ADHESION BEHAVIOR AND FUNCTIONAL STUDIES ON NORMAL AND TUMORAL CELLS EXPOSED TO *ARISTOLOCHIA CLEMATITIS* AQUEOUS EXTRACTS

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Keywords: *Aristolochia clematitis*, cell culture, flow chamber adhesion assay, MTT assay

Abstract *Aristolochia clematitis*, member of the ***Aristolochiaceae*** family is a medicinal plant used from immemorial time in folkloric medicine to succesfully treats various ailments, from snake bites to cancer. Recently *Aristolochia* plant use as a natural or pharmacologic remedy was banned by the Food and Drug Administration (FDA), because it contains aristolochic acids, carcinogenic and nephrotoxic compounds. The purpose of this study is to assess the effects of different concentrations of *Aristolochia clematitis* aqueous extract on human breast adenocarcinoma cell lines (MDA-MB-231 and MDA-MB-468), tumor-associated fibroblasts (TAFs), and mesenchymal stem cells (MSCs). Viability and proliferation MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, and a flow chamber adhesion assay were used in order to test the adhesion capacity of TAFs and MSCs treated with *A. clematitis* extracts on MDA-MB-231 or MDA-MB-468 cellular coatings. Our studies demonstrated a significant inhibitory effect of *A. clematitis* extracts on the viability and function of normal cells (MSCs) compared to tumor cells.

INTRODUCTION

Aristolochia clematitis (Birthwort), member of the ***Aristolochiaceae*** (Birthwort) family, popularly named in Romania *„Mărul Lupului”, „Curcubetica”, „Buruiana de remf”*, is a medicinal plant that has a very long history of medicinal use (Arlt et al., 2002; Nortier and Vanherweghem, 2007). It is an aromatic tonic herb that stimulates the uterus, reduces inflammation, controls bacterial infections, and promotes wound healing (Steenkamp and Stewart, 2005). Herbal drugs derived from *Aristolochia* species have been known since antiquity and were used in the treatment of skin infections, and in the treatment of snake bites in ancient Egypt and India. Contemporary traditional medicine has used *Aristolochia* plant extracts for the therapy of arthritis, gout, and rheumatism (Stiborova´ et al., 2008). Externally, various parts of the plant are used in the treatment of slow-healing cuts, eczema, infected toe and finger nails, but internal consumption can cause uterine bleeding and especially damage to the kidneys (Steenkamp and Stewart, 2005).

The plant contains a complex of acids so called “aristolochic acids” (Shibutani et al., 2007), which stimulate white blood cell activity and speeds the healing of wounds if it is used in the right concentration (Steenkamp and Stewart, 2005). However these acids are also known as compounds with carcinogenic and nephrotoxic effects (Wu et al., 2004).

Consumption of products containing aristolochic acid has been associated with permanent kidney damage, sometimes resulting in irreversible kidney failure that has required kidney dialysis or kidney transplantation. In addition, some patients have developed certain types of cancers, most often occurring in the urinary tract (Nortier et al., 2000).

Aristolochia clematitis and aristolochic acids are considered to be responsible for the development of Aristolochic Acid Nephropathy (AAN) (formerly known as the Chinese Herbs Nephropathy, CHN) (Cosyns et al., 1994) and induction of upper urinary tract tumors. An interesting correlation has also been made between *Aristolochia clematitis* and the etiology of Balkan Endemic Nephropathy (BEN) (Slade et al., 2009; Grollman and Jelaković, 2007; Grollman et al., 2007).

In 2001, the Food and Drug Administration (FDA) issued warnings and an import alert that herbal products are unsafe if they contain or are suspected to contain aristolochic acids. Despite the action of the FDA and the effort of many countries, 19 products containing AAs and 95 products suspected to contain AAs were for sale on the Web in 2003, and are currently sold in many open markets in Romania. The International Agency for Research on Cancer also classifies products containing *Aristolochia* species as group 1 human carcinogen.

The aim of the present study is to investigate the effect of different concentrations of an aqueous extract of *Aristolochia clematitis* on human breast adenocarcinoma cell lines, tumor-associated fibroblasts (TAFs), and mesenchymal stem cells (MSCs) functions, using a viability and proliferation MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, and of the adhesion capacity of TAFs and MSCs treated with *A. clematitis* extracts on MDA-MB-231 or MDA-MB-468 cellular coatings, using a flow chamber adhesion assay.

MATERIALS AND METHODS

Cell culture

In our experiments human tumor-associated fibroblasts (TAFs), human mesenchymal stem cells (MSCs) and two human breast adenocarcinoma cell lines, MDA-MB-231 and MDA-MB-468 were used.

