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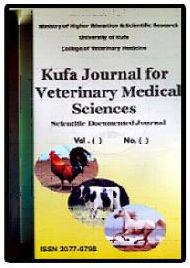

In vitro: anticancer effect of oily and methanolic extracts of Al-Zahdi (*Phoenix dactylifera L.*) from dry dates and leaves on AMN3, Hela and Ref cancer cell cultures

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Abstract

The antioxidant and anti-tumor effects of extracts from various herbs and medicinal plants were measured using various in vitro and in vivo methods. The current study investigates and compare the anticancer effects of the two palm part extracts against cancer cell line in cultured cell line of AMN3, Hela and Ref. The results showed a concentration dependent inhibitory cytotoxic effects during 72 hrs of exposure for methanolic and oily crude extracts of date and leaves on AMN3, Hela and Ref cell lines. The highest significant effect of both dry date and leave methanolic extracts were achieved at concentration 2500 µg/ ml that causing highest growth inhibition percentage (GIP) of (73.3 %, 66.4%) for AMN3 and (76.7 %, 55.4%) for Hela respectively. The concentration 5000 µg/ ml showed nearly less effect after 72 hrs exposure. Both methanolic extracts tested concentrations didn't cause any inhibitory effect on the REF cell line. The effect of date and leaves oily extracts at different concentrations caused on tumor Cell Line AMN3, Hela and Ref highly significant in difference at exposure periods 72 hrs for concentrations gradient ranged from (15.62- 250 µg/ml). The concentration 125 µg/ml showed higher inhibition percentage compared to the higher concentration of 2500 µg/ ml causing on AMN3 (85.1%, 66%), Hela (79.1%, 77%), and Ref (76%, 61.7%), respectively. Plateau were noticed for all highest palm extracts concentrations GIP effect on all cell line cultures. The study concluded that the superiority of oil extract for both date and leave over alcoholic extracts one in inhibition of in vitro the same cell lines AMN3, Hela, Ref.

Keywords: Anticancer, dry date and leave extracts, Phoenix dactylifera, in vitro

Introduction

The date palm (*Phoenix dactylifera L.*) is one of oldest cultivated plants of human kind and used as food for 6000 years. There are more than two hundred varieties of dates available worldwide, especially in Iraq [1].

Moreover, recent Studies have shown that date fruits are an excellent source of phenolics and therefore possess an extremely high antioxidant capacity. Dates have potent anthocyanins, carotenoids, and phenolics compounds (protocaechuic, p-hydroxy benzoic, vanillic, syringic, caffeic, coumaric, ferulic, hydroxy benzoic, mainly cinnamic acids) and flavonoids (flavones, flavonols and flavanones). As of today, dates also have the unique distinction of being the only food to contain flavonoid sulfates, which like most other fruits, have antioxidant properties [2].

The antioxidant and anti-tumor effects of extracts from various herbs and medicinal plants have been proved experimentally and clinically. Several in vitro or in vivo studies have proved the anticancer potential of the extracts from several medicinal plants [3]. Regular consumption of bioactive compounds from plants and fruit may be associated with protection against oxidative damage and lowered risk of chronic diseases, such as cancer, heart disease, and cerebrovascular disease. [4].

In vitro, tissue-based models are common and widely used for screening and ranking chemicals, especially in testing of drugs at the preclinical stages. The toxic effects include general cytotoxicity, genotoxicity, mutagenesis and carcinogenesis. Cell-based assays are currently considered central to toxicity testing, biomaterial testing, and environmental material exposure testing [5].

The objective of this research is to determine the anticancer effects of alcoholic and oily extract of phoenix dactylifera in vitro cell lines AMN3, Hela, Ref.

Material and methods

Preparation of solutions:

Trypsin versin was prepared according to [6].

Phosphate buffer saline (PBS), the following materials should be prepared in advance (0.8 gm of NaCl, 0.2 gm of KCl, 1.15 gm of Na2HPO4 and 0.2 gm of KH2PO4). These materials are dissolved in 500 ml of distilled water, then the volume is completed to 1000 ml. After adjusting the acidity of the solution to pH= 7.2 then it is sterilized (121 ºC, 1 bar for 20 minutes) and kept at 4ºC. [7].

