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Terrassa
Biology Department :
Molecular and Industrial Biotechnology group

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Study of Rutin Nanocarriers for Skin Care

Presented by Tony Marceline

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In front of the panel’s members:

Pr. Tzanko Tzanov	Universitat Politecnica de Catalunya, Terrassa	Director
Dr. Kristina Ivanova	Universitat Politecnica de Catalunya, Terrassa	Co-supervisor
Dr. Eva Ramon	Universitat Politecnica de Catalunya, Terrassa	Co-supervisor

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Abstract

Rutin (quercetin-3-rhamnosyl glucoside) is a natural flavonoid widely distributed in vegetables and fruits which has many therapeutic properties, mainly attributed to its potent antioxidant and antimicrobial activities. These benefits are used in different fields namely in the pharmaceutical to treat some infectious diseases or in the cosmetics to fight against premature aging. Rutin plant pigments have multiple effects on skin, one of the most important being to promote redness-free skin. Furthermore, rutin has showed an increase in skin elasticity and a decrease in the length, area and number of wrinkles. The consequences of human aging are primarily visible on the skin, such as increased wrinkling, sagging and decreased elasticity. It also enhances the action of vitamin C on the body, on the face particularly, by increasing its absorption, delaying its elimination then limiting wrinkles. [1]

The main objective of this study was first to prepare Rutin-loaded polymersomes which have the particularity to be nanoparticles. An important goal of this research was to develop antioxidant and antibacterial mechanism of rutin-loaded nanoparticles. Different concentrations (e.g. 1, 5 and 10 mg/mL) and amplitudes (35 and 50%) during the preparation of polymersomes using single step ultrasound process. Radical scavenging assays with DPPH and ABTS were then employed as tools to further assess the antioxidant efficacy of the nanoformulated rutin. The other objective was to analyze the antimicrobial effects of rutin polymersomes with on common Gram-positive and Gram-negative bacteria according to Minimum Inhibitory Concentration (MIC) method. A strategy was elaborated to increase the antimicrobial performance of rutin NPs by combining them with another antimicrobial agent (colistin).

Even if, the experiments have showed very good antioxidant activities thanks to ABTS assay but not relevant antimicrobial activities, we will try to find the good concentration and amplitude during the preparation of rutin nanoparticles to incorporate into a skin care cream to minimize the development of bacteria responsible for skin infections such as *Staphylococcus* and *Streptococcus* and the destruction of bacteria from skin flora (*S.epidermis*, *S.hominis*) as well as maximize the radical scavenging inhibition for more antioxidant properties.

Introduction

Nowadays, some skin diseases such as Acne, triggered by *Cutibacterium acnes* (C.acne) bacteria, remain a societal issue. Even if there are efficient medical treatments like clindamycin and erythromycin, this disease can reappear because of bacteria resistance with some active agents and the treatment may not be natural. Natural extracts in the polyphenols have been introduced as alternative and more effective solutions for managing skin diseases [2].

The influence of polyphenols on bacterial growth and metabolism depends on the polyphenol structure, the dosage assayed and the microorganism strain [3]. But some bacteria like Gram-negative bacteria are more resistant to polyphenols than Gram-positive bacteria, possibly due to the differences found in their wall composition [4]. Recent findings suggest a variety of potential mechanisms of action of polyphenols on bacterial cells ; rutin is one of them.

Rutin has been widely used in treating disease such as skin redness, its several pharmacological activities including antiallergic (Rosane, Oliveira, Fernandes, & Vieira, 2006), anti-inflammatory and vasoactive, antitumor, antibacterial, antiviral and antiprotozoal properties (Calabrò et al., 2005). In addition, hypolipidemic, cytoprotective (Casa, Villegas, Alarcón de la Lastra, Motilva, & Martín Calero, 2000), antispasmodic and anticarcinogenic (Webster, Gawde, & Bhattacharya, 1996) activities have also been reported. Rutin is a kind of flavonoid glycoside known as Vitamin P and has antiplatelet, antiviral and antihypertensive properties, as well as strengthening the capillaries of blood vessels, which are the results of its high radical scavenging activity and antioxidant capacity (Guo, Wei, & Liu, 2007). These properties are potentially beneficial in preventing diseases and protecting the stability of the genome.

Moreover, the nanoparticles (NPs) used contain lipid moieties and effectively using in many biomedical applications. Generally, a lipid NP is characteristically spherical with diameter ranging from 10 to 1000 nm. Like polymeric NPs, lipid NPs possess a solid core made of lipid and a matrix contains soluble lipophilic molecules. Surfactants or emulsifiers stabilized the external core of these NPs (Rawat et al., 2011). Lipid nanotechnology (Mashaghi et al., 2013) is a special field, which focus the designing and synthesis of lipid NPs for various applications such as drug carriers and delivery (Puri et al., 2009) and RNA release in cancer therapy (Gujrati et al., 2014). [5]

Keywords

Rutin ; polymersomes ; nanoparticules ; antioxidant activity ; flavonoids ; antimicrobial activity

I. Characterization of rutin

I.1. Rutin origins

Rutin also known as rutoside, quercetin-3-O-rutinoside and sophorin is a citrus flavonoid glycoside, which is a low molecular weight polyphenolic compound [6]. Flavonoids are natural compounds belonging to the polyphenol family, they have fifteen carbon atoms, with two aromatic rings linked by a three-carbon bridge (Figure 2). These are the major compounds of polyphenols. They are involved in various processes such as UV protection, pigmentation, stimulation of nitrogen-fixing nodules and disease resistance [6]. They are also what give them their color and taste.

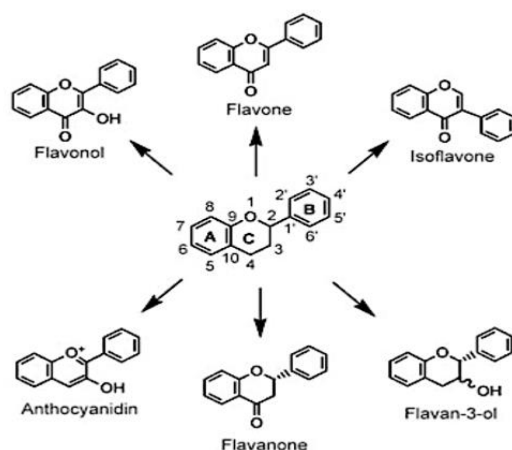

Figure 1 : Generic structures of the main flavonoids (Crozier et al., 2006).

Rutin is synthesized in higher plants, and it was found in various fruits and fruit rinds, especially citrus fruits and berries such as *Ruta graveolens* and *Morus alba*. Rutin is also called vitamin P, which is widely distributed in vegetables and medicinal herbs such as asparagus and buckwheat (*Fagopyrum esculentum*) which is considered to be one of the best dietary sources of rutin. [6]

The molecule structure of Rutin shows that Rutin has polyphenols because the molecule has many phenolic hydroxyl groups (10) and aromatic rings.

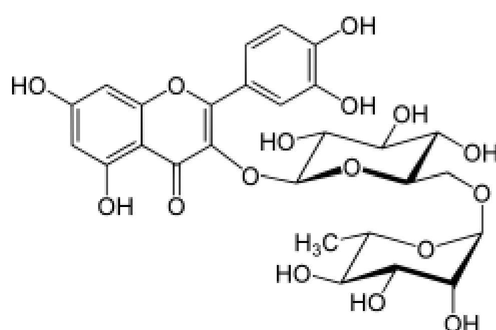

Figure 2: Molecular structure of Rutin

Rutin has various pharmacological activities such as antimicrobial, antiprotozoal, antitumor, antiallergic, antiviral, cytoprotective, vasoactive, hypolipidemic, antiplatelet, antispasmodic and antihypertensive. Rutin is used in different forms such as colorant, antioxidant, preservation, stabilizer and UV absorbent. It is also used as an active component in various herbal medicines, multivitamin preparations, the cosmetics and chemical industries [3].

Thus, flavonoids possess several antimicrobial and antioxidant activities, thus flavonoids are considered the most effective antioxidants with higher antioxidant activities than tocopherols and ascorbic acid (Ferhoum, 2010; Benyahia, 2017).

When human absorbs them, some of their properties are beneficial. They are natural antioxidants that are attracting more and more health interest in the prevention, treatment of cancer and inflammatory skin diseases [7].

I.2. Rutin and its anti-inflammatory activity

Inflammation is a series of well-coordinated immune system reactions to an attack caused by a set of foreign agents (physical, chemical, microbiological) in order to eradicate these pathogens by returning to the state of homeostasis (Ricciotti et al. , 2011). But sometimes the inflammation can be harmful due to the aggressiveness of the pathogen, its persistence, the site of the inflammation, by anomalies in the regulation of the inflammatory process, or by quantitative or qualitative anomaly of the cells involved in inflammation. On the other hand, the overproduction of reactive oxygen species (ROS) such as nitrous oxide, hydroxyl, and superoxide beyond the antioxidant capacities of biological systems gives rise to oxidative stress, which is involved in the onset of chronic inflammatory diseases. Rutin has been identified as an exerted protective effect under conditions of oxidative stress [8].

Consequently, several substances of plant origin such as rutin have proven anti-inflammatory activity which inhibits the synthesis of prostaglandin, a chemical modulator of inflammation (vasodilatation and pain) by inhibiting the synthesis of cyclo-oxygenase (COX), thus rutin can inhibit the activation of IL-6 which is a pro-inflammatory cytokine which participates in the action of inflammation, other enzymes involved in inflammation are inhibited by rutin such as lipo-oxygenase, myeloperoxidase, NADPH-oxidase, ornithine decarboxylase (Severine, 2014; Billel et al., 2018) [8].

I.3. Rutin: many benefits for human body

All the benefits of Rutin are described in the Figure 3.

