Effect alkaloid Narciclasine Extraction of Zephyranthes candida On Microtubulues of HepG2 Hepatic Cell Line

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Abstract

Background: Alkaloid narciclasine extraction of *Zephyranthes candida* are microtubular toxins of chemically similar nature that disrupt microtubule function by binding to a site on β-tubulin and suppressing microtubule dynamics.effect in hepatic cell line.

Objective: To evaluate biological activity of alkaloid narciclasine extraction of *Zephyranthes candida* on microtubule HepG2 cell line by used GF tubulin with alcoholic extraction.

Patients and Methods: The experiments work in China (Wuhan) 2016 need about 6 months , we used TLC technology to extraction alkaloids narciclasine from by used different concentration 20,40,80,100,200,400 µg/ml for one hours to microtubule cell line of HepG2 cell line and used high concentration 1000,2000 µg/ml to investigate the action of alkaloids narcilacsine onto the network of microtubule to one hours .

Results: Increased the number of the cell effect by extraction in microtubule by used GF tubulin inside the cell by alkaloid or alcoholic extraction see the results by immunofluorescence microscope, the microtubule is effect by high concentration of extraction see apoptosis and thinned down, and individual fibres have a wavelike shape. Anther experimental work was conducted to determine the biological activity of alkaloids Narcilasine on microtubule in concentration 20 µg/ml in (12,10,8,6) hours incubation and 400 µg/ml for 10 min to study cell line and the recovery of its disruption.Cells were treated with alkaloids at various concentrations from 20 µg/ml to 400 µg/ml for 60 min microtubules were recovered and network is nearly restored.the results show the microtubulesis back normal after reduce extraction alcoholic and alkaloids in 12 hours by used indirect immunofluorescence.

Conclusion: The cells showed changes in the arrangement of microtubules even at the 80 µg/ml concentration of cytostatics after 60-min exposition. Its damage increased with increasing concentration of cytostatics.

Key words: Cytoskeleton, microtubule disruption, cytoskeleton recovery, alkaloids.

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Introduction

In eukaryotic cells the cytoskeleton is formed by three major structural elements– microtubules, microfilaments, and intermediate filaments [1]. The cytoskeleton plays a specific role in cell division, maintenance and changes of cell 20 shape, in intracellular contacts, interaction with membranes, extracellular matrix, and in cell motions.Microtubules are filaments with the

largest diameter of all cytoskeletal components.Microtubular network is important for the execution of many cell functions.They play an important role in cell division. The microtubular diameter measures about 25 nm. Microtubules are composed of 13 equally spaced protofilaments [2].

Tubulin is the basic protein of the microtubules and molecules of tubulin are arranged in dimers containing both its forms: α-tubulin and β-tubulin. Microtubules are continuously changeable structures [3]. A polymerisation and depolymerisation of MTs is regulated by extracellular and intracellular factors [4]. Guanosine triphoshate (GTP) at microtubule ends is necessary to maintain the stability of the polymer [5].

The cytoskeleton can be damaged through the effect of many external factors or chemical agents [6]. The opposite ends of free microtubules show different sensitivities to microtubule depolymerising agents such as low temperature, Ca++ or colchicine[7]. The mitotic spindle is a self-organising structure that is constructed primarily from microtubules. Among the most important spindle microtubules are those that bind to kinetochores and form the fibres along which chromosomes move. Vinca alkaloids vincristine and vinblastine are microtubular toxins of chemically similar nature [8] that disrupt microtubule function by binding to a site on β-tubulin and suppressing microtubule dynamics. Although they are closely related in physical and chemical properties, they have various effects on the human body. When microtubules fail to attach to one or more kinetochores as a result of drug treatment, the checkpoint components continue to generate signals that inhibit the metaphase/anaphase transition that delays cell cycle progressionand induces programmed cell death [9].

At higher drug concentrations, vinca alkaloids induce the assembly of spiral filaments of tubulin, which, consequently, can interact laterally and form paracrystals [10]. This action is similar to the action of colchicine, but is different from that of paclitaxel, which promotes the polymerization of tubulin polymers to form abnormal stable. microtubule structures. Treatment with high concentrations (100 nM) of vincristine cause disruption of microtubule organization, which, in turn, prevent accumulation of p53 in the nucleus. By contrast, treatment with lower drug concentrations (3 nM) that are known to suppress microtubule dynamics but do not alter microtubule organization enhance nuclear accumulation of p53 and its downstream transactivated elements above physiological levels [11]. Vincristine and vinblastine have been widely used to treat cancer (e.g. acute leukaemia, rhabdomyosarcoma, neuroblastoma, Hodgkin's disease), to synchronise cell cycle, or to look for defects in the mitotic[13].

