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## Ph.D. Thesis

# Herbal-extracts and nanoparticles technologies to prevent or address bacterial biofilm formation in dentistry


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# INDEX.

**PREFACE** pag.3

**CHAPTER 1. Introduction** pag. 7

References pag. 31

**CHAPTER 2. Exploring African medicinal *Pelargonium sidoides* root extract for potential anti-bacterial evaluations.** pag. 37

Introduction pag. 38

Materials and Methods pag. 41

Results pag. 48

Discussion pag. 55

References pag. 58

**CHAPTER 3. Polyphenols extract from *Padina pavonica* algae for surface functionalization of bioactive glasses: physico-chemical characterization, biological response and in situ reduction of metal ions.** pag. 61

Introduction pag. 62

Materials and Methods pag. 65

Results pag. 69

Discussion pag. 77

References pag. 80

**CHAPTER 4.** **The significance of Redox potential measurments for pathogen activity versus algal extractions** pag. 84

Introduction pag. 85

Materials and Methods pag. 93

Results pag. 99

Discussion pag. 111

References pag. 114

## PREFACE.

- **Background:**

Herbal medicine and green chemistry is a very attractive branch of modern medicine. In fact, it represents not only a promising vehicle to face the "the utilization and generation of dangerous substances and antibiotics" requests, but it is also a very sophisticate tool for the in vitro studies of complex biological systems such as the design of new natural products to demonstrate and being good for human health and the environment. Herbal medicine - also called botanical medicine, phytomedicine or herbalism indicates using a plant's derivatives such as leaves, bark, seeds, berries, roots, or flowers for medical therapeutic issues.

Antibiotic failure due to increasing antibiotic resistance is a worldwide threat to public health. According to the Center for Disease Control (CDC). The negative impacts of antibiotic resistance on healthcare systems as a whole are substantial, as resistance adds to the number of infections that occur.

A great step forward in Herbal medicine advancement is represented by the improvement of discovering new biomaterial order to provide a natural antimicribial substances. From this point of view, herbal extracts are nowadays considered as a very interesting materials thanks to their biocompatibility and controllable good features for human health.

- **Aim and presentation of the Thesis.**

In this PhD Thesis, it will be reported the three years activities of the candidate. The main purposes has been the development of a novel herbal extracts and the discovery of new infection treatment strategies in the race with increasing bacterial virulence and resistance is among the most important goals of contemporary science and healthcare. In addition to this, the strategy is expected not to destroy the part of human microbiota that positively contributes to skin, oral or intestinal physiology as well as the immune system. The constantly increasing average age of the society of industrialized countries is turning the lack of efficient solutions in this field into a major medical problem. This challenge forces scientists and pharmacists to re-evaluate the significance of complex plant-derived antibacterial substances with mild yet multi-targeted activity.

Therefore, the Thesis has been divided into 4 different chapter in order to better detail the succeeding steps that are all related to each others. In turn, each of the 4 chapters is characterized by a specific introduction, materials and methods and results and discussion sections, with the purpose to minutely explain the biological hypothesis encountered and the strategies used to face

them. As support of the selected techniques, a chapter specific references section was spent to each one.

- **In Chapter 1**, it will be described general introduction to discuss the history of traditional herbal medicines, the traditional healing by using herbal medicines with different herbs and Regulatory Situation of Herbal Medicines. Then in details the twelve principles of green chemistry were discussed with its framework main subjects to reduce the hazardds of chemical products by designing the essential category. After this background, the purpose was cleared to select the best herbs byproduct of polyphenols as a secondary metabolites and considering as one of the most outstanding contributions to the treatment of life-threatening infectious diseases, also discribed the natural resources, the bioactivity, antioxidant activity, anticancer, antimicrobial, anti-inflammatory, cardioprotection and some other bioactivity of polyphenols with considering the dark side of potential toxicity of polyphenols.
- Bacterial resistance to antibiotics and the disruption of beneficial microbiota are key problems in contemporary medicine and make the search for new, more efficient infection treatment strategies among the most important tasks in medicine. Multicomponent plant-derived preparations with mild antibacterial activity created by many simultaneous mechanisms together with anti-inflammatory, innate immune and regenerative capacity-stimulating properties are good candidates for this therapy, and proanthocyanidins are among the most promising compounds of this sort. **In Chapter 2**, proanthocyanidins have isolated from Pelargonium sidoides DC root extract and characterized and compared the composition, antioxidant properties and antibacterial activity of the proanthocyanidin fraction with those of the whole extract. The results revealed that proanthocyanidins had significantly stronger antioxidant capacity compared to the root extract and exhibited a unique antibacterial action profile that selectively targets Gram-negative keystone periodontal and peri-implant pathogenic strains, such as *Porphyromonas gingivalis*, while preserving the viability of beneficial oral commensal *Streptococcus salivarius*. The finding suggests that proanthocyanidins from Pelargonium sidoides root extract are good candidates for the prolonged and harmless treatment of infectious diseases.

  Experimental procedures of this part were performed in the Biomedical Materials Laboratory of the Health Sciences Department in Novara for the biological part, this chapter of thesis is related to the work done by the cooperation with Lithuanian University of health sciences within the funded grant project Pelargodont.

- **In Chapter 3**, It included the work on bioactive glasses (BGs) as an attractive materials for bone replacement due to their tailorable chemical composition that is able to promote bone healing and repair. Accordingly, many attempts have been introduced to further improve BGs' biological behavior and to protect them from bacterial infection, which is nowadays the primary reason for implant failure. Polyphenols from natural products have been proposed as a novel source of antibacterial agents, where as silver is awell-known antibacterial agent largely employed due to its broad-ranged activity. Based on these premises, the surface of a bioactive glass (CEL2) was functionalized with polyphenols extracted from the Egyptian algae *Padina pavonica* and enriched with silver nanoparticles (AgNPs) using an in situ reduction technique only using algae extract. The composite's morphological and physical-chemical characteristics were analyzed using FE-SEM, EDS, XPS and Folin–Ciocalteau; all analyses confirmed that both algae polyphenols and AgNPs were successfully loaded together onto the CEL2 surface. Antibacterial analysis revealed that the presence of polyphenols and AgNPs significantly reduced the metabolic activity (>50%) of *Staphylococcus aureus* biofilm in comparison with bare CEL2 controls. Finally, we verified the composite's cytocompatibility with human osteoblasts progenitors that were selected as representative cells for bone healing advancement.

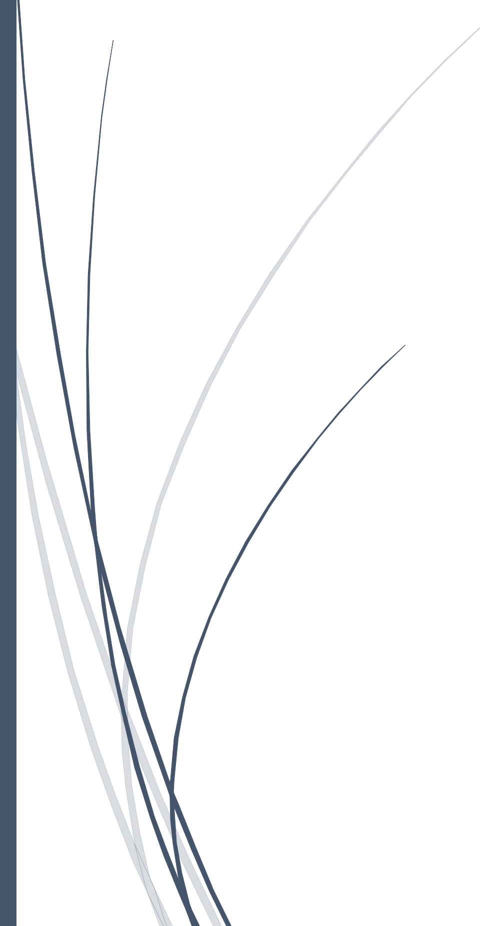
  The Egyptian algae *Padina pavonica* was isolated from the Red Sea in Egypt, collected, cleaned and prepared to analysis in South Valley University, Egypt. Experimental procedures of this part were performed in the Biomedical Materials Laboratory of the Health Sciences Department in Novara for the biological part, this chapter of thesis is related to the work done by the cooperation with Depatment of Applied Science and Technology, Politecnico Di Torino, Italy.
- **In chapter 4**, This chapter of the thesis is related to the work done in International Center for Material Nanoarchitectonics (WPI-MANA), National Institute For Materials Sciences, Tsukuba, Japan.

  Research mainly focusses on: 1. Established electrochemical system for pathogen activity detection (optional). 2. Checked the effect of your materials to the pathogens in the system (electrochemistry). 3. Studied the mechanism how it affected (electrochemistry, LC-MS, microscopy etc.). Most of the modern techniques of electrochemical system for pathogen activity detection were mastered, checked the effect of algal bioactive compounds materials to the pathogens in the system (electrochemistry) that considered new report to use this technique to detect the anti-activity of bacteria. Firstly this chapter

containing the procedures for the extractions of polyphenols from four different species of Egyptian macroalgae (*Padina pavonica*, *Sargassum muticum*, *Halimeda opuntia* and *Jania rubens*)., followed by determination of polyphenols, carotenoids, and chlorophylls from macroalgae extracts befor studied their electrochemistry properties on the current production of two oral pathogenic bacteria (*Streptococcus mutans* Clarke (ATCC® 700610™) and *Porphyromonas gingivalis* (Coykendall et al.) Shah and Collins (ATCC® BAA-308™)).

The Egyptian four species of macroalgae were isolated from the Red Sea in Egypt, collected, cleaned and prepared to analysis in South Valley University, Egypt. The determination of polyphenols and other secondary metabolites done in the Department of Agricultural Sciences, University Of Milan, Italy.

# Chapter 1: Introduction



## 1.1) Herbal medicine:

Herbal medicine is called botanical medicine, phytomedicine and herbalism indicates the using a plant's derivatives such as leaves, seeds, berries, flowers or roots for medical therapeutic issues. The traditional Hindu system of medicine is called Ayurveda, that is based on the idea of balance in bodily systems, herbal treatment, uses diet and yogic breathing. This traditional Hindu system has been known for nearly 5000 years also it is emphasizing the body, spirit and mind in disease prevention or treatment. An herb is considering a plant or just part of a plant used for its therapeutic properties or flavor and scent. Herbal medicines are one type of dietary supplement used by people to maintain or improve their health. They have a belief that natural products are always good and safe for their health. This is not necessarily true, there are many Shreds of evidence that some herbs can cause serious harm, such as comfrey and ephedra [1].

### *1.1.1)* History of traditional herbal medicines:

A large proportion of the population in many developing countries, in order to meet health care needs, adhering to their old customs or habits on traditional practitioners and their armamentarium of medicinal plants. Herbal medicines had maintained their popularity and may exist side-by-side with such traditional practice for historical and cultural reasons modern medicine.

Particularly in developed countries, such products of herbal medicines became additional wide offered commercially, these products were marketed for uses that were never contemplated from which they emerged, probably that related to the region or countries. An example, in Germany, herbal products are sold as ‘phytomedicines’ and reacted like any other drug products in the same criteria, safety and quality. By contrast happening in United States of America, where most herbal products found simply in the supermarket and all the products do not require pre-approval by the physicians.

Approximately 60 000 years ago, evidence indicates that plants have been cultivated like drugs (3). From 5000 years ago, in India, China and Egypt, researches about medicinal plants and at least 2500 years in Central Asia and Greece (4). It is clear that at that time there was insufficient information about useful plants for treating the causes of the different disease, and the ways of using these herbs for such purpose. Over time, the handling of using certain medicinal plants were discovered for the treatment of certain diseases; consequently, the use of medicinal plants gradually rejected the empirical framework and was limited to the facts. The earliest written evidence of the use of

medicinal plants for the preparation of drugs has been found on a Sumerian clay slab from Nagpur dating back to nearly 5000 years ago (5). For more than 27 centuries BC; according to some inscriptions; Egyptians and Chinese that used plants as medicine were the earliest human beings who did so. Also, ancient Greek people were familiar with the some medicinal plants for medicinal properties. Hippocrates, the founder of Greek medicine and Aristotle, used medicinal plants for the treatment of diseases. After that, Theophrastus, a Greek scientist, founded the School of Medicinal Plants. Then, Pedanius Dioscorides a physician and surgeon in the years 75-45 BC, wrote an encyclopedia, (He lived in the first century A.D), called De Materia Medica, to describe 600 therapeutic medicinal plants in the form of a series of scientific studies on medicinal plants (6-7).

### *1.1.2)* The traditional healing by using herbal medicines:

The first way was used for many centuries for the healing and pharmacological treatment of disease is the herbs. The methods of healing were different according to the tradition surrounding the world. In traditional Chinese medicine, Chinese people usual to use traditional medicine, their primary source as is botanical remedies but also animal and mineral materials have been used. The botanical products pass through many preparing processing before used, for example which may include, soaking in wine or vinegar or stir-frying. In China, traditional Chinese medicine is still in common used. More than 5000 traditional products are available in China, also, near to half of the population regularly uses traditional medication. With Japanese traditional medicine, Japanese herbs were classified in the first Japanese traditional medicine pharmacopeia (8). Many herbal treatments used in the Japanese systems of traditional healing, similar or found their way from China. On the other hand, nearly 5000 years, in Indian medicine, for example Ayurveda is considered as a medical system primarily practiced in India, it is involving diet and herbal treatments that can maintain the mind, the body and spirit away from diseases (9).

Many herbs work on different pathways in the body. Some of them are commonly used in plant medicine with their traditional uses; involving:

**Echinacea** – for increase the body fighting infection and stimulation the immune system. Also used to treat diseases such as fever and herpes.

**Dong quai** – older studies indicate that this herbs has ability to low blood pressure. In addition, they also used for some gynaecological complaints such as premenstrual tension and period pain, menopause symptoms.

**Garlic** – It is so famous for lowering levels of blood fats and cholesterol, used to reduce the risk of heart disease. It is also used to fight sinusitis, colds and other respiratory infections with its antimicrobial properties mean that.

**Ginger** – some studies proved that it is powerful in treating nausea, including morning sickness and motion sickness.

**Ginseng** – mostly is used during recovery from illness to treat fatigue. In addition of that is used to cholesterol levels and decrease blood pressure. On the other hand, overuse of ginseng has been associated with raised blood pressure.

**Ginkgo biloba** – used to treat tinnitus and poor blood circulation.

**Hypericum** – many studies have suggested that Hypericum is just as effective in treating mild to moderate depression as some pharmaceutical antidepressants. Hypericum also used for insomnia and anxiety. However, Hypericum can interact with some prescription medications, including the oral contraceptive pill, and stop them from working properly.

### *1.1.3)* Regulatory Situation of Herbal Medicines :

The legal situation regarding herbal preparations different from country to another. In some countries, phytomedicines were established as medications, but in others they are regarded as kind of food and therapeutic claims are not allowed. A great number of traditionally used herbal medicines found in developing countries, they have much folk-knowledge about them. On the other hand, they haven't any criteria to legislative establish herbal medicines as part of the drug legislation. For the classification of traditional medicinal products or herbal products, many criteria was included such as: prescription status, description in a pharmacopeia monograph, periods of use, claim of a therapeutic effect, scheduled or regulated ingredients. Many countries design a variation between "officially approved" products and "officially recognized" products, by which the products can be marketed without any authority scientific assessment [10]. Where the related products of herbal medicines are neither registered nor, a special licensing system is needed which would enable health authorities to screen the constituents, demand proof of quality before marketing, ensure safe use and correct, and also to oblige license holders to report suspected adverse reactions within a post-marketing surveillance system [11].

A number of resolutions drawing attention by the World Health Assembly (WHA) that has adopted to the fact that a large section of the population in many developing countries still relies on traditional medicine. Also guidelines has issued by WHO for the assessment of herbal medicines. These guidelines mainly defined scientific organizations, the assisting national regulatory authorities and manufacturers with respect of such products and criteria for the evaluation of herbal medicines's efficacy, quality and safety in assessing documentation, submissions, and dossiers.

The below [Table 1] mentioned WHO guidelines discribed the need for assessment of efficacy including the determination of pharmacological and clinical effects of the active ingredients, cultivation and collection of the medicinal plants, and labeling which includes a quantitative list of active ingredient, dosage, and contraindications.

**Table 1:**

**Different WHO guidelines with their major resolutions and year of establishment** [12]:

| WHO guidelines | Major resolutions taken | Year |
| --- | --- | --- |
| Quality control methods for medicinal plant materials | Emphasized the need to ensure the quality of medicinal plant products by using modern control techniques and include suitable standards and limits for contaminants are included. | 1998 |
| WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems | Provide technical guidance on the principles of good pharmacovigilance and the inclusion of herbal medicines in existing national drug safety monitoring systems. | 2004 |
| Guidelines for the Regulation of Herbal Medicines in the South-East Asia Region | This guideline aims to facilitate the registration and regulation of herbal medicines by establishing the foundation for a harmonized regulatory standard to meet the common demands of the region. | 2003 |
| General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine | Harmonize the use of certain accepted and important terms in TM, summarize key issues for developing methodologies for research and evaluation of TM, improve the quality and value of research in TM, and provide appropriate evaluation methods to facilitate the regulation and registration of TMs. | 2000 |
| National policy on TM and regulation of herbal medicines Report of a WHO global survey | Main objectives of this report are framing policy for safety, efficacy, and quality of herbal medicines and its promoting rational use. | 2005 |
| WHO guidelines on good agricultural and collection practices for medicinal plants | Quality assurance of medicinal plant materials used as the source for herbal medicines, and encourage and support the sustainable cultivation and collection of medicinal plants of good quality. | 2003 |

## 1.2) Green chemistry:

Chemistry science considered as a dangerous science and often the public associates the word "chemical" with "toxic" In the 1990s nearly 30 years ago. The main definition or discerption of the green chemistry is the designing of chemical products and all processes to scale back or eliminate the utilization of dangerous substances [13, 14]. During these 20 years, the primary definition of green chemistry was first formulated [15]. Many leading programs and governmental initiatives located in the U.S., Italy, and United Kingdom [16] have adoption that resulted in a significant role in informing and well-

established a sustainable design for Green Chemistry [17]. Green chemistry was carefully characterized by molecular design and chemical synthesis to reduce adverse consequences. As a result of this characterization, the Twelve Principles of Green Chemistry were designed as “design rules” to help scientists achieve the knowing goal of sustainability. This design it has been applied to all branches of industry such as energy, pharmaceutical, electronics, household products, aerospace, automobile cosmetic, to agriculture.

The concept of green chemistry has demonstrated to be good for human health and the surrounding environment by designing the next chemical generation products and processes.

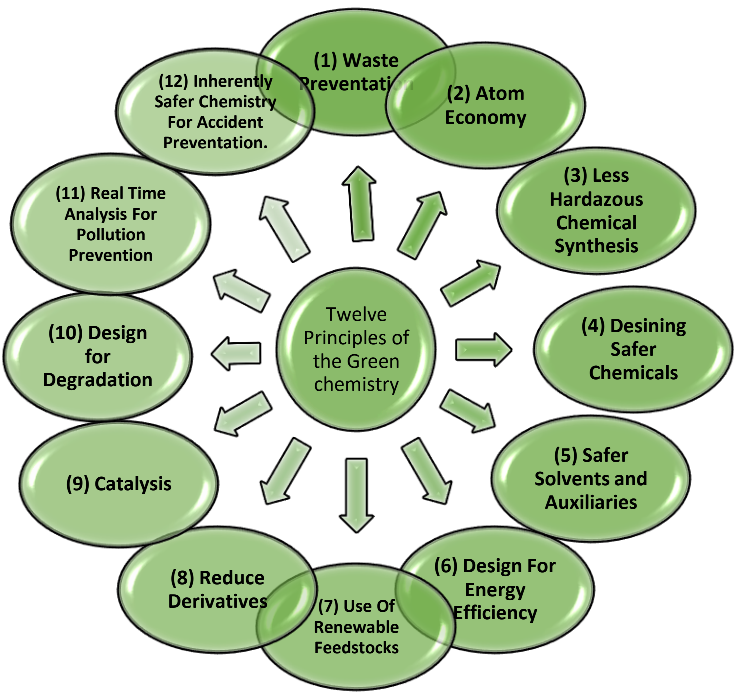
There are three main subjects for the green chemistry framework can be summarized as follows:

- Green chemistry designs for all the chemical life-cycle stages.
- Green chemistry aims to reduce chemical products and processes intrinsic hazards, by designing the essential category.
- Green chemistry disgined as a tenacious system of principles and/or design criteria.

### *1.2.1)* **The twelve principles of green chemistry:**

Figure 1 shown the twelve principles of green chemistry. They are a framework of guiding for the design of new chemical products and their processes, applying to all sides of the life-cycle process from the raw materials used to the efficiency, the toxicity and biodegradability of products, safety of the transformation as well as the used reagents. Recently, they were summarized recently into the more convenient and memorable acronym [18]. The following figure introduces different sections to provide general knowledge about green chemistry. The twelve principles of green chemistry set up a construction for the design of chemical transformations and safer chemicals. Chemistry has long been perceived as a dangerous science and often the public associates the word “chemical” with “toxic.” There are ways to reduce risk by using safety precautions such as protective gear.

**Figure 1: The twelve principles of green chemistry wre introduced by Paul Anastas and John Warner in 1998:**




**(1) Waste prevention:**

Waste prevention is the first of the twelve principles of Green Chemistry. Better to prevent waste than to clean up or treat waste after it is formed. The generation of any material that does not have realized value or the loss of unutilized energy can be considered a waste. waste can take many forms and may impact the environment differently depending on its nature, its toxicity, its quantity, or the way it is released. In 1992, the concept of what is now widely accepted as the E-Factor, or Environmental Impact Factor, was introduced by Roger Sheldon [19]. This metric helps to quantify the amount of waste generated per kilogram of product. It is a means to assess the "environmental acceptability" of a manufacturing process. In some new chemical industry such as the synthesis of ethylene oxide, The new process was generating more than 16 times less waste than the original one [20].

**(2) Atom economy:**

The material incorporation should be designed by synthetic methods. The concept of synthetic efficiency of Atom Economy (AE) or Atom Efficiency [21]. It refers to maximize the use of raw materials so that the final product contains the maximum number of atoms from the reactants. The ideal reaction would incorporate all of the atoms of the reactants. The AE is measured as the ratio of the molecular weight of the desired

product over the molecular weights of all reactants used in the reaction. It is a theoretical value meant to quickly assess how efficient a reaction will be.

**(3) Less hazardous chemical synthesis**:

The concept of that is to generate new chemical materials with pose little or no toxicity to human health and the environment. The synthetic toolbox of organic chemists has been improved with many of the new green reactions. Reactions based on cycloaddition rearrangement or multi-component coupling reactions. Also, Cascade or tandem reactions C–H activation metathesis and enzymatic reactions [22] are rather new approaches and illustrate strong examples of cleaner, more efficient synthetic tools available to organic chemists.

**(4) Designing safer chemicals:**

With a significant focus on designing new chemicals for many functions ranging from medicines to materials, there are a surprising lack of consideration hazards in the design process of chemical materials. The understanding of the molecule properties which have an impact on the transformations that take place in the biosphere and the surronding environment is essential to sustainability. Through this understanding, chemistry will be able to creat new molecules that are safer for humans or for the environment. Recently progressively towards the incorporation of an in-silico component. Because of that transition, it has been possible to create correlations, equations, and models that relate structure, properties, and function. For instance the existing understanding of medicinal chemistry can already help establish some ground rules for designing less toxic chemicals via the incorporation of specific design features that block their access into humans and many animal organisms. [23].

**(5) Safer solvents and auxiliaries:**

Solvents are perhaps the most active area of Green Chemistry research. They represent an important challenge for Green Chemistry because they often account for the vast majority of mass wasted in syntheses and processes [24]. Moreover, many conventional solvents are toxic, flammable, and/or corrosive. Their volatility and solubility have contributed to air, water and land pollution, have increased the risk of workers' exposure, and have led to serious accidents. Recovery and reuse, when possible, is often associated with energy-intensive distillation and sometimes cross-contamination. In an effort to address all those shortcomings, chemists started a search for safer solutions. Where possible, the ideal situation would be to not use any solvent. Water is the most abundant molecule on the planet and is sometimes referred to as a benign "universal solvent". Another example of

greener solvents would be ionic liquids, they have virtually no vapor pressure and very low flammability [25].

**(6) Design for energy efficiency:**

Rising concerns over the depletion of petroleum feedstocks and the increase in energy consumption have pushed the development of more energy-efficient processes and for the search for renewable energies; non-depleting resources in a time frame relevant to human scale [26]. Increasing the energy efficiency of a chemical system is merely one part of the solution.

**(7) Use of renewable feedstocks:**

Turning towards renewable feedstocks both for material and fuel has now become more urgent. The major renewable feedstock on the planet both for material and energy is bio-mass, the material available from living organisms. This includes wood, crops, agricultural residues, food, etc. [27].

**(8) Reduce derivatives:**

Should be minimized or avoided if possible, the using of blocking groups, protection/ deprotection, temporary modification of physical/chemical processes as unnecessary derivatization, because such steps require additional reagents and can generate waste.

**(9) Catalysis:**

By avoiding the use of a stoichiometric number of reagents and also by lowering the energy input required, catalysis can improve the efficiency of a reaction, and also by greater product selectivity. This implies less energy, less feedstock and less waste [28]. Moreover, it often opens the door to innovative chemical reactions and brings unconventional solutions to traditional chemical challenges.

**(10) Design for degradation:**

Is not a simple task the designing biodegradable materials and chemicals as illustrated by continuing problems of environmental pollution.

**(11) Real-time analysis for pollution prevention**:

There is currently a huge emphasis in industrial settings to monitor and control large-scale processes in real-time.4 Changes in the physical criteria such as temperature, pressure and/or pH are monitored by internal thermometers, barometers or pH meters. With the help of these analytical tools, it’s easy to verify if a reaction’s conditions exceed the safe limits, and subsequently, halt the process before anything gets out of hand.

**(12) Inherently safer chemistry for accident prevention:**

It is meaning that the substances and its forms which used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

## 1.3) Polyphenols:

Natural polyphenols are secondary metabolites of plants and mostly found in plant-based foods. Polyphenols were considered as a class of chemical compounds which are mainly biosynthesized naturally. Polyphenols are consisted of multiple phenols units according to the phenol units number and binding structure; branched into four major classes; flavonoids, stilbenes, phenolic acids, and lignans; with different chemical, physical and biological properties. Polyphenols have performances different acts in human life and biology of plant, inclusive of UV protective agents, defensive compounds against herbivores and pathogens, pharmaceuticals, plant colors and the taste of food and drink. The generic term “polyphenol” containing more than 8000 types of phenolic structures, the more common one are: phenolic acids, flavonoids, lignans and stilbenes. These phytochemicals play important therapeutic and antioxidant properties which may promote oral health by lowering the risk of tumor production and oxidative stress (the body’s inability to remove free radicals) and reducing the risk of inflammation and infections [29].

The introduction of antibiotics to clinical practice considering as one of the most important contributions to the treatment of infectious diseases. In spite of, bacterial disease-causative agents is the various resistance mechanisms that emerged and rapidly spread, due to the extensive use of these valuable therapeutics. Currently, novel medicine for some types of infectious diseases, is the pre-antibiotic era. The rapid spread of multidrug-resistant (MDR) or extremely drug-resistant (XDR) bacterial strains seems to be the most frightening development [30].

Nowadays, there is an increasing interest in the plant-derived functional foods in their bioactivities provided by these phytochemicals and considerable attention due to their safety and therapeutic potentials. Polyphenols are the most plentiful and widely distributed group of bioactive molecules. Polyphenols with high antioxidant capacity make as an important key factor that was involved in the chemical defense against pathogens and predators. They have two general classes by the number of phenol rings that they contain and the basis of structural elements that bind these rings [31]; one is

flavonoids and phenolic acids. Flavonoids are divided into flavones, flavanones, flavonols, flavanols, isoflavones. However phenolic acids are generally classified into hydroxybenzoic and hydroxycinnamic acids. There are two other sub-general classes of polyphenolic compounds; Tannins and stilbenes. All these phenolic compounds with their chemical structures can basically be categorized and shown in Table. 2. Polyphenols containing many molecules and a different set of biological activities that are mainly attributed to their structure. Polyphenols play a key role to prevent various diseases according to previous investigations [31].

### *1.3.1)* Natural resources of polyphenols:

Mostly 10,000 polyphenol compounds have been identified from various plants. The main characteristic structural of most polyphenols was built from a common intermediate structure. The polyphenols occurred with one or more sugar residues linked to hydroxyl groups, Also sugar linked directly to an aromatic carbon. There are linkage with other compounds, such as carboxylic acids, amines, organic acids, lipids and association with other phenol were also common. Polyphenols divided into many variou groups by the number of phenol rings that they contain and the basis of structural elements that bind these rings, which were classified into several sub-classes, the different groups of polyphenols and their chemical structures [32] as shown in Table 2.

A number of factors such as environmental condition, storage, and food processing could affect the content of polyphenols in daily food. Generally, phenolic acid concentrations decreased during ripening, whereas anthocyanins concentrations increased. Many cooking methods caused a reduction in the contents of polyphenols. Carrots completely loose their polyphenols after boiling, while steaming and frying had a less negative effect. Boiling and frying considered as the main reasons for a higher loss of polyphenols than steaming. However, the evidence was emerging that bioavailability of many protective compounds was enhanced when the vegetables are cooked [33]. Storage also affected the content of polyphenols. After 11 months of storage, the content of phenolic acids decreased by 5% to 21% in apple juices. A decrease in the content of free p-coumaric acid was also observed in frozen red raspberries [34].

