

**Cytotoxic Effect of Alkaloid Extract of *Equisetum arvense* plant on Human lymphocytes and MCF7 Cancer Cell line**

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**Cytotoxic Effect of Alkaloid Extract of *Equisetum arvense* Plant on Human lymphocytes and MCF7 Cancer Cell Line**

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**Abstract**

This study aimed to investigate the effect of alkaloids extracted from *Equisetum arvense* on the proliferation of lymphocytes, breast cancer cell line (MCF7) and mice embryo fibroblast normal cell line (MEF) by exposing them for different concentrations for a period of 24 hours. The results showed that in the absence of phytohaemagglutinin (PHA), the alkaloid extract did not stimulate the division of human lymphocytes at all concentrations used. In contrast in the presence of PHA, the alkaloid extract decreased the division of human lymphocytes at all concentrations used. This decrease was significant compared with the control group, which gave a division ratio of 4.20%. The effect of alkaloid extract on the growth of MCF7 cancer cell line demonstrated that the lowest inhibition rate 20.07% was at the concentration 15.1 µg/ml, and the higher inhibition rate was 64.1% at the concentration 500 µg/ml. While in the MEF normal cell line, the higher inhibition rate was 11.3% at the concentration 500 µg/ml. In conclusion, this study showed that the alkaloid extract of *E. arvense* plant affects the growth of MCF7 cancer cell line and have a lower effect on the growth of lymphocytes and MEF normal cell line at high concentrations, making this plant promising candidate for the treatment of cancer but this needs more studies to prove that.

**Keywords:** *Equisetum arvense*, Lymphocyte, Alkaloids, MCF7 cancer cell line.

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### التأثير السمي الخلوي للمستخلص القلويدي لنبات ذنب الخيل *Equisetum arvense* على الخلايا اللمفاوية للإنسان والخط الخلوي السرطاني MCF7

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#### الخلاصة

هدفت الدراسة إلى اختبار تأثير القلويدات المستخلصة من نبات ذنب الخيل *Equisetum arvense* على الخلايا اللمفاوية للإنسان وخط خلايا سرطان الثدي البشري MCF7 والخط الخلوي الطبيعي للخلايا الليفية في جنين الفأر MEF باستخدام سبعة تراكيز ولمدة تعريض 24 ساعة. اوضحت نتائج دراسة تأثير المستخلص القلويدي على انقسام الخلايا اللمفاوية في دم الانسان بدون المادة المشطرة PHA عدم وجود أي تحفيز لأنقسام الخلايا اللمفاوية ولجميع التراكيز المستخدمة بينما اظهرت نتائج دراسة تأثير المستخلص القلويدي على انقسام الخلايا اللمفاوية في دم الانسان بوجود المادة المشطرة PHA ان المستخلص القلويدي لنبات ذنب الخيل *E. arvense* قد سبب انخفاض في نسبة انقسام الخلايا اللمفاوية في التراكيز المستخدمة جميعها وكان هذا الانخفاض معنويًا ( $p \leq 0.05$ ) مقارنة مع مجموعة السيطرة التي اعطت نسبة انقسام بلغت 4.20%. بينما اظهرت نتائج تأثير المستخلص القلويدي على نمو الخط الخلوي السرطاني MCF7 أن اقل نسبة تثبيط كانت 20.07% بتركيز 15.1 مكغم / مل و اعلى نسبة تثبيط 64.1% بتركيز 500 مكغم / مل. اما عند الخط الخلوي الطبيعي فكانت اعلى نسبة تثبيط 11.3% بتركيز 500 مكغم/مل. الاستنتاج: اوضحت هذه الدراسة بأن للمستخلص القلويدي لنبات ذنب الخيل تأثير عالي على خط خلايا سرطان الثدي MCF7 مقارنة مع الخلايا اللمفاوية للإنسان والخط الخلوي الطبيعي لجنين الفأر MEF في التراكيز العالية مما يجعل لهذا النبات دورا واعدا في علاج السرطان.

