STUDIES REGARDING THE CYTOTOXICITY OF ANTIMICROBIAL GELS FORMULATED WITH NATURAL BIOPOLYMERS

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STUDIES REGARDING THE CYTOTOXICITY OF ANTIMICROBIAL GELS FORMULATED WITH NATURAL BIOPOLYMERS

ABSTRACT. Two antimicrobial gels, formulated with natural biopolymers (collagen and chitosan), limonene, and an imidazole derivative, were subjected to cytotoxicity tests. In these two compositions, the limonene content was 0.4% and 0.67%. The cytotoxicity tests were performed *in vitro*, using MTT methodology, and a standardized human normal cells line, HUVEC type. These cells were exposed to different levels of gel concentrations in the culture media. The final concentration of each gel type in culture media was situated between (0-0.125) µL/mL. The cell viability was determined after 24, 48 and 72h of exposure. The analyses showed that after 24h of exposure, the viability of the cells is greater than 91%, after 48h the viability is greater than 80%; after 72 h of exposure, the viability of the cells is greater than 74%. These values reveal that both selected gels exhibit no cytotoxicity for the normal cell line.

KEY WORDS: antimicrobial gels, biopolymers, cytotoxicity

STUDII PRIVIND CITOTOXICITATEA UNOR GELURI ANTIMICROBIENE FORMULATE CU BIOPOLIMERI NATURALI

REZUMAT. Două tipuri de geluri antimicrobiene, formulate cu biopolimeri naturali (colagen și chitosan), limonene și un derivat de imidazol, s-au testat *in vitro* din punct de vedere al citotoxicității. În aceste două compoziții, conținutul de limonene a fost de 0,4% și 0,67%. Testele de citotoxicitate au fost efectuate folosind metodologia MTT și o linie standardizată de celule umane normale, de tip HUVEC. Celulele normale au fost expuse la diferite niveluri de concentrații de gel, situate între (0-0,125) µL/mL. Viabilitatea celulară a fost determinată după 24, 48 și 72 de ore de expunere. Rezultatele obținute au arătat că după 24 de ore de expunere, viabilitatea celulelor este mai mare de 91%, după 48 de ore viabilitatea este mai mare de 80%; după 72 de ore de expunere, viabilitatea celulelor este mai mare de 74%. Aceste valori arată că niciunul din gelurile selectate nu prezintă citotoxicitate pentru linia celulară normală.

CUVINTE CHEIE: geluri antimicrobiene, biopolimeri, citotoxicitate

ÉTUDES SUR LA CYTOTOXICITÉ DE GELS ANTIMICROBIENS FORMULÉS AVEC DES BIOPOLYMÈRES NATURELS

RÉSUMÉ. Deux types de gels antimicrobiens, formulés avec des biopolymères naturels (collagène et chitosane), du limonène et un dérivé d'imidazole, ont été testés *in vitro* du point de vue de la cytotoxicité. Dans ces deux compositions, la teneur en limonène était de 0,4 % et 0,67%. Des essais de cytotoxicité ont été effectués en utilisant la méthodologie MTT et une lignée cellulaire normale humaine standardisée, HUVEC. Les cellules normales ont été exposées à divers niveaux de concentrations de gel allant de (0-0,125) µL/mL. La viabilité cellulaire a été déterminée après 24, 48 et 72 heures d'exposition. Les résultats obtenus ont montré qu'après 24 heures d'exposition, la viabilité cellulaire est supérieure à 91%, après 48 heures la viabilité est supérieure à 80% ; après 72 heures d'exposition, la viabilité cellulaire est supérieure à 74%. Ces valeurs montrent que les deux gels sélectionnés ne présentent pas de cytotoxicité vis-à-vis de la lignée cellulaire normale.