**Table 2: Different groups of polyphenols, their chemical structures and their natural sources:**

| Class | Basic structure | Natural source | |
|---|---|---|---|
| | | Plant | References |
| **Simple phenolics** | OH | Essential oils of plants like vanilla, wintergreen, thyme, cloves | **35** |
| **Benzoquinones** | O<br>O | leaves of *Miconia lepidota,* alga *Perithalia capillaries*, *Polygonatum alte-lobatum*, *Maesa lanceolata*, *Cyperus alopecuroids*, *Saccharomyces cerevisiae* and *Heliotropium ovalifolium* | **36** |
| **Phenolic acids** | HOOC | Strawberries, raspberries, blackberries, coffee, blueberries, kiwis, plums, cherries, apples and cereals | **37** |
| **Hydroxybenzoic acids** | OH<br>O<br>HO | Blackberries, Tea leaves, olive oil. | **38** |
| **Acetophenones** | O<br>$CH_3$ | Apple, cheese, apricot, banana, beef, and cauliflower | **39** |
| **Phenylacetic acids** | OH<br>O | Guava, wines, papaya, raspberry, strawberry, potato, tomato, gruyere, Swiss and cheddar cheeses, mango, passion fruit, honey and tea, balsamic vinegar and cocoa powder. | **40** |

| | | | |
|---|---|---|---|
| **Hydroxycinnamic acids** | | Ripe fruits such as apples, various berries, plums, cherries, some citrus fruits, peaches, cereals, carrots, salad, eggplants, cabbage, artichoke, Coffee AND TEA | **41** |
| **Phenylpropanoids** | | Cereal bran, Wheat grain bran and germ, rice seeds and Nipponbare" seeds | **42** |
| **Coumarins** | | Tonka bean, Apiaceae, Asteraceae, and Rutaceae | **43** |
| **Isocoumarins** | | Flowers of Hydrangea hortensia Smith, flowers of Hydrangea macrophylla Seringe var. thunbergii Makino, Aloe sp., Scorzonera sp., Crassocephalum sp. and Tragopogon. | **44** |
| **Chromones** | | *Eremophila georgei, Bothriochloaischae mum, Imperata cylindrica, Cucumis melo L.,* and Aquilaria spp. | **45** |
| **Napthoquinones** | | Avicenniaceae, Acanthaceae, Balsaminaceae, Bignoniaceae, Boraginaceae, and Plumbagnaceae. | **46** |
| **Xanthones** | | Gentianaceae, Guttiferae, Moraceae, Clusiaceae, and Polygalaceae | **47** |

| | | | | |
|---|---|---|---|---|
| **Stilbenes** | | | root of *Polygonum cuspidatum*, *Polygonum multiflorum*, *P. lactiflora, P. multiflorum* and *oxyresveratrol*, fruit of *Morus alba*, *Shorea hemsleyana*, *Vatica rassak*, *Vatica indica* and *Kobresia nepalensis.* | **48** |
| **Anthraquinones** | | | Polygonaceae, Rhamnaceae, Rubiaceae, Fabaceae, and Xanthorrhoeaceae | **49** |
| **Flavonoids** | **Flavones** | | Parsley, celery, Cereals such as millet and wheat and skin of citrus fruit | **50** |
| | **Flavonols** | | Onions, curly kale, leeks, broccoli, blueberries, Red wine, tea, glucose, rhamnose, galactose, arabinose, xylose and glucuronic acid | **37** |
| | **Flavanones** | | Tomatoes, aromatic plants, grapefruit, oranges and lemons | **51** |
| | **Flavanonols** | | leaves of *Paepalanthus argenteus var. argenteus* | **52** |
| | **Isoflavones** | | Soybean-derived products and leguminous plants | **53** |

| | Flavanols | | Apricots, red wine, green tea, chocolate, Black tea, leguminous plants, grapes, apples and cider | **54** |
|---|---|---|---|---|
| | **Anthocyanidins** | | Red wine, cabbage, beans, onions, radishes, cherries and strawberries. | **55** |
| | **Chalcones** | | Citrus, apple, tomato, etc), vegetables (shallots, bean sprouts, potatoes, etc.), and also some edible plants (licorice) | **56** |
| **Isoflavonoids** | | | *Papilonoidae*, subfamily of *Leguminosae,* red clover (*Trifolium pratense*), leaves of *Millettia taiwaniana* (Leguminosae) | **57** |
| **Lignans** | | | Seeds, grains, fruits and vegetables, and in higher concentrations in sesame and flax seeds | **58** |
| **Neolignans** | | | *Forsythia koreana*, *Carthamus tinctorius*, *Anthriscus Sylvestris, Forsythia* leaves, *Sesamum radiatum* and *Sesamum alatum.* | **59** |

| **Biflavonoids** | | Pulp and white core that runs through the centre of citrus fruits, green peppers, lemons, limes, oranges, cherries, and grapes. | **60** |
|---|---|---|---|
| **Lignins** | | Is noticed in all vascular plants, commonly between the cells, within cells and cell walls | **61** |
| **Proanthocyanidins or flavolans** | | Grapes, peaches, kakis, apples, pears, berries, wine, cider, tea, beer and chocolate. | **62** |

### *1.3.2)* The bioactivity of polyphenols:

Many epidemiological studies shown many health-promoting actions of the absorbed polyphenols such as anti-inflammatory, anticancer and anti-diabetes. A variety of the standard phenolics and phenolic extracts bioactivities that extracted from natural origins such as cereals, legumes, grains, seeds and fruits. These bioactivities are studied by focusing on their preventive effects against chronic ailments and summarizing clinical evidence.

**Figure 2: Numerous biological activities of polyphenols:**



Natural polyphenols showing many health benefits and biological activities for prevention and treatment of diseases, cancers, heart diseases as following:

#### *1.3.2.1)* Antioxidant activity:

The antioxidant activity have been widely studied among many bioactivities of phenolic compounds, including inhibition of lipid oxidation, scavenging of free radicals, reduction of hydroperoxide formation, and so on [63]. Phenolic compounds were usually major contributors of antioxidant capacities of plants, proven by many experiments founded in vitro. Polyphenols may also function as antioxidants through its effects on membranes, plasma, transcription factors and enzyme activities in vivo. Polyphenols could be used food preservatives in the food industry not only as antioxidant for human.

Polyphenol antioxidants in low concentration can prevent biomolecules (proteins, nucleic acids, polyunsaturated lipids, sugars) from undergoing oxidative damage through free radical mediated reactions. They can inhibit oxidizing chain reactions in several ways,

including direct quenching of reactive oxygen species, inhibition of enzymes, and chelation of metal ions (Fe3+, Cu+).

Two main mechanisms of polyphenols as antioxidants can play their protective: In first one, the free radical removes a hydrogen atom from the antioxidant (ArOH) that itself becomes a radical. This mechanism is referred to as H-atom transfer. A higher stability of the radical ArO• corresponds to a better efficiency of the antioxidant ArOH, so that it is unlikely to react with the substrate. Generally, hydrogen bonds, resonance, and conjugation make it a nonreactive phenoxyl radical. In this mechanism, the bond dissociation enthalpy (BDE) of the O-H bonds is an important parameter in evaluating the antioxidant action, because the weaker the OH bond the easier will be the reaction of free radical inactivation. In the second mechanism (the one-electron transfer), the antioxidant can give an electron to the free radical becoming itself a radical cation [64].

#### *1.3.2.2)* Anticancer activity:

Effect of polyphenols as anticancer agent on human cancer, mostly by induce a reduction of the number of tumors cell lines or their growth. This evidence has been observed at various sites, including mouth, stomach, duodenum, colon, liver, lung, mammary gland or skin. Many polyphenols have been studied; although their mechanisms of action were found to be different, all of them showed protective effects in some models [65].

Development of cancer or carcinogenesis is a microevolutionary or multistage process. It can divided into three major stages of carcinogenesis: firstly: initiation, Secondly: promotion and finally: progression. Initiation is a heritable aberration of a cell. Cells so initiated can undergo transformation to malignancy if promotion and progression follow. Promotion, on the other hand, is affected by factors that do not alter DNA sequences and involves the selection and clonal expansion of initiated cells.

Chemoprevention effect action mechanisms of polyphenols have been identified, these include antiproliferation, estrogenic/antiestrogenic activity, induction of cell cycle arrest or apoptosis, induction of detoxification enzymes, prevention of oxidation, regulation of the host immune system, anti-inflammatory activity and changes in cellular signaling. Polyphenols influence the metabolism of pro-carcinogens by modulating the expression of cytochrome P450 enzymes involved in their activation to carcinogens. By increasing the expression of phase II conjugating enzymes, polyphenols may also facilitate their excretion. Polyphenols which considered as a substrates of these enzymes, can form potentially toxic quinones in the body. The intake of polyphenols could induce a general

boosting of our defenses against toxic xenobiotics by activation these enzymes for their own detoxication. It has been demonstrated that tea catechins in the form of capsules when given to men with high-grade prostate intraepithelial neoplasia (PIN) demonstrated cancer preventive activity by inhibiting the conversion of high grade PIN lesions to cancer [66].

As a example as anticancer agent, the abundant polyphenols in black tea have also been shown to possess strong property. Black tea polyphenols were found to inhibit proliferation and increase apoptosis in Du 145 prostate carcinoma cells. Higher level of insulin like growth factor-1 (IGF-1) was found to be associated with a higher risk of development of prostate cancer. IGF-1 binding to its receptor is a part of signal transduction pathway which causes cell proliferation. Black tea polyphenol addition was found to block IGF-1 induced progression of cells into S phase of cell cycle at a dose of 40 mg/ml in prostate carcinoma cells.

Quercetin has also been reported to possess anticancer property against benzo(a)pyrene induced lung carcinogenesis in mice, an effect attrtibuted to its free radical scavenging activity.Resveratrol prevents all stages of development of cancer and has been found to be effective in most types of cancer including lung, skin, breast, prostate, gastric and colorectal cancer. It has also been shown to suppress angiogenesis and metastasis. Extensive data in human cell cultures indicate that resveratrol can modulate multiple pathways involved in cell growth, apoptosis and inflammation. The anti-carcinogenic effects of resveratrol appears to be closely associated with its antioxidant activity, and it has been shown to inhibit cyclooxygenase, hydroperoxidase, protein kinase C, Bcl-2 phosphorylation, Akt, focal adhesion kinase, NFκB, matrix metalloprotease-9 and cell cycle regulators. These and other in vitro and in vivo studies provide a rationale in support of the use of dietary polyphenols in human cancer chemoprevention, in a combinatorial approach with either chemotherapeutic drugs or cytotoxic factors for efficient treatment of drug refractory tumor cells [66].

#### *1.3.2.3)* **Antimicrobial activity:**

Besides brevious established antioxidant activity of polyphenols, many of them may exhibit significant antibacterial activity. Since many plant extracts are rich in phenolic compounds, this is of particular interest for the development of natural alternatives to synthetic preservatives in food and cosmetic applications.

The mechanisms of antibacterial action of phenolic compounds are not yet fully deciphered but these compounds are known to involve many sites of action at the cellular level. Several authors explained this activity by the modification in permeability of cell membranes, the changes in various intracellular functions induced by hydrogen binding of the phenolic compounds to enzymes or by the modification of the cell wall rigidity with integrity losses due to different interactions with the cell membrane. Irreversible damages may induces of the cytoplasmic membrane and coagulation of the cell content that can even lead to the inhibition of intracellular enzymes, by the elevation of the lipophilic character of phenolic compounds which enhances their antimicrobial activity by favoring their interaction with the cell membrane. For example, condensed phenylpropanoids-tannins may induce damages at the cell membrane and even inactivate the metabolism by binding to enzymes while phenolic acids have been shown to disrupt membrane integrity, as they cause consequent leakage of essential intracellular constituents. Flavonoids may link to soluble proteins located outside the cells and with bacteria cell walls thus promoting the formation of complexes. Some articles reviewed that, by affecting protein and RNA syntheses, flavonoids also may act through inhibiting both energy metabolism and DNA synthesis [67].

Despite the complexity of the mechanisms of action involved, different authors have investigated the antibacterial activity of phenolic compounds by Quantitative Structure-Activity Relationship (QSAR) studies. Some studies have shown the importance of the contribution of the octanol-water partition coefficient (Log P) in relation with the hydrophobic and amphiphilic character of the molecule, the role of the number and the position of OH groups (, the role of size and type of alkyl groups, and the contribution of the presence of acetate groups and aldehydes in the antibacterial efficacy of phenolic compounds. The existing reviwes concerning QSARs for the prediction of the antibacterial activity of polyphenols of slightly higher molecular weight such as phenolic acids, flavonoids, stilbenes, coumarins and quinones still remains limited. Although a QSAR study has not been conducted regarding the antibacterial effect of polyphenols, descriptors related to the number of hydroxyl functions, electronic effects and lipophilicity are the most common in QSAR models involving polyphenols.efficacy, to lower antibiotic dose, and therefore to reduce antibiotic adverse reactions [67].

- **Antibiofilm Phenolic Compounds:**

A biofilm is a sessile form of bacterial existence on solid surfaces or air-liquid interfaces, in which bacteria multiply covered by a self-produced biofilm matrix, composed of

bacterial intercellular polysaccharides, proteins, and extracellularly released nucleic acids. The protective effect of bacterial biofilm phenotypes is multifactorial. It includes decreased penetration of antimicrobial agents into the deep layers of biofilms, the capture of positively charged molecules by the extracellular polymeric biofilm matrix, or the ability of biofilm matrices to concentrate bacterial enzymes which inactivate antibiotics [68].

Multiple mechanisms of antibacterial activity for phenolics compounds have been described: they may cause damage to cytoplasmic membranes, inhibit nucleic acid synthesis, cell wall synthesis, reduce membrane fluidity, or energy metabolism by their interaction with bacterial proteins and cell wall structures. Besides their destructive activity on bacteria, also “softer” activities leading to biofilm have been released; suppression by affecting the bacterial regulatory mechanisms such as quorum sensing or other global regulator systems , without an effect on bacterial growth [69].

To the most extensively studied bacteria from the point of view of biofilm production belong *Staphylococcus aureus* and coagulase-negative staphylococci, which play a crucial role in medical device-associated infections and one of the most important dental caries agents -*Streptococcus mutans*.

Roccaro *et al.* referred to the modulation effect of catechin gallates to bacterial drug resistance. Epigallocatechin gallate (EGCg) shown that it had several antibacterial activities such as; limiting bacterial growth and invasion and acting in synergy with some antibiotics. Sub-inhibitory concentrations of EGCg were able to reverse tetracycline resistance in *staphylococci* by inhibition of the Tet(K) efflux pump, in addition to further sensitizing of the susceptible staphylococcal isolates to this antibiotic. EGCg at subinhibitory concentrations has shown to decrease slime production of *S. aureus* biofilm formation. Without inhibiting bacterial growth, Tannic acid from black tea (*Camellia sinensis*) inhibited *S. aureus* biofilm formation via a mechanism dependent upon the putative transglycosylase IsaA [70]. Recent research showed a good activity against *S. aureus* in planktonic and biofilm growth forms by gallotannins such as gallic acid and methyl gallate that rich in *Cotinus coggygria* leaves. Biofilm formation by *Escherichia coli* inhibited by ellagic acid and tannic acid. Both compounds reduced biofilm formation significantly.

In several studies it was described that the inhibition of biofilm formation on surfaces covered by plant products may be significant in the future techniques which prevent medical device biofilm-associated infections. As a major phenolic compound in many

antimicrobially active plants, e.g., in the genera *Mentha*, *Melissa*, *Lycopus*, *Origanum*, *Thymus*, *Salvia*; Rosmarinic acid, also known as Lamiaceae tanning compound, was identified to be a candidate topical antimicrobial agent with killing activity on planktonic forms of clinical *S. aureus* strains and suppressing activity in the early stages of biofilm development [71].

#### *1.3.2.4)* Anti-inflammatory activity:

Polyphenols have significant anti-inflammation effects in vivo and in vitro. Excessive inflammation is considered as a critical factor in many human diseases, including obesity, type II diabetes, cardiovascular diseases, neurodegenerative diseases and aging. In vivo and in vitro, polyphenols have shown significant anti-inflammation effects. Chronic and acute inflammation processes are implicated in the development of chronic diseases. Therefore, interventions that modify the inflammatory cascade associated with diseases may be regarded as potential targets in the prevention of such conditions. Some phenolic compounds have exhibited anti-inflammatory properties. There is a correlation between a downregulation of the inflammatory response and the high intake of food rich in phenolic compounds, although the precise mechanisms of this anti-inflammatory activity are not fully dilucidated [72].

The structural requirements associated of phenolic structure and anti-inflammatory activity has have been established using different targets of inflammation:

- A planar ring system is essential in the flavonoid molecules to exhibit the activity.
- Unsaturation in the C ring as ketonic carbonyl at C4 and/or C2-C3 double bond.
- Hydroxyl groups in B ring and at C5 and C7 of A ring are necessary.
- The number and position of hydroxyl groups as the catechol group at ring B.
- The flavones and flavonols having a hydroxyl group at 4′ position of B ring showed higher activity than those do not.
- The methylation of the hydroxyl groups at 3, 5, or 4′ positions improved the activity.
- The methylation of the 3-hydroxyl group reduced the cytotoxicity.
- Flavones exhibited higher activities than the corresponding isoflavones, flavonols, and flavanones.
- The non-glycosylation of the molecule, aglycones have a bigger effect than

glycosides.

Every class of phenolics present in the extracts could have a different effect on pro-inflammatory mediators according to several studies that focused on the evaluation of extracts containing a mixture of many compounds. As we can see in the analysis of *C. ternatea* phenolic extract, the ternatin anthocyanins inhibited nuclear NF-κB translocation, iNOS protein expression, while flavonols showed strong inhibition of COX-2 activity and partial ROS suppression and NO production through a non-ROS suppression mechanism.

The mechanism of action of phenolic compounds is executed by a variety of inhibitory activities of pro-inflammatory mediators and/or gene expression. While drugs work in a single one, an individual phenolic compound or a mixture of them can exert anti-inflammatory activity through many ways; The flavonol kaempferol have shown inhibitory activity of LPS-induced NF-κB activations. Anti-inflammatory effects of flavonoids: Genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-κB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-κB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. Mediators of Inflammation, Anti-inflammatory effects of flavonoids: Genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-κB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-κB activation [72].

#### *1.3.2.6)* **Cardioprotection activity:**

Postprandial hyperlipemia and oxidative stress, a well-defined risk factor for atherosclerosis, could be reduced by polyphenols. Atherosclerosis develops in lesion-prone regions of medium-sized arteries. Atherosclerosis might be present or clinically silent for decades before becoming active and producing pathological conditions such as acute myocardial infarction, unstable angina, and sudden cardiac death. The rate of myocardial infarction is continually increasing. Numerous studies have shown that dietary polyphenols could reduce the risk of thrombosis, which is one of the leading causes of myocardial infarction, ischemic heart disease, etc.. In addition, epidemiologic studies provided strong evidence that the incidence of cardiovascular disease was low in the Mediterranean area. Both epidemiological and experimental studies found that the incidence of mortality and morbidity from coronary heart disease reduced by the mild-to-moderate consumption of wine, particularly of red wine. The cardioprotective effects of

red wine were thanks to its high content of antioxidant polyphenols including resveratrol and proanthocyanidins. Many experiments have been carried out to prove the antioxidant activity of resveratrol (Resveratrol is a polyphenols abundantly found in grapes and wine) in vitro and in vivo. Many studies investigated two main working mechanisms of five new potential antioxidant analogues of cis-resveratrol, H atom and single-electron transfer. The results indicated that the number of hydroxyl groups and the presence of the catechol moiety were the most significant features in determining the order of radical scavenging potentiality [73].

#### *1.3.2.7)* **Other bioactivities of polyphenols :**

Except for the above-introduced bioactivities, polyphenols have shown several other health beneficial effects like antiaging activity and in cosmetic. Some studies showing that polyphenols had a protective response to skin damage, erythema and lipid peroxidation from UV exposure. The protective effects of (−)-epigallocatechin-3-gallate on the ultraviolet-induced skin damage were studied in guinea pigs, hairless mice and human dermal fibroblast cultures. Epigallocatechin-3-gallate reduced the phenomenon of skin damage, such as roughness, sagginess and the decrease of dermal collagen, induced by UVA in hairless mouse skin. Epigallocatechin-3-gallate treatment could also block the UV-induced increase of collagen secretion and collagenase mRNA level in fibroblast culture. In addition, consumption of green tea polyphenols prevents photocarcinogenesis in mice.

On the other hand, polyphenols can protect the kidney after glycerol injection. Also, polyphenols might protect against obstructive lung disease. A high intake of the soy isoflavone and genistein was associated with better lung function in asthmatic patients. This investigation found out that genistein, a soy isoflavone, was the only nutrient that had a consistent association with asthma severity. None of the other nutrients evaluated were related to asthma rate when adjusted for known confounders. Increasing consumption of genistein was associated with better lung function in patients with asthma.

Insulin resistance, a hallmark of metabolic disorders, is a risk factor for diabetes and cardiovascular disease. Concordant results were reported that polyphenols, such as resveratrol and epigallocatechin-3-gallate, in vivo had a beneficial effect on energy metabolism in diseases such as diet-induced obesity and insulin resistance [74-75].

### *1.3.3)* Potential toxicity of polyphenols:

In recent years, the potential toxicity of some polyphenols was reported, such as catechin to damage DNA in mice spleen cells. Noticeable DNA damage was induced in mice spleen cells disposed by higher concentration of catechin. Moreover, grape extracts were found to promote mitomycin C inducing sister chromatid exchange at the concentration from 75 to 300 μg/mL in human peripheral blood lymphocytes. At the same concentrations, the situation of mitomycin C-induced clastogenicity was enhanced by a mixture of caffeic acid, gallic acid and rutin hydrate. In addition, notably negative effects were observed in fibroblast and keratinocyte cell lines after exposure to high concentration of epicatechin for 24 h or more time. Furthermore, the compounds with a gallate group exhibited more potential toxicity than those without the gallate group. The results indicated that positive effects could be obtained from polyphenols in a safe concentration range. However, the concentrations of polyphenols were not the only crucial factor; negative effects of polyphenols were related to the synergistic effect of the mixture of polyphenols contained in each food matrix tested and exposure time. Therefore, the dose and composition of polyphenols should be investigated further for a secure and healthy application [72].

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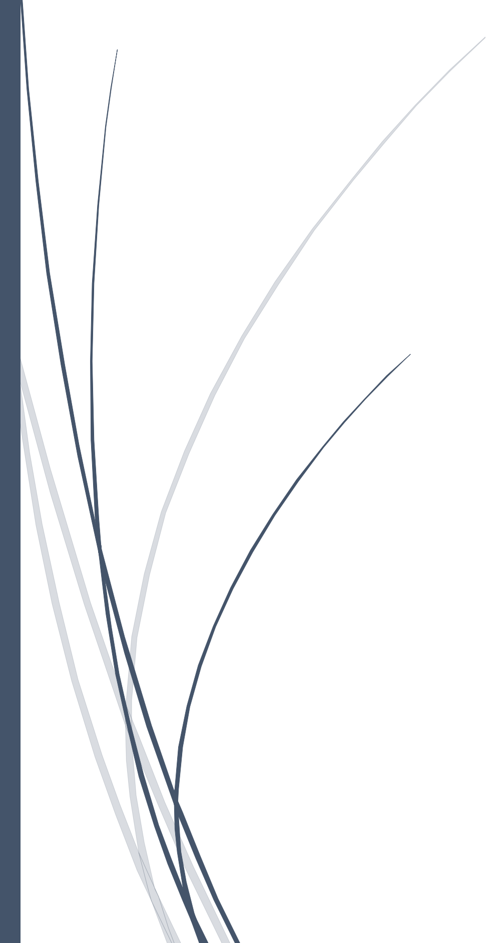
# Chapter 2:

# Exploring African medicinal *Pelargonium sidoides* root extract for potential anti-bacterial evaluations.



THIS CHAPTER OF THE THESIS IS RELATED TO THE WORK DONE BY THE COOPERATION WITH LITHUANIAN UNIVERSITY OF HEALTH SCIENCES WITHIN THE FUNDED GRANT PROJECT PELARGODONT (NO. S-M-ERA.NET-17-2) FROM THE RESEARCH COUNCIL OF LITHUANIA, THE STATE EDUCATION DEVELOPMENT AGENCY OF LATVIA, AND ITALIAN MINISTRY OF EDUCATION, UNIVERSITY AND RESEARCH.

THE RESULTS OBTAINED HAS BEEN PUBLISHED:

Savickiene, N.; Jekabsone, A.; Raudone, L.; **Abdelgeliel, A.S.**; Cochis, A.; Rimondini, L.; Makarova, E.; Grinberga, S.; Pugovics, O.; Dambrova, M.; Pacauskiene, I.M.; Basevičiene, N.; Viškelis, P.. Efficacy of Proanthocyanidins from *Pelargonium sidoides* Root Extract in Reducing *P. gingivalis* Viability While Preserving Oral Commensal *S. salivarius*. Materials

## 2.1) Introduction:

### *2.1.1)* African medicinal plant *Pelargonium sidoides:*

Medicinal plants are commonly used for the therapy of various diseases. People have been appealing to nature for curing diseases since ancient times and the use of medicinal plants has been somewhat instinctive and based on experience. Geranium *Pelargonium sidoides* DC. (Family: Geraniaceae, syn. *Pelargonium sidaefolium* Thunb.), also known by the traditional names of *Umckaloabo*, *Uvendle* or *Kalwerbossie*, is a perennial plant originating from South Africa, where it mainly grows in the Eastern Cape Province and Lesotho Highlands [1]. The roots of this geranium are succulent and red, the leaves have a round cordial shape, featuring a long footstalk and glandular trichomes. The flowers are quinate, dark red to black colored Figure 1 [2-3].

**Figure 1:**

**A) Flower of *Pelargonium sidoides***

Proprietary extract from *Pelargonium sidoides* roots known as EPs®H7630 or UmckaloaboH has been evaluated in numerous clinical trials for safety and alleviation of symptoms associated with acute bronchitis and is licensed in Germany as herbal medicine for the treatment of upper respiratory tract infections. PS extract contains numerous different metabolites and has been shown to inhibit viruses associated with respiratory diseases like influenza viruses [4].

### *2.1.2)* ***Pelargonium sidoides*** **extracts:**

Clinical trials have shown that a modern aqueous - (Ethanolic, Methanolic or Acetonic) formulation of *P. sidoides* extracts is an efficacious treatment for disorders of the respiratory tract, for example bronchitis and sinusitis [5]. The pharmacological efficacy of *Pelargonium sidoides* has been partly attributed to the biological activity of highly oxygenated coumarins (7-hydroxy-5,6-di-methoxycoumarin;6,8-dihydroxy-5,7-dimethoxycoumarin), gallic acid derivatives, flavonoids, phenolic and hydroxy cinnamic acid-derivatives [5]. Recently, 6-Methoxy-7-(sulfooxy)-2H-1-benzo- pyran-2-one and 6, 8-Bis (sulfooxy)-7-methoxy-2H-1-benzopyran-2-one were identified in *Pelargonium sidoides* for the first time. Most significantly researches characterized two novel compounds, 7-Hydroxy-6-methoxy-8-(sulfooxy)-2H‑1-ben-zopyran-2-one and 8-Hydroxy-7-methoxy-6-(sulfooxy)-2H‑1-ben-zopyran-2-one. These novel compounds have to be screened for pharmacological activity as they may represent the individual active constituents that have so far remained elusive.

### *2.1.3)* **Bacterial antibiotic resistances:**

Antibiotic failure due to increasing antibiotic resistance is a worldwide threat to public health. According to the Center for Disease Control (CDC), in 2013 in the United States alone, about 2,000,000 people were infected with antibiotic-resistant bacteria, and 23,000 died as a result (CDC, 2013). Globally, current antibiotic resistance accounts for at least

700,000 lives lost per year, and it is projected that an unabated rise in antimicrobial resistance may lead to 10 million deaths per year by 2050 [6]. Long-term applications of antibiotics in the protection of humans and animals and in agricultural growth promotion have exerted a major impact on bacterial communities. These applications have resulted in bacteria possessing various resistances to antibiotics that are generally controlled by ARGs. The following mechanisms create increasing resistance in a bacterial community: (a) target bypass, which allows some bacteria to become refractory to specific antibiotics by bypassing the inactivation of a given enzyme; (b) efflux pump, which is the mechanism that prevents the antibiotic from penetrating the outer and/or cytoplasmic membrane by decreasing the uptake of the antimicrobial molecule, antibiotic inactivation, which inactivates the active antibiotic molecule directly; and (d) target modification, which modifies action sites of antibiotics. Furthermore, the resistance of certain antibiotics might be associated with more than one mechanism. Generally, several types of antibiotic resistance genes (ARGs) have been found in the environment, including genes resistant to tetracycline, sulfonamide, aminoglycoside, macrolide–lincosamide–streptogramin (MLS), chloramphenicol, vancomycin, and β-lactam genes [6].

The negative impacts of antibiotic resistance on healthcare systems as a whole are substantial, as resistance adds to the number of infections that occur, to expense, to interrupted hospital activity and to limitation of treatment options. Resistant bacterial spread reflects both additional infections caused by resistant strains and replacement of susceptible strains by resistant strains. There is evidence of additional infections caused by resistant strains rather than merely a replacement of susceptible strains. Increasing antibiotic resistance potentially threatens the safety and efficacy of surgical procedures and immunosuppressive chemotherapy. It is estimated that between 38·7% and 50·9% of pathogens causing surgical site infections and 26·8% of pathogens causing infections after chemotherapy are resistant to standard prophylactic antibiotics in the USA [7].

### *2.1.4)* The efficacy of Pelargonium sidoides extracts as antibacterial agents:

*Pelargonium sidoides* root extract (PSRE) is powerful in the treatment of various respiratory system infections. PSRE is not accompanied by the risk of the evolvement of treatment-resistant pathogenic strains. However, considered as a good candidate as an antibiotic and chemical bactericide therapy substitute and/or additive in cases of chronic and repeated infectious diseases [8].

On the other hand, the antibacterial effects of PSRE are so mild for the treatment of many infections. So, the power of the antibacterial activity of PSRE is mostly assigned to the second extract proanthocyanidins (PACN) which have many efficacies like as antioxidant, anti-inflammatory, anti-aging and anticancer properties. Many studies found that PACN-extracts has ability to inhibit or address bacterial biofilm formation and the adhesion of period onto pathogenic species and suppress bacterial proteolytic activity. On the other hand, it is likely that PACN isolated from PSRE and concentrated has stronger antibacterial activity yet preserves the same advantage of no risk of resistance because of its multiple action pathways. To test this hypothesis, we have isolated a PACN fraction from PSRE, analyzed its composition and compared its antibacterial efficiency with that of PSRE. To investigate the selectivity of antimicrobial activity, two bacterial strains were selected to assay PSRE and PACN antibacterial efficacy: (i) The Gram-negative biofilm former anaerobic keystone pathogen *Porphyromonas gingivalis*, a well-known player in the development of periodontal disease and peri-implantitis; and (ii) the Gram-positive aerobic oral commensal *Streptococcus salivarius* [9].

## 2.2) Material and Methods:

### *2.2.1)* Chemicals and reagents used for purifications and analysis:

All chemicals and reagents that used were of analytical and chromatographic grade.