Methyl thiazolyl tetrazolium (MTT) solution, the stain 3-(Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazoliumbromide (0.024g) is dissolved in 12 ml of PBS in order to prepare a 2 mg/ml concentration of the dye. The solution is then filtered using 0.2 µm Millipore filter to remove any blue formazan product, and stored in sterile, dark, glass bottle covered with foil at 4˚C. The solution should be used within no longer than 2 weeks of preparation [6].

Crystal violet stain was prepared by dissolving 5 g of crystal violet powder in 200 ml methanol, and the solvent filtered by Whatman No.1 filter paper. Then 50 ml of formaldehyde (37%) were added and the volume of mixture was completed to 1000 ml by distilled water. Excess solid residue was filtered off using Whatman No.1 filter paper. [8].

Media with serum:

Penicillin/streptomycin solution from (Capricorn/ Germany) and filtered by Millipore and poured to media RPMI from (S,p.A./Italia) 100 microliter / ml from antibiotic then incubated in incubator 37 C for

24 hours to be sure that media not contamination then add bovine calf serum from (Capricorn/ Germany) 10% in media and filtered by Millipore 0.2 micrometer pore size.

Cell lines:

The cell lines used in this study were supplied by tissue culture unit/ Iraqi Centre for Cancer and Medical Genetics Research (ICCMGR) Baghdad, Iraq maintained in RPMI- 1640. Ahmed-Mohammed-Nahi-2003 (AMN-3 cell line), was supplied by tissue culture unit / ICCMGR, (passage number 190-204). The origin and description of this cell line was first mentioned by Al-Shammari [9]. The specimen was taken from murine mammary adenocarcinoma.

Human cervical cancer cell line (HeLa), was primarily established by (George Gey) from a 31 years old mother, named (Henrietta Lacks) [10]. This cell line was supplied by tissue culture unit/ ICCMGR, Baghdad, Iraq (passage number 50-70).

Rat Embryo fibroblasts (REFAM3), was established and kindly provided by Dr. A. Al-Shamery from ICCMGR. Cells of this normal murine cell line were a mixture of fibroblastic and epithelial cells with normal chromosomal picture.

Cell Maintenance and Culture Procedures:

AMN3, Hela and Ref cells are routinely grown as a monolayer in tissue culture grade flasks (dimeter 75 - 80 cm^2) kept in CO_2 incubator at 37ºC ± 1ºC, 90 % ± 5 % humidity, to detect 5.0 % ± 1 % CO2/ air. The cells examined on a daily basis under a phase contrast microscope, and any changes in morphology. Depending on protocol described by [6] cell lines used in this study were sub-cultured by the following procedure:

a. Decanting of medium RPMI, then briefly rinsing of cultures with 5 mL PBS. Followed by wash cells by gentle agitation to remove any remaining serum that might inhibit the action of the trypsin-versin.
b. Discarded the washing solution and repeating the rinsing procedure and discarding the washing solution was done later
c. Added of 1-2 ml trypsin-versin solution per 25 cm2 to the monolayer for a few seconds (e.g., 15-30 seconds).
d. Removed excess trypsin-versin solution and incubating the cells at room temperature.
e. Incubated for (2-3) minutes at 37ºC until they had detached from the falcon and detached the cells into a single cell suspension.
f. We added 15 ml of growth medium (10% fetal calf serum) to stop the effect of trypsin-versine and dispersed cell by pipetting with growth medium.
g. Approximately 12 ml of this growth medium containing cell suspension was transferred into sterile container used for seeding and the other as left to grow and incubated at 37ºC in presence of 5% CO_2.