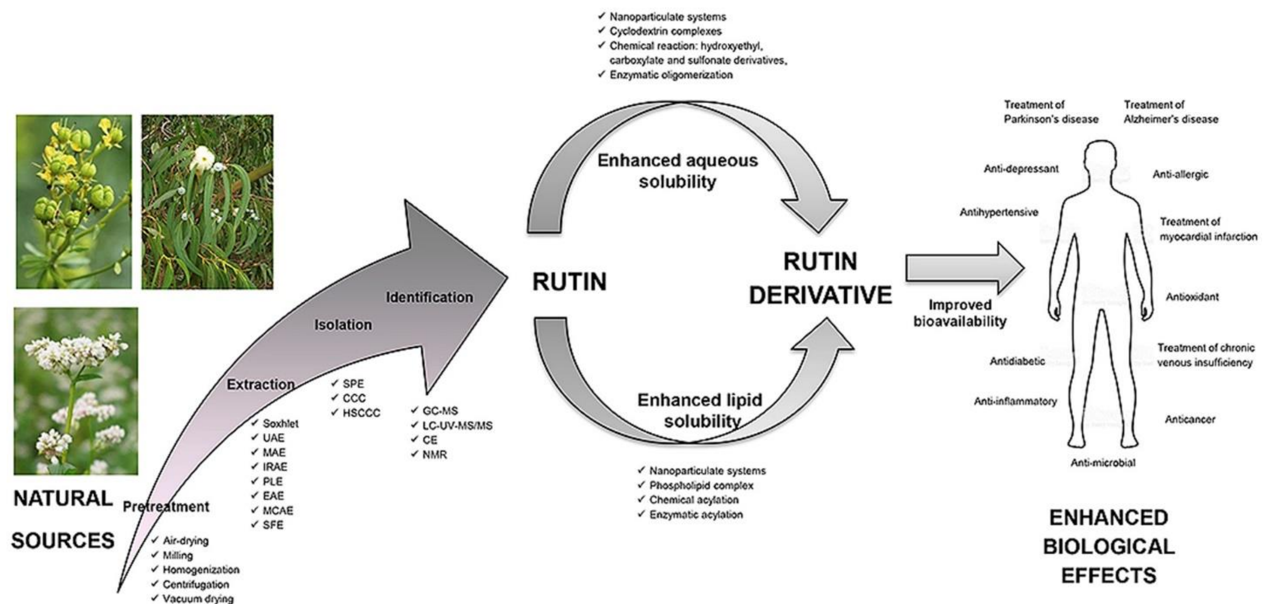

Figure 3 : Rutin from natural sources to enhanced biological effects on body

II. Nanoformulation of rutin

II.1. Polymersomes and liposomes

Nanoformulation changes the chemical and physical properties of natural actives, endowing them with superior antibacterial performance and long-term stability when compared to their free form [2].

Polymersomes are nanoscopic (e.g. nanometer-sized) nanostructures formed by amphiphilic block copolymers. They represent the more robust and versatile macromolecular counterparts to the well-established lipid vesicles or liposomes. They are produced in a uniform and functional manner [9].

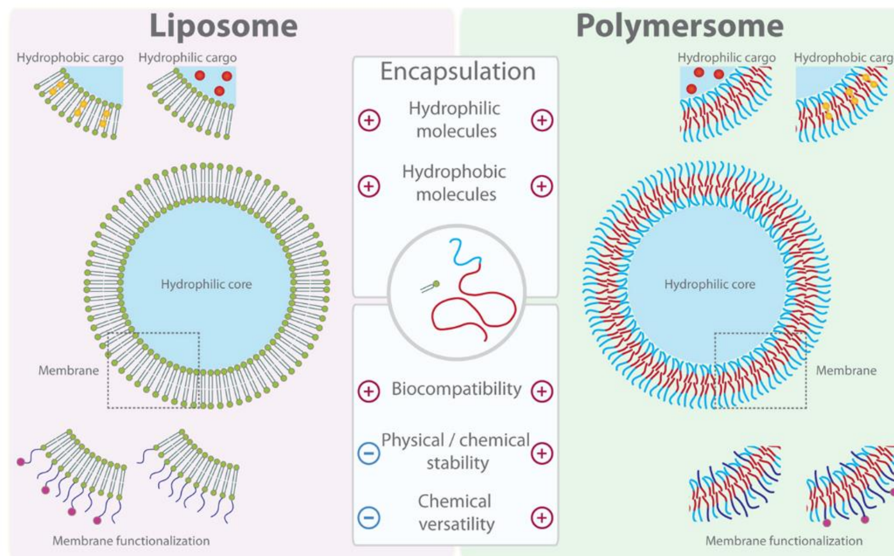

Figure 4 : Comparison between a liposome and a polymersome (ScienceSet)

Liposomes are vesicles resulting from self-assembly of natural phospholipids, which make them fully biocompatible. As depicted in Figure 4, their structure consists of an aqueous core entrapped by a bilayer membrane, which is composed of bilayer hydrophobic tails stabilized by hydrated hydrophilic heads both at the inner and outer shells.

These features make liposomes very attractive for encapsulation of therapeutic molecules because, contrary to other existing nanomedicines, they combine the unique ability to encapsulate both, hydrophilic compounds in the aqueous core, and hydrophobic and amphiphilic molecules within the membrane. However, many limitations have emerged since the first liposomal formulation appeared. In particular, liposomes have shown to exhibit low encapsulation efficiency, poor physical and chemical stability (e.g. high critical aggregation concentration) and low chemical versatility.

Only amphiphilic block copolymer vesicles also known as polymersomes offer the unique opportunity to combine both advantages of liposomes and block copolymer assemblies. Essentially, they comprise a hydrophilic core surrounded by a hydrophobic layer of entangled polymer chains, which are stabilized by hydrophilic polymer brushes on both internal and external side. Polymersomes display enhanced stability and chemical versatility of polymer assemblies. Thus, they also offer the possibility of liposomes to encapsulate hydrophobic and hydrophilic therapeutics [10].

III. Equipment

III.1. Ultrasonic processor for polymersomes preparation

Sonication can be used to produce nanoparticles, such as nanoemulsions, nanocrystals, liposomes and wax emulsions, as well as for wastewater purification, degassing, extraction of seaweed polysaccharides and plant oil, extraction of anthocyanins and antioxidants. It is applied in pharmaceutical, cosmetic.

Figure 5 & 6 : 750 Watt ultrasonic processor

III.2. Ultracentrifuge for nanoparticles cleaning

The centrifuge uses the centrifugal force to separate particles from a solution according to their size, shape, density, medium viscosity and rotor speed.

There is a correlation between the size and density of a particle and the rate that the particle separates from a heterogeneous mixture, when the only force applied is that of gravity. The larger the size and the larger the density of the particles, the faster they separate from the mixture.

Figure 7 : Ultracentrifuge (Beckman Coulter (Optima LE-80K))

The centrifuge (Figure 7) uses the centrifugal force to separate particles from a solution according to their size, shape, density, medium viscosity and rotor speed.

The denser components of the mixture migrate away from the axis of the centrifuge, while the less dense components of the mixture migrate towards the axis.

There is a correlation between the size and density of a particle and the rate that the particle separates from a heterogeneous mixture, when the only force applied is that of gravity. The larger the size and the larger the density of the particles, the faster they separate from the mixture.

III.3. Ultrasonic processor for polymersomes preparation

Figure 8 : Ultrasonic processor

Ultrasonic bath (Figure 8) enables sonication which is the act of applying sound energy to agitate and clean particles in a sample, for various purposes such as the extraction of multiple compounds from plants, microalgae and seaweeds.

III.4. Zetasizer Nano Z for particles characterization

Figure 9 **:** Zetasizer Nano Z (Malvern Instruments)

Zetasizer (Figure 9) is used to measure the particle size of dispersed systems from sub-nanometer to several micrometers in diameter, using the technique of Dynamic Light Scattering (DLS). Zetasizer is also used to analyze particle mobility and charge (Zeta potential) using the technique of Electrophoretic Light Scattering (ELS), and the molecular weight of particles in solution using Static Light Scattering (SLS).

IV. Materials and methods

IV.1. Preparation of Rutin-loaded polymersomes

500 µL of 1, 5 and 10mg/mL Rutin solution in DMSO (Dimethyl sulfoxide) was mixed with 0.5 g polymer (*DOWSIL™ OFX-5329 Fluid*) and 40 mL PBS. The mixture was left for 1 h at room temperature and then sonicated for 5 min at 35 % or 50 % amplitude using *750 Watt ultrasonic processor*. The samples were centrifugated at 45 000 revolutions per minutes for 40 minutes to remove the non-encapsulated rutin.

During this project, we will prepare 8 samples and two parameters will be modified to be compared: the concentration of Rutin and the amplitude at sonication during the polymersomes' preparation.

Name of the sample	Notation	Rutin concentration (mg/mL)	Amplitude during sonication (%)
Rutin 1 mg/mL 35%	R135	1	35
Rutin 5 mg/mL 35%	R535	5	35
Rutin 10 mg/mL 35%	R1035	10	35
Rutin 1 mg/mL 50%	R150	1	50
Rutin 5 mg/mL 50%	R550	5	50
Rutin 10 mg/mL 50%	R1050	10	50
Control	C35	0	35
Control	C50	0	50

Table 1 : Description of the samples

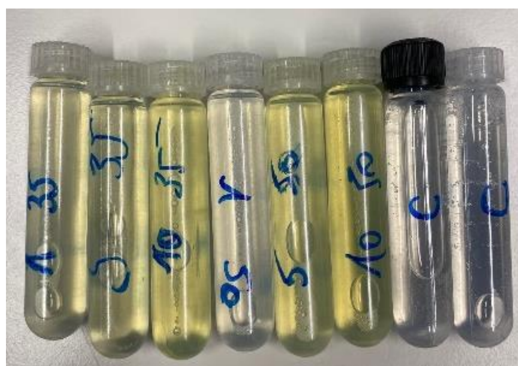

Figure 10 : Rutin solutions in minitubes

IV.2. Polymersomes characterization

IV.2.1. Zeta potential

IV.2.1.1. Principle

Zeta potential is a physical property which is exhibited by any particle in suspension, macromolecule or material surface.

It can be used to optimize the formulations of suspensions, emulsions and protein solutions, predict interactions with surfaces, and optimize the formation of films and coatings. Knowledge of the zeta potential can reduce the time needed to produce trial formulations. It can also be used as an aid in predicting long-term stability [11].