- The *Pelargonium sidoides* root extract (PSRE) was purchased from UNILAB, LP (Rockville, MD, USA) (extraction medium 50% methanol).
- From Sigma-Aldrich (Steinheim, Germany) used the following solvents and reagents:
  - (+)-catechin and (−)-epicatechin.
  - -hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox).
  - 2,2-azinobis (ethyl-2,3-dihydrobenzothiazoline-6-sulphonic acid) diammonium salt (ABTS).
  - Iron (III) chloride hexahydrate.
  - 2,4,6-tripyridyl-s-triazine (TPTZ).
  - Acetonitrile.
  - Methanol.
  - Epigallocatechin.
  - Epigallocatechin gallate.
  - Gallic acid.

- Quercetin.
- Delphinidin.
- Cyanidin.
- 1-butanol.
- Sephadex LH-20.
- Hydrochloric acid.
- Ferric ammonium sulphate.
- Phosphoric acid.
- Sodium acetate trihydrate.
- Potassium persulphate were purchased Fluka.

o From Carl Roth (Karlsruhe, Germany), 99.8% trifluoracetic acid.

o From Vilniaus degtine AB (Vilnius, Lithuania), 96% ethanol.

o The purified deionized water (18.2 mΩ/cm) was produced using the Millipore water purification system.

### *2.2.2)* Preparation of *Pelargonium sidoides* root extract (PSRE) and Proanthocyanidins from Extract (PACN):

o **For PSRE:**

- Two grams of root extract powder were resuspended in 10 mL of 60% methanol (Sigma, in ultra-pure water) and vortexed for 2 min to allow for a homogeneous powder dispersion (Figure. 2.)
- This solution was incubated for 2 h at 50 °C with agitation in the dark.
- The tube containing powder solution was collected and centrifuged at 2000 ×g (at room temperature) for 20 min to pellet unsolved powder. The supernatant was collected, 0.45 µm filtered to remove debris, covered with tinfoil, and stored at 4 °C until use.
- To prepare freeze-dried powder, the solution was first evaporated by speed vacuum (SCANVAC-Scan speed 32/40) under vacuum pressure (1.013 mbar) for 4 h; the final yield was approximately 50% (≈4 mL).
- Finally, the concentrated solution was solved with 10 mL of ultra-pure water, stored overnight at - 80 °C and then freeze-dried using a Scanvac machine (SCANVAC LABOGENE/COOL SAFE 55-4 with vacuum pump; T = -52 °C; P = 0.500 hpa) for 7 h.
- The obtained powder was stored at 4 °C until used for experiments; a fresh freeze-

dried powder was prepared prior to each experiment. The powder was then solvated in ultra-pure water and filtered through a 0.22 µm filter prior to use on bacteria.

**Figure 2:**

**Soulbility of *Pelargonium sidoides* root extract.**

- **For PACN:**
  - A 0.05g of PACN fraction powder was dissolved in 50 mL of ultra-pure water (Millipore, Vimodrone, Italy), vortexed for 2 min. to allow complete dissolution.
  - Filtered with a 0.22 µm filter. A fresh solution was prepared prior to each experiment and stored at 4 °C.

### *2.2.3)* Purification of PACN:

The purification methods were used as described by Hellström, J [10], as follow: in 200 mL of 50% methanol dissolve 4 g of PSRE powder, the solution was centrifuged at 2000 ×g for 20 min then the supernatant filtered through 0.45 µm nylon filters (Roth Gm.bH, Karlsruhe, Germany). By using gel adsorption over Sephadex LH-20, the solution was purified. The PACN was released from the gel with 70% aqueous acetone (500 mL) and concentrated under vacuum at 35 °C. The aqueous aliquot was freeze-dried.

- Acid/n-Butanol Hydrolysis of Extracts:

PACN was hydrolysed according to the method described by Porter et al. [11], as follows: for the extract [the powder of PACN extract was weighed to 0.05 g into a conical flask added 50 mL of 50% methanol, vortexed for 2 min and filtered by using 0.22 µm filters]. For the analysis: 6 mL of the n-butanol/HCl reagent (950 mL of n-butanol and 50 mL concentrated HCl), 1.0 mL of the extract, and added 0.2 mL of the iron reagent. The flask was connected under reflux in a boiling water bath for 60 min. Then, the solution was cooled and transferred to a volumetric flask and adjusted to 25 mL with the n-butanol/HCl reagent.

### *2.2.4)* Qualitative and quantitative analysis:

#### *2.2.4.1)* HPLC analysis:

- Chromatographic analysis was carried out using a Waters Alliance e2695 Separations Module equipped with a Waters 2998 PDA Detector (Milford, CT, USA).
- For the HPLC analysis of extracts:
  - Sample preparation: an accurate sample from powder extract (0.05 g) was placed into a conical flask with 50 mL of methanol soln. [1:1], vortexed for 2 min and then filtered through 0.22 µm filters and subjected to analysis.
  - The separation was performed at 25 °C on an ACE Excel 3 Super C18 analytical column (Aberdeen, Scotland) (250 × 4.6 mm, 3 µm). The mobile phase consisted of 0.1% TFA in deionized water (A) and acetonitrile (B). The gradient elution was as follows: 0-30 min, 15-30% B; 30-50 min, 30-60% B; 50-55 min, 60-90% B; and 55-60 min, 90-15% B. The injection volume was 10 µL and the flow rate was 0.5 mL/min. The detector was set in the 200-400 nm range. The chromatographic data were acquired and processed with Empower 3 software (Milford, CT, USA).
  - For the HPLC analysis of extracts after butanol hydrolysis, the separation was performed on an ACE Excel 5 Super C18 analytical column (Aberdeen, Scotland) (250 × 4.6 mm, 5 µm) at 25 °C. The mobile phase consisted of 4% Phosphoric acid in deionized water (A) and acetonitrile (B). The gradient elution was as follows: 0-10 min, 15-30% B; 10-15 min, 30-90% B; 15-17 min, 90-90% B; 17-18 min, 90-15% B; and 18-25 min., 15% B. The injection volume was 10 µL and the flow rate was 1 mL/min. The detector was set at the 550 nm. The chromatographic data were acquired and processed with Empower 3 software (Milford, CT, USA).
  - According to the analyte and reference compound retention time, as well as by comparing the UV absorption spectra of the reference compounds and analytes obtained with a diode array detector, chromatographic peak identification was carried out.

#### *2.2.4.2)* UPLC-ESI-MS conditions:

Phenolic compounds were separated using an Acquity H-class UPLC system (Waters, Milford, CT, USA) equipped with a Xevo triple quadrupole tandem mass spectrometer

(Waters, Milford, CT, USA). An electrospray ionization (ESI) source was used to obtain MS and MS/MS data. An Acquity BEH C18 column (50 × 2.1 mm, 1.7 µm) (Waters, Milford, CT, USA) was used for analysis. The column temperature was maintained at 40 °C. Gradient elution was performed with a mobile phase consisting of 0.1% water solution of formic acid (solvent A) and acetonitrile (solvent B), with the flow rate set to 0.5 mL/min. A linear gradient profile was applied with the following proportions of solvent A: initial 95%, 1 min. 80%, 7.5 min. 75%, 8 min. 0%, 10 min. 95%. Negative electrospray ionization was applied with the following settings: capillary voltage 2.5 kV, source temperature 150 °C, desolvation temperature 500 °C, desolvation gas flow 800 L/h, cone gas flow 20 L/h. MS spectra were recorded in the range of 80–2000 m/z.

#### *2.2.4.3)* **UPLC-MS/MS conditions:**

Sample analysis for the determination of the mean degree of polymerization was carried out on an Acquity UPLC system (Waters, Milford, CT, USA) equipped with a Quattro micro triple quadrupole tandem mass spectrometer (Waters, Milford, CT, USA). An electrospray ionization (ESI) source in negative mode was used to obtain MS/MS data. An Acquity HSS T3 column (50 mm × 2.1 mm, 1.8 µm) (Waters, Milford, CT, USA) was used for analysis. The column temperature was maintained at 30 °C. Gradient elution was performed with a mobile phase consisting of 0.1% water solution of formic acid (solvent A) and acetonitrile (solvent B), with the flow rate set to 0.25 mL/min. A linear gradient profile was applied with the following proportions of solvent A: initial 95%, 0.5 min 2%, 4.5 min 2%, 4.7 min 95%, 6 min 95%. Negative electrospray ionization was applied with the following settings: capillary voltage 3.0 kV, source temperature 150 °C, desolvation temperature 400 °C, desolvation gas flow 800 L/h. For the detection of procyanidin and prodelphinidin terminal and extension units following multiple reaction monitoring (MRM) transitions 287.1→125.4, 289.1→245.4, 303.2→125.1, 305.2→125.1 at five cone voltage energy values (50, 75, 85, 110 and 140 V for procyanidins and 55, 80, 110, 130 and 150 V for prodelphinidins) were used. The optimal collision energy for all transitions was 15 eV. Measured MRM peaks areas were used for calculation of mPD [12].

### *2.2.5)* **Determination of antioxidant activity:**

- Sample preparation: (0.05 g) of the extract powder was placed into a conical flask with 50 mL of 50% methanol, then filtered through 0.22 µm filters and subjected to analysis.

- According to the methodology described by [13], an ABTS·+ radical cation decolorization assay was applied. A volume of 3 mL of ABTS·+ solution (absorbance 1.00 ± 0.005) was mixed with 20 µL of the methanol extract of PSRE or PACN powder. A decrease in absorbance was at a wavelength of 734 nm after keeping the samples in the dark for 60 min. Antioxidant activity was expressed as Trolox equivalents (TE) in µmol/g DW of the extract.
- The ferric reducing antioxidant power (FRAP) assay was applied according to the methodology described by Benzie et al. [14]. A volume of 3 ml of the prepared FRAP reagent (consisting of TPTZ (0.01 M in 0.04 M HCl), FeCl3·6H2O (0.02 M in water), and acetate buffer (0.3 M, pH 3.6) at the ratio of 1:1:10) was mixed with 20 µL of the methanol extract of PSRE or PACN powder. An increase in absorbance was recorded after 60 min at a wavelength of 593 nm. Antioxidant activity was expressed as Trolox equivalents (TE) in µmol/g DW of the extract.

### *2.2.6)* Bacteria and the growth conditions:

- Two bacterial strains were tested in the experiments (*P. gingivalis* DSM 20709 and *S. salivarius* DSM 20067; DSZM, GmbH, Braunschweig, Germany). The bacterial strains were purchased from the (Deutsche Sammlung von Mikroorganismen und Zellkulturen).
- By using manufacturer's instructions for equilibrating lyophilized bacteria in specific media: brain heart infusion (BHI, Sigma Aldrich) supplemented with freshly prepared hemin and menadione (final concentration 5 mg/L and 0.5 mg/L, respectively).
- Tryptic soy agar (TSA) enriched with 5% sheep blood for *P. gingivalis*; tryptic soy broth (TSB, Sigma Aldrich) and TSA plates for *S. salivarius*.
- By using Anaerobox (Thermo Fisher Scientific, Monza, Italy) for anaerobic conditions, *P. gingivalis* was grown for 3 days at 37 °C.
- Once round-single colonies were formed, fresh broth-cultures were prepared prior to each experiment by dissolving 2–3 colonies in 30 mL of each specific medium, as previously described. Bacteria concentration was adjusted until $1 \times 10^5$ cells/mL by diluting in fresh media until the optical density was adjusted to ($OD_{600}$= 0.001) as determined by spectrophotometer (Victor, Packard Bell, Lainate, Italy).

#### *2.2.6.1)* PSRE and PACN antibacterial activity:

- PSRE and PACN were tested for their antibacterial activity at different concentrations from the original solutions prepared as described previously.

(i) PSRE water solution: 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08 and 0.09 g/mL.

(ii) PACN water solution: 0.01, 0.03, 0.05, 0.07 and 0.09 mg/mL.

- Individually, PSRE and PACN were mixed directly into each complete medium to obtain a final volume of 200 μL containing $1 \times 10^5$ exponentially growing bacteria/mL into sterilized 96 well-plate.
- Plates were then incubated at 37 °C for 2 days in anaerobic conditions for *P. gingivalis* and for 1 day in aerobiosis for *S. salivarius*, respectively. Anaerobic conditions were maintained for 2 days prior to test viability due to the longer growth time of *P. gingivalis*. In this way, both the tested strains were allowed to produce biofilm in a comparable manner prior to undergoing metabolic analysis [15].
- Bacteria viability was evaluated by the colorimetric Alamar blue assay (alamarBlue®, Thermo-Fisher, Waltham, MA, USA); briefly, after incubation, all the supernatant contained the planktonic cells of bacteria is removed carefully without touch surrounding area from the biofilm that built on the walls of each well in 96 plate. The ready-to-use solution of Alamar blue was added to each well by 100 μL and incubated for 4 h in the dark at 37 °C. After incubation, 100 μL were collected from each test specimens into 1.5 mL tubes, centrifuged to remove any residual debris and finally spotted to a new black-bottom 96-well plate. Fluorescence was recorded at 590 nm using a spectrophotometer. Wells containing only extract + Alamar blue were used as blanks while bacteria cultivated with each complete medium without the extract were considered as positive untreated controls and considered to have 100% viability.

### *2.2.7)* Statistical Analysis:

- The data were processed using Microsoft Office Excel 2010 (Microsoft, Redmond, WA, USA) and SPSS 20 (IBM, Armonk, NY, USA) software. For antibacterial efficiency evaluation, the experiments were performed in 16 replicates. Data were analyzed by the SPSS software (v20, IBM) using the one-way ANOVA and the Sheffè tests as post hoc. Significance was set at $p < 0.01$.

- The amounts of phenolic compounds were expressed as a mean ± standard deviation (SD) of three replicates. The statistical data analysis was evaluated by applying the ANOVA with Tukey HSD post hoc test. Differences were considered statistically significant when $p < 0.05$.

## 2.3) Results:

### *2.3.1)* Composition of Phenolic Compounds of PSRE:

Six phenolic compounds were identified and quantified in PSRE as follow per mg/g: catechin, epicatechin, epigallocatechin, epigallocatechin gallate, gallic acid, and quercetin Figure 3.

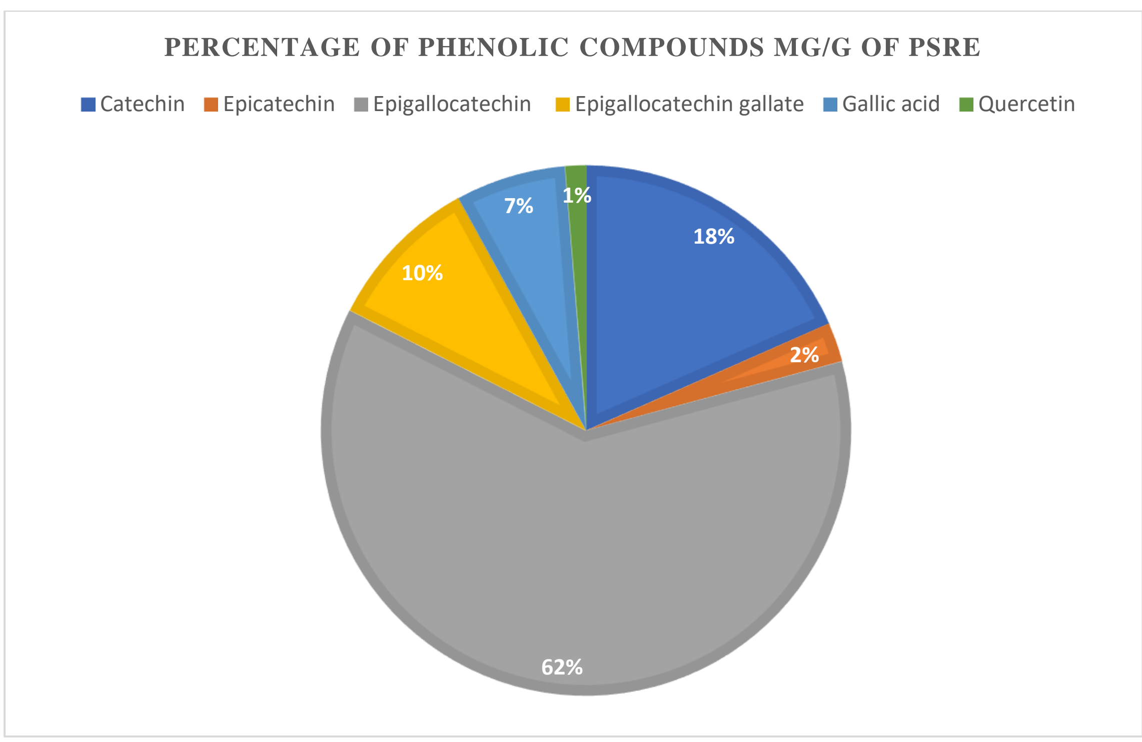



**Figure 3:** Percentage of phenolic compounds (mg/g) determined in *Pelargonium sidoides* root extract (PSRE).

All identified phenolic compounds were detected in the methanol eluate after purification of the PSRE extract, and as shown in Figure 3, Figure S1; Epigallocatechin (7.71 ± 0.15 mg/g, $p < 0.05$) was the foremost phenolic compound, and the amounts of other identified and quantified phenolic compounds were lower.

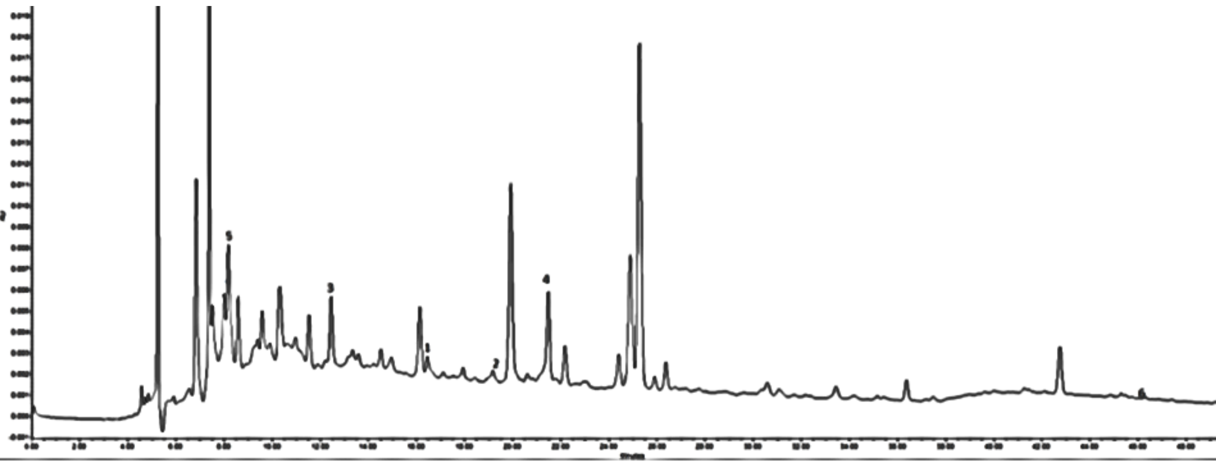


**Figure S1.**: HPLC phenolic profile of methanol extract of *Pelargonium sidoides* root extract ($\lambda$ = 280 nm). Numbers indicate the peaks of analytes: (1) catechin, (2) epicatechin, (3) epigallocatechin, (4) epigallocatechin gallate, (5) gallic acid, (6) quercetin. HPLC system: Waters Alliance e2695 Separations Module equipped with a Waters 2998 PDA Detector (Milford, USA). The column: ACE Excel 3 SuperC18 analytical column (Aberdeen, Scotland) (250 × 4.6 mm, 3 $\mu$m) at 25∘ C. The mobile phase: 0.1% TFA in deionized water (A) and acetonitrile (B). The gradient: 0–30 min, 15%–30% B; 30–50 min, 30%– 60% B; 50–55 min, 60%–90% B; and 55–60 min, 90%−15% B. The flow rate was 0.5 mL min−1, and the injection volume was 10 $\mu$L.

To present a more detailed view of the PSRE composition, it was analyzed using UPLC-MS/MS (data presented in Table 1).

**Table 1: List of phenolic compounds identified in *Pelargonium sidoides* root extract (PSRE) methanol fraction:**

| PSRE | Compound | [M-H]- m/z | product ions m/z |
|---|---|---|---|
| 1 | epicatechin gallate | 441 | 289 |
| 2 | Epigallocatechin gallate | 457 | 305 |
| 3 | catechin | 289 | |
| 4 | epicatechin | 289 | |
| 5 | epigallocatechin | 305 | 179, 221, 261 |
| 6 | prodelphindin dimer | 609 | 305, 423, 441, 483, 565, 591 |
| 7 | PD trimer | 913 | 303, 423, 533, 483, 559 |
| 8 | PD tetramer | 1217 | 955, 1133, 1155, 1064, 1144, 1133, 732, 661 |

| 9 | PD pentame | 1521 | 1421, 1283 |
|---|---|---|---|
| 10 | PD hexamer | 1825 | 609 |

A UPLC-ESI-MS system was applied for the identification of phenolics. Components were identified according to their retention time and a mass fragmentation spectrum that was compared to literary data. Compounds 1–10 were found in PSRE. Compound 1 represents a molecular ion [M − H] − at m/z 441 with a fragment ion at m/z 289, which is a typical mass in the negative mode of epicatechin. Compound 2 showed a molecular ion at m/z 457 and was identified as epigallocatechin-gallate due to the presence of fragment at m/z 305, as the result of a loss of galloyl moiety. Compounds 3 and 4 were identified as catechin and epicatechin, and their identities were confirmed by the standards. Compound 5 showed an ion m/z 305 and was identified as epigallocatechin; the fragment ion at m/z 261.760 was due to the loss of $CO_2$ [16, 17]. Compounds 6–10 were identified as derivatives of prodelphinidins.

### *2.3.2)* Composition of phenolic compounds of PACN:

For the identification of the composition of the phenolic compounds, PACN was separated from PSRE and collected as an acetone fraction (AF) by means of Sephadex LH-20. The freeze-dried PACN preparation (from AF) yielded 1.37 ± 0.07 g and comprised approximately 34.25% of the loaded PSRE. PACN was characterized by different degrees of polymerization, and therefore the application of reverse phase HPLC is very complicated and a colorimetric assay based on butanol/HCL hydrolysis was employed [11, 18]. After hydrolysis, the HPLC profiles of PSRE and a freeze-dried PACN sample revealed that all of the PACN can be ascribed to prodelphinidins as the peak detected in the chromatogram was that of delphinidin. The total amount of prodelphinidin (expressed as delphinidin equivalents) was 570.29 ± 9.49 mg/g, with 782.60 ± 6.50 mg/g and 9.87 ± 0.21 mg/g of PSRE, PACN and methanol eluate, respectively. The highest ($p < 0.05$) prodelphinidin content was found in PACN (Figure S2).

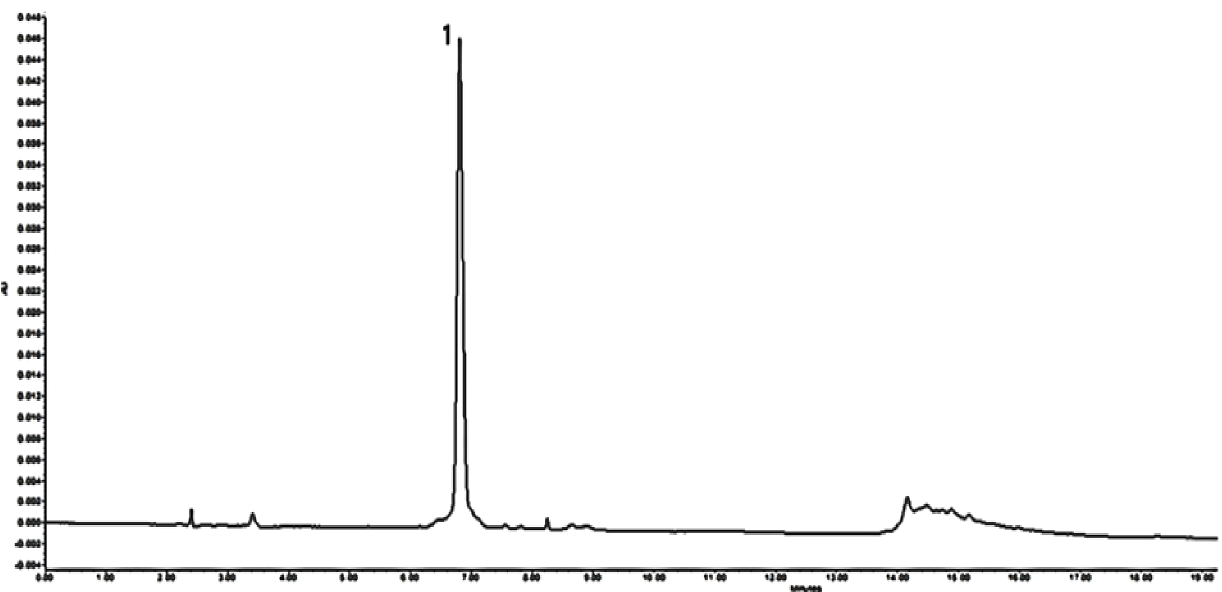



**Figure S2.**: HPLC chromatogram of prodelphinidins hydrolysed using an n-butanol/HCl reagent. ($\lambda$ = 550 nm). Numbers indicate the peaks of analytes: (1) delphinidin. HPLC system: Waters Alliance e2695 Separations Module equipped with a Waters 2998 PDA Detector (Milford, USA). Column: ACE Excel 5 SuperC18 (250 × 4.6 mm, 5 $\mu$m) at 25∘ C. The mobile phase: 4% Phosphoric acid in deionized water (A) and acetonitrile (B). The gradient: 0–10 min, 15%–30% B; 10–15 min, 30%– 90% B; 15–17 min, 90%–90% B; 17–18 min, 90%–15% B; and 18-25 min, 15% B. The flow rate: 1 mL min−1, and the injection volume was 10 $\mu$L.

To present a more detailed view of the PACN composition, it was analyzed using UPLC-MS/MS (data presented in Table 2).

**Table 2: List of phenolic compounds identified in PACN:**

| PACN | Compound | [M-H]- m/z | product ions m/z |
|---|---|---|---|
| **6** | PD dimer | 609 | 305, 423, 441, 483, 565, 591 |
| **7** | PD trimer | 913 | 303, 423, 533, 483, 559 |
| **8** | PD tetramer | 1217 | 955, 1133, 1155, 1064, 1144, 1133, 732, 661 |
| **9** | PD pentamer | 1521 | 1421, 1283 |
| **10** | PD hexamer | 1825 | 609 |

Compounds 6–10 were identified as derivatives of prodelphinidins. The characteristic fragmentation pathways for PACN are the quinone methide mechanism, heterocyclic ring

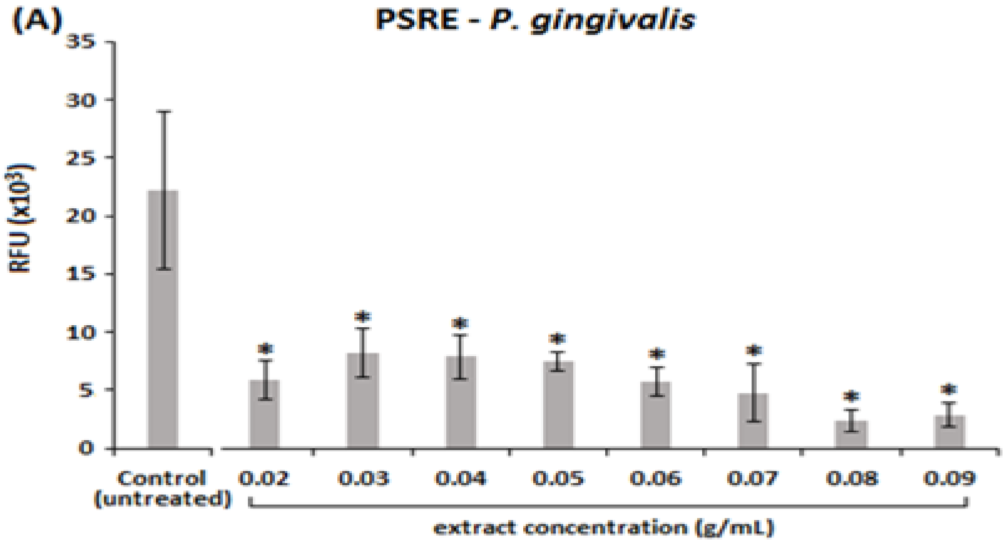
fission, and retro-Diels-Alder (RDA) fission with neutral losses of 168 Da [11]. These compounds produced m/z ions of 609, 913, 1217, 1521, 1825, respectively, with various fragment ions (Table 2) obtained by the loss of a galloyl and RDA fragments [17].

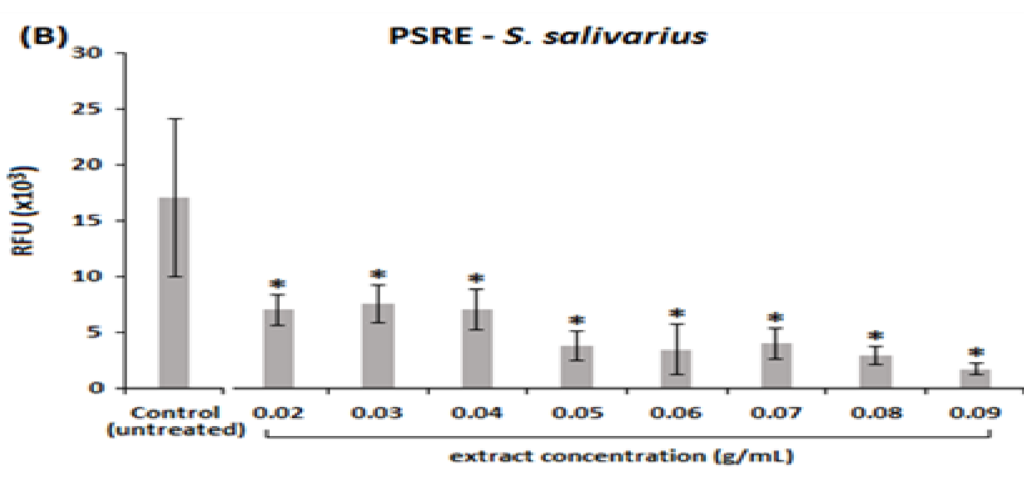
UPLC-MS/MS analysis was used for the estimation of the mean degree of polymerization (mDP) for the oligomers and polymers in Pelargonium sidoides root extract. The determination of mDP is based on the mMRM quantification of both terminal an extension units of the most of the common PACN subclasses—e.g., procyanidins (m/z 287 and 289 respectively) and prodelphinidins (m/z 303 and 305 respectively)—according to a method described by Engstrom et al. [19]. The calculated mean degree of polymerization for the analysed PACN sample was 6.0.

