Use of plant extracts to block bacterial biofilm formation

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SUMMARY

We live surrounded by bacteria; in fact, in only one gram of soil we can find millions of bacterial cells. Our body houses more than 10^{14} bacteria. Even though some of these microorganisms can cause us problems, such as caries, actually most of them help in the proper functioning of our organism. Generally, bacteria coexist setting up communities associated to solid superficies, this is to which we refer as biofilms, that serve as a survival strategy. This type of formation cause serious sanitary problems for both humans and animals. Nowadays, chemical or natural compounds able to block this formation are looked for. In this project, we have set out how to use extracts of different plants with the purpose of testing their effects against biofilms of two bacterial species: *Escherichia coli* and *Pseudomonas putida*.

INTRODUCTION

Bacteria are single-cell prokaryotic organisms, that is to say, they can only be observed with a microscope and they are constituted by only one autonomous cell that has no nuclear membrane. They are widely spread all around Nature; we find them in all environments with life possibility: air, water, land, inside the human body, animals and plants. Even some bacteria have been found in meteorites. Their seniority on earth is very noticeable, since the most archaic fossils known are bacteria. Depending of environmental circumstances, bacteria can suffer modifications that are generally not definitive, because when the causes that motivated them disappear, bacteria return to their normal unicellular forms and properties.

In nature, generally we find bacteria living in communities associated to solid surfaces, called biofilms. This fact was already reported by Arthur Henrici in 1933, when he observed that most aquatic microorganisms were not in the form of individual cells swimming freely but aggregated over solid submerged surfaces [1]. However, in the last decade the importance of biofilm formation as a microbial survival strategy has been recognized in a generalized manner, as well as its huge impact on many human activities [2,3]. The ability of microorganisms to colonize solid surfaces in the biofilm form is a serious problem in human and animal health as these populations are more resistant to antibiotic action [4]. This fact allows pathogenic bacteria to survive and wait for the occasion to invade a new host, or to multiply when the immune system decreases its activity. Thereby, the importance of biofilms in infections arising from prostheses and medical implants, keratitis associated to contact lens wearing, or colonization of lung tissue in patients with cystic fibrosis has been highlighted [5]. Biofilms can also generate deterioration of materials and cause health risks in the food industry. Nevertheless, not all is negative; the ability of microorganisms to colonize solid surfaces has been taken advantage of in industrial process, such as the case of wastewater treatment.

All these factors, especially its clinical importance, explain the great interest in studying biofilms and the search for new naturally or chemically synthesized compounds capable of blocking its formation. One promising approach is to identify compounds of plant origin that can have antimicrobial or antibiofilm effects. Throughout human history, many plants have been used because of their healing effects. One example is *Aloe vera*. In historical documents from the ancient Romans, Greeks, Indians and Arabs it is possible to read about its medicinal use to treat several diseases, such as skin or hair diseases, or even about its use in the embalming process. Aloe vera has proven to be quite useful in cases of acne and bronchitis. Chamomile (*Chamaemelum nobile*) flowers, are another paradigm of traditional remedy, in the form of infusion, to treat eye irritation and infection. Another well-known example is garlic (*Allium sativum* L.). It has been used in almost every culture. In ancient history, this plant was known for its beneficial effects on controlling heart diseases, bites, intestinal parasites and tumors, and also for being a great meat and fish preservative. Some of its presumed or known properties are: it improves blood circulation, it is a bactericide because of its high sulphur content, it protects against cancer and is a great skin sanitizer in case of bites, fungi or burns. Recently, it has been reported that a compound present in garlic, ajoene, can interfere with signaling beween bacterial cells [6], and by this mechanism it could possibly block infection and alter biofilm formation.

In this project we have focused on the effects of several plant extracts on biofilm formation, taking advantage of the botanical diversity at the Estación Experimental del Zaidín or using commercial dry plant material. We have identified plants that reduce biofilm formation by two different gram-negative bacteria.

