Antimicrobial activity of plant natural extracts and essential oils

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The use of natural products with curative aims is a common practice in any culture worldwide. It is mainly due to the activity of some extracts that they contain (terpenes, essential oils, coumarins, flavonoids, etc.). The main aim of this chapter is to describe the techniques that allow evaluating the antimicrobial activity of plants extracts and their essential oils against bacteria and fungi. The different methodologies will be differentiated in: Techniques on broth culture media, techniques on solid culture media, microwell techniques, aromatogram technique and bioautographies. These techniques also allow establishing the Minimum Inhibitory Concentration (MIC) of the assayed products.

Keywords Natural extracts; essential oils; MIC; aromatogram; techniques; bioautography

1. Introduction

The use of natural products with curative aims is a common practice in any culture worldwide. The discovery of America and the expansion of the European Empires in Africa and Far East made possible the finding of new plants with medicinal properties, and therefore were used to treat many different diseases. These plants were consumed in many different ways: infusions, steam and intake, among others. Nowadays, more than 1340 plants are known to be potential sources of antimicrobial compounds [1], but more than 250000 species have been described for the diversity of the bio-active compounds that contain.

Most of the times, natural extracts owe their biologic activity to the synergism between their different compounds, as their activity is lesser than when these substances are together. It is also considered that the toxicity of the extracts is higher if the substances are purified whereas is reduced when considering the pool of compounds. This phenomenon is known as buffering [2, 3].

Regarding their antimicrobial properties, these are mainly attributed to some of the compounds such as terpenes, essential oils, coumarines and flavonoids [4-7].

Natural extracts obtained from plants have been widely used since many centuries ago in order to treat many pathologic processes. Going back in time, we can pay attention to the discovery of a frozen corpse in the Alps. That person died more than 5300 years ago, and among his personal belongings, several fruits of Piptous betulimus were found. These fruits are known for their antifungal and antiparasitic activity, as well as their activity against Mycobacterium species [8]. It means that at that moment, that person was aware of the properties of those fruits, proving therefore the empiric knowledge regarding the benefits of consuming some plants.

The first text about medicinal plants is dated on 3000 BC and was written by the Sumerians [9]. Ancient Greeks also used those plants for their treatments, especially because Crete has a large variety of plants. It helped this civilization carry out an important role in the development of medicine in the Mediterranean area between the 1st and 2nd centuries AC. The first known complete Hellenic treaty regarding medicinal plants was written by Diocles of Karystos (IV AC). This treaty, named Phizotomikon, verses about the origin, acknowledgement and medicinal value of several plants.

The discovery of the New Continent and the expansion of the European Empires throughout Africa and Orient, allowed the knowledge of many new plants with medicinal properties and this their therapeutic properties were applied to the treatment of several diseases. These plants were consumed in different ways: infusions, steams and/or intake among others.

Probably, one of the first drugs of the traditional folklore that became a modern drug by the end of the XVIII century was Digitalis purpurea L., and it means the beginning of the modern pharmacology [10].

Subsequently, the era of modern chemotherapy started in 1928, when Sir Alexander Fleming form Mary’s Hospital in London, found out that a substance produced by the fungus Penicillium notatum that had contaminated a plate with growth of Staphylococcus aureus, was able to inhibit the growth of that bacterium [11, 12]. As a result of that discovery, phytotherapy was left aside, especially in the developed countries, and in contrast, chemotherapy had a meteoric development thanks to the discovery of new antibiotics. These antibiotics were most of the times isolated, purified and industrially processed from natural sources. The use of antibiotics was extended especially in animal production, mainly in pork and poultry production. These substances were used not only to treat diseases but also were routinely added in feed as growth promoters.

Nowadays, plant natural extracts is a business worth millions of euros worldwide. More than 1340 plants are known as potential antimicrobial compounds sources, but more than 25000 plant species are known to contain a wide range of
bio active compounds. Only in 1999, the global business of plant natural supplement sales for human consumption, reached $15 billion, from which $7 billion were in Europe, $2.4 in Japan, $2.7 in the rest of Asia and $3 in North America [13].