**الكلمات المفتاحية:** نبات ذنب الخيل، الخلايا اللمفاوية، القلويدات، الخط الخلوي MCF7.

#### Introduction

Cancer is a group of diseases that can lead to death and characterized by uncontrolled growth and the proliferation of abnormal cells [1]. Traditional methods for treating cancer include chemotherapy, radiation, surgery, immunotherapy and hormone therapy [2]. All chemotherapy drugs kill the tumor and normal cells without discrimination, and surgery does not have the ability to remove the invisible nests, as well as tumor cells can develop resistance against these

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drugs [3]. Normal cells are killed by oxidative stress and cause damage or mutation in DNA in radiation therapy [4].

Recent studies have shown that many medicinal plants have anticancer activity [5]. *Equisetum arvense* belongs to the family Equisetaceae and its common name is horse tail. It is a 100 cm long herb containing alkaloids, amino acids, phytosterols, proteins, saponins, ascorbic acid, flavonoids and many other biological active components [6]. This plant is traditionally used to stop bleeding, healing of ulcers and wounds and in the treatment of tuberculosis and kidney problems [7]. The new approach in using this plant was its cytotoxic effects, and it has been reported that the crude proteins extracted from *E. arvense* inhibit the proliferation of human leukemic U 937 cells [8].

A cytotoxic study performed to assess the effect of different extracts from *E. arvense* on the growth of human cervical carcinoma cells (HeLa cells) by using ethyl acetate, ethanol and aqueous extracts showed that all extracts have a detectable effect on cell growth inhibition of HeLa cells [9]. The pharmacological studies showed that it contains antioxidant, anticancer, antimicrobial, dermatological, immunological, anti-inflammatory, antidiabetic, diuretic, inhibition of platelet aggregation, anti-leishmanial and many other effects [10]. This study aimed to study the effect of alkaloid extract from *E. arvense* on the growth of human lymphocytes, MCF7 cancer cell line and MEF normal cell line.

### Materials and Methods

#### **The plant**

*Equisetum arvense* plant Figure 1 was collected from Diyala river at the end of July 2017, and classified according to its physiological characteristics by Prof. Dr. Nidal Idris Sulaiman at the Department of Biology \ College of Education (Ibn Al-Haitham) \ University of Baghdad.

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Figure 1: *Equisetum arvense* plant

### Extraction of alkaloids

The extraction was carried out according to the method described previously [11], by taking 20g of plant powder and placed in a cylindrical container called thimble, then put in the place assigned as the Soxhlet, then Hexane was added to remove fat and chlorophyll. Extraction was performed for 12 hours at 40-60°C, then the plant powder was transferred to methanol 70% for 3hours. The extract was then sprayed with a filter paper, after which it was concentrated in the incubator for one to two days. The methanol extract was treated with 1% HCl and filtered again with a filter paper (Whatman 1) and diethyl ether was added in the funnel and left for one day. The mixture was separated into two layers, the upper layer was diethyl ether layer which was removed and the lower layer was the aqueous layer which was collected and then the pH was adjusted to 8. This layer was then divided into two layers by adding chloroform in the separation funnel with mixing. The upper layer was the aqueous layer and the bottom one was the chloroform layer which contains the primary, secondary and tertiary alkaloids. This process was repeated three times, the chloroform layer was then placed in the incubator to evaporate and isolate the alkaloids as a final product.

### Detection of alkaloids

The presence of alkaloids was detected by Dragendroff's reagent. The appearance of brown orange color after exposure of extract to Dragendroff's reagent indicated the presence of

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alkaloids. The concentrations used in this study were (15.1, 31.2, 62.5, 125, 250, 400 and 500)  $\mu\text{g/ml}$ .

