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Antiproliferative Effects and Apoptosis induction of Ethanolic Extract of *Andrachna aspera* on Human Rabdomyosarcoma Cells

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Abstract:

Arial part of Andrachna aspera ethanolic extract was evaluated by studying their effect on human rabdomyosarcoma (RD) cell line. The cytotoxic activity was determined by anti-proliferative effects when the human rabdomyosarcoma cells were cultured under different concentrations. After 24, 48 and 72h when treated with the ethanolic extract, using cell viability assay. The plant extract showed the strong cytotoxic affect upon the cell like RD with IC50 of 25 µg/ml concentration after 72h, Furthermore, cell cycle analysis showed a significant increase in the accumulation of RD- treated cells at G0/G1 phase ($p<0.01$), and apoptotic rate also shows significantly increasing in a dose- time dependent manner.The extract of A. aspera induced apoptosis and possessed the most robust anticancer activity, against RD cells

Keyword: *Andrachna aspera, Rabdomyosarcoma, Ethanolic extract, Antiproliferative*

Introduction:

During the last 20 years, more than 25% of drugs were derived from plant species while the other 25% were chemically altered natural product. It was highlighte that only 5-15% of approximately 250.000 higher plants have been investigated for bioactive(1). Plants based natural constituents can be derived from any part of plant like bark, leaves, flowers, roots, fruits, seeds, etc i.e. any part of the plant may contain active components. On the other hand, plants have been a source of medicine for thousands of years, and phytochemicals are play an essential role in medicine (2). The goal of screening medicinal plant is to search for the best anticancer agent avertable to human malignancies. In defiance of astonishing advances to modern medicine, such as surgery, radiotherapy, chemotherapy and hormone therapy, cancer disease remains a worldwide health problem. The nature as a huge valuable contributor of potential source for chemotherapeutic agents has recently been reviewed (3).

Andrachna aspera belong to the family Euphorbiaceae, about 15 species of this genus are known From the aerial parts a number of piperidine alkaloids were isolated: aspertine A–D, andrachcinine, andrachcinidine, andrachamine, andrachcine, as well as the alkaloids (+)-allosedridine, (–)-8-epi-8-ethylnorlobelol I and (–)-8-epihalosaline. Terpenes isolated from the aerial parts are lupeol acetate, α-amyrin, β-amyrin, α-taraxerol, stigmasterol, β-stigmasterol, lupeol, oleanolic acid and germanicol.r3

Medically, this plant is used to improve eyesight and to treat eye sores2−3. A crude alkaloidalmixture has been reported to show various biological activities4. The biological significance of A. aspera motivated us to reinvestigate for further alkaloidal constituents fourteen years later (4).

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Material and Methodes:

Plant material

The fresh aerial parts of the plants Andrachna aspera was collected in Augast of 2013 from Erbil Province, Kurdistan region, Iraq, and the species was identified in College of Education , Biology Department, University of Salahaddin, Erbil, Iraq.

Preparation of extract

The dried and powdered of the aerial parts of the plants (4 kg) were percolated with 95% ethanol. (5). The volumetric flask was incubated in Ultrasonic bath for two hours (6). Then the suspension was filtered through a Bückner funnel several times. The residues were extracted for 48 and then 72 hours. The filtrations were performed daily, until the solution appeared

as colorless.Furthermore , the solution was collected in a clean bottle, then dried in a rotary evaporator at 40 °C. Then the final crude extract stored at -20°C until using in the experiment.

Cell culture

The human Rabdomyosarcoma (RD) cells were provided by Tissue Culture Unit/ Iraqi Center for Cancer and Medical Genetic Re-search (ICCMGR, Baghdad/ Iraq) was cultured in RPMI medium and supplemented with 10% fetal bovine serum, Penicillin (100 unit/ ml) and streptomycin (100µg/ml). The cells were maintained at 37C° in humidified atmosphere containing 5% CO2, confluent cells were detached using 0.25% (w/v) trypsin- EDTA cell number and viability were determine by using haemocytometer after staining with trypan blue.