The present study aims to evaluate biological activity of alkaloid narciclasine extraction of *Zephyranthes candida* on microtubule HepG2 cell line by used GF tubulin with alcoholic extraction.

Materials and Methods

Cell line: China hamster ovary cells line (CHO))(from the department of Biology, faculty of Medicine, Wuhan University - China) used in vivo were grown in monolayers at 37°C without antibiotics in 5%C02/95% air [14] Cell proliferation was determined by countingcells by hemocytometer at the time of alkaloids addition and20 h later. Mitotic index, cell morphology, and spindle interpolar distances were determined by immunofluorescence microscopy [15].

Preparation of alcohol and alkaloids extract of Zephyranthes candida: For the preparation of the aqueous extract, 50 gram of soft plant was weight and added in to 500 ml of distilled water, and then put 5-10

minutes in a blender for mixing until homogeneity. Extract was put in to a clean sterile bottles and transferred to the Shaker for an hour, then centrifuged for 10 minutes (2000 rpm / min), get rid of the sediment and then taking supernatant which then distributed in the clean dry dishes, and left in an incubator to get the dry extract to prepare concentrations used in the study [11]. crude alcoholic extract gave 5 gm out of 75 grams dry, i.e. the extraction ratio was 6.66 % of crude. The extract was dark brown to black color, thick and little viscous[16].

For preparation of alkaloids extract from plant , Soxhlet is used. Twenty 20 g of dry powder was took and placed in Thimble, then put Thimble in the space provided in the Soxhlet device and hexane was added to remove fat and chlorophyll and conducted extraction for 12 hours at a temperature (40-60°C) which is the temperature of the evaporation of solvent used. Then after that, the powder was transferred to Reflex device with 70% alcohol methanol for three hours, and then the ex-tract was filtered by piece of gauze and filter paper then incubated for 24 hours for evaporation of alkaloids[17].

Exposure to alkaloids Narciclasine.

In the first series of experiments, to investigate the action of alkaloids narciclasine on to the microtubule network, a solution containing 1 mg of alkaloids sulfas in 1 ml of medium an original solution containing 5 mg of alkaloids sulfas in 5 ml of medium was mixed with 3 ml of growth medium in each of the Petri dishes so that the final concentration of alkaloids, or was 20, 30, 40, 80, 100, 200, 400, 800 µg/l, or 4000 µg/l and 8000 µg/l (alkaloids only). Each concentration was in two dishes. The cells were exposed to the drugs for 60 min at 37 °C. After the treatment[18]. The samples were washed three times for 4 minutes concurrently with control samples in phosphate-buffered saline (PBS, Ph=7) and processed for immunofluorescence microscopy[18].

In the second series of experiments the cells were cultivated for 5, 10, 20, 30,and 60 minutes at 37 °C in media with a final alkaloids concentration of 20 µg/l, or for 5 minutes in a medium containing alkaloids at a concentration of 800 µg/l. The samples were washed three times for 4 minutes concurrently with control samples in PBS, pH 7, and processed for immunofluorescence microscopy. The third series of experiments was performed with alkaloids at a final concentration of 20 µg/l. The cells were exposed to the drug for 60 min. After the treatment, the drug-containing medium was poured off and monolayers were subjected to three washing procedures concurrently with control samples with phosphate-buffered saline (PBS) (pH 7)[19].