### Cell lines

In this study, the human breast cancer MCF7 cell line was used in passage (50). It was isolated in 1970 from a 69-year-old woman. While the mice embryo fibroblast (MEF) normal cell line was used in passage (1). These cell lines were obtained from the Unit of tissue culture \Iraqi Centre for Cancer and Medical Genetics Research (ICCMGR) \ AL-Mustansiriah University, Baghdad, Iraq.

### Effect of alkaloid extracted from *E. arvense* on human lymphocyte proliferation

The effect of alkaloid extract on lymphocytes were studied using short-term culture, based on the method of Verma and Babu [12]:

#### I- Incubation of blood with the extract

Half ml of blood collected from healthy persons was added to each tube using 5 ml disposable syringe and treated with seven concentrations of alkaloid extract (15.1, 31.2, 62.5, 125, 250, 400 and 500  $\mu\text{g/ml}$ ) using RPMI-1640 culture tubes with three replicates per a concentration. Another group of tubes were left without adding alkaloid extract to serve as a control group. This experiment was divided into two groups:

**The first group:** includes the treatment of alkaloid extract with lymphocytes without the Phytohaemagglutinin (PHA).

**The second group:** includes the treatment of alkaloid extract with lymphocytes in the presence the PHA.

To the second group of tubes, (0.1) ml of the PHA was added in a concentration of 15  $\mu\text{g/ml}$ , then the mixture was gently mixed and incubated at 37°C for 72 hours.

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### II. Harvesting of the cells

1. To each control tube, 0.1 ml of colchicin at a concentration of 10  $\mu\text{g/ml}$  was added 150 minutes before the original implant time, but colchicin was not added to the treated tubes. The tubes were then returned to the incubator.
2. The tubes containing the samples were centrifuged for 10 minutes at 1500 rpm, then the suspension was removed and the precipitate was mixed with RPMI-1640.
3. Then 5-10 ml of low-tension solution (KCL) 0.075 M were added and warmed up to 37°C gradually and with shaking.
4. Tubes were incubated in a water bath (37°C) for 30 minutes.
5. The tubes were centrifuged at 1500 rpm for 10 minutes.

### III. Fixation

1. The precipitate was shaken and few drops of cold fixative (methanol: glycial acetic acid) by 3: 1 (volume \ volume) were added on the wall of the tube with continuous shaking and the size was completed to (5) ml.
2. The samples were mixed with vortex, then the tubes were put in (4°C) for 30 minutes.

### IV. Washing

1. The tubes were centrifuged for 10 minutes at 1500 rpm and the suspension was removed while the precipitated cells were kept.
2. The process was repeated several times until the clear color of the suspension was observed. The precipitate was suspended by 1mL of the fixative and stored at -20°C.

### V. Dropping

Cold clean glass slides were prepared, the cells were mixed well and dropped on the cold slides using a Pasteur pipette from a distance of (0.5-1) m and left to dry.

### VI. Staining and microscopical examination

The slides were stained with Giemsa stain for 2 to 3 minutes, then washed with Sorensen buffer and left to dry.

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After that, they were examined by the microscope to calculate mitotic Index (MI). The cell division was calculated according to the following equation [13]:

$$\text{Mitotic Index (MI)} = (\text{Number of dividing cells} \setminus \text{Total number of cells } 1000) \times 100.$$

