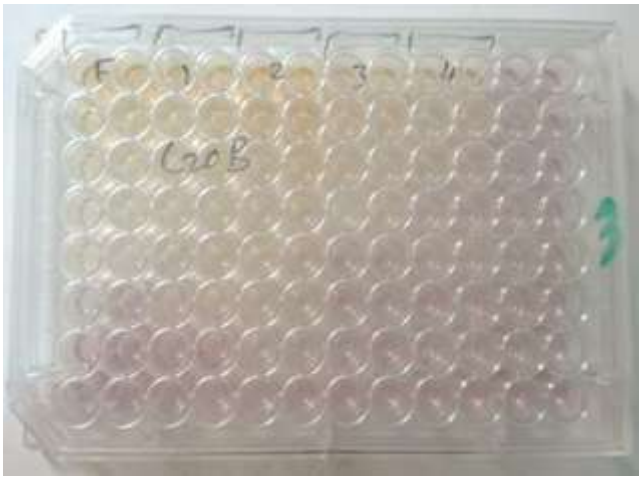

Figure 2: $L_{20}B$ cell line after treated them by date palm seeds extract (24 hour).

Table 1
Sequences of the eluted material of the standard (Concentration 25 µg / ml).

Seq.	Subjects	Retention time / minute	Area µvolt
1	Gallic acid	1.28	34652
2	Genstic acid	2.12	70529
3	Cafeic acid	3.04	95867
4	Chlorogenic acid	5.53	81401
5	p-coumaric acid	6.35	106120
6	Ferulic acid	7.43	118751
7	p-hydroxy benzoic acid	8.52	98922
8	Sinapic acid	9.24	84973
9	Protocatechuic acid	10.39	119875

Table 2
Estimation of some active compounds (phenolic compounds) in Palm pit aqueous extracts

Phenolic compounds	Concentration µg / ml
Caffeic acid	276.33
Ferulic acid	235.12
Chlorogenic acid	164.26
p-coumaric acid	145.16
Sinapic acid	95.09
Genstic acid	91.42
p-hydroxy benzoic acid	91.11
Gallic acid	78.57
Protocatechuic acid	19.44

Table 3
Cytotoxic effects of date palm seed (aqueous and methanol) extract against Hela cell line.

Extract Concentration (µg/ml)	Growth Inhibition (%)	
	Aqueous Extract	Ethanol Extract
6.5	40.3	48.8
12.5	43.8	50.5
25	66.8	58.3
50	70.0	83.8
IC_{50}	7.38	7.80

This research investigated the anti-tumor activity of the ethanolic and aqueous extract (in a concentration of 6.5 -12.5 -25 -50 µg/mL) on HELA and $L_{20}B$ cell lines. A dose-dependent inhibition was seen in treated cell line. For functional antagonist and competitive binding assays, the

most common measurement of the dose-response curve is the IC_{50}. The half maximal inhibitory concentration (IC_{50}) is an estimation of the effect of a substance in inhibiting a specific biological or biochemical function.

Table 4
Cytotoxic effects of date palm seed extracts (aqueous and ethanol) on $L_{20}B$ cell line.

Extract Concentration (µg/ml)	Percentage of Growth Inhibition	
	Aqueous extract	Ethanol extract
6.5	26.0	38.7
12.5	34.6	48.6
25	35.7	70.0
50	77.1	85.7
IC_{50}	5.3	6.2

The cell proliferation was significantly lower when compared to untreated control cells after 24 h of treatment but lower concentrations had lower anti-tumor activity. Maximum inhibition of proliferation was seen at the highest concentration (50 µg/mL). The anti-tumor activity may be attributed to the presence of compounds that can inhibit cell cancer found naturally in this plant especially the most active antioxidative compounds of date palm pits are phenolic compounds pointed in detection (Caffeic acid, Ferulic acid, Chlorogenic acid, Sinapic acid, p-coumaric acid, Genstic acid, p-hydroxy benzoic acid, Gallic acid, Protocatechuic acid) in different concentrations[11,22]. Many researches showed that phenolic compounds have a significant role in cancer treatments[18,26,29].

Conclusion

Many active compounds were detected in the aqueous and ethanolic extracts of date palm pits including Caffeic acid, Sinapic acid, Ferulic acid, Chlorogenic acid, p-coumaric acid, Genstic acid, p-hydroxy benzoic acid, Gallic acid, Protocatechuic acid. Anti-tumor activities of both date palm pits extracts were *in vitro* detected with different concentrations but the ethanolic extract was more efficient than aqueous extract.

Acknowledgement

This work was done in educational laboratory of Collage of Biotechnology and in Biotechnology Research Center / Alnahrain University. Many thanks go to them for providing the space and tools to accomplish this work.

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