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THE CYTOLOGICAL EFFECTS OF CHLORHEXIDINE COMPOUNDS WITH COPPER ION TESTED ON *ALLIUM* ROOT MERISTEM

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ABSTRACT. The chlorhexidine complexes with metallic ions of copper are newly synthesized in order to obtain topical pharmaceutical preparations with antiseptic and anti-inflammatory activities, but without adverse effects or allergic reactions on the mucous membranes or epithelia. The testing of the cytological activities on the radix meristem of *Allium cepa* completes the previous tests accomplished on microorganism. Bulbs (2 cm in diameter), with 5-10 mm radices, were introduced into solutions of the complexes. The used complexes have ethanol (10^{-3}) as solvent, therefore the control samples were: water, ethanol (250 µL, 500 µL) in order to record possible influences of the solvent volume that could cover the real effects of the compound. The radices were measured and then the meristems were harvested at 24 hours and 96 hours respectively. The microscope preparations were fixed in acetic acid 45% and then colored with Schiff reagent. The results emphasized that all the compounds have a cytostatic effect, but do not manifest genotoxicity.

Keywords: cytostatic, cytotoxic, mitotic index, root cells

INTRODUCTION

Many pharmaceutical studies aim at finding substances with antimicrobial or antiseptic activity, but with few adverse effects on the epithelia or mucous membranes. The newly synthesized substances are chlorhexidine complexes with copper ions, tested from the point of view of the antibacterial and fungicide activity. The test of onion meristem cells was chosen in order to complete the testing of this new substance.

The *Allium* test is a method used for the biological monitoring and the determination of substance toxicity (Adegbite et al., 2009, Evandri et al., 2001) such as the metallic ions (Fiskesjö, 1998) or in cases of pollution. Also, it is a widely used method for the evaluation of the cytotoxic activity and fungicide activity (Fiskesjö et al., 1981) of a large variety of compounds antitumor substances (Oloyede et al., 2009), vegetal extracts used in the pharmaceutical field (Amalia L., 2008, Rhathore, et. al 2005, 2006, Çelik, et al., 2010, Ragunathan et al., 2007, Kuras et al, 2009), synthetic oils (Awodele et al., 2010).

The test also has a wide spectrum of applications and it is preferred to other testing systems for eukaryotes and prokaryotes (Fiskesjö, 1994, 1998). The most important advantage of this test is that the results are reliable and can be obtained very quickly (Fiskesjö, 1994, 1998). The *Allium* meristem cells represent activated enzymatic systems, therefore the test has advantages for the screening of genotoxicity. It is considered that the oxidases it displays are similar to those of the mammalian liver cells (Fiskesjö, 1998).

MATERIALS AND METHODS

Allium cepa bulbs with 5 cm diameter were placed in distilled water. When 1-2 cm radices emerged, the experiment was begun.

Four bulbs were used for each version and two experimental replications were accomplished. The used complexes have ethanol (10^{-3}) as solvent, therefore the control samples were: water, ethanol (250µL, 500 µL) in order to record possible influences of the solvent volume that could cover the real effects of the compound.

The solution for each container was added on filter paper and, after evaporation, a volume of 4 ml of distilled water was added. The collection of meristems was done at 24 hours and 96 hours from the beginning of the exposure. The solutions were changed at 24 and 48 hours.

For the microscopic observations, the collected radices (2 mm from the distal end of the radix) were fixed in acetic acid 45%, while the coloring was accomplished with Schiff reagent.

The preparations were realized by the squash method. For the microscopic observations, a NOVEX optical trinocular microscope was used, as well as a EUROMEX photo camera with objective 20x, 40x and 100x.

The index of root inhibition was calculated (Ir) as the ratio M-P/M x100, where M is the average length of the radices from the witness water sample (M1) and P is the average length of the radices in each experimental version (P1, P2, P3). The mitotic index (MI) was calculated as the ratio between the number of cells in a division and the total number of the counted cells.

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Table 1

Experimental protocol used in the *Allium cepa* test.