Cell Viability Assay:

Ethyle extract of A. aspera was dissolved in maintenance media to obtain serial dilutions of (0, 5, 10, 15, 20, 25µg/ ml) and 0 µg/ml as a control to study the effects of ethyle extract on cell line. Briefly, cell were plated at a density of (55000 cells/ well) in the a 96-well cell culture plate which treated with ethyle extract at different, concentrations for 24, 48, 72 hrs. Absorbance at 595 nm was measured by microplate enzyme-linked immunosorbent assay (ELISA) reader. Each point repressents the mean of the experiments. The difference of absorbance between the treated and untreated control groups was calculated to determine cell viability. The viability percentage was calculated with the following formula (7) :-

%Growth Inhabition =absorbance of sample – absorbance of control / absorbance of control×100

Determination of IC50:

A curve of percentage of viability cell versus concentrations was plotted from 5 replication of experiment. Inhibitory concentration (IC50), defined as concentration of the tested material that results in 50% of cell death, that was determined from the cell viability curve (8)

Cell cycle analysis by flow cytometer :

RD cells were treated with A. aspera ethanolic extract for 24, 48 and 72hours. The cells were harvested, washed, and fixed in 70% ethanol overnight at -20 C°, ethanol fixed cells were pelleted, washed ice-cold PBS, and resuspended in staining solution containing 50 µg/ml PI. 0.1% Triton-X100, 0.1% sodium citrate, and 100µg/ml RNase. After incubation for 30 min, Cell cycle phase distribution of nuclear DNA was determined by flow cytometry (Becton Dickinson FACS Calibur) by analyzing at least 20,000 cells per sample. The percentage of cells in the G0/G1, S and G2/M phases were analyzed by Guava PCA CytoSoft software, version 2.1.4 for Cell Cycle analysis software (Guava Nexin).

Phase contrast microscopy:

RD cells were seeded in a density of 5×104 cell/ml into sterile culture plate and left overnight for adherence. Then, cells were incubated with different concentrations of A. aspera for 24, 48 and 72 hours. Chang in the cytomorphology of the cells were studied.

Statistical Analysis:

The results were analyzed with a prepared program of SPSS, version 18 (9). Factorial experiment with a completely randomized design (CRD) were used for the reason of using different factors; such as two types of ethanol crude extracts RD, three periods; (24, 48 and 72) Hrs. Six concentrations of the extracts; (0µg/ml as control, 5, 10, 15, 20, 25 µg/ml) and five replications for each concentrations.

Results:

The effect of *A. aspera* ethanolic extract on the cell viability of RD cells

The effect of aerial parts of the A. aspera extracted successively with ethanol extract was studied on RD cell line. RD cells were treated with a serial concentrations and IC50 was evaluated after 24, 48 and 72h of continuous exposure of the cells cultivated in suspension ethanolic extract, the plant effect showed a significant cytotoxic against the RD cell line also showed a significant anti proliferative activity.

The effect of Ethyle extract of A. aspera on the proliferative of human rabdomyosarcoma cell by cell viability assay, in [fig-1] inhibition of proliferation produced after 72h after cell were treated with ethanol extract at 20, 25 µm/ml concentration was (50.5± 0.54), (33± 0.67) respectively when compared with control($p<0.05$). Morever, the same inhibition effect was observed after 48h treatment by ethanolic extract only 25 µg/ml showed significant inhibition ($p<0.05$). Ethyle extract of A. aspera causes 50% cells death in the RD cancer cell line tested. The best IC50 values obtained were (50%, 67%) mµ/ml for two concentrations (20, 25 mµ/ml) a respectively after 72h.

