

Original Research Article

Convolvulus Arvensis crude Alkaloids extract induces apoptosis through microtubules destruction in mice CHO(China Hamster) cell line

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A B S T R A C T

Keywords

Cytoskeletal,
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This study evaluated the ability of crude alkaloids extracted from the leaves of *Convolvulus arvensis* to distract the microtubule network of mice cell line (CHO), which is an invasive metastasis cell line. This assessment was carried out using the immunostaining technique. The extract was able to distract the microtubules of the cells under investigation after 60 min of exposure in a concentration as little as 20 µg/ml. when DAPI staining used, the cells apoptosis was not detected in this concentration and time. The apoptotic cell have been observed when the concentration of the alkaloid extract elevated up to 80 and 100 µg/ml during the mentioned exposure time. The cells were capable of recovering there native microtubules constriction after 12 hr of the alkaloid removal from the media. The extract concentration of 1mg/Kg/Bw efficiently inhibited CHO cell line tumor growth in vivo to 97.14% in mice after three weeks treatment compared to untreated control animals.

Introduction

In eukaryotic cells the cytoskeleton network consists of three major structural elements, microtubules, microfilament, and intermediate filaments (1). This network plays specific role in cell division, intracellular contacts, interaction with membranes, extracellular matrix, cell motion and maintenance or changes of cell shape (2). The diameter of microtubules (MTs) is about 25 nm they are composed of 13 equally spaced pro-filaments (2). Tubulin is the basic protein of the MTs, molecules of tubulin arranged in dimmers consisting of

two forms, α - tubulin and β -tubulin. They are continuously changeable structures (3), polymerization and depolymerization of MTs is regulated by extra and intra-cellular factors (4). The presence of GTP at MTs ends is necessary to maintain the stability of the polymer (5). Because of their key role in cell function, microtubules emerged as important targets for cancer therapy. Taxanes and vinca alkaloids are microtubule inhibitors that destabilize microtubules, thereby suppressing their dynamics which required for proper mitotic function and effectively blocking cell cycle progression

resulting in apoptosis. In spite of their antitumor activity, drug resistance to such MTs inhibitors is common, limiting their overall clinical efficacy. Therefore the discovery of novel agents that may overcome resistance to conventional MTs inhibitors and provide higher efficacy of microtubule-targeting with limited toxicity is actually need (6). In addition, despite the success of taxanes and vinca alkaloids to inhibit the progression of some cancers in clinical use, resistance to antimicrotubule agents is encountered in many tumor types, particularly during multiple cycles of therapy. Therefore, there has been great interest in identifying and developing novel anti-microtubule drugs. (7) Moreover the most widely used Vinca alkaloids such as vinblastine, vincristine, and vindesine, often induce some intractable side effects including neurological and hematological toxicities and in particular, experience with both acquired focusing on the development of new semi synthetic Vinca alkaloids, with the aim of overcoming current restrictions. The vindoline coupled with catharanthine were chemically manipulated in an effort towards the finding of the promising therapeutics. Of that BM6 stood out as the most potent new Vinca alkaloids derivative. In comparison with classical Vinca alkaloids, BM6 had its distinct antitumor activities in vivo due to its better pharmacokinetics profiles and its more specificity towards tubulin (8).Natural products have provided key leads for drug discovery. Many interesting biological properties have been characterized for novel natural products. Alkaloids are a large group of secondary metabolites containing usually basic nitrogen derived from amino acids, purines, pyrimidine or other source such as transamination (9). Most alkaloids are classified chemically according to the nitrogen-containing ring system. (10).*Convolvulus arvensis* is bindweed

native to the countries of the eastern part of the Mediterranean. The species name *Convolvulus* is derived from the Latin, convolare, meaning “to entwine”. The genus *convolvulus* contains about 250 species. *Convolvulus* is an ubiquitous weed (11). On the contrary to the species *Convolvulus avensis* which is understood to contain alkaloids that appear nontoxic in animal studies and have potent tumor-inhibitory effects and anti-angiogenetic effects (12) (13) (14). The alkaloids of the species are not yet investigated. This study is the first to assess the activity of locally harvested *Convolvulus arvensis* alkaloids against the microtubules of aggressive mice cell line CHO, and induction of apoptosis.

