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CELL DIFFERENTIATION PROCESS OF *Artemia* sp. LARVAE TOOLS FOR NATURAL PRODUCTS TESTING

Verginica SCHRÖDER[1], Mariana ARCUS[1], Andreea Hortanse ANGHEL[2, 3], Florica BUSURICU[1], Anca Cristina LEPADATU[2]

[1]Ovidius University, Faculty of Pharmacy, Campus C, 6 Capitan Aviator Al. Serbanescu Street, Constanta, Romania
[2]Ovidius University, Faculty of Natural and Agricultural Sciences, Campus B, 1 University Street, Constanta, Romania
[3]ICDCOC Palas, Constanta, Romania

Corresponding author email: ahanghel@yahoo.com

Abstract

Morphogenesis and cellular differentiation present a remarkable interest in studies of cellular regeneration, cellular responses to xenobiotics or the evaluation of tumorigeneses and cytotoxicity induced by substances with therapeutic potential. The study of the process of cellular differentiation and its modifications under the influence of natural extracts was made on the larvae (naupliar stages) of Artemia salina. As a result of high sensitivity and accessibility in laboratory manipulation, the larvae of Artemia are used as animal models in the aquaculture, the assessment of acute cytotoxicity and brine shrimp lethality assay (BSLA). The larvae in the growth period pass through a lot of moulting processes which are associated with epithelium cell divisions and the rearrangement of these in tissues structures within a period of several hours. The assessment of cellular differentiation was followed after the exposure of freshly hatched larvae to natural extracts. The effects of the compounds were measured by determining the survival rate of larvae and microscopically observing visible changes. Natural extracts from Taraxacum officinale F.H. Wigg, Chelidonium majus L., Tragopogon dubius Scop, Usnea barbata L., Galanthus elwesii Hook. were analyzed. A cytological study performed on these biotesters indicates a significant correlation between cell differentiation and metabolism. Some potentially cytotoxic compounds induce changes in organogenesis. The results highlight shape changes of the epithelial cells, alteration at intercellular connections, rearranging and reorganizing the primordium appendices cells and altering membrane and blocking cellular differentiation.

Key words: *Artemia sp., biotester, cell differentiation.*

INTRODUCTION

The evaluation of cytotoxicity on *Artemia salina* larvae (BSLA) is a test developed and adapted as one of the fastest and most effective due to larval sensitivity to a variety of applications (Michael et al., 1956; Togulga et al., 1998; Telens-Perales et al., 2017). The test is an efficient, cheap and relatively quick way to detect the effect of toxic compounds, requiring only small amounts of samples, i.e., <20 mg.

The species is used in studies aimed at testing the potential cytotoxic activity of various plant extracts (Solýs et al 1993; Meyer, 1982; Rajeh et al., 2012; Sirinthipaporn et al., 2016) and the toxic activity of some mycotoxins (Prior, 1978; Harwig and Scott, 1971). The test also allows rapid and meaningful information to be obtained in cases of teratogenic or potentially mutagenic phenomena (Kerster, 1983; Milhem et al., 2008).

The method has the advantage that the larvae used as a tester have a rapid growth rate. These larvae, in 24 hours, pass to another stage through moulting which activates cell division. For these reasons, larval mortality is associated with phenomena that block the cell cycles during the moulting period.

Furthermore, the simplicity of the larva makes this system comparable to a cellular stem cell complex. In the first 24 hours of hatching the cells are not differentiated, and also, the digestive tract is not open, which strictly involves membrane changes. The larva's transparency allows for the visualization of morphological and cytological details in vivo, which is a significant advantage for the evaluation process.

The study has attempted to test plant extracts known in traditional therapy, and that are described as phytochemical: *Taraxacum officinale* F.H. Wigg, *Chelidonium majus* L., *Tragopogon dubius* Scop*, Usnea barbata* L., *Galanthus elwesii* Hook.

