BIO-ACTIVITY OF ORGANIC/INORGANIC PHYTO-GENERATED COMPOSITES IN BIO-INSPIRED SYSTEMS

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Received October 9, 2017

Abstract. The present study aimed to design in a "green" approach, novel biohybrids based on silver-copper particles and biomimetic membranes. These hybrids presented good physical stability, high antioxidant activity, good antimicrobial properties and exhibited *in vitro* antiproliferative effect on human colorectal cancer cells, and no citotoxicity on normal cell lines.

Key words: Silver-copper biocomposites, phytosynthesis, biohybrids.

1. INTRODUCTION

The *green* nanotechnology approaches have been increasingly used in the last ten years due to a series of advantages such as the use of cheap, inexpensive and non-toxic reactants, simplified synthesis routes and mild reaction parameters. The use of plants as raw materials for biosynthesis of metallic particles was preferred in the last decades, due to their low cost and high abundance in nature, but also for the biodegradability and reusability properties of the vegetal wastes [1].

Banerjee and co-workers [2] highlighted the huge potential of fruit peels and other fruit wastes as renewable sources for production of biofuels or as a feedstock for recovery of many bioactive compounds (flavonoids, pectin, dietary fibres, lipids, etc.). It is well known that *Citrus* species contain a wide range of active phytocompounds (vitamin C, pectin, flavonoids, acids and volatile oils) [3, 4].

Matrix-embedded metal nanoparticles possess advantage of their favorable mechanical, chemical and optical properties [5]. However, for many bioapplications, the preferred biological matrix are *liposomes* (known as model of biomembranes, or biomimetic membranes) which are lipid vesicles that can be formed spontaneously when lipids are dispersed in aqueous solutions, the inner volume being surrounded by a membrane made of a lipid bilayer, structure mimicking the cell membranes. The lipid coating of metallic nanoparticles ensures biocompatibility and stability in suspensions for applications in life sciences [6]. On the other hand, biohybrids based on liposomes and metallic nanoparticles possess high antioxidant and antimicrobial activities [7–9].

Bimetallic composite nanoparticles are of a greater interest than monometallic one, having enhanced properties [10]. It has been found that antimicrobial activity increases when bimetallic nanoparticles are used [11, 12]. The "green" Ag-Cu nanoparticles are effective agents with high potential for purifying water contaminated with pesticides [13, 14]. Bionanocomposite films based on fish skin gelatine and bimetallic nanoparticles (Ag-Cu NPs) showed strong antibacterial activity against gram-positive and gram-negative bacteria [15].

In this paper, we developed two types of lipid-based biohybrids (with and without chlorophyll) containing silver/copper particles phyto-generated using *Citrus reticulata* peel aqueous extract. These hybrids were characterized by spectral (UV-Vis absorption spectroscopy) and microscopical (AFM) methods, and their bioperformances (antioxidant, antimicrobial, antiproliferative activities) were further evaluated.

2. MATERIALS AND METHODS

2.1. MATERIALS

Tris (hydroxymethylaminomethane base), luminol (5-amino-2,3-dihydro-phthalazine-1,4-dione), soybean lecithin (Calbiochem), H_2O_2, KH_2PO_4, Na_2HPO_4, were supplied by Merck (Germany). Copper sulphate pentahydrate **(**$CuSO_4 \cdot 5H_2O$) was purchased from Sigma Aldrich (Germany), and silver nitrate ($AgNO_3$) from Gatt Koller – GmbH (Austria).

Chlorophyll a (Chl*a*) was prepared in our laboratory from fresh spinach (*Spinacia oleracea* L.) leaves by the chromatographic approach described in [16], and the photopigment purity was monitored by Vis absorption spectra.

Citrus reticulata (tangerine) fruits were purchased from a local market of Magurele, Romania.

In order to evaluate the antibacterial activity, biohybrids were tested against pathogenic *Escherichia coli* ATCC 8738 bacterium. *Escherichia coli* was grown in Luria Bertani Agar (LBA) plates at 37°C with following composition: 10 g/L peptone (Merck, Germany), 5 g/L yeast extract (Biolife), 5 g/L NaCl (Sigma Aldrich, Germany) and 20 g/L agar (Fluka). The stock culture was maintained at 4°C.