### *2.3.3)* Antibacterial activity of PSRE and PCANs:

- **For PSRE:**

The last aim of the study was to test the antibacterial properties of PSRE and PACN. The effect of a concentration range of PSRE and PACN solutions on the viability of a Gram-negative biofilm former periodontal keystone pathogen *P. gingivalis* and a Gram-positive aerobic commensal (*Streptococcus salivarius*) was evaluated by colorimetric Alamar blue assay. Bacteria cultivated with the fresh untreated medium were used as control.
Results related to PSRE antibacterial efficacy are reported in Figure 4. In general, PSRE extract was effective in reducing the viability of both *P. gingivalis* (Figure 4A) and *S. salivarius* (Figure 4B) in a significant manner in comparison with untreated control starting from the lowest 0.02 g/ml concentration (a-b, $p<0.01$, indicated by the *). Accordingly, all the increasing tested concentrations demonstrated high efficacy in reducing bacteria viability that resulted in a range between 12-36% for *P. gingivalis* and between 9-44% for *S. salivarius* as summarized in Figure 4C. However, no differences were noticed by comparing *P. gingivalis* and *S. salivarius* results; so, despite its high efficacy, PSRE extract did not show a selective activity towards pathogen or commensal strains.




(C)

*P. gingivalis* viability

| extract (g/mL) | viability (% vs cnt) |
|---|---|
| 0.02 | 26,200 |
| 0.03 | 36,927 |
| 0.04 | 35,212 |
| 0.05 | 33,443 |
| 0.06 | 25,911 |
| 0.07 | 21,398 |
| 0.08 | 10,693 |
| 0.09 | 12,674 |

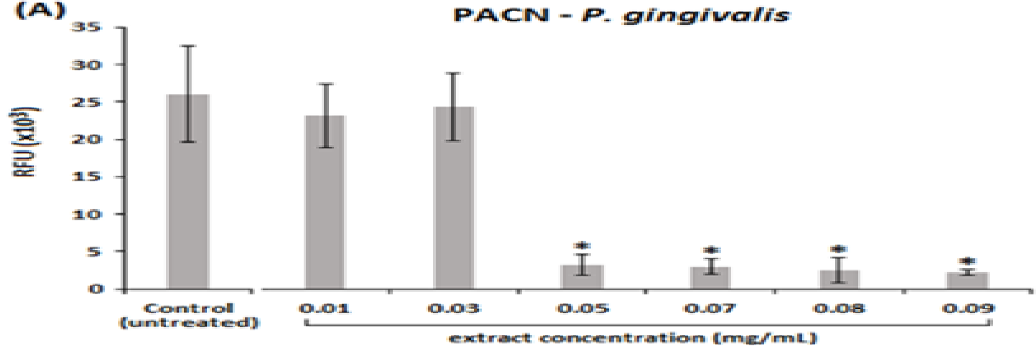
*S. salivarius* viability

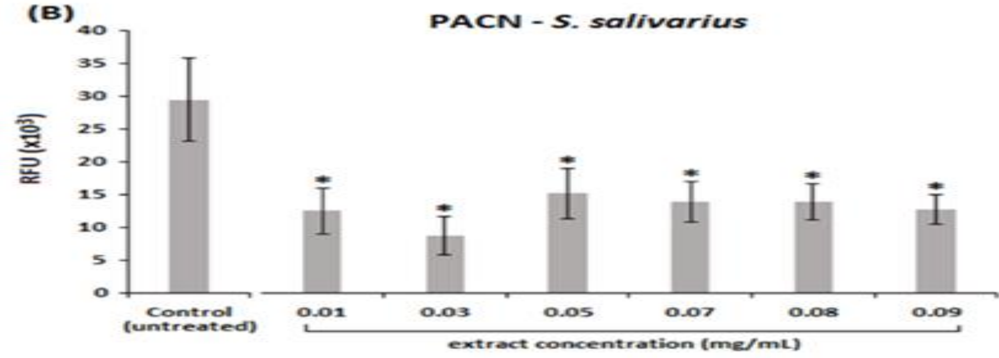
| extract (g/mL) | viability (% vs cnt) |
|---|---|
| 0.02 | 40,978 |
| 0.03 | 44,290 |
| 0.04 | 41,539 |
| 0.05 | 21,930 |
| 0.06 | 20,081 |
| 0.07 | 23,278 |
| 0.08 | 16,933 |
| 0.09 | 9,717 |

**Figure 4.** PSRE antibacterial activity. The extract at all tested concentrations (expressed as g/ml) induced a significant reduction of *P. gingivalis* (A) or *S. salivarius* (B) viability in comparison with untreated control ($p<0.01$, indicated by the *). Accordingly, assuming control as 100% viability, the surviving rate of *P. gingivalis* resulted between 12-36% (C, left panel) while *S. salivarius* between 9-44% (C, right panel). Bars represent means and standard deviations.

- **For PACN:**

Results related to PACN antibacterial efficacy are reported in Figure 3. As opposed to results obtained with PSRE extract, PACN treatment resulted in different effects on viability between *P. gingivalis* and *S. salivarius*. In fact, the lower 0.01 and 0.03 mg/ml concentrations were not sufficient to determine a significant reduction of *P. gingivalis* viability in comparison with control (Figure 5A, p>0.05); on the opposite, the same amount was effective in significantly decrease *S. salivarius* viability (Figure 5B, p<0.01, indicated by the *). However, when the concentrations were increased to 0.05-0.09 mg/ml, a strong decrease of *P. gingivalis* was observed (p<0.01 in comparison with control, indicated by the *) while *S. salivarius* values were almost stable. Accordingly, looking at the viability rate (Figure 5C), it was noticed that the increase of PACN in solution reduced *P. gingivalis* viability from ≈90% (0.01-0.03 mg/ml) to ≈10% (0.05-0.09 mg/ml), while *S. salivarius* viability was always in the range between 40-50%. Thus, the activity of PACN extract was less effective against the commensal *S. salivarius* viability; in fact, even if the viability of the bacteria was significantly reduced in comparison with untreated controls, the values have never fallen below 50%. Thus, PACN displayed a strong activity towards oral pathogen *P. gingivalis* while preserving the viability of the commensal *S. salivarius*.






(C)

*P. gingivalis* viability

| extract (mg/mL) | viability (% vs cnt) |
|---|---|
| 0.01 | 88,919 |
| 0.03 | 93,379 |
| 0.05 | 12,299 |
| 0.07 | 11,436 |
| 0.08 | 9,612 |
| 0.09 | 8,448 |

*S. salivarius* viability

| extract (mg/mL) | viability (% vs cnt) |
|---|---|
| 0.01 | 42,345 |
| 0.03 | 39,408 |
| 0.05 | 51,330 |
| 0.07 | 47,193 |
| 0.08 | 47,156 |
| 0.09 | 42,964 |

**Figure 5.** PACN antibacterial activity. A different and strain-selective activity was noticed for various concentrations (expressed as mg/ml). For *P. gingivalis* (A) only concentrations >0.03 mg/ml were effective in significantly reducing viability ($p<0.01$, indicated by the *) that resulted in 10% (as summarized in C, left panel). On the opposite, all tested concentrations determined a significant reduction of *S. salivarius* viability (B, $p<0.01$, indicated by the *) but surviving rate values always ranged between 40-50% (C, right panel). Bars represent means and standard deviations.

## 2.4) Discussion:

The discovery of new infection treatment strategies in the race with increasing bacterial virulence and resistance is among the most important goals of contemporary science and healthcare. In addition to this, the strategy is expected not to destroy the part of human microbiota that positively contributes to skin, oral or intestinal physiology as well as the immune system. The constantly increasing average age of the society of industrialized countries is turning the lack of efficient solutions in this field into a major medical problem. This challenge forces scientists and pharmacists to re-evaluate the significance of complex plant-derived antibacterial substances with mild yet multi-targeted activity. Multiple antibacterial properties of PSRE are well known in both folk and modern medicine and are usually attributed to the phenolic compounds of the herbal preparation. Literature evidence indicates that phenolic content of PSRE is mainly comprised of

coumarins, phenolic acids, flavonols, flavan-3-ols and oligomeric PACN [14, 17, 20]. The increasing evidence about strong antioxidant and anti-inflammatory but yet immunity-stimulating antibacterial activity of PACN [20] suggests the compounds could be potential candidates to replace or add to antibiotics therapy. Our study was dedicated to PACN from PSRE preparation known for mild anti-infection efficiency and aimed to isolate, analyze and test the compounds for antioxidant ant selective antibacterial action.

Because of the polymeric nature of PACN, the application of reverse phase HPLC is very complicated and the content of PACN was determined by colorimetric assay based on butanol/HCL hydrolysis. The experiments revealed that PACN isolated from PSRE was composed of prodelphinidins, the phenolic compounds characterized by 3,4,5-trihydroxyphenyl groups that are reported to be responsible for antioxidant and antibacterial activity [20, 21].

Oxidant activity is implicated in the pathways of the etiopathogenesis of various diseases, such as cancer and cardiovascular, neurodegenerative, pulmonary, ocular disorders, and many others [22]. Thus, the search for efficient antioxidant compounds that could potentially be used for the prevention and treatment of these disorders or as a therapy-additive is of particular importance. Pereira et al. (2015) determined the superoxide, nitric oxide and peroxyl radical, DPPH scavenging and reducing activities of a *Pelargonium sidoides* commercial tincture [23]. The first candidates for antioxidant activity in PSRE are expected to be PACN, as there are numerous reports about the very potent antioxidant effects of these compounds extracted from different sources [24, 25]. The significantly increased free radical scavenging activity of the PACN fraction compared to total PSRE preparation indicate that in pure PACN fraction, the activity is more concentrated and unmasked from other substances of PSRE. This result confirms that the compounds are suitable for the suppression of tissue-deteriorating inflammatory conditions. Additionally, besides the direct effect of preventing free radical-induced molecular injury, antioxidative effects are responsible for the induction of signal cascades supporting innate immunity, preventing cancerogenesis as well as autoimmune disorders [22, 26]. Moreover, antioxidant activity can be related to tissue regenerating properties enhancing the anti-stress ability of human mesenchymal stem cells, stimulating intracellular self-renewal pathways and preventing cell senescence [22, 27, 28]. One of the most widely-spread infectious diseases that does not have an effective treatment strategy is periodontal disease. Periodontitis results from the unbalanced interaction between a subgingival biofilm and the host immune response [22, 28]. Similarly, bacterial infection is

considered to be the most important factor for implant failure [9]. Changes in biofilm composition are thought to disrupt homeostasis between the host and subgingival bacteria, thus resulting in periodontal/peri-implant tissue damage. In this scenario, some strains are strongly related to periodontal/peri-implant disease, whereas others are associated with healthy teeth and gums. Accordingly, it must be considered that, in the oral microenvironment, bacteria continuously interact with each other; therefore, the balance between the healthy and the diseased state should be considered to be a dynamic phenomenon where changes in ecology in the oral niche result in a shift towards the healthy or diseased state. Therefore, an ideal long-term antibacterial agent should be effective against putative pathogens and safe for commensals in order to preserve the balance. The most interesting discoveryof the study is that PACN at a certain concentration range exerts strain-selective antibacterial action, killing *P. gingivalis* much more efficiently than *S. salivarius*. This is a very important finding since S. salivarius is considered to be a beneficial bacterial strain [28], while *P. gingivalis* is recognized as a pathogen [29, 30]. This strain-specific activity shown by the PACN preparation is very promising in terms of meeting the aim of selectively reducing pathogen viability within a complex microenvironment without compromising beneficial bacteria such as S. salivarius. To the best of our knowledge, this is the first case reporting such strain-selective activity of PACN. The antibacterial action of PACN is attributed to biofilm disrupting properties by interfering with a N-acylhomoserine lactone-mediated quorum sensing of the bacteria [31, 32]. PACN have also been shown to compromise adhesion to host cells by mimicking cell surface signalling [33] and lipopolysaccharide (LPS) binding [31]. LPS binding might lead to the destabilization of the outer membrane of the bacteria as reported in the study dedicated to the innate immunity protein lactoferrin action mechanism. Thus, LPS binding might be related not only to host protection against bacterial attachment and LPS-induced toxicity, but also to making the membranes of the pathogens more permeable and their inner systems more vulnerable. However, the exact mechanism of selective antibacterial activity of PACN from PSRE is yet to be determined, and this might provide a key to the new therapeutic strategy. The most important feature of a good anti-infection therapeutic candidate is that it does not exert toxicity against the cells of the host. An in vivo study performed with rats revealed that PACN are non-toxic for gastric mucosal cells [34]. The antioxidant activity of the compounds, confirmed also in this study, should supposedly contribute to the reduction of inflammation and regeneration of the infected tissue. This adds to the value of PACN

from PSRE as a possible therapeutic agent for the treatment of infections. However, more comprehensive studies on various cell and tissue types as well as prolonged treatment tests should be done to confirm the biocompatibility of PACN.

## 2.5) References.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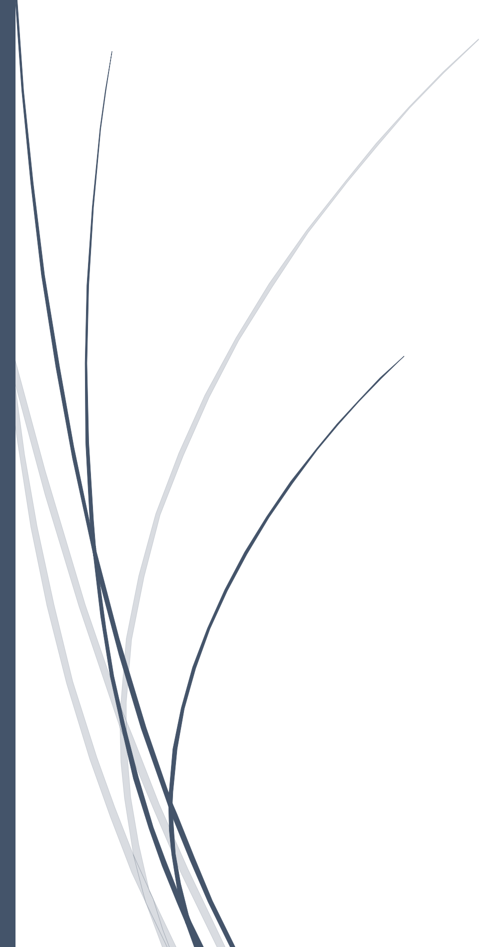
# Chapter 3:

# Polyphenols extract from *Padina pavonica* algae for surface functionalization of bioactive glasses: physico-chemical characterization, biological response and in situ reduction of metal ions



THIS CHAPTER OF THE THESIS IS RELATED TO THE WORK DONE BY THE COOPERATION WITH DEPARTMENT OF APPLIES SCIENCE AND TECHNOLOGY, POLITECNICO DI TORINO, ITALY AND DEPARTMENT OF AGRICULTURAL AND ENVIRONMENTAL SCIENCES, UNIVERSITÀ DEGLI STUDI DI MILANO, ITALY.

THE RESULTS OBTAINED HAS BEEN PUBLISHED:

**Asmaa Sayed Abdelgeliel**, Sara Ferraris, Andrea Cochis, Sara Vitalini, Marcello Iriti, Hiba Mohammed, Ajay Kumar, Martina Cazzola, Wesam M. Salem, Enrica Verné, Silvia Spriano and Lia Rimondini. Surface Functionalization of Bioactive Glasses with Polyphenols from *Padina pavonica* Algae and In Situ Reduction of Silver Ions: Physico-Chemical Characterization and Biological Response. Coatings 2019, 9, 394; doi:10.3390/coatings9060394.

## 3.1) Introduction:

### *3.1.1)* Red Sea *Padina pavonica* algal extract:

Red Sea algae characteristics and bioactivities are less investigated and just few information regarding their particular activities were communicated. In the coral reefs of Red Sea, marine algae are exposed to many defiance including space competition high salinity, high water temperatures in addition to intensive sun radiation. Such drastic circumstances have forced these organisms to evolve various adaptational possibilities in order to be able to survive; this adaptability gave rise to the development of wealthy algal bioactive compounds [1]. Red Sea was first explored in the 18th century when the 1st algal record was published by Strand [2]. Then, the first considerable algal gathering was made by Forsskal in 1775 during his journey to Arabia and Egypt where new specimens were described and investigated by botanists then revised by Borgesen in 1932. Consequently, more than 500 benthic algal taxa were discovered from the Red Sea. The northern and central areas of the Red Sea are characterized by seasonally abundance of small brown, filamentous green, and tuft-forming (calcareous-coralline) red algae in the shallow coral reefs. While the southern areas of Red Sea are characterized by the

abundance of perennial brown algae, like *Hormophysa*, *Cystoseria* and *Sargassum* that present over shallow hard substrates. Usually, the hard substrates aremainly covered by macroalgae where these areas appear very turbid due to coral growth. Unfortunately, there is no detailed information or clear background about the conditions and situations of the Red Sea algal communities, but it is known that algal reefs in Farasan Islands might be built under low energy environment by calcareous algae on rugged deposits at depth between 2 and 4 m [3].

The brown alga *Padina pavonica* belongs to the family Dictyotaceae, order Dictyotales, subclass Isogeneratae, class Phaeophyceae. There are limited data found on the chemical composition of the algae from the genus *Padina*. For the fatty acids, containing 14 – 22 carbon atoms have been identified in different *Padina* species. Significant differences in the sterol composition were found within the different *Padina* species. Fucosterol predominated in *Padina gym-nospora* and no cholesterol was found. Fucosterol was also the mainsterol in *Padina crassa*. In *Padina vickersiae* (from Senegal waters and in *Padina pavonica* from the Aegean Sea, themain sterol was cholesterol. The biogenetic precursorof fucosterol, 24-methylene cholesterol, was found in significant amounts in *Padina vickersiae* and in *Padina gymnospora* from the Qatar coast. In the Mediterranean *Padina pavonica* the main sterol appearedto be fucosterol instead of cholesterol. Several oxidised sterol derivatives have beenfound in some *Padina* species, 24-hydroperoxy-24-vinyl-cholesterol in *Padina pavonica*, 7-ketocholesterol in *Padina tetrastromatica* and 7α-hydroxy-fucosterol in *Padina crassa* [4].

Few terpenoids have been found in *Padina* species such as; Halogenated terpenoids, Lo-liolide , Hexa-hydrofarnesy lacetone and Dimethyl sulfide and dimethyl-β-propiothetin. Pigments were investigated in *Padina pavonica*. The characteristic brown algal polysaccharides (alginates and laminarans) were found in *Padina pavonica* [4].

**Figure 1: *Padina pavonica* Temniskova et al., 2008 [5].**

Marine algae have been proved to be able to provide valuable impacts in therapeutics and pharmaceuticals as a natural renewable origin, wealthy of a variety of bioactive compounds [6]. In particular, the algal polysaccharides, proteins, polyunsaturated fatty acids, pigments as well as polyphenols such as flavonoids can serve as bioactive bases of novel medical products [7]. Between them, *Padina pavonica* is a famous genus of marine algae that demonstrated antioxidant properties due to its wealth of polyphenols [8]. Previous literature demonstrated that some algal-derived phytochemicals including polyphenols and flavonoids exhibited antioxidant, anti-inflammatory as well as antimicrobial activities [9]. These antioxidant activities rely on the polyphenol's potential in reducing the production of reactive oxygen species (ROS) through inhibiting oxidases, decreasing the superoxide production, ameliorating mitochondrial oxidative process and inhibiting the formation of oxidized low-density lipoprotein [10]. From the antibacterial point of view, flavonols displayed remarkable activity against several Gram-positive bacteria, such as Staphylococcus aureus as well as Gram-negative ones [11]. The above-mentioned antibacterial effect is due to the polyphenols antioxidant activity that causes damage of the cell membrane and leakage of the biomolecules into the bacteria cell [12-14].

### *3.1.2)* Bioactive glasses:

The need for replacing damaged parts of the body in order to restore their physiological functionality has always been the driving force which has supported research into the discovery and the design of new materials able to perform this task as efficiently as possible. Actually, Bioglass® represents the first example of a biomaterial belonging to the third generation, because of the biological role played by its ionic dissolution products, directly released in the physiological environment, as well as its capability to strongly bond the host tissue (primarily bone) with the formation of an interfacial calcium phosphate layer. The optimization process of the glass composition led to the final choice of the so-called 45S5 formulation, 45% $SiO_2$–24.5% $Na_2O$–24.5% CaO–6% $P_2O_5$ (wt %), characterized by high amounts of $Na_2O$ and CaO, as well as a relatively high $CaO/P_2O_5$ ratio that make the surface of the material very reactive in a physiological environment [15].

Bioactive glasses (BGs) are biocompatible materials, currently applied for rapid induction of bone tissue regeneration due to their tailorable chemical composition that is able to promote bone healing and repair. This process is promoted by BGs osteoconduction and osteoinduction properties: once a BG is introduced into the defect, an immediate and

continuous dissolution of critical concentrations of soluble P, Ca, Si, and Na ions stimulates the self-renewal healing of the healthy bone surrounding the defect [16, 17]. In particular, once the BG comes into contact with the physiological environment, it starts to release $Na^+$and $Ca^{2+}$ ions and to exchange $H^+$, thus forming a hydrated silica gel on the surface. This gel turns into an amorphous $CaO$-$P_2O_5$-$SiO_2$ layer with a continuous consumption of $Ca^{2+}$ and $PO_4^{3-}$, and subsequently crystallizes into a hydroxycarbonate apatite (HCA) layer through constant incorporation with $Ca^{2+}$, $PO_4^{3-}$, $OH^-$, and $CO_3^{2-}$ [18, 19]. The growing HCA layer provides an ideal environment for osteoblasts colonization, proliferation, and differentiation [20, 21].

Another attractive broad-range antibacterial treatment adopted as an alternative to conventional antibiotics is represented by inorganic metal ions and nanoparticles. The principle advantages of the ions' use are their broad range activity toward both Gram-positive and Gram-negative bacteria, as well as their ability to avoid most of the resistance mechanism exploited by pathogens to counteract drug activity. Amongst the large class of antibacterial metal ions, silver (Ag) is probably the most used and characterized as its wide range of antibacterial e
ects has been widely demonstrated. In particular, when a biomaterial surface is coated by silver nanoparticles (AgNPs), these particles provide better surface contact between Ag and the microorganism. As such, the cell membrane is impaired, allowing AgNPs to invade the cell causing oxidative stress. As a consequence, DNA damage, genotoxicity, and chromosomal abnormality occur, thus leading to apoptosis [22].

## 3.2) Materials and methods:

### 3.2.1) *Padina pavonica* macroalgae selection, collection, and storage:

*Padina pavonica (P. pavonica)* macroalgae were collected by hand picking from the Red Sea in Hurghada, Egypt. Then, healthy algae samples were immediately cleaned of epiphytes and extraneous matter, and necrotic algae were discarded. Specimens were washed thoroughly with sterile distilled water, air dried, cut into small pieces, and then strongly pressed in a tissue grinder until obtaining a fine powder (IKA A 10, IKA®-Werke GmbH & Co. KG, Staufen im Breisgau, Germany).

### *3.2.2)* Determination of polyphenols, carotenoids, and chlorophylls from *P. pavonica* extract:

Polyphenols were extracted and characterized from the *P. pavonica* powder using solvent technique (95% ethanol, v/v in ultrapure water) and the colorimetric Folin–Ciocalteau method, respectively [23–25]. Briefly:

- Algae powder was added to the ethanol solution in a ratio of 1:50 (w/v).
- Then, the solution was heated to 60 °C for 1 h under continuous stirring (120 rpm).
- Afterward, the solution was 0.45-µm filtered to remove unsolved debris and ethanol was fully evaporated into an incubator (60 °C, 2 h).
- Finally, the obtained extract was resuspended in ultrapure water, freeze-dried (using a Scanvac CoolSafe 4, from LaboGene, Lillerød, Denmark) and stored at -20 °C until use.

Total polyphenol content (TPC) of the powdered algae was determined calorimetrically by the Folin–Ciocalteau method with slight modifications. Briefly:

- 0.5 mL of the ethanolic solution was added to 2.5 mL of 10% Folin–Ciocalteau reagent, previously diluted with distilled water.
- After 3 min, 2 mL of 7.5% sodium carbonate solution was added.
- The mixture was incubated in the dark for 1 h at room temperature.
- The absorbance was measured at 765 nm using a UV-Vis spectrophotometer (Jenway 7205, Cole-Parmer, Sta_ordshire, UK).
- A calibration curve was prepared with gallic acid standard solution at various concentrations (10 to 100 mg/L).
- The results are expressed as mg gallic acid equivalent (GAE)/g dry weight (DW). Total carotenoid ©, chlorophyll-a (Chl-a) and chlorophyll-b (Chl-b) contents of the extracts were determined spectrophotometrically (Jenway 7205, Cole-Parmer, Sta_ordshire, UK) according to Sumanta et al. [24].

### *3.2.3)* Specimen preparation and physico-chemical characterization:

#### *3.2.3.1)* Specimen preparation:

- The bioactive glass **(CEL2)**, was prepared by the melt molar composition of 45% $SiO_2$, 3% $P_2O_5$, 26% CaO, 7% MgO, 15% $Na_2O$, and 4% $K_2O$ and quenching route to obtain the bulk form.
- Glass bars were then cut into 2-mm thick slices and polished with SiC abrasive papers up to 4000 grit.

- Polished specimens were gently washed with ultrapure water prior to undergoing surface functionalization following a procedure prior described by Vernè et al. [26, 27]. Briefly, specimens were first washed with acetone in ultrasonic bath for 5 minutes and then with ultrapure water for 5 minutes in ultrasonic bath, 3 times.
- After this washing step, glasses were functionalized using a 1 mg/mL or 5 mg/mL *P. pavonica* polyphenols solution (5 mL/specimen, resuspended in ultrapure water) by soaking technique 3 h at 37 °C in the dark **(CEL2 + pad)**.
- At the end of the soaking step, samples were gently washed twice in ultrapure water and dried in a laminar flow cabinet.
- Specimens were soaked 1 h at 37 °C in a 0.005 M silver nitrate (AgNO3) aqueous solution in the dark prior to being finally washed in ultrapure water, to achieve in situ reduction of silver nanoparticles (AgNPs) on the surface of functionalized glasses previously grafted with 1 mg/mL of *P. pavonica* extract **(CEL2 + pad + Ag)** [28].

#### *3.2.3.2)* Specimens surface characterization:

- To determine the total adhered phenol content, The Folin–Ciocalteau test was performed on the functionalized specimens [29].
- To investigate the presence of molecules on the glass surfaces, means of X-ray photoelectron spectroscopy (XPS; PHI 5000 VersaProbe, Physical Electronics, Chanhassen, MN, USA) were used on samples functionalized with the 1 mg/mL solution before and after in situ reduction of AgNPs.
- Both survey spectra (for the determination of the chemical composition) and high-resolution spectra of the most important elements (C, O, and Ag to determine specific functional groups as well as the chemical state of silver) were acquired.
- The precipitation of AgNPs after in situ Ag reduction was investigated by means of FE-SEM equipped with an EDS camera (FE-SEM-EDS, Supratum 40, Carl Zeiss, Oberkochen, Germany) after Pt surface sputter coating.

### *3.2.4)* Antibacterial evaluation:

#### *3.2.4.1)* Strain:

- The orthopedic-infections-related, multi-drug resistant (MDR) certified biofilm former strain *S. aureus* (SA, ATCC 43300, purchased from the American Type Culture Collection, Manassas, MA, USA) was used to assay the specimens' antibacterial activity.
- SA was cultivated into selective blood agar medium (Sigma Aldrich, Milan, Italy); bacteria were cultivated at 37 °C until round colonies formed on the agar surface.

- Plates were maintained at 4 °C prior to experiments.
- Fresh broth-cultures were prepared prior to each experiment by dissolving some colonies in 10 mL of Luria Bertani (LB) broth culture (Sigma Aldrich, Milan, Italy). Finally, bacteria concentration was adjusted until $1 \times 10^5$ cells/mL by diluting in fresh media until the optical density of 0.001 at 600 nm was reached as determined by a spectrophotometer (Victor, Packard Bell, Lainate, Italy).

### *3.2.4.2)* **Biofilm formation:**

- Sterile specimens were gently paced into a 12 multi-well plate using sterile tweezers avoiding any surface damage.
- Each specimen was submerged with 1 mL of the broth bacteria culture prepared as described in Section 3. 2. 5.1.
- The plate was incubated for 90 minutes in agitation (120 rpm) at 37 °C to allow the separation between adherent biofilm cells and non-adherent floating planktonic cells (separation phase) [30, 31].
- Afterwards, supernatants containing planktonic cells were removed and replaced with 1 mL fresh media to cultivated surface-adhered biofilm cells (growth phase). Biofilms were grown at 37 °C for 1 to 3 days prior to evaluations.

### *3.2.4.3)* **Metabolic evaluation:**

At each time-point, bacterial biofilm metabolic activity was evaluated by the colorimetric Alamar blue assay (alamarBlue®, Life Technologies, Milan, Italy) following the manufacturer's instructions. Briefly, specimens were gently moved to a new 12 multi-well plate and washed 3 times with sterile phosphate buffered saline (PBS, from Sigma, Milan, Italy) to remove non-adherent cells; then, specimens were submerged with 1 mL of the ready-to use Alamar blue solution and the plate was incubated at 37 °C in the dark for 5 h. Finally, 100 µL of supernatant was collected from each well, transferred to a black-bottom 96-well plate and the fluorescent signal was detected using a spectrophotometer (Victor, Packard Bell, Lainate, Italy) at a 590 nm wavelength. Results are expressed as relative fluorescence unit (RFU).

### *3.2.5)* Cytotoxicity evaluation:

#### *3.2.5.1)* Cells:

- Human primary osteoblasts progenitors (hFOB 1.19, CRL-11372, ATCC, Manassas, MA, USA) were selected as test cells to evaluate specimens' cytocompatibility in vitro.
- hFOB cells were cultivated in MEM/F12 mix medium (50:50, Sigma, Milan, Italy) 10% fetal bovine serum (FBS, Sigma), 1% antibiotics, and 3 mg/mL neomycin (G418 salt, Sigma, Milan, Italy).
- Cells were cultivated until 80–90% confluence, detached with trypsin/ethylene diamine triacetic acid solution (trypsin/EDTA, Sigma, Milan, Italy) and used for experiments.