In similar studies, the biotest was used to identify plant extract activities by analysing larval survival, and the results have shown a correlation with cytotoxicity on human cell cultures (Jamieson et al., 2014; Carballo et al., 2002).

Our study specifically focuses on the rapid identification of cytological changes induced by plant extracts in a much simpler and more efficient economical way. The aim is to use these tests as preliminary cytotoxicity studies in the evaluations of substances with toxicological and pharmacological activity, in animals.

The method can be adapted in approaches to assessing the effects of substances such as additives, dietary supplements, medicines or disinfectants used in aquaculture. It also has multiple applications in understanding the effects that certain substances with toxicity risks have when accidentally releasing in the environment.

MATERIALS AND METHODS

Plant hydroalcoholic extracts are either marketed (*Taraxacum officinale*, *Chelidonium majus*) or obtained through specific laboratory extraction procedures from three vegetables species *Tragopogon dubius, Usnea barbata* (acetone extract), *Galanthus elwesii*. Approximately 100 grams of dried and ground vegetable product was extracted with a solvent (alcohol, acetone) in a Soxhlet installation at about 70°C for 8 hours. After evaporating the solvent through rotavapor, a dry extract was obtained. It was stored in a freezer at a temperature below -20°C until it was used. Stock solutions for each extract were prepared by dilution in saline water (3‰).

Artemia cysts hatched in water at 35-36‰, at 25°C (thermostat), with continuous bubbling and artificial illumination (2000 lx). When the larvae appeared, bubbling was interrupted. The larvae were placed in plexiglass for testing (1 ml volume, culture plates). There were 10-20 specimens/samples on each plate. For each concentration, four repetitions were performed. For testing, the extracts were diluted in saline water (Table 1), depending on predicted effects of the extracts.

Table 1. The plant extracts protocol prepared for test

Plant analysed extracts	Extract type (w:v ratio and solvent used)	Dilutions in saline water
Taraxacum officinale	40% alcoholic (1:1 ratio)	1:100
Chelidonium majus	40% alcoholic (1:1 ratio)	1:100
Tragopogon dubius	40% alcoholic	1:2, 1:6; 1:10
Usnea barbata	DMSO (0,1%)	1:2, 1:4, 1:10
Galanthus	40% hydroalcoholic (1:3)	1:2; 1:5, 1:10

The larvae that were exposed in the analysed solutions for 24-48 h were subsequently quantified for survival and cytological changes. Results on cytotoxicity assessment were expressed by larval mortality in 24 h. These were compared with control samples, meaning salt water or solvents (alcohol, acetone, DMSO) depending on the extract, in a volumetric solvent: water ratio, comparable to samples containing vegetable extract.

For *in vivo* evaluation and cytological analysis, the nauplii were placed on the slide without fixation and colouring since evaluation though transparency was possible. An Optika 350 microscope with Optikam photo capture System was used.

RESULTS AND DISCUSSIONS

Survival of larvae measured at 24 h revealed the following:

The cytotoxic activity measured by the BSLA test indicates higher toxicity in the case of *T. officinale, C. majus, G. elwesii* extracts. The correlation between the quantified effects (mortality in 24 h) and the dose is evident in all performed analyses.

As a reference in the cytological evaluation, the visible organogenesis processes in the primordial region of appendages (Figure 1 a, b), as

well as the characteristics of the subcuticular cell lines visible in the posterior part of the larva (Figure 1, a, c) were taken into account.
These epithelial cells are disposed in a monolayer, and the cells are polarized and attached to the cuticle. Microscopic observations allow visualization of the layout in longitudinal or cross-sectional lines (Figures 1, c).