Dullbecco's modified Eagle's medium (DMEM), *Fetal bovine serum* (FCS), Trypsin-EDTA, *Phosphate-buffered saline* (PBS) and antibiotics (penicillin and streptomycin) were purchased from Sigma-Aldrich (St. Louis, MO). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), and CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) kit was procured from Promega Corporation (Madison, WI).

Colorectal cancer cells (Caco-2 ATCC HTB-37) and normal murine fibroblast (L929 ATCC CRL-6364) cell lines were purchased from ATCC (LGC Standards, Germany). They were maintained at 37°C and 5% CO_2 humidified atmosphere with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Invitrogen, Waltham, MA, USA), and 1% antibiotics (10 000 units/mL penicillin and 10 000 μg/mL streptomycin in 0.90% saline).

2.2. PHYTO-GENERATION OF BIMETALLIC STRUCTURES

The bimetallic particles were generated from an aqueous extract of *Citrus reticulata* peels (Fig. 1). Clean fresh fruit peels were cut into small pieces and then mixed with distilled water in a final mass ratio peels/distilled water of 1:3 (w/w). This mixture was boiled for 5 minutes, then cooled at room temperature in dark, and filtered with Whatman filter paper no. 1. Copper sulphate was added to 100 mL of this aqueous extract under continuously stirring (at 80ºC for 1 hour, and at room temperature for another 2 hours). In this resulting solution, under magnetic stirring, silver nitrate was then added to the final mass ratio silver:copper of 1.59. Obtaining of the bimetallic composite structures was visually observed *via* color change of this mixture: yellow → dark green → khaki → dark brown, therefore C. *reticulata* peels' extract acted as both a reducing and a stabilizing eco-friendly agent.

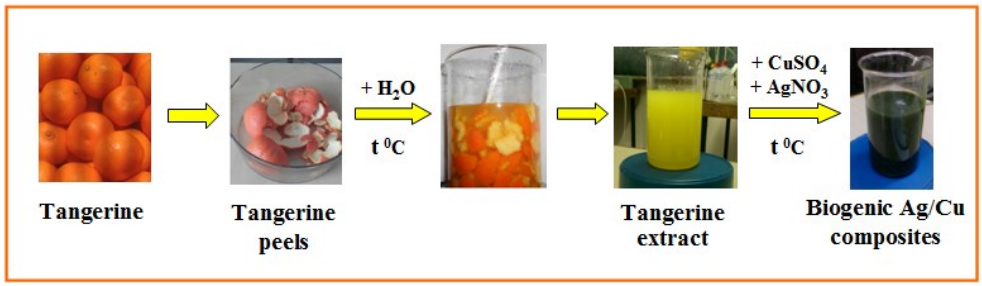

Fig. 1 (Color online) – Phyto-generation of Ag/Cu composites.

2.3. PREPARATION OF BIOHYBRIDS SILVER-COPPER-BIOMIMETIC MEMBRANES

The bio-inspired lipid membranes were achieved by using the procedure previously described [17, 18]. Briefly, two kinds of mimetic biomembranes with and without chlorophyll *a* were obtained by hydrating a soybean lecithin film (Chl*a* containing in the molar ratio of 0 or 1: 100) with a buffered phosphate solution (PB, KH_2PO_4–Na_2HPO_4 pH 7.4), followed by a vigorous mechanical stirring (VIBRAX stirrer – OHIO 43230 USA, 200 rpm) for 40 min, and then subjected to ultrasound treatment (Hielscher Ti probe sonicator, UP 100 H – Hielscher Ultrasonics GmbH, 14513 Teltow, Germany). These bio-inspired lipid bilayers were used to develop two hybrid systems containing the bimetallic structures achieved through the method described in the section 2.2. Specific aliquots from the suspension of bimetallic particles were mixed with appropriate volumes of soybean lecithin suspensions, in a volume ratio of 1:3. These mixtures were subjected to an ultrasound treatment (Hielser titanium probe sonicator, UP 100 H) in order to obtain the *silver-copper-liposomes* biohybrids.

