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EVALUATION OF ANTIOXIDANT, CYTOTOXICITY AND APOPTOSIS OF ALKALOID EXTRACTS OF *EQUISETUM ARVENSE* PLANT ON CANCER CELL LINES

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ABSTRACT : This study aimed to investigate the effects of the alkaloids extracted from *Equisetum arvense* on the proliferation of human breast cancer (AMJ13 cell line) and mice liver cancer (HC cancer cell line). In this study, the antioxidant activity of *E. arvense* was measured using DPPH test, the results showed that this plant have activity to remove free radicals similar to ascorbic acid activity, this activity depend on concentration that used in this study, the inhibition rate was 94.91% in the concentration 500 µg/ml, while in ascorbic acid was 95.43% in the same concentration. The results of effect alkaloid extract on growth of tumor cell lines AMJ13 and HC showed that alkaloid extract revealed cytotoxicity on *different* cell line and this effect depend on concentration and type of cells. The inhibition activity for tumor cell line increase with increase concentration of extract. The higher inhibition rate in AMJ13 cell line was 79.26% in concentration 500 µg/ml and lower inhibition rate was 34.87% in concentration 15.1µg/ml, while in HC cell line, the highest inhibition rate was 83.2% in 500µ g/ml and lower inhibition rate was 65.31% in 15.1µ g/ml. The concentration IC_{50} obtained from effect the alkaloid extract on growth inhibition of cancer cells after 24 hours of exposure was employed to study the effect of crude alkaloid extract on genes that coded for apoptosis (*P53*, *BAX*, *Caspase8* and *Caspase9*) and also by staining with fluorescence dye mix acridine orange/propidium iodide for nucleus. The results showed that fold gene expression of apoptosis gene (*P53, BAX, Caspase8* and *Caspase9*) was (0.05, 0.65, 0.15 and 2.98, respectively) for AMJ13 Cancer cell line and (1.36, 0.12, 0.19 and 3.45, respectively) for HC cancer cell line. These results showed that the fold change in gene expression of caspase9 was higher than other genes in cell lines. The all results showed that crude alkaloid extract was induced apoptosis by intrinsic mitochondrial pathway after 24 hours of exposure. According to acridine orange/propidium iodide mixed fluorescent staining assay, the used alkaloid extract was able to induce highest apoptosis percentage in the treated cells after 24 hours of exposure. The study shows that alkaloid extract of *E. arvense* is rich in antioxidants may prove beneficial in tumor treatment. This study also shows that the concentration IC_{50} of alkaloid extract was induction of apoptosis for AMJ13 and HC cancer cell lines through intrinsic mitochondrial pathway.

Key words : *E. arvense*, antioxidant activity, AMJ13 cell line, HC cell line, apoptosis.

INTRODUCTION

Plant metaboliates products play an important role in chemotherapy, over than 60 available chemotherapeutic agents are plant derived agents (Al-Hili, 2009). Studies are in progress to understand how these compounds may or may not provide protection against mutagenic and carcinogenic activities of chemical compound (Towensend and Gest, 1985). *Equisetum arvense*, common name hoarse tails is a perennial herb with 10 cm high (Kokate *et al*, 2008). Phytochemical studies of this plant showed the presence of different compounds like flavonoids (isoquercetine and apeginin), caffeic acid and alkaloids (nicotine and palustrine) (McCaleb and Kronenbery, 2004).

This plant is used traditionally to heal ulcers, stop bleeding, treat kidney problems and tuberculosis (Peter, 2006). The new approach in using of this plant is the cytotoxic effects. Regarding *E. arvense*, different types of extracts can inhibit cellular growth, this variation is controlled by the type of the cell line, type and procedure of extraction, and on the concentration incubated with cells. A notable anti proliferative activity on human leukemia cells is seen with ethyl acetate extract of the plant, this cytotoxic action is believed to be in a dose dependant manner (Uslu *et al*, 2013). Screening of some plant extracts for inhibitory effects on HIV-1 and its essential enzymes shows that water extract of aerial parts of *E. arvense* possesses inhibitory effect on HIV-1

induced cytopathy (Ashutoshkar, 2007). The majority of anticancer drugs presently used in clinical settings have been described to induce cell death by apoptosis (Cheah *et al*, 2011). Due to the critical role of apoptosis in tissue homeostasis and cancer development, the modulation of apoptosis has become an exciting target in therapeutic of cancer (Zhu and Zhang, 2013). Apoptosis is a tightly regulated and at the same time highly efficient cell death program which requires the interplay of a multitude of factors. The components of the apoptotic signalling network are genetically encoded and are considered to be usually in place in a nucleated cell ready to be activated by a death inducing stimulus. Apoptosis can be triggered by various stimuli from outside or inside the cell, e.g. by ligation of cell surface receptors, by DNA damage as a cause of defects in DNA repair mechanisms, by a lack of survival signals, treatment with cytotoxic drugs or irradiation, contradictory cell cycle signaling or by developmental death signals. Death signals of such diverse origin nevertheless appear to eventually activate a common cell death machinery leading to the characteristic features of apoptotic cell death (Weil *et al*, 1996).