Human mesenchymal stem cells (MSCs) were obtained from bone marrow from healthy Orthopedics patients undergoing bone surgery. Approximately 10 ml of bone marrow were placed in culture plates, and the fibroblastic-like, plastic adherent fraction, was isolated following multiple passages and used in our experiments. The MSCs were further cultured and expanded in alpha-minimum essential medium (MEM; Gibco BRL, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany), 10 ng/mL basic fibroblast growth factor (FGF; Sigma, St. Louis, MO, USA) and 2% Penicillin/Streptomycin mixture (Pen/Strep, 10,000 IU/ml; PromoCell), by incubation at 37ºC in 5% CO_2 atmosphere. Medium was replaced every three days and when the confluence was 80-90% the cells were passed using 0.25% Trypsin-EDTA solution (Sigma) followed by centrifugation (10 minutes, 300g) and replated in T75 culture flasks at a density of 10,000 cells/cm^2 to ensure optimal proliferation.

Human tumor-associated fibroblasts (TAFs) were isolated using both the explant and collagenase type IV-S from *Clostridium histolyticum* (Sigma) methods. Breast cancer surgical pieces of approximately 5 cm^2 were obtained form 8 female patients, with the histopathological diagnosis of infiltrative ductal mammary carcinoma. Tissue-isolated cells were washed several times with phosphate buffered saline (PBS, Sigma) solution, and passed through 0.70/0.40 μm strainer filters and were replated as single-cell suspension in adherent plastic culture plates. TAFs were further subcultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma), supplemented with 10% fetal calf serum (FCS; PromoCell) and 2% Penicillin/Streptomycin mixture (Pen/Strep, 10,000 IU/ml; PromoCell), by incubation at 37ºC in 5% CO2 atmosphere and the medium was replaced every three or four days.

Both MSCs and TAFs were used in the experiments at passages four and five.

All tissue samples were obtained after signing the informed consent elaborated under an approved protocol, according to the World Medical Association Declaration of Helsinki.

The two human breast adenocarcinoma cell lines, MDA-MB-231 and MDA-MB-468 were purchased from ATCC, USA. Both cell lines were cultured in ATCC-formulated Leibovitz's L-15 Medium, supplemented with 10% fetal calf serum (FCS; PromoCell) and 2% Penicillin/Streptomycin mixture (Pen/Strep, 10,000 IU/ml; PromoCell) by incubation at 37ºC in a humidified atmosphere of 100% air, without CO_2.

Extracts

We prepared our extracts following the traditional recipes used in Romanian villages, where decoctions are made from dried and crushed leaves. *Aristolochia clematitis* plants were harvested from BEN non-endemic areas in the summer of 2009, during the plant flowering season. For our assays, we used 2 grams of dried leaves of *Aristolochia*, crushed and mixed with 200 mL of distilled water and refluxed for 30 minutes and then cooled in order to obtain the aqueous extract. All the plant extracts were sterile filtered using 0.2 μm polytetrafluoroethylene (PTFE) filters before using them in our experiments.

MTT- based toxicology Assay

Toxicity of the extracts was determined using a standard MTT assay (Sigma). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is based on the assessment of mitochondrial activity due to the conversion of the yellow tetrazolium salt MTT by mitochondrial dehydrogenase of live cells to the purple MTT formazan (Mosmann, 1983). According to manufacturer's protocol, 15 μl of the 5 mg/ml MTT reagent stock solution (prepared in phosphate-buffered saline) were added to each well of the microtitre plate and plates were incubated for an additional 3 h in the dark at 37^0C; the resulting intracellular purple formazan product were dissolved in 150 μL of Detergent Reagent. The plates were allowed to stand for 10 minutes at room temperature in the dark, and absorbances were read at 570 nm using a spectrophotometric plate reader (Bio-Rad, Tokyo, Japan) with a reference wavelength of 655 nm to subtract background. The results are the average values from quadruplicate readings and the subtracted average values for the blank. The percent of cell viability (% cell viability) values, computed as mean absorbance of sample*100/mean absorbance of control, that are lower than those of the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher % cell viability rate indicates an increase in cell proliferation.

The cells were seeded on 96-well culture plate at a density of 7 x 10^3 cells /well in quadruplicate. The cells were incubated for 24 hours and then the medium was changed with fresh medium completed with four different concentrations of *Aristolochia clematitis* aqueous extracts: 10%, 15%, 20%, 30% and sterile distilled water (30%/well) was used as control. Cells were exposed to *Aristolochia clematitis* aqueous extracts for 48 hours, before the viability and proliferation assays.