The schematic representation of biohybrid preparation is presented in Fig. 2.

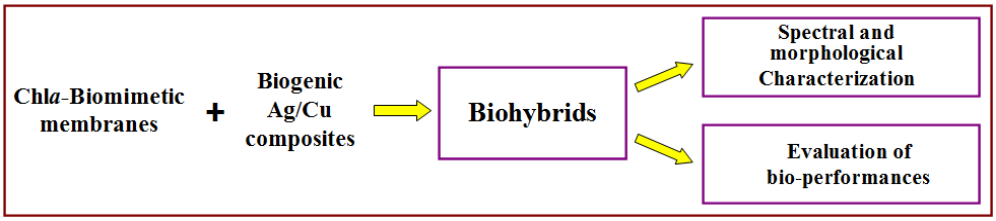

Fig. 2 (Color online) – Schematic representation of biohybrid preparation.

These experiments were performed in dark to prevent the photodegradation of the samples. The abbreviations of all the samples prepared in our work are displayed in Table 1.

Table 1

The abbreviations of all the samples

Sample code	Code
Citrus reticulata peel extract	E
Citrus-Ag/Cu composites	M
Soybean lecithin-liposomes	L1
Chl*a*-Soybean lecithin-liposomes	L2
Citrus-Ag/Cu-Soybean lecithin liposomes (hybrids)	ML1
Citrus-Ag/Cu-Chl*a*-Soybean lecithin liposomes (hybrids)	ML2

2.4. CHARACTERIZATION METHODS

The absorption spectra were recorded in the 200–800 nm wavelength range on a double beam JASCO V-570 UV/Vis Spectrophotometer, operated at a resolution of 0.5 nm.

The bimetallic particles were investigated by *Energy-dispersive X-ray* spectroscopy (EDX), on a FEI Inspect S scanning electron microscope equipped with EDX spectrometer.

Measurements of ***zeta potential*** **(ZP)** were carried out in an appropriate device of Zetasizer Nano ZS (Malvern Instruments Ltd., U.K.), by applying an electric field across the analyzed aqueous suspensions.

AFM images were recorded on an integrated platform, AFM/SPM (NTEGRA Prima, NT-MDT) in semi-contact mode (scanning area range of 10× 10 μm^2) using an NSG01 cantilever with a typical radius of curvature of 10 nm. All AFM measurements were obtained on samples deposited on quartz glass substrates, and the images obtained were processed with the AFM system image processing software.

The experiments of ***bioperformance testing*** were performed in triplicate, and the standard deviations were calculated as the square root of variance using STDEV function in *Excel* 2010:

a) ***The in vitro antioxidant activity (AA%)*** of the samples was investigated through chemiluminescence method on a Chemiluminometer Turner Design TD 20/20 (USA), as described in [19].

b) ***Antibacterial assay.*** The agar disc diffusion method was used to determine the antibacterial activities of the biocomposites. Disc-assay was found to be a simple, and reproducible practical method [20]. A suspension of *Escherichia coli* bacterium (1 mL) was spread on a solid agar medium in Petri dishes (LBA). Agar plate was punched with a sterile cork borer of 6 mm size and 50 μL of each sample was poured with micropipette in the bore, and then incubated at 37°C for 24 h. The pure distilled water was used as a control. The diameters of the inhibition zones (IZ) were measured in millimetres [21].

c) ***Cell viability test.*** For all experiments, cells were plated in 96-well plates at a density of 5000 cells per well in 100 μL of culture medium. Cells were allowed to attach and achieve approximately 80% confluence prior to starting the experiments. The particle suspensions were prepared in complete culture media consisting of DMEM supplemented with 10% FCS and 1% antibiotic solution. After reaching confluence, the cells were detached from the flask with Trypsin-EDTA. The cell suspension was centrifuged at 2000 rpm for 5 min and then resuspended in the growth medium. The effects of samples on the viability of were evaluated using MTS reduction, which is a colorimetric test based on the selective ability of viable cells to reduce the tetrazolium component of MTS into purple coloured formazan crystals [22].