Two slips with the cells were then subjected immediately to fixation and detection of the microtubular network as well as two slips which were cultivated for 60 min in alkaloids -free growth medium as a control. The other Petri dishes were refilled with fresh growth medium and incubated for another 6, 7, 8, 9, and 10 hours in order to evaluate the recovery processes. Recovery progressed at 37 °C in a humidified atmosphere of 95 % air and 5 % CO2. The other control monolayers treated with a growth medium only containing alkaloids-free DMEM were cultured in the same conditions as the alkaloids -treated cells recovered for 10 hours. After fixation, the microtubular components were visualised and viewed in a fluorescence microscope. A similar attempt was provided for a alkaloids concentration of 400 µg/l. The cells were exposed 22 to the drug for 60 min, and after the washing procedure, they were recovered for 8 or 12 hours in the same condition as last mentioned. Microtubular components were visualised and examined in a fluorescence microscope.To quantify the cytoskeleton

changes, cells were incubated in a fourth series of experiments at 37 °C in a medium containing alkaloids at a concentration of 20 µg/l for 60 minutes. They were cultivated on two coverslips in two Petri dishes. The control cells on two slips were cultivated parallelly. The medium was poured off and the cells were subjected to three washing procedures with PBS (pH 7)[20].

Visualisation of microtubular network

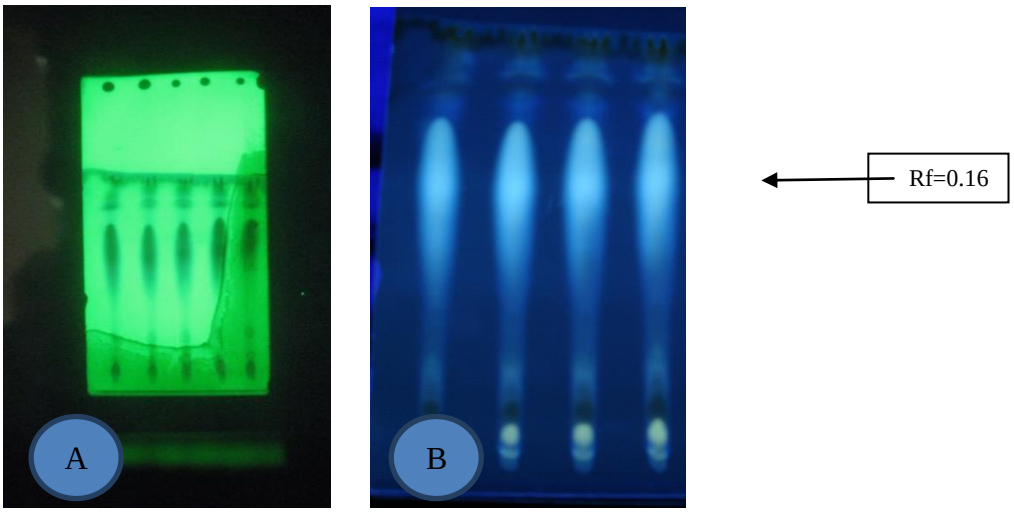
Cells were washed three times for 4 min in the phosphate-buffered saline (PBS, pH 7) and fixed by 3 % paraformaldehyde in PBS. Thereafter, the cells were permeabilised by 0.2 % Triton X-100 solution in PBS. The microtubules were detected by means of the tubulin monoclonal antibody TU-. diluted 1:300 by PBS, and a secondary antibody SwAM/FITC (conjugated swine anti-mouse globulin/fluorescein isothiocyanate; Institute for Sera and Vaccines, Prague, Czech Republic), diluted 1:100 by PBS. The cells were washed in the phosphate buffer three times for 5 min between the application of individual agents[21].

Statistical analysis

The differences are compared by using Data acquired with Duncun [22].

Result

The results of alkaloids was extracted by using several techniques which includes chromatographic techniques, and thin layer chromatographic (TLC). According to the melting point, absoration measuring and alkaloid spot colour the conclusion was made that, this alkaloid is the narciclasine extraction of *Zephyranthes candida.*



Figure(1): Detection of alkaloid by TLC, A –In 254 nanometer and B- In 365 nanometer.

Table 1, shows considerable changes in the distribution of microtubules at high concentration in 60 min and untreated control cells. Cells exposed to drugs at concentrations of 20 µg/ml and 40 µg/ml for 60 min did not show considerable changes in the distribution of microtubules. but when cells exposed to drugs at concentrations of 80 µg/ml to 400 µg/ml for 60 min There were significant differences in all other groups. The quantity of microtubules in the cytoplasm was significantly higher ($P<0.05$).

