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Effect crude alkaloids extraction of *Iastis tinctoria* induces apoptosis through microtubules destruction in mice H_{22} (Hepatic cell line)

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Abstract

This study evaluated the ability of crude alkaloids extracted from the leaves of *Isatis tinctoria* to distract the microtubule network of mice cell line (H_{22}), 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 60min of exposure in a concentration as little as 20µg/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 100µg/ml during the mentioned exposure time. The cells were capable of recovering there native microtubules constriction after 12hrs of the alkaloid removal from the media. The extract concentration of 1mg/Kg/BW efficiently inhibited H_{22} cell line tumor growth in vivo to 97.14% in mice after three weeks treatment compared to untreated control animals.

Keywords: Cytoskeletal, Microtubules, Alkaloids, *Isatis tinctoria*.

Introduction

In eukaryotic cells the cytoskeleton network consists of three major structural elements, microtubules, microfilament and intermediate filaments (Schliwa, 1986). This network plays specific role in cell division, interaction with membranes, extracellular and maintenance or changes of cell shape (Toshio *et al*., 2008) The diameter of microtubules (MTs) is about 25nm they are composed of 13 equally spaced pro-filaments (Toshio *et al*., 2008). Tubulin is the basic protein of the MTs, molecules of tubulin arranged in dimmers consisting of two forms, α- tubulin and β-tubulin (Schmidt and Bastians, 2007), polymerization and depolymerization of MTs is regulated by extra and intra-cellular factors (Jordan, 2002). The presence of GTP at MTs ends is necessary to maintain the stability of the polymer (Lopus, and Panda, 2006) 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,. In spite of their antitumor activity, drug resistance to such MTs inhibitors is common, limiting their overall clinical efficacy. (Perez, 2006). In addition, despite the success of taxanes and vinca alkaloids to inhibit the progression of some cancers in clinical use, resistance to anti-microtubule 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. (Tian *et al*., 2010) Moreover the most widely used vinca alkaloids such as vinblastine, effects including neurological and hematological toxicities and in particular, vinca alkaloids, its distinct antitumor activities in vivo due to its better pharmacokinetics profiles and its more specificity towards tubulin (Klener and Protinádorová, 1996). 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 (Li, *et al*., 2007). Most alkaloids are classified chemically according to the nitrogen-containing ring system. (Usui, *et al*., 1998). *Isatis tinctoria* is glastum is flowering plant it is commonly called dyers woad. Woad is native to the steppe and desert zones of the Caucasus, (Sajedi, and Asadi, 2005). On the contrary to the species *isatis tinctoria* which is understood to contain alkaloids that appear nontoxic in animal studies and have potent tumor-inhibitory effects and anti-angiogenetic effects (Honda, 1980; Sasan, 2009; Hoi, 2009) The alkaloids of the species are not yet investigated. This study is the first to assess the

activity of locally harvested *Isatis tinctoria* alkaloids against the microtubules of aggressive mice cell line H_{22}, and induction of apoptosis.

Material and Methods

The plant: The plat used in these experiments was gathered from gardens in Erbil (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, *Isatis tinctoria* Family Brassicaceae.

Figure (1): Photograph of the plant used in this study

Alkaloid extraction: Crude alkaloids extraction from the leafs of this plant was extracted as described by (Hassan, 2009; Cannell, 1998).

Cell line: In our experiments we used the mouse H_{22} cell line. This cell line was obtained from the Department of Biology, Faculty of Medicine, Wuhan University, China.

Characterization of invasive and metastasis properties *In vivo*: For invasive assay, group of five mice were injected in the intraperitoneal cavity with H_{22} 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 H_{22} tumor cells in the right leg muscle. After tumor development, right femur (the nearest boon) 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 *Isatis tinctoria* 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 and 100μg/ml), over one hundred (200, 400 and 800μg/ml), and over one thousand (4000 and 9000μg/ml), the crud alkaloids was prepared in1 ml of 2% SDS. The cells were exposed to the prepared solutions for 60min 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 20μg/ml for 15, 30 and 60min at 37°C in DMEM media. Cells were also treated for 5min in a medium containing alkaloids at a concentration of 800μg/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 20μg/ml for 60min. 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 12hrs in order to evaluate the recovery processes. Recovery progressed at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Visualization of microtubules network: *Iastis tinctoria* crude alkaloid extract treated cells were cultivated over microscopic cover slips, washed three times for 4min 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 antibodyTU-01 (Institute of Molecular Genetics, Prague, Czech Republic). 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 H_{22} 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} mm^2, 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 1mg/Kg/BW for deferent period of time. The treatment dose was determined according to the LD_{50} (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. 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.