MATERIALS AND METHODS

Bacteria and culture medium used

To check the extracts' inhibition capacity upon biofilms we tested them with two different bacteria:

- *Escherichia coli* MG1655. It is an inocuous laboratory strain, but some strains of this species are of medical interest because they cause several intestinal illnesses [8].
- *Pseudomonas putida* KT2440. It is a plant-beneficial bacterium that can colonise different environments [9]. Certain *Pseudomonas* species, such as *P. aeruginosa*, are opportunistic human pathogens.

Cultures were grown were grown at 30°C (*P. putida*) or at 37°C (*E. coli*) in LB medium [10].

Plant extracts

Plants used in this work are summarized in Table 1. Certain plant materials are not identified in the table for results protection reasons. We collected most plant material (leaves, fruits, petals...) from the gardens at the Estación Experimental del Zaidín. Besides, we included other plants according to their medical attributes, based on information found on the internet. These were obtained from commercial preparations as dry material, or from potted plants. Each extract was assigned a number.

To obtain the extracts, we put the collected material (between 0.5 and 5 g) in sterile tubes with 10 ml of an etanol:water solution (1:1). We added 10 glass beads (3 mm diameter), to help in the grinding. The tubes were agitated in a vortex at maximum velocity for two minutes and we took the extract solution out with a syringe. We used Whatman filters (0.45 µm) to remove particles and ensure the extracts were sterile.

Table 1. Extracts used in this work

Extract number	Common name	Scientific name	Material	Known attributes or traditional medicine uses [7]
1	Cypress	*Cupressus sempervirens*	Leaf	antiseptic
2	Undisclosed			
3	Lavender	*Lavandula stoechas*	Leaf	antiseptic
4	Rose	*Rosa sp.*	Petals	treatment of bronchial infections
5	Cherry laurel	*Prunus laurocerasus*	Leaf	antispasmodic
6	Sago palm	*Cycas revoluta*	Leaf	-
7	Rose	*Rosa sp.*	Leaf	-
8	Wall cotoneaster	*Cotoneaster horizontalis*	Leaf (Fruit)?	-
9	Olive	*Olea europaea*	Fruit	-
10	Undisclosed			
11	Olive	*Olea europaea*	Leaf	reduction of fever
12	Aloe vera	*Aloe vera*	Leaf	treatment of skin diseases
13	Chamomile	*Chamaemelum nobile*	Dry flowers	treatment of eye inflammation
14	Ginkgo	*Ginkgo biloba*	Dry leaves	circulatory system
15	Lime flower	*Tilia sp.*	Dry leaves	sedative

Biofilm assays

Biofilm formation was studied in plastic 24-well plates or in glass test tubes. In 24-well plates, 1 mL of liquid LB medium was inoculated with 5 µl of an overnight culture of the chosen bacterium and we added increasing volumes of extract. As a control, the same volumes of ethanol:water (1:1) were used. Plates were incubated at 30°C or 37°C for 4, 7 or 24 h and then the liquid was removed and the biofilms stained. In test tubes, we mixed 2 mL of liquid medium (LB), 20 µL of extract and 40 µl of an overnight culture of the chosen bacterium. As a control, 20 µL of ethanol:water (1:1) was used instead of the extract. The tubes were incubated with orbital agitation at 30°C. After 2h, the cultures were taken and photographed on a black background. We then removed the liquid of the tube and stained the biofilms as described in the next section before photographing on a white background. This process was repeated at 3 and 5 hours.

Staining techniques

To visualize and quantify biofilms we used a 0.4% crystal violet solution, a dye that colours bacteria's polysaccharides. 1.5 mL or 5 mL of crystal violet were added to each well or tube, respectively. We let the solution rest for 10 minutes and then the dye was removed. The tubes and plates were washed three times with water and allowed to dry. The intensity of the violet colour that remains on the tube/plate is indicative of the amount of bacterial biomass attached to the surface. To obtain quantitative data, the dye was solubilized with 70% ethanol and colour intensity was measured in a spectrophotometer as absorbance at a wavelength of 540 nm (A_{540}).