Materials and methods

The plant

The plant used in these experiments was gathered from general local gardens in Baghdad (Figure 1). Plant specimens (leaves, stems and flowers) were taken to the College of Science, Botany Department, University of Dayala and was identified by botanist doctor Khazal D. Wady as *Convolvulus arvensis*, Family Convolvulaceae

Alkaloid extraction

Crude alkaloids extraction from the leafs of this plant was extracted as described by Hassan 2009 (15) and Cannell (1998)(16).

Cell line

In our experiments we used the mouse CHO cell line. This cell line was obtained from the Department of Biology, Faculty of Medicine, Wuhan University, China. The cells were grown in Dulbecco's Minimal Essential Medium (DMEM) (PAA

Laboratories GmbH, Linz, Austria), supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml-1 penicillin, and 100 µg/ml streptomycin (PAA Laboratories, Austria) in a humidified atmosphere of 95 % air and 5 % CO₂ at 37°C.

Characterization of invasive and metastasis properties in vivo

For invasive assay, group of five mice were injected in the intraperitoneal cavity with CHO tumor cells and observation for ascites formation was conducted through cavity volume measurement. For the evaluation of metastatic potential mouse bone metastatic model was adopted, a group of five mice were injected with CHO tumor cells in the right leg muscle. After tumor development, right femur (the nearest bone) was extracted and evaluated morphologically (differences between normal and abnormal bone shape). Exposure to Crude Alkaloid extract In order to assess the kinetics and mechanism of *Convolvulus arvensis* crude alkaloid extract effect on the MTs network of the cell line under investigation, three sets of experiments were conducted. In the first experiments, three different concentrations sets were used, sub-one hundred (20, 40, 80, 100 µg/ml), over one hundred (200, 400, 800 µg/ml), and over one thousand (4000 µg/ml and 9000 µg/ml), the crud alkaloids was prepared in 1 ml of 2% SDS. The cells were exposed to the prepared solutions for 60 min at 37 °C. Each concentration was tested in two replicates, the control samples were exposed to phosphate-buffered saline (PBS, pH 6.9). In the second experiment, the cells were exposed to final crude alkaloid concentration 20 µg/ml for 15, 30, and 60 minutes at 37 °C in DMEM media. Cells were also treated for 5 minutes in a medium containing alkaloids at a concentration of 800 µg/L. control samples

were treated with PBS(pH 6.9).The third series of experiments was performed with the crude alkaloid extract at a final concentration of 20 µg/ml for 60 min. After the time of the treatment was over, the drug containing medium was poured off and cells were subjected to three washings with PBS (pH 6.9). Plates were refilled with fresh growth medium and incubated for another 6, 7, 8, 9 and 12 hour in order to evaluate the recovery processes. Recovery progressed at 37 °C in a humidified atmosphere of 95 % air and 5 % CO₂.

Visualization of microtubules network

Convolvulus arvensis crude alkaloid extract treated cells were cultivated over microscopic

Visualization of microtubules network

Convolvulus arvensis crude alkaloid extract treated cells were cultivated over microscopic cover slips, washed three times for 4 min with PBS (pH 6.9) and fixed by 3% paraformaldehyde in PBS. Thereafter, the cells were permeabilised by 0.2 % Triton X-100 solution in PBS. The microtubules were detected by means of the mice antitubulin monoclonal antibody TU-01 (Institute of Molecular Genetics, Prague, Czech Republic), diluted 1:300 by PBS, and a secondary swine antimouse IgG conjugated with Taxes Red (TR) or conjugated with green fluorescent protein (SwAM/TR,GFP ;Institute for Sera and Vaccines, Prague, Czech Republic) diluted 1:100 by PBS. Cells were washed with phosphate buffer three times for 5 min between the applications of individual agents. The samples were then closed in the Vectashield mounting medium (Vector Laboratories, Inc., Burlingham, CA, USA) and visualized with fluorescent microscope. Staining with 4,6 - diamidino -2-

phenylindole (DAPI) In order to assess the incidences of DNA condensation as an indicator of apoptosis, treated and control cells was stained with DAPI.