Results and Discussion

The cell line under investigation was described to be an invasive and metastasis cell line (Liberatore, 2008). 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 and C). The aggressiveness of the H_{22} cells was substantial, the tumor cells was able to induce ascites tumor aggressively after two days of intraperitoneal injection (Figurer 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 ells exposed to alkaloid crude extract from leaves of *Isatis tinctoria* at concentrations from 2 to 10μg/ ml for 60min, 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 800μg/ ml for 60min they showed changes in the arrangement of the microtubule network (Figures 4 and 5). The network of cytoplasm microtubules at the lowest concentration used (20μg/ml) was clearly and obviously thinned down, and the treated cell individual microtubules fibers had a destructed and granulated wavelike shape.

Figure (1): The H_{22} 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 injection in the mice.

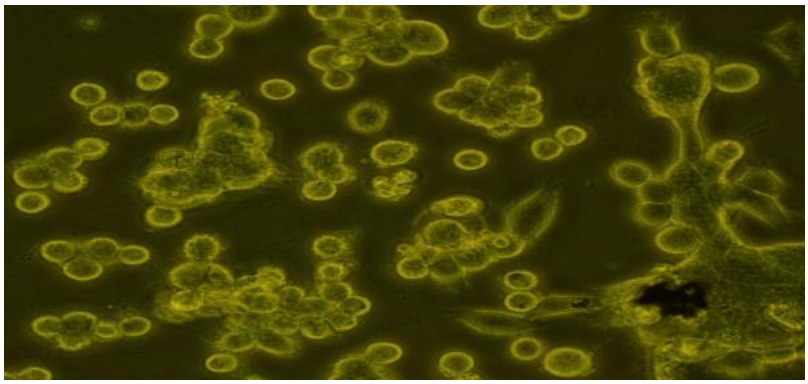

Figure (2): H_{22} cell line photograph in bright fielded microscopic.

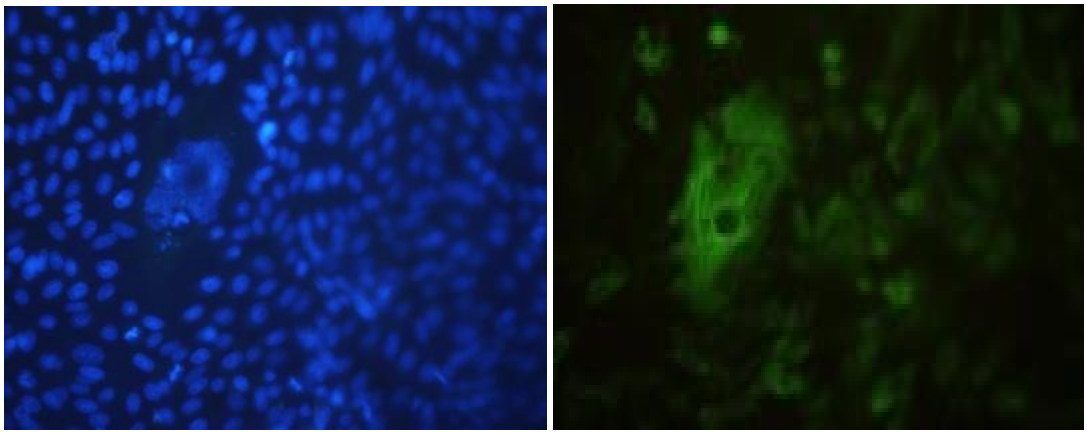

Figure (3): Visualizing H_{22}cells microtubules: untreated cells (control) and stained with secondary GFP conjugated swine anti-mouse IgG (upper panel), nuclei stained with DAPI (lower panel).