Microscopy

Biofilm structure was studied using phase contrast microscopy. For *E. coli*, the crystal violet stained plates were directly observed with an inverted microscope (Euromex). In the case of *P. putida*, cultures were grown in LB in 6-well plates with extract or control (ethanol:water) with a 40 mm glass coverslip placed in the well. We let biofilms form on the coverslip for 6 hours and then we observed them with a Zeiss microscope.

Swimming motility

Bacterial motility was tested in Petri plates with semi-solid medium (LB + agar 0.3%), containing plant extract or ethanol:water as control. 2 µl of a grown culture were inoculated in the center of the plate and then plates were incubated at 30°C for 16 h. Swimming halos were visualized, and their diameter was measured.

RESULTS AND DISCUSSION

Biofilms on glass

First we tested the effect of adding extracts on *P. putida* biofilm formation in glass tubes (40 µl of extract in 2 ml). Bacterial cultures were observed at different times and then stained with crystal violet as described in Materials and Methods. *P. putida* normally forms a ring on the tube surface, in the area where there is more oxygen. Results obtained with some extracts are shown in Figure 1. At two hours, the biofilm with extract 2 is a bit more diffuse than in the control. With extracts 6 and 10 results are similar to the control. Extracts from *Olea europaea* and *Aloe vera* at this time produce a diffuse biofilm that covers all the surface in contact with the culture. At three hours, without staining there is less biomass visible on the surface with extracts 2, 9 and 10 than in the control. After staining, with extract 2 some of the biofilm has shed. With extract 9 we observed spread of biofilm and less biomass. There is less staining with extract 10 than in the control. No clear differences were seen with extracts 6 and 12.
Finally, at five hours, clear reduction in the attached biomass is only observed with extract 10. Surprisingly, at this time *O. europaea* extract seems to cause increased adhesion.

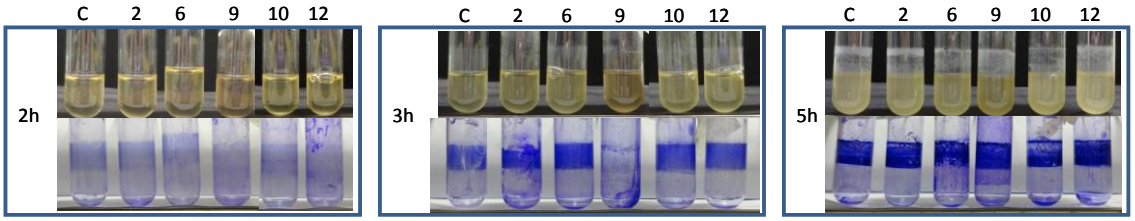

Figure 1. Biofilm formation by *Pseudomonas putida* in glass tubes in the presence of plant extracts or ethanol:water as control (C).

Next we tested the effect of increasing concentrations of plant extracts on biofilm formation by *E. coli* in glass tubes. 40, 60 or 80 µl of extract were added to 2 ml of medium and cultures were incubated for 16 h. Biofilms formed were stained with crystal violet. Results with two extracts are presented in Figure 2. Both of them blocked completely biofilm formation when 80 µl were added.

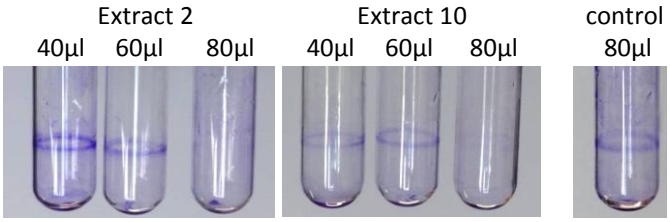

Figure 2. Increasing concentrations of extracts 2 and 10 block biofilm formation by *E. coli*.

Biofilms on plastic: quantification

We tested biofilm formation by *P. putida* in 24-wells plates to measure at the same time the effect of several extracts and concentrations. To do this we stained biofilms formed after 7 hours with crystal violet, and then solubilized the dye with ethanol 70%. We then measured absorbance at 540 nm in a spectrophotometer to quantify the color intensity. The amount of dye is proportional to the biomass attached to surface. Results are shown in Figure 3.