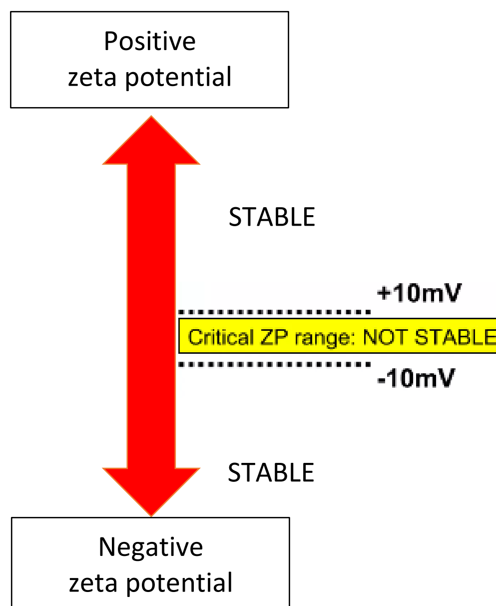

Figure 11 : Stabality and unstability in zeta potential

As illustrated in the figure 11, the more or the less the zeta potential is, the more stability there is. When the zeta potential tends towards zero, the inter-particle forces decrease. The attractive forces then become preponderant and the particles aggregate: the system is destabilizing [12].

To obtain a stable suspension, the objective is therefore to obtain the highest zeta potential (in absolute value) [12].

IV.2.1.2. Methods

The zeta potential of all the samples were measured before and after cleaning the suspensions by the centrifuge using Zetasizer Nano Z.

IV.2.2. Size of particles

IV.2.2.1. Principle

Properties and applications of nanoparticles (NPs) exist in different forms. NPs are tiny materials having size ranges from 1 to 100 nm. NPs possess unique physical and chemical properties due to their high surface area and nanoscale size. Their reactivity, toughness and other properties are also dependent on their unique size, shape and structure. Nanoparticles (NPs) are wide class of materials that include particulate substances, which have one dimension less than 100 nm at least (Laurent et al., 2010).

They are mostly nanospheres or nanocapsular shaped (Mansha et al., 2017). The former are matrix particles whose overall mass is generally solid and the other molecules are adsorbed at the outer boundary of the spherical surface. In the latter case the solid mass is encapsulated within the particle completely (Rao and Geckeler, 2011).

IV.2.2.2. Methods

The size of the nanoparticles of all the samples were measured before and after cleaning the suspensions by the centrifuge using Zetasizer Nano Z.

IV.3. Antioxidant activity of Rutin polymersomes

IV.3.1. DPPH radical scavenging assay

IV.3.1.1. Principle

The method is based on the reduction of the radical DPPH (1,1-Diphenyl-2-picrylhydrazyle), which has a free electron on a nitrogen atom (see the figure below) which gives it a blue-violet color characteristic of the DPPH solution at room temperature, an antioxidant (in our case rutin) has the ability to donate a singlet electron to the DPPH•II radical to return it to its reduced form, with a gradual color change to yellow (Figure 12), the discoloration is measured using a UV-VIS spectrophotometer at 517 nm (Severine, 2014).

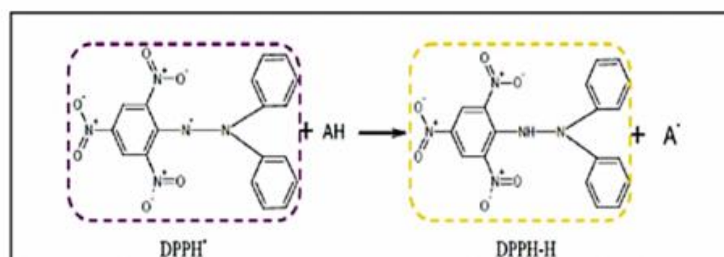

Figure 12 : Reduction of DPPH radical (Amrouche and Koko, 2017)

IV.3.1.2. Materials

Rutin hydrate (94%), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, PBS, methanol, distilled water.

IV.3.1.3. Methods

An amount of 2.3 mg of DPPH was added to 100 mL of methanol. Then the solution was homogenized and incubated in the dark for 30 min. Different dilutions of polymersomes loaded with rutin were prepared in PBS and mixed with DPPH.

Then, the samples at amplitude 35% during sonication were distributed in the same way as the Figure 13. The distribution was the same for the samples at amplitude 50%.

Dilution 0x	50 µL R135	50 µL R135	50 µL R135	50 µL R535	50 µL R535	50 µL R535	50 µL R1035	50 µL R1035	50 µL R1035
Dilution 2x	25 µL R135 + 25 µL PBS	25 µL R135 + 25 µL PBS	25 µL R135 + 25 µL PBS	25 µL R535 + 25 µL PBS	25 µL R535 + 25 µL PBS	25 µL R535 + 25 µL PBS	25 µL R1035 + 25 µL PBS	25 µL R1035 + 25 µL PBS	25 µL R1035 + 25 µL PBS
Dilution 4x	12.5 µL R135 + 37,5 µL PBS	12.5 µL R135 + 37,5 µL PBS	12.5 µL R135 + 37,5 µL PBS	12.5 µL R535 + 37,5 µL PBS	12.5 µL R535 + 37,5 µL PBS	12.5 µL R535 + 37,5 µL PBS	12.5 µL R1035 + 37,5 µL PBS	12.5 µL R1035 + 37,5 µL PBS	12.5 µL R1035 + 37,5 µL PBS
Dilution 8x	6,25 µL R135 + 43,75 µL PBS	6,25 µL R135 + 43,75 µL PBS	6,25 µL R135 + 43,75 µL PBS	6,25 µL R535 + 43,75 µL PBS	6,25 µL R535 + 43,75 µL PBS	6,25 µL R535 + 43,75 µL PBS	6,25 µL R1035 + 43,75 µL PBS	6,25 µL R1035 + 43,75 µL PBS	6,25 µL R1035 + 43,75 µL PBS
Control	50 µL C35	50 µL C35	50 µL C35						

Figure 13: Distribution of the samples at amplitude 35% in the microplate

A negative control was also tested with 50 µL of an ascorbic acid solution, made with 1.1 mg of ascorbic acid powder mixed in 10 mL of PBS.

Then, 50 µL of DPPH were added in each well and the absorbance has been measured at 734 nm after 1 hour of incubation at room temperature.

Figure 14 : DPPH solution

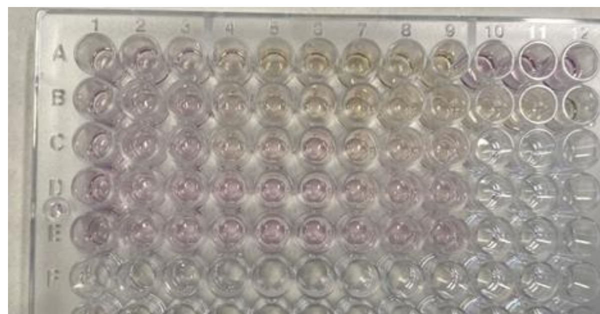

Figure 15: microplate with the different dilutions of Rutin and DPPH

The samples were incubated in a dark for 1 hour. Then the absorbance was measured at 517 nm. The radical scavenging activity was determined spectrophotometrically measuring the decrease in the absorbance of DPPH in a *Infinite M200 multiwell plate reader (Tecan)*. Ascorbic acid surve as negative control. A control with no active agent (no rutin nanoparticles) is also realized.

Then, we put 100 µL of Rutin polymerosomes from each sample (diluted 0x). We dilute it 8 times with PBS and we mix it with 100 µL of DPPH. A control with no active agent (no rutin nanoparticles) is also realized.

The percentage inhibition is calculated according to the following formula:

$$(1-(S_{abs}/Ctrl_{abs}))*100$$

Where S_{abs} represents the absorbance in presence of gel sand $Ctrl_{abs}$ the absorbance of the control DPPH in MilliQ water, without any scavenging agent.

IV.3.2. ABTS radical scavenging assay

IV.3.2.1 Principle

ABTS is a compound used to measure the antioxidant capacities. In this assay, ABTS is converted to its radical cation by addition of sodium persulfate. This radical cation is blue in color and absorbs light at 734 nm. The ABTS radical cation is reactive towards most antioxidants including phenolics, thiols and Vitamin C. During this reaction, the dark green ABTS radical cation is converted back to its colorless neutral form. The reaction may be monitored spectrophotometrically. This assay is often referred to as the Trolox equivalent antioxidant capacity (TEAC) assay.

IV.3.2.2. Materials

3-ethylbenzothiazoline-6-sulfonic acid (94%), potassium peroxodisulfate (99%), MilliQ water, PBS.

IV.3.2.3. Methods

A potassium persulfate stock solution (0.66 mg/mL) was prepared in MilliQ water and homogenized at room temperature. Then, 3.8 mg/mL of ABTS work solution in $K_2S_2O_8$ (0.66 mg/mL) was prepared and was kept at dark under stirring for overnight. After the incubation, ABTS work solution was prepared by mixing 1.2 mL of ABTS in 18.8 mL of MilliQ water.

The samples at amplitude 35% during sonication were distributed in the same way as the Figure 13, in a microplate. The distribution is the same for the samples at amplitude 50%.

Then, 50 µL of ABTS were put in each well and the absorbance has been measured at 734 nm after 1 hour of incubation at room temperature.

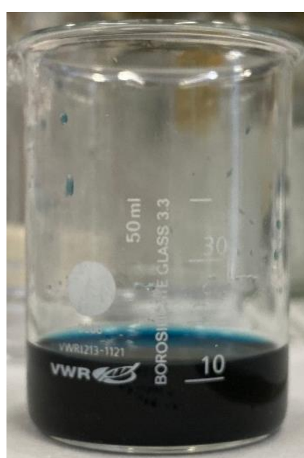

Figure 16. : ABTS solution after incubation

Figure 17 : ABTS diluted and microplate

IV.4. Evaluation of Cytotoxicity in HaCaT Cell Line with AlamarBlue

IV.4.1. Principle

AlamarBlue Cell Viability Reagent is a ready-to-use ressaurin-based solution that functions as an indicator of cellular health by utilizing the reducing power of living cells to quantitatively measure viability. Resazurin, the active ingredient of the alamarBlue reagent, is a non-toxic, cell-permeable compound that is blue in color and practically non-fluorescent. Upon entering living cells, resazurin is reduced to resoruphin, a highly fluorescent red compound. Changes in viability can be easily detected using an absorption or fluorescence plate reader. AlamarBlue Cell Viability Reagent has wide applicability and can be used with various human and animal cell lines, bacteria, plants and fungi [14].