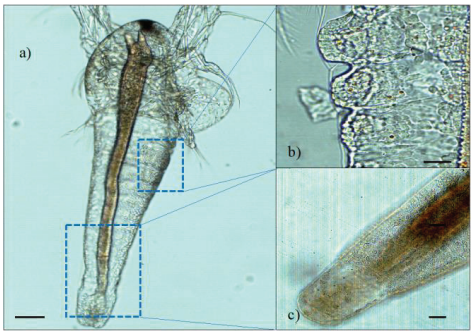

Figure 1. *Artemia* larvae in the control sample: (a) larvae body (the black bar correspond to100 μm), (b) Morphogenesis details of the thorax segment region (the black bar correspond to 20 μm), and (c), subcuticular epidermal cells from the posterior region, arrangements details (the black bar correspond to 40 μm)

Exposure in analysed solutions allowed the tracking and evaluation of cellular changes (arrangement, cell appearance, cell and nuclear volume changes, intercellular attachment loss) induced in these areas and visible *in vivo*. All of these changes were classified as major by affecting cellular differentiations and organogenesis and implicitly stopping larvae from passing through subsequent stages of development (stage II and III).
The visible processes induced by the *C. majus* and *G. elwesii* extracts consisted of stopping the formation of the limb primordium (Figure 2 a, c). The explanations of this phenomenon are related to the content of the analysed extracts.
Thus, phytochemical studies show that *Chelidonium majus* contains little known toxicological alkaloids. Human cell studies that indicate antiproliferative activity for human keratocytes (Vavreckova et al., 1996), and anti-leukaemic activity of protoberberine alkaloids (Smekal et al., 1984) could correlate to our studies.
Other studies indicate that chelidonins are identified as telomerase inhibitors in tumour cells. The alkaloid also inhibits tubulin polymerization by inducing mitosis blockade (Biswas, 2013).
These studies examples correlate with the results of observations on the effect of *Chelidonium* extract analysed on *Artemia* larvae and support the claim that the organogenesis of these larvae is a possible model of cytotoxicity analysis.
The *Galanthus* hydroalcoholic extract exhibits very high acute toxicity in 24-30 hours. The *Galanthus* extract also induces major effects on cell activity in the thoracic segment (Figure 2, c) at the larvae II and III stages.
In addition, there is a loss of contact between the cuticle and the subcuticular cells. Explanations could be related to the combined effect of the complex mixture of alkaloids contained in the *Galanthus* extract.
Phytochemical studies indicate more than 90 alkaloids in the *Galanthus* species.
Of these, some are studied and are known to have a pharmacological activity such as galantamine, which is a competitive inhibitor to acetylcholinesterase (AChE) and an allosteric nicotinic receptor modulator for acetylcholine (ACh). For these properties, the extract finds applications in the treatment of Alzheimer's.
Tyramine is another alkaloid that exhibits structural similarities to adrenaline and leads to cortisol release, inducing high toxicity in animals (Clement et al., 1998).
Lycorine is one of the most common alkaloids in Amaryllidaceae and possesses a wide range of biological properties. It has been reported as a potent inhibitor in ascorbic acid synthesis, in cell growth and division, and organogenesis in higher plants, algae and yeasts, and it inhibits cell cycles during interphase (Bastida et al., 2006).
In addition, lycorine exhibits antiviral, antifungal (*Saccharomyces cerevisiae*, *Candida albicans*), and antiprotozoal (*Trypanosoma brucei*) activity and is more potent than indomethacin acting as an anti-inflammatory agent (Citoglu et al., 1998).
Cellular effects induced by *Taraxacum officinale* and *Usnea barbata* indicate changes in the rearrangement of epidermal invasion (Figure 2, b and d) and blocking morphogenesis mechanisms activity.
Extracts of *Taraxacum* and *Usnea*, tested on human cell cultures, are known to have

inhibitory effects on cancer cells (EMA/HMPC, 2008; Lei Guo, 2008). These inhibitions are related to decreasing phosphorylation at FAK and src levels and reducing the extracellular matrix (*T. officinale)* or blocking mitochondrial activity in the *Usnea* extract (Lei Guo, 2008).