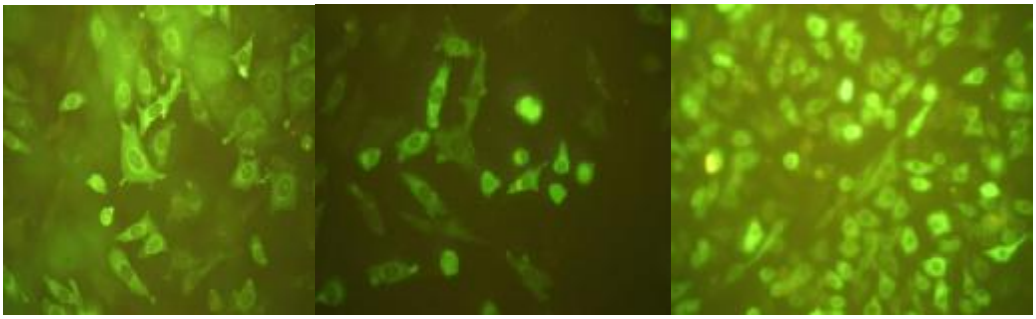

Figure (4): H_{22} cells treated with different sub-one hundred concentrations of *I. tinctoria* alkaloids extract as indicated for 60min is a representative microscopic fields.

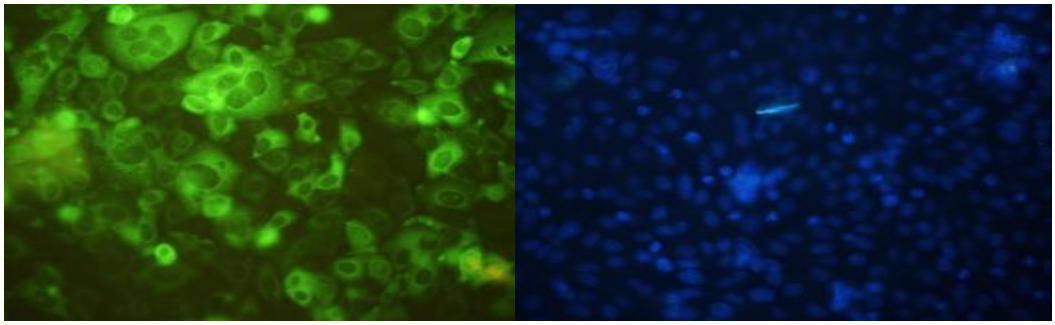

Figure (5): H_{22} cells treated with different over-one hundred concentrations of *I. tinctoria* alkaloids extract as indicated for 60min.

The network damage increased with the increasing of *I. tinctoria* alkaloid extract concentration from 20 to 800μg/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 9000μg/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 20min, no noticeable changes occurred in the microtubule network (data not shown). The 30min treatment at concentration of 20μg/ml did not cause an obvious disruption of the treated cell microtubules (Figure 7). When exposed to *I. tinctoria* alkaloid extract at a concentration of 800μg/ml for 5min, 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 6hrs in a drug-free growth medium following *I. tinctoria* alkaloid extract exposure, showed no recovered but damaged microtubules. The cells after 7hrs recovery period had their microtubules network either partially restored or still damaged. After recovery for an 8hrs 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 9hrs, the microtubules was also damaged, only several cells showed nearly restored microtubules. After a recovery period of 12hrs, 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 *I. tinctoria* leafs in vivo was considerably substantial, the tumor volume was reduced significantly ($P<0.01$) ten times after only two week of crude alkaloid extract treatment, and significantly ($P<0.01$) 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 and 13). A huge differences ($P<0.01$) were observed in tumor mass between treated and non-treated control animals after three weeks of 1mg/Kg/BW administration (Figure 14).