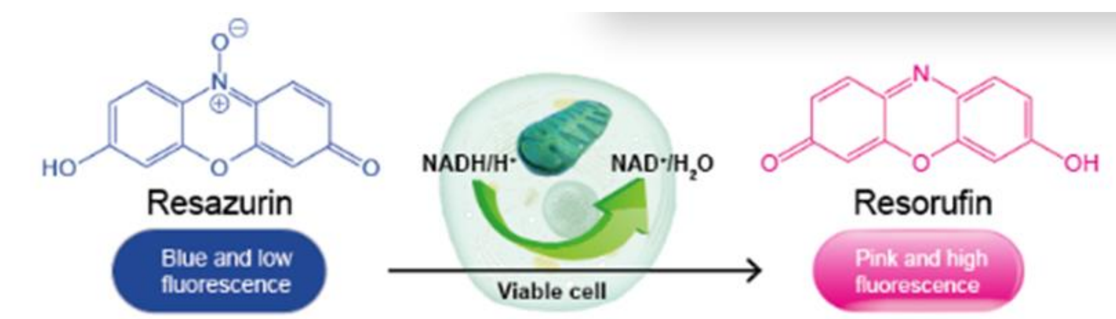

Figure 18 : Colorimetric change and fluorescent signal in response to metabolic activity thanks to AlamarBlue (Cell-Quant™ AlamarBlue Cell Viability Reagent | ABP Biosciences)

IV.4.2. Materials

HaCaT cells, crystal violet, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin (Invitrogen), PBS

IV.4.3. Methods

For determination of the polyphenols' cytotoxic effect, a crystal violet assay was used, with modifications. The cytotoxic effect of polyphenols was analyzed on spontaneously immortalized human skin keratinocytes (HaCaT) cell lines. HaCaT cells are keratinocytes that have been spontaneously immortalized in vitro from a histologically normal human epithelial cell line from adult skin that still has comprehensive epidermal differentiation capacity.

Keratinocytes constitute a major part of the human skin epidermis and and form a barrier against the damage of xenobiotics, heat, UV radiation, pathogenic bacteria, fungi, parasites, and viruses. [15]

Cells were grown in a high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin and streptomycin (Invitrogen) at 37 °. Cells (2×10^4 cells/well) were seeded in a 96-well plate, 24 h before treatment.

After the medium was aspirated, fresh medium supplemented with different concentrations of the polyphenols (0.0039–1 mg/mL) dissolved in PBS was added to the cells. Cells were incubated with the compounds for 24 h. The medium was removed and the cells were washed twice with PBS and stained for 15 min at room temperature with 0.5% crystal violet. After crystal violet was removed, the plates were washed in a stream of tap water and left to air-dry at room temperature for 24 h. The absorbance was measured in a microplate reader at 570 and 600 nm.

IV.5. Antimicrobial activity of Rutin polymersomes

IV.5.1. Minimum Inhibitory Concentration methodology (MIC)

Minimum Inhibitory Concentration (MIC) is the lowest concentration (expressed as mg/L or µg/µL) of an antimicrobial agent that inhibits the visible in-vitro growth of microorganisms. The MIC test determines the antimicrobial activity of a test agent against specific bacteria. E-test, tube dilution, and agar dilution methods are employed to determine MIC value.

While performing the dilution method, the antimicrobial's lowest concentration (highest dilution) preventing the appearance of turbidity (growth) is considered MIC. At this dilution, the antimicrobial agent is bacteriostatic, i.e. some bacteria may still be alive. [16]

IV.5.2. Preparation of the bacteria

The first step to do before doing the MIC method, we must remove colonies of bacteria and put in Petri dishes. The organism must first be isolated in pure culture. A pure culture is one that contains a single strain of microorganism.
Then, we prepare the culture medium made with Nutrient Broth (NB). To do so, we prepare 1.3 g of Nutrient Broth powder and we mix it with 100 mL of distilled water. We sterilize this solution by autoclaving at 121°C for 1 hour [17].

Lastly, a quantity of 5 mL of Nutrient Broth solution with a single strain of microorganisms are put in each tube, according to the bacteria we want to test.

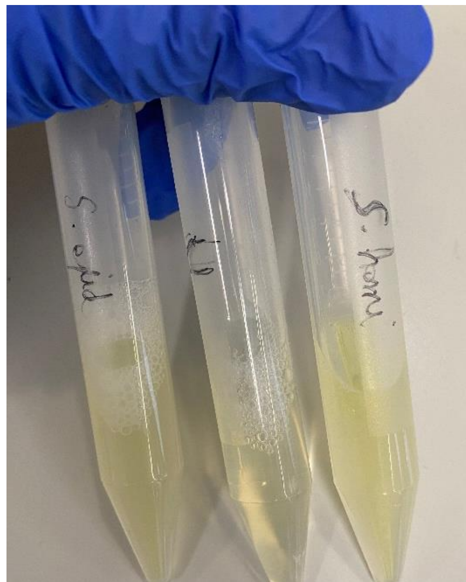

Figure 19 : Bacteria strains with medium

We put the mix {strain+culture medium} (Figure 32) in tubes according to the name of the bacteria and we incubate it for 24 hours.

Afterwards, we measure the absorbance at 600 nm thanks to the microplate reader of each medium with bacteria and we dilute them to have a concentration of 0.01 for 20 mL of culture medium.

V. Results and discussion

V.1. Zeta potential

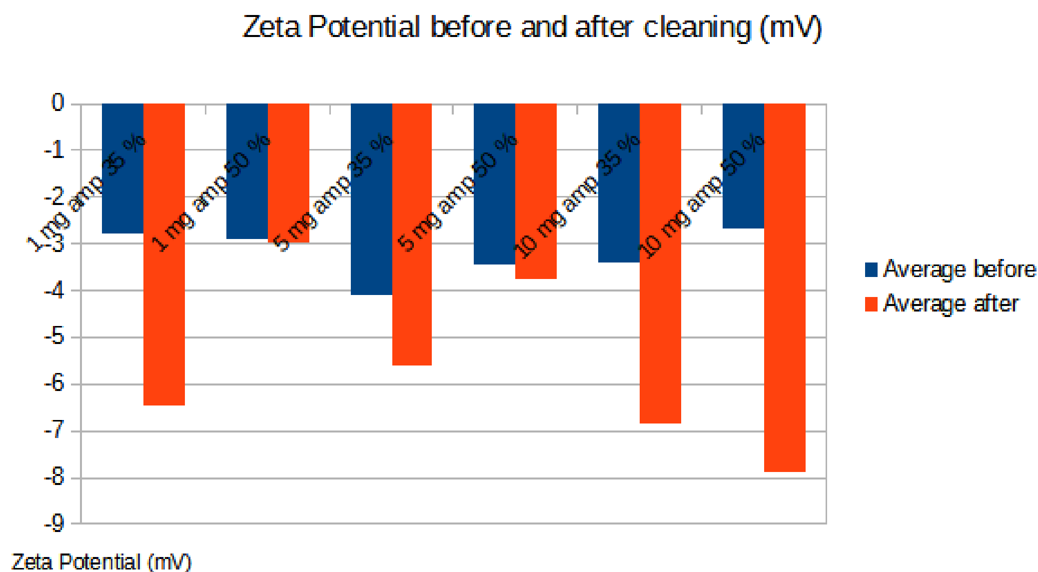

Figure 20 : Measures of zeta potential before and after cleaning the polymersomes at centrifuge

Before cleaning at centrifuge, the zeta potential of all the samples tends towards 0 and are globally homogeneous but after cleaning, there are instabilities of behavior of the colloid particles because of some discrepancies in the values. This can be explained by the addition of 2.5 mL of PBS to the nanoparticles after centrifuge and the insolubility of these nanoparticles with water. Therefore, the mix of the nanoparticles with PBS is heterogeneous, hence the instability in zeta potential.

V.2. Nanoparticles size

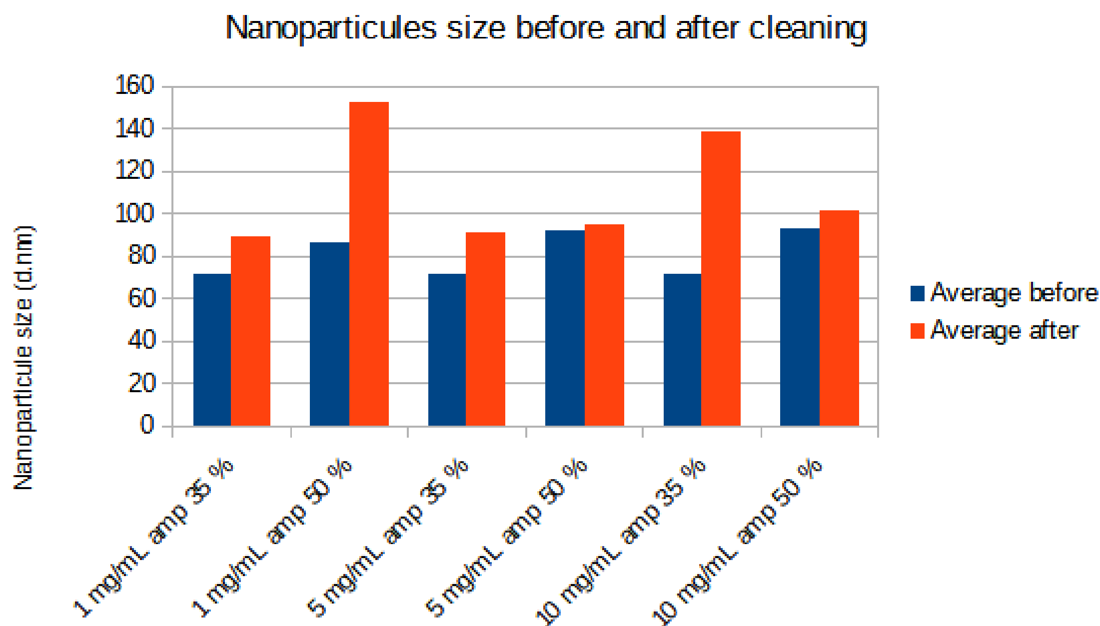

Figure 21 : Measures of the nanoparticles size before and after cleaning the polymersomes at centrifuge

Before cleaning at centrifuge, at any concentration and amplitude, Rutin NPs are globally small with an average of 79 nm +/- and stable (<100 nm)

Nevertheless, after cleaning at centrifuge, Rutin NPs are less stable and bigger than those before cleaning (85-156 nm). This can be explained by the fact that 2.5 mL of PBS were added to the Rutin nanoparticles after centrifuge and these NPs are hydrophobic, therefore not soluble with PBS. Hence the instability after the cleaning of the particles.