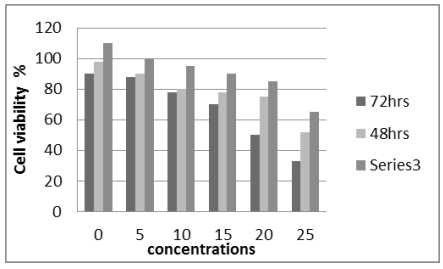

Fig 1. The cytotoxic effects of *A. aspera* ethanolic extract toward different concentrations of RD cell line where plotted in different periods and the value are expressed as mean ± SD

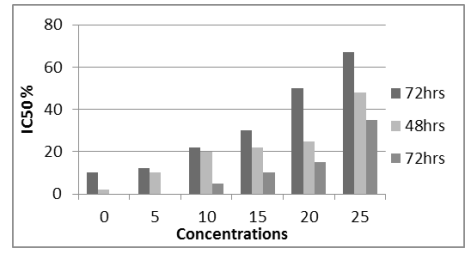

Fig 2. IC50% values of ethanolic extract of *A. aspera* toward different concentrations of RD cell line where plotted in different periods

Change in cell and nuclear morphology

Phase contrast microscopy demonstrated dose- and – time dependent detachment of cell from the surface of culture plate. Andrachna aspera were qualitatively analyzed at their IC50 values to investigate their influence on the RD cells. The mechanism of action of plant extract was determined by observing the change in cell morphology after exposure of the cell line to the extract and comparing them to the control. In figure [3-a] normal proliferation seemed to take place and typical morphology was observed monolayer confluent , and figure [3-b] showed disruption of monolayer after treated with 15µg/ml for 72hrs. However, in figure [3-c] [3-d] normal cell proliferation was absent and signs of cell death and growth disorders started to appear. In these figures the RD cells were exposed to 20, 25µg/ml for 72hrs of A. aspera ethanol extract, in both these figures the typical morphology feature of apoptosis started to appear such as condensed and DNA fragment.

