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Study the *in vitro* effect of alcoholic extract of Prunus aremasia kernels, Methotreaxte, amygdalin and the combination between them on Hela cancer cell line

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Abstract:

This study was done to evaluation the cytotoxicity of each apricot kernels ethanolic extract at concentration (100-500) µg/ml, amygdalin and methotreaxat at concentration (0.1-1000) µg/ml, (plant extract plus methotreaxat) and (amygdalin plus methotreaxat) at half the original concentration for each one on Hela (human cervical cancer cell line) in vitro .

A mixtures of (extract plus Methotreaxte) and (amygdalin plus Methotreaxte) was used together to evaluated its cytotoxicity on cancer cell line for indentify about the possibility of synergistic effect between them.

The results shows the cytotoxicity of (plant extract and methotreaxat) on Hela cancer cell line was same as the cytotoxicity of plant extract alone and more than the cytotoxicity of Methotreaxte alone, while the cytotoxicity of (amygdalin and methotreaxat) on Hela cancer cell line is similar to the cytotoxicity of amygdalin alone and more than the cytotoxicity of Methotreaxte alone.

Key word: *amygdalin, apricot kernels, methotreaxat, Hela cancer cell line.*

Introduction:

Medical plants have been identify and used through the history of human , plants have ability to synthesize of many different types of chemical compounds which useful to performed the important biological functions , there is at least 12.000 kinds of compounds have been isolated (25,12).

A lot of recent investigations about medical Plants have been done for advancements in the treatment and control of cancer progression; the main disadvantages of synthetic drugs are the associated side effects. However natural therapies, such as the use of the plants or plant derived natural products are being beneficial to kill cancer cells , The search for anti-cancer agents from plant sources started in the 1950s when discovery and development of the vinca alkaloids (vinblastin and vincristine), and the isolation of the cytotoxic podophyllotoxins were carried out (9) prunus aremasia kernels shows also a promising anticancer activity where is several study done for evaluated the anticancer activity of apricot kernels where (2) found an apricot kernels extract has a significant cytotoxic effect on human hepatoma and human lung carcinoma cancer cell lines , while another study found the maximum cytotoxic effect of serial apricot kernel extract concentration on rhabdomyosarcoma cell line was (500, 750 and 1000) µg/ml at 72 hr, incubation time (5) , the major role of apricot kernels anticancer related to its amygdalin contains activity , where amygdalin was reported to selectively kill cancer cells without systemic toxicity and effectively relief pain in cancer patients (28) with founded of several phytochemical that related to its anticancer activity as (falvonoids, tannins and terpenoids) which have anticarcinogenic and antioxidant activities (20) ,

Amygdalin and modified form named laetrile or vitamin B17 , both promoted as cancer cures (13) , amygdalin is a glycoside separated from the seeds of prunus dulcis , also known as bitter almond , there are many different species of prunus genus such as apricot (prunus aremasia) and black cherry (prunus serotina) contains amygdalin also (24) ,

Chemotherapy was one form the routine treatments of cancer, side effects that resulting from harmful effect on normal cells was the major problem that associated with the treat-

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ments with chemotherapy resulting from the killing effect of chemotherapy on each cancer cells and highly proliferated normal cells (6) there was several medical plants show ability to ameliorated the chemotherapy side effect , as apricot kernel which show ability to prevents the undesirable effect of Methotreaxte related to its ability to induce antioxidant activity via increase of catalyze superoxide dismutase and glutathione level (26)

Material and Methods:

1- plant Extraction:

Iraqi Apricot fruits kernels (Prunus armeniaca) were collected from local Iraqi market, the individual stone were hammered to obtain the seed kernel, the kernels were dried in oven at 40 C˚ than grounded to powdered by using an electric blender (the powder then kept in dry sealed container), the plant that used in this study was Iraqi Prunus armeniaca .

2- Preparation of ethanolic extracts 70% of apricot kernels:

According to (10), alcoholic extract of plant was prepared as follows: 100 gram weight of plant powder were suspend in 1000 ml 70% ethanol (4% acetic acid), extraction was done by soxhlet apparatus which last for 24 hrs.

3 - Chemotherapeutics agent:

Methotrexate vial at concentration (vial 50 mg/5cc - kocak / turkey) was used at different concentration ranged between (0.1-1000) µg/ml, after diluted with media without serum

4 - Standard agent (amygdalin):

Amygdaline were purchased from Santa Cruz (Santa Cruz, CA, USA), and used at different concentration which ranged between (0.1-1000) µg/ml, these concentration achieved by dilution the amygdaline with free serum media.