V.3. DPPH assay

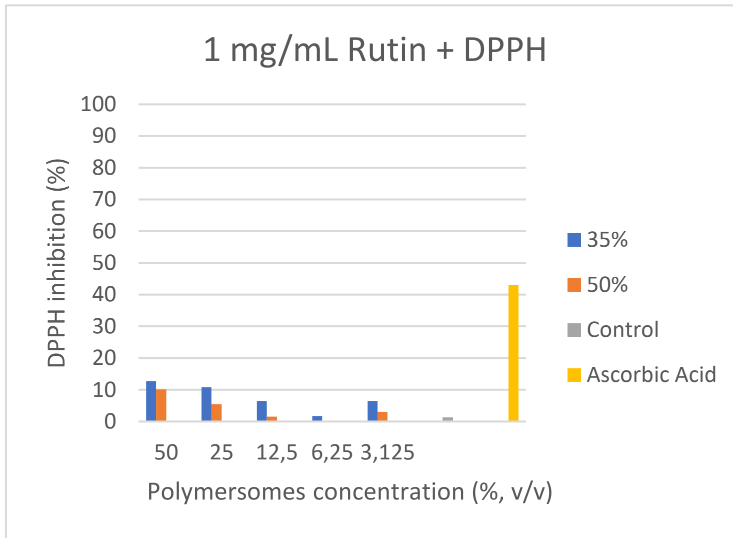

Figure 22 : DPPH inhibition depending on Rutin polymersomes concentration (1 mg/mL)

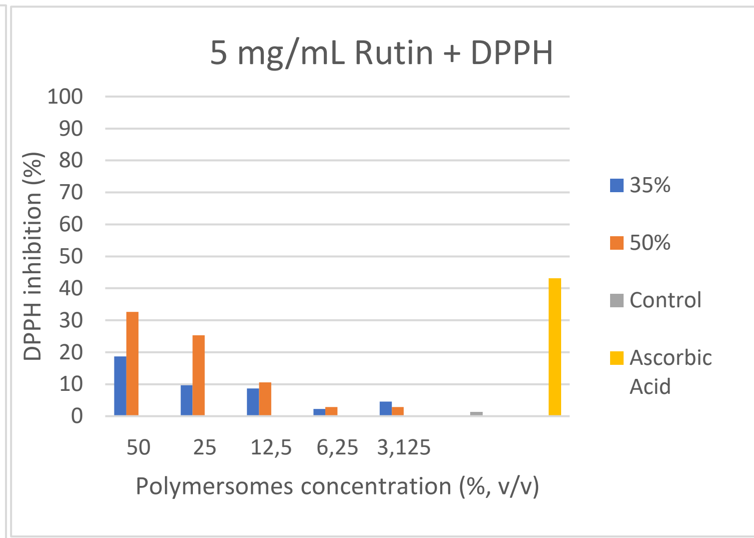

Figure 23 : DPPH inhibition depending on Rutin polymersomes concentration (5 mg/mL)

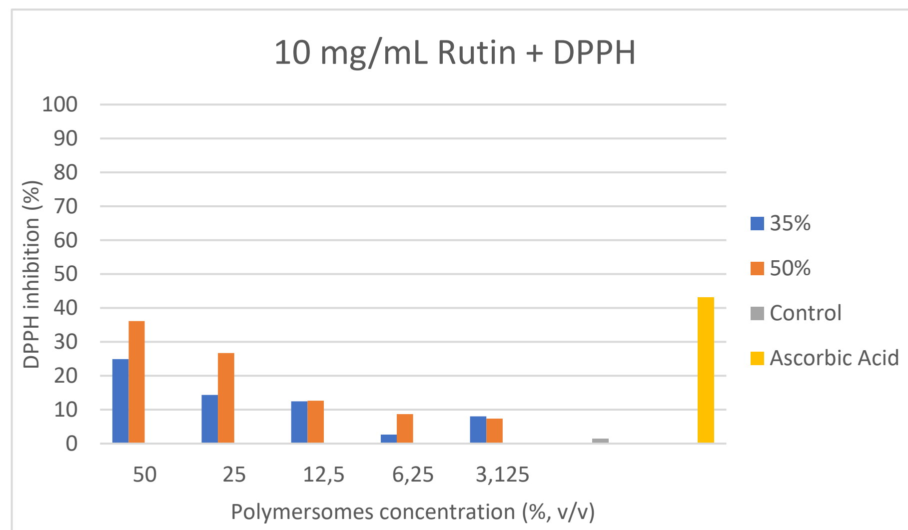

Figure 24 : DPPH inhibition depending on Rutin polymersomes concentration (10 mg/mL)

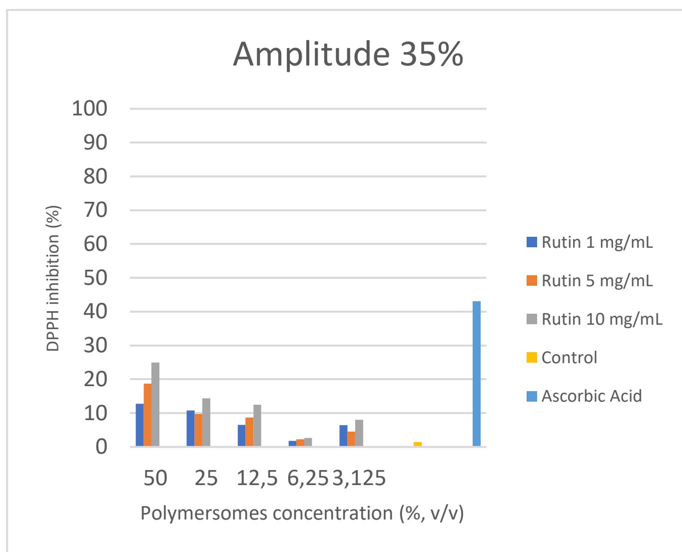

Figure 25 : DPPH inhibition depending on Rutin polymersomes concentration at amplitude 35%

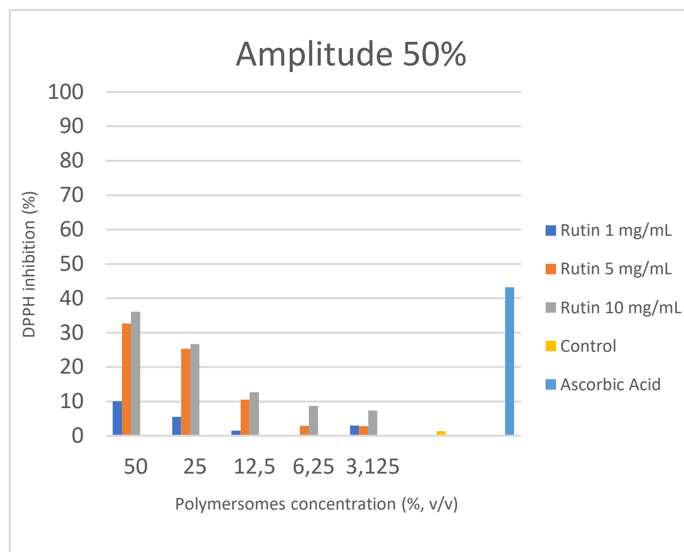

Figure 26 : DPPH inhibition depending on Rutin polymersomes concentration at amplitude 50%

Visually, it was noticed that the more the concentration of rutin polymersomes increased, the more a change in color towards the yellow was observed.

Rutin polymersomes presented some DPPH inhibitions, therefore some antioxidant properties comparing with the control (no rutin polymersomes) where a very low DPPH inhibition was observed. Although Rutin at 25% and 50% of polymersomes concentration presented the highest values of DPPH inhibition for 5 mg/mL and 10 mg/mL of Rutin, they were globally low (<40%), no matter the amplitude.

The results were better for the polymersomes prepared at amplitude 50%, showing better antioxidant activities.

Therefore, the antioxidant activity of Rutin was very low when assessed with DPPH, despite the fact that Rutin is well known as an antioxidant agent. Therefore, we decided to check the antioxidant potential of polymersomes with ABTS.

V.4. ABTS assay

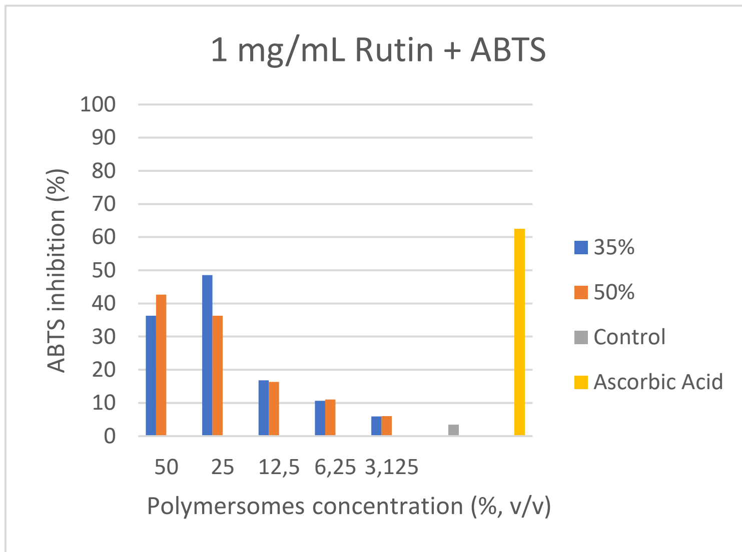

Figure 27: ABTS inhibition depending on Rutin polymerosomes concentration for 1 mg/mL