d) In vitro *antiproliferative test.* The cells were incubated in the presence of samples for 48 hours at 37°C in 5% CO_2 atmosphere, after which the cell viability was determined by a colorimetric method using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega, USA). Cells from the Caco2 colon cancer line were seeded in 96-well culture plates at a cell density of 104 cells/mL, in a final volume of 200 µL/well. After 24 hours of incubation, medium was removed and fresh medium added. Next, the cells were incubated for 48 hours at 37°C in 5% CO_2 atmosphere in the presence of biohybrids at concentrations of 100 µg/mL, 50 µg/mL, 10 µg/mL and 5 µg/mL, the dilutions being made in the culture medium, after which the cell viability was determined by the MTS test [23].

3. RESULTS AND DISCUSSIONS

3.1. SPECTRAL AND MORPHOLOGICAL CHARACTERIZATION OF THE SAMPLES

Spectral (UV-Vis absorption, EDX spectrum) and morphological characterization by AFM indicated formation of Ag/Cu bimetallic particles using *Citrus reticulata* peel aqueous extract.

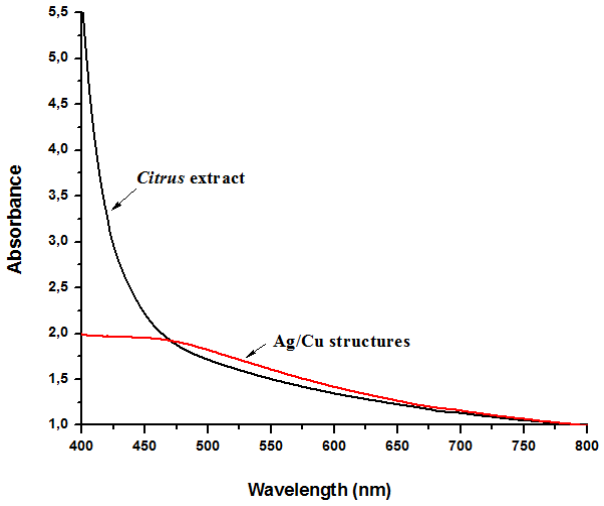

Fig. 3 (Color online) – The Vis absorption spectra of *Citrus reticulata* extract and biogenic Ag/Cu bimetallic structures.

The biosynthesis of bimetallic structures was firstly observed with the naked eye, *via* colour change over time, of *Citrus* extract upon addition of metal salts (Fig. 1), and then confirmed by absorption spectroscopy (Fig. 3) and AFM images. *Citrus* extract alone presented no absorption peak in VIS domain between 400–650 nm,

whereas the peels' extract exposed to metal ions (arising from $AgNO_3$ and $CuSO_4$ solutions) showed a distinct absorption band (known as a SPR band) centred at around 470 nm. This broad band suggested the formation of larger bimetallic particles. Due to its content rich in active phyto-ingredients like: proteins, pectin, polyphenols [3, 18], the aqueous extract from peels of *C. reticulate* acted both as reducing and capping agent in Ag/Cu particles' synthesis.

In Fig. 4 the EDX analysis obtained from bimetallic particles deposited on glass substrate is presented. The revealed composition is 11% Cu, 21% Ag, indicating that the Ag/Cu bimetallic particles were enriched in Ag.

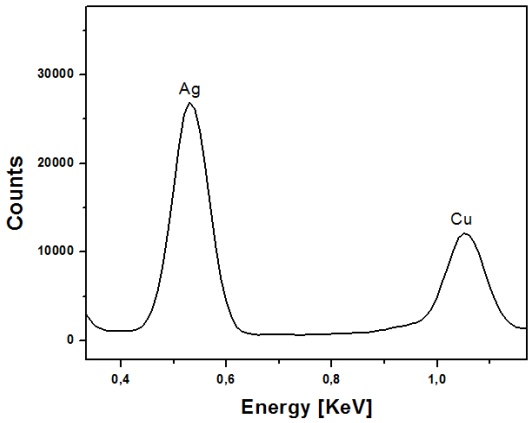

Fig. 4 – EDX spectrum of sample M onto glass substrate.

Morphological characterization highlighted the spherical shape of the samples containing bimetallic particles (Fig. 5).