#### *3.2.5.2)* Direct metabolic evaluation:

Cells were cultivated in direct contact with the specimens' surface to reveal eventual toxic compounds on the coating [32]. Sterile specimens were gently placed into a 12 multi-well plate using sterile tweezers, avoiding any surface damage. Then, $2 \times 10^4$ cells/specimens were dropwise (100 µL) seeded directly onto specimens' surfaces and allowed to adhere for 2 h; afterwards, 1 mL of fresh medium was gently spotted into each well to fill specimens. Cells were cultivated for 1 to 3 days onto specimens' surfaces and viability was evaluated at each time point using the Alamar blue assay (alamarBlue®, Life Technologies, Milan, Italy) as previously described in Section 3.2.4.2. Results are expressed by means of RFU.

### *3.2.6)* Statistical analysis of data:

Data were analyzed using SPSS software (v25, IBM, New York, NY, USA) by means of one-way ANOVA followed by the Tukey' test as a post hoc analysis. Significance level was set to $p < 0.05$.

## 3.3) Results:

### *3.3.1)* Polyphenols, carotenoids, and chlorophylls quantification from *P. pavonica* extract:

Prior to undergoing CEL2 surface functionalization, the total polyphenol content in *P. pavonica*

extract was evaluated by means of the Folin–Ciocalteau test to determine whether the selected source was sucient to provide an adequate bulk of polyphenols. This step was performed as natural extracts often differ considerably from each other in terms of polyphenols content due to the different geographical origin. So, *P. pavonica* extract was

expected to differ from previous sources of polyphenols were previously proposed [25]. Results confirmed that the selected *P. pavonica* algae was rich in polyphenols as the total amount resulted in 75.6 mg GAE/g DW (the polyphenols in the extract have the same redox activity of a gallic acid solution: 75.6 mg GAE/g in distilled water). By the same analysis, E/we determined that the carotenoids amount was quantified in 0.9 µg/g DW and that Chlorophyll b µg (Chl-b) was the most abundant pigment in *P. pavonica* extract (129.6 µg/g DW).

### *3.3.2)* Specimens' physico-chemical characterization:

After analyzing the chemical features of the raw extract, we functionalized the bioactive CEL2 glass. The success of the entire procedure is based on different parameters that start from the extract stability that can be affected by pH variations. Accordingly, the pH of the applied *P. pavonica* solutions (1 and 5 mg/mL) was evaluated before and after the soaking step; the results are summarized in Table 1.

**Table 1: *Padina pavonica* solutions pH evaluation before (pad) and after (CEL2 + pad) soaking.**

| Sample | pH |
|---|---|
| pad (1 mg/ml) | 6.76 |
| pad (5 mg/ml) | 7.44 |
| CEL2 + pad (1 mg/ml) | 7.53 (± 0.06) |
| CEL2 + pad (5 mg/ml) | 8.28 (± 0.04) |

The starting pH of the *P. pavonica* solution was close to seven, which is less acidic than that recorded for other natural extracts such as gallic acid, grape polyphenols, and tea polyphenols that we previously investigated [25].As a further macroscopic confirmation of the extract stability, no color changes were recorded, nor for solutions, nor for samples after algae functionalization, thus suggesting that no significant changes in the pH occurred due to bioactive glass contact.

Once the surface functionalization was completed, the quantification of total polyphenols onto the CEL2 surface was verified by means of the Folin–Ciocalteau test. The results are reported in Table 2.

**Table 2: CEL2 surface polyphenols amount after functionalization.**

| Sample Polyphenols (GAE mg/mL) | Sample Polyphenols (GAE mg/mL) |
|---|---|
| CEL2 + pad (1 mg/mL) 0.001 | CEL2 + pad (1 mg/mL) 0.001 |
| CEL2 + pad (5 mg/mL) 0.001 | CEL2 + pad (5 mg/mL) 0.001 |

As far as functionalized glass samples are concerned, about 0.001 mg/mL GAE was quantified by the Folin–Ciocalteau test. The Folin–Ciocalteau test is commonly used for the quantification of the total amount of polyphenols in solutions.

Different from polyphenols functionalization, surface darkening was evidenced after AgNPs in situ reduction. As a confirmation of this macroscopic evidence, the atomic percentages of elements on the surface of bare CEL2, CEL2 + pad 1 mg/mL, and CEL2 + pad 1 mg/mL + Ag obtained by XPS survey analysis are reported in Table 3.

**Table 3: Atomic percentages of elements from XPS survey analyses:**

| Element | CEL2 | CEL2 CEL2 + pad (1 mg/mL) | CEL2 + pad (1 mg/mL) + Ag |
|---|---|---|---|
| O | 43,3 | 46,9 | 35.1 |
| C | 36,9 | 28 | 41.9 |
| Si | 13,5 | 12 | 8.0 |
| Na | 2,1 | 2 | 2.0 |
| Ca | 1,8 | 3,7 | 3.9 |
| Al | 1,4 | - | - |
| Mg | 0,9 | 7,5 | 4.8 |
| Ag | - | - | 3.3 |

The appearance of silver was clearly detected after in situ reduction, confirming the surface ability to facilitate Ag precipitation; this behavior and the amount of silver are in accordance with the results we previously obtained on the same glass functionalized with gallic acid, grape, and tea polyphenols [28]. The high-resolution spectra of carbon and oxygen regions for bare and *P. pavonica*-polyphenols-functionalized CEL2 samples are reported in Figure 1.

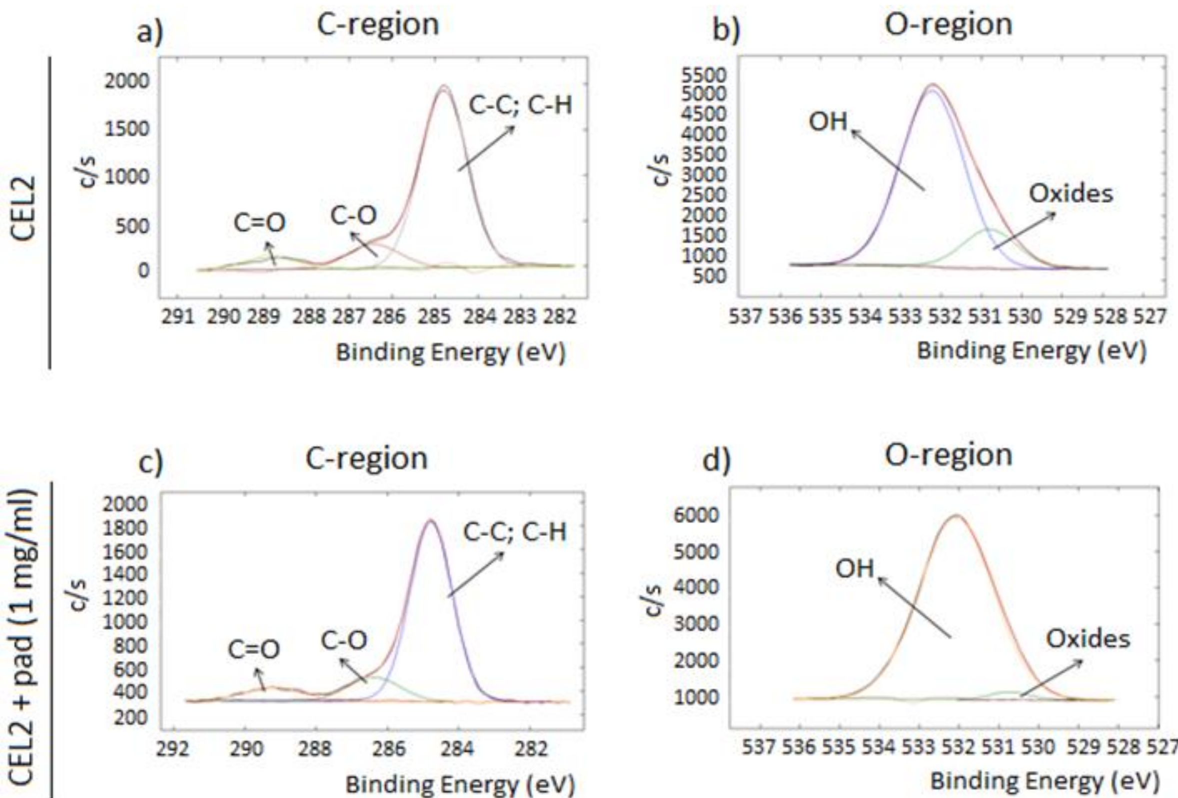



**Figure 1.** High resolution XPS spectra of (a,c) carbon (C-region) and (b,d) oxygen (O-region) for bare (CEL2) and *P. pavonica* extract grafted (CEL2 + pad (1 mg/mL) CEL2 specimens.

Once AgNPs were introduced into the CEL2 surfaces containing polyphenols, the typical Ag signal was clearly detected by high resolution spectra as a confirmation of the corrected co-grafting between *P. pavonica* polyphenols and AgNPs. The high-resolution spectrum of silver detected on CEL2 pad (1 mg/mL) +Ag is reported in Figure 2.

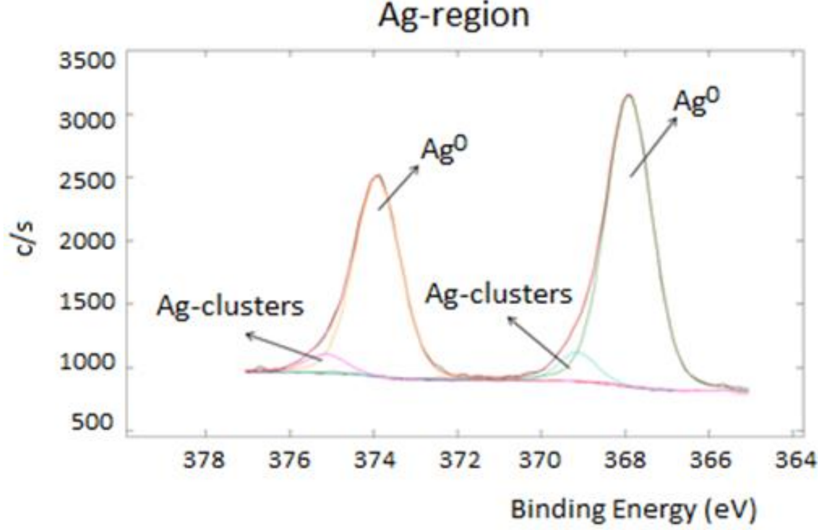



**Figure 2:** High resolution XPS spectrum of the Silver region for CEL2 + pad (1mg/ml) +Ag.

The main doublet was detected at 367.92–373.92 eV, close to the typical binding energy of metallic silver [33, 34]; these findings are in line to the hypothesis of an in situ reduction induced on the glass surface by algae polyphenols, in a similar manner as previously observed on bioactive glasses functionalized with natural polyphenols [30]. Then, a second small doublet was detected at 369.15–375.15, which can be probably associated with the presence of silver clusters [33]. To verify this hypothesis, FE-SEM images of CEL2 + pad (1 mg/mL) + Ag specimens were collected and reported in Figure 3.

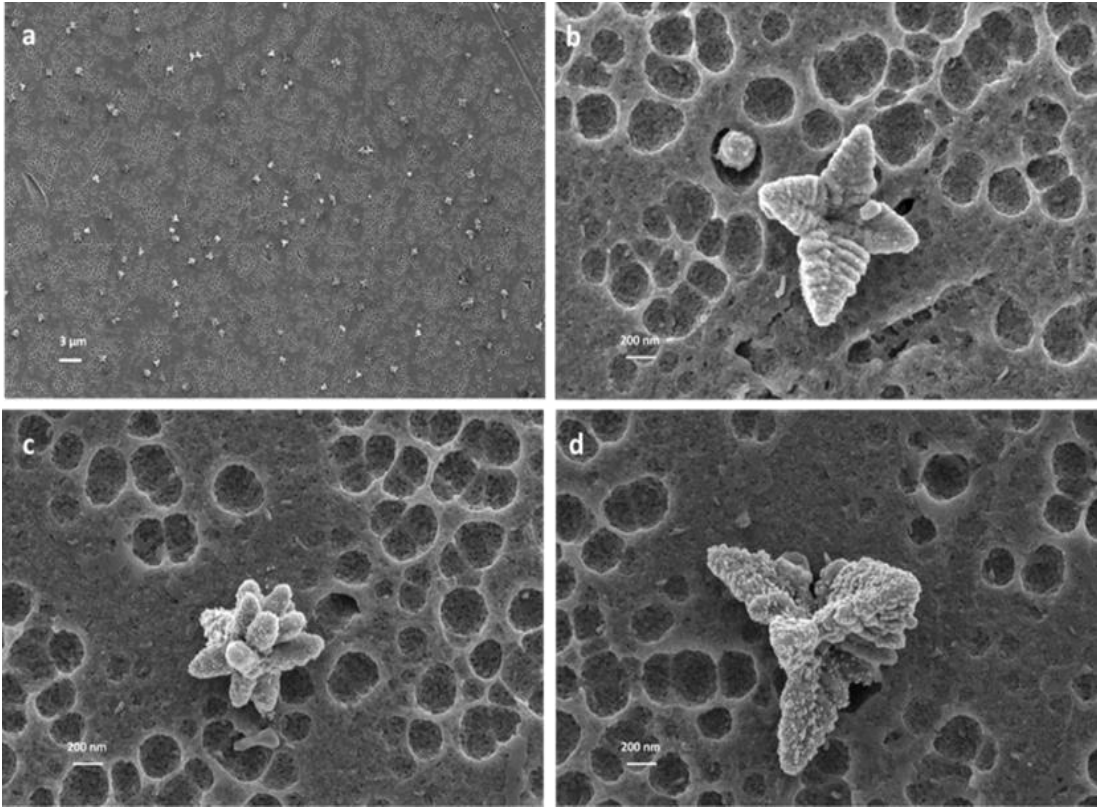



**Figure 3:** FE-SEM observations of CEL2 + pad (1 mg/mL) + Ag. (a) Low magnification overview of surface; (b–d) Ag precipitates as dendrimeric nanoflowers and holes on the glass surface due to reaction in the functionalization media (higher magnification).

As a further confirmation of this hypothesis, EDS analyses (Figure 4) confirmed that observed precipitates were mainly constituted of silver. Silver signals were only detected within the precipitates whereas no other signals were observed when analyzing other surface areas. This result is in accordance with the metallic nature of silver, which selectively precipitates in metallic form due to the reducing action of surface grafted polyphenols on Ag ions within the solution.

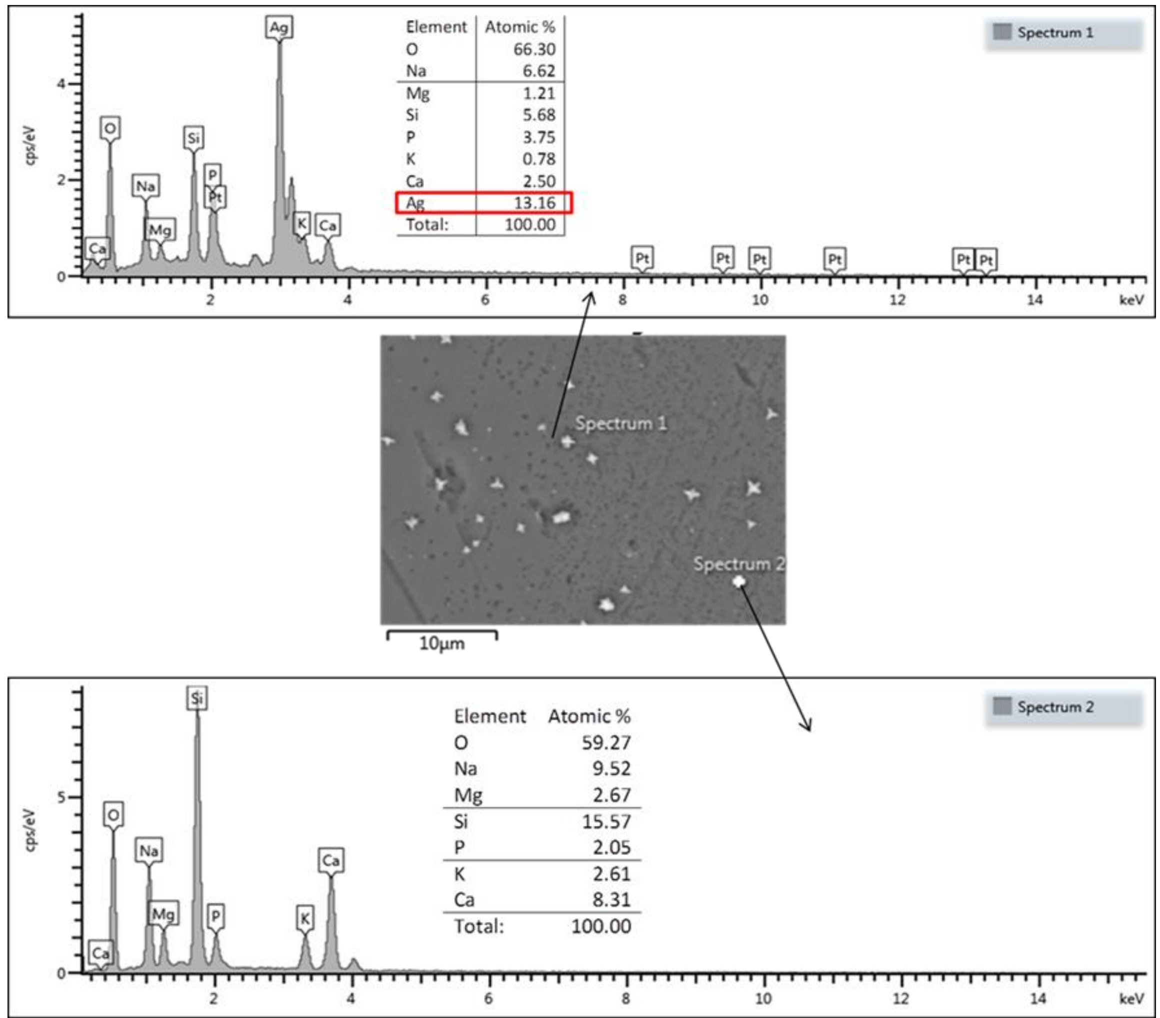



**Figure 4.** EDS analyses on CEL2 + pad (1 mg/mL) + Ag.

### *3.3.3)* Antibacterial Evaluation:

The results concerning antibacterial activity obtained by contaminating doped and control specimens directly in contact with $1 \times 10^5$ cells/mL Staphylococcus aureus suspension are reported in Figure 5. Here, we selected a certified multi-drug resistant strain (ATCC 43300) to test specimens' antibacterial performance towards bacteria resistant to conventional drug treatments.

The use of polyphenols from *P. pavonica* extract only (1 mg/mL, named CEL2 + pad) was not sufficient to determine a significant decrease in bacteria metabolism ($p > 0.05$) at each tested time-points in comparison with untreated CEL2 controls. Conversely, when silver nanoparticles (1 mg/mL, labelled CEL2 + pad + Ag) were coupled with the algae extract, a strong significant decrease was observed after 24 (Figure 5a) and 48 (Figure 5b) h, whereas the effect decreased after 72 h (Figure 5c), probably due to the limited amount

of antibacterial compounds still available for release. The amounts of grafted Ag ions and polyphenols are not unlimited and thus the efficacy is strongly related to the release period. So, our hypothesis is that once all the polyphenols and the Ag ions are completely delivered into the medium, their effects conclude and the bacteria that survived until that moment started to proliferate again. This behavior is analogous to that we previously observed for bioactive glasses functionalized with grape and tea polyphenols-the presence of the polyphenols alone is not able to induce a strong antibacterial behavior but the in situ reduction of silver nanoparticles significantly increases this activity.

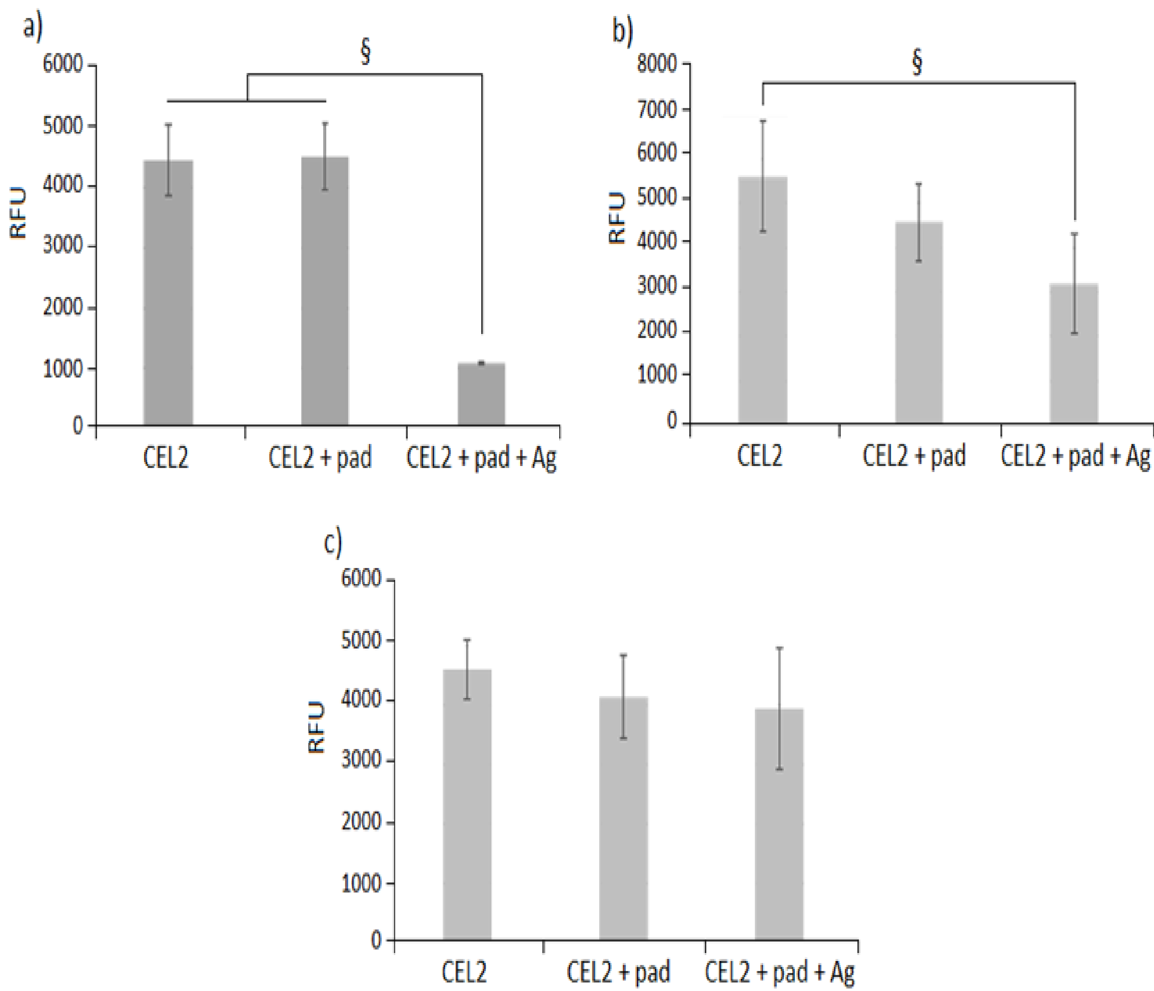



**Figure 5.** Specimens' antibacterial activity in direct contact with *S. aureus* biofilm for 24 (a), 48 (b) and 72 (c) hours. The doping with algae extract only (CEL2 + pad) was not effective in decreasing bacteria viability ($p>0.05$) in comparison with untreated controls (CEL2). The introduction of silver in combination with extract (CEL2 + pad + Ag) determined a significant decrease of bacteria metabolism that was significant after 24 and 48 hours cultivation (c, $p<0.05$ indicated by the §) in comparison to both CEL2 and CEL2 + pad. The effect was lowered after 72 hours probably due to saturation. Bars represent means and standard deviations.

### *3.3.4)*: Cytocompatibility Evaluation:

The results obtained by seeding cells directly in contact with doped and control specimens are shown in Figure6. In general,the doping realized with algae extract only (1mg/mL,called CEL2+pad) did not have any toxic effect on the bioactive glass as results were comparable with controls (CEL2) at each time point($p>0.05$). Differently, the combination of the extract and silver nanoparticles (1mg/mL, called CEL2 + pad + Ag) produced a decrease in cell viability that was evident, in particular, after three days' cultivation (Figure 6c). The results are significant in comparison with both CEL2 control and CEL2 + pad ($p < 0.05$, indicated by §). Accordingly, looking at the cell metabolism as a function of time (Figure 6d), values were increasing for CEL2 and CEL2 + pad specimens, thus prompting us to hypothesize that cell number increased during the experimental time-points. Conversely, results from CEL2 + pad + Ag showed a stationary plateau phase between 24 and 48 h and a slight decrease from 48 to 72 h (Figure 6c).

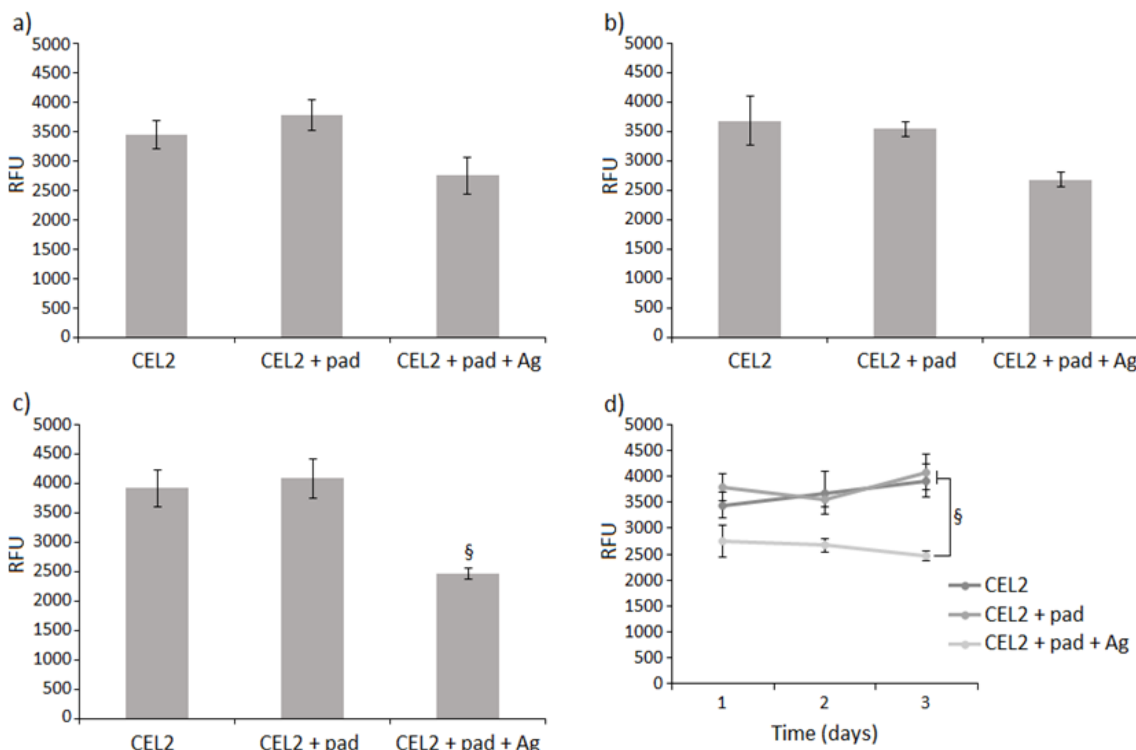



**Figure 6.** Specimens' cytocompatibility in direct contact with hFOB cells for 24 **(a)**, 48 **(b)** and 72 **(c)** hours. The doping with algae extract only (CEL2 + pad) did not introduce any toxic effect as results were comparable ($p>0.05$) with untreated controls (CEL2). The introduction of silver in combination with extract (CEL2 + pad + Ag) determined a decrease of cells metabolism that was significant after 72 hours cultivation (c, $p<0.05$ indicated by the §) in comparison to both CEL2 and CEL2 + pad. Accordingly, cells metabolism was lowered during the 72 hours as summarized in **(d).** Bars represent means and standard deviations.

### 3.4) Discussion:

A moderate basification was detected after glass soaking due to the ion release from the glass surface; these variations in pH were less evident than that we previously observed for gallic acid, grape polyphenols, and tea polyphenols, thus demonstrating a higher stability of the grafting procedure [25]. A strong shift to a basic environment can favor both a further hydroxylation of the glass and the oxidation of catechol groups of phenol stoquin one groups [26].

We adapted the method [25, 27] to solid surfaces. All the values can be considered as analogous samples of 1 cm2 area and are comparable. Measurements were recorded on a small set of samples (n = 3) obtaining the same results (this is why standard deviation is not reported). This value is comparable with that previously obtained by functionalizing the CEL2 surface with grape skin extracts [25]. However, the final volume of grafted polyphenols seems to be independent of the concentration of the source solution (1 or 5 mg/mL of freeze-dried extract in ultrapure water), different from what we previously observed for grape and tea polyphenols. This difference can be associated with the different typologies of the molecules grafted onto the surface, which is worthy offurther investigations. As the amount of polyphenols grafted on the surface didnot increase the uptake solution concentration, further investigations were performed on glass samples functionalized with 1 mg/mL of *P. pavonica* solution.

No evident modifications of the chemical composition of the glass were detected after surface functionalization. This phenomenon can be ascribed to the moderate amount of biomolecules grafted and to the fact that they mainly constituted of carbon and oxygen, which are already present in the glass and on its surface due to unavoidable atmospheric contaminations. On the other hand, no significant differences were detected between bare (CEL2) and functionalized (CEL2 + pad 1 mg/mL) bioactive glass in the carbon region (Figure 1a,c). An increase in the OH signal was highlighted in the oxygen region for the functionalized specimens. This increase can be attributed to the OH groups belonging to polyphenols as previously described [25].

Looking at the images, a uniform distribution of precipitates was observed all over the surface (Figure 3a). Their morphologies are similar and can be defined as dendrimeric nanoflowers (Figure 3b–d) with dimensions varying around 400–800 nm. It is possible to speculate that they are derived from nanometric particles aggregation as previously suggested by the presence of silver clusters detected by XPS. Some holes were also observed on the surface and can be ascribed to the surface reaction of the glass (ion

release and partial dissolution associated with the bioactivity mechanisms) in the functionalization media.