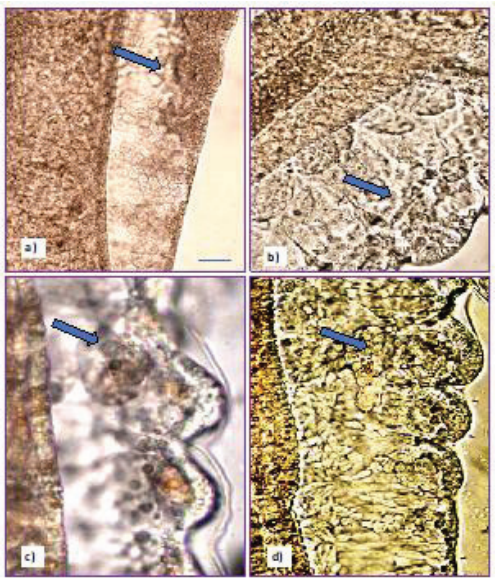

Figure 2. The region of morphogenesis of the thorax segment at brine shrimp larvae, in 24 h exposure to extracts; the arrow indicates the region with amplified abnormal cell arrangement in the lateral germs: *C. majus*; b) *T. officinale* extracts; c) *G. elwesii.* (bulbs extracts; d) *U. barbata* (the blue bar corresponds to 20 μm)

Examination of epidermal lines reveals a loss of arrangement and cell density in solutions with *Chelidonium* extract comparative with control samples (Figure 3, a, b).

Also, phenomena such as: exacerbation of cellular volume and nuclear deformation at exposure to *Tragopogon dubius* extract (Figure 3, c) occur. In traditional Romanian medicine, the *Tragopogon* decoction is used for the skin smoothing (Moromete at al., 2016).

Our observations of the osmotic changes that resulted in cellular growth could be based on the same mechanisms as those revealed in the human skin.

Other cytological phenomena noted at the epithelial level are: cell deformation and loss of intercellular adhesion or linkages between the cuticle and the cellular layer (Figure 3, d) respectively. Observations were made on nauplii introduced into solutions containing the *Usnea barbata* extract.

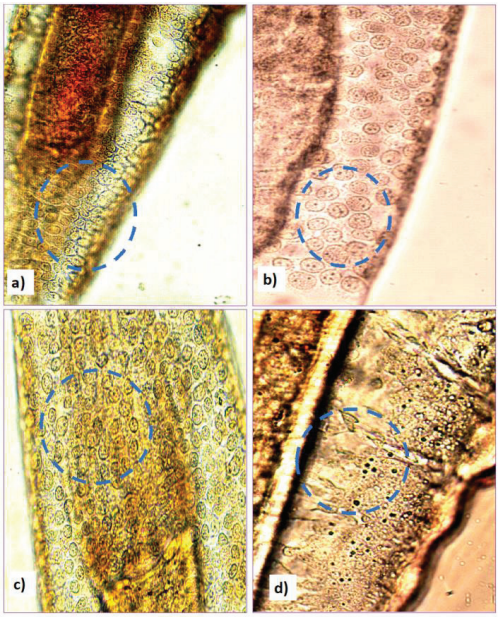

Figure 3. The epidermal cells details with alteration of cell neighbours at 24h of exposure in extracts (mark circle): a) control sample; b) *C. majus;* c) *T. dubius* and loss of connections between cells; d) *Usnea barbata* (the blue bar corresponds to 20 μm)

CONCLUSIONS

Our results describe a cytotoxic evaluation of plant extracts using the *Artemia salina* biotest. The technique highlights the fact that extracts penetrating into the larvae's body alters the growth and differentiation of cell populations in the area of the thoracic appendix buds and subcuticular epithelial cells.

The rapid highlighting of cellular changes related to shape, density, and the reorganization of elements targeting organogenesis stages by microscopic evaluation, without special preparation techniques, makes the *Artemia* test a fast cytotoxicological test with comparable relevance to animal cell cultures.

The test allows in vivo evaluation of possible substances with cytotoxic and teratogenic potential, providing a good insight into applications in aquaculture, ecotoxicology and pharmacology.

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