MATERIALS AND METHODS

The plant

The plant used in these experiments was gathered from Diyala river (Fig. 1). Plant specimens (leaves, stems and roots) were taken to the Department of Biology, College of Education (Ibn Al-Haitham), University of Baghdad and was identified by botanist Nidal Idris Sulaiman as *Equisetum arvense*, family Equisetaceae.

Alkaloids extraction

Crude alkaloids extraction from this plant was extracted as described by Cannel (1988). 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 alkaloids. The concentrations used in this study were (15.1, 31.2, 62.5, 125, 250, 400 and 500 µg/ml).

Antioxidant assay

The antioxidant activity of *E. arvense* was determined by DPPH assay according to the method introduced by Smita and Swati (2017). Briefly, equal volume (0.5 ml) of DPPH (60 µM) and each concentration of *E. arvense* (15.1, 31.2, 62.5, 125, 250, 400 and 500 µg/ml) were mixed in acuvette and allowed to settle at room temperature for 30 min. The absorbance was read at a wavelength of 517nm. Ascorbic acid (vitamin C) was used as positive control. The DPPH free radical scavenging activity of alkaloid extract was expressed as

Fig. 1 : *Equisetum arvense* plant.

percentage inhibition of DPPH, which was calculated using the following equation.

Inhibition of DPPH (%) = (AC – AS/AC) × 100

Where, AC and AS are the peak intensity for DPPH and test sample solvent, respectively.

Preparation of cell line and culture

This *in vitro* method was used to evaluate the effect of the alkaloid extract of *E. arvense* on (AMJ13 and HC cell lines). Solutions were prepared according to Iraqi center of medical and genetic researches standard method. These cells were maintained in RPMI–1640 media with 10% (v/v) bovine serum and incubated at 37^{0}C in a humidified atmosphere containing 5% CO_2.

Cytotoxic assay

The cytotoxic assay was measured using the crystal violate stain (Freshney, 2012). In brief, the extract redissolved in DMSO then diluted by serum free media (SFM) and (15.1, 31.2, 62.5, 125, 250, 400 and 500 µg/ml) concentrations, then tumor cells were seeded in 96-well microplate and incubated for 24 hours at 37^{0}C, then old media was changed with new media SFM containing the serial concentrations of each extract, and then plate incubated for 24 hours of all cell lines and humidified incubator at 37^{0}C containing 5% CO_2. After finishing the exposure periods, the media was discarded and the wells treated with 100 µl/well of crystal violate dye and incubated the plate for 20 min at 37^{0}C, the wells were washed with phosphate-buffered saline (PBS) and the plates left for 15 min at room temperature and then the absorbenc (O.D.) of wells measured by ELISA reader at 492 nm wave length.

Apoptosis test

Real-Time Polymerase Chain Reaction (qRT-PCR)

A. Cell seeding

Two falcon were seeded with AMJ13 cell line and two falcon were seeded with HC cell line as 10^6 cell\falcon and incubated in 5% CO_2 incubator at 37°C and humidity for 24 hours to allow cell attachment, proliferation and

Table 1 : The Primers used in the present study.

Primer	Human /Mice	F/R	Sequence 5´..... 3´
P53	F	H	CCG TCC CAA GCA ATG GAT G
		R	GAA GAT GAC AGG GGC CAG
	F	M	GAT CTG TAG CTG CCC CAG GAT
		R	AGA TGA CAG GGG CCA TGG AGT
BAX	H	F	CCT CTC CCC ATC TTC AGA TCA
		R	TCA AGT CAA GGT CAC AGT GAG
	M	F	CGC AAG AGA GGC CAG AAT GA
		R	TGT GGA GAG AAT GTT GGC GT
Caspase 8	H	F	GAC CAC GAC CTT TGA AGA GCT
		R	CAG CCT CAT CCG GGA TAT ATC
	M	F	GCT CTG AGT AAG TTT AAG G
		R	GAT CTT GGG TTT CCC AGA C
Caspase 9	H	F	CTC TTG AGC AGT GGC TGG TC
		R	GCT GAT CTA TGA GCG ATA CT
	M	F	GCT GTT TCT GCG AAA GGG ACT
		R	AGG GCA CAA TCC CTA ACC AC
GAPDH	H	F	GGG TTC TTT GTG CTG AGC GG
		R	TGC AGA TAG GAA GGG CTT TG
	M	F	TCT CCA TGG TGG TGA AGA
		R	TGG CCG TAT TGG GCG CCT

confluent monolayer is achieved.

B. Exposure stage

For each cell line (AMJ13 and HC) 24 hours of exposing was done after monolayer formation. One falcon was exposing with alkaloid extract in IC_{50} concentration (32.5 µg/ml for AMJ13 and 1.75 µg/ml for HC) and other one save as control falcon (Which was treated with SFM only).