The *P. pavonica* polyphenols extract + AgNPs combination was here tested to improve the antibacterial properties of the bioactive CEL2 glass for the first time, to the best of our knowledge. The obtained results are encouraging because the combined effect of these two antibacterial agents was effective at significantly reducing *S.aureus* viability onto bioactive glasses. The hypothesisat the base of this combination was related to the possibility of coupling different broad-range antibacterial activities derivates from a natural compound and a broad range antibacterial metal in terms of nanoparticles.

Ag is known to be effective towards a large class of both Gram-positive and Gram-negative strains due to its ability to anchor to the bacterial cell membrane by electrostatic reaction; as such, bacteria die due to the irreversible membrane damage [35, 36]. Ag was demonstrated to be able to trigger the formation of free ROS that leads to a strong increase in the microenvironment oxidative stress. This condition causes unrepairable damage to bacteria by directly targeting their DNA, thus irreversibly stopping the replication cycle, causing bacterial death [35, 36].

Differently, the role of polyphenols in counteracting bacterial infection is less understood. Themore accredited hypothesis is related to the possibility that polyphenols can link bacteria membrane phospholipids [37, 38]; once this interaction occurs, the integrity of the membrane lipid bilayer is irredeemably compromised, thus leading to an increase in permeability, a loss of membrane fluidity, and animpairment of ions inside/outside transportation [37, 38]. All the seconditions are not compatible with correct metabolism, thus leading to bacteria death. According to this evidence, despite a more evident role of silver in comparison with polyphenols, we hypothesize that the obtained results are due to a combined activity that potentially irreversibly damages the bacteria membrane. This effect seems to be attractive, as it is not dependent on the Gram-positive or negative classification of the strain; moreover, it involves so many pathways (oxidative stress, DNA replication, membrane integrity, and ions trafficking) that bacteria may not easily develop resistance to all these mechanisms, thus making the treatment probably effective toward most bacterial strains. From the materials point of view, the presence of polyphenols can have a double effect on the surface modification: acting as reducing agents to catalyze the precipitation of antibacterial silver nanoparticles and, imparting other properties to the surface specific to polyphenols (e.g., antioxidant, anti-inflammatory, bone stimulating, and anti-cancer) to make the surface multifunctional.

The obtained results can generally be considered as being in agreement with previous literature. The bare CEL2 bioactive glasses were considered as a control due to their well-known cytocompatibility towards osteoblasts. They can be considered as superior in terms of bioactivity in comparison with glass based on the $SiO_2$–CaO–Na2O–$Al_2O_3$ system (named SCNA) that were previously applied to test polyphenols and silver antibacterial properties [25]. They were shown to be affective in supporting primary osteoblasts colonization and proliferation through their ordered mesoporous channels configuration that permits proper transportation of nutrients as well as released ions [18–20]. The subsequently formed HCA layer serves as an ideal environment for the osteoblast's growth, thus promoting bone self-healing [18–20].

The introduction of polyphenols from *P. pavonica* algae extract did not decrease cells metabolism, thus demonstrating cytocompatibility. Besides their recognized antibacterial activity, polyphenols are known to be effective at free radicals scavenging [39–42]. In cell cultures, they can directly interact with cell cytoskeletons through the phenolic hydroxyl groups [39–41]. Due to this tight interaction, polyphenols seem to be effectiveat protecting cells from damage due to oxidative stress, thus supporting proliferation [43].

However, when the algae extract was coupled with silver nanoparticles, we detected a significant increase in specimens' cytotoxicity. As the extract itself is not toxic aspreviously debated, wespeculate that this toxic effect can be attributed mainly to the silver (Ag). Ag is known to be a potential toxic element for cells [44–47]; it can trigger the formation of ROS by preventing intracellular antioxidants and cause DNA damage that results in cell death [47, 48]. This mechanism can be initiated by the Ag uptake by the cellular clathrin-dependent endocytosis and micropinocytosis; as a consequence, physiological impairment can be achieved due to the ROS increase [46, 47].

Another explanation of Ag potential toxicity is due to the employment of AgNPs [48–52]. Due to their nano-size, AgNPs can easily interact with cellular organelles, producing a lack of functioning due to direct contact or accumulation [48–52].

The combined polyphenols from *P. pavonica* algae and silver nanoparticles (AgNPs) surface doping of bioactive glasses CEL2 was successfully achieved using an in situ reduction technique. The specimens' chemical-physical analysis confirmed that both polyphenols and AgNPs were homogeneously spread onto the glass surface. The obtained composites showed a strong ability to prevent *S. aureus* biofilm contamination due to a combined activity mainly targeted toward the bacteria membrane. However, a certain specimen toxicity was observed toward human progenitor cells.

The proposed procedures for surface modification allows the tailoring of the silver content, so in the future, the amount of silver should be reduced to balance antibacterial activity and biocompatibility.

## 3.5) References: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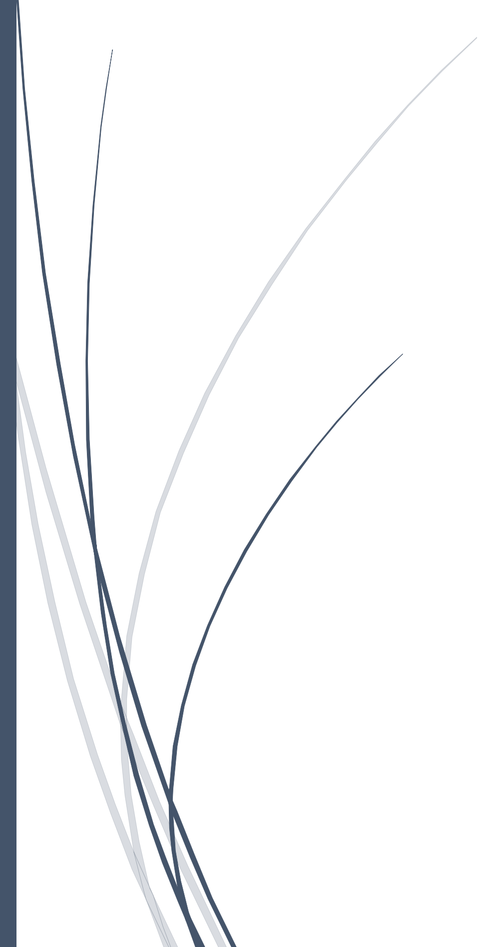
# Chapter 4:

# The significance of Redox potential measurments for pathogen activity versus algal extractions



THIS CHAPTER OF THE THESIS IS RELATED TO THE WORK DONE IN INTERNATIONAL CENTER FOR MATERIALS NANOARCHITECTONICS (WPI-MANA), NATIONAL INSTITUTE FOR MATERIALS SCIENCE, TSUKUBA, JAPAN. 30 AUGUST 2018 – 24 FEBRUARY 2019.

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## 4.1) Introduction:

### *4.1.1)* Electromicrobiology

#### *4.1.1.1)* A bit of history:

Electromicrobiology is rapidly morphing into a multidisciplinary area of its own. It was ‘born’ in the early 1900s with experiments showing that microbes could metabolically convert organic carbon into low levels of electricity [1, 2] and revived with the discovery of dissimilatory metal oxide reducing microbes [3, 4]. This included first, the demonstration, of extracellular electron transport (EET), and second, the demonstration that EET-capable microbes produced moderate levels of electricity in microbial fuel cells (MFCs) [5, 6, 7, 8]. This has led to renewed interest in both MFCs and the electrochemical interactions of microbes and minerals. More recently, the reports of electron uptake from insoluble electron donors, including cathodic electrodes, has spurred interest in electrosynthesis, cathodic bioelectrodes for remediation of metal pollution [9, 10], and the growth of autotrophic microbes with electricity as their sole source of energy. Taken together, these reports provide a window into environmental microbiology that did not exist only a few years ago. The merger of electrochemical

approaches, with microbiology and in some cases environmental microbiology (outlined in Figure 1) has already had a huge impact on understanding microbial physiology and characterizing the diversity of organisms capable of EET.

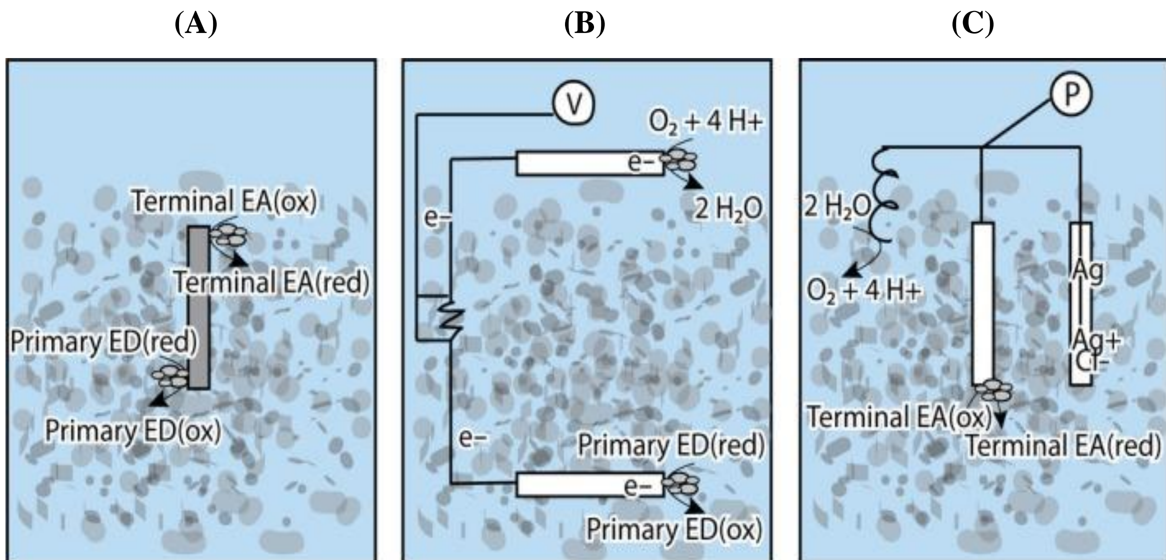



**Figure 1.** [11] Overview of electrochemical techniques used to study microbial EET including: (A) Oxidation or reduction in insoluble minerals or metals (solid grey bar) analogous to reactions causing corrosion; (B) microbial fuel cell or two electrode (solid white bars) system for quantifying current generation through oxidation of an electron donor (ED) at the anode and reduction in electron acceptor (EA, commonly O2) at the cathode and (C) three electrode or half-cell system for poising electrode potential relative to a reference electrode. A platinum counter electrode, Ag/AgCl reference electrode, and general working electrode (white bar) are illustrated (cathode depicted). Symbols represent: (P) potentiostat and (V) voltmeter.

### *4.1.1.2)* Summary and background:

• Electromicrobiology is a subdiscipline of microbiology that involves extracellular electron transfer (EET) to (or from) insoluble electron active redox compounds located outside the outer membrane of the cell. These interactions can often be studied using electrochemical techniques which have provided novel insights into microbial physiology in recent years. The mechanisms (and variations) of outward EET are well understood for two model systems, *Shewanella* and *Geobacter*, both of which employ multihaem cytochromes to provide an electron conduit to the cell exterior. In contrast, little is known of the intricacies of inward EET, even in these model systems. Given the number of labs now working on EET, it seems likely that most of the mechanistic details will be understood in a few years for the model systems, and the many applications of electromicrobiology will continue to move forward. But emerging work, using electrodes

as electron acceptors and donors is providing an abundance of new types of microbes capable of EET inward and/or outward: microbes that are clearly different from our known systems. The extent of this very diverse, and perhaps widely distributed and biogeochemically important ability needs to be determined to understand the mechanisms, importance, and raison d'etre of EET for microbial biology [11].

• Recent discoveries in electromicrobiology have pointed to the fact, we are only 'scratching the surface' in terms of understanding microbe–electrode interactions, with important implications for environmental microbiology. In marine sediments, it has been demonstrated that so-called 'cable bacteria' can provide a conductive link between anoxic and oxic levels of stratified sediments; adding, quite literally, a new (cm level) dimension to the potential impact of electron transfer reactions [12, 13]. How this spatially uncoupled redox process provides energy to a whole population chain of cells remains to be seen but has expanded the spatial scale of electron transfer reactions. In addition, EET is clearly not limited to organisms of the same species. Several reports have suggested that microbes can transfer electrons to metabolic partners in syntrophic reactions [14, 15, 16, 17]. In several, cases we are also broadening the taxonomic breadth, as well as structural variation in organisms capable of EET (Gram-positive, Archaeal cell walls, etc.), which further highlights the potential diversity of EET mechanisms that have yet to be discovered [10]. There may be even more EET variations; for example, incorporation of melanin as a conductive element [18] and the use of cellbound minerals as electron carriers and others, waiting to be discovered.

• Many microorganisms are thought to transfer electrons between their cytoplasmic membranes and extracellular minerals through a network of redox and structural proteins. Some of these proteins are well characterized in a few model microorganisms. These proteins often form pathways that electrically and physically connect intracellular metabolic processes with redox transformations of extracellular mineral associated metal ions. However, the components of these pathways are phylogenetically diverse13 and cannot always be identified from genomic data. The development of a mechanistic understanding of microbial extracellular electron transfer pathways requires the identification and functional characterization of their components [19].

#### *4.1.1.3)* **Principles of Reduction/Oxidation (Redox) reactions:**

Redox reactions involve the transfer of electrons from one chemical species to another. The reduced plus the oxidized form of each chemical species is referred to as an

electrochemical half cell. Two half cells having at least one common intermediate comprise a complete, coupled, redox reaction. Coupled electrochemical half cells have the thermodynamic properties of other coupled chemical reactions. If one-half cell is far from electrochemical equilibrium, its tendency to achieve equilibrium (i.e., to gain or lose electrons) can be used to alter the equilibrium position of a coupled half cell. An example of a coupled redox reaction is the oxidation of NADH by the electron transport chain:

$$NADH + ½O_2 + H+ \longrightarrow NAD+ + H2O$$

The thermodynamic potential of a chemical reaction is calculated from equilibrium constants and concentrations of reactants and products. Because it is not practical to measure electron concentrations directly, the electron energy potential of a redox system is determined from the electrical potential or voltage of the individual half cells, relative to a standard half cell. When the reactants and products of a half cell are in their standard state and the voltage is determined relative to a standard hydrogen half cell (whose voltage, by convention, is zero), the potential observed is defined as the standard electrode potential, Eo. If the pH of a standard cell is in the biological range, pH 7, its potential is defined as the standard biological electrode potential and designated Eo′. By convention, standard electrode potentials are written as potentials for reduction reactions of half cells. The free energy of a typical reaction is calculated directly from its Eo′ by the Nernst equation as shown below, where n is the number of electrons involved in the reaction and F is the Faraday constant (23.06 kcal/volt/mol or 94.4 kJ/volt/mol):

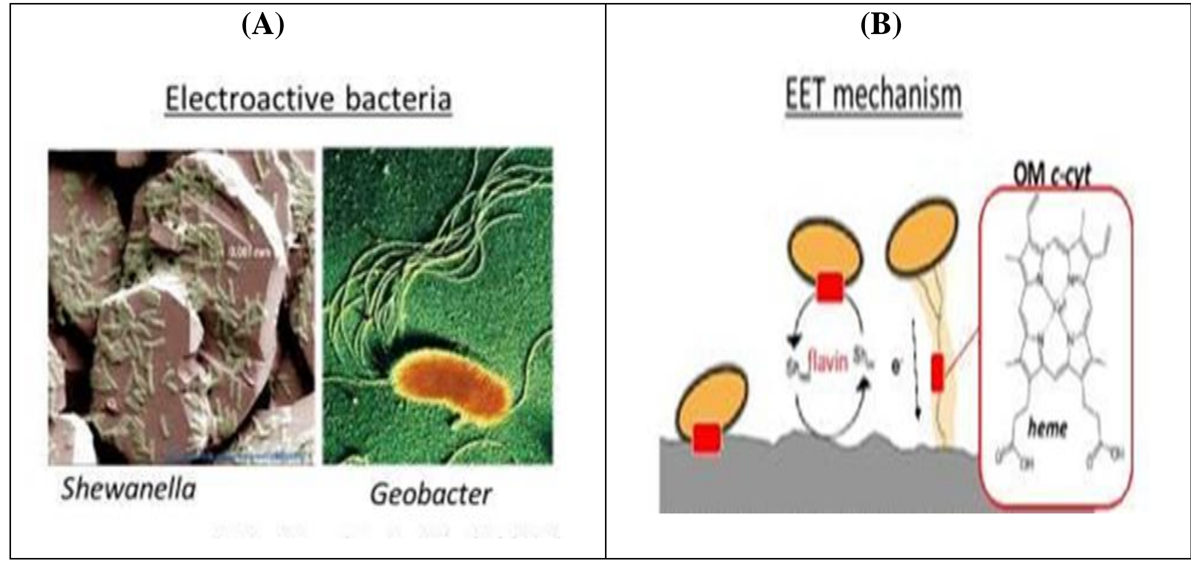
$$\Delta G^{\circ\prime} = -nF\Delta Eo'$$

For the oxidation of NADH, the standard biological reduction potential is –52.6 kcal/mole. With a free energy change of –52.6 kcal/mole, it is clear that NADH oxidation has the potential for driving the synthesis of a number of ATPs since the standard free energy for the reaction below is +7.3kcal/mole:

$$ADP + Pi \longrightarrow ATP$$

Classically, the description of ATP synthesis through oxidation of reduced electron carriers indicated three moles of ATP could be generated for every mole of NADH and two moles for every mole of $FADH_2$. However, direct chemical analysis has shown that for every two electrons transferred from NADH to oxygen, 2.5 equivalents of ATP are synthesized and 1.5 for $FADH_2$. Despite these new measurements many texts still use the historical values of 3 moles of ATP per mole of NADH and 2 moles of ATP per mole of $FADH_2$ [20].

In anaerobic environments with a substantially reductive condition such as the human gut, fermentation is the primary mechanism of microbial metabolism, in which the redox cycling of biological electron carriers, such as nicotinamide adenine dinucleotide (NADH), drives the intracellular oxidation and reduction of organic substrates. As fermentation does not require extracellular electron acceptors for the termination of metabolism, the energy gain under such conditions is potentially lower than that of respiratory metabolism; therefore, the possibility for EET to increase the rate of NAD+ regeneration and fermentative metabolism may be important for these microbes to increase their net energy gain and compete with other respiratory bacteria. In fact, a few studies have shown that fermentative gut microbes are capable of EET using soluble electron carrier molecules. However, by simply studying isolated bacterial cultures, it is impossible to examine which bacteria primarily rely on EET-coupled metabolism in the human gut and to study the ecophysiological importance of EET coupled with fermentation, compared to anaerobic respiration, which is also abundant in the gut environment. Here, we examined the growing competition between fermentative and respiratory bacteria on an electrode surface that enriches for EET-capable bacteria. Specifically, we performed electrochemical enrichment, which was initiated using a diluted gut microbial community, by employing two different medium conditions that biased for either fermentation or anaerobic respiration. The isolated bacterial strains were characterized by electrochemical assays for their metabolism associated with current production and the EET mechanism [20]. All these mechanisms are represented in Figure 2 (A, B, C, D and E).



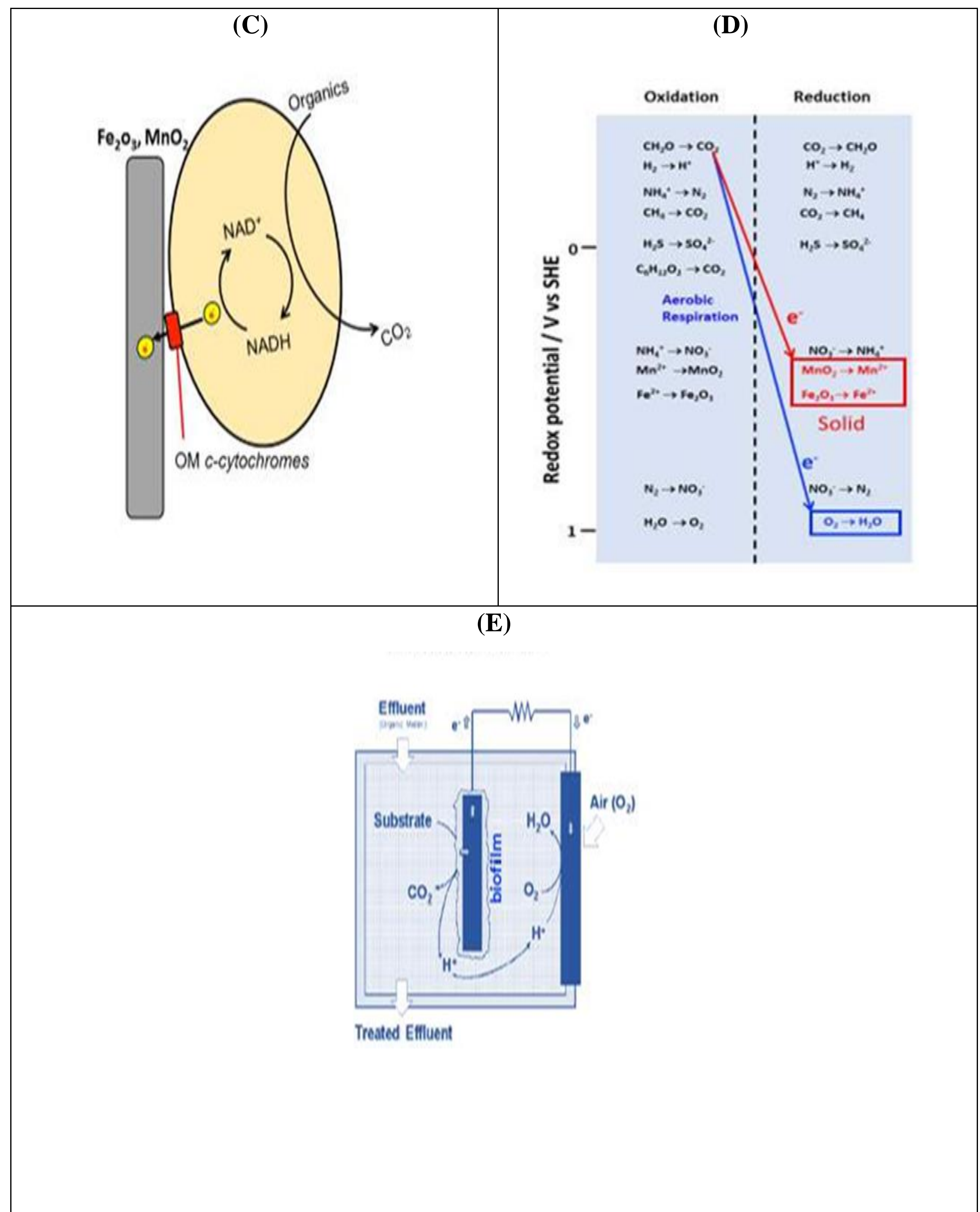



**Figure 2:** [20]. Overview of electrochemical techniques used to Extracellular Electron Transfer EET, metabolically generated electrons are transferred from the cell interior to the exterior to an external electron acceptor for anaerobic respiration, including: (A) *Shewanella* and *Geobacter* are well studied electroactive microbes, forms conductive biofilm matrix. (B) Transfer electrons through cytochromes or via redox shuttles (flavins) or conductive nanowires. (C) EET-associated anaerobic respiration. (D) EET capable microbes can reduce the external insoluble electron acceptors. (E) Implications of EET process; Microbial Fuel Cells (MFC) for energy conversion and waste treatment.

### *4.1.2)* Monitoring the metabolic activity of bacterial biofilm:

Biofilm formation is a major issue in various field. In civil engineering it accelerates the metal corrosion, in medical science it promotes diseases and contaminate medical devices in contact with a human body, in the water network and pool it allows the development of pathogenic bacteria such as *Legionella pneumophylla*. Whereas planktonic bacteria are usually easy to count, easy to observe, easy to manipulate and easy to kill, bacteria inside biofilm are resistant to biocides, their concentration is difficult to evaluate, and they are difficult to study at the laboratory scale. This is why biocide efficiency is generally evaluated in planktonic form, leading in an over estimation of the efficiency when the biocide should be used on a biofilm. In this study, *Streptococcus mutans*, ubiquitous bacteria in teeth plaque is growing as a biofilm, and the current generated by the biofilm is regularly recorded. Using ampicillin as a growth inhibitor and ethanol as a biocide, we show that the current is directly related with the metabolic activity. Therefore, we provide an accurate, in live, and non-destructive method to evaluate a biofilm activity.

Provided that the biofilm studied is able to produce current, such a method might help to evaluate the real efficiency of any biocide.

### *4.1.3)* The Red Sea Algae:

Algae are global; they are not only abundant in oceans and seas, but they also grow in fresh water streams like rivers lakes or ponds. Moreover, they are very capable of growing on other moist surfaces such as rocks, soil, snow, ice and even plants and animals. Algae photosynthesis consists of about 40% of the whole ubiquitous process. Algae are considered as microscopic plants that are extremely diverse; their size ranging from 1/1000 to 2 mm and they are split into four groups: (i) brown algae, (ii) green algae, (iii) red algae, and (iv) diatom. They exist as macroalgae, microalgae and cyanobacteria. Microalgae are peculiar. They are photosynthetic organisms that can be prokaryotic or eukaryotic. They are capable of growing in a broad domain of ecological conditions. In contrast, macroalgae (seaweed) are multicellular photoautotrophic organisms and are classified into three main categories; Chlorophyta (green algae), Rhodophyta (red algae) and Ochrophyta-Phaeophyceae (brown algae). In addition to the role of these organisms as primary producers, they serve significantly in marine ecosystem structuring and preservation. So far, Red Sea algae characteristics and bioactivities are less investigated and just a few information regarding their particular activities were communicated. In the coral reefs of the Red Sea, marine algae are exposed to many defiance including space competition high salinity, high water temperatures in addition to intensive sun radiation.

Such drastic circumstances have forced these organisms to evolve various adaptational possibilities in order to be able to survive; this adaptability gave rise to the development of wealthy algal bioactive compounds [21].

*4.1.3.1)* **Marine Algae as a Source of Industrial Exploitable Compounds**:

Marine algae significantly contribute to the vast array of daily life applications; not only as a primary food source for different ecosystems on our planet but they also provide marine-derived bioproducts which are therapeutically effective due to their pharmacologically active content representing multiple cytotoxic, antifungal, antiviral as well as anti-inflammatory and antioxidant capacities. Thus, vigorous investigative researches have been carried out to obtain bioactive compounds from marine origins as natural sources of drugs Marine algae produce a vast diversity of distinguishable natural components known as secondary metabolites that don't affect the basic machinery of life. However, they provide a primary role for the organism's integrity and survival such as growth and reproduction in spite of the fact that these molecules predominantly contribute to an extremely tiny portion of the organism's total biomass. The noticeable halogenation of numerous secondary metabolites reflects the abundance of chloride and bromide ions in seawater. These two halogens are the most utilized ones to enhance the bioactive properties of secondary metabolites whilst fluoride and iodine appear to be less commonly included within these chemical compounds although chlorine concentrations are higher than those of bromine, marine algae usually use bromine more frequently to produce organ halogen. Thus, all marine halogenated compounds such as phenols, indoles, peptides, terpenes, polyketides acetones as well as volatile halogenated hydrocarbons have their own bioactive properties that render the compound interestingly applicable in different industrial fields [21].

*4.1.3.2)* **Marine Algae: A novel biomaterial in Dentistry:**

Marine algae compounds provide many beneficial contributions in stomatology. Furans, which consist of a variety of polysaccharides and sulfated galactans, show their efficacy in minimizing the opportunity for dental caries incidence inrats by preventing oral bacterial adherence and colonization. Calcium alginate is another essential component representing hemostatic characteristics by which alginate fibers are capable of imbibition and turning gelatinous as soon as they are applied to the tooth surface. This gelatinous nature acts as a coagulant matrix which renders alginate applicable as a dressing material in packing the tooth cavity as well as fistulas and sinuses. Moreover, an alginate-based drug "polaprezinc-sodium alginate suspension" is produced as specific treatment for

severe gingivostomatitis associated with hemorrhagic erosions and ulcers.This drug acts via two principal mechanisms: first, sodium alginate serves as a hemostatic agent and secondly, polaprezinc in addition to its ability of bind the free radicals, stimulates healing and reparation of the mucous membrane. In prosthetic dentistry, alginates are essentially used as impression materials to produce the negative impressions of intraoral structures for the purpose of diagnosis as well as the construction of restorative appliances such as fillings, crowns, dentures and orthodontic apparatus [21].

## 4.2) Materials and methods:

### *4.2.1)* Macroalgae selection, collection, and storage:

Four different strains of macroalgae; (*Padina pavonica*, *Sargassum muticum*, *Halimeda opuntia* and *Jania rubens*) as showen in Figure 3; were collected by hand picking from the Red Sea in Hurghada, Egypt. Then, healthy algae samples were immediately cleaned of epiphytes and extraneous matter, and necrotic algae were discarded. Specimens were washed thoroughly with sterile distilled water, air dried, cut into small pieces, and then strongly pressed in a tissue grinder until obtaining a fine powder (IKA A 10, IKA®-Werke GmbH & Co. KG, Staufen im Breisgau, Germany).

**Figure 3:**

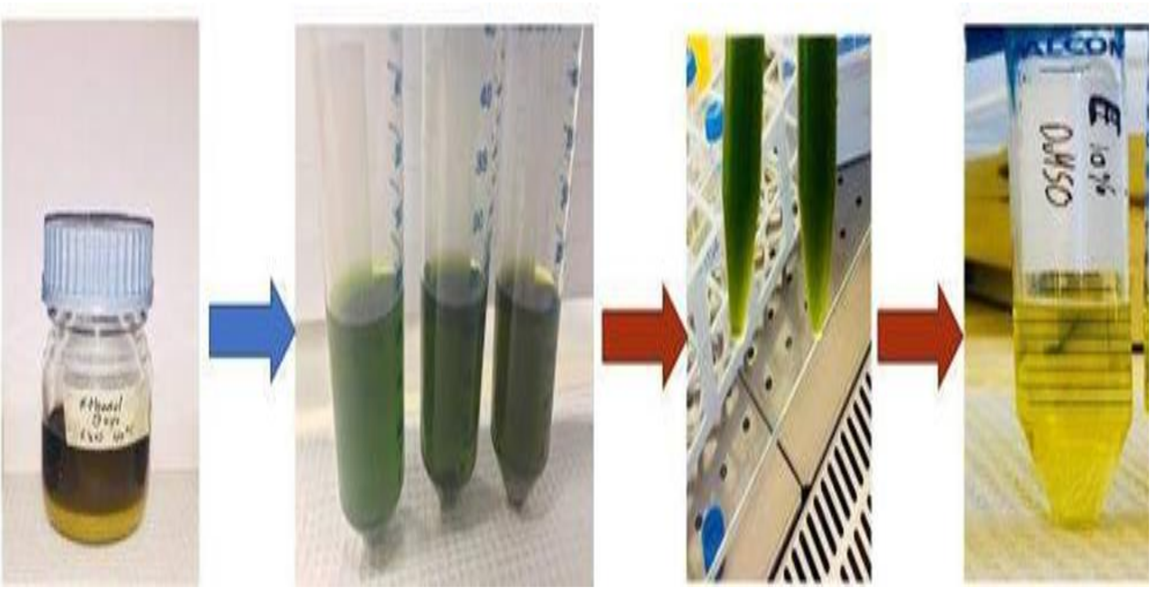
**Four different strains of macroalgae:**

### *4.2.2)* Extraction of polyphenols:

1. An aliquot of seaweed powder is extracted in Ethanol/water (80:20, v/v). Then, placed in a water bath with constant shaking at 300 rpm and 40°C for 6 h.
2. All samples after incubations were centrifuged at 2000 xg for 20 mins. and the supernatant was filtered.
3. Filterated supernatant of the extractions were evaporated in water bath at 45°C for 7-8 hrs with constant shaking at 200 rpm; the final yield was approximately 20%.
4. Finally, concentrated solutions were solved with 10% DMSO (as a co-solvent) [22] diluted in 10 ml of ultra-pure water then stored at 4°C for further analysis. Figure 4.