### Cytotoxicity of alkaloids extracted from *E. arvense* in cell lines

The toxicity of the alkaloid extract was tested in the MCF7 cancer cell line and MEF normal cell line by adding trypsin-versin solution to the tissue culture flask (size 25 cm<sup>2</sup>), then 20 ml of the RPMI-1640 medium was added with fetal calf serum (FCS). The cells were mixed and 0.2 mL was transferred from each mixture into each of the 96 wells using a fine automatic pipette. The plates were left in the incubator at 37° C for 18-24 hours until the adhesion of cells in the well, then the old media from wells were removed, 0.2 ml of the prepared concentrations of alkaloid extract were added using the Serum-free media (15.1, 31.2, 62.5, 125, 250, 400, 500 µg/ml) with four replicates per a concentration. Four replicates were performed using DMSO (dimethyl sulfoxide) as a negative control and four replicates were added to PBS supplemented with 0.2 ml of serum free medium as a positive control, and plates were incubated at 37°C for 24hours according to the method mentioned by [14]. The plate was taken out from the incubator and (50µl) of crystal violet stain per well was added then returned to the incubator for 20 minutes. After that, the contents were removed and washed with (PBS) until the excess dye was removed and the cells were allowed to dry. The results were read using the optical spectroscopy device at a wave length of 492 nm. The rate was determined to inhibit the growth of cancer cells according to the following equation:

$$\text{Inhibition rate (IR)\%} = (A - B \setminus A) \times 100$$

Where: A= Control reading

B= Treatment reading for each concentration

Statistical analysis: The results were statistically analyzed using the Graph pad Prism Version 6 analysis system and ANOVA test. The means were compared with the Duncan Multiplex experiment with significant difference at a probability level of  $P \leq 0.05$  [15].

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### Results and Discussion

#### Effect of alkaloid extract of *E. arvense* on human lymphocyte proliferation

##### I- Effect of alkaloid extract as lymphatic stimulate

Human lymphocytes were treated with alkaloid extract of *E. arvense* without PHA using seven concentrations, varying between (15.1, 31.2, 62.5, 125, 250, 400 and 500)  $\mu\text{g/ml}$ . The results showed that there was no stimulation for the division of lymphocytes in all concentrations and found undivided cells only. This is an evidence that the extracts do not have the ability to stimulate the division of lymphocytes.

##### II- Effect of alkaloid extract as anti-lymphatic stimulate

The results in Table 1 showed that the alkaloid extract of *E. arvense* in the presence of the phytohaemagglutinin (PHA) caused a decrease in the in lymphocytic division in all concentrations used. This decrease was significant compared with the control group, which gave a divided ratio of 4.20%.

The ratios of blocked cells in the metaphase were 0.56%, 1.60%, 2.33%, 3.33%, 3.50%, 3.83% and 3.93% at the concentrations of (15.1, 31.2, 62.5, 125, 250, 400 and 500)  $\mu\text{g/ml}$  respectively. The difference was significant at  $P \leq 0.05$  between the concentrations (15.1, 31.2, 62.5)  $\mu\text{g/ml}$  respectively. While the difference was not significant  $P \geq 0.05$  between the concentrations (125 and 250  $\mu\text{g/ml}$ ) and between the concentrations (400 and 500  $\mu\text{g/ml}$ ) as shown in figure 2.