5- Cell culture:

Human cervical cancer Hela cell line and Rat embryo fibroblast REF cell line were purchased from tissue culture unit/ Iraqi Centre for Cancer and Medical Genetics Research (ICCMGR), The cells were cultured in 75 cm2 tissue culture flasks under humidified 5% CO2 atmosphere at 37°C in RPMI-1640 medium (Sigma chemicals, England) with 10% fetal bovine serum (FBS), and penicillin- streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin) During the course of the experiment (7).

6 -Cytotoxicity Assay

Cells cultures in microtiter plate (96wells) were exposed to range of (plant extracts, amygdalin, methotreaxat, (plant extract with methotrexate) and (amygdalin with methotrexate) concentration during the log phase of growth and the effect determined after several incubation time. (7) , each well contain 1X104cells/well, Serum calf medium 10% used for seeding, Plates were then incubated for 24hrs in 37□C for achieve cell attachment, then By using maintenance medium, fivefold serial dilution were prepared starting from (100-500 µg/ml) for ethanolic extract of prunus armeniaca (4) and at concentration ranged between (0. 1-1000 µg/ml) for amygdalin and Methotreaxte, while the concentration for (plant extract with methotreaxat) ranged between (50+0.05 to 250+500 µg/ml) for each plant extract and Methotreaxte respectively, and at (0.05- 500 µg/ml) for (amygdalin with methotreaxat) .

After incubation for 24 hrs, cells were exposed (Six replicate at 200µl for each tested concentration), 200 µl of maintenance medium added to each well of control group, the times of exposure were 24, 48 and 72 hrs. The plates were sealed with self adhesive film then returned to incubator, cells where staining with MTT stain.

The optical density of each well was read by using a micro-ELISA reader at a transmitting wavelength on 550 nm (15) (7).

The inhibition rate was calculted according to (8) as follows:

IR% = ((A – B)/A) x 100

IR=inhibitor rate, A= the optical density of control, B= the optical density of test.

7 - Statistical Analysis

The Statistical Analysis System (19) was used to identify the effect of different factors in study parameters. Least significant difference –LSD test was used to compare between means in this study significantly, all experiments were performed in the Iraqi centre for cancer and medical genetic research

Results and Discussion:

1-plant Extraction:

After end of hot ethanolic extraction of Prunus armeniaca kernels, the product was dried by using of rotary evaporator, the result of drying was a material appearance like dark brown sticky material, with extraction ration about 12% resulting from getting 23 gram of extract from 100 gram of dry powder of prunus armeniaca kernels , the method of apricot kernels extraction was done by using (ethanol 70% , citric acid 4%) and that for ensure getting a maximum chemical constituents of apricot kernel , in addition of , the extraction ratio was 12 % , using of 4% acetic acid in procedure of extraction that for prevent epimerization of amygdalin into neoamygdalin (27) ,

3 - The cytotoxicity study on Hela cancer cell lines

1- The cytotoxicity of *Prunus aremasia* extract :

The result reveled to the maximum cytotoxic effect occur at (300,400, and 500) µg/ ml with a significance variation comparing with control at level ($p<0.05$), without a significant variation between the cytotoxic effect for each (400and 500) µg/ ml between the three incubation period, the maximum growth inhibition rate for all incubation period occur at 300 µg/ ml. table (1)

Table (1) Effect of extract exposure on growth inhibition rate on HELA cancer cell line

Inhibition effect of Extract on cell viability %			
Concentration (µg/ml)	**24 hr. Mean ± SE**	**48hr. Mean ± SE**	**72hr. Mean ± SE**
100	**19 ± 0.82 C b**	**29 ± 1.14 C a**	**17 ± 0.67 B b**
200	**39 ± 1.48 B a**	**36 ± 1.84 B a**	**22 ± 0.52 B b**
300	**54 ± 2.75 A a**	**45 ± 2.39 A b**	**50 ± 2.58 A ab**
400	**45 ± 2.09 B a**	**45 ± 2.47 A a**	**48 ± 2.61 A a**
500	**42 ± 1.69 B b**	**43 ± 1.94 A b**	**49 ± 1.88 A a**

Different capital letter represents significant differences ($P<0.05$) between means of the same column; Different small letters represent significant differences ($P<0.05$) between means of the same row.

The results was compatible with the study that done by (2) by using a serial concentration of apricot kernels extract ranged between (100-400) µg/ml they found the maximum cytotoxic effect on Human hepatoma and human lung carcinoma cancer cell lines occur at 400 µg/ml on incubation time 72hr.