Often, natural extracts owe their biological activity to the synergism among their different compounds, as separately they show less activity than when being together. It is considered that the toxicity of the extracts is much less when the compounds are together than when they are purified. This phenomenon is known as buffering.

Regarding their antimicrobial properties, these are mainly due to the action of some compounds like terpenes, essential oils, cumarines and flavonoids [14-16].

The exact mechanism of action of many natural extracts is not well known, but it has been proved that owe their bacteriostatic or bactericide activity to the overload that the bacterial cellular wall undergoes. This fact determines the loss of control and integrity of the wall. Apart from the antimicrobial activity of some natural extracts, these may possess other beneficial biologic activities [14]: Allium species are tested because of their anticancerous properties [17], or for the action over the enzymatic system, thus improving appetite and optimizing nutrient absorption [18].

Other uses that natural extracts may possess are due to other properties such as: anti-inflammatory, immunomodulatory, spasmylytic and sedative.

The bioflavonoids present in some plants, mainly citrus fruits, have beneficial effects on the animal performance. It can be explained taking into account the following facts: reduce amino acid oxidation, exert antimicrobial action against some intestinal micro-organisms, promote intestinal absorption, stimulate enzyme secretion, increase feed palatability [19] thus stimulating intake, and improve the immunologic status of the animal.

Natural extracts are part of a group of substances considered as “tolerated” but not admitted as additives in a strictly legal way. Vegetal extracts would be included in the additive group classified as “aromatic and flavouring substances”, in which are included all the natural products and relevant synthetic products and that can be used on all animal species, without any restriction regarding age of product dosage. Due to the fact that these products are well accepted by consumers, they are a promising alternative to growth promoter antibiotics, and the search of new substances represents an important research area in the field of food additives.

The most common active principles obtained from plants are: essential oils (volatile), resins, alkaloid glycosides and fixed oils.

1.1 Essential oils

According to the 8th Edition of the French Pharmacopeia (1965), essential oils are products of complex general composition that contain volatile principles present in plants, more or less modified during their preparation. Essential oils are mainly found in superior plants. It is calculated that there are more or less 17500 aromatic species. These substances are chemical products that constitute the odoriferous essences of many plants, and are widely distributed in approximately 60 plant families from which we can point out Asteraceae, Lamiaeceae, Lauraceae, Myrtaeae, Pinaceae, Rosaceae, Rutaceae, Umbeliferae, etc. [20].

2. Methodology to evaluate the antimicrobial activity of plant extracts and organic acids

Currently, there is not a clear regulation and/or standardization of the methodology to evaluate the inhibitory activity of plant extracts (PE) as it is established for antibiotics. Most of the methods are based on the ones used to evaluate the resistance and/or susceptibility to antibiotics.

However, the methods that allow evaluating the activity of plant extracts against bacteria and fungi are similar, differing in the inoculum preparation, culture media, temperature and incubation time [21].

The most commonly used methods in the laboratory for their simplicity and quickness are:
- Agar diffusion disk technique: used to obtain qualitative results.
- Dilution in broth and agar: used to obtain quantitative results [22].

2.1 Agar Methods

Among these methods, the most used due to the simplicity and quickness of results interpretation, is Agar diffusion disk method, based on the methodology used by Bauer et al. [23]. This method consists of applying a fixed amount of an antimicrobial substance or similar on a substrate (usually paper disks) onto the agar surface where an inoculum with the micro-organism to be tested has been previously spread. An inhibition halo will appear around the disk, and the sensitivity of the microorganism to the tested product will be measured according to the diameter of the halo, where no microbial growth is observed.

This diameter will depend not only on the sensitivity of the microorganism but also on the concentration on the disk, the thickness of the agar, the pH and the composition of the culture medium, the ability of the tested product to diffuse
in that medium, the temperature, the incubation atmosphere, the speed of bacterial growth, the amount of inoculum and the phase of the bacterial growth [24].

In order to evaluate antibiotics, the most used medium is Agar Mueller Hinton, but for plant extracts, many different media are used. The most commonly used are Agar Mueller Hinton, Tryptone Soya Agar, Nutritive Agar and Brain Heart Infusion Agar [25-27].