**Table 1:** Effect of different concentrations of alkaloid extract of *E. arvense* plant on human lymphocytes.

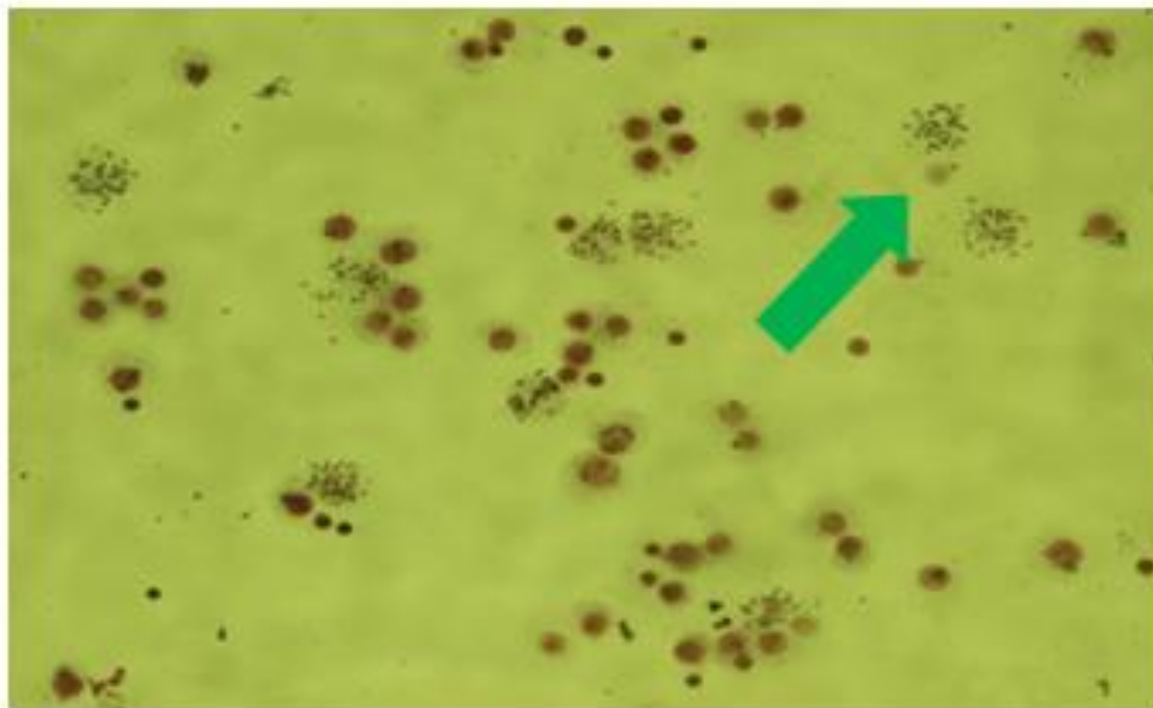
Concentrations of alkaloid extract ( $\mu\text{g/ml}$ )	Mitotic Index (MI %) $M \pm SD$	The Percentage of control %
Control	4.20 $\pm$ 0.01 a	
15.1	0.56 $\pm$ 0.56 f	13.33
31.2	1.60 $\pm$ 0.11 e	30.09
62.5	2.33 $\pm$ 0.08 d	55.47
125	3.33 $\pm$ 0.08 c	79.28
250	3.50 $\pm$ 0.40 c	83.33
400	3.83 $\pm$ 0.03 b	91.19
500	3.93 $\pm$ 0.05 b	93.33

The different letters mean significant difference at ( $P \leq 0.05$ ).



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**Figure 2:** Blocked human lymphocytes in metaphase, treatment with alkaloid extract of the *E. arvense* plant in concentration 500µg/ml

Al-Shaebany [16] used crude extracts (water, methanol, acetone) for the leaves of the *Nerium oleander* plant where the compound characterized from this plant was Oleandrin instead of colchicin, and found that cells stopped in the metaphase in the rate of 1.14%. This finding indicates that the number of stopped cells in the metaphase was lower than that shown in this study. On the other hand, the inhibition action of *Salix* extracts which were examined on normal lymphocytes using seven concentrations by counting the percentage of mitotic index and blastotic index showed a non-significant effect on MI at low concentrations but showed significant effect at high concentrations in comparison with the control group [17].

**Cytotoxicity of *E. arvense* alkaloid extract on cell lines (MCF7 cancer cell line and MEF normal cell line)**

Table 2 showed that the alkaloid extract had an inhibitory effect on the MCF7 cancer cell line which began with 22.07% at a concentration of 15.1µg/ml and increased to 26.45%, 29.41%,

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33.32%, 36.37%, 56.83% and 64.1% at concentrations (31.2, 62.5, 125, 250, 400 and 500  $\mu\text{g/ml}$  respectively). There were no significant differences at  $P \leq 0.05$  between the concentrations (15.1, 31.2 and 62.5)  $\mu\text{g/ml}$ , between the concentrations (125 and 250  $\mu\text{g/ml}$ ) and between the concentrations (400 and 500)  $\mu\text{g/ml}$  as shown in Figure (3A-B). Table 3 showed that MEF cell line had no inhibitory effect at low concentrations (15.1, 31.2, 62.5, 125 and 250)  $\mu\text{g/ml}$ , while there was an inhibitory effect at high concentrations (400 and 500)  $\mu\text{g/ml}$  which was 10.2% and 11.3% in the concentrations (400 and 500)  $\mu\text{g/ml}$  respectively. There was a significant difference at  $P \leq 0.05$  between low and high concentrations as illustrated in figure (4A-B).