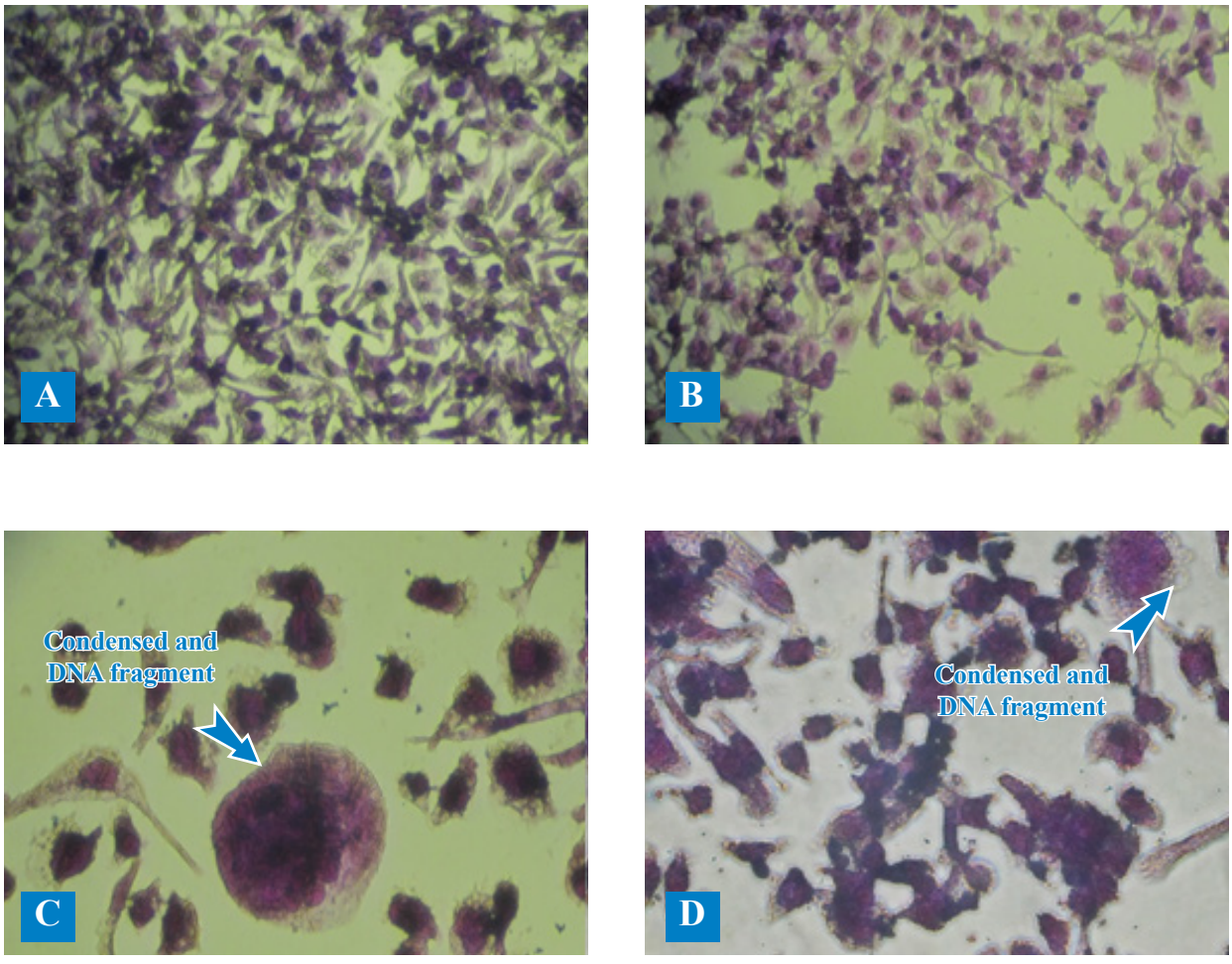

Figure 3. RD cells the treated with the ethanol extract of Andrachna aspera (a) the control monolayer confluent (b) 15µg/ml disruption of monolayer (c) 20µg/ml (d) 25µg/ml both condensed and DNA fragment.

Cell cycle analysis by flow cytometer

Figure -4-showed the effects A. aspera ethanolic extract on the cell cycle of RD cells. A significant ($P< 0.01$) increase in the cell population at sub-G1 phase was observed at 25µg/ml of A. aspera ethanolic extract (7.52± 0.62) and was more pronounced at the higher concentration (15, 20, 25 µg/ml). After 72hrs, the cell death was increased significantly up to (77.15 ±1.13) compared to the control (3.46± 0.08) in cells treated with ethanol extract of A. aspera.