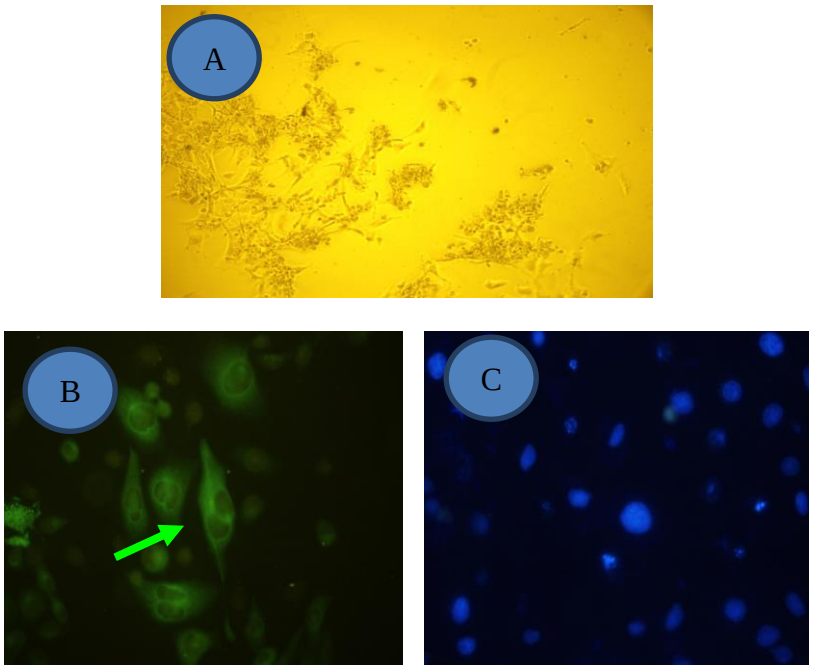
Table (1): Effect alkaloids Narciclasine at various concentrations with alcoholic extraction of *Zephyranthes candida* in HepG2 cell line in considerable changes in the distribution of microtubules.

	Narciclasine alkaloids		Alcoholic extraction	
Concentration µg\ml	%	Changes Microtubules	%	Changes Microtubules.
control		0.00±4.20 a		0.00±4.20 a
20	13.33	0.56±0.56 f	7.14	0.11±0.30 f
40	30.09	0.11±1.60 e	26.19	0.20±1.10 e
*80	55.47	0.08±2.33 d	36.42	0.17±2.21 d
*100	79.28	0.08±3.33 c	50.00	0.11±2.53 c
*200	83.33	0.40±3.50 c	57.82	0.17±2.43 c
*400	91.19	0.03±3.83 abc	69.04	0.051±2.90 b

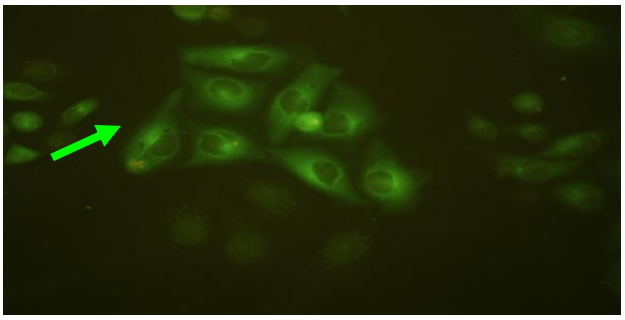
*Different letters means the presence of significant different at (P<0.05).

Untreated control cells (Figure 2) showed a microtubule network regularly distributed along the whole cell content. When cells exposed to drugs at concentrations of 20 µg/ml for 60 min did not show considerable changes in the distribution of microtubules.

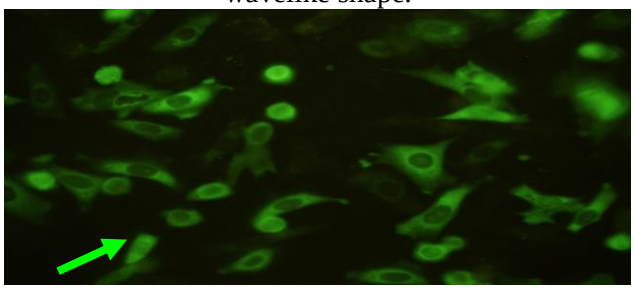
Cells exposed to alkaloids or alcoholic extraction at concentrations of 80,100, 200and 400 µg/ml for 60 min showed changes in the arrangement of the microtubular network (Figurs. 3, 4).



Figure(2): HepG2 cell line and microtubules of untreated control cells of HepG2 cell line. The network is regularly distributed along the whole cell content A,B,C .