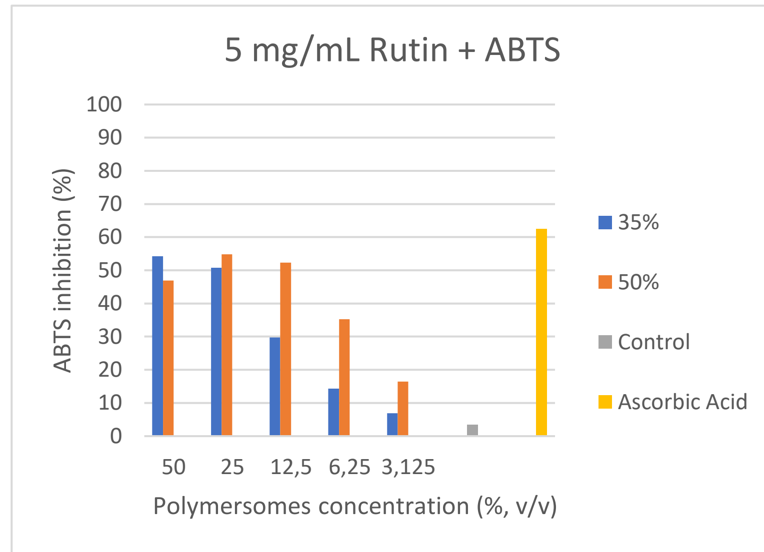

Figure 28: ABTS inhibition depending on Rutin polymerosomes concentration for 5 mg/mL

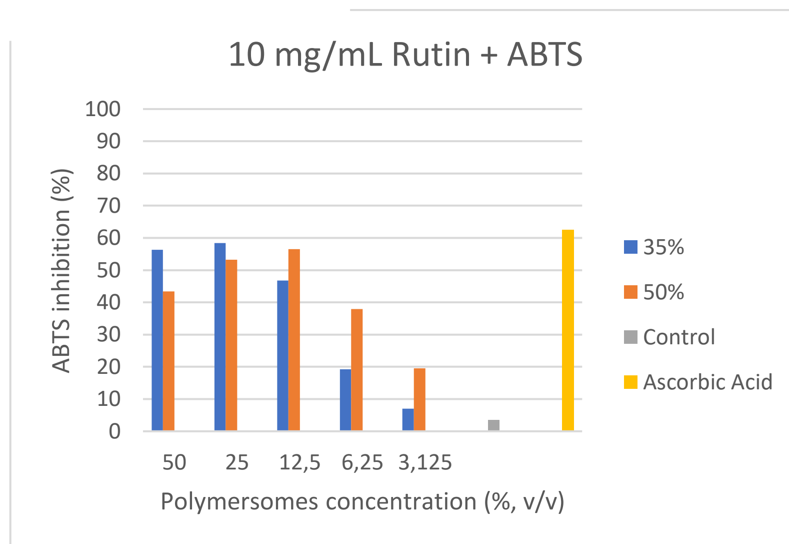

Figure 29 : ABTS inhibition depending on Rutin polymersomes concentration for 10 mg/mL

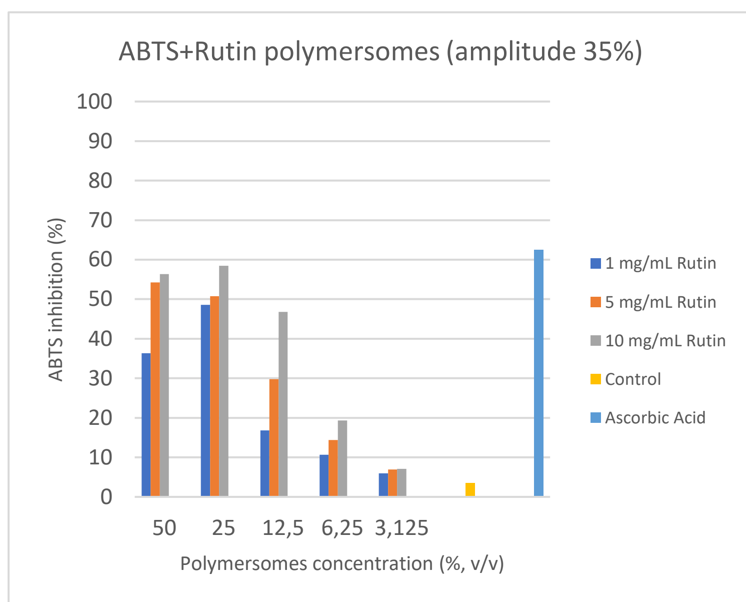

Figure 30 : ABTS inhibition depending on Rutin polymersomes at amplitude 35%

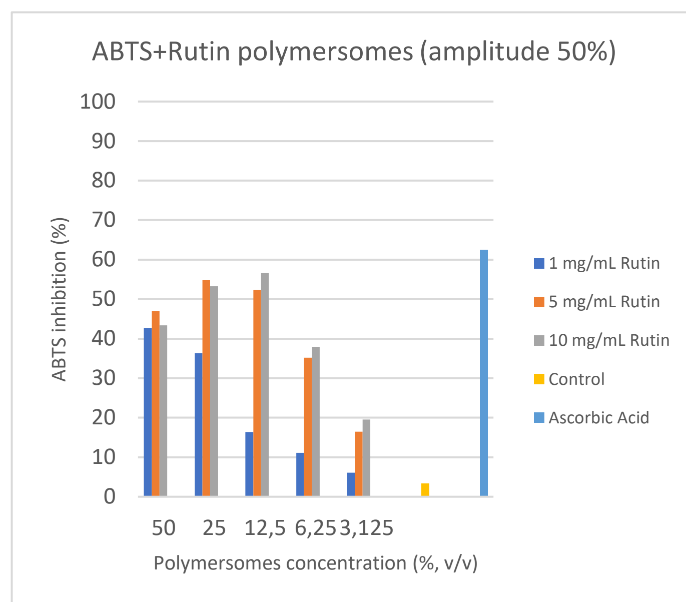

Figure 31 : ABTS inhibition depending on Rutin polymersomes at amplitude 50%

With the eyes, it was noticed that the more the concentration of rutin polymersomes increased, the more a change in color towards the yellow was observed.

It was observed better antioxidant activities than the DPPH assay because some values are above 50% ABTS inhibition.

Therefore, ABTS radical scavenging assay was a more efficient one and showed that Rutin polymersomes are very good antioxidants, especially at amplitude 50% for 5 mg/mL and 10 mg/mL of Rutin and at 25% polymersomes concentration. They can inhibit more easily the pollution (free radicals) from the skin and regenerate the skin cells. Then, the present antioxidant properties in the samples R550 and R1050 can reduce ROS harmful effects the most.

V.5. Cytotoxicity and Biocompatibility of rutin

V.5.1. AlamarBlue test with BJ cells

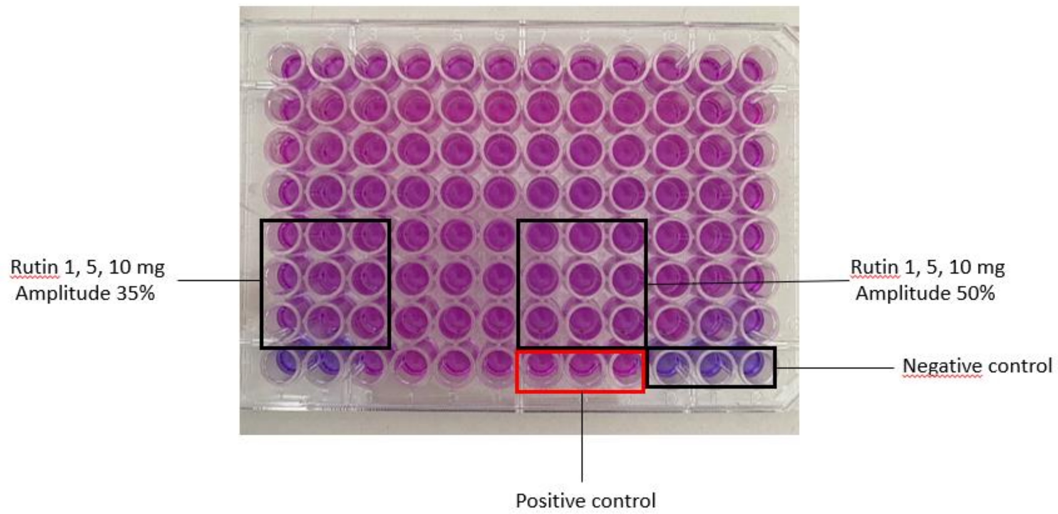

Figure 32 : BJ cells with different rutin solutions and AlamarBlue

Thanks to this AlamarBlue test, in presence of Rutin polymersomes at different concentrations (1, 5, 10 mg/mL) and different amplitudes (35, 50%), we see that all the BJ cells which are skin cells are pink.

The negative control with medium, AlamarBlue reagent, no cells and without rutin has a purple color and the positive control (same composition but with cells) has a pink color.

Therefore, our samples are not toxic for the skin no matter the concentration and the amplitude.