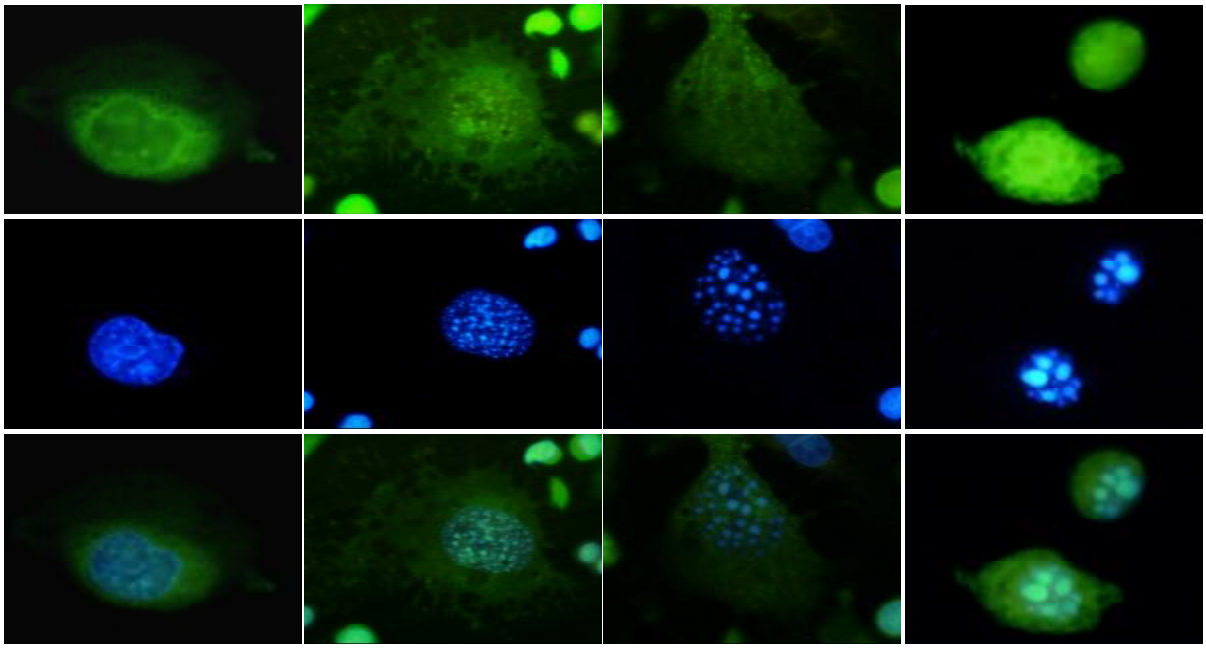

Figure (6): H_{22} cells treated with highest concentrations of *I. tinctoria* alkaloids extract as indicated for 60min stained with secondary GFP conjugated swine anti-mouse IgG, nuclei stained with DAPI, apoptosis form interphase after treatment of alkaloids.

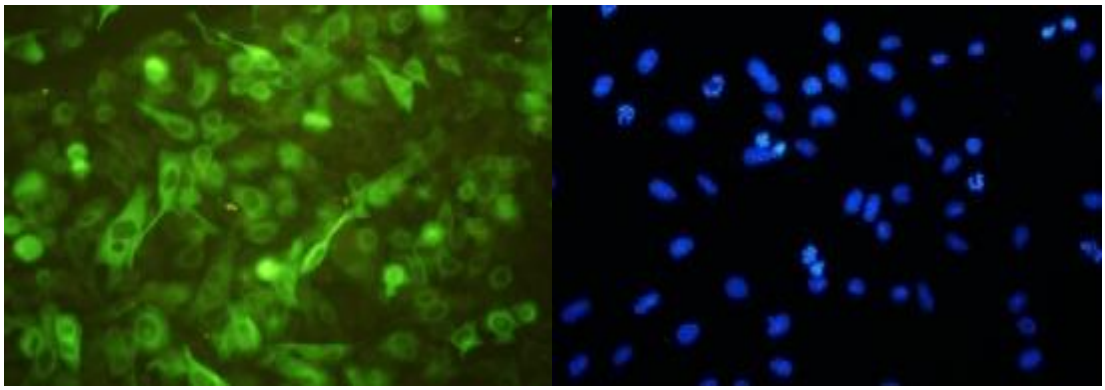

Figure (7): H_{22} cells treated with 20μg/ml of *I. tictoria* alkaloids extract as indicated for 30 min, stained with secondary GFP conjugated swine anti-mouse IgG (middle panels) nuclei stained with DAPI (lower panels).

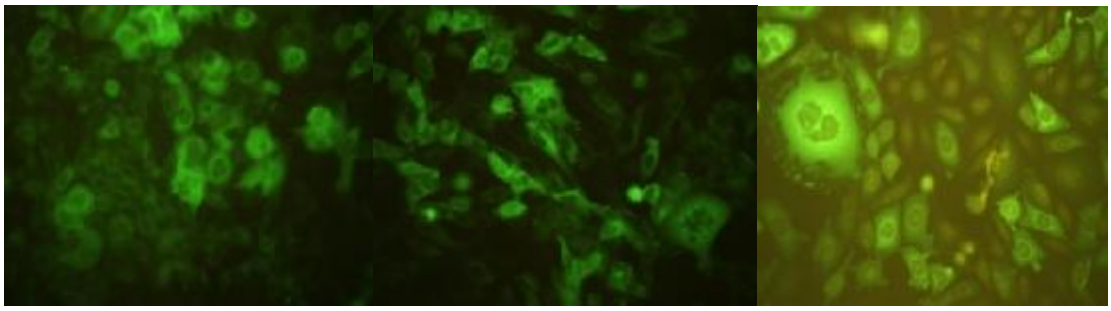

Figure (8): H_{22} cells treated with 800 μg/ml of *I. tinctoria* alkaloids extract as indicated for 5min, stained with secondary GFP conjugated swine anti-mouse IgG.