C. Harvesting of cells

After the exposure time, the medium was removed from falcons. The cells were washed with 5 ml of PBS. Each cell populations were harvested by scraping of the cell plate bottom in 50 µl cold PBS, according to the abm EXCellenCT Lysis Kit with a subsequent transfer of respective solution to a separate 200 µl eppendrof tube. The tubes were immediately placed in a box with ice to avoid as much degradation as possible of the biological material then it was kept at -80°C.

D. RNA Extraction

Freezed cells were thawed at room temperature for RNA extraction from cultured cells using abm EXCellenCT Lysis Kit (Abm, Canada) according to manufacture in-structions. Pure extracted RNA was used for the synthesis of cDNA using First Chain cDNA Synthesis Kit (TonkBio, U.S.A) according to instructions. The concentration and purity of cDNA was also checked with nano-drop spectrophotometer.

E. Quantitative Real time-PCR analysis of genes expression

The mRNA levels of (*P53, BAX, Caspase8, Caspase9* and *GAPDH* genes) in AMJ13 and HC cancer cell lines were determined by real-time PCR. Primers used in this study were synthesized by IRAQI BIOTECHNOLOGY COMPANY matched with the NCBI (National Center for Bio-technology Information), stored lyophilized at -20°C. All mRNA sequences ob-tained are curated sequences. Primer-Blast tool at NCBI was used to design all genes including in this study (Table 1).

The PCR amplification reaction was carried out using kappa syber green master mix kit (Kappa, U.S.A). The reaction was performed in a 20 µl vol-ume Each 20 µl of the qRT–PCR reactions contained 2 µl, cDNA (100 ng), 10 µl KAPA SYBR Green Master Mix, 6µl RNase free water and 1 µl of each primer (Forword and Reverse) in the concentration (100µM).

The thermal profile of genes expression in this study is shown in Table 2.

The expression ratio was calculated without acalibrator sample $2^{-\Delta Ct}$ according to the following equation:

ΔCT (test) = CT gene of interest (target, test) - CT internal control.

Finally, the expression ratio was calculated according to the formula :

$2^{-\Delta Ct}$ = Normalized expression ratio.

To compare the transcript levels between different samples the $2^{-\Delta\Delta Ct}$ method was used. The CT of gene of interest was normalized to that of internal control gene. The difference in the cycle threshold (Ct) values between the *GAPDH* (internal control gene) and genes of apoptosis *P53*, *BAX*, *Caspase8* and *Caspase9* (interest genes) was calculated as the following formula:

ΔCT(test) = CT gene of interest (target, test) - CT internal control

ΔCT (calibrator)= CT gene of interest (target, calibrator) - CT internal control.

The calibrator was chosen from the control samples.

ΔΔCT was calculated according to the following equation:

ΔΔCT = ΔCT (test) - ΔCT (calibrator)

Finally, the expression ratio was calculated according to the formula:

$2^{-\Delta\Delta Ct}$ = Normalized expression ratio (Livak and Schmittegen, 2001).

Acridine Orange/Propidum Iodide (AO/PI) staining to detect morphological changes

The principle : Dual-Fluorescence staining using acridine orange (AO) and propidium iodide (PI) is a nuclear DNA staining method to assess apoptotic cell morphology using Acridine Orange/Propidum Iodide (AO/PI) kit (US biological, U.S.A). AO is cationic dye which is able to permeate into both live and dead cells to stain all nucleated cells and excite green florescence, while PI only enters dead cells with damaged membranes and generates red florescence.

Procedure

1. A12-well culture plate should be used for this method. Add 200 µl of cultured medium and incubate in CO_2 incubator at 37^0C for 24 hours.
2. Discard the cultured media and treat the cells with alkaloid extract in IC_{50} concentration and incubate for 24 hours.
3. Remove mixture exposed after the end of incubation period.
4. Add 50 µl of the mixture stain AO/PI to each well and incubate in a CO_2 incubator at 37^0C for 20-30min in dark.
5. After that, remove mixture stain by aspirating contents of the wells and rinse the wells with 100 ìl of PBS then removed by aspirate.
6. Repeat pervious step one more time.
7. Finally, add 50 µl of PBS to each well directly. Microphotographs were taken with a Leica inverted fluorescent microscope DMI6000 with the blue and green filters.

RESULTS AND DISSCUSION

Antioxidant activity of alkaloid extract

The results obtained for the antioxidant activity showed effective free radical scavenging by alkaloid extract. Seven different concentrations were monitored as shown in Table 3. In general, a concentration dependent inhibition was observed, showing that 500 µg/ml was better than the other concentrations. There were no significant differences at level P≤ 0.05 between the concentrations (15.1, 31.2 µg/ml) and between the concentrations (62.5, 125, 250, 400, 500 µg/ml and ascorbic acid) as shown in Fig. 2.

Table 2 : Thermal profile of qRT-PCR.