Antitumor activity in vivo

The ability of alkaloid extract to inhibit CHO tumor cells growth in vivo was assessed; forty mice were injected subcutaneously in the right dorsal with this tumor cells, after the tumor developed to 8-1 mm², and eliminating the animals that did not develop tumors, the animals were divided to three groups each one with ten individuals (of each group,five animals served as control untreated and five animals was treated with the alkaloid extract). the treated animals in each group was injected four time a week (every other day) subcutaneously with 1 mg/Kg/Bw for deferent period of time.The treatment dose was determined according to the LD₅₀ (data not shown) of the crud alkaloid extract. The first group injected with the alkaloid for one week, the second group for two weeks, and the third group for three weeks. The control animals of the three groups were injected subcutaneously with DMSO. After the treatment times were over, animals were sacrificed and tumors were extracted and tumor mass was determined according to the relation $Tv=L(W)^2 / 2$, where Tv = Tumor volume, L = Length of tumor, and W = Width of tumor (Ge et al., 2003) .Tumor growth inhibition was calculated according to the relation GI% = (A-B/A)100, where GI = Growth inhibition, A= tumor volume in untreated animals, and B = Tumor volume in treated animals.

Result and Discussion

The cell line under investigation was described to be an invasive and metastasis cell line (17). The cell line metastasis was

tested in vivo, this was emphasized when the tumor cell injected in the right leg of a group of mice. The tumor cells were able to develop a secondary bone tumor in the right femur of the injected animals only after one week of the injection time (Figure 1, A, B, C.). The aggressiveness of the CHO cells was substantial, the tumor cells was able to induce ascites tumor aggressively after two days of intraperitoneal injection (Figure 1, D). Bright field microscope and fluorescent microscope The used fluorescent dye stains the cell nuclei with green color.The microtubule of untreated control cells (Figure 3) showed a network regularly distributed along the whole cell content.

When cells exposed to alkaloid crude extract from leaves of *Convolvulus arvensis* at concentrations from 2 to 10 μ g/ ml for 60 min, it did not show considerable changes in the distribution of microtubules (data not shown). Cells exposed to concentrations of 20, 40, 80, 100, 200, 400, and 800 μ g/ ml for 60 min they showed changes in the arrangement of the microtubule network (Figures 4 and 5). The network of cytoplasm microtubules at the lowest concentration used (20 μ g/ml) was clearly and obviously thinned down, and the treatedcell individual microtubules fibers had a destructed and granulated wavelike shape.

The network damage increased with the increasing of *Convolvulus arvensis* alkaloid extract concentration from 20 to 800 μ g/ml. In these concentrations DNA fragmentation was observed in the treated cells as indicated by DAPI staining cells. The microtubules were more thinned down and fragmentation of microtubule fibers occurred at a higher concentration of alkaloid extract (4000 and 9000 μ g/ml),sometimes blebs were formed in this elevated concentration and DNA fragmentation was indicated (Figure 6).When cells were exposed to alkaloid at a

concentration of 20 µg/l for 2, 5, 10, 15 or 20 minutes, no noticeable changes occurred in the microtubule network (data not shown). The 30 min treatment at concentration of 20 µg/ml did not cause an obvious disruption of the treated cell microtubules (Figure 7).

When exposed to *Convolvulus rvensis* alkaloid extract at a concentration of 800 µg/ml for 5 minutes, the treated cells showed a severely defected microtubules network. In this time and concentration the network was thinned down, and individual fibers had a granulated wavelike shape (Figure 8).

In the microtubules recovery experiments, all the cells with the recovering period of 6 hours in a drug-free growth medium following *Convolvulus arvensis* alkaloid extract exposure, showed no recovered but damaged microtubules. The cells after 7 hours recovery period had their microtubules network either partially restored or still damaged. After recovery for an 8 hours period, some cells showed a partially defective (thinned-down) network, but the majority of the cells showed restored microtubules (Figure 9). When the cells were allowed to recover for 9 hours, the microtubules was also damaged, only several cells showed nearly restored microtubules. After a recovery period of 12 hours, microtubules were spread out comparably to those observed in untreated control cells (Figure10). The control cells showed their microtubule network regularly distributed along the whole cell volume.