**Table 2:** Inhibition rate in MCF7 cancer cell line influenced by different concentrations of the alkaloid extract of *E. arvense* plant after 24-hour exposure

Concentration in $\mu\text{g/ml}$	Cancer cell line MCF7 (I.R)
	$\pm$ SD M
15.1	20.07 $\pm$ 2.6 c
31.2	26.45 $\pm$ 5.1 c
62.5	29.41 $\pm$ 0.9 c
125	33.32 $\pm$ 8.9 b
250	36.67 $\pm$ 6.1 b
400	56.83 $\pm$ 9.1 a
500	64.1 $\pm$ 8.8 a

The different letters indicate that there are statistical differences at the level of  $P \leq 0.05$ .

**Table 3:** Inhibition rate in MEF normal cell line influenced by different concentrations of the alkaloid extract of *E. arvense* plant after 24-hour exposure

Concentration in $\mu\text{g/ml}$	MEF normal cell line (I.R)
	M $\pm$ SD
15.1	0 b
31.2	0 b
62.5	0 b
125	0 b
250	0 b
400	10.2 $\pm$ 1.5 a
500	11.3 $\pm$ 1.5 a

The different letters indicate that there are statistical differences at the level of  $P \leq 0.05$ .

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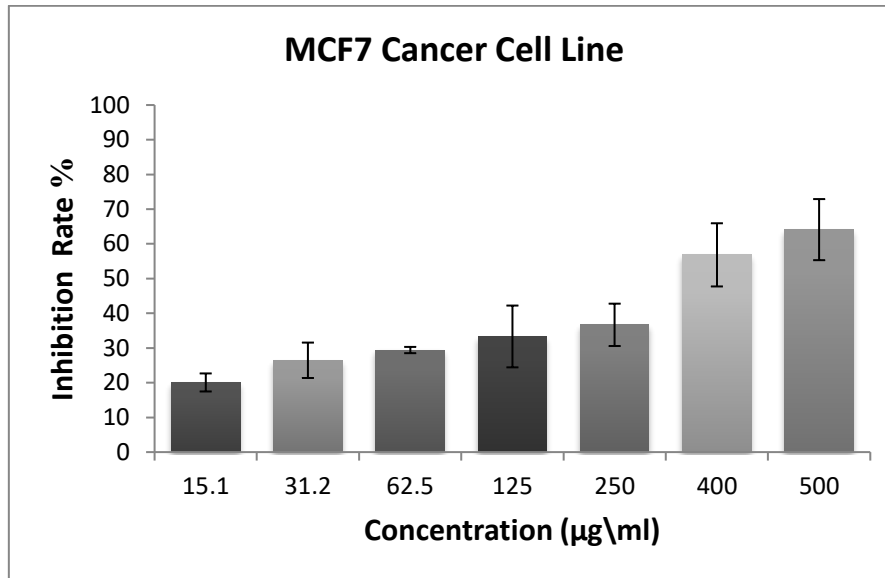


Figure 3A: Effect of the alkaloid extract of *Equisetum arvense* plant on the MCF7 cancer cell line after 24-hour exposure