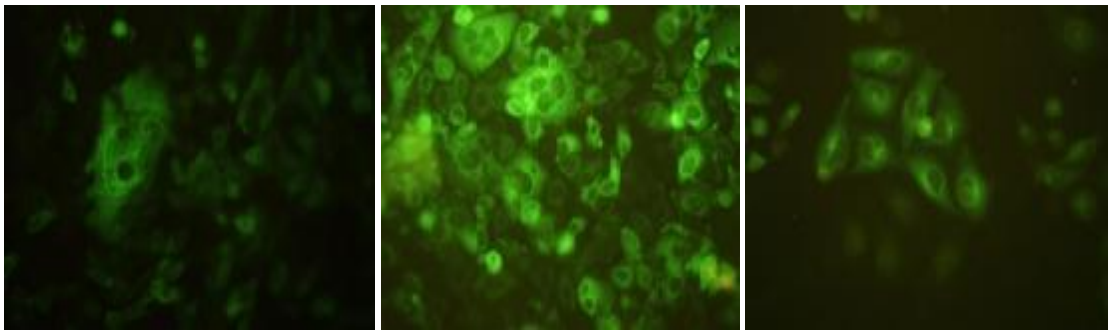

Figure (9): H_{22} cells treated with 20μg/ml of *I. tinctoria* alkaloids extract as indicated for 60min as indicated in the methods and recovered with fresh media for 6, 7 and 8hrs, stained with secondary GFP conjugated.

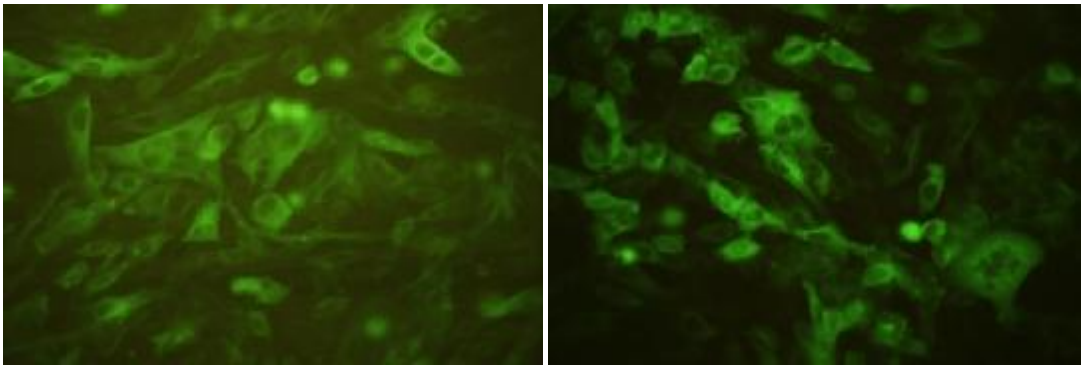

Figure (10): H_{22} cells treated with 20μg/ml of *I. tinctoria* alkaloids extract for 60min as indicated in the methods and recovered with fresh media for 9 and 12hrs.

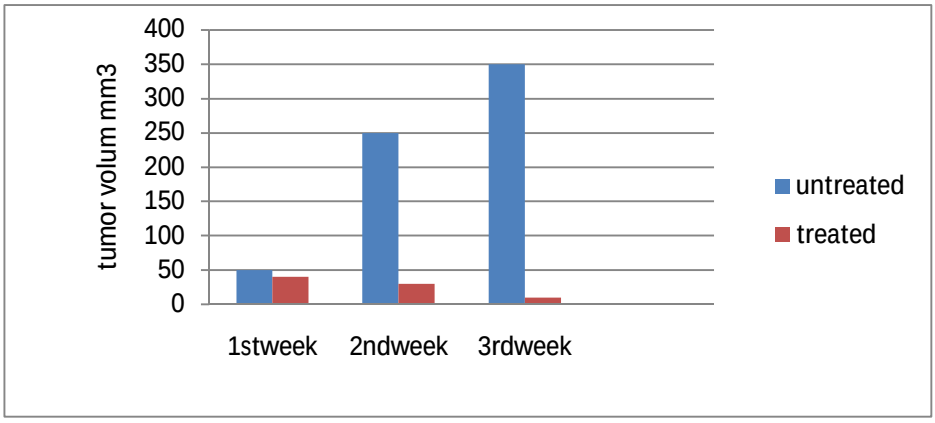

Figure (11): Tumor volume in mice treated with alkaloids extract of *I. tinctoria* for deferent periods of time compared to untreated control mice.