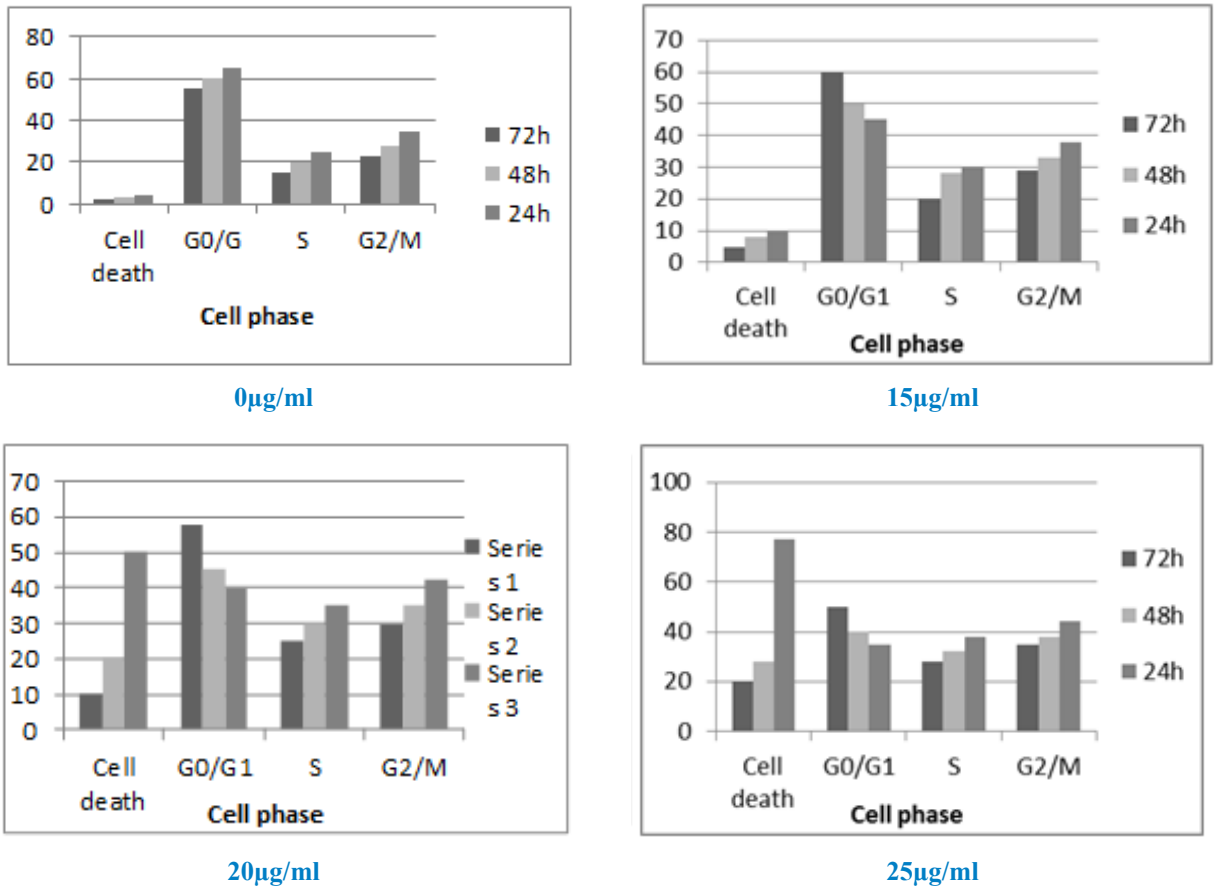

Fig 4. Cell cycle analysis of RD treated with (a) 0µg/ml (b) 15µg/ml (c) 20µg/ml (d) 25µg/ml for 24h, 48h and 72h.

Discussion:

In this study, we further demonstrated that ethyle extract of A. aspera has anti proliferative effects on RD cells in a dose-dependent manner, and the morphological analysis showed that RD cells were sensitive to ethanol extract in 72h periods especially at 20, 25µg/ml.

The anti-proliferative activity of A. aspera is due to the presence of bioactive compounds such as alkaloids and terpenes were isolated from aerial parts. The ethanolic solubale part of Andrachna aspera yielded four new trans-2,6-disubstituted-piperidine alkaloid(3). In fact, alkaloids are among the most important active components in natural herbs, and some of these compounds have already been successfully developed into chemotherapeutic drugs, of the plants such as camptothecin (CPT), a famous topoisomerase I (TopI) inhibitor (9, 10), and vinblastine, which interacts with tubulin (11).

The source of alkaloids with anticancer potentials is very extensive. Most of the are mentioned alkaloids are from different families, and the biosynthesis of these compounds is also varied. For example, berberine is isolated from Ranunculaceae and roots in phenylalanine and tyrosine, whereas evodiamine is isolated from Rutaceae and roots in tryptophan (2). In 1986-87 two new piperidine type of alkaloids from Andrachna aspera namely andrachcine and andrachamine (12 ,13) were reported. These results suggest that different extraction reagents may result in different formulations that generate different functions (10). Similar to our finding of the anti-oral cancer effect of EEGT, several ethanolic extracts of natural products have demonstrated potential antiproliferative effects in cancer; such as Gracilaria tenuistipitata against oral cancer cells, Corydalis yanhusuo against breast cancer (14).

A piperidine alkaloid isolated is a compound found in famous spices that have been used for centuries (15). It exhibits antioxidant, antiinflammatory, antidiarrheal, anticonvulsant, antimutagenic, hypolipidemic, promoting bile secretion, and tumor inhibitory activities (16, 17, 18). It has been demonstrated that piperine induced apoptosis and increased the percentage of cells in G 2 / M phase in 4T1 cells and induced K562 cells to differentiate into macrophages/monocytes (19 , 20). Piperine also

has very good antimetastatic properties against lung metastasis induced by B16F-10 melanoma cells in mice (200 μM/kg) (21) and suppresses phorbol-12-myristate-13-acetate (PMA)-induced tumor cell invasion (22).