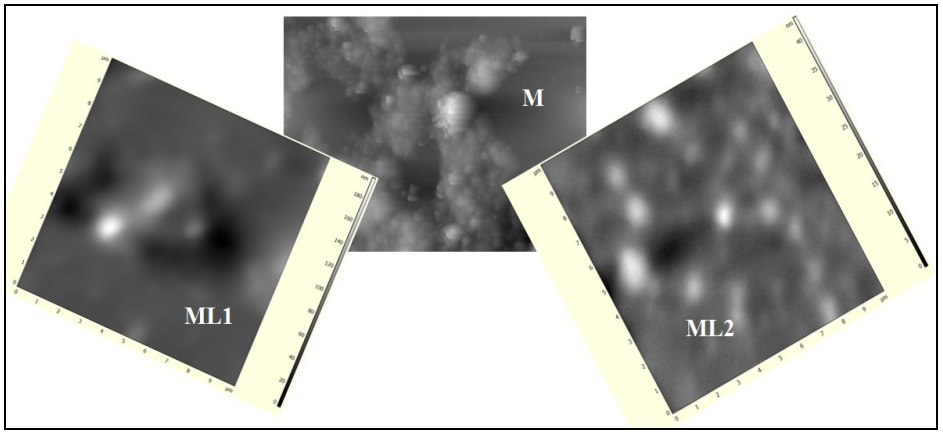

Fig. 5 – AFM analysis of the nano-bioarchitectures: silver-copper particles (M) and the biohybrids (ML1 and ML2).

AFM images revealed also the formation of the nano-sized hybrids (ML1 and ML2) well dispersed as compared to silver-copper particles (M) which are more aggregated. Multilayered Ag-CuNPs clusters were observed in the sample M, demonstrating its poor stability, and therefore the tendency to aggregate.

3.2. THE EVALUATION OF PHYSICAL STABILITY

The dispersions of particles having zeta potential values more negative than −30 mV or more positive than +30 mV are considered to be physically stable [10]. Moreover, knowing the value of ZP (that is closely related to stability) is very useful tool for understanding and predicting the biological performances of the samples [10].

As seen in Fig. 6, the lipid vesicles have low zeta potential values (–12.2 mV for L1 and –16.3 mV for L2), therefore weak repulsion forces to prevent the particles coming together.

The obtained bio-hybrids exhibited the highest physical stability assured by electrostatic repulsion forces between the particles due to high negative ZP values (–30.1 mV for ML1 and –35.1 mV for ML2). On the contrary, the bimetallic particles (sample M) present low to moderate stability (ZP = –18 mV).

These results are good correlated with AFM images.

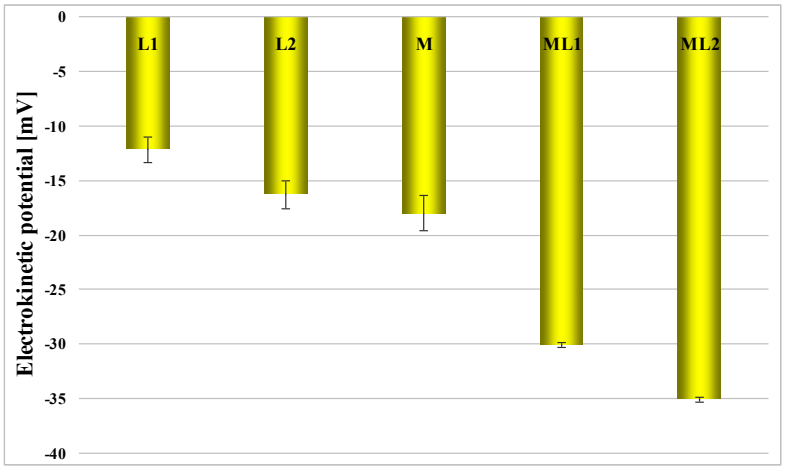

Fig. 6 (Color online) – Comparative electrokinetic potential for the samples prepared.