MOTS CLÉS : gels antimicrobiens, biopolymères, cytotoxicité

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INTRODUCTION

Bioproducts with antimicrobial properties, once developed, must also be evaluated from the point of view of cytotoxicity. The first step for this is represented by the cytotoxicity evaluations performed *in vitro*, on standardized normal cell lines. There are several methods for this, methods that are based both on the evaluation of the cytotoxicity of each individual component and the evaluation of the end bioproduct. Cytotoxicity studies are initiated in the first stage *in vitro*, on normal cells [1-4]. Many natural or synthetic components are used in the development of topical antimicrobial formulations. This category includes citrus essential oils, respectively the predominant compounds of citrus essential oils such as limonene, and some synthetic antibiotics such as imidazole derivatives (i.e., clotrimazole) which cannot be administered intramuscularly or orally due to serious adverse effects [5]. Due to its properties, limonene is intensively used in dermato-cosmetic formulations, but its use in topical formulations is limited due to the fact that it can induce the appearance of epidermal sensitization phenomena. Thus Shah *et al*. [6], in the cytotoxicity studies carried out on standardized cell lines or on primary cell lines initiated from animal tissue, with D-Limonene, reported that at 24 h the cytotoxicity of limonene for the K562 cell line is similar to that of the antitumor reagent doxorubicin (used as a positive control). In this experiment, at concentrations of D-limonene = 4 mM, the viability of K562 cells was 32%, while the viability of cells treated with 0.8µM doxorubicin was 4.1%. If the concentration of D-limonene in the medium increased to 8mM, then cell viability was reduced to 17%. After 48 h of exposure, the best results in terms of cell viability are obtained for doxorubicin. The tests initiated by the same authors on hepatocytes (primary cell line initiated from liver cellular tissue, taken from laboratory animals) showed that for doxorubicin concentrations between (2-32) µM, hepatocyte cell viability is between (100-28)% after 48 h of exposure. If the hepatocyte cells are exposed to concentrations of D-limonene between (2-32)mM, the cell viability obtained is between (100-28)% after 48 h of exposure [6]. The cytotoxicity of limonene can be modified by incorporating it into various lipid formulations such as nanoliposomes or niosomes (niosomes are vesicles made of a nonionic surfactant and cholesterol [7] which are similar to liposomes because they are formations composed of a double lipid start [8]. Another compound often used in topical formulations is clotrimazole, which is a non-biodegradable chemical compound. The half-life of this compound is 60 days, being considered a chemically persistent chemical compound [9]. Regarding clotrimazole, its cytotoxicity can be increased by complexing with ruthenium, gold, or platinum [10]. In the documentary studies carried out by Wang [10], it was reported that the complex combination formed between clotrimazole and ruthenium $[RuCl_2CTZ]_2$ inhibits the proliferation of a parasitic protozoan that causes Chagas disease (i.e., *T. cruzi*) by 90%, in while free clotrimazole does not have this effect [11, 12]. Reducing the cytotoxicity of clotrimazole can also be achieved by microencapsulating it in vegetable oils [13]. Other studies carried out *in vitro* [4, 14-16] showed that the cytotoxicity of some components can be reduced by introducing them into collagen matrices, matrices have the effect of protecting normal cells.

EXPERIMENTAL

Materials and Methods

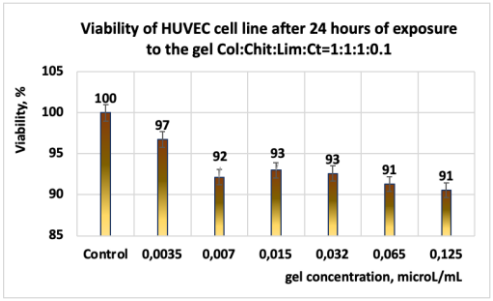
Two types of gels formulated with chitosan, collagen (both from Sigma-Aldrich), limonene, and clotrimazole (named Col:Chit:Lim: Ct = 1:1:1:0.1 and Col:Chit:Lim:Ct=1:0:1:0.1, respectively) with

antimicrobial activity on microorganisms such as *Staphylococcus aureus*, *Staphylococcus aureus* MRSA, and *Candida albicans*, respectively [17], were tested *in vitro* from the point of view of cytotoxicity in order to further reveal these types of bioproducts, the obtained gels. For this aim, a standardized cell line of human cells named HUVEC ATCC PCS 100-010 was used using the methodology presented by Zaharie *et al*. [1]. The biological determinations were made by exposing the cell line to different concentrations of gels, concentrations that varied between 0.0035 µg/mL and 0.125 µg/mL, for 24h, 48h, and 72h.