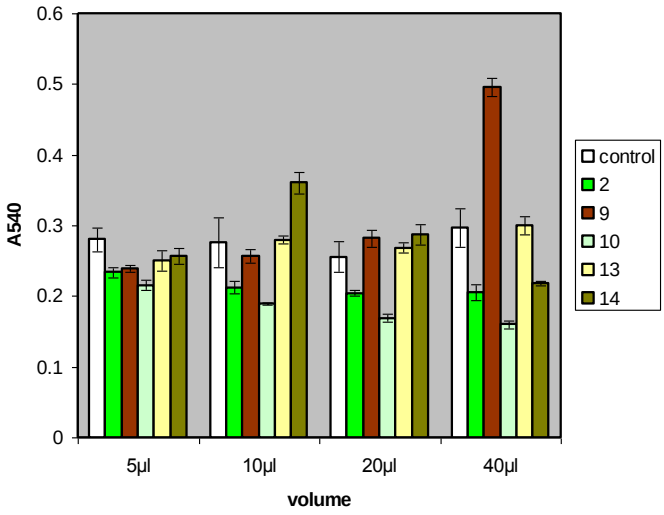

Figure 3A. Biomass of *P. putida* attached to plastic surface after 7 hours of growth in the presence of increasing amounts of extracts, or of ethanol:water as control.

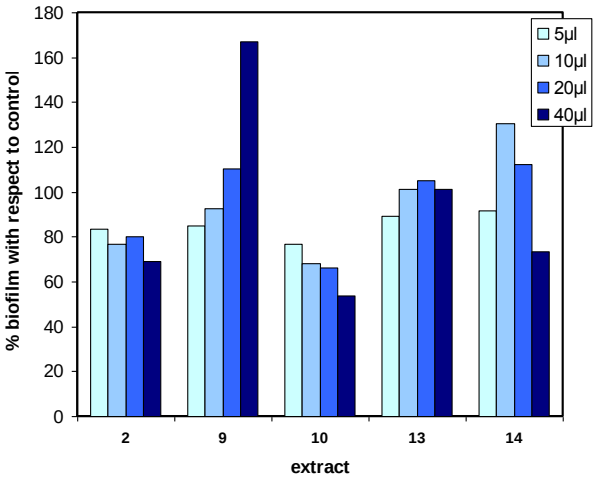

Figure 3B. Results from figure 3A are represented as relative biofilm formed with each extract and concentration, compared to the same volume of ethanol:water (value of 100%).

Extract 2: We observe how the amount of biofilm decreases with increasing volume of extract in relative absorbance values. It is without a doubt the 2nd more effective in these tests.
Olive (fruit): Although low doses tend to slow the progression of biofilm (10 µl, 20 µl), with increasing volume of extract its formation is stimulated.
Extract 10: It is the most efficient extraction of those analyzed. As increasing extract is supplied, the biofilm diminishes, reaching 55 % reduction (with 40 µl of extract).
Camomile: It is the extract that has less effect on the biofilm. It does not reduce or stimulate it.
Ginkgo biloba: At low doses (5 µl) it has no effect to the biofilm. When the quantity increases (a number between 10 µL and 15 µl), it tends to stimulate the formation to a certain point, from which it has a inhibitory power.

With this information we put attention to the two extracts that appear to give better results: 2 and 10. It was with them with which we did the following test with *E. coli*. We measured the percentage of biomass relative to the control in two time periods, at 4 h and 7 h. The results were very similar to the previous test (Figure 4): Extract 10 gives the best results in both periods (55% vs. 71% at 4h and 56% vs. 74%. at 7h). Through the use of this extract we could reduce the biofilm by almost half at four hours and seven hours.