Flow chamber adhesion assay

MDA-MB-231 and MDA MB-468 adenocarcinoma cell lines were cultured as cellular layer in 6-channels μ-Slide VI ibiTreat flowchamber (Ibidi Integrated BioDiagnostics, Munich, Germany), while adherence and optimal confluence occurred 24 hours after the initial plating. At this time point, 10%, 15%, 20%, and 30% *Aristolochia clematitis* aqueous

extracts replaced the initial growth medium of cellular substrates in 4 different channels of the flowchamber plate, while sterile distilled water (30%) and specific medium were used as control in the other 2 channels of the plate. The layer cells were exposed to *Aristolochia clematitis* aqueous extracts for 48 hours, incubated at 37ºC in a humidified atmosphere of 100% air, without CO_2, according to the supplier's protocol.

Adhesion capacity of MSCs and TAFs on tumor cells substrates treated with various concentrations of *Aristolochia clematitis* aqueous extracts was tested by infusing 100 µl of 100,000 suspension cells on top of the attached MDA-MB-231 and MDA MB-468 cell lines. MSCs and TAFs were left to interact with the cellular substrate for 3 minutes and then underwent progressively increasing shear stress of 0.35, 2, 5, 8 and 15 dynes/cm^2 generated using an ISMATEC pump - IPC High Precision Multichannel Dispenser (IDEX Corporation, Glattburg, Switzerland), for a total time of 3 minutes. Pictures of the centered microscopic field were taken every 30 seconds, for every value of shear stress, and total cell count was compared with the control. Variations of at least 15% in cell count were considered significant when compared with control cells for the same values of the shear stress.

RESULTS AND DISCUSSIONS

The MTT assay revealed important differences in viability and proliferation of mesenchymal stem cells (MSCs) exposed to different concentrations of *Aristolochia clematitis* aqueous extract. Thus, the proliferation rate of MSCs decreased proportionally with increasing concentrations of the extract. Cells treated with 20% and 30% extract presented the same proliferation rate for these two concentrations ($p>0.05$) and it was decreased about seven fold compared to the control cells (untreated cells and cells treated with 30% distilled water) (Figure 1).

Tumor-associated fibroblasts (TAFs) exposed to *Aristolochia clematitis* aqueous extract, evaluated using the MTT assay presented a different behavior comparative to the MSCs, meaning that proliferation rate increases when *Aristolochia* extract concentration is augmented. However, all cells treated with *Aristolochia* extract or exposed to 30% distilled water express lower proliferation rate compared to the untreated cells, with significant statistical differences between cells treated with high concentration (20%, 30%) of extract and cells treated with distilled water ($p<0.01$) (Figure 2).

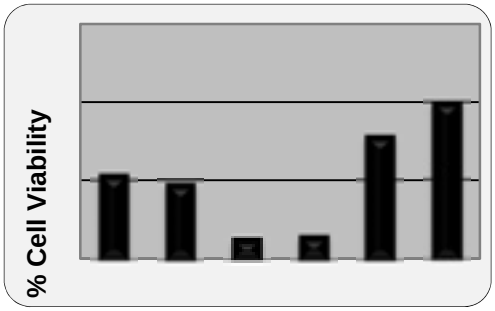

Fig.1. The effect of *A.clematitis* extract (E) on MSCs proliferation

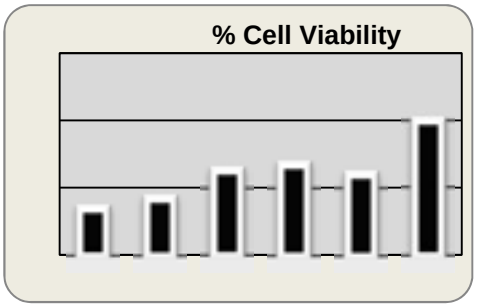

Fig.2. The effect of *A.clematitis* extract (E) on TAFs proliferation

Although TAFs could be a subset of MSCs, they exhibit a different behavior under the experimental conditions, probably due to influences they received *in vivo* from the tumoral microenvironment, which can change their gene expression and physiology (Paunescu *et al*., 2010). Thus, TAFs become more resistant and more adapted to toxic compounds, maintaining a good viability and expansion potential.

When analyzing the MTT assay results for the two breast adenocarcinoma cell lines, MDA-MB-231 and MDA-MB-468, an increased proliferation at low concentrations of extracts was observed. The tumor cells treated with 10% or 15% of *Aristolochia* extracts exhibited proliferation rates which were almost double comparative to untreated cells, and decreased

proportionally with concentrations of the extract. We may conclude that low concentrations of the extract have a stimulating effect on cellular proliferation, most likely due to other compounds than the aristolochic acids, while high concentrations will inhibit proliferation and cellular expansion (Figures 3 and 4).