3.3. BIOPERFORMANCES OF THE BIOHYBRIDS

The antioxidant properties of the two prepared biohybrids were tested through the chemiluminescence technique by using a free radicals' generator system

based on luminol (1 mM), Tris-HCl buffer solution (pH 8.6), and H_2O_2 (10 μM). The values of AA% expressed as: AA = $[(I_0–I)/ I_0]\cdot100\%$, where I_0 is the maximum CL intensity at t = 5 s, for the reaction mixture without the sample, and I is the maximum CL intensity for each sample at t = 5 s [19], are comparatively presented in Fig. 7.

As expected, the *Citrus* extract showed high value of antioxidant activity (AA = 98.3%) due to its content rich in vitamin C and other active phytocompounds like: polyphenols, pectin, with antioxidant role [3, 18], while the liposomes alone presented the lowest AA%, with higher value for Chl*a* – loaded lipid vesicles (see sample L2), due to the antioxidant properties of this photopigment [24].

It is worth noting that the addition of bimetallic structures in a percent of 25% vol., to liposomes' suspensions, resulted in huge increase of liposomal AA%, from 51.85 to 98.51% for Chl*a*-free lipid bilayers, and from 62 to 99.56% for Chl*a*-containing membranes, due to the synergistic effect exerted by the *C. reticulata* peels' extract and the biogenic Ag/Cu bimetallic structures (AA = 99.43%). Other explanation is the nanodimension and spherical shape of the ML1 and ML2 biohybrids (Fig. 5), which offer high total surface area providing many reaction centers that improve the capacity of free radical scavenging [7].

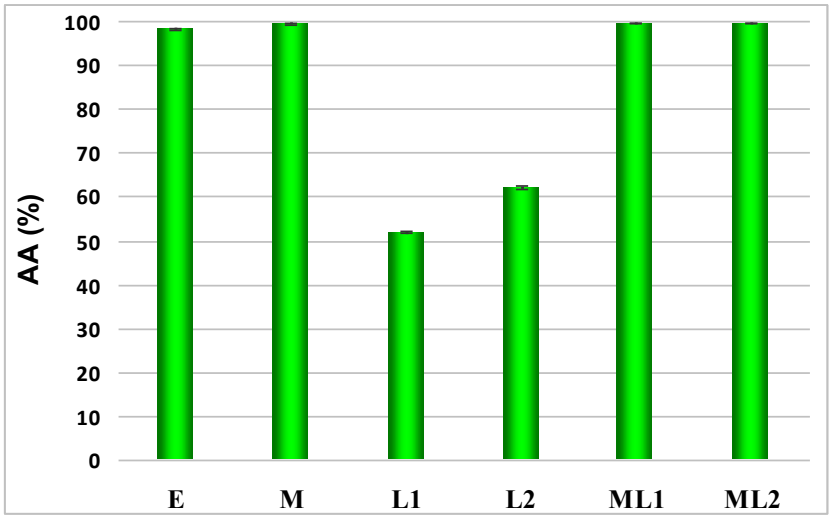

Fig. 7 (Color online) – Comparative presentation of the antioxidant activity of the samples.

Antibacterial testing against *E. coli* ATCC 8738 highlighted a good biocidal activity of the samples ML1 and ML2, exhibiting inhibition zones with diameters values of 15 mm and 17 mm, respectively (Fig. 8).

A significant synergistic antimicrobial effect was observed when *E. coli* cultures were exposed to a *Citrus*-Ag/Cu-Chl*a*-Soybean lecithin-liposomes hybrids

(ML2). This sample was the most potent against *E. coli*, due to two main contributing factors:

1) its content in many antimicrobial agents, like: *Citrus reticulata* extract [18, 25], Chl*a* [26], copper [27], silver [28] and also, a lipid component that facilitates interaction with bacterium cell membrane.

2) the high ZP value (Fig. 6) which is directly proportional to the antibacterial activity [29].

3) its nano-dimension allowing better interaction of these nanomaterials with bacterial cells resulting in changes in the cells/lipid membranes at the molecular level [30].