Complex notation	Ligand	Formulae	Volum solution (µL) complexes + water (mL)	Number of used bulbs	Harvesting period (h)
P1	Chlorhexidine	$[Cu_2(CHX)Br_4]\cdot 2C_2H_5OH$	250 µL + 4ml	4x2	24-96
P2	Chlorhexidine	$[Cu_2(CHX)Cl_4]\cdot 2C_2H_5OH$	250 µL+4mL	4x2	24-96
P3	Chlorhexidine	$Cu_2(CHX)(CH_3COO)_2](NO_3)_2\cdot 2C_2H_5OH$	250 µL+4mL	4x2	24-96
M1	Distilled water	-	4ml	4x2	24-96
M2	Ethanol 250 µL	-	4ml	4x2	24-96

RESULTS AND DISCUSSIONS

The used complexes with ethanol (500µL) as solvent caused vacuolization of nuclei and autolysis in the cytoplasm and were eliminated from final analyses. These solvent concentrations showed lethal action on cells of *Allium* test. No different observations were showed between M1 control (distillated water) and M2 (ethanol 250 µL), also.

The results obtained emphasize modifications of the rood growth, compared to the witness (M1) in all the analyzed samples. High values of the inhibition were registered in the case of complex P3, sample P1 and P2, with low percentages of 20.9% and 13.9% respectively (Fig. 1).

The presence of the Br^- ion, in complex P1, can explain the inhibition percentage of only 20.9%, as this ion stimulates the absorption of Ca^{2+}, and when they are absorbed together with K^+ they are incorporated in more stable structures and this is reflected in the increase of the absorption rate (Overstreet, 1957).

This ionic interrelation probably facilitates the cellular division and maintains the enzymatic system active so that the radices growth should be inhibited less.

Despite this, the cells exposed to this compound display very large vacuoles (Fig.2, B), probably due to the homeostatic imbalance, and cells in apoptosis are indeed evident (96 hours from the exposure).

The microscopic observations emphasized in P2 case, numerous cells with considerably enlarged nucleoli (Fig. 2, C). These observations suggest either the blocking of the nuclear pores, or dysfunctions of the transporting proteins between nucleus and cytoplasm, which leads to the accumulation of the intranuclear material.

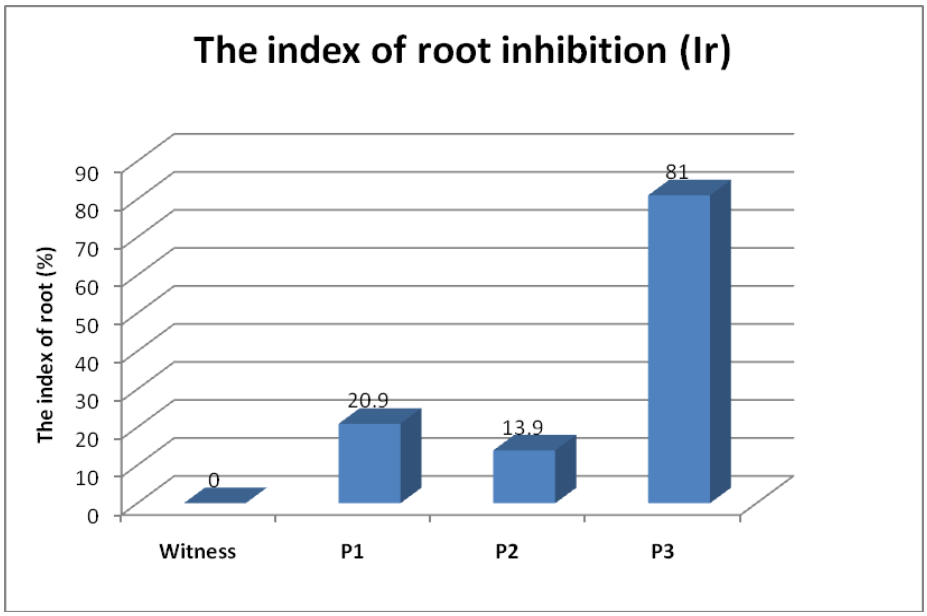
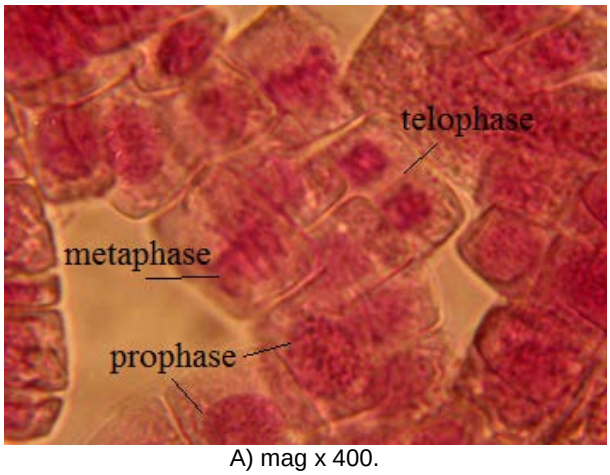