The growth inhibition effect of the alcoholic extract of prunus aremasia on cancer cell lines may attributed to their phytochemical (amygdalin) contains as induce of apoptosis effectively (3)

Amygdalin is composed of two molecule of glucose, one benzaldehyde which induce an analgesic action and on hydrocyanic acid, which is an antineoplastic compound (17), cyanide act by minimized the activity of cytochrome C oxidase in the respiratory electron transport chain of mitochondria, impairing both oxidative metabolism and associated process of oxidative phosphorylation thereby it cause death through energy deprivation (18).

In addition of hydrocyanid, benzaldehyde that liberated from hydrolysis of amygdalin by β- glucosidase inside the cancer cells show antineoplastic effect , (14) find benzaldehyde and its derivatives have antitumor activity in mice against Ehrlich carcinoma, adenocarcinoma and colon carcinoma by mechanism of action included the ability of (β-cyclodextrin benzaldehyde inclusion compound) to induce internucleosomal DNA fragmentation in human myelogenous leukemia cell line and Caspase 3,8,9 activation , which induce the destruction of mitochondrial structure and digestion of broken organelles by secondary lysosomes in all of these cells , also induce autophagic cell death in cancer cell lines.

several study were done to evaluated the antineoplastic effect of amygdalin , where (11) find amygdalin ability to reduce proliferation potential , minimize mitochondrial activity of cervical cancer cells and accumulation cells in G1 phase leading to cells death .

Beside amygdalin the antineoplastic effect of extract related to other phytochemicals found in the extract such as (falvonoids, tannins and terpenoids) which have anti-carcinogenic and antioxidant activities (20) where the mechanism of falvonoids and its derivatives as anticancer depends on presence of iron in the cells where falvonoids and its derivatives come to contact with iron by chemical reaction ensures spawning charged atoms that effect free radical , because cells need iron to replicate DNA when they divided and since cancer cells is characterized by out of control cell division , cancer cells have much higher iron concentration comparing with normal cells pumping up cancer cells with even more iron and then introduce falvonoids selectively kill these cells (23)

2- The cytotoxic effect of amygdalin:

The result revealed the maximum cytotoxic effect occur at (1000) µg/ ml for each three incubation period with a significance variation comparing with control at level ($p<0.05$), with a significance variation between the (0.1) µg/ ml comparing with other concentration for each (48 and 72) hr. with a significant variation for (1, 10, 100, 1000) µg/ ml between (48, 72) hr comparing with 24 hr. table (2)

Table (2): Effect of concentration and time in growth inhibition rate for Amygdalin on Hela cancer cell line

Inhibition effect of Amygdalin on cell viability %			
Concentration (µg/ml)	24 hr. Mean ± SE	48hr. Mean ± SE	72hr. Mean ± SE
0.1	0 ± 0.00 D a	0 ± 0.00 B a	0 ± 0.00 B a
1	7 ± 0.25 C b	34 ± 1.41 A a	33 ± 1.38 A a
10	10 ± 0.48 C b	35 ± 1.60 A a	35 ± 1.27 A a
100	19 ± 0.71 B b	36 ± 1.29 A a	35 ± 1.54 A a
1000	30 ± 1.46 A b	38 ±2.05 A a	37 ± 1.16 A a

Different capital letter represents significant differences (P<0.05) between means of the same column; Different small letters represent significant differences (P<0.05) between means of the same row.

This result showed, there is an increase in the cytotoxicity occur with increasing of the (concentration and time of incubation) i.e. its (dose depend and time depend) , another study was done have a compatible result by using the same concentration of the present study , where (2) find ,by using amygdalin at concentration ranged between (10-10000) µg/ml exert dose depend cytotoxic effect on prostate cancer cells by induce apoptotic cell death by Caspase-3 activation through the down regulation of anti apoptotic Bcl-2 protein and the up regulation of proapoptotic of Bax protein in prostate cancer cells . another study show that amygdalin dose dependently reduce growth and proliferation in three bladder cancer cell lines, reflected in a significant delay in cell cycle progression and G0/G1 arrest (16) ,

3-The cytotoxic effect of methotrexate:

The result revealed the maximum cytotoxic effect occur at (1000) µg/ ml for (24 and 72) hr. incubation period significantly comparing with control at level (p<0.05), and without a significance variation for 1000 µg/ ml, between (24 and 72) hr comparing with 48 hr incubation period, table (3)

Table (3): Effect of concentration and time in growth inhibition rate for methotrexate on Hela cancer cell line