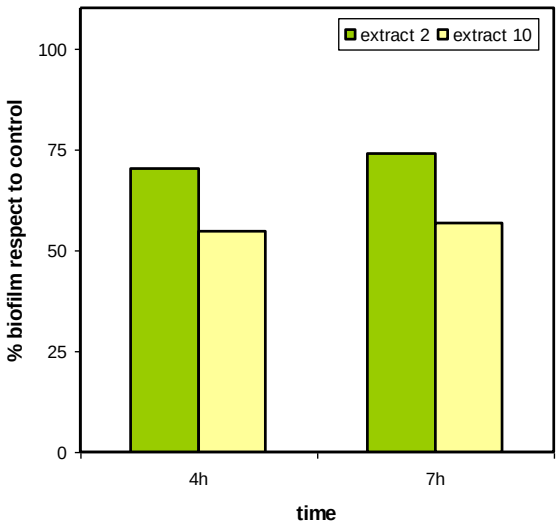

Figure 4. Biomass of E. coli attached to plastic at different times of growth in the presence of extracts. Results are represented as relative biofilm formed with each extract, compared to the ethanol:water control at that time (value of 100%).

Observation of biofilms under the microscope

We used the microscope to observe the structure of the biofilms formed by *E. coli* and *P. putida* (Figure 5). For *E. coli* we observed biofilms on plastic that were stained with crystal violet, and for *P. putida* we used contrast phase microscopy to observe biofilms on glass coverslips.

In *E. coli*, in the case of the control most areas are covered with biofilm, with areas where bacteria are very compacted. With extract 2 the results are good (the colored areas are the minority) but with extract 10 they are excellent (much less biofilm), although some areas show also compacted aggregates of cells. With other extracts such as that of olive fruit there is hardly difference with the control (result not shown).

In *P. putida* the results are similar, but extract 10 gave irregular results with most areas with very few bacteria and some others showing compact aggregates (not shown).

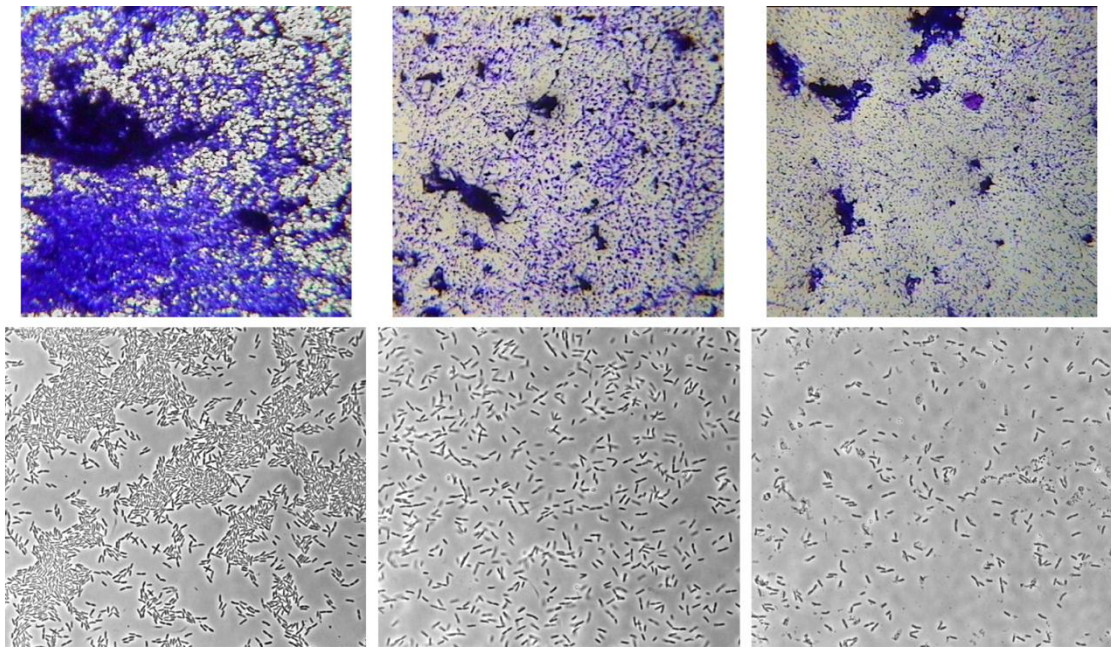

Figure 5. Microscopy analysis of biofilms of *E. coli* (top) and *P. putida* (bottom). Left: control without extract. Center: extract 2. Right: extract 10.