Another evaluation method in agar is known as agar dilution. In this method, the product to be tested is added in a medium with agar. The product is added when the medium is still liquid. In order to achieve the desired dilution range, several plates are prepared, each one containing a fixed concentration of the product. The plates are then inoculated once the medium is solid [22].

2.1.1 Agar diffusion disk method

This method is used in order to evaluate the antimicrobial activity of natural extracts and organic acids against bacteria and fungi.

2.1.1.1 Methodology for bacteria

This method was described by Bauer et al. in 1966 [23] and modified by Calvo and Asensio in 1999 [25]. The culture medium was Tryptone Soya Agar (TSA). The bacterial inocula used had a concentration of 1-2x10^8 CFU/ml. The medium was poured onto 90mm diameter Petri plates until the thickness of the agar was 4mm so that possible problems of diffusion of the tested products could be prevented [24].

0.1ml of each bacterial solution was inoculated onto the plates and by means of sterile swabs was uniformly distributed. Plates were allowed to stand for 15 minutes.

At the same time, 6mm diameter disks (Schleicher & Schuell) were soaked with the products to be tested at different concentrations. After discarding the excess of product, the disks were symmetrically placed onto the medium by means of sterile tweezers. One of the disks was soaked with sterile distilled water as a control.

The plates were incubated for 24±2h at 37ºC under anaerobic conditions. The results were evaluated by measuring the areas with no bacterial growth. These results were obtained after using the following formula:

\[
\text{Inhibition value} = \frac{\text{Inhibition diameter in mm} - \text{Disk diameter (6mm)}}{2}
\]

These experiments were carried out in triplicates and control cultures were prepared for all the strains.

2.1.1.2 Methodology for fungi

The used culture medium was Sabouraud Dextrose Agar (SDA). The bacterial inocula had a concentration of 1-2x10^6 CFU/ml. The medium was poured onto 90mm diameter Petri plates until the thickness of the agar was 4mm so that possible problems of diffusion of the tested products could be prevented [24].

0.1ml of each fungal solution was inoculated onto the plates and by means of sterile swabs was uniformly distributed. Plates were allowed to stand for 15 minutes.

At the same time, 6mm diameter disks (Schleicher & Schuell) were soaked with the products to be tested at different concentrations. After discarding the excess of product, the disks were symmetrically placed onto the medium by means of sterile tweezers. One of the disks was soaked with sterile distilled water as a control.

The plates were incubated for 48±2h at 28ºC under aerobic conditions. The results were evaluated by measuring the areas with no fungal growth. These results were obtained using the same formula than for bacteria:

\[
\text{Inhibition value} = \frac{\text{Inhibition diameter in mm} - \text{Disk diameter (6mm)}}{2}
\]

These experiments were carried out in triplicates and control cultures were prepared for all the strains.

2.1.2 Aromatogram

This technique allows the evaluation of the antimicrobial activity due to some compounds volatilization.

If plate diffusion methods are used, it may happen that some of the compounds of essential oils are volatile or/and their solubility in water is very low (hydrophobic). In order to evaluate this parameter, the extracts of this study were tested by means of the method described by Ross et al. in 2001 [28]: TSA plates were prepared and inoculated with Escherichia coli FVB467 as representative bacterial strain, and SDA plates inoculated with Aspergillus ochraceus ATCC 2948 as representative fungal species. The plates were allowed to stand for 15 minutes. Afterwards, the plates were inverted and a sterile disk soaked with 15μl of the raw extract to be tested was placed in the inner side of the lid.

The assays were carried out in triplicates and control plates were also prepared. The plates were incubated at 37°C for 24±2h for bacteria and at 28°C for 48h for fungi. All plates were carefully sealed with Parafilm. After the incubation time, the possible growth inhibition was evaluated by means of comparing the growth with the control plates.
2.1.3 Micro-well plate dilution technique

This methodology is applied in order to establish Minimum Inhibitory Concentrations (MIC) of different products, against bacteria and fungi.