There are some possible reason supporting the different toxicity effect of the three ethanolic extract of A. aspera towards RD cells. One of them is due to differing the levels alkaloid compound that have been proven to posses anticarcinogenic effect. Alkaloid, mainly bisbenzilisoquinoline, have been known to have cytotoxic/antitumor activity (23, 24,25). Therefore, we are concluded that the alkaloid level may have a significant role of or effects of ethanolic extracts of A.aspera towards its cytotoxicity effects on RD cells.

In this study, typical apoptosis in RD cell induced by ethanolic extract was confirmed by flow cytometer method, which implied the involvement of apoptosis-elicited process in the ethanolic extract-triggered the growth inhibition. Apoptosis is a normal physiological process serving to eliminate unwanted cells and maintain homeostasis in healthy tissue (26,27). Failure to undergo apoptosis leads to tumor development and resistance to cancer therapy (28). It has been found that most of the cancer chemoterapy drugs exert cytotoxic effects on malignant cells by inducing apoptosis (29). Indeed, some studies have demonstrated that cervical cancer cells show sensitivity to apoptosis induced by chemotherapeutic

agents (30).

Cancers and many human disease have been known as cell cycle disease. This is due to common alteration and many regulatory factors of checkpoints are lost or arrested during the process of tumorigenesis. Cell cycle checkpoints are important control mechanism that ensure the proper execution of cell cycle events. However, there are several chemopreventive phytochemicals could restore and alter regulatory checkpoints through inducing cell cycle arrest (31). Recent studies also suggest that anticancer agents arrest the cell cycle at the G0/G1, S or G2/M phase and then induce cell apoptosis (32). In this study, cell analysis revealed RDcell treated with ethanolic extract induced cell cycle arrest at G0/G1 at concentration below IC50, whereas at high (IC50) concentration, RD cells was arrested at S-phase.

The present result of ethanolic root extract mediated cell cycle arrest in the G0/G1 phase may be well correlated with the up-regulation of CDK inhibitor such as p21 and down-regulation of CDK4, CDK 6, and cyclin D1. Inducing cell cycle arrest at G0/G1 phase in RD cells accompanied by p53- dependent p21 accumulation and down-regulation of cyclin D1. However, future study is needed to determine whether ethanolic extract down regulate or up regulate the expression of the proteins (33).

CONCLUSIONS:

The ethanolic extract of A. aspera inhibited the growth of RD cells. And the ethanolic extract exhibited both the most potent and most selective towards RD cells. The ethanolic extract inhibited the growth of RD cells through induce apoptosis and cell cycle arrest at G0/G1.

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التأثير التثبيطي و تحفيز موت الخلايا لمستخلص الايثانولي لنبات Andrachna aspera على خط الخلايا السرطانية RD

هازه جمال هدايت

مركز البحوث/ قسم علوم الحياة كلية التربية/ جامعة صلاح الدين

الخلاصة:

الدراسة الحالية لتقيم و التاثيرات السمية للمستخلص ايثانولى لنبات *Andrachna aspera* في نمو الخلايا السرطانية لخط RD . و أختبرت الفعالية السمية للمستخلص الايثانولي بثلاث التراكيز مختلفة و ضمن مدد تعريض مختلفة (24 و 48 و 72) ساعة. كانت النتيجة وجود تأثير سمي واضح, وبمعنوية عالية و تم ايجاد التركيز السمي لنصف عدد الخلايا السرطانية (IC50) وكان التركيز 25مايكروغرام/ ملليتر أكفاء التراكيز سمية بعد 72ساعة. و تبين في تحليل دورة الخلية أزدياد تراكم الخلايا معاملة بمستخلص النباتي في طور G0/G1. و كذلك زيادة نسبة موت الخلايا معنويا بزيادة التركيز و مدة التعريض.