RESULTS AND DISCUSSION

The results obtained in the case of the bioproduct named Col:Chit:Lim:Ct = 1:1:1:0.1 showed the following:

- after 24 hours of exposure to the bioproduct to be tested, for the range of antimicrobial gel concentrations studied, cell viability was between (91-97)% (Figure 1a); the corresponding cytotoxicities obtained for the studied concentration range are below 9% (Figure 1b);



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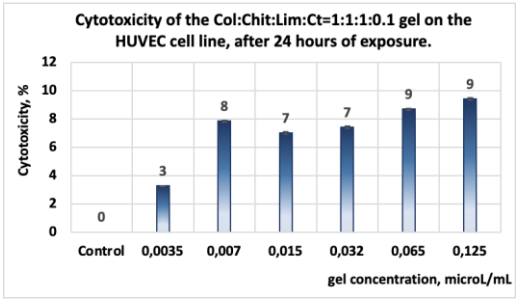

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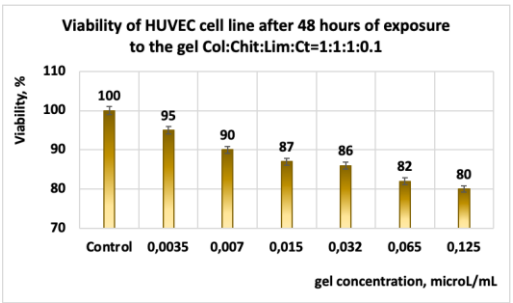
Figure 1. Cytotoxicity studies performed at 24 h on the product Col:Chit:Lim:Ct = 1:1:1:0.1; a) cell viability; b) dead cells (cytotoxicity)

- from a mathematical point of view, the obtained cytotoxicities can be evaluated with a logarithmic function, but in this case, the correlation coefficient is small (Table 1);

- when the normal cells are exposed to the antimicrobial bioproduct for 48 h, their viability ranges between (80-95)% (Figure 2 a, b). The corresponding cytotoxicities were situated between (5-20)% and can be mathematically described by an exponential function (Table 1).



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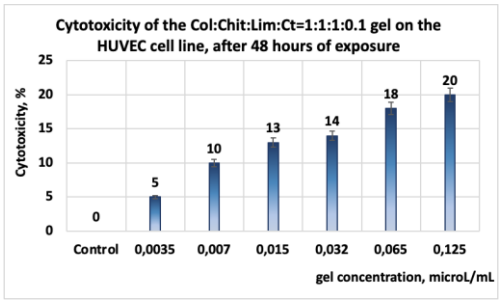


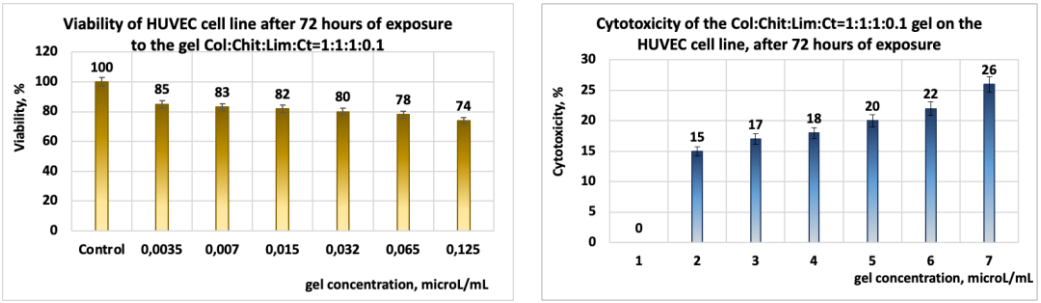
b

Figure 2. Cytotoxicity studies performed at 48h on the product Col:Chit:Lim:Ct = 1:1:1:0.1; a) cell viability; b) dead cells (cytotoxicity)

Table 1: Math function used for cytotoxicity evaluation of antimicrobial gels *in vitro*