**Figure 4:**

**Procedure of polyphenols extraction:**




### *4.2.3)* Determination of polyphenols, carotenoids, and chlorophylls from macroalgae extracts:

Polyphenols were extracted and characterized by the Four different strains of macroalgae powder using the solvent technique (95% ethanol, v/v in ultrapure water) and the colorimetric Folin–Ciocalteau method, respectively [23, 24]. Briefly:

- Algae powder was added to the ethanol solution in a ratio of 1:50 (w/v).
- Then, the solution was heated to 60 °C for 1 h under continuous stirring (120 rpm).

- Afterward, the solution was 0.45-μm filtered to remove unsolved debris and ethanol was fully evaporated into an incubator (60 °C, 2 hrs.).
- Finally, the obtained extract was resuspended in ultrapure water, freeze-dried (using a Scanvac CoolSafe 4, from LaboGene, Lillerød, Denmark) and stored at -20 °C until use.

Total polyphenol content (TPC) of the powdered algae was determined calorimetrically by the Folin–Ciocalteau method with slight modifications. Briefly:

- 0.5 mL of the ethanolic solution was added to 2.5 mL of 10% Folin–Ciocalteau reagent, previously diluted with distilled water.
- After 3 min, 2 mL of 7.5% sodium carbonate solution was added.
- The mixture was incubated in the dark for 1 h at room temperature.
- The absorbance was measured at 765 nm using a UV-Vis spectrophotometer (Jenway 7205, Cole-Parmer, Sta_ordshire, UK).
- A calibration curve was prepared with the gallic acid standard solution at various concentrations (10 to 100 mg/L).
- The results are expressed as mg gallic acid equivalent (GAE)/g dry weight (DW). Total carotenoid ©, chlorophyll-a (Chl-a) and chlorophyll-b (Chl-b) contents of the extracts were determined spectrophotometrically (Jenway 7205, Cole-Parmer, Sta_ordshire, UK) according to Sumanta et al. [23].
- Statistical treatment of data. Data were subjected to one-way analysis of variance (ANOVA) and comparison among means was determined according to Tukey's honestly significant difference (HSD) test. Significant differences were accepted at $p < 0.05$ and represented by different letters.

### *4.2.4)* **Electrochemical cell operation and medium composition** [20]**:**

#### *4.2.4.1)* **Electrochemical cell operation:**

Electrochemical measurements were performed in a single chamber (Bioreactor) and three-electrode reactors. Tin-doped $In_2O_3$ (ITO) grown on a glass substrate by spray pyrolysis deposition was used as the working electrode (WE) having a surface area of 3.1 cm2, and thickness 1.1 mm. The WEs were placed at the bottom of the reactor with sealing gaskets to avoid any leakage. A platinum wire (approximate diameter of 0.1 mm) and Ag/AgCl (sat. KCl) were used as counter and reference electrodes, respectively. Electrochemical experiments were conducted in a Coy anaerobic chamber filled with

100% N2. Electrochemical analysis techniques such as single-potential amperometry (SA) and differential pulse voltammetry (DPV) were measured with an automatic polarization system (VMP3, Bio-Logic Science Instruments). DPV was measured under the following conditions: pulse increment, 5.0 mV; pulse amplitude, 50 mV; pulse width, 300 ms; and pulse period, 5.0 s. The electrochemical cell was maintained at 37°C throughout the experiment and the WE was poised at +0.2 V [vs. Ag/AgCl (sat. KCl)] reference electrode for SA.

#### *4.2.4.2)* Medium Composition:

Gifu Anaerobic Medium (GAM Broth) and Brain heart infusion (BHI), which are known for providing reducing conditions and adequate anaerobiosis, was used as an energy-rich medium for the enrichment of gut microbes. Defined medium (DM), used as a minimum medium for EET strains enrichment and initial electrochemical characterization experiments had the following composition ($L^{-1}$): $NH_4Cl$: 1 g; $MgCl_2$ $6H_2O$: 0.2 g; $CaCl_2.2H_2O$: 0.08 g; yeast extract: 0.5g; NaHCO3: 2.5g; NaCl: 10 g; HEPS: 1.2 g. Volume completed with osmosed water up to 1L. The final medium was autoclaved at 121°C and deaerated by purging it with 100% N2 for 15 min prior to use for experiments.

#### *4.2.4.3)* Bacteria and the growth conditions:

- Two bacterial strains were tested in the experiments (*Streptococcus mutans* Clarke (ATCC® 700610™) and *Porphyromonas gingivalis* (Coykendall et al.) Shah and Collins (ATCC® BAA-308™)).
- By using manufacturer's instructions for equilibrating lyophilized bacteria in specific media: brain heart infusion (BHI, Sigma Aldrich) for *S. mutans* and Gifu Anaerobic Medium (GAM Broth) for *P. gingivalis*.
- By using a COY anaerobic chamber filled with 100% N2, Electrochemical experiments were conducted for anaerobic conditions, *S. mutans* was grown for 24 hrs and *P. gingivalis* was grown for 2 days at 37 °C.
- The bacteria are harvest and washed 2 times in DM using centrifugation 2000 xg for 10 min.
- Washed bacteria in DM are added in a final concentration of 0.1 $OD\lambda600$.

### *4.2.5)* Monitoring of biofilm metabolic activity by using the electrochemical system:

#### *4.2.5.1)* Electrochemical characterization of commercial chemicals and its antibacterial activity (as a control) for the system:

- Two commercial antibiotics (Ampicillin sodium powder (FUJIFILM Wako Chemicals USA Corporation,Wako 016-23301) and Triclosan (Pubchem, USA)), and etheyl alcohol were used as antibacterials.
- Four bioreactors were used in this experiment, in the first and second bioreactors it was as a control. The bioreactor well washed and sterilized by ethanol, acetone and UV is filled with 5mL of autoclaved DM medium, 100µl of glucose 250 mM is mixed with DM, and the air is eliminated by N2 bubbling for 15 min. Washed *S. mutans* in DM are added in a final concentration of 0.1 ODλ600. One left without connection of the electricity and the other is connected and after 150 hrs of running the current, Ethyl alcohol was used as 20% from the final volume.
- Ampicillin is an antibiotic used to stop the bacteria growth by inhibition of the cell wall formation. A mother solution of Ampicillin sodium 0.1g/L is prepared from ampicillin sodium powder (Wako 016-23301). In the third bioreactors, the same like control plus 80µl of the mother solution is added in the bioreactor for a final concentration about 1.6 µg/ml with is 10 times higher than the minimum inhibitory (MIC) and 5 time more than the minimum bactericidal (MBC) for both planktonic or biofilm-detached cells according to Liu et al 2012 [25].
- Triclosan (M 289.56 g/mol) is used at 0.03mmol/L that is to say 8.67µg/mL which provide a D value of 3 minutes (Phan and Marquis 2011) [26]. Mother solution if first prepared with 8.67mg of triclosan solubilized in 10ml of methanol 10% (osmosed water 90%). In the fourth bioreactors, 5µl of Triclosan is then added in the bioreactor for a final concentration of 0.003 mmol/l to observe the reaction of a sublethal dose for 3 hours, then 45µl is added for a final concentration of 0.03mmol/l.

#### *4.2.5.2)* Microscopic observation of dead and/or alive bacterial cells:

The kit back light (Propidium Iodide (PI) to stain dead bacteria and Cyto9 to stain alive bacteria) is used to evaluate the amount of dead or alive cells: working electrode is washed with 1mL of NaCl 10g/L. 90µL of NaCl 10g/L mix with 5µL of PI and 5µL of Cyto9 is put on the biofilm and cover by a cover glass. Observation is performed using a microscope with a filter range: 540-580 excitation wavelength and 592-668 emission wavelength for PI, 460-500 excitation wavelength and 512-542 emission wavelength for Cyto9.

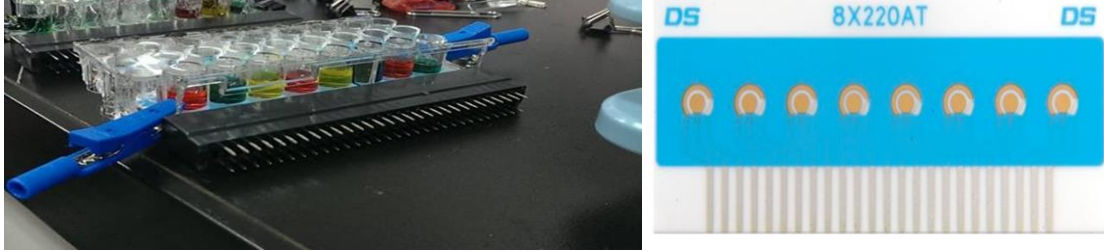
exposition duration 200ms. To show the PI and cyto9 photos on the same image, imageJ was used first to adjust the scale from 0 to 50 instead of 0 to 255 in such a way to make the cells more visible on both PI and Cyto9 photos, and then to merge the red and green channel.

### *4.2.6)* Electrochemical characterization of algal extracts:

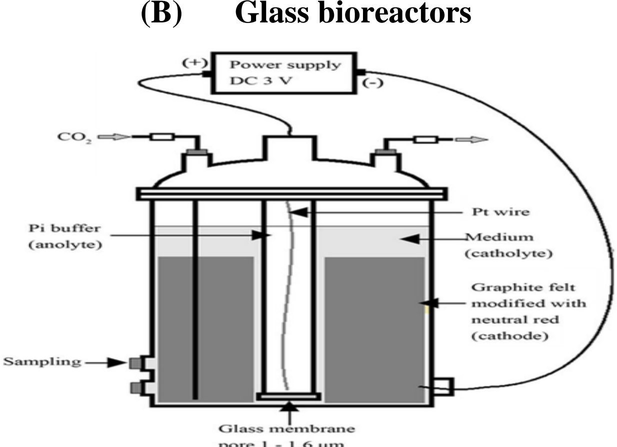
- Individually, the extractions from the four different strains of algae (*Padina pavonica*, *Sargassum muticum*, *Halimeda opuntia* and *Jania rubens*) were tested to detect the electrochemical characterization for their antibacterial activity at different concentrations from the original solutions prepared as described previously.
- The final extract was filtered using 0.22-µm pore-size filters and deaerated by purging it with 100% N2 for 15 min prior to use for experiments.
- Two different kind bioreactors were used in this experiment, 8 channels gold printed electrode (8X220AT- Spain) and glass bioreactors as Figures 5 (A, B).
- The bioreactor well washed and sterilized by ethanol, acetone and UV is filled with 5mL of autoclaved DM medium. A bioreactor composed by an ITO working electrode on the bottom, a cylinder of a volume near 7ml as a middle part, and a cover with reference (Ag/Ag Cl in saturated KCl) and platin counter electrode on the top. All the system is airtight by silicon sheet. 4.5 ml of DM is used to fill the bioreactor, 200µl or 100µl of glucose 250 mM is mixed with DM, and air is eliminated by N2 bubbling for 15 min.
- The working, reference and counter electrodes are connected on a potentiostat (ref) in such a way to continuously apply 0.2V on the working electrode and to record the current harvested as a so-called chronoamperometry measurement, and the bioreactor is incubated at 37°C. After stabilization of the current record, bacteria were washed in DM is added in a final concentration of 0.1 Odλ600.
- By injection, the filtered extract added to this bioreactor, with different conc. As will describe in the results.

**Figure 5:**

**(A) 8 Channels printed electrode bioreactors**



**(B) Glass bioreactors**




## 4.3) Results:

### *4.3.1)* Polyphenols, carotenoids, and chlorophylls quantification from algal extract:

The total polyphenol content in the four different strains of algae (*Padina pavonica*, *Sargassum muticum*, *Halimeda opuntia* and *Jania rubens*) extracts were evaluated by means of the Folin–Ciocalteau test to determine whether the selected source was sufficient to provide an adequate bulk of polyphenols. This step was performed as natural extracts often differ considerably from each other in terms of polyphenols content due to the different geographical origins. So, these macroalgae extract were expected to differ from previous sources of polyphenols were previously proposed. Results confirmed that the selected macro algae were rich in polyphenols as the total amount resulted in the following figures (6, 7).

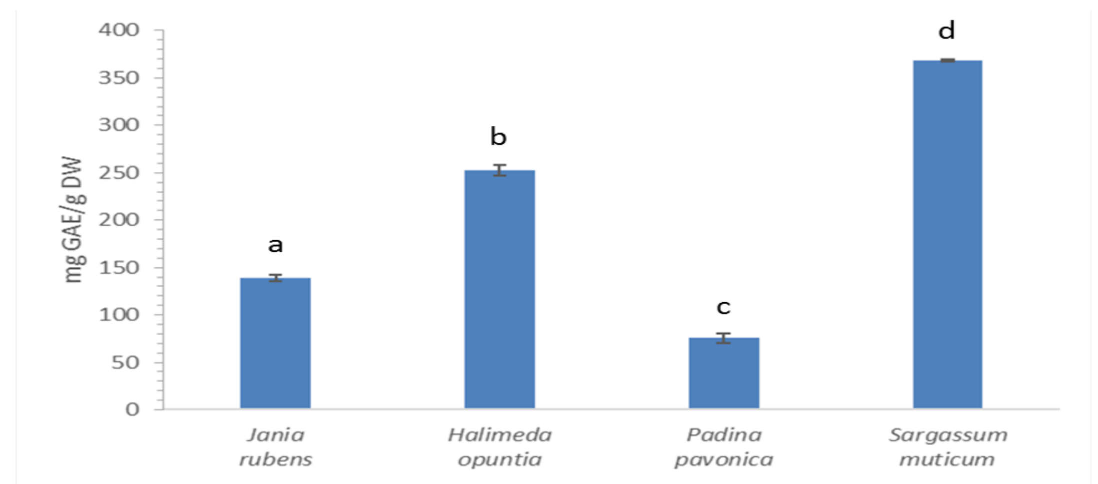



**Figure 6:** Total polyphenol content in algae. Results are presented as the mean of triplicate measurements. Error bars represent the standard deviation (SD). Significant

differences were accepted at $p < 0.05$ and represented by different letters, according to Tukey's honestly significant difference (HSD) test.

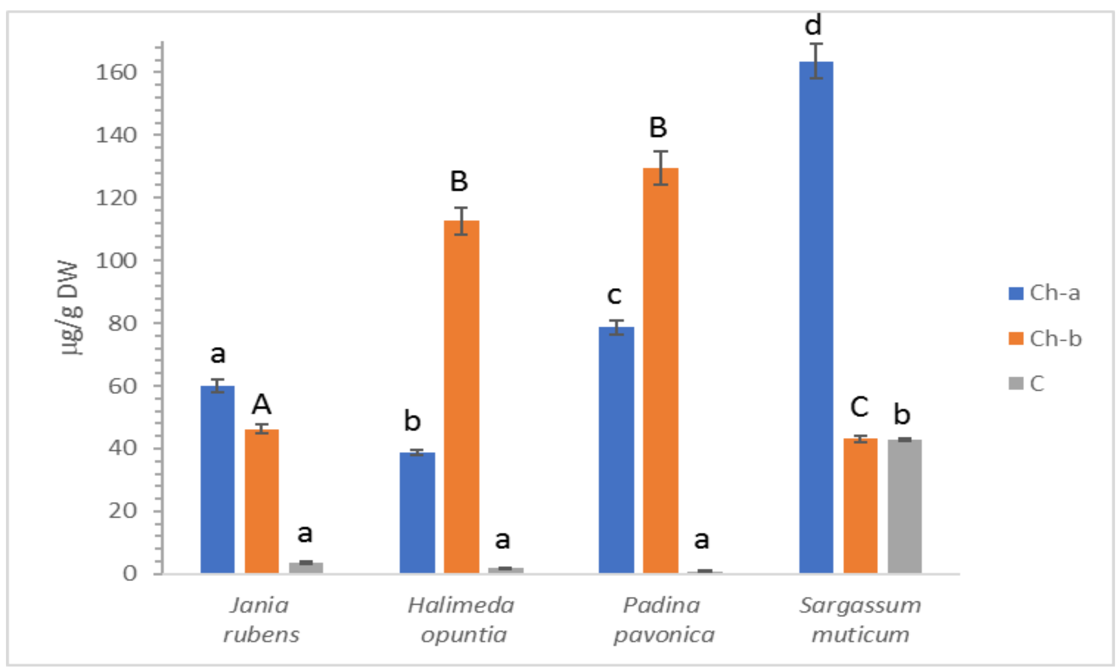



**Figure 7:** Concentrations of chlorophyll-a (Ch-a), chlorophyll-b (Ch-b) and total carotenoids (C) in algae. Results are presented as the mean of triplicate measurements. Error bars represent the SD. Significant differences were accepted at $p < 0.05$ and represented by different letters, according to Tukey's HSD test.

***4.3.2):*** **Monitoring of biofilm metabolic activity:**

***4.3.2.1)*** **Detection electrochemistry properties of *S. mutans* with antibacterial agents; by using the electrochemical system:**

The aim of this study was to test the electrochemistry properties of oral pathogenic bacteria *S. mutans* by measuring the current production (Ic) to be approximately 0 – 0.3 µA cm−2 for each cycle at around 200 hrs and to check the effect of a commercial antibacterial agent on the current production of *S. mutans*. For the control, the current production is started to increase at the beginning of the experiment to reach approximately 0.22 µA and stay stable for many hrs. At the end running of this experiment; ethanol 20% of the final volume equals 1 ml of 98% ethyl alcohol; The current production of *S. mutans* sharply decreased from 0.21 µA to reached no current production.

By using Ampicillin 2 µl/ml, the current production of *S. mutans* is increased at the beginning of the experiment to reached 0.2 µA then after added the Ampicillin it starts to decrease 0.16 µA and finished at the end of the running to be 0.1 µA. On the other hand; by adding Triclosan 3 µmol, the current production of *S. mutans* is increased at the

beginning of the experiment to reached 0.15 µA then after added the Triclosan it starts to decrease 0.12 µA and finished at the end of the running to be 0.05 µA.

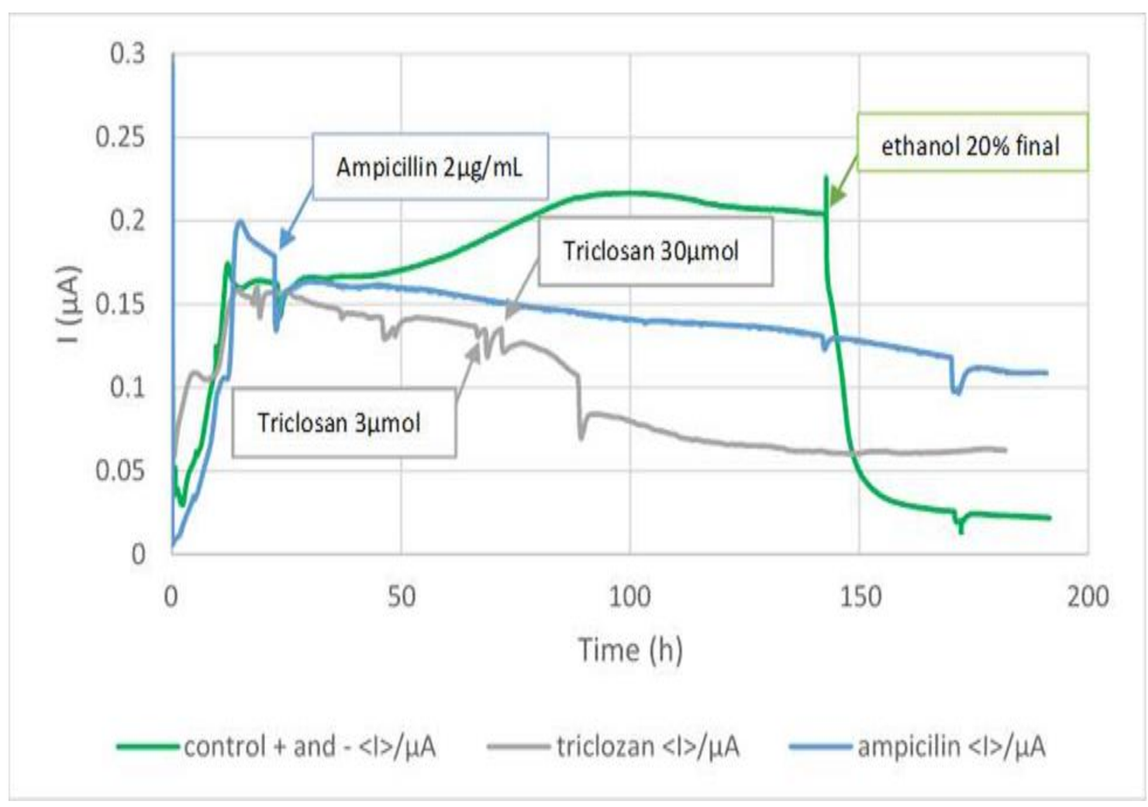



**Figure 8:** Current production of *S. mutans* as control and after adding Ampicillin, Triclson and ethyl alcohol.

### *4.3.2.2)* Microscopic observation dead/alive:

Staining procedures have been used to differentiate living cells from dead cells, fluorescent stains measuring oxidation-reduction activity target the electron transport chain and various catabolic and anabolic pathways.

Figure 9 shows photos by the microscope for only the biofilm of pathogenic *S. mutans* that formed on the surface of the working electrode in the control bioreactor and the biofilm that formed of pathogenic *S. mutans* on the working electrode in the bioreactor with Ampicillin & Triclson treatments. The green color represents the live cells while the red color represents dead cells. By using Cyto9 to stain alive bacteria, it is so clear the difference to evaluate the amount of live bacterial cells in the control sample with the comparison with the sample of Ampicillin & Triclson treatments. On the other hand, by using Propidium Iodide (PI) to stain dead bacteria to evaluate the number of dead cells no difference between treatment and controls in cell counts.

**Figure 9:**

**Photos of Floresences microscope for the biofilm of *S. mutans*:**

| Sample | Microscope power | Whight Light | Alive staining | Dead staining |
|---|---|---|---|---|
| Control | X10 | | | |
| | X63 | | | |
| With Ampicillin | X10 | | | |
| | X63 | | | |
| With Triclosan | X10 | | | |
| | X63 | | | |

***4.3.3)* Effect of polyphenol extracted from *Padina pavonica* on *S. mutans* current production after forming the biofilm:**

**By using two different measurements:**

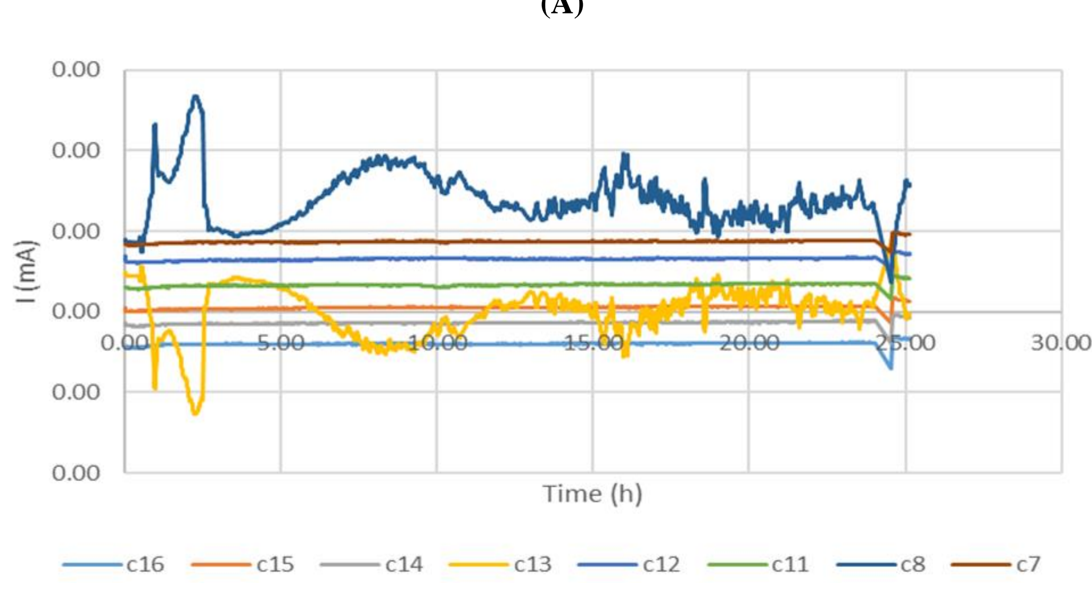
- **Firstly by using 8 channels electrode:**

As will show in Figure 10 (A, B and C), the experiment at the beginning was performed by using 8 channels gold printed electrode (8X220AT- Spain) and with using the DM medium inculated with *S. mutans* without the addition of any polyphenols extract of *Padina pavonica* to check the current production of *S. mutans* alone without any external effect for the first 24 hrs from running the experiment and during the metabolism of *S. mutans*. Measuring the current production of *S. mutans* (Ic) was approximately 0 – 0.05 µA cm−2; clearly at Figure 10 B and Figure 10 C. To our surprise, upon addition of different concentrations of polyphenol extracted by *Padina pavonica,* the current production immediately sharply decreased followed by short current recovery, suggesting that polyphenol might damage *S. mutans* it spent more than one hour with this limt of no current production. Also as clearly in Figure 10 B, the percentage of decreasing the current production was increased gradually by increasing the percentage of addition of polyphenol.

After more than 1 hr from added polyphenol, the current production return back to increase but with different behavior from the beginning one.

At Figure 10 C, the same experiment was run in another time to check the same characters but to check also the DMSO 10% (co-solvent) to see if it has a current production or have any similar effect like the polyphenol from the algae. It also clear that the polyphenol has sufficient effect on the current production of *S. mutans.*

**(A)**



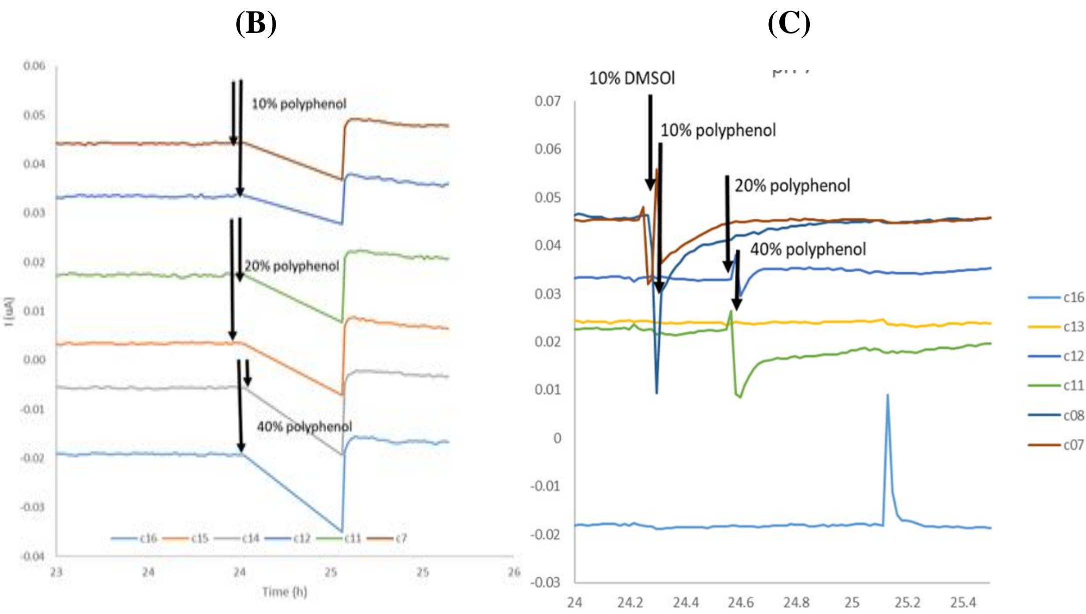



**Figure 10 (A, B and C):** Representative current production versus time of *S. mutans* in 8 Channels gold printed electode by using DM medium then added different concentrations of *Padina pavonica* polyphenol.

- **Secondly by using bioreactors:**

  It is the same characters like in the 8 channels printed electrode experiment; but here in the bioreactors and by using three different electodes inside the bioreactor; it makes the experession of measuring the current production so clear to see the different after adding different concentraions of the polyphenols. Also, it is difficult to notice the decrease in the current production after adding the polyphenols after forming the biofilm. Figure 11. That make us to the need to check the polyphenols before forming the biofilm like a protection mode from contamination.