Fig 1. Modifications of the index of root inhibition Ir (%) in the analyzed samples, compared to the control, distillated water, sample (M1) - 24 hours from the beginning of the exposure

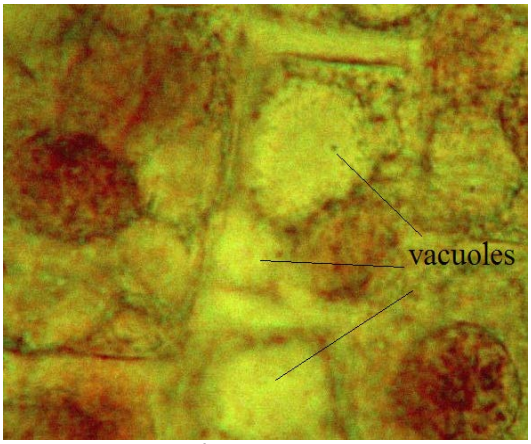
This aspect of the nucleus was encountered in the case of other compounds of chlorhexidine which contain NO_3^- and Cl^- in their structure (unpublished data) and it can be associated to the role of these ions in the vegetal cells at vacuolar level (Trebacz et al. 1994).

The highest inhibition (81%) is remarked in the case of the compound that contains NO_3^- as counter

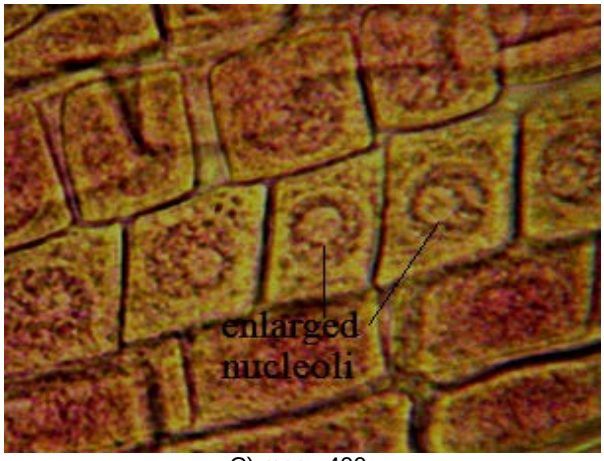
ion. In these samples, microscopic observations showed vacuolizations of nuclei and autolysis in the cytoplasm (Fig.2, E and F). The appearance of the nuclei with strongly condensed chromatin (probably apoptotic cells) was present in P3 samples (Fig.2, D).



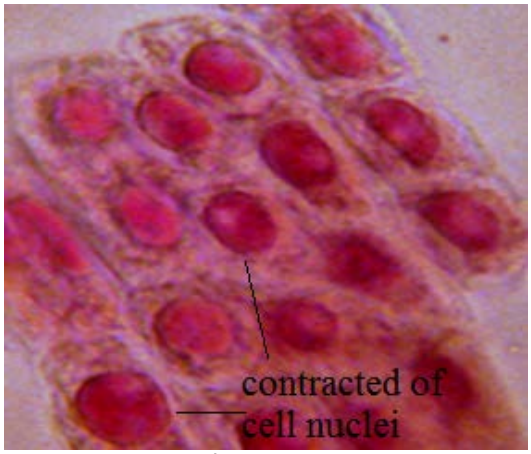
A) mag x 400.



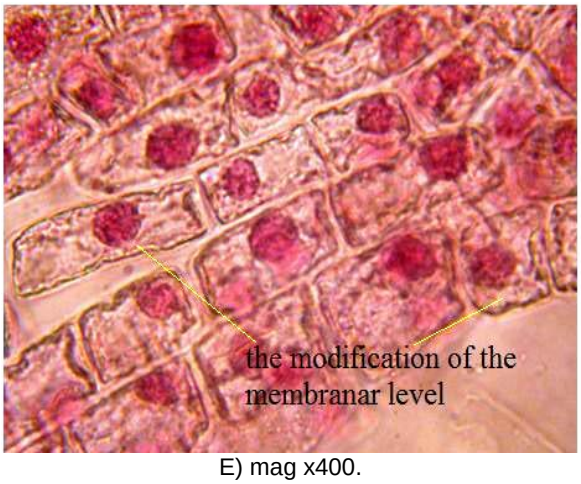
B) mag x1000.