Bioproduct Col:Chit:Lim:Ct =1:1:1:0.1		
Time	Math function	Correlation coeficient
24h	$y = 1.2961\ln(x) + 12.15$	$R^2 = 0.6199$
48h	$y = 46.17x^{0.3464}$	$R^2 = 0.915$
72h	$y = 33.51x^{0.1428}$	$R^2 = 0.9706$
Bioproduct Col:Chit:Lim:Ct =1:1:1:0.1		
24h	$y = -90.264x$	$R^2 = 0.9421$
48h	$y = -1.552\ln(x) - 4.1348$	$R^2 = 0.889$
72h	$y = 12.651e^{-14.34x}$	$R^2 = 0.943$

If the exposure of the cell line is prolonged for 72 h, the corresponding cell viability is between (74-85)% (Figure 3a). The corresponding cytotoxicity ranges between (15-26)% (Figure 3b). From a mathematical point of view, the cytotoxicity, in this case, can be characterized by an exponential function (Table 1).



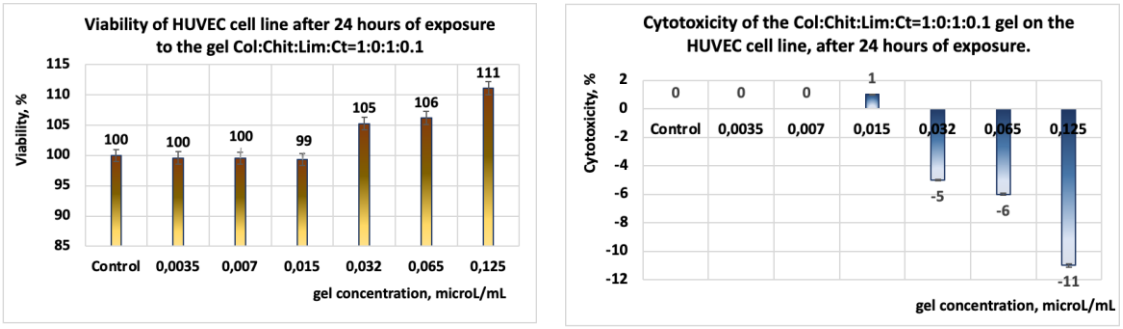
a b

Figure 3. Cytotoxicity studies performed at 72h on the product Col:Chit:Lim:Ct = 1:1:1:0.1; a) cell viability; b) dead cells (cytotoxicity)

The cytotoxicity studies made in the case of the bioproduct named Col:Chit:Lim:Ct = 1:0:1:0.1, showed the following:

- after 24 hours of exposure to the biopreparation to be tested, for the antimicrobial gel concentrations studied, cell viability was between (100-111)% (Figure 4a). The cytotoxicity obtained for the concentration range studied have negative values (Figure 4b), which suggests that this bioproduct favors the proliferation of normal cells and is not cytotoxic;

- when the cells are exposed to the antimicrobial bioproduct for 48h, the cell viability ranged between (95-107)% (Figure 5a). The cytotoxicity obtained after 48 hours of exposure to the antimicrobial gel is below 5% (Figure 5b). If the exposure of the cell line is prolonged up to 72 h, the cell viability was situated between (87-98)% (Figure 6a).



a b

Figure 4. Cytotoxicity studies performed at 24h on the product Col:Chit:Lim:Ct = 1:0:1:0.1; a) cell viability; b) dead cells (cytotoxicity)

The corresponding cytotoxicity obtained in this case are below 13% and can be evaluated mathematically with an exponential function (Table 1). Results obtained are in agreement with studies performed by Babeanu *et al*. [4] and Ioan *et al*. [16] which obtain a considerable reduction of cytotoxicity in the case of solid formulation between collagen and clotrimazole, formulation with antimicrobial activity against the same microbial pathogens. Similar results were obtained by Hajidadeh [18] with niosomes based on limonenes, on three types of tumor cells such as HepG2, MCF-7, A549, with the MTT technique. These results showed that at a concentration of 5µM of niosomes with limonenes, in the culture medium, the viability of Hep2G cells is 58%, a much lower value comparison with the viability obtained when the cells were exposed to a culture media in which the D-limonene concentration was 5µM (viability = 95% in this last case). Yip *et al*. [13], in their cytotoxicity studies performed on a normal keratinocyte cells line (HaCaT), exposed to concentrations of 5 µg microcapsules with clotrimazole/mL, reported that after 24 h of exposure, cell viability was 91%, compared to the viability obtained at exposure to solid clotrimazole, when cell viability was 71% after 24 h of exposure.