Step	Temperature	Duration	Cycles
Enzyme activation	95ºC	30 sec	Hold
Denature	95ºC	5sec	
Anneal/extend	62ºC	20 sec	40
Dissociation	1min /95ºC-30 sec/ 5ºC-30sec/95ºC		

Table 3 : Antioxidant activity for alkaloid extract of *E. arvense*.

Concentrations (µg\ml)	SD ± Mean
15.1	74.57 ± 6.29 a
31.2	85.20 ± 4.39 ab
62.5	87.67 ± 2.55 b
125	88.60 ± 2.30 b
250	90.17 ± 2.27 b
400	91.30 ± 2.74 b
500	94.50 ± 2.71 b
Ascorbic Acid	95.43 ± 1.46 b

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

E. arvense plant was contained high levels of copper and zinc and rich in vitamins C and E. These were essential elements for superoxide dismutase to act against active oxygen species (Nagai *et al*, 2005).

DPPH scavenging activity of *E. arvense* for root and rhizomatous stem was 94.7% at 4.0 mg\ml (Huh and Han, 2015). The antioxidative activity of *E. arvense* extracts was tested by reactive hydroxyl radicals and DPPH. The results showed that the free radical scavenging activity depended on the type and concentration of applied extracts; the highest DPPH (EC_{50} = 0.65 mg/ml) and hydroxyl radical scavenging activities (EC_{50} = 0.74 mg/ml) were obtained in the case of *n*-butanol extract. (Conadanovic-Brunet *et al*, 2009). Onitin and luteolin isolated from the methanolic extract of *E. arvense* showed DPPH free radical scavenging effect was (IC_{50} of 35.8±0.4 microM and 22.7±2.8 microM, respectively) (Oh *et al*, 2004).

Cytotoxicity of *E. arvense* alkaloid extract on cell lines (AMG13 and HC cancer cell lines)

Table 4 showed that the alkaloid extract has an inhibitory effect on the AMJ13 cancer cell line which began with 34.87% at a concentration of 15.1µg\ml and increased to 49.98%, 61.11%, 68.92%, 70%, 78.60% and 79.26% at concentrations (31.2, 62.5, 125, 250, 400 and 500 µg/mL, respectively). There were no significant differences at level P≤ 0.05 between the concentrations (62.5, 125, 250 µg/ml) and between the concentrations (400 and 500 µg/ml) as shown in Figs. 3 and 4. Table 5 showed that HC cell line had inhibitory effect, which

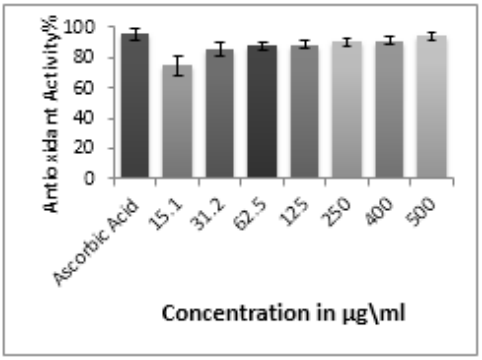

Fig. 2 : Antioxidant activity of alkaloid extract of *E. arvense*.

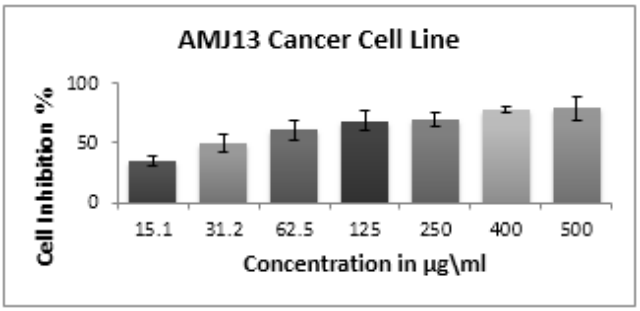

Fig. 3 : Effect of the alkaloid extract of *E. arvense* on the AMJ13 cell line after 24 hour of exposure.

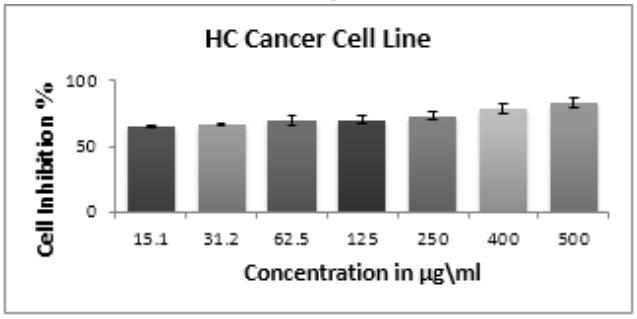

Fig. 4 : Effect of the alkaloid extract of *E. arvense* on the HC cell line after 24 hour of exposure.

began with 65.31% at a concentration of 15.1 µg\ml and increased to 66.54%, 69.64%, 70.20%, 72.79%, 78.46% and 83.20% at concentrations (31.2, 62.5, 125, 250, 400 and 500 µg/mL, respectively). There were no significant differences at level P≤ 0.05 between the concentrations (15.1, 32.2, 62.5, 125 µg/ml), between the concentrations (31.2, 62.5, 125, 250 µg/ml) between the concentrations (250, 400 µg/ml) and between the concentrations (400 and 500 µg\ml) as shown in Figs. 5 and 6.