The antitumor activity of the crude alkaloid extract of *Convolvulus arvensis* leafs *in vivo* was considerably substantial, the tumor volume was reduced significantly (< 0.01) ten times after only two week of crude alkaloid extract treatment, and significantly

(< 0.01) thirty-five times after three weeks of alkaloid treatment (figure 11). The alkaloid extract tumor growth inhibition ability reached almost 95% of that in control untreated animals (figure 12 & 13). A huge differences (significant < 0.01) were observed in tumor mass between treated and nontreated control animals after three weeks of 1mg/Kg/Bw administration (figure 14) week of crude alkaloid extract treatment, and significantly (< 0.01) thirty-five times after three weeks of alkaloid treatment (figure 11). The alkaloid extract tumor growth inhibition ability reached almost 95% of that in control untreated animals (figure 12 & 13). A huge difference (significant < 0.01) were observed in tumor mass between treated and non treated control animals after three weeks of 1mg/Kg/Bw administration (figure 14).

Interaction of antitumor agents with components of the cytoskeleton is a theme studied in many researches (5–12). Targeting cancer cells microtubules is one of many strategies utilized to defeat deferent types of this disease (17). Natural products were of the realist chemicals to be recognized as potent antitumor drugs as a result of their interaction with cancer cells microtubules (18-26). The cell line under this study was highly invasive and metastatic, its ability to induce ascites tumor in the peritoneal cavity of the mice after 48 hr after its injection indicted such conclusion. Moreover when it injected in the right leg of mice group, it metastasized to the nearest bone (femur) in period of one week in all individuals of the injected animals. The huge femur bone morphological deformations in the injected animals were clearly indicted as a result of bone tumor formation. These *in vivo* results confirm what have been characterized about this cell line in *in vitro* experiments , it was found that these cells can detached from the mother tumor nuclide to invade basement

membrane and extracellular matrix by adhering to fibronectin for movement and migration, thus leading to tumor diffusion and metastasis. It metastasized to the lung when injected intravenously (27).

Phytochemicals such as alkaloids compounds elicit various biological effects including cancer chemoprevention and treatment (28). Meng (29) studied the interaction of alkaloids with tubulin, and compared alkaloid and aqueous extract from leaves of *Convolvulus arvensis*. He studied the affinity of the drug to tubulin heterodimers. Alkaloid exhibited a higher overall affinity for porcine brain tubulin than aqueous extracts. Under the present experimental conditions, a similar affinity was marked. The 20 concentration used in this study indicates a specific affinity of the to this invasive cell line. The minimum time required for this concentration to induce microtubules deformation was 60 min, where it induces no effect in exposure time less than that.

Elevating the concentration to 800 shortened the time needed to induce microtubules deformation in this cell line down to 5 minute. All these results indicated the specific targeting of the microtubules by alkaloid extract of *Convolvulus arvensis* leafs. Cells with apoptotic characteristics started to appears in alkaloid concentrations started from 100 µg/ml and higher during exposure time (60 min). This refer to another possible effector mechanism that alkaloid extract exercise towered CHO cells. Nagappan and co-workers found that Carbazole Alkaloids have antitumor activity with much higher concentration, the significant minimum inhibition concentration (MIC) values was 25.0–175.0 mg/mL against MCF-7, Hela and P388 cell lines (30). Some authors also studied the recovering processes of the cytoskeleton

after treatment of cell cultures with physical factors or agents interfering with cytoskeleton compounds. Alkaloid caused a sequence of morphological changes insensitive cells from three pleiotropic resistant MCF-7 human breast carcinoma cell lines mixed with vaginal adenocarcinoma cells. The cells were selected in serially increasing drug concentrations.

These changes included precipitation of tubulin and disappearance of tubular structure. The changes occurred initially within 3 hours of incubation, but were expressed in all cells after 6 hours. After 3 hours of drug exposure, the cells were subcultured in drug-free media, the cells cytoskeletal structure reformed within 10 hours. The maximal recovery of the cytoskeletal structure occurred 22 hours after drug removal and was sustained up to 36 hours (13). Treatment of hippocampus neurons with alkaloid compounds eliminated the microtubule bundles, leaving only tubulin paracrystals.

Within 24 hours after washing out the alkaloid, the microtubule bundles repolymerised in cultured cells (14). In competence with this, alkaloid extract of *Convolvulus arvensis* leafs demonstrate the same mechanism effect on the cell line under this study. Its antitubules effect was eliminated after 10-12 hr of in vitro cultivation in drug free media. The effect of the alkaloid extract toward this cancer cells was significant in vivo as well. The used dose was capable to inhibit tumor growth in 26.8%, 92.16% and 97.14% after one, two and three weeks of treatment respectively compared to nontreated control animals. This explains that action of the alkaloid extract on distraction of microtubules of this cancer cells is active in vivo as well in vitro in addition to other possible mechanisms.