Cell Counting:

After detaching the cells, 0.1- 0.2 ml of pre-warmed (37ºC) Routine Culture Medium/cm2 was add to the flask (e.g., 2.5 mL for a 25 cm2 flask). The monolayer was dispersed by gentle triturating. It is important to obtain a single cell suspension for exact counting. Counting of the cell suspension sample obtained using by a hemocytometer or RBC counter detected concentration of cell about 10000 cell for each well.

Subculture of Cells

After determination of cell number, the culture sub-cultured into other flasks or seeded into 96-well microtiter plates, then 100

µl of cell suspension was added to each well and incubated at 37 ºC for 24 hours.

Extract dilution

Alcoholic extract dilution:

Stock solution of the fruit and leaves methanolic extracts will add to RPMI media without serum to prepare the concentration 50000 µg /ml, then filtered by Millipore 0.2 mm. One ml of this concentration was taken and added to 9 ml media without serum that continued by serial one fold for 4 dilutions. To prepare the following concentrations 5000 µg /ml, 2.500 µg /ml, 1.250 µg/ml, 625 µg /ml and 312.5 µg /ml

Oily extract dilution

Stock solution of fruit and leaves oily extracts was added to RBMI to prepare the concentration 25 mg /ml, then 1 ml of this concentration was taken and added to 99 ml media without serum that continued by serial one fold for 4 dilutions. To prepare the following concentrations 250 µg /ml, 125 µg /ml ,62.5 µg /ml, 31.25 µg /ml and 15.625 µg /ml

Cytotoxicity assay method

When the cells are in exponential growth (approximately 70-80% confluent monolayer), treated cells with crude extracts has been done by removing the medium from the micro-titration plate and 200 µl of each of the serial dilutions of each oil and methanol extracts was add to each micro- titration plate. Three replicates were used for each concentration of either extract, and the plates were re-incubated at 37°C for the (72 hours). After that the medium was withdrawal and PBS was added for each well to relief remnant extract this was replaced with 50 µl MTT for alcoholic extract and a further 2 hr incubation at 37°C, the MTT was removed and the purple formazan product was dissolved in 50µl DMSO according to Mosmann [11]. While 50 µl crystal violet for oily extract after 20-minute incubation 37°C after that the dyes were washed by Distilled water and dried. Optical density (OD) of each well after treatment was read using Enzyme Linked. Immunosorbent Assay (ELISA) reader at a transmitting wavelength of 544 nm. The percentage of cytotoxicity was calculated as (A-B)/A X100, where A was the mean O.D of untreated wells (control) and B is the O.D of wells with plant extract according to Saotome et al [12] and modified by Itagaki [13].

Results

Cytotoxicity of AMN3 cell line

The result of AMN3 cell line inhibitory growth percent caused by different methanolic fruit and leaves palm extract showed a significant ($P< 0.05$) positive concentration dependent effect since the lowest and intermediate proportional. The concentration of 312.5 µg/ml, 625 µg/ ml, 1250 µg/ml caused growth inhibition percentage (GIP) by 41%, 47.3%, and 54.7% respectively, while the concentration of 2500 µg/ml showed the highest effect with inhibition growth of 73.3 % then a plateau effect was noticed at the highest concentration of 5000 µg/ml that caused 70% growth inhibition percent (GIP) as presented in table 1.

Table 1. Growth inhibition effect of fruit and leaves methanolic extract different concentrations in AMN3 cancer cell line after period of 72 hours exposurea.

Cell line	AMN3 cell	AMN3 cell
Concentration Alcoholic extract	growth inhibition % of Fruit methanolic extract	growth inhibition % of leaves methanolic extract
312.5 µg/ml	41 ± 1.04 C a	39.2 ± 1.02 C a
625 µg/ml	47.3 ± 2.1 BC a	44.6 ± 3.01 B C a
1250 µg/ml	54.7 ± 1.03 B a	51.5 ± 4.2 B a
2500 µg/ml	73.3 ± 4.01 A a	66.4 ± 2.03 A a
5000 µg/ml	70.1 ± 5.2 A a	60.3 ± 3.01 A b

LSD= 7.8
Different capital letters to significant result between concentrations ($p< 0.05$)
Different small letters to significant result within groups ($p< 0.05$

In comparison same pattern of significantly increasing ($P< 0.05$) in GIP accordingly with increasing concentration of methanolic leaves extract since the same concentration as in fruit extract caused in AMN3 less GIP effect of (39.2%, 44.5%, 51%, 66%) noticed at the highest concentration figure (1). The fruit and leaves oily extract showed the same pattern of concentration dependent GIP effect at much lower concentration (15.62, 31.25, 62.5, 125 and 250 µg/ml) with a plateau effect at higher concentration (table 2).