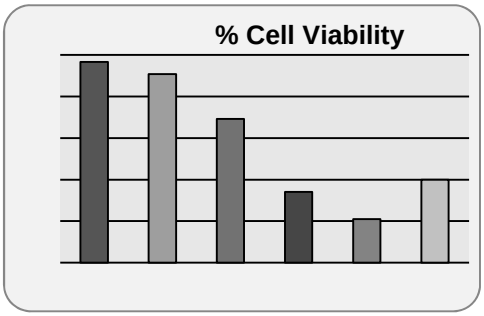

Fig.3. The effect of *A.clematitis* extract (E) on MDA-MB-231 cells proliferation and viability

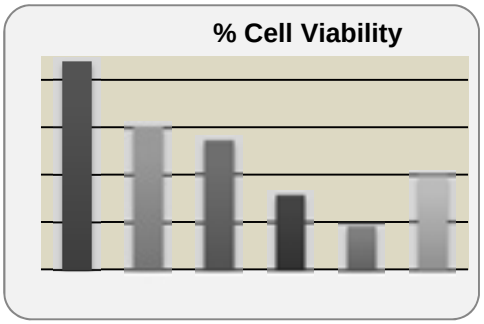

Fig.4. The effect of *A.clematitis* extract (E) on MDA-MB-468 cells proliferation and viability

Adherence capacity of MSCs on tumor cells layer was dependent upon shear stress generated and the type of layer cell line. When MSCs were tested for adhesion on MDA-MB-231 cell line, significant differences ($p<0.01$) were revealed for lower shear stress values (0.35, 2, and 5 dynes/cm^2) and for all extract concentrations initially used on layer cells (Figure 5). When shear stress reached 8 dynes/cm^2, there were no significant differences between extract concentrations, and only 50-60% of the initially adherent cells remained on the layer cells. MDA-MB-468 cellular layer, although having a similar behavior with MDA-MB-231 when treated with *Aristolochia* extracts, did not have any influential role on MSCs adherence. MSCs adhesion maintained similar values for all extract concentrations used and for all values of the shear stress, and approximately 100% of initially adherent cells remained on the cellular substrate (Figure 6).

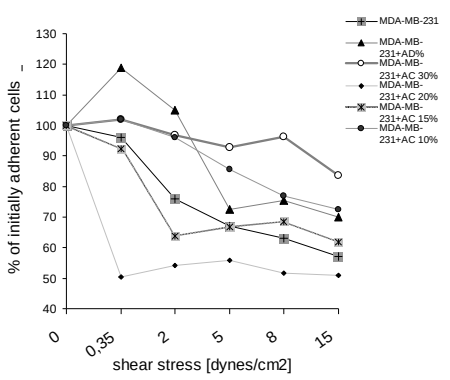

Fig.5. The effect of *A. clematitis* extract on MSCs adhesion capacity to MDA-MB-231 layer

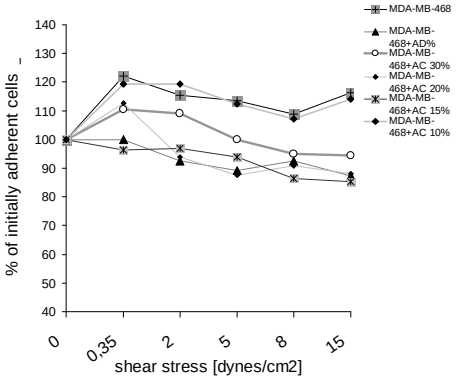

Fig.6. The effect of *A. clematitis* extract on MSCs adhesion capacity to MDA-MB-468 layer

MSCs and TAFs could be endowed with immunosuppressive properties (Paunescu et al., 2010), and they are attracted towards the tumor sites due to various signals transmitted by the cancer cells. Chemical compounds such as those found in *A. clematitis* extracts could decrease the adherence of these cells to the tumor stroma, thus facilitating the immune response triggered by the organism against the tumor formation. TAFs presented a decreased adhesion on tumor

substrate when treated with *A. clematitis* extracts, demonstrated by objective cell count of adhered cells (Figures 7 and 8).

