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Development of an antimicrobial compound with silver nanoparticles synthesized from rosemary (rosmarinus officinalis) leaf extract, to be applied on fruits

Elaboración de un compuesto antimicrobial con nanopartículas de plata sintetizadas a partir del extracto de hojas de romero (rosmarinus officinalis), para ser aplicado en frutas

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Abstract
This research aims the study of nanotechnology taking advance the natural resources of Ecuador. By using a plant to get nanoparticles friendlier with nature. It was used as a reducing agent obtained from rosemary leaves (Rosmarinus Officinalis) which facilitated the formation of nanoparticles without residues of toxic contaminants. The (AgNPs) were characterized by UV-VIS spectroscopy presenting an absorption in the range 400-418 nm, which corresponds to the surface plasmon resonance of the (AgNPs), also scanning electron microscopy (SEM) was used to determine the size and morphology of the (AgNPs) determining a spherical shape of 10 nm in diameter. It was verified through strains of E. coli, S. aureus and B. subtilis, the inhibitory activity producing halos of approximately 3.2 cm in diameter product of the (AgNPs) synthesized. Therefore, nanoparticles were applied to a traditional fruit (apple), a natural preservative (beeswax) was incorporated together with the (AgNPs). It caused efficient results proving its antimicrobial effect and maintaining its initial characteristics, concluding that the nanoparticles in this research are very high, due to their dimensions, inhibitory effect and excellent power in food preservation.

Keyword
Silver nanoparticles, reducing agent, green synthesis, activity antimicrobial, Rosmarinus officinalis.

Resumen
El presente trabajo de investigación se realizó con la finalidad de contribuir al estudio de la nanotecnología en el Ecuador aprovechando los recursos naturales que nos brinda nuestro país, mediante la utilización de una planta para la obtención de nanopartículas de plata que sean más amigables con la naturaleza. Se usó como agente reductor un extracto acuoso obtenido a partir de las hojas de romero (Rosmarinus officinalis), el cuál facilitó la formación de nanopartículas sin residuos de contaminantes tóxicos. Las (AgNPs) fueron caracterizadas mediante Espectroscopía UV-VIS presentando una absorción en el rango 400-418 nm, que corresponde a la resonancia del plasmón superficial de las (AgNPs), también se empleó la microscopía electrónica de barrido (SEM) para determinar el tamaño y morfología de las (AgNPs) determinando una forma esférica de 10 nm de diámetro. Se comprobó a través de cepas de E. coli, S. aureus y B. subtilis, la actividad inhibitoria produciéndose halos de aproximadamente 3.2 cm de diámetro producto de las (AgNPs) sintetizadas, por lo consiguiente dichas nanopartículas se la aplicó a una fruta tradicional (manzana), se incorporó un conservante natural (cera de abejas) conjuntamente con las (AgNPs), el mismo dio un resultado eficiente comprobándose su efecto antimicrobiano y manteniendo sus características iniciales. Se concluye que las nanopartículas realizadas en esta investigación son muy altas, debido a su estructura en tamaño, su efecto inhibitorio y su excelente poder en la conservación de alimentos.

Palabras Claves
Nanopartículas de plata, agente reductor, síntesis verde, actividad antimicrobiana, Rosmarinus officinalis.

1. Introduction

Since ancient times, plants have been used in various parts of the world for their nutritional and medicinal properties. Although the use of plants for therapeutic purposes was, for many years, associated with religious and attractive rituals, the emphasis on their use has led to the subsequent ancestral knowledge of plants. The origin of the use of essences and aromas is as old as agriculture itself. It began with the observation and collection of plants used by animals, followed by selective gathering of the best organoleptic characteristics such as fruit flavor, size, and aroma, eventually leading to the domestication of the most useful plants until they were widely cultivated [1].

Today, plants are utilized in the food industry, medicine, cosmetics, and more. In recent years, the effect of bioactive compounds present in plants has been studied for use in medicine, leading to several reports on their functional,

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medicinal, and/or toxicological properties [1]. However, the current knowledge of plants and their bioactive compounds is very limited compared to the potential they offer in the development of new compounds. [1] Various research sources indicate that the most important byproducts of rosemary are its aqueous extract and essential oil. [2]. In recent years, there has been a substantial amount of scientific contributions providing extensive information on the applications of rosemary. [3]

Considering that synthesizing silver nanoparticles through physical and chemical methods has a significant environmental and economic impact, ongoing research aims to find eco-friendly and efficient methods, known as green synthesis, that produce fewer contaminants compared to other already established methods. [4]

Nanotechnology has been applied in various fields such as medicine, the textile industry, agriculture, etc.; likewise, some recent studies have utilized silver nanoparticles derived from plant extracts. [5]

Some reports have been made on the synthesis of silver nanoparticles from fruit peels, such as bananas, as well as from plants like garlic, aloe vera, basil, matico, coriander, and rosemary, which contain bioactive components with natural antimicrobial properties capable of reducing silver and synthesizing nanoparticles [6]. Plant extracts like rosemary (Rosmarinus officinalis) have been shown in several studies to possess a wide variety of antioxidant and antimicrobial compounds and properties used in traditional medicine [7].