Table 2. Growth inhibition effect of fruit and leaves oily extract different concentrations in AMN3 cancer cell line after period of 72 hours exposure.

Cell line	AMN3 cell	AMN3 cell
Concentration oily extract	growth inhibition % of Fruit oily extract	growth inhibition % of leaves oily extract
15.62 µg/ml	49.7 ± 2.4 C a	40.5± 4.1 C b
31.25 µg/ml	54.2 ± 5.2 C a	47.1± 3.6 C a
62.5 µg/ml	75 ± 3.2 B a	56.2 ± 2.4 B b
125 µg/ml	85.1 ± 1.5 A a	66 ± 2.1 A b
250 µg/ml	83.9 ± 4.2 A a	65.7 ± 3 A b

LSD = 9.1
Different capital letters to significant result between concentrations ($p< 0.05$)
Different small letters to significant result within concentrations ($p< 0.05$)

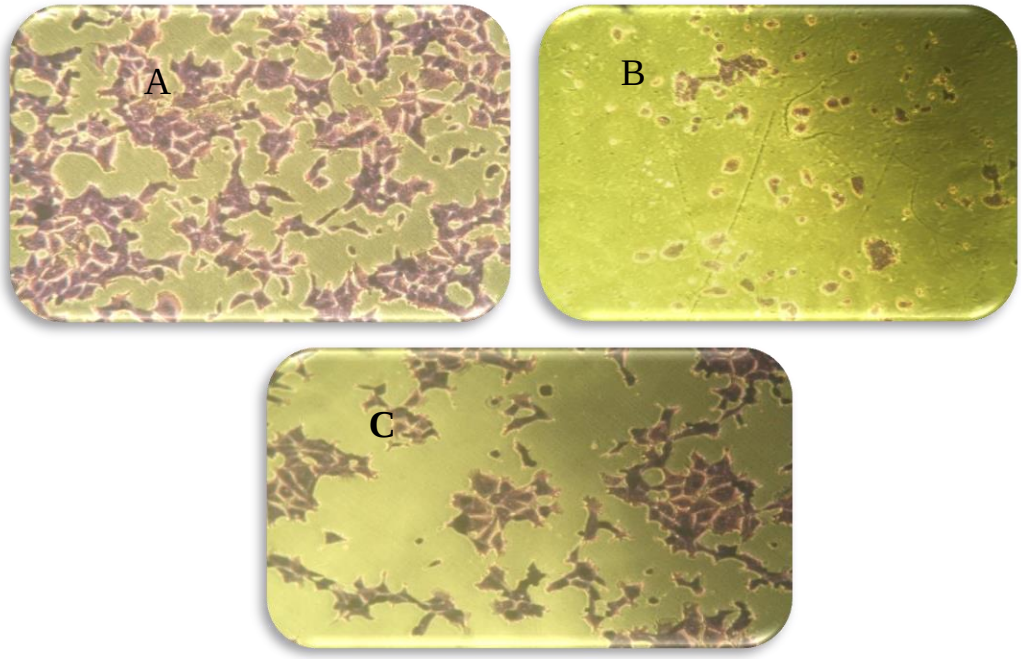

Figure 1. Images for AMN3 cells after exposure to different concentrations for 72 hr at 37ºC. (A) control untreated cells (B) treated with of date methanolic extract at concentration 2500 µg/ ml, in GIP: (73.3). (C) cells treated with leaves oily extract at concentration 15.62 µg/ml cells, in GIP (40.5). used crystal violet magnification 20X