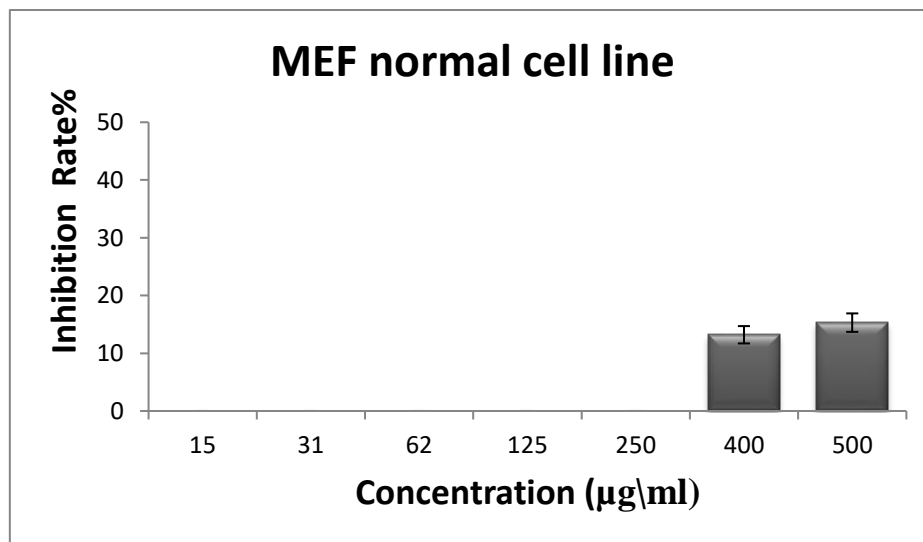
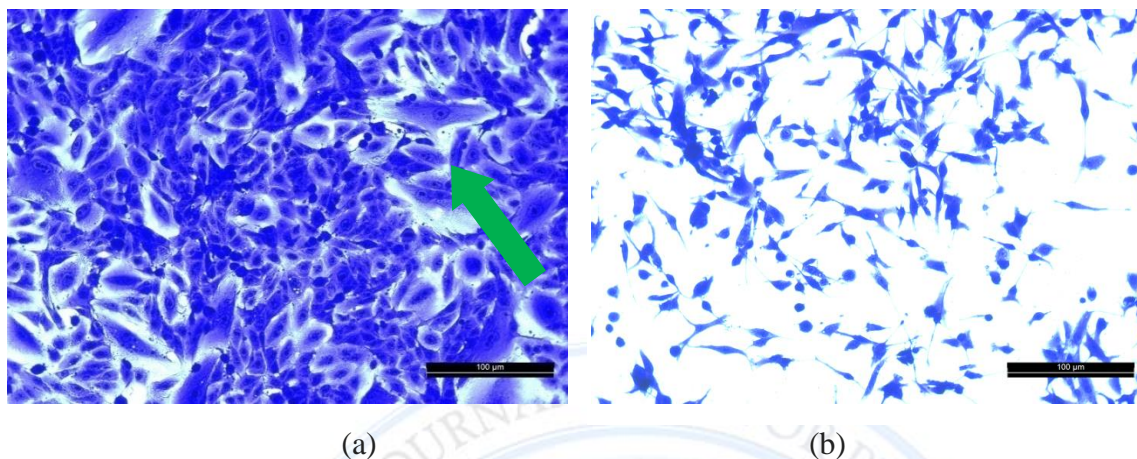