Swimming motility

Biofilm formation starts with bacteria swimming towards a surface. We tested if plant extracts altered motility of *E. coli* in plates with a semi-solid medium (LB + agar 0.3%), where bacteria can swim, forming a halo from the point of inoculation. Results are presented in Figure 6. With extract 10 the size of the halo is smaller, indicating less motility. Olive and ginkgo stimulate the expansion of the bacteria, whereas extract 2 has no apparent effect.

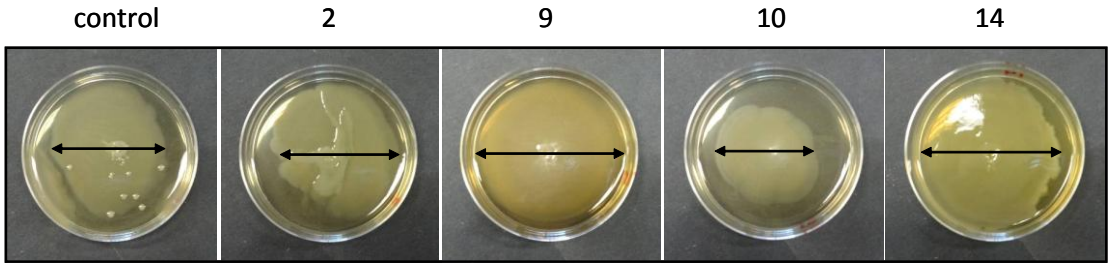

Figure 6. Swimming motility of *E. coli* in the presence of different extracts.

CONCLUSIONS

We have identified two plant extracts that have inhibitory effect on bacterial biofilms on different surfaces. Future experiments are required to study these extracts in detail and their potential as antimicrobials.

ACKNOWLEDGEMENTS

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MY OWN IDEAS

Diego Velasco IES Alhambra

This project has been a great experience because I could learn a lot about microbiology and how scientists work with different types of bacteria in the laboratory.

In our project we worked with two types of bacteria and thirteen types of plants. I've learned about the scientific method and I'm very satisfied with our work and results, it's true that only two extracts have a real and important effect in the inhibition of biofilm but my research team have done a good work. Also we learned to use some of the laboratory equipment such as pipettes and microscopes.

In conclusion, I think PIIISA is a great idea for made young children get closer to the scientific comunity. This project can help us to improve our knowledge of the scientific methods, materials and experiments.

José Alberto Lizana IES Alhambra

My experience in PIIISA 2014 has been very positive. This project opened the doors to enter a well equipped laboratory where there are many researchers working, and we have also worked in this laboratory. We have had the opportunity to work as if we were the researchers of truth, being in first person with laboratory and working with him. PIIISA opened me the mind to my future studies and also to mark me a goal in my career. I hope this project to proceed, and I recommend everyone try to access PIIISA, because I assure you that you will love.

Ana Marchal IES Francisco Ayala

The PIIISA project has seemed to me a very interesting, useful thing which shouldn't stop being organized. In my opinion, it means an opportunity to put into practice the theory taught at the high school, to strengthen that knowledge, furthermore it also shows us how one part of the day-to-day laboratory work is, which helps us to know more about this job.

I've learnt that patience, for example, is something required and indispensable in this job, as the team working. Also the constancy is very important because there will be days when you will get excellent results nevertheless, it might also be days when results won't be as good as the others. Despite of that, you have to keep on trying and trying.

On my project in particular, I believe that the idea of using plants against the micro organism action is something very useful and beneficial. I also found curious the fact of plants have been used against diseases for a thousand years.

Úrsula Serrano CDP Compañía de María

I am really grateful for being given the opportunity of participating in the PIIISA 2014 project. It has been an unique opportunity to be able to participate in activities related to the microbiology field, with professional researchers, suitable equipment and real research projects. Researchers have helped us with loads of patience and dedication, showing us some lab working techniques and how interesting science can be.