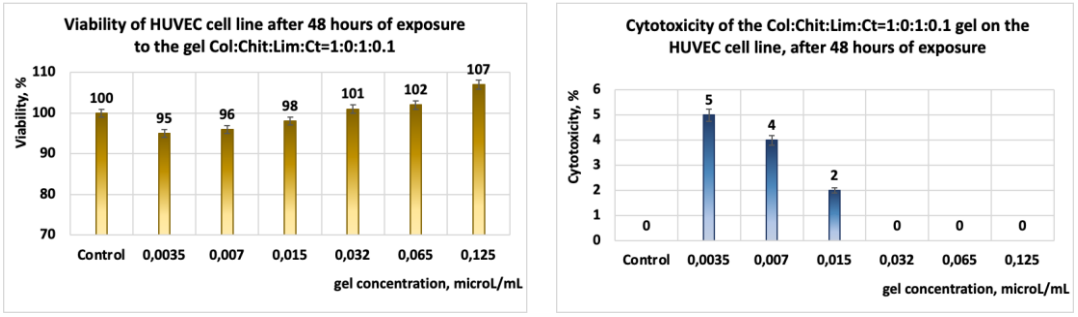


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Figure 5. Cytotoxicity studies performed at 48 h on the product Col:Chit:Lim:Ct = 1:0:1:0.1; a) cell viability; b) dead cells (cytotoxicity)

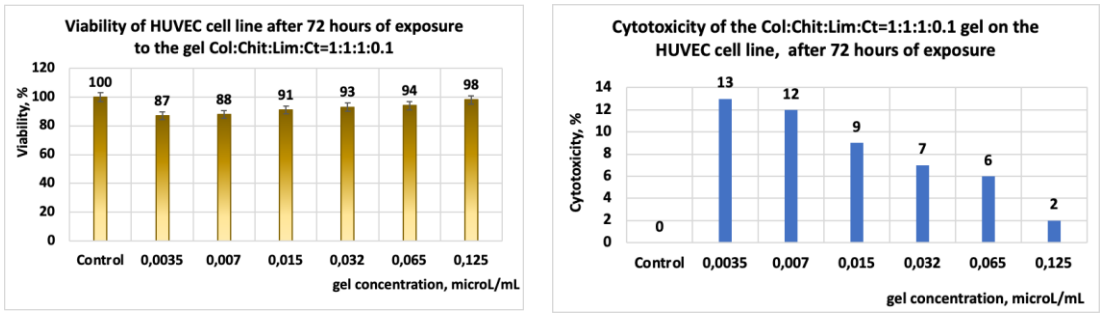

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Figure 6. Cytotoxicity studies performed at 72 on the product Col:Chit:Lim:Ct = 1:0:1:0.1; a) cell viability; b) dead cells (cytotoxicity)

The studies carried out *in vitro* by Adinolfi *et al*. [19] on a melanoma cells line and a normal keratinocyte cells line, showed that the exposure of these cells to a concentration of 10µM clotrimazole has the effect of reducing tumoral cell viability up to 50%, while the viability of normal cells had values higher than 85%.

CONCLUSIONS

The cytotoxicity studies made with two gels with antimicrobial properties, formulated with raw materials obtained from by-products from the food industry (collagen obtained from cattle hides, chitosan obtained from crab exoskeletons, citrus peels) and an imidazole derivative (clotrimazole), have demonstrated that the selected antimicrobial biopreparations are not cytotoxic. The cytotoxicity obtained after exposing a normal cell line to the antimicrobial bioproducts for 72h is situated below 25% and is probably due to the more accentuated decomposition of the culture medium as well as the influence of the accumulation in the environment of the products resulting from the cellular metabolism. The cytotoxicity obtained after 72 h of exposure to the antimicrobial bioproducts that do not contain chitosan is 13%, is lower, which suggests a less pronounced decomposition of the culture medium.

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