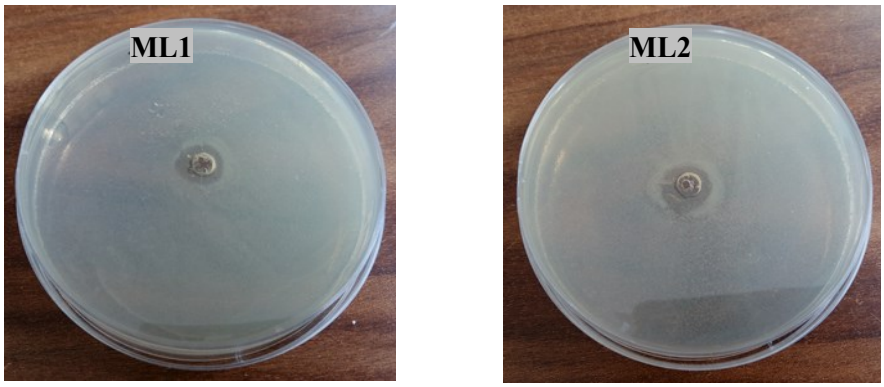

Fig. 8 (Color online) – Bacto-agar plates inoculated with the biohybrids.

In the experiment conducted on the L929 cell line (Fig. 9a), for the two biohybrids (samples ML1 and ML2), there are no cytotoxic effects on these cells when biohybrid concentrations of 1–10 μg/mL were applied. With increasing concentrations of the sample to values of 50–100 μg/mL, cell viability is diminished, as shown in Fig. 9a. Given that the high IC_{50} values: 178.33 ± 7.61 μg/mL for ML1 and 197.79 ± 3.94 μg/mL for ML2 (Table 2), we can conclude that ML1 and ML2 samples *are essentially free from cytotoxicity* against normal cells tested [31].

The antiproliferative effect of the two biohybrids on the Caco-2 ATCC HTB-37 cell line (Fig. 9b) can be explained by *Citrus reticulata* extract content, whose specific chemical composition (especially carotenoid compounds: beta-cryptoxanthine and flavonoids: hesperidins), causes antiproliferative action in the case of colon cancer [32, 33].

The antiproliferative effects of the two biohybrids are closely related. *In vitro* human cell culture studies on Caco-2 colon cancer demonstrated that, they inhibit the proliferation of tumour cells at an IC_{50} value of 93.05 ± 0.11 µg/mL for ML1, and an IC_{50} of 102.96 ± 0.23 µg/mL for ML2 (Table 2).

These results are promising and could be exploited in biomedical applications.