Cytotoxicity of Hela cell line

The result of GIP listed in table (3) and figure (2-B) showed positively proportional effect according the increasing concentration of fruit and leave methanolic extracts (312.5 µg/ ml, 625 µg/ ml, 1250 µg/ml, 2500 µg/ml, and 5000 µg/ml) that caused significant $p< 0.05$ GIP of (46.1%, 54.3%, 62.2%, 76.7 % and 73.5%) for fruit methanolic extract respectively and GIP of (41%, 47.2%, 51.3%, 53± 1.1%, 53% and 55.4%) for leaves methanolic extract respectively. The same pattern was seen for the effect of fruit and leaves oily extract in which much less concentration was used (15.62, 31.25, 62.5, 125 and 250 µg/ml) to exert significant GIP effect in hela cell line as listed in table (4) and figure (2 - C). Plateau in GIP effect were also seen at the higher concentration for both fruit and leave oil and alcoholic extracts.

Table 3. Growth inhibition effect of fruit and leaves methanolic extract different concentrations in Hela cancer cell line after period of 72 hours exposure.

Cell line	Hela cell	Hela cell
Concentration methanolic extract	growth inhibition % of Fruit methanolic extract	growth inhibition % of leaves methanolic extract
312.5 µg/ml	46.1± 1.2 C a	41± 2.05 B a
625 µlg/ml	54.3± 3.3 CB a	47.2± 1.1 AB a
1250µg/ml	62.2± 2.03 B a	51.3± 4.04 A b
2500µg/ml	76.7± 3.1 A a	55.4± 3.02 A b
5000 µg/ml	73.5 ± 1.05 A a	53± 1.1 A b

LSD =9.4
Different capital letters to significant result between concentrations ($p< 0.05$)
Different small letters to significant result within concentrations ($p< 0.05$)

Table 4. Growth inhibition effect of fruit and leaves oily extract different concentrations in Hela cancer cell line after period of 72 hours exposure.

Cell line	Hela cell	Hela cell
Concentration oily extract	growth inhibition % of Fruit oily extract	growth inhibition % of leaves oily extract
15.62 µg/ml	53.2±2.2 CB a	50.1±2.01 C a
31.25 µg/ml	60.3±1.07 B a	56±1.04 CB a
62.5 µg/ml	70±3.03 A a	65.2±4.3 B a
125 µg/ml	79.1±2.4 A a	77±1.1 A a
250 µg/ml	78 ± 1.06 A a	76.1±3.07 A a

LSD =10
Different capital letters to significant result between concentrations ($p< 0.05$)
Different small letters to significant result within concentrations ($p< 0.05$)