Figure.1 Photograph of the plant used in this study



Fig.1 The CHO cells were injected subcutaneously in the right leg of mice. A and B; tumor development.C; right femur deformation after secondary bone tumor formation after one week of injection.D; ascites tumor formation only solid after three week of intraperitoneal injectionin the mice



Fig.2: CHO cell line photograph in bright fielded microscopic .

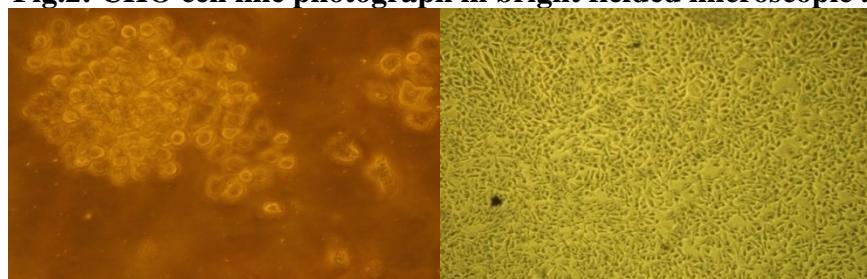


Fig.3 Visualizing CHOCells microtubules: untreated cells (control) and stained with secondary GFP conjugated swine anti-mouse IgG (upper panel), nucleostained with DAPI(lower panel).

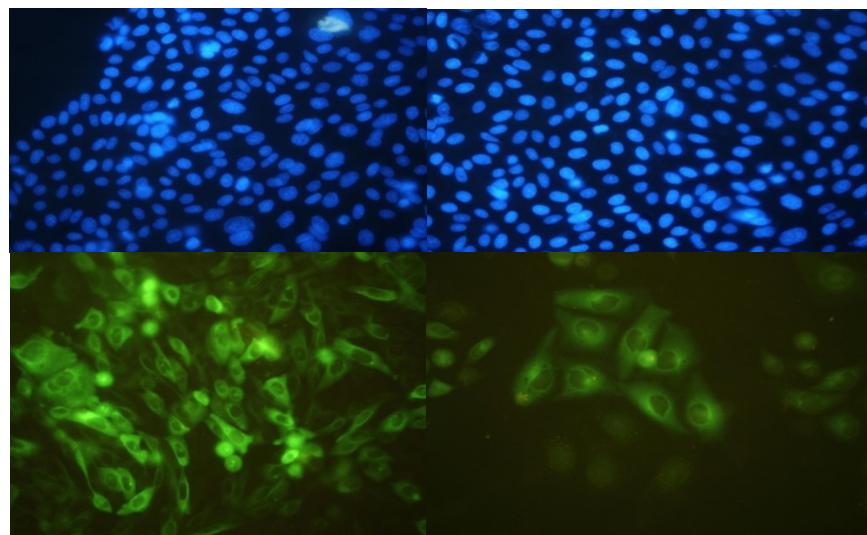


Fig.4 CHO cells treated with different sub-one hundred concentrations of *C. arvensis* Alkaloid extract as indicated for 60 min. is a representative microscopic fields)

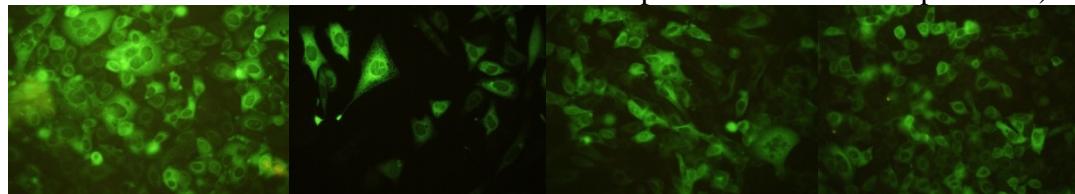


Fig.5 CHO cells treated with different over-one hundred concentrations of *C. arvensis* alkaloid extract as indicated for 60 min



Fig.6 CHO cells treated with highest concentrations of *C. arvensis* alkaloid extract as indicated for 60 min. Stained with secondary GFP conjugated swine anti-mouse IgG. Nuclei stained with DAPI,. Apoptosis form interphase after treatment of Alkalioids