Inhibition effect of Methotreaxat on cell viability %			
Concentration (µg/ml)	24 hr.	48hr.	72hr.
0.1	0 ± 0.00 C a	0 ± 0.00 C a	0 ± 0.00 C a
1	17 ± 0.62 B a	4 ± 0.06 C b	0 ± 0.00 C b
10	19 ± 0.59 B a	10 ± 0.37 B b	0 ± 0.00 C c
100	30 ± 1.24 A a	14 ± 0.51 AB b	17 ± 0.55 B b
1000	37 ± 2.49 A a	19 ± 0.62 A c	31 ± 1.07 A b

Different capital letter represents significant differences (P<0.05) between means of the same column; Different small letters represent significant differences (P<0.05) between means of the same row.

The result of study shows the maximum cytotoxicity occur at (24)hr. incubation period comparing with 48 hr and 72 hr incubation period , may related to the resistance of the cancer cells to the methotrexate activity , where there is several mechanism explain the resistance of cancer cells to the methotrexate effect included impaired transport of methotrexate into cells , production of altered form of DHFR that have decrease affinity for the inhibition , increase concentration of intracellular DHFR through gene amplification or altered gene regulation and decrease ability to synthesis methotrexate polyglutamat (1).

4 - The cytotoxic effect of (alcoholic extract of prunus aremasia and methotrexate) :

The result revealed the maximum cytotoxic effect occur at (150/5) µg/ ml for (24 , 48 and 72) hr. incubation period with a significance variation comparing with control at level ($p<0.05$), without a significance variation for each (200/50-250/500) µg/ ml Between (24 , 48 and 72) hr incubation period , table (4)

Table (4): Effect of concentration and time in growth inhibition rate for (Extract, methotrexate) on Hela cancer cell line

Inhibition effect of (Extract, methotrexate) on cell viability %			
Concentration (µg/ml)	**24 hr. Mean ± SE**	**48hr. Mean ± SE**	**72hr. Mean ± SE**
50/0.05	**28 ± 1.35 C a**	**27 ± 0.62 B a**	**13 ± 0.52 C b**
100/0.5	**45 ± 2.63 B a**	**37 ± 1.47 A b**	**35 ± 1.36 B b**
150/5	**54 ± 2.75 A a**	**41 ± 1.08 A b**	**52 ± 2.73 A a**
200/50	**47 ± 1.89 B a**	**43 ± 2.15 A a**	**48 ± 2.48 A a**
250/500	**43 ± 1.64 B a**	**43 ± 1.72 A a**	**48 ± 2.36 A a**

Different capital letter represents significant differences (P<0.05) between means of the same column; Different small letters represent significant differences (P<0.05) between means of the same row.

This result indicated there is an additive effect between the extract and methotrexate as anticancer. with a less Occurrence of antagonism between the extract and methotrexate that may related to the mechanism of mixture anticancer activity which occur by several unrelated mechanism involved the action of amygdalin (cyanide & benzaldehyde), ,phytochemicals (flavonoids and terpenoids) and Methotreaxte , Where decrease in the cytotoxicity at concentration (250/500) µg/ml when comparing with (150/5) µg/ml at 24hr. incubation time, may related to the high variation in the methotreaxat concentration from 5 µg / ml to 500 µg / ml comparing with the narrow variation between extract concentration which elevated from 150 µg / ml to 250 µg / ml where this variation between the elevation in methotreaxat comparing with extract may lead to impaired ability of methotreaxat to extract cytotoxicity , that may related to minimized of extract influx into the cells by forming of chemical complex between Methotreaxte and specific chemical constituents in the extract that related to it the anticancer activity or by Methotreaxte blocking activity to the specific cellular receptors by which the active chemical constituents of extracts influx to the cells , that related to the large gap between the plant extract and methotreaxat concentration from 150/5 to 250/500 µg / ml.