The cytotoxic activity of *E. arvense* alkaloid extract was studied on the (AMJ13 and HC cell lines). This cytotoxic activity was depended on cell line and extract concentration. The water extract from sterile stems of *E. arvense* exerted dose dependent cytotoxic effects on human leukemic U937 cells. DNA fragmentation, externalisation of phosphatidilserine, the colapse of mithocondrial transmemhrane potential, were all observed in cells cultured for 48 hours due to apoptosis (Alexandru

Table 4 : Cytotoxicity of alkaloid extract during 24 hours of exposure on growth inhibition percent in AMJ13 cell line.

Concentrations (µg\ml)	SD ± Mean
15.1	34.87 ± 3.7 d
31.2	49.98 ± 8.2 c
62.5	61.11 ± 8.6 b
125	68.92 ± 7.6 b
250	70 ± 6.1 b
400	78.6 ± 2.4 a
500	79.26 ± 10.4 a

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

Table 5 : Cytotoxicity of alkaloid extract during 24 hours of exposure on growth inhibition percent in HC cell line.

Concentrations (µg\ml)	SD ± Mean
15.1	1.2±65.31 d
31.2	0.8 ± 66.54 cd
62.5	3.9± 69.64 cd
125	3.5± 70.2 cd
250	3.1± 72.97 bc
400	4.3± 78.46 ab
500	3.9±83.2 a

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

et al, 2007). The cytotoxicity of the methanolic extract of the dried aerial part of *E. arvense* was tested against various cancer cell lines including cervical adenocarcinoma, lung fibroblast, breast adenocarcinoma, and human embryonic kidney cells. After 72 hours treatment, the cells were assayed to determine the relative percentages of dead and live cells. The extract induced death on the four tested cell lines with the greatest effect on human embryonic kidney cells followed by breast adenocarcinoma. However, the extent of toxicity varied depending on the cell type and the concentration of the used extract. Compared to untreated cells, the plant extract had a profound cytotoxic effect on the breast cancer cell line (Aldass, 2011).

Quantitative Real Time PCR

Real time PCR quantification of *GAPDH* and other genes expression, The Ct value of *GAPDH* (the housekeeping gene) used in this study is shown in Table (6) Range of Ct value for *GAPDH* in AMJ13 cell line, in the treatment group was (25.09) and for control group was (26.90) and the fold of gene expression of each gene (P53, BAX, Caspase8 and *Caspase9*) in AMJ13 cell line were (2.98, 0.05, 0.65 and 0.15) respectively. Table 7 shows the Ct value of *GAPDH* Range in HC cell line in the treatment group was (21.84) and for control group was (21.61) and the fold of gene expression of each gene

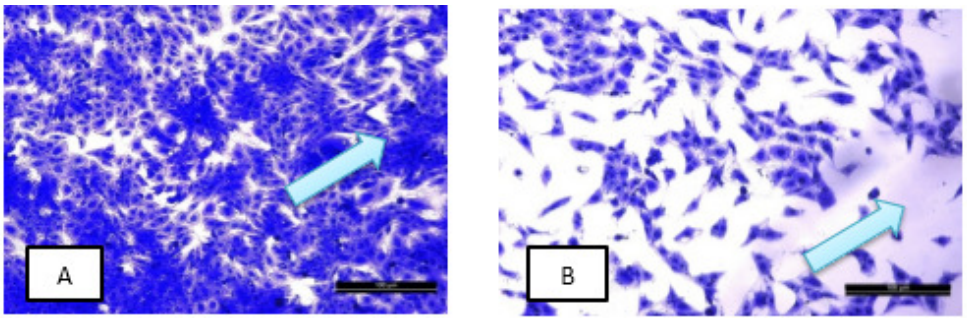

Fig. 5 : Effect of the alkaloid extract of the *E. arvense* on the AMJ13 cell line after 24 hour exposure at 37°C using crystal violet dye. (A) AMJ13 cells as a control showed dense cells. (B) AMJ13 Cells line treated with alkaloid extract at concentration of 500 µg\ml showed intercellular space.

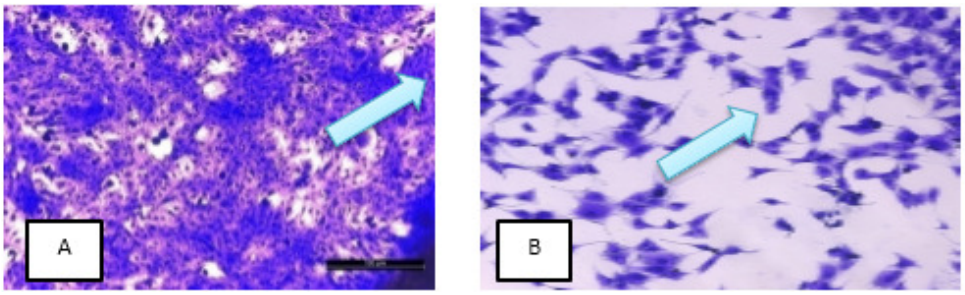

Fig. 6 : Effect of the alkaloid extract of the *E. arvense* on the HC cell line after 24 hour exposure at 37°C using crystal violet dye. (A) HC cells as a control showed dense cells. (B) HC Cells line treated with alkaloid extract at concentration of 500 µg/ml showed intercellular space.