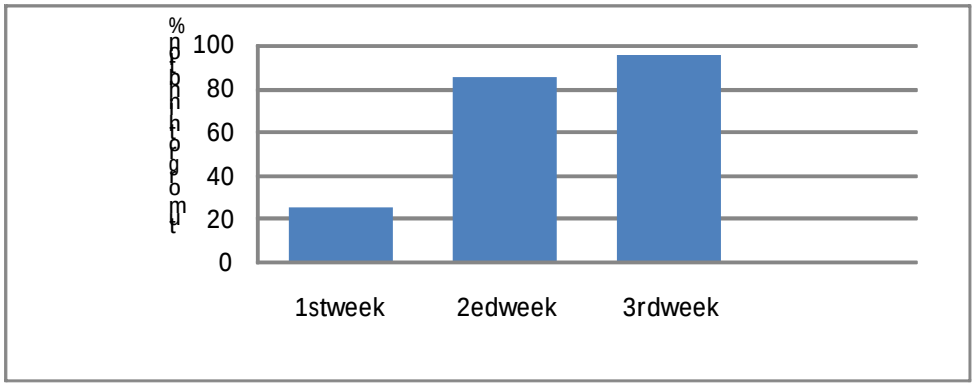

Figure (12) : Tumor growth inhibition percentage in mice treated with the alkaloids extract of *I. tinctoria* during the three different time of administration *In vivo*.

Figure (13): Antitumor activity of *I. tinctoria* alkaloids 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.

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

The antitumor activity of the crude alkaloid extract of *I. tinctoria* leaves *In vivo* was considerably substantial, the tumor volume was reduced significantly ($P<0.01$)ten times after only two week of crude alkaloid extract treatment, and significantly ($P<0.01$) the 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 and 13). A huge differences ($P<0.01$) were observed in tumor mass between treated and non-treated control animals after three weeks of 1mg/Kg/BW administration (Figure 14). Targeting cancer cells microtubules is one of many strategies utilized to defeat deferent types of this disease (Liberatore, 2008). Natural products were of the realist chemicals to be recognized as potent antitumor drugs as a result of their interaction with cancer cells microtubules (Zhao *et al.*, 2009). 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 48hrs after its injection indicted such conclusion. Moreover when it injected in the right leg of mice group, it metastasized to the nearest boon (femur) in period of one week in all individuals of the injected animals. The huge femur boon morphological deformations in the injected animals were clearly indicted as a result of boon 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 (Farombi and Owoeye, 2011). Hoi *et al.,* (2009) studied the effect and action mechanisms of try ptanthrin on murine myloid leukemia cells interaction of alkaloids with tubulin, and compared alkaloid and aqueous extract from leaves of *I. tinctoria*. 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 60min, 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 5min. All these results indicated the specific targeting of the microtubules by alkaloid extract of *I. tenctoria* leaves. Cells with apoptotic characteristics started to appears in alkaloid concentrations started from 100μg/ml and higher during exposure time (60 min). This refer to another possible effecter mechanism that alkaloid extract exercise towered H_{22} cells. Nagappan and co-workers found that carbazole alkaloids have antitumor activity with much higher concentration, the significant minimum inhibition concentration (MIC) values was 25.0–175.0mg/ml against MCF-7, Hela and P388 cell lines (Jordan, 2002). 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 3hrs of incubation, but

were expressed in all cells after 6 hours. After 3hrs of drug exposure, the cells were sub-cultured in drug-free media, the cells cytoskeletal structure reformed within 10hrs. In competence with this, alkaloid extract of *I. tintoria* leafs demonstrate the same mechanism effect on the cell line under this study. Its antitubules effect was eliminated after 10-12hrs 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 non-treated 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.

Conclusions

The invasive H_{22} cells showed changes in the arrangement of their microtubules at a concentration of *I. tinctoria* leaf crude alkaloids extract as low as 20µg/ml concentration after 60min of exposure time. Its damage increased with increment of the alkaloids extract concentration. Increasing exposure dose may reduce the exposure time; disruption of the microtubules was also time-dependent. The extract was able to reduce tumor growth *In vivo* up to 95% of control untreated animals and inhibit tumor growth in the treated mice to 97.14% compared to control untreated animals.

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