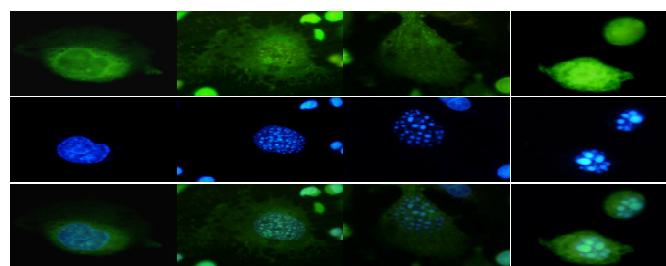


Fig.7 CHO cells treated with 20 µg/ml of *C. arvensis* alkaloid extract as indicated for 30 min. Stained with secondary GFP conjugated swine antimouse IgG (middle panels).Nuclei stained with DAPI (lower panels)

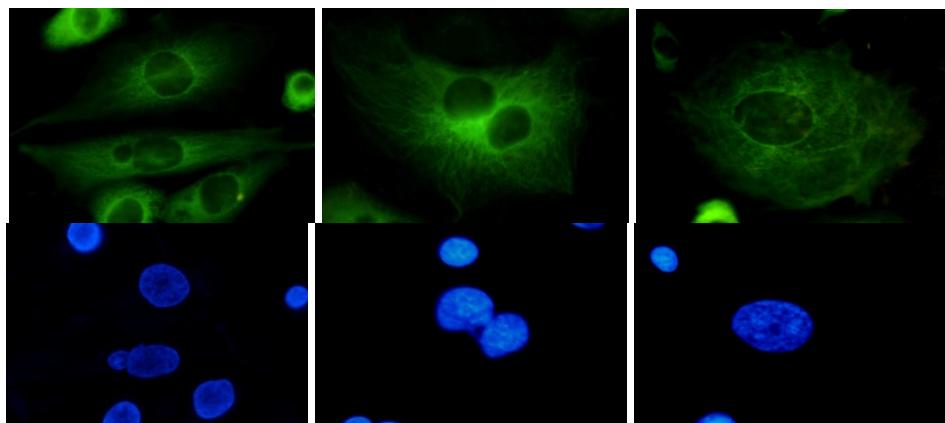


Fig.8 H22 cells treated with 800 µg/ml of *C. Arvensis* alkaloid extract as indicated for 5 min. stained with secondary GFP conjugated swine anti-mouse IgG

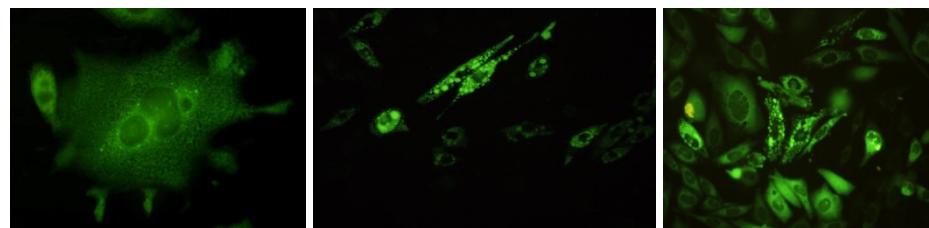


Fig.9 CHO cells treated with 20 µg/ml of *C. arvensis* alkaloid extract as indicated for 60 min as indicated in the methods and recovered with fresh media for 6, 7 and 8 hr, stained with secondary GFP conjugated

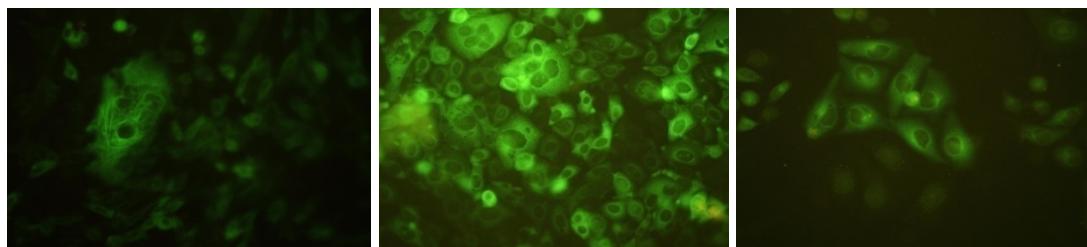


Fig.10 CHO cells treated with 20 µg/ml of *C. arevensis* alkaloid extract for 60 as indicated in the methods and recovered with fresh media for 9 hr and 12 hr