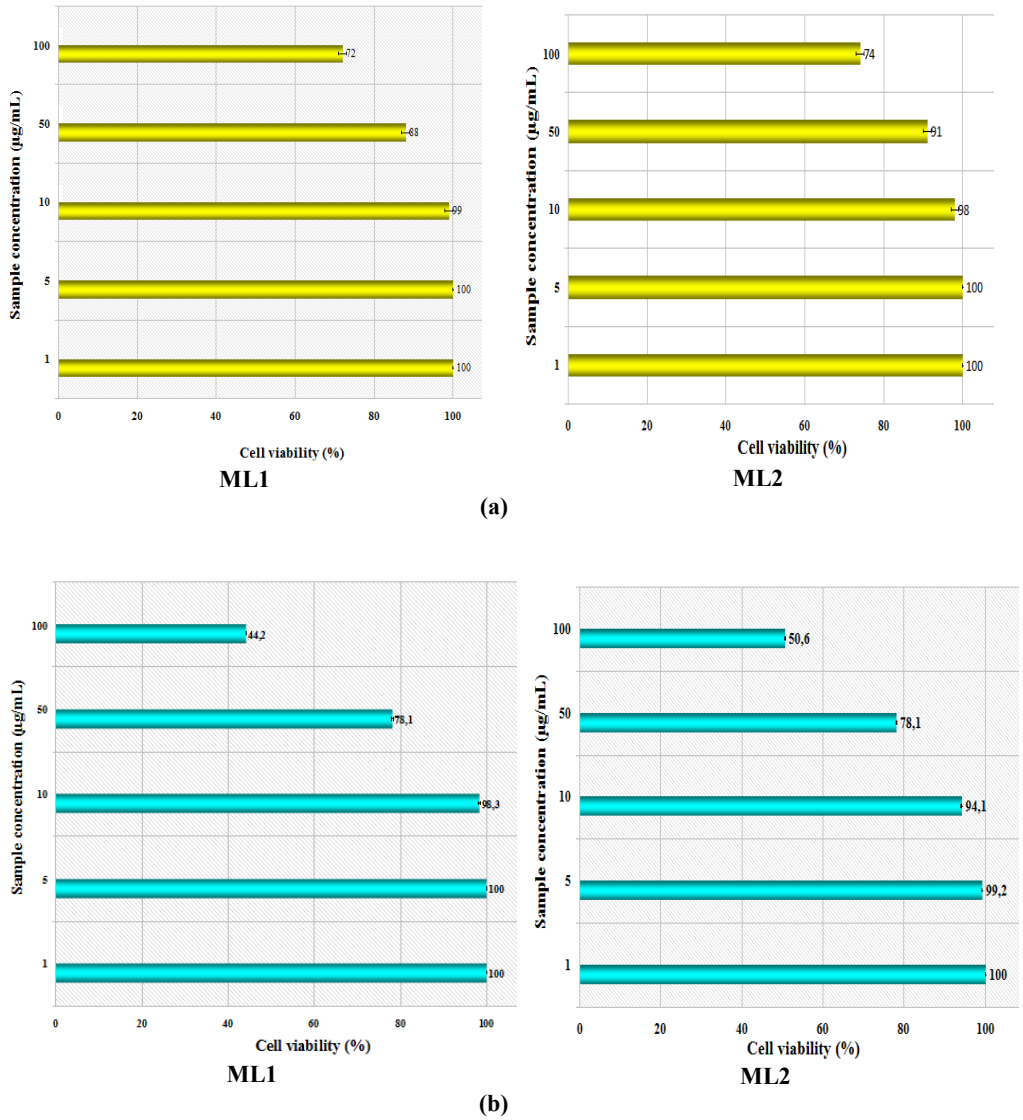

Fig. 9 (Color online) – Cytotoxicity of biohybrids against normal cells (a) and the antiproliferative effect on the Caco2 malignant cell line (colon cancer) (b).

Table 2

IC_{50} value for cytotoxicity against normal cell line murine fibroblast cells (L929 ATCC CRL-6364) and IC_{50} value for the antiproliferative effect on the Caco2 malignant cell line (colon cancer)

Sample	IC_{50} L929 [µg/mL]	IC_{50} Caco-2 [µg/mL]
ML1	178.33 ± 7.61	93.05 ±0.11
ML2	197.79 ± 3.94	102.96 ± 0.23

4. CONCLUSIONS

This paper presented a simple, low cost, time efficient and "clean" *bottom-up* approach to achieve biohybrids based on biomimetic membranes and silver-copper particles, following the *Green Chemistry* principles.

The obtained *bio-green-hybrids* showed good physical stability (with high negative zeta potential values), high antioxidant activity, and good antibacterial activity against *Escherichia coli* ATCC 8738. In addition, all bio-based hybrids had more toxic effects against colorectal cancer cells Caco-2 ATCC HTB-37 and does not show toxicity to normal murine fibroblast (L929 ATCC CRL-6364) cell lines. Both biohybrids prepared with and without chlorophyll, exhibited *in vitro* antiproliferative activity with close values of IC_{50} (93.05 ±0.11 µg/mL for Chl*a* – free hybrid systems, and 102.96 ± 0.23 µg/mL for Chl*a* – loaded hybrids), but first seems to induce more inhibition on the proliferation of tumour colorectal cancer cells.

These findings are promising; the obtained *green* biohybrids could be used as adjuvants in colon cancer therapy.

Acknowledgements. This work was co-financed from the European Social Fund through the Sectorial Operational Programme, Human Resources Development 2007–2013, Contract Code: POSDRU/187/1.5/S/155559, multidisciplinary doctoral research on European competitiveness (CdocMD). The work was supported also by the Romanian National Authority for Scientific Research, Project PN16270303, by grants from the Romanian National Authority for Scientific Research, CNCS-UEFISCDI: PN-II-PT-PCCA-2013-4-1268 (142/2014), and by Projects PN-II-RU-TE-2014-1550.

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