V.5.2 Alamar Blue test with HaCaT cells

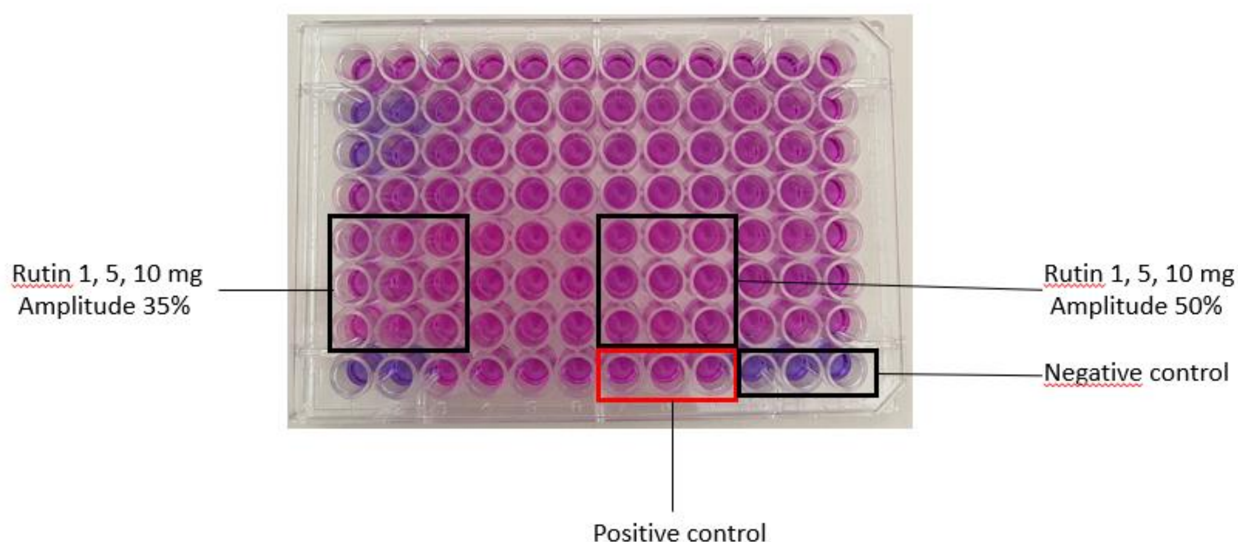

Figure 33 : HaCaT cells with different rutin solutions and AlamarBlue

Thanks to this second AlamarBlue test, in presence of Rutin polymersomes at different concentrations (1, 5, 10 mg/mL) and different amplitudes (35, 50%) , it was observed that all the HaCat cells (skin cells) are pink.

The negative control with media, AlamarBlue reagent and no cells has a purple color, and the positive control (same composition but with cells) has a pink color.

According to the previous results, both HacaT and BJ cells are still alive because of the pink color of these cells. Therefore, our samples containing rutin polymersomes are not cytotoxic for skin applications.

V.6. Antimicrobial activity of Rutin polymersomes

V.6.1. Bacteria and antibacterial effects

The antibacterial property of rutin is one of the most famous and studied among the antimicrobial activities. Rutin acts against Gram-positive and Gram-negative bacteria as well as aerobic and anaerobic bacteria with a more apparent sensitivity for Gram-positive bacteria (Severine, 2014). Among the bacteria sensitive to rutin, mention can be made in the form of table 1.

Bacterial strain	
Gram-positive	Gram-negative
- *Staphylococcus aureus* - *Staphylococcus hominis* - *Staphylococcus epidermidis*	- *Pseudomonas aeruginosa* - *Escherichia Coli*

Table 2: Examples of bacterial strains sensitive to rutin

Polyphenols, such as rutin, act on different bacterial species (E. coli, Bordetella bronchiseptica, Serratia marcescens, Klebsiella pneumonie, Salmonella choleraesis, Pseudomonas aeruginosa, Staphylococcus aureus and Bacillus subtilis) by generating hydrogen peroxide and by altering the permeability of the microbial membrane.

The test involved mixing solutions of bacteria with the 8 different samples of Rutin polymersomes. The bacteria tested were P. aeruginosa, E.coli and S.aureus, S.hominis and S.epidermis.

V.6.2. Results

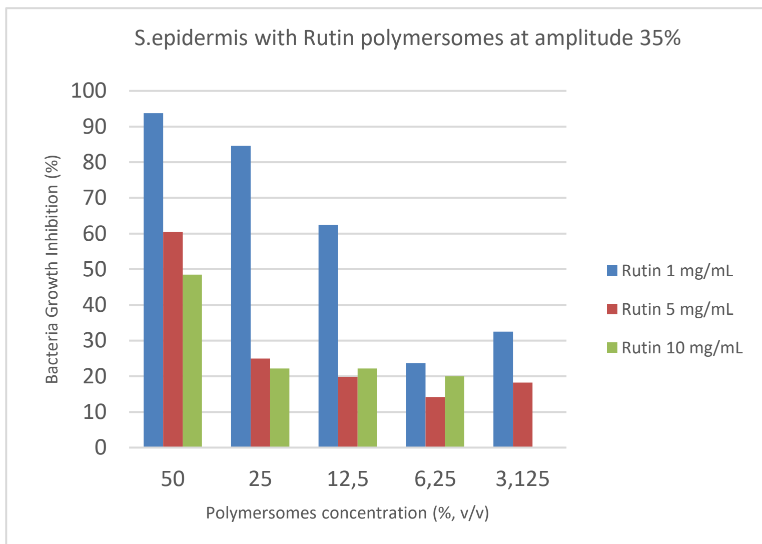

Figure 34: S.epidermis Growth Inhibition depending on Rutin polymersomes concentration at amplitude 35%

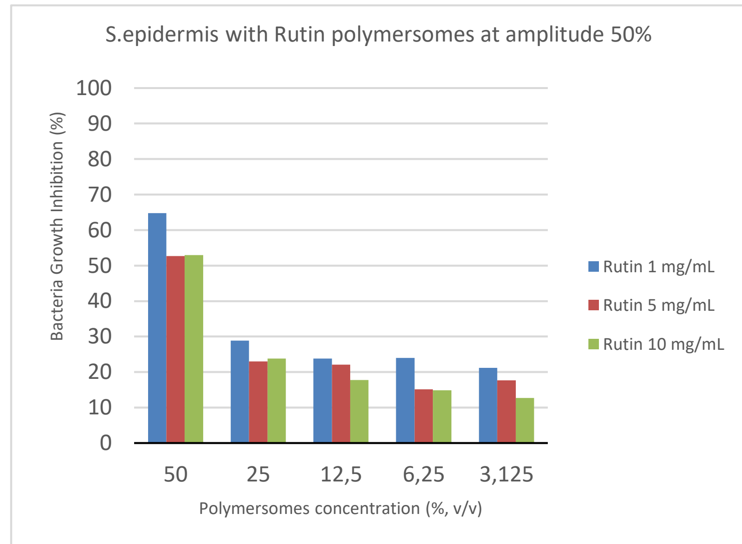

Figure 35: S.epidermis Growth Inhibition depending on Rutin polymersomes concentration at amplitude 50%

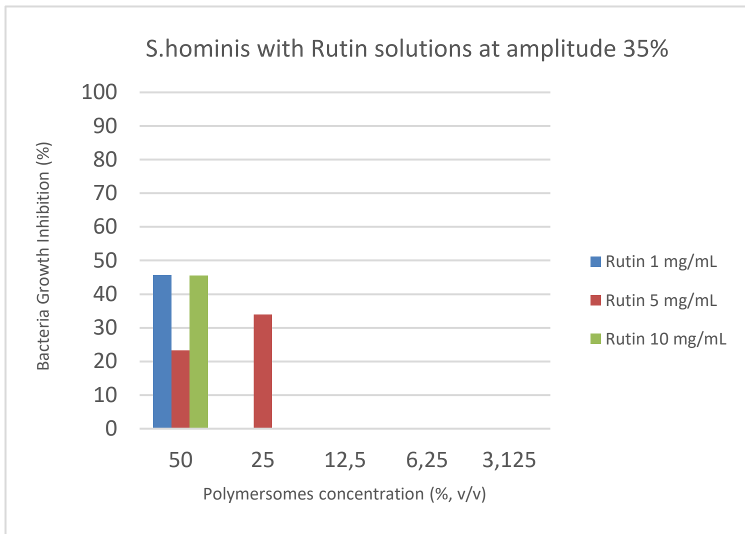

Figure 36: S.hominis Growth Inhibition depending on Rutin polymersomes concentration at amplitude 35%

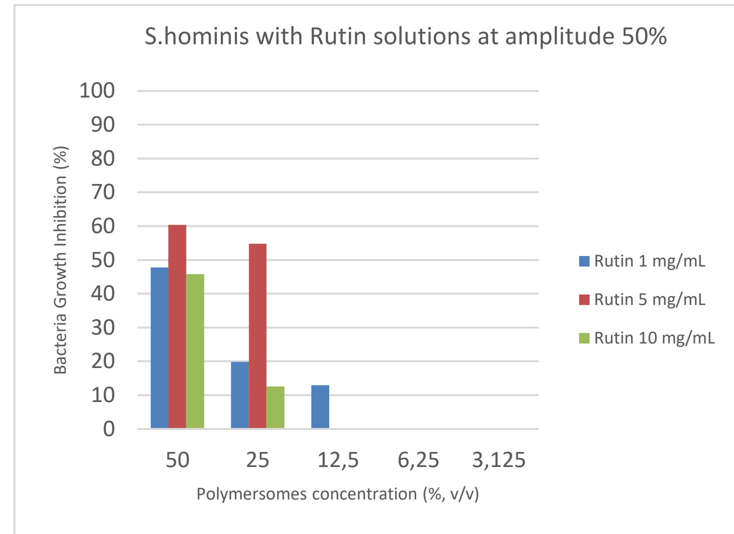

Figure 37: S.hominis Growth Inhibition depending on Rutin polymersomes concentration at amplitude 50%

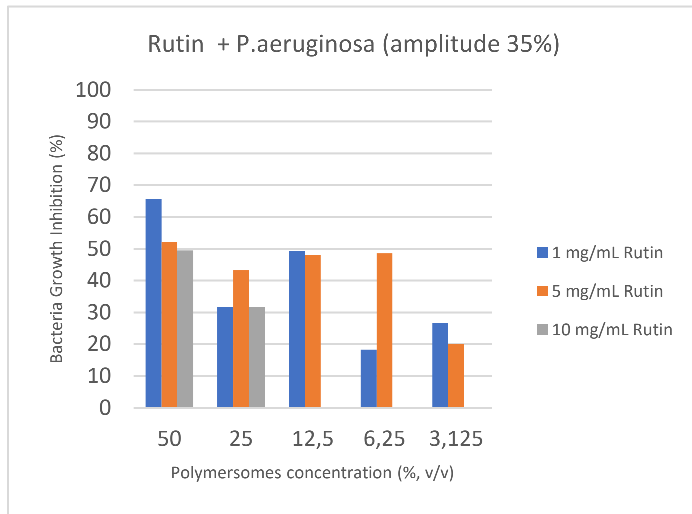

Figure 38 : P.aeruginosa Growth Inhibition depending on Rutin polymerosomes concentration at amplitude 35%