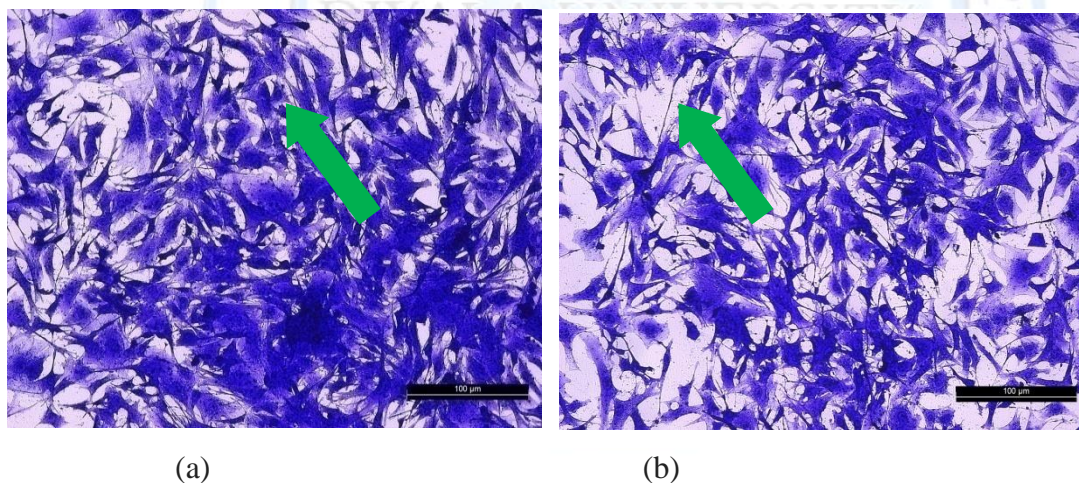
Figure 4A: Effect of the alkaloid extract of *E. arvense* plant on the MEF normal cell line after 24-hour exposure

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**Figure 3B:** Effect of the alkaloid extract of the *E. arvense* plant on the MCF7 cancer cell line after 24-hour exposure at 37°C using crystal violet dye. (a) MCF7 cells as control showed dense cells. (b) MCF7 cells treated with alkaloid extract at concentration of 500 µg/ml showed intercellular space and dead cells.



**Figure 4B:** Effect of the alkaloid extract of the *E. arvense* on the MEF normal cell line after 24-hour exposure at 37°C using crystal violet dye. (a) MEF cells as a control showed dense cells. (b) MEF Cells line treated with alkaloid extract at concentration of 500 µg/ml showed few intercellular spaces.

The results of this study reinforced the findings of many researchers in their local studies on the activity of plant extracts on cancer cells [18]. The pharmacological studies on *E. arvense* showed that it possessed antioxidant, anticancer, antimicrobial, dermatological immunological,

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anti-inflammatory, antidiabetic, diuretic, inhibition of platelet aggregation, anti-leishmanial and many other biological activities [10]. A local study reported that the crude proteins extracted from *E. arvense* inhibited the proliferation of human leukemic U 937 cells [8]. A cytotoxic study was performed to assess the effect of different extracts of *E. arvense* on the growth of human cervical carcinoma cells (HeLa cells) which used ethyl acetate, ethanol and aqueous extracts show that all of plant extracts have the ability to inhibit the cells growth of HeLa cells [9]. Crude extracts generally showed inhibitory effects on tumor cells depending on the concentration, the type of extract and sensitivity of cancer cells. The inhibition rate was increased with increasing of the concentrations.

This is because these extracts contain compounds that affect the physiological state of these cells, and contain compounds that stop the cycle of cancer cells (arrest cell cycle) at a certain stage and prevent the reproduction or contain compounds that stimulate cancer cells to apoptosis [19]. Alkaloids extracted from *Lsatis tinctoria* inhibited H22 cell line (hepatic cell line) proliferation by inducing mitotic index at the metaphase\ anaphase boundary [20]. Another study also showed inhibitory effects of alcoholic and alkaloids crude extracts of *M. azedarach* fruits on tumor cell lines HepG2 and SK-GT2. Using six concentrations and for a period of 24-hour exposure both extracts revealed cytotoxicity on different cell lines, and this effect depends on the concentration and type of cells and extracts [21]. The tumor cells are unique in their ability to invade cells, to proliferate as well as to change their proteins and surface antigens, characterized by the permeability of its membranes and this feature facilitates the process of entering the materials into the cells irregularly, which negatively affects those cells and makes it easier to respond to the anticancer to which they are exposed [22].

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