There are also several reports where research has been conducted on rosemary's compounds, showing the presence of secondary metabolites and essential oils. Some compounds found in rosemary include flavonoids, terpenes, and phenolic acids, among others [3]

Based on the previously described specifications, this thesis proposes synthesizing silver nanoparticles from rosemary leaf extract due to its various properties, such as polyphenolic compounds (PFCs), which are the main reducing agents and are crucial for nanoparticle development.

In recent years, there has been an increase in microbiological diseases, which is attributed to the resistance of certain pathogenic microorganisms to conventional antibiotics, often used as additives in the food industry. Numerous reports suggest these antibiotics are considered carcinogenic and teratogenic due to their residual toxicity. For this reason, there has been a growing trend towards the use of natural-origin preservatives, which have gained market space as a viable alternative in antimicrobial substances. [3]

Additionally, previous studies indicate that rosemary leaf extract (Rosmarinus officinalis) exhibits antimicrobial activity. However, very few studies have been conducted on the Minimum Inhibitory Concentration (MIC), and it has been noted that the quantities of non-visible organisms evaluated are scarce. Determining the MIC is essential for evaluating the effects of non-traditional substances and for demonstrating the inhibitory values of pathogens, considering changes in their characteristics or parameters that need to be preserved. [2]

Rosemary (Rosmarinus officinalis L.) has been known since ancient times as an aromatic plant, with uses ranging from medicinal purposes to being used as a valuable seasoning. Its name is derived from the Greek "rhops" and "myrinos," meaning "marine shrub," due to its growth near coastal regions. The rosemary plant has been significant due to its medicinal properties, providing great benefits to humanity and its culture. [8]

For thousands of years, humans have had a close relationship with plants, mainly due to their medicinal uses. These plants have been crucial in the development of natural medicines and functional foods, as they are an important source of bioactive compounds. Research into the properties of rosemary, as well as its applications, has been established for many years. In addition to its geographically advantageous location for easy consumption and studies, making it easily accessible, its cultivation extends along coasts worldwide. Among rosemary's most important byproducts, which have also been highly beneficial and given added value, are its aqueous extract and essential oil. [9]

In the year 1330, rosemary essential oil was obtained for the first time by Ramón Llull, and since then, it has been used in perfumery. According to [9] this plant was used as a natural aromatic, and currently, industries have taken these ideas into account to create products aimed at satisfying needs. For example, it is used in the pharmaceutical industry due to its medicinal properties and also to alleviate discomfort caused by the ingestion of spoiled food. The food industry uses it as both an aromatic and flavoring agent.. [9]

Phytotherapy is the name given to the use of plants for therapeutic purposes. This science studies the use of medicinal plants with the goal of preventing, relieving, or curing diseases. Research indicates that since ancient times, herbs have been one of the main healing remedies used by humans. However, phytotherapy is currently an alternative that complements certain treatments. [10]

1.1. Origin of Rosemary

The plant species Rosmarinus officinalis, belonging to the family Lamiaceae and commonly known as rosemary, is a plant native to the Mediterranean region, cultivated in many countries worldwide. It is used as a condiment and aromatic agent in the preparation of some foods, as it is rich in active compounds that act on most organs of the human body. [3]. It contains sources of secondary metabolites and essential oils, with active compounds distributed among flavonoids, terpenes, phenolic acids, and other functional groups.. [3] The most relevant byproducts are its extracts, including aqueous extracts and essential oil. [2]. Additionally, it is used in traditional medicine for its digestive, antispasmodic, and carminative effects. Recently, a great deal of scientific contributions have provided extensive information about rosemary's applications. [3]

Its origin traces back to the Mediterranean region of southern Europe and northern Africa, where rosemary grows either spontaneously or through cultivation. It can generally be found in the Balearic Islands and in most of the Iberian Peninsula, except for the north, with its highest production occurring in warm lowland areas. Today, it is cultivated worldwide. [11]

Medicinal plants have gained significant demand as alternative or complementary therapies in various regions around the world. Medicinal plants are characterized by their high content of active substances or compounds with multiple chemical, biochemical, and organoleptic properties, which allow their better use for therapeutic purposes. Aromatic plants or essential oils, such as those from rosemary, possess antioxidant capabilities and are primarily used in the pharmaceutical or therapeutic industries. Their use in the food industry is also becoming increasingly prevalent, with their toxicological applications now being part of that industry as well. [12]