5 - The cytotoxic effect of (amygdalin and methotrexate):

The result revealed the maximum cytotoxic effect occur at (500/500) µg/ ml for each (24 , 48 and 72) hr. incubation period with a significance variation comparing with control at level ($p<0.05$) , without a significance variation between (50/50) (500/500) µg/ ml for reach (24 , 48 and 72) hr incubation period, table (5)

Table (5): Effect of concentration and time in growth inhibition rate for (amygdalin, methotrexate) on Hela cancer cell line

Inhibition effect of (amygdalin, methotrexate) on cell viability %			
Concentration (µg/ml)	**24 hr.**	**48hr.**	**72hr.**
0.05l0.05	**24 ± 0.62 B b**	**33 ± 0.93 A a**	**17 ± 0.63 B c**
0.5/0.5	**27 ± 0.88 B c**	**35 ± 1.47 A b**	**49 ± 2.48 A a**
5/5	**28 ± 0.79 B c**	**36 ± 1.39 A b**	**52 ± 2.36 A a**
50/50	**44 ± 2.55 A b**	**37 ± 1.62 A c**	**53 ± 1.89 A a**
500/500	**48 ± 1.37 A b**	**38 ± 2.09 A c**	**55 ± 2.38 A a**

Different capital letter represents significant differences (P<0.05) between means of the same column; Different small letters represent significant differences (P<0.05) between means of the same row.

This result show an increase in the cytotoxicity on cancer cells occur with increase in each (concentration of amygdalin and methotrexate) and (time incubation), according to the result the cytotoxic effect of the mixture (amygdalin and methotrexate) was more than the cytotoxic effect of each amygdalin or methotrexate alone ,without resistance developments from the cancer cells toward the mixture effect , these effects may related to a additive effect between amygdalin and methotrexate resulting from the mixture anticancer effect occur by multiple mode of action without interruption between these mechanism , where the anticancer activity of mixture related to cyanide , benzaldehyde and Methotreaxte activity .

In addition of the less occurrence of resistance toward the methotreaxat in the mixture causing increase in the cytotoxicity of mixture comparing with methotreaxat alone and that may related to low concentration of methotreaxat that used in the mixture comparing with the concentration of methotreaxat alone ,where gene amplification consider as the common mechanism for cell line resistance related to high dose of Methotreaxte exposure (**22**) where increase in the level of DHFR activity in human methotrexate resistance cancer cell line occur due to gene amplification (**21**) .

Conclusion:

The study result indicated to the cytotoxicity of apricot kernels extract and the cytotoxicity of the mixture of (extract and methotreaxat) is more than the cytotoxicity of methotreaxat alone on Hela cancer cell line, the cytotoxicity of (extract with Methotreaxte) may related to the ability of apricot kernels extract to minimized the occurrence of resistance of cancer cells to methotreaxat activity, related to less concentration that used in methotreaxat.

The result concluded also the anticancer activity of a mixture of (amygdalin and Methotreaxte) shows more cytotoxic effect toward cancer cell line Hela comparing with the cytotoxicity of each one alone, this elevation in the mixture cytotoxicity may related to the ability of mixture to overcome the resistance that developments toward methotreaxat cytotoxicity , and that mainly associated by less concentration of methotreaxat in the mixture comparing with methotreaxat concentration alone and related to the ability of amygdalin in minimized of methotreaxat resistance .

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دراسة تأثير المستخلص الكحولي لبذور نبات المشمش ومزجة مع كل من الميثوتركسيت و الامكدالين في تثبيط نمو خلايا سرطان عنق الرحم (Hela) النامية في الزجاج

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الخلاصه:

تم اجراء هذة الدراسة لتقييم السمية الخلوية لكل من المستخلص الكحولي لبذور نبات المشمش وبتراكيز تراوحت مابين (100-500) مايكروغرام / ملل و الامكدالين و الميثوتركسيت وبتراكيز تراوحت مابين (0,1-1000) مايكروغرام / ملل و المزيج مابين (المستخلص الكحولي لبذور نبات المشمش مع الميثوتركسيت) والمزيج مابين (الامكدالين والميثوتركسيت) وبأستخدام نصف التراكيز المستخدمة لكل علاج منفردا على نمو خط خلايا سرطان عنق الرحم (Hela) النامية في الزجاج .

تم استخدام المزيج مابين كل من (المستخلص الكحولي لبذور نبات المشمش مع الميثوتركسيت) والمزيج مابين (الامكدالين والميثوتركسيت) لاجل التحري عن وجود ظاهرة التعاضد مابين العلاجات المختلفة في تثبيط نمو الخلايا السرطانية .

وقد اظهرت النتائج ان السمية الخلوية لمزيج (المستخلص الكحولي لبذور نبات المشمش مع الميثوتركسيت) في تثبيط نمو الخلايا السرطانية مقاربة الى السمية الخلوية للمستخلص النباتي منفردا واعلى من السمية الخلوية للميثوتركسيت منفردا , بينما السمية الخلوية للمزيج (الامكدالين والميثوتركسيت) فقد كانت مقاربة الى السمية الخلوية للامكدالين منفردا واعلى من السمية الخلوية للميثوتركسيت منفردا .