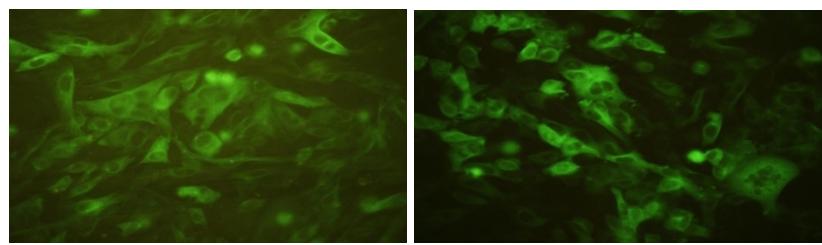


Fig.11 Tumor volume in mice treated with alkaloid extract of *C. arvensis* for defferent periods of time compared to untreated control mice

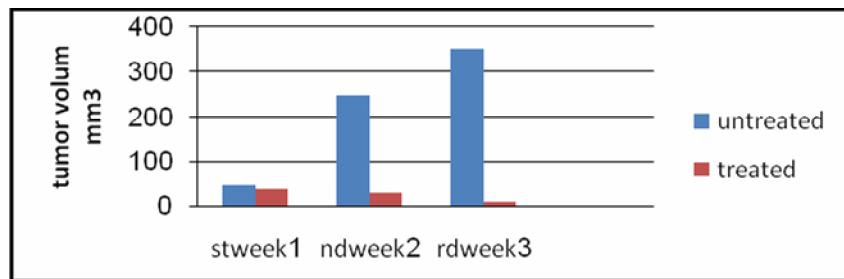


Fig.12 Tumor growth inhibition percentage in mice treated with the alkaloid extract of *C. arvensis* during the three different time of administration in vivo

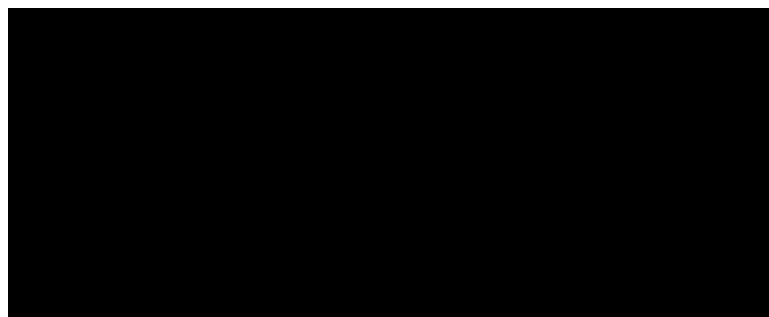
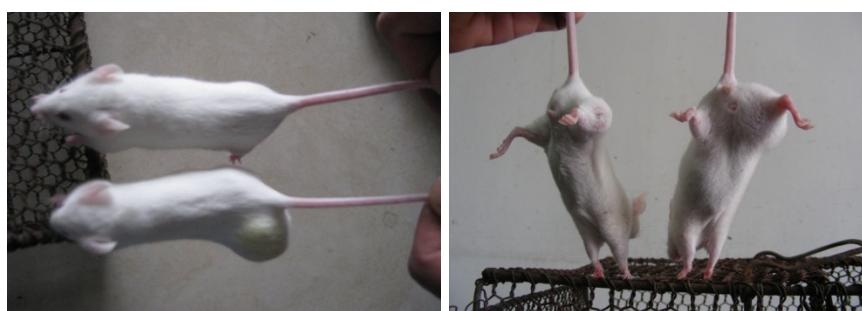


Fig.13 Antitumor activity of *C. Arevensis* alkaloid extract, animals was injected for four times a week with 1 mg/Kg/Bw for A; one week treatment, B; two weeks treatment C; three weeks treatment. Upper row is for untreated control animals and lower row is for treated animals



Fig.14 The appearance of representative control and treatment animals of group three (after three weeks of 1mg/Kg/Bw crude alkaloid extraction administration)



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