2.1.3.1 Methodology for bacteria

The used technique was a variation of the described by Eloff in 1998 [29]. The assay was carried out using 96 U-bottom microwell plates (Rubilabor S.L.). In order to improve the solubility of some essential oils, a mixture of TSB (Tryptone Soya Broth) and agar 0.15% was used as emulsificant.

The first row of wells was inoculated with the product to be tested previously diluted in TSB or TSB + Agar 0.15% to reach 32000ppm (100 μl) and the rest of the wells with 50 μl TSB. Serial dilutions were prepared using the first row of wells and afterwards 50 μl of the microorganism (1-1.5x10⁶ CFU/ml) was added, so that the final concentration of the microorganism ranges between 5 and 7x10⁵ CFU/ml and the product is diluted until 16000ppm (final volume for the wells containing 100 μl). The last row contains positive and negative controls.

Before incubation, in order to control that the final concentration is correct, 10 μl of one of the positive controls are taken and diluted in 10ml of Ringer solution. Afterwards, 100 μl are taken and 3 TSA plates were inoculated. The incubation was at 37°C for 24h, and the valid plates were those in which 50 to 70 CFU could be counted.

All assays were carried out in duplicate. It was considered that the result was valid if for each replicate there was no variation in one dilution (above or below) and if the positive controls had those values as well.

The microplates were incubated at 37°C under aerobic conditions for 24-48h. Afterwards, 40 μl of p-iodonitrotetrazolium violet salts solution (INT) were added so that the final concentration was 0.2mg/ml INT. The microwells were allowed to stand at 37°C for 30-60 minutes and results were observed. This salt solution is an indicator of biological activity since its components act as electron acceptors and are reduced. It produces color when there is microbiological activity, and where no activity is present, there is no color.

2.1.3.2 Methodology for moulds

This methodology is based on the technique described for bacteria. In this case, the culture medium is Sabouraud Dextrose Agar (SDA), except for the insoluble essential oils, which needed a mixture of Sabouraud Dextrose Broth (TSB) + 0.15% agar. The final mould concentration was 5-7x10⁴ CFU/ml.

The incubation time was 48h at 28°C, and it was no necessary to add vital colorants in order to see the results, which were carried out visually. It was considered the minimum inhibitory concentration as the lowest concentration in which growth was not observed.

2.2 Liquid culture media techniques

The quantification of the in vitro activity of the essential oils is commonly carried out by means of one of the variations of the dilution methods. These methods are based on the determination of the growth of the microorganism when present with increasing concentrations of the essential oil which is diluted in the culture medium (broth).

These assays may be carried out using many tubes containing broth culture media with a fixed range of oil (macrodilution). This methodology is not practical as much equipment is necessary and is time consuming. The use of micropipettes and microwell plates makes easier the broth microdilution method.

This method has been used to determine Minimum Inhibitory Concentration (MIC) of plant extracts. MIC is known as the lowest concentration that keeps or reduces the viability of the inoculum after 24h in contact. In most of the cases, different dilutions of the product to be tested are prepared in geometric progression in base 2 using the suitable culture medium; Afterwards that medium is inoculated and after incubation, it is determined which is the concentration that inhibits the growth of the microorganism.

Keeping in mind that most of the microwell plates have 96 wells (12x8), up to 11 dilutions can be assayed for a product, and the last row may be used for the positive and negative growth control and sterility control.

The standard culture medium used to evaluate antibiotics by means of the broth dilution method is Mueller Hilton Broth (MHB) although for the evaluation of MIC of plants extracts other culture media are used such as Nutritive Broth (NB), Tryptone Soya Broth (TSB) and sometimes adding supplements like serum [30-33].
Fig. 1 Example of the results obtained regarding the study of the antimicrobial activity by means of the agar diffusion method. Left: Growth control plate. Right: Inhibition haloes produced by the action of natural extracts.

Fig. 2 Example of micro-well plate in which the assay to establish MIC has been carried out.
Control plate

Effect produced by citral

Fig. 3 Left plate: *Aspergillus ochraceus* ATCC 2948 positive growth control. Right plate: effect of citral vapours against this species (complete inhibition after 3 days incubation at 28°C).

References