Numerous chemical compounds present in rosemary (R. officinalis L) have been identified and generally grouped by various authors. These include phenolic acids, flavonoids, essential oil, triterpenic acids, and triterpenic alcohols. [12]

Fig. 1. Rosemary
Source: [13]

The branches of rosemary have a dry appearance, typical of the areas where it grows, as can be seen in Figure 1. It is native to the Mediterranean region, where it is also cultivated. The main producing countries are Spain, Morocco, and Tunisia, but it can also be found in Oceania, Asia, South America, Central America, and some parts of North America. Its harvesting usually takes place from April to July, and it is stored in cardboard boxes or paper bags. [8]

1.2. Distribution of Rosemary

The distribution of rosemary (Rosmarinus officinalis L.) is located in the Mediterranean region of southern Europe and northern Africa, where the plant grows spontaneously. This is due to the sandy and calcareous soils, and agroecologically, the warm lowlands are the most suitable for its development. Nowadays, it is cultivated worldwide. [11]

The following image shows the global distribution of rosemary. It is important to note that it can generally be found in southwestern Europe, northern Africa, southwestern Asia, and Latin America. The introduction of rosemary to Latin America is attributed to the journeys made by the Spanish during the colonial era, where it was used for medicinal purposes as a substitute for Aztec remedies.

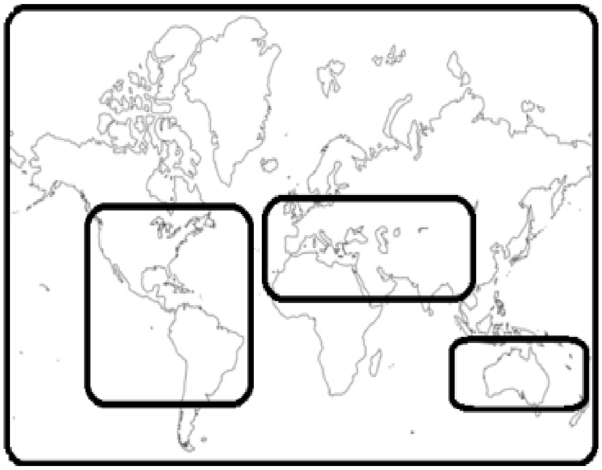

Fig. 2. Global Distribution of *R. officinalis*
Source: [12]

There are many countries where rosemary is cultivated, with Spain, Morocco, and Tunisia being the main producers. However, countries with relatively moderate production, such as Russia, England, France, Portugal, China, Australia, and the United States, also stand out. Rosemary grows within a temperature range of 2°C to 35°C; outside of this range, it does not grow adequately.

1.3. Characterization and Morphology

Rosemary is a shrub-like plant with small, spike-shaped leaves that have a shiny green coloration. It is a shrub that can grow up to two meters in height and blooms twice a year. This plant is very resistant to temperature changes, and its upright position is generally maintained. However, at times, it can be collapsed by other plants. Its average lifespan ranges between 5 and 15 years. Flowering can vary, as some authors state that flowers may be present on the plant year-round, while others claim there is no flowering during the hottest months of the year. Part of the stem grows straight with a velvety texture and has numerous spheroidal glands. [14]

The leaves have a simple structure, regularly measuring less than 4–6 mm in size, although there are branches that differ from others. Characteristics such as the outer edge of the leaf show it to be sharp and entire, with a linear and concave oscillation, and the margins are flat. As for the leaf surface, young leaves are generally attached to the stem, although some frequently appear woolly. [14]

Fig. 3. Morphology of Rosemary
Source: [15]

1.4. Medicinal Properties of Rosemary

According to [16], antioxidants are compounds that delay self-oxidation in such a way that they inhibit or prevent the formation of free radicals. In the medical industry, antioxidant activity is of great importance. Data from INEN, in Ecuador, show that in 2015 the use of products made from this plant increased by 15% compared to previous years, due to the antioxidant potential of rosemary.

In chemistry, natural antioxidants derived from plants are found in parts such as stems, leaves, and flowers. Nowadays, they are a conventional alternative due to their high content of phenolic compounds, which can prevent the propagation of oxidation reactions. [17]

Industries such as pharmaceuticals use rosemary in health and beauty products. Fields like cosmetology state that incorporating infusions or oils of this plant into commercial shampoos enhances the nutritional properties of rosemary, with the goal of preventing hair loss and scalp damage. [18]