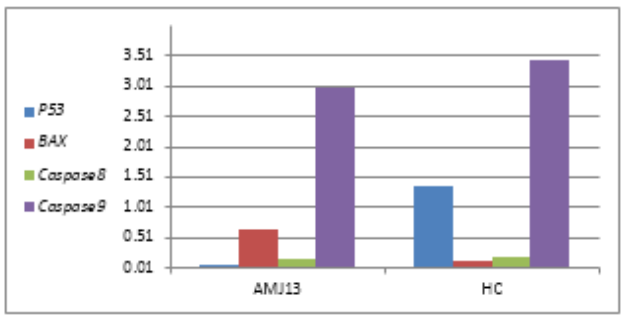

Fig. 7 : Fold of genes expression in (AMJ13 and HC cell lines).

(*P53, BAX, Caspase8* and *Caspase9*) in HC cell line were (3.45, 1.36, 0.12 and 0.19) respectively as shown in Fig. 7.

Morphological change observations using Acridine Orange and Propidium iodide

In this study, using the acridine orange\Propidium Iodide (AO\PI) double staining test is well documented procedure to detect the apoptotic, which was induced by alkaloid extract of *E. arvense* with IC_{50} concentration in (AMJ13 and HC) cell lines after exposure 24 hours, the alkaloid extract had shown inducing apoptosis in each cancer cell tested. The morphology of apoptotic and living cells were identified separately using fluorescence microscopy based on the cell membrane integrity. Characteristic of each type of cells was scored. From these notes, the 24 hours incubation period with alkaloid extract was fairly enough to bring high percentage of the cell population to the late stage of apoptosis.

The results obtained with AO/PI shown in the Figs. 8 and 9. After staining the cells under effect with the mixture of these dyes, three types of colored cells could be seen under fluoresces microscope. All nuclei in control live cells (untreated cells) test appeared as green (in blue fluorescence) and disappeared (in green fluorescence), showed a regular spherical structure and chromatin organization due to the penetration of AO into cell membrane and band of DNA. Where as nuclei in early apoptotic cells were observed as green-yellow or orange colored (in blue fluorescence) and appeared in light color (in green fluorescence) with either condensed or fragmented nuclei. Late apoptosis were observed as an orange or reddish-orange colored (in blue fluorescence) and appeared brightly color (in green fluorescence) and had condensed or fragmented nuclei due to the PI band of DNA cell deaths. The cells under necrosis event would stain red (in blue fluorescence) and appeared brightly (in green fluorescence) and had uncondensed or not fragmented nuclei.

Several bioactive agents plant-derived exhibited induction of apoptosis in many of experimental models of carcinogenesis (Taraphdar *et al*, 2001). Apoptosis: a key event in many biological processes, was very different from another type of cell death, known as necrosis which followed massive tissue injury. Whereas necrosis involved the swelling and rupture of the injured cells, apoptosis