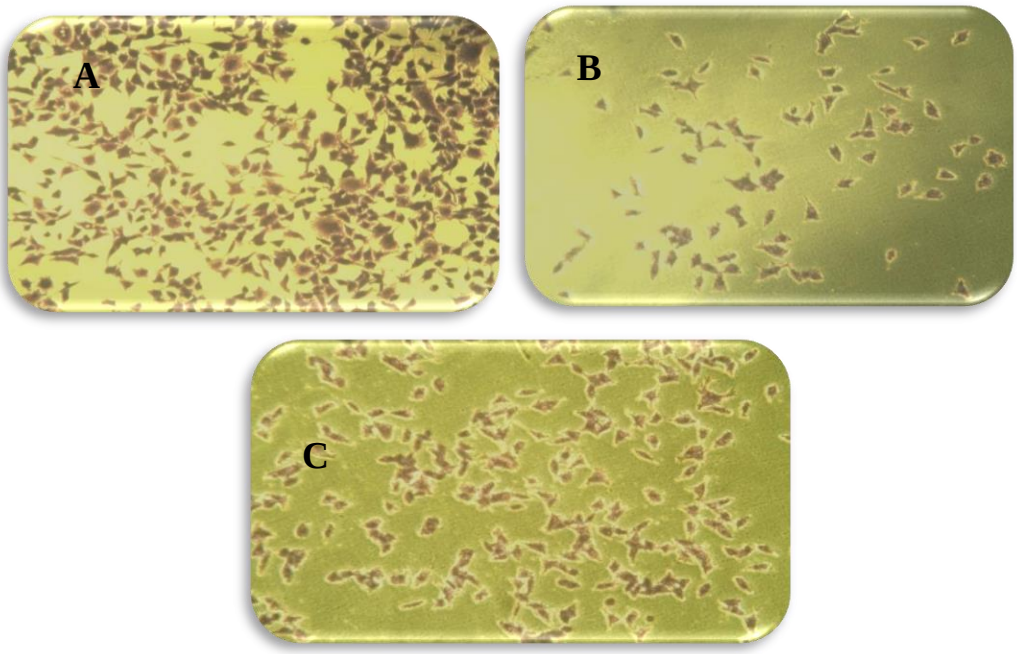

Figure 2. Images for Hela cells after exposure to different concentrations for 72 hr at 37ºC. (A) control untreated cells, (B) cells treated with date methanolic extract at concentration 2500 µg/ ml, in GIP: 76.7%. (C) cells treated with leave oily extract, at concentration 15.62 µg/ml, in GIP: 50.1%. used crystal violet magnification 20X.

Cytotoxicity of Ref cell line

The result of GIP in Ref cell line culture for palm fruit and leaves methanolic extracts showed no effect in all concentration used, while the concentrations of fruit and leave oily extracts of (15.62 µg/ml, 31.25 µg/ml 62.5 µg/ml125 µg/ml and 250 µg/ml) exert significant growth inhibition percentage (P< 0.05) of (36.2 %, 37 %, 46.3 %, 61.7 % and 52.3%) for leave oily extract and (45 %, 46.1 %, 51.2 %, 76 % and 68.4 %) for fruit oily extract respectively table (5) and figure (3).

Table 5. Growth inhibition effect of fruit and leaves oily extract different concentrations in Ref cancer cell line after period of 72 hours exposure.

Cell line	Ref cell	Ref cell
Concentration oily extract	growth inhibition % of Fruit oily extract	growth inhibition % of leave oily extract
15.62 µg/ml	45 ± 2.3 BC a	36.2 ± 1.03 BC a
31.25 µg/ml	46.1 ± 4.2 B a	37 ± 0.38 CB a
62.5 µg/ml	51.2 ± 1.06 B a	46.3 ± 2.01 B a
125 µg/ml	76 ± 3.02 A a	61.7 ± 1.05 A b
250 µg/ml	68.4 ± 2.1 A a	52.3 ± 2.4 AB b

LSD =12.7
Different capital letters to significant result between concentrations (p< 0.05)
Different small letters to significant result within concentrations (p< 0.05)