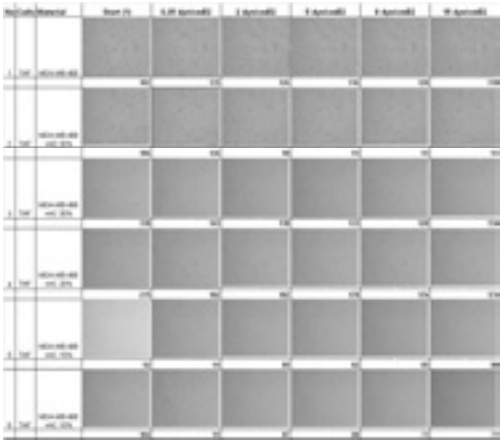

Fig.7. TAFs adhered on MDA-MB-231 cells substrate for different shear stress values

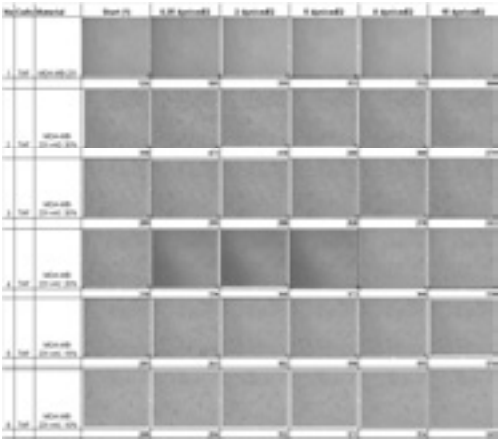

Fig.8. TAFs adhered on MDA-MB-468 cells substrate for different shear stress values

TAFs' adherence on the tumor cell line coatings, after treated with *A. clematitis* extract for 48 hours, presented significant differences depending on the extract concentration and the shear stress that was applied. When TAFs were placed on MDA-MB-231, significant differences were revealed for all values of shear stress, especially for prior treatment of tumor layer with 20% *A. clematitis* extract ($p<0.01$). Similar values were obtained in this case even for 10% *A. clematitis* extract ($p<0.05$) (Figure 9).

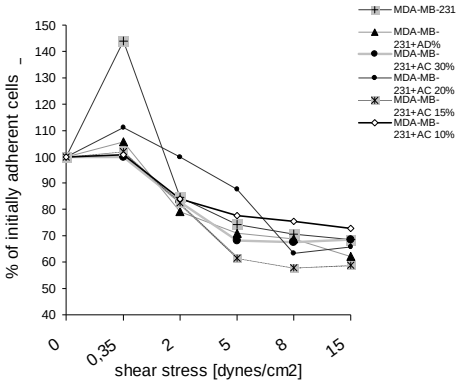

Fig.9. The effect of *A. clematitis* extract on TAFs adhesion capacity to MDA-MB-231 layer

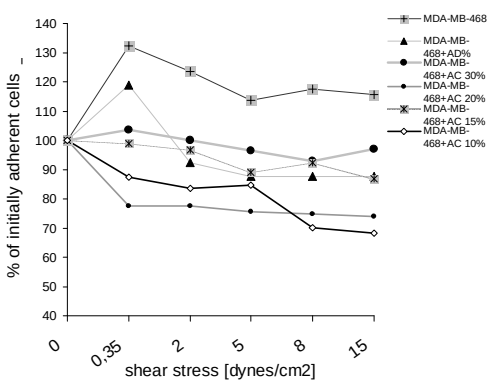

Fig.10. The effect of *A. clematitis* extract on TAFs adhesion capacity to MDA-MB-468 layer

Adherence of TAFs on MDA-MB-468 substrate was significantly reduced for all values of the shear stress, and proportionally correlated with the concentration of *A. clematitis* extracts (Figure 10), but still maintaining approximately 70-80% of the initially adherent cells. It is interesting to note that both MSC and TAFs show somehow a similar pattern of response to shear stress, when in contact with MDA-MB-231 and MDA-MB-468 cell lines, respectively, perhaps due to their common ontogenetic origin.

CONCLUSIONS

Our studies demonstrated a significant inhibitory effect of *A. clematitis* extracts on the viability and function of normal cells (MSCs) compared to tumor or peritumoral cells (tumor cell lines and TAFs), which are less affected by the extracts. Adherence of MSCs and TAFs on tumor cells layer exposed to *A. clematitis* extracts decreases, mainly at high concentrations combined with augmented values of shear stress, thus showing that accumulation of cells with immunosuppressive properties can be inhibited by appropriate concentration of the extracts, under conditions that could be similar to the *in vivo* ones.

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Acknowledgements: This work was supported by PNCDI-II – Parteneriate, project number 41-019/2007 and by CNCSIS –UEFISCSU, project number PNII – IDEI 198/2007.

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Date of manuscript submission: 24.08.2010