1.5. Nanotechnology

The term "nanotechnology" comes from the Latin word nanus (meaning "dwarf" - Royal Academy, 1895) and was coined in 1974 by Norio Taniguchi, a Japanese researcher from the University of Tokyo. It was introduced to refer to any technology applied at the atomic and molecular scale. Today, two general paths for nanotechnology development are recognized: the top-down and bottom-up approaches. One refers to the “dilated version, meaning the use of technology related to other types of substances or tiny objects smaller than 100 nanometers (nm),” while the other focuses on "designing, modeling, and constructing

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machines capable of observing every atom and chemical bond when specifically outlined" (Storrs). The first is the lighter (soft) version, and the second is the more complex (hard) version. [19]

Nanosciences encompass a diverse set of disciplines that seek to understand the laws governing this tiny universe. Their goal is to study the properties of matter at both atomic and molecular levels (thermal, electrical, magnetic, optical, and chemical characteristics). Nanosciences significantly contribute to research and scientific studies in areas such as mechatronics, engineering, biology, organic and inorganic chemistry, and various branches of physics (optics, thermodynamics, electronics, hydraulics, etc.). Nanotechnologies refer to the set of techniques and processes that allow manipulation at the atomic and molecular level, enabling the intentional configuration and alteration of matter's characteristics and performance (Royal Society: 2004).

When combined with microelectromechanical systems (MEMS), computing power, and design concepts based on biological systems, nanotechnology creates conditions for the development of nanomaterials capable of responding to their environment. [19]

Nanotechnology is considered a "horizontal" science because it influences virtually all sectors, either directly or indirectly. This interdisciplinary approach suggests that nanotechnology could become a central, converging element in the science, economy, and society of the future. Advocates predict that nanotechnology will not only improve the industrial world but also replace it. Nanotechnological progress is seen as a phenomenon, evidenced by the increasing number of patents and scientific literature, and will undoubtedly positively impact the demand for nanostructured materials [20]

1.6. Nanotechnology in Ecuador

Although nanotechnology has seen significant global development, scientific information in this field is still limited in Ecuador. However, some products based on nanotechnology are already being used in the country. Research into nanoparticles is highly important due to their applications in fields like environmental science, space exploration, communication technologies, energy, textiles, construction, architecture, medicine, instrumentation, agriculture, livestock, electronics, cosmetics, military industries, automotive sectors, personal and road safety, hygiene, public health, sports, espionage, and more. [21]

Silver nanoparticles have garnered significant interest over the years due to their unique physicochemical properties at the nanoscale, which can be applied in various areas, such as their use as antibacterial and antiviral agents. Their size and shape are particularly attractive for these applications. [21]

Currently, various methods for synthesizing these nanomaterials have emerged, allowing control over the shape and size of silver nanoparticles (AgNPs) for different applications. However, most synthesis methods are expensive and harmful to the environment, prompting a search for biological or biosynthetic pathways involving living organisms such as bacteria, fungi, and plants. Ecuador, being considered a megadiverse country in terms of plant resources, presents a valuable opportunity to study the potential of Ecuadorian plants as reducing and stabilizing agents in the green synthesis of nanoparticles [22].

According to research by the aforementioned authors, the aim is to determine the feasibility of using a plant (rosemary) with antimicrobial properties—specifically rosemary—in the synthesis of silver nanoparticles. Several synthesis processes will be conducted, considering variables like the concentration of plant extract, temperature, and other factors. The results obtained from the synthesis will be characterized using the available techniques in the country [23].

1.7. Nanomaterials

These are defined as materials that contain particles within the nanoscale or nanometric scale, meaning they range from one nanometer to 100 nm. A nanometer (nm), when expressed in another unit (m), is equivalent to one-billionth of a meter ($1\ nm = 10^{-9} m$). According to the European Commission, in 2011 a recommendation on the definition of nanomaterial was adopted. It is understood as a natural, accidental, or manufactured material that contains particles, either free or as an aggregate, where 50% or more of the particles have one or more external dimensions within the size range of 1 to 100 nanometer [24].

1.8. Silver Nanoparticles

According to Faraday and Turkevich (2009), they proposed one of the mechanisms by which silver nanoparticles are formed. This mechanism consists of three fundamental steps: nucleation, growth, and stabilization, as shown in Figure 4.

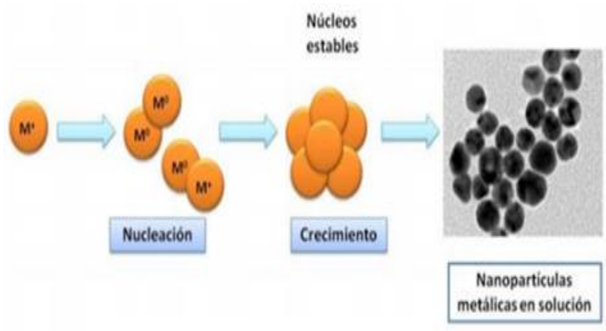

Fig. 4. Structure of Silver