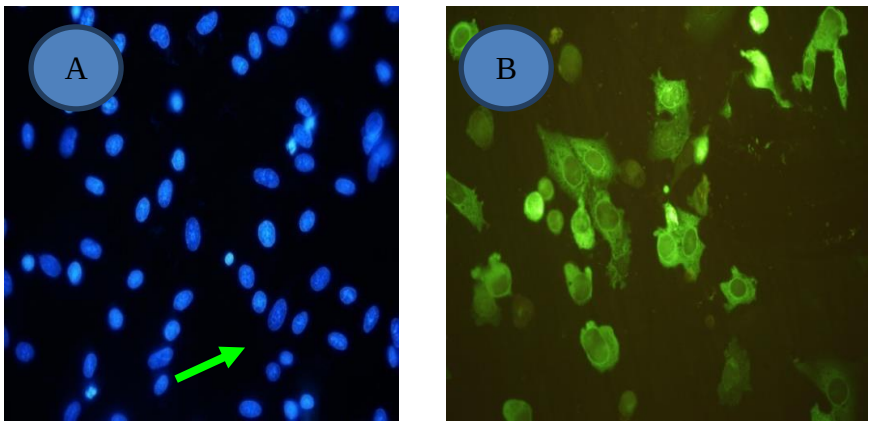


Figure(3): Microtubules of HepG2 cell line treated with alkaloids at a concentration of 80 µ/ml for 60 min. The network of cytoplasmatic microtubules is thinned down, and individual fibres have a wavelike shape.

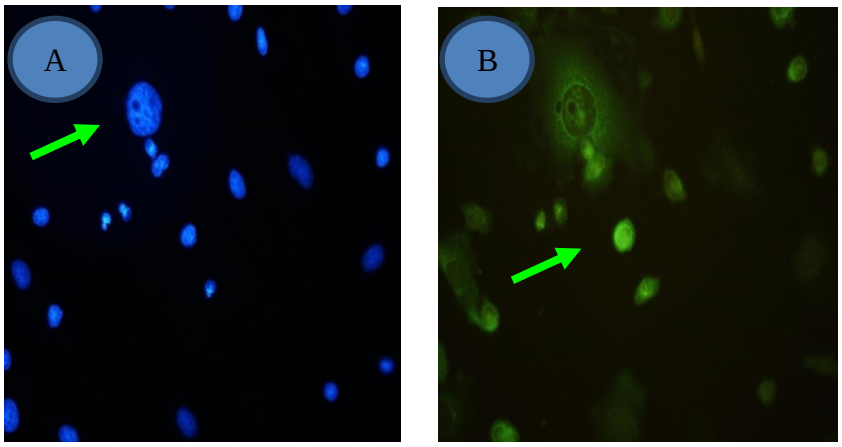


Figure(4): Microtubules of HepG2 cell line treated with alkaloids at a concentration of 400 µ/ml for 60 min. The network of cytoplasmatic microtubules is thinned down, and individual fibres have a wavelike shape.

The network of cytoplasmatic microtubules at concentrations of 10, 80 µg/ml was thinned down, and individual fibres showed a wavelike shape. The network damage increased with increasing concentration of cytostatics. The microtubules were more thinned down and fragmentation of fibres occurred. At a higher concentration of 400 µg/ml, sometimes blebs were formed (Fig. 5). Cells exposed to alkaloids at concentrations of 1000 µg/ml and 2000 µg/ml formed paracrystals (Fig. 6),No significant difference was detected between alkaloids and alcoholic extraction treated cells.



Figure(5): Cells of HepG2 cell line treated with alkaloids at a concentration of 400 µg/ml for 60 min. There are blebs formed on the periphery of the cells A,B.



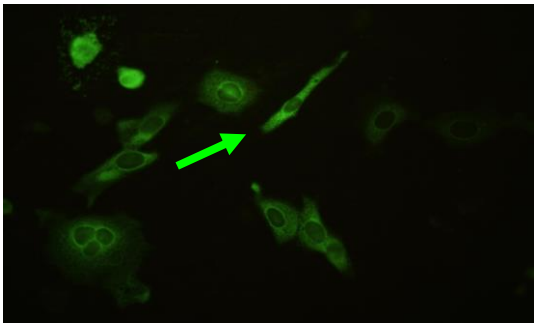
Figure(6): HepG2 cells treated with alkaloids at a concentration of 1000 µg/ml for 60 min. Paracrystals are formed A,B.