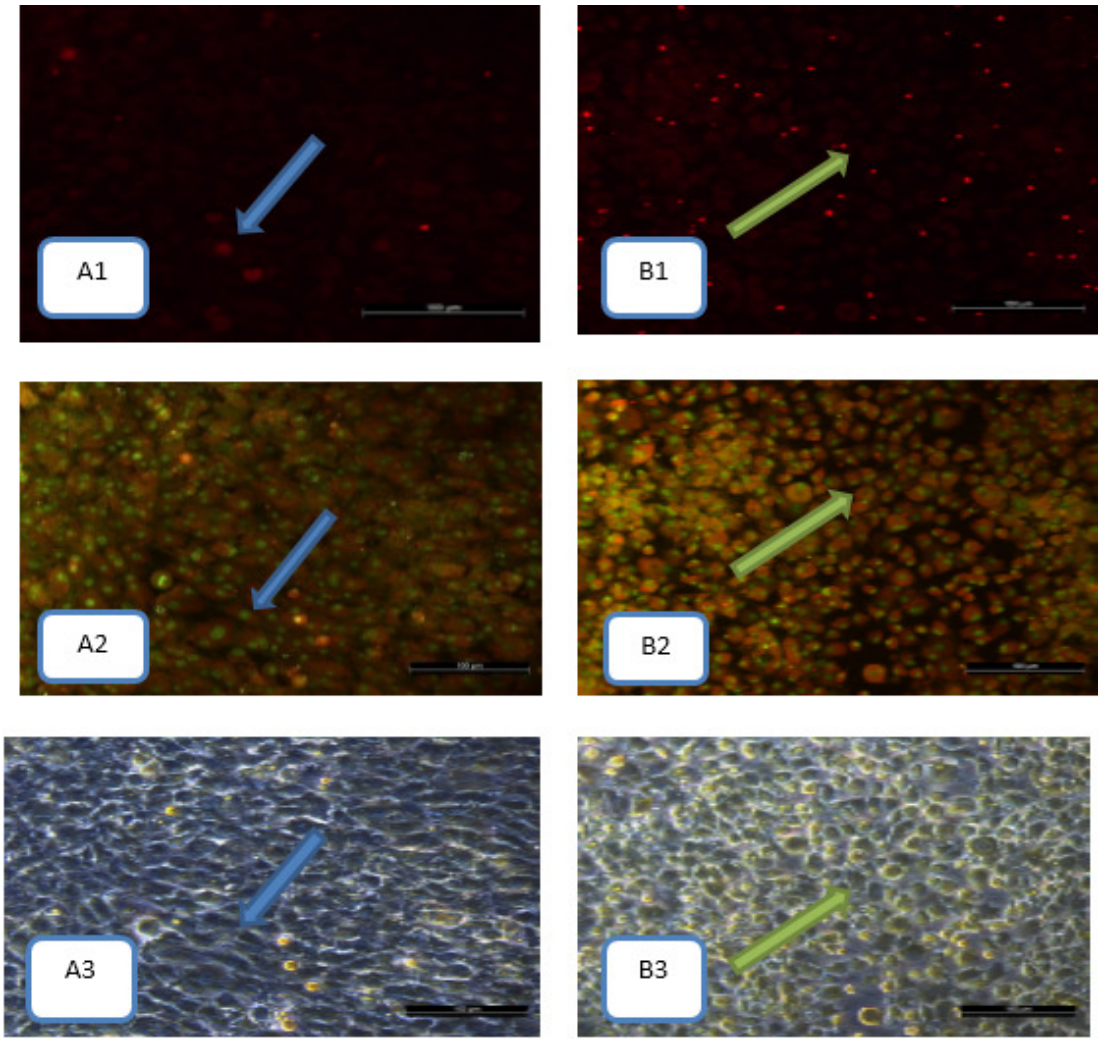

Fig. 8 : Microphotographs with a Leica inverted fluorescent microscope; Detection of Apoptosis (AO/PI) dual staining in AMJ13 cells that treated with IC50 of alkaloid extract. A) Control; B) Treatment: (A1, B1 "in blue fluorescence") and (A2, B2 "in green fluorescence") and (A3, B3 "in visible fluorescence"). Blue arrow=living cells; green arrow = apoptosis.

Table 6 : Fold of genes expression in AMJ13 cell line.

Gene	T/C	CT(Gene)	CT (GAPDH)	ΔCTT	ΔCTC	ΔΔCT	Fold of gene expression
P53	T	24.79	25.09	-0.30	-	-4.19	0.05
	C	22.41	26.90	-	-4.49	0.00	1.00
BAX	T	32.80	25.09	7.71	-	0.62	0.65
	C	33.99	26.90	-	7.09	0.00	1.00
Cas8	T	29.90	25.09	4.81	-	0.92	0.15
	C	29.02	26.90	-	2.12	0.00	1.00
Cas9	T	21.20	25.09	-3.89	-	-1.58	2.98
	C	24.59	26.90	-	-2.31	0.00	1.00

involved a specific series of events that lead to the dismantling of internal contents of the cell (Wayne *et al*, 2009). There are two major pathways that lead to apoptosis, both of which culminate in a common death program: The mitochondrial (intrinsic) pathway and the death receptor (extrinsic) pathway. The mitochondrial pathway involved the induction of specialized protein that induce mitochondrial leakiness, leading to release of cytochrom C which binds to a protein called apoptosis-activating factor-1 (APaf-1) a death-inducing protein. In the death receptor pathway, the triggering of cell surface receptors of the TNF-receptor family results in activation