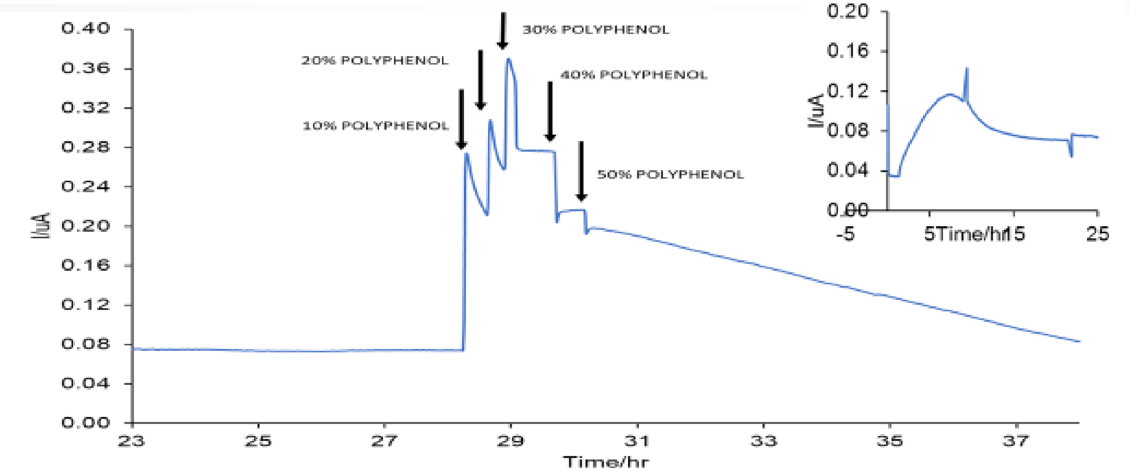



**Figure 11:** Representative current production versus time of *S. mutans* in bioreactor by using DM medium then added different concentrations of *Padina pavonica* polyphenol.

### *4.3.4)* **Effect of polyphenol extracted from four species of algae on *S. mutans* current production before forming the biofilm:**

On contrast from the last experminet, As will show in Figure 12 (A, B, C, D and E), this experminet from the beginning was performed by using the DM medium inculated with *S. mutans* 0.1 OD with the addition of two concentrations 10% and 20% of polyphenols extracted from four different species of algae (*Padina pavonica*, *Sargassum muticum*, *Halimeda opuntia* and *Jania rubens*) to check the current production of *S. mutans* mixed with polyphenols from zero point of running the experiment and during the metabolism of *S. mutans*.

This experminet consist of 8 bioreactors, each two bioreactors represent *S. mutans* with one of polyphenol extracted from one algae, but with different concentration; Figure 12 **A** represent *S. mutans* with polyphenol extracted from *P. pavonica* (one bioreactor containing 4.5 ml of DM with *S. mutans* 0.1 OD plus 0.5 ml of *P. pavonica* polyphenol (10%), the other bioreactor containing 4 ml of DM with *S. mutans* 0.1 OD plus 1 ml of *P. pavonica* polyphenol (20%)). The same in Figures 12 B with *Sargassum muticum* polyphenol, Figure 12 C with *Halimeda opuntia* polyphenol and in Figure 12 D with *Jania rubens* polyphenol.

Measuring the current production of *S. mutans* (Ic) was approximately 0 – 0.6 μA cm−2; clearly at Figure 12 A, upon addition of 20% of polyphenol extracted by *P. pavonica*, the current production immediately sharply lower, suggesting that polyphenol might damage *S. mutans* it spent more than 30 hour with this limt of no current production. Also as clearly in the same Figure 12 A; with the other concentration 10% of *P. pavonica* polyphenol; the current production was increased gradually by running the experiment without any effect on the normal current production of *S. mutans.* The same behavior in Figures 12 B and C, no current production by using 20% of polyphenols from *Sargassum muticum* and

*Halimeda opuntia,* with 10% polyphenol no any effect on the current production.

On completely different behavior, the 20% of polyphenol from *Jania rubens* gave the same effect like 10%, No significant change was observed in the current generation of *S. mutans* current production. That's refer to the sensitivity of this system with different orign of polyphenols.

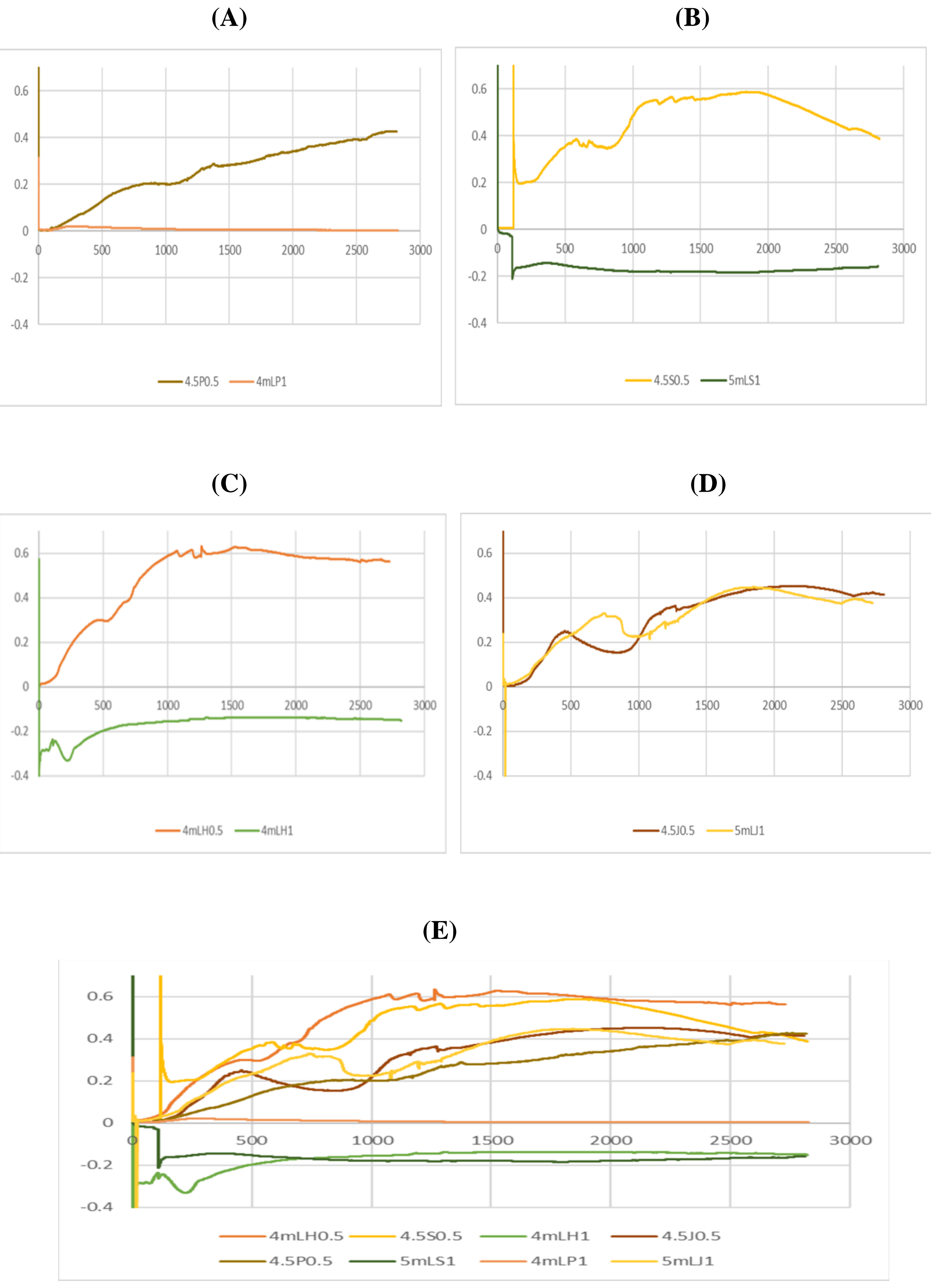



**Figure 11:** Representative current production versus time of *S. mutans* in bioreactor with different algal polyphenol (A) *P. pavonica*, (B) *S. muticum*, (C) *H. opuntia* and (D) *J. rubens* polyphenols with two concentrations 10% and 20%. (E) represent collection of four algal

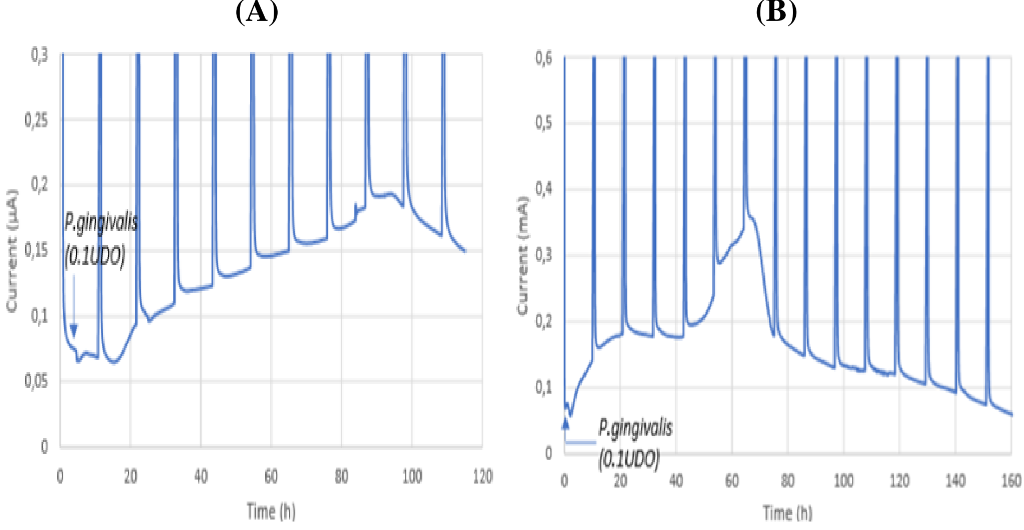
polyphenols together to see the different from different species algal polyphenol effect on *S. mutans* current production.

### *4.3.5)* Effect of polyphenol extracted from *Padina pavonica* on *Porphyromonas gingivalis* current production After forming the biofilm:

- **Firstly, the controls:**

*P.gingivalis* is grown anaerobically in 10mL of Gam medium where 100% N2 was bubbled for 20 min. After 24h of incubation at 37°C without agitation, *P.gingivalis* is washed 2 times in Defined Medium (DM). The DO is measured in such a way to add 0.1 UDO in a bioreactor fill with 5mL of DM. Bacteria are incubated 10 hour in DM at 37°C before being add in the bioreactor. After 5 hour of potentiostat measurement without bacteria, bacteria inoculation in the bioreactor is performed without opening the box nor the bioreactor, thanks to 2 septum (one on the box, the other one on the bioreactor) and a siring with a long needle. Two different experments were performed to check the current production of *P.gingivalis* as a control only in Dm medium without any adding of polyphenols. The current production start after a 10 hours latency (5 to 15h). It is explainable by the time required to the bacteria to change their metabolism to assimilate saccharose in DM medium, instead of GAM medium as will show in next Figure 13 (A and B).

For Figure 13 C, it is ultra control to check the effect of DMSO 10% (that was used as a co-solvent for polyphenol) on the current production. As will show in the figure, there is no affect from DMSO 10%.



**(C)**

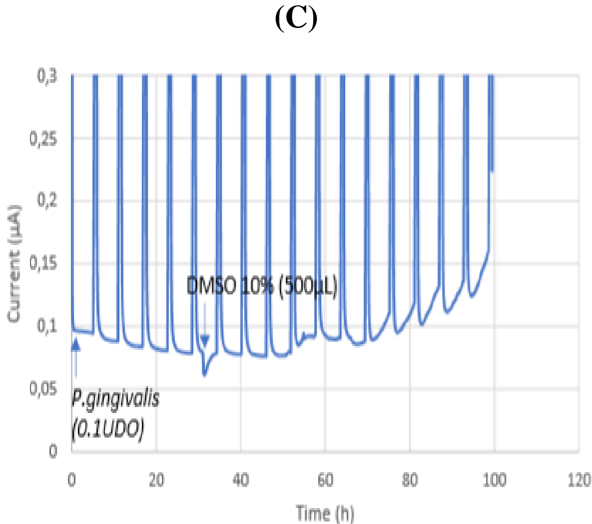
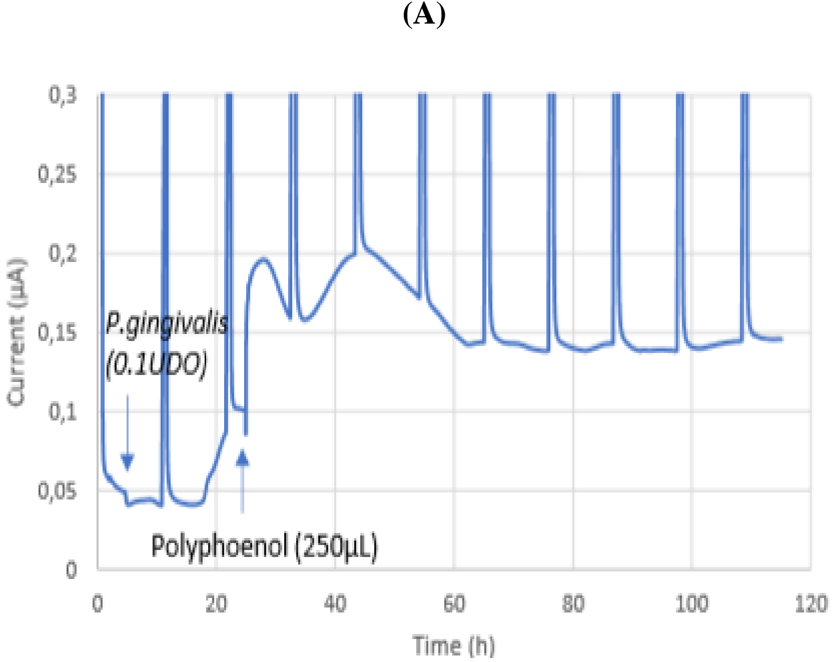



**Figure 13:** (A and B) Representative current production versus time of *P.gingivalis* by using DM medium, for (C) the same but with adding DMSO 10%.

- **Secondly, Adding *Padina pavonica* on *Porphyromonas gingivalis*:**

Adding *Padina pavonica* polyphenol induce an early decreasing of the current production showing a reduction of the bacterial metabolism: in condition without polyphenol, the pick of activity is rush after 94 hours, whereas when Polyphenol is added in two different concentrations 250 µl (5%) and 500 µl (10%) after 25 hours of incubation, activity decrease from 45h (20h after polyphenol inoculation). Figure 14 (A and B).

**(A)**



**(B)**

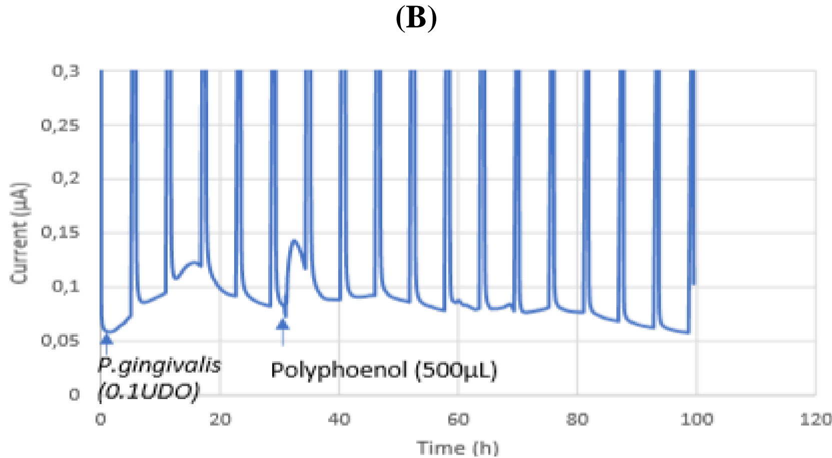



**Figure 14 (A and B):** Representative current production versus time of *P.gingivalis* by using DM medium then added different concentrations of *Padina pavonica* polyphenol.

### *4.3.5)* Effect of polyphenol extracted from four species of algae on *Porphyromonas gingivalis* current production before forming the biofilm:

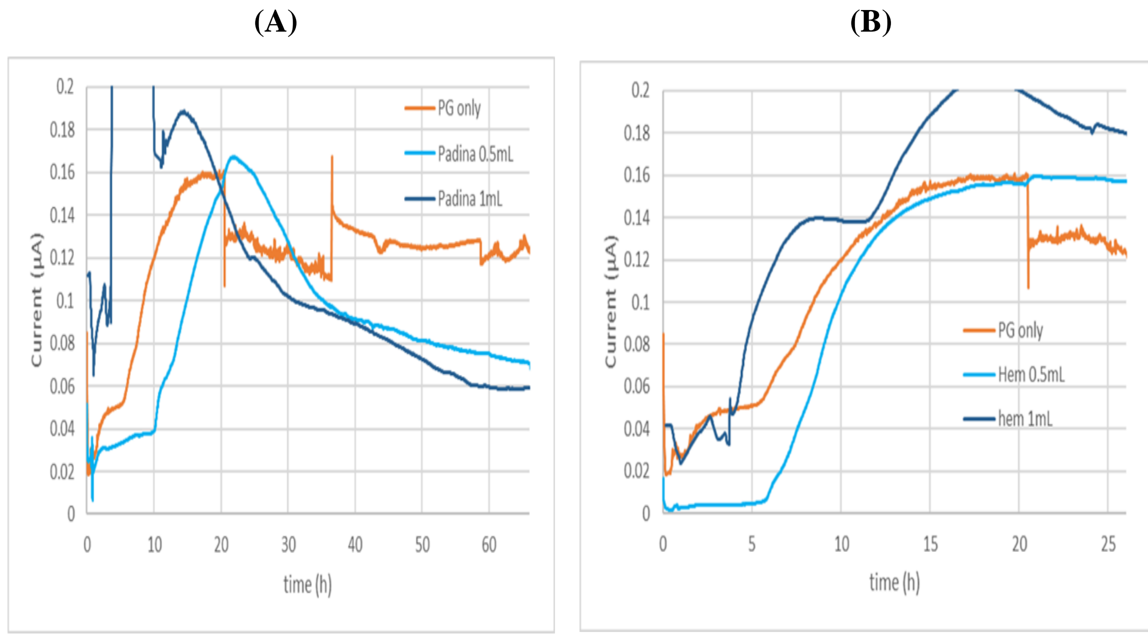
On contrast from the last experminet, and with the similar of experminet in part (**4**.3.5). As will show in Figure 15 (A, B, C and D), this experminet from the beginning was performed by using the DM medium inculated with *P.gingivalis* 0.1 OD with the addition of two concentrations 10% and 20% of polyphenols extracted from three different species of algae (Padina pavonica, Halimeda opuntia and Jania rubens) to check the current production of *P.gingivalis* mixed with polyphenols from zero point of running the experiment and during the metabolism of *P.gingivalis*.

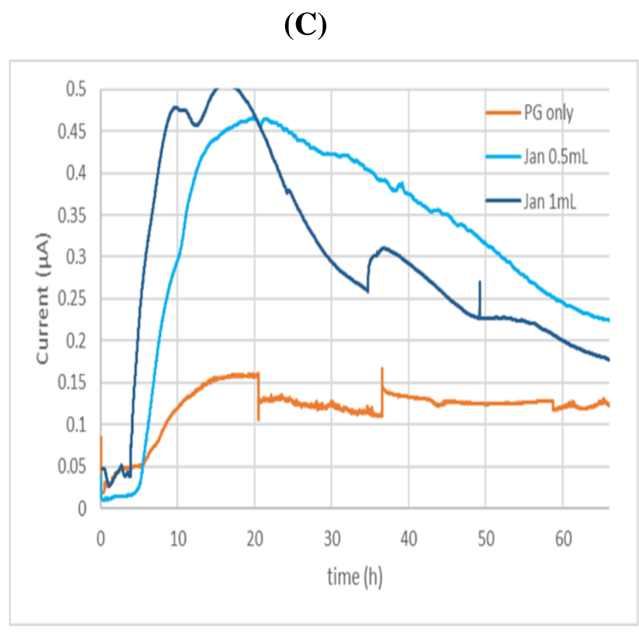
This experminet consist of 7 bioreactors, one bioreactor was contained 5 ml DM medium inoculated with 0.1 OD of *P.gingivalis* "running as control". For the others 6 bioreactors each two represent *P.gingivalis* with one of polyphenol extracted from one algae, but with different concentration; Figure 15 A represent *P.gingivalis* with polyphenol extracted from *P. pavonica* (one bioreactor containing 4.5 ml of DM with *P.gingivalis* 0.1 OD plus 0.5 ml of *P. pavonica* polyphenol (10%), the other bioreactor containing 4 ml of DM with *P.gingivalis* 0.1 OD plus 1 ml of *P. pavonica* polyphenol (20%)). The same in Figure 15 B with *Halimeda opuntia* polyphenol and in Figure 15 C with *Jania rubens* polyphenol.

Measuring the current production of *P.gingivalis* (Ic) was approximately $0 - \geq 0.6$ µA cm−2. For the current production of *P.gingivalis* alone as a control, it started from zero and increased to reached 0.16 µA then alomstly stay stable between range 0.16 – 0.12 µA during 60 hrs of its life cycle. Clearly at Figure 15 A, upon addition of 10% and 20% of

polyphenol extracted by *P. pavonica*, the current production started with the normal way of the control, but after 20 hrs from running the experiment it started to decreased sharply than the control.

On completely different behavior, in Figures 15 B and C, both concentrations 10% and 20% of polyphenol from *Halimeda opuntia* and *Jania rubens* increased the current production of *P.gingivalis* than in the control. That's refer also to the sensitivity of this system with different orign of polyphenols.



**(E)**

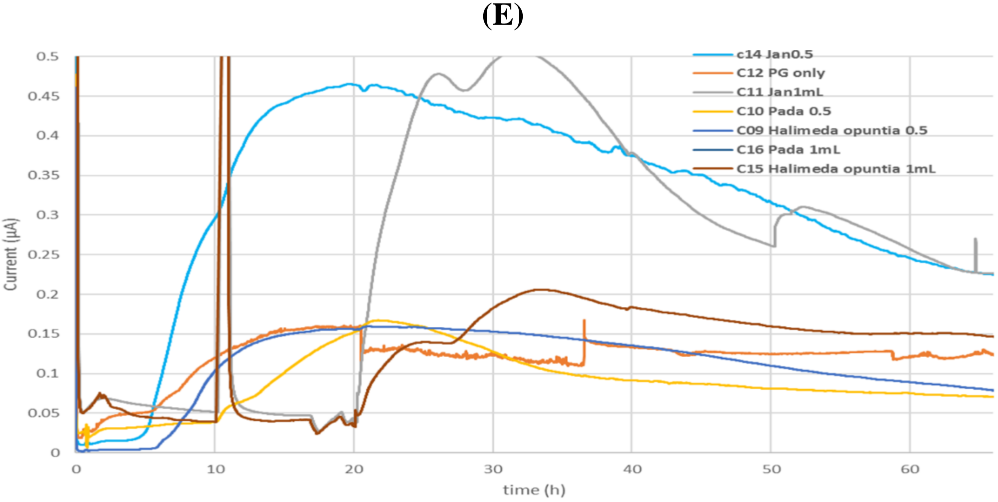



**Figure 15:** Representative current production versus time of *P.gingivalis* as control with different algal polyphenol **(A)** *P. pavonica*, **(B)** *H. opuntia* and **(C)** *J. rubens* polyphenols with two concentrations 10% and 20%. **(E)** represent the collection of three algal polyphenols together to see the different from different species algal polyphenol effect on *P.gingivalis* current production.

## 4.4) Discussion:

The discovery of a new infection treatment strategies is a very important goal now with increasing bacterial virulence and resistance. In addition to that, the discovery of a new technique or first report of a novel method to check the new property of bacteria to produce electrons as a part of its metabolism. These interactions can often be studied using electrochemical techniques which have provided novel insights into microbial physiology in recent years with understanding microbe-electrode interactions, with important implications for environmental microbiology. On the other hand, the other goal is to re-evaluate the significance of complex plant-derived antibacterial substances with mild yet multi-targeted activity from natural sources. On the other hand, the marine environment is a good reservoir of bioactive natural products, many of these seaweeds exhibit structural features that didn't find in terrestrial natural products. every year there are an increasing number of new marine metabolites were reported in the literature, indicating that the marine environments will be a prolific source of several natural products for many years to come. For two decades, Marine algae were found to be a vital source for useful bioactive substances. Several studies have demonstrated that seaweeds are an excellent source of components with biological activity such as antibacterial [27].

In this study, the EET ability of the extractions from the four different strains of Egyptian algae (*Padina pavonica*, *Sargassum muticum*, *Halimeda opuntia,* and *Jania rubens*) were investigated to detect the electrochemical characterization for their antibacterial activity at different concentrations from the original solutions prepared. Red Sea algae characteristics and bioactivities are less investigated and just a few information regarding their particular activities were communicated [21]. Moreover, Marine algae compounds provide many beneficial contributions in Dentistry by preventing oral bacterial adherence and colonization.

Firstly; total polyphenol content in the four different strains of algae extracts was evaluated by means of the Folin–Ciocalteau test to determine whether the selected source was sufficient to provide an adequate bulk of polyphenols. Total carotenoid (C), chlorophyll-a (Ch-a) and chlorophyll-b (Ch-b) contents of the extracts were determined spectrophotometrically (Jenway 7205) according to Sumanta and co-workers (2014) [28]. All samples were analyzed in triplicate and results expressed as mean ± SD. The total polyphenol content of algae ranged from 368.3 mg GAE/g DW in *S. muticum* to 75.6 mg GAE/g DW in *P. pavonica* ($p < 0.05$) (Figure 6). *S. muticum* exhibited higher levels of carotenoids (42.8 mg/g DW) than *J. rubens*, *H. opuntia* and *P. pavonica* (3.6, 1.8 and 0.9 mg/g D, W respectively) ($p < 0.05$) (Figure XX). In *S. muticum*, the Ch-a content was higher (163.6 mg/g WD) than that of Ch-b (43.2 mg/g DW) ($p < 0.05$) (Figure 7). Ch-b was the most abundant pigment in *H. opuntia* and *P. pavonica* (112.6 and 129.6 mg/g DW, respectively) ($p < 0.05$) (Figure 7).

Secondly: the electrochemistry properties of oral pathogenic bacteria *S. mutans* were studied with some commercial antibacterial agents (as a control); by using the electrochemical system by measuring the current production. *Streptococcus mutans* is frequently associated with dental caries, bacterial fermentation of food debris generates an acidic environment on the tooth surface, ultimately resulting in tooth deterioration [29]. Therefore, two antibiotics have been used to reduce and prevent *Streptococcus mutans* current production, these antibiotics are commonly prescribed by dentists. In our study, we observed that by using Ampicillin 2 µl/ml, the current production of *S. mutans* is started to decrease with 30 % less than the current production of the control *S. mutans.* Also, by using Triclosan 3 µmol, the current production of *S. mutans* is decreased by 70% less than the control of current production. There is no other previous report for that to check the antibacterial effect of antibiotics by using the electrochemistry system but there are many previous studies checked that by using disc diffusion methods and other

classic microbiological methods [30]. This experiment was studied to just check the electrochemical system how it is working by using the antibacterial agent.

Secondly: data provide evidence that the use of different concentrations from different strains of algae gave different EET, some recorded results of EET are significantly lower than those typically reported as a control in this investigations, others more than the EET of control samples. The low currents observed here also point to the lower ability of these isolated strains to gain cellular energy from external redox-active surfaces. The physiological role of this mode of energy acquisition from external substrates should be further investigated in detail.

Results from several laboratories indicate that extracellular electron transfer may be a general mechanism whereby microorganisms generate energy for cell growth and/or maintenance. Specifically, bacteria can use redox-active organic small molecules, generated outside or inside the cells, to shuttle electrons between reduced and oxidized compounds. Electron shuttling has now been reported for several different bacterial species, and exchanges of shuttling compounds may even syntrophically link diverse organisms in nature. Biofilm systems in both geological and clinical settings are likely to be important environments for metabolisms that employ extracellular electron transfer [31]. The effect of polyphenol extracted from *Padina pavonica* on *S. mutans* current production after forming the biofilm showed that the current production strongly effected by coupling with the *Padina pavonica* polyphenol addition by eight channels electrode or by bioreactor. It is difficult to notice the decrease in the current production after adding the polyphenols after forming the biofilm Figure (10 &11). That makes us to the need to check the polyphenols before forming the biofilm-like a protection mode from contamination. So, Effect of polyphenol extracted from four species of algae on *S. mutans* current production before forming the biofilm was studied, but the EET capability of these specific strain has not been reported,

Older studies reported that Seaweeds or macroalgae are available in the intertidal, shallow and deep waters in the marine environment. They have been reported to contain many important compounds that act as antibiotics, laxatives, anticoagulants, antiulcer products. Seaweeds are known to produce a variety of secondary metabolites that have been characterized as a broad spectrum of antibacterial agents antiviral, anticancer compounds antioxidant compounds, antifouling compounds, pharmaceutical preparations [32]. According to that, the EET capability of *S. mutans* before forming the biofilm coupling with polyphenol extracted from four species of algae with two concentrations

10% and 20% of polyphenols was studied and showed good evidence of reducing the current production of *S. mutans* by using 20% of *P. pavonica*, *Sargassum muticum* and *Halimeda opuntia* aqueous solution of polyphenols, suggesting that polyphenol might damage *S. mutans.* But by using 10% of the same previous strains plus 10% and 20% of *Jania rubens* the current production was increased gradually by running the experiment without any effect on the normal current production of *S. mutans*.

In our study, we checked also the EET of other oral pathogenic Gram-negative bacteria; *Porphyromonas gingivalis,* it is found in the oral cavity, where it is implicated in periodontal disease, as well as in the upper gastrointestinal tract, the respiratory tract and the colon [33]. Our results have shown that the EET of *Porphyromonas gingivalis* coupling with 10% of polyphenol from *Padina pavonica* after forming the biofilm, is reduced with 30%, but not sufficient reduction in the current production by using 10% from the same polyphenol.

While when we used the EET capability of *Porphyromonas gingivalis* before forming the biofilm coupling with polyphenol extracted from three species of algae (*Padina pavonica*, *Halimeda opuntia,* and *Jania rubens*) with two concentrations 10% and 20% of polyphenols was studied and the results shown strongly effect from *Padina pavonica* with both concentrations to reduce the current production than in control bioreactor. On completely different behavior, both concentrations 10% and 20% of polyphenol from *Halimeda opuntia* and *Jania rubens* increased the current production of *P.gingivalis* than in the control. That refers also to the sensitivity of this system with the different origins of polyphenols. Using these natural polyphenols before forming the biofilm is considering as the antiinfective agents in preventing recolonization of bacteria, chlorhexidine digluconate (CHX) is a widely used agent in periodontitis treatment. Development of resistance against antibiotics and side effects of the drugs implicate search for alternatives. Among others, plant-based therapy including the combination with antibiotics or the usage of honey might be one option [34].

We consider that this new EET mode, coupled with new natural antibacterial agents, may open new windows for biotechnological applications and pathogenicity control models. It should be noted that while the focus here was on gut microbes, similar electrochemically active microorganisms with EET capability may be of interest in the vast range of other human pathogens and the external environment.

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