When cells were exposed to alkaloids at a concentration of 20 µg/ml for 6, 8, or 10 hrs, no noticeable changes occurred in the microtubule network.but in the 12 hrs treatment at a concentration of 20 µg/ml caused disruption of microtubules. The network was thinned down, and individual fibres had a wavelike shape.

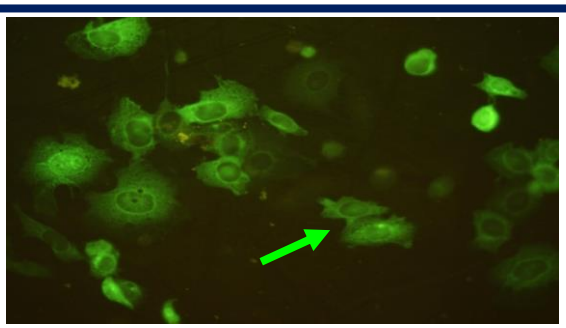
The cells exposed to alkaloids at a concentration of 400 µg/ml for 10 minutes showed a severely defective microtubular network. All the cells with the recovering period of 12 hours in a drug-free growth medium following alkaloids treatment showed damage of microtubules. The cells after a 12 hours recovery period had their microtubular network either fully restored or still damaged. After recovery for an 12 hrs period, some cells showed a partly defective (thinned-down) network, but the majority of the cells showed restored microtubules.

When the cells were allowed to recover for 12 hours, the microtubules were spread out comparably to those observed in untreated control cells. The control cells showed their microtubule network regularly distributed along the whole cell volume.

The microtubules were thinned down, and individual fibres had a wavelike shape. After a recovery period of 12 hrs, the network was also damaged (Figure 7). When cells were recovered after treatment with alkaloids at a concentration of 400 µg/ml for 10 min, the cytoskeleton was partially restored afterwards (Figure 8).



Figure(7): Microtubules of HepG2 cell line treated with alkaloids at a concentration of 20 µ/ml.Microtubules recovered for 12 hrs. The network is nearly restored.



Figure(8): Microtubules of HepG2 cell line treated with alkaloids at a concentration of 400 µ/ml for 10 min.Microtubules were recovered and network is nearly restored.

Discussion

The interaction of antitumour agents with compounds of the cytoskeleton is a theme studied in several studies [23]. Lobert [24] studied the interaction of Vinca alkaloids with tubulin, and compared vinblastine and vincristine.She studied e.g. the affinity of the drug for tubulin heterodimers.

Alkaloids exhibited a higher overall affinity for porcine brain tubulin than alcoholic ectraction, but the affinity of the drug for tubulin heterodimers was identical for the two drugs. Under our experimental conditions we did not mark any differences between the two drugs. Some authors also studied the recovering processes of the cytoskeleton after treatment of cell cultures with physical factors or agents interfering with cytoskeleton compounds. Alkaloids caused a sequence of morphological changes in sensitive 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[25].

The changes occurred initially within 6 hrs of incubation, but were expressed in all cells after 12 hrs. If, after 6 hrs of drug exposure, the cells were subcultured in drug-free media, the cytoskeletal structure reformed within 12 hrs. The maximal recovery of the cytoskeletal structure occurred 12 hrs after drug removal and was sustained up to 12 hrs eliminated the microtubule bundles, leaving only tubulin paracrystals. Within 12 hrs after washing out the alkaloids [26].

Treatment with alkaloids [27] the microtubule bundles repolymerised in cultured hippocampal neurons. [28] they investigated quantitative changes after application of the microtubule inhibitor nocodazole. They presented a decrease of the assembled tubulin after treatment with nocodazole and a less delicate structure of the remaining microtubules. This was indicated by a reduction of the parameters used. They also showed significant differences between the high and low metastatic cell lines. They used confocal laser scanning microscopy.

We have no possibility of comparing the mathematical approach because the description of image analysis is not detailed in this paper, but we suppose to have probably a more appropriate approach on account of using histogram expansion, gamma correction [29].

In conclusion, the cells showed changes in the arrangement of microtubules even at the 80 µg/ml concentration of cytostatics after 60-min exposition. Its damage increased with increasing concentration of cytostatics.

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