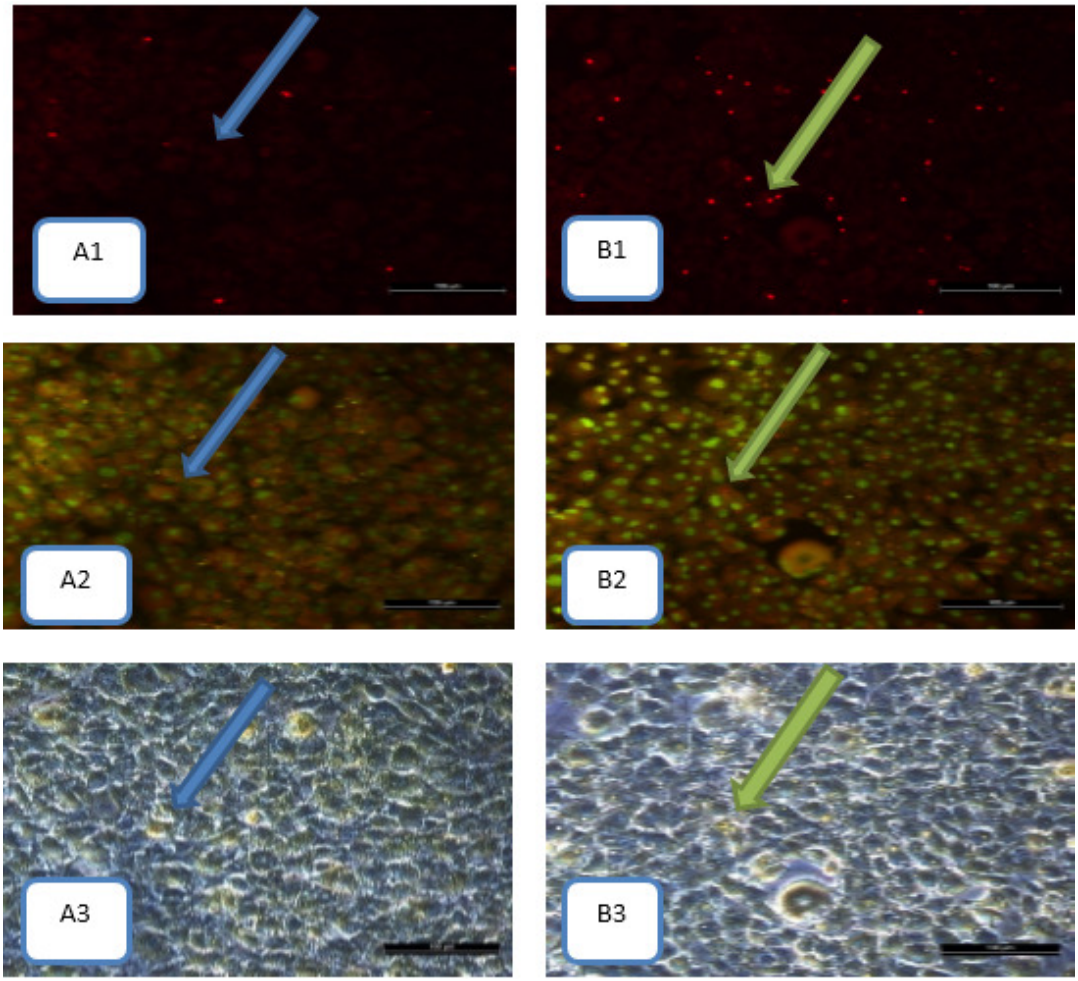

Fig. 9 : Microphotographs with a Leica inverted fluorescent microscope; Detection of Apoptosis (AO/PI) dual staining in HC cells that treated with IC_{50} of alkaloid extract. A) Control; B) Treatment: (A1, B1 "in blue fluorescence") and (A2, B2 "in green fluorescence") and (A3, B3 "in visible fluorescence"). Blue arrow = living cells; green arrow = apoptosis.

Table 7 : Fold of genes expression in HC cell line.

Gene	T/C	CT(Gene)	CT (GAPDH)	ΔCTT	ΔCTC	ΔΔCT	Fold of gene expression
P53	T	30.00	21.84	8.16	-	-0.45	1.36
	C	29.77	21.16	-	8.61	0.00	1.00
BAX	T	31.86	21.84	10.02	-	2.96	0.12
	C	28.22	21.16	-	7.06	0.00	1.00
Cas8	T	33.07	21.84	11.23	-	2.37	0.19
	C	30.02	21.16	-	8.86	0.00	1.00
Cas9	T	29.07	21.84	7.23	-	-1.79	3.45
	C	30.18	21.16	-	9.02	0.00	1.00

of common death pathway (Abbas *et al*, 2008).

There are several studies that show the toxicity of plant extracts on cancer cells. The water extract of the roots of *Vetineria zizanioides* showed cytotoxicity towards MCF-7 cells by stimulating the fragmentation of nuclear material and the intensification of chromatin (Chitra *et al*, 2014). The effect of the methanolic extract of the Cinnamomum zeylanicum on cancer cells illustrates morphological programmed apoptosis, such as cell shrinkage, nuclear material intensification and fragmentation of HepG2 cancer cells (Varalakshmi *et al*, 2014). While, Okubo *et al* (2017) noted that Berberine has a inhibitory effect on HL-60 leukemia cells because of its ability to condense chromatin and the fraction of nuclear material. It was noted in Zhang *et al* (2018) that the alkaloid extract of *Peganum harmala* has an

inhibitory effect on cancer cells by stimulating the fragmentation of nuclear material as well as the release of cytochrome C cells.

All of these studies confirm the effect of plant extracts on the induction of programmed cell death of cancer cells through its effect on many cellular indicators, some of which were detected in the current study. The results of these studies agree with the current study that the extract of alkaloids have a clear effect on the induction apoptosis in cancer cell lines.

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