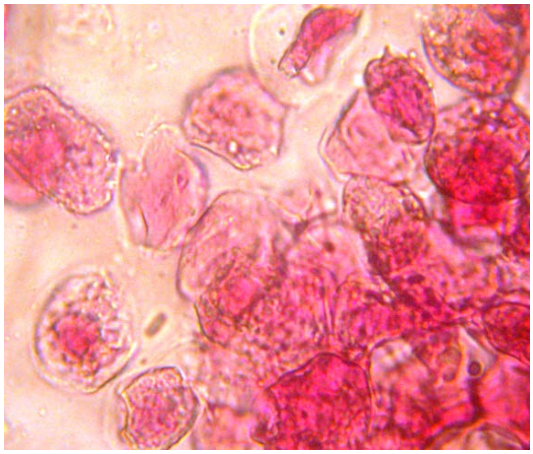
C) mag x400.



D) mag x400.



E) mag x400.



F) mag x 400.

Fig 2. Microphotographs of control meristematic cells of *Allium* test (A) and after incubation in solutions (B-F). Squeezed preparations observed in light microscope (Mag x400). Stain acetic acid (45%). A- The cellular divisions from control samples; B- Vacuolated cells (P1 complex samples); C- The different shape of nuclei and enlarged nucleoli (P2 complex samples); D - The marked contraction of cell nuclei, cromatin condensation (P3 complex samples); E,F - The membrane modifications and cell death (P3 complex samples).

The values of the mitotic indexes are below 50% of the value registered in the case of the witness (Table 2) and below the value of the cytotoxic limit (Çelik, 2010), which supports the observation regarding the radices growth and emphasizes the cytostatic effect of the analyzed compounds.

The presence of prophases suggests that the DNA replication occurs and the related enzymatic systems are functional. It is also important to remark that the treatment with the second complex P2 (which contains Cl^- as counter ion) induces the appearance of certain relatively small nuclei with considerably enlarged nucleoli as the effects of alkaloid (Kuras et al, 2009).

Table 2

The variation of the mitotic index (MI) and the proportion of the different stages of the mitotic division in the condition of treating the radices with solutions of the chlorhexidine complexes

Samples	Total counted cells	Total cells in division	The mitotic index (MI) compared to the witness (%)	Prophase %	Metaphase %	Anaphase %	Telophase %
M1	2010	271	13.4(100%)	64.2	3.69	11.8	20.99
P1	3760	47	1.25 (9.32%)	79	2	2	17
P2	2670	97	3.63 (27%)	84.5	0	1	14.5
P3	1850	0	-	-	-	-	-

The metaphases and anaphases are very few or even absent from samples P1 and P2. This indicates the decrease of cellular proliferation and even cellular death (P3). Similar aspects were observed in the case of the effect of certain vegetal extracts (Eleftheriou et al.,1992, Kuras et al., 2009).

It is possible that these stages are absent either as a result of cellular death or as a result of the blocking of the synthesis of microfilaments or microtubules, which are important structures in the subsequent functioning and triggering of cellular division. These protein structures are those that prepare the plan of cellular division and ensure the organization of the preprophase plaque.

Another possibility is the modification of the molecular transport system as a result of the homeostasis modified at vacuolar level consequent to the content of anions of the complexes (Cl^-, NO_3^-).

In what regards the genotoxic effect, we remarked the absence of chromosomal aberrations or of modified mitoses and this is an important aspect which supports the possible pharmaceutical qualities of the all analyzed complexes.

The presence of a significantly low mitotic index, compared to the witness, in the case of all the analyzed complexes suggests the cytostatic effect, favorable in the case of using this compound for the synthesis of new antiseptic or disinfectant pharmaceutical compounds.

CONCLUSIONS

This study demonstrates the cytostatic effects of chlorhexidine compounds which contain the Cu^{2+} ion in their structure and Br^- and Cl^- anions, the values of the mitotic index being below the cytotoxic limit.

The highest inhibition of divisions is remarked in the case of the compound that contains NO_3^- anions as counter ion.

The presence of the prophases in all the analyzed cases, in similar percentages to those in the witness sample, can be a result of the maintaining of the enzymatic system and of the DNA synthesis within normal limits.

The influences of blocking the cellular proliferation or even the lethal effects are probably due to the system of intra-vesicular transport or to the modifications suffered by the cytoskeletal elements with role in cell division (microfilaments and microtubules). Apparently, the affected elements are those that allow the passing into the next stages of the cellular cycle, namely metaphase and anaphase.

The presence of morphological differences of the cells depending on the anions present in the complexes indicates, also modifications of cellular homeostasis.

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