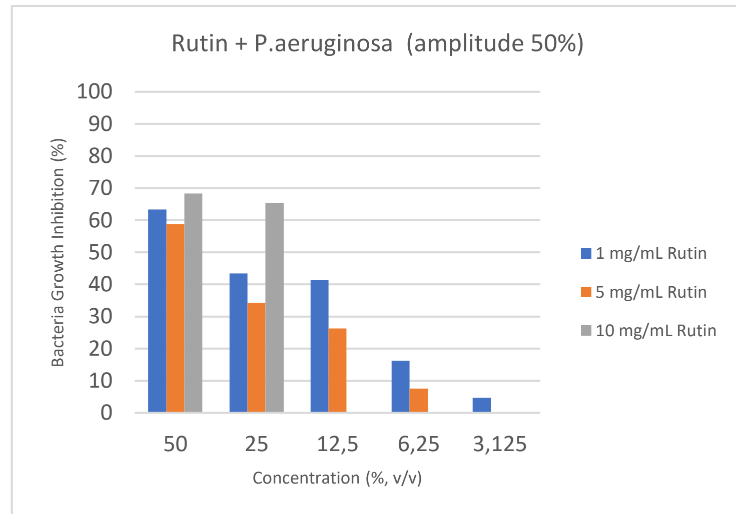

Figure 39 : P.aeruginosa Growth Inhibition depending on Rutin polymerosomes concentration at amplitude 50%

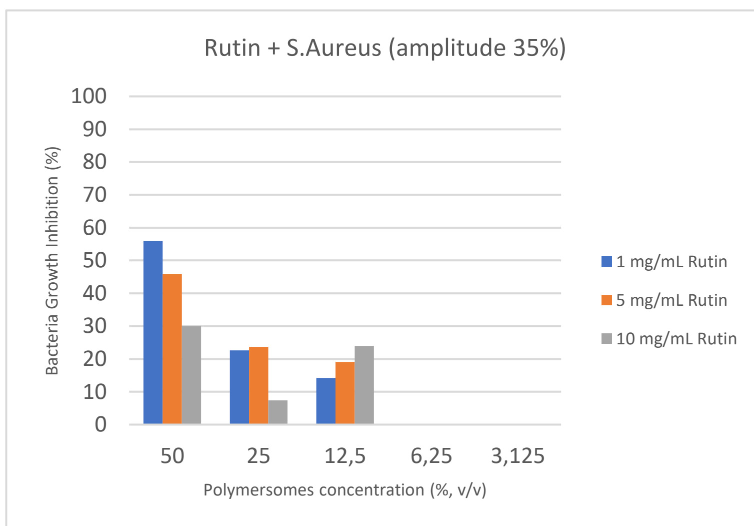

Figure 40: S.aureus Growth Inhibition depending on Rutin polymersomes concentration at amplitude 3

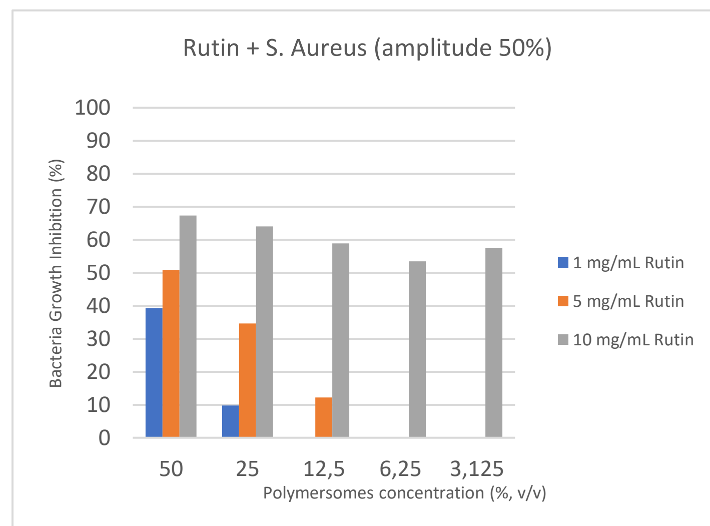

Figure 41: S.aureus Growth Inhibition depending on Rutin polymersomes concentration at amplitude 50%

According to the results, it was observed that Rutin polymersomes can inhibit bacteria growth but cannot kill them totally.

The objective here was that Rutin polymersomes can inhibit the bacteria responsible for skin diseases like *E.coli, S.aureus* and *P.aeruginosa* and protect as much as possible the bacteria from the skin microbiome (*S.hominis* and *S.epidermis*).

Rutin at 10 mg/mL, at 25% of polymersomes concentration and at amplitude 50% seems to be a good choice to incorporate into creams for skin cares because it inhibited the less possible the bacteria from skin microbiome ; *S.epidermis* Growth Inhibition : 22% and *S.hominis* Growth Inhibition : 12% (Figures 35 & 37) and can lower *S.Aureus* and *P.aeruginosa* development (resp. 63% and 67%).

Finally, it was noticed that rutin alone did not show much activity in itself.

Another hypothesis is that the antibacterial activities of flavonoids like rutin may be enhanced by combining or mixing them with another active agent.

VI. A new strategy

VI.1. Mixing different active agents

Antibacterial activities of rutin may be enhanced in the presence of colistin. The antibacterial activities of the combination colistin and rutin, were evaluated, based on the minimum inhibition concentration (MIC) in a liquid culture, by using *P. aeruginosa* and *S.aureus*, *E.coli* as the test bacteria.

VI.2. Protocole for the preparation of Rutin and colistin nanoparticles

A {Rutin (10 mg/mL) + Colistin (10 mg/mL)} solution at amplitude 50% during sonication has to prepared. To do so, the same protocole for the preparation of Rutin-loaded polymersomes with a concentration of 10 mg/mL has been established but 500 µL from a colistin solution, made with 10 mg of colistin powder and 1 mL of distilled water, have been added.

VI.3. Results and discussion

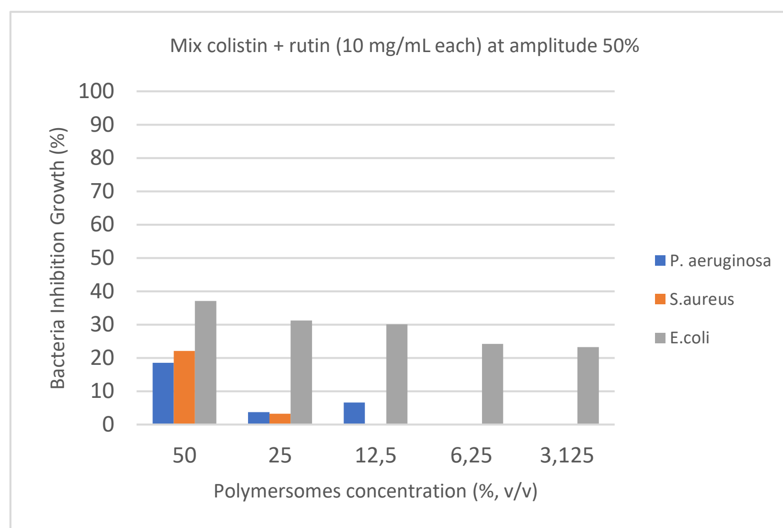

Figure 42: Bacteria Inhibition Growth depending on colistin and rutin polymersomes concentration

According to the results of this experiment, *E.coli* (gram-negative bacteria) was inhibited in all the dilutions for different concentrations of polymersomes : 50, 25, 12.5, 6.25, 3.125% . *P.aeruginous* (gram-negative bacteria) was also inhibited but at higher polymersomes concentration (50, 25, 12.5%) in very small quantities.

Likewise, rutin and colistin did not seem to have an effect on *S.aureus* growth.

In all the cases, the mix colistin and rutin faintly inhibited all the bacteria and presented some resistance to the active agents.

The resistance to colistin is linked to changes in the LPS composition of gram-negative bacteria. These modifications at all points because of the reduction in the negative charge of LPS, essentially via the addition of positively charged residues, thus leading to a reduction in the affinity of colistin (itself positively charged) for its target of action. [18]

This experiment was less relevant than Rutin ones.

VII. Nanosafety of Anti-Ageing Rutin-Loaded NCs Containing Cosmetic Formulations

The inclusion of nanomaterials such as rutin polymersomes in cosmetic products has raised some discussion about their toxicity due to small size, safe dosage administration, and material composition. The high surface/volume ratio of these materials makes them more reactive than their pristine forms.

The nano-size allows them to easily pass through cell membranes and potentially interact with molecules that are important for cell viability (e.g., DNA and proteins).

Therefore, it is critical to assess whether the developed anti-acne creams are safe toward skin cells. An in vitro viability test using keratinocytes (HaCaT cell line) was carried out to confirm the biocompatibility of the nano-enabled cosmetic products. Keratinocytes are the major cell type of the protective thin outer layer of skin epidermis, where the creams are intended to be applied. The results showed that the anti-acne formulation did not cause significant side effects on the human HaCaT cells, confirming that the NCs and the compounds employed for the preparation of the cosmetic base formulation are safe for use. After 24 h of exposure, the cells exhibited intact membranes, allowing the intracellular retention of the green dye calcein and showing a similar behavior as the control creams with the non-encapsulated active.

VIII. Conclusion

Rutin is considered and selected as the polyphenol with the most promising antimicrobial and antioxidant activity, along with lack of cytotoxicity, based on research. Nevertheless, practical limitations exist regarding the effectiveness of flavonoids like rutin as antimicrobial, possibly due to their poor solubility and miscibility in lipidic environments [19].

The previous experiments have concluded that Rutin polymersomes in each sample were not cytotoxic, therefore suitable on skin without having inflammation or redness after the fitting. Moreover, their small sizes enable them to penetrate the skin more easily and spread their antioxidant and antimicrobial properties more effectively.

Thanks to the results, it is showed that Rutin polymersomes are a great promise to control the skin aging thanks to their high capacities to absorb free radicals and skin infections even if they had lower antimicrobial effects than expected.

To increase the efficiency of rutin for skin application to cure diseases like redness, it is favorable to prepare rutin polymersomes with 10 mg/mL of rutin, at amplitude 50% during the sonication and diluted them twice to have a concentration of polymersomes of 25%, for faster effects. This sample (R1050) was the most efficient one and have showed one of the best ABTS inhibition and revealed strong antioxidant and antimicrobial activities by keeping as much as possible the bacteria from the skin microbiome and reducing the bacteria responsible for skin diseases.

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