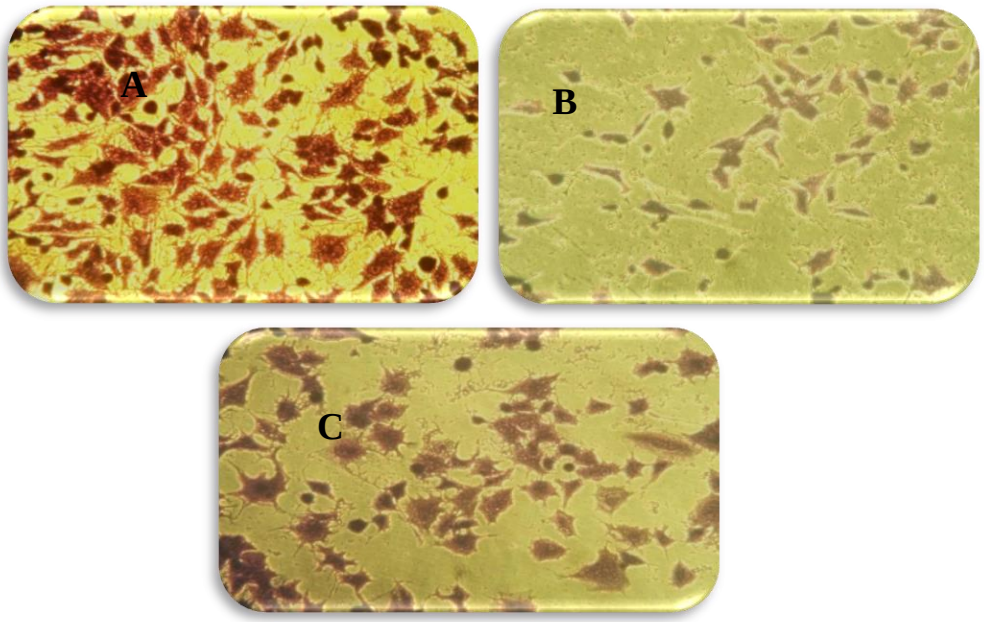

Figure 3. Images for Ref cells after exposure to different concentrations for72 hr at 37ºC. (A) control untreated cells (B) cells treated of date oily extract at concentration 125 µg / ml, in GIP : 76% (C) cells treated with leaves oily extract at concentration 15.62 µg/ml, in GIP: 36.2%.used crystal violat stain magnification 20X.

Discussion

Current results of the inhibitory effect of different palm date and leaves extracts against in vitro growth of cell lines culture (AMN3, Hela, and Ref) that showed dose dependent inhibitory cancer growth for all extracts against all cell line especially AMN3 and Hela with the superiority of the oil palm extracts for date and palm leaves in opposing cancer cell growth over their alcoholic one since the concentration used was 1/20 less than that of alcoholic indicating their potent effect. This possibly because oily extract contain fat soluble content with more potent anticancer effect like some essential oils and fatty acids [14] with other antioxidant phytochemicals (flavonoid, saponin) that present more in alcoholic than oily one according to the phytochemical results [15], so one can conclude from such results that the extract mechanism of inhibitory action of extracts against cancer cell growth were not only antioxidative effect but possibly other reported mechanisms like receptor mediated inhibitory effect (16) or hypermethylation or induction of suppressor gene , apoptosis , DNA repair or increasing cellular gap junction.

The tested cancer cell inhibitory effect induced by all extracts showed a plateau effect at the higher concentration at 5000 µg/ml for alcoholic extracts and 250 µg/ml for oily extracts indicating saturative kinetic with nonlinear effect.

P. dactylifera (PD) fruits have been documented possess antioxidant activity due to the presence of water-soluble compounds with potent free radical-scavenging effects, such as phenolic compounds (mainly cinnamic acids) and flavonoids (flavones, flavonols and flavanones [17]. PD fruits are proved to possess carotenoid content, consumption of which has been related to prevention of cancer, cardiovascular diseases and other degenerative processes involving oxidative stress [18].

The quantification of the amounts of the phytochemicals present in oil palm leaves confirmed flavonoid, tannin and phenolic are the main phytochemical constituents in oil palm leaves. [15]. Based on the study, the high content of phenolic, tannin and flavonoid compounds may contribute to its antioxidant activity [19].

The result of the gas chromatography in palm oil indicated that Linoleic acid was the most predominant fatty acid in this plant oil; oleic acid and linolenic acids were also present. There was a significant presence of palmitic and stearic acids as well [20].

Meanwhile, not only the oil palm fruit containing many bioactive compounds such as fatty acid and essential oil, but the leaves also contain leveled amount of bioactive compounds, in fact in much diverse and higher in concentration oil contains mainly palmitic and oleic acids and is about 50% saturated [21].

Constituents of medicinal plants such as flavanoid and phenol play a significant role in cancer control through the regulation of genetic pathways without any side effects [22]. So daily consumption of antioxidants enhances immunity of the body against free radicals production and serves as anticancer [23]. Dietary polyphenols may exert their anticancer effects via a variety of mechanisms such as removal of carcinogenic agents, modulation of cancer cell signaling and antioxidant enzymatic activities, and induction of apoptosis and cell cycle arrest. Some of these effects may be related, at least partly, to their indirect antioxidant activities. For example, the enhancement of GPx, catalase, NQO1, GST and/or phase II enzyme activities by polyphenols could help the detoxification of carcinogenic agents [24].

Several observations have suggested that natural flavonoids have growth inhibitory effects on various kinds of cancer cells mediated by different molecular targets and acting through diverse metabolic pathways. However, the precise mechanisms responsible for the antitumor effect of flavonoids are still not thoroughly understood. Flavonoids can easily bind to the cell membrane, penetrate *in vitro* cultured cells, and modulate the cellular metabolic activities. Mitigation of oxidative damage, inactivation of carcinogen, inhibition of proliferation, promotion of differentiation, induction of cell cycle arrest and apoptosis, impairment of tumor angiogenesis, and suppression of metastasis contribute to the anticarcinogenic activities of flavonoids [25].

Flavonoids have been found to arrest cell-cycle progression at either G_1/S or G_2/M boundaries by modulating multiple cell cycle regulatory proteins. Somewhat conflicting results have been reported with regard to the stage-specific arrest caused by one and the same compound, and several studies have indicated the ability of flavonoids to block the cell growth at more than one stage of the cell cycle [26]. Quercetin could suppress the viability of Hela cells in a dose-dependent manner by inducing G2/M phase cell cycle arrest and mitochondrial apoptosis through p53-dependent mechanism and quercetin may induce apoptosis by direct activation of caspase cascade through mitochondrial pathway and by inhibiting survival signaling in Hep-G2 [27].

In vitro anticancer study performing by Al- juraisy, et al. [28] which examined crude extracts of fruits and pits of date (*Phoenix dactylifera L*. Zahdi) on two malignant cell lines (human laryngeal carcinoma-Hep2 and murine mammary. The in vitro cell growth assay showed that there were time- and concentration dependent cytotoxic effects of crude extracts of fruits and pits on Hep2 and AMN3 cell lines.

An in vitro study was conducted to evaluate the antioxidant potential of *P. dactylifera* fruit aqueous extract by determination of its hydroxyl-radical-scavenging potential. There was a significant dose dependent relationship in the inhibition of hydroxyl-radical inhibition by *P. dactylifera* extract with complete inhibition shown at a concentration of 4.0 mg/mL. The antioxidant effects of *P. dactylifera* were found to hinder lipid peroxidation and inhibit free radical-mediated oxidation of lysine, arginine, and proline residues of proteins, leading to prevention of the formation of carbonyl derivatives. [25].

Dietary polyphenols help reduce oxidative damage, and the related chronic diseases risk. Many of them interfere with signal transduction regulation at different levels modulating hormones/growth factors activities, inhibit oncogenes and activate tumour suppressor genes, induce terminal differentiation, activate apoptosis (cancer cell death), restore immune response, inhibit angiogenesis and decrease inflammation [27]. Conclude from such results that the extract mechanism of inhibitory action of extracts against cancer cell growth were not only antioxdative effect but possibly other reported mechanisms like receptor mediated inhibitory effect [16] or hypermethylation or induction of suppressor gene, apoptosis, DNA repair or increaseing cellular gap junction.

Conclusion:

The main goal of the current study was to determine the effects of alcoholic and oily extract of phoenix dactylifera an anticancer in vitro cell lines AMN3, Hela, Ref.

The study concluded that alcoholic extracts of date and leaves of phoenix dactylifera inhibition of in vitro cancer cell lines AMN3, Hela, Ref and the superiority of oil extract for both date and leave over alcoholic one in inhibition of in vitro the same cell lines, indicated presence of effective phytochemical not present in alcoholic extract possibly essential oil, alkaloid and resin as well as fat soluble vitamins. The concentration dependent effects and plateau were noticed in high concentrations of palm extracts against in vitro cell line growth indicating either maximum effect or hormesis effect.

Conflict of interest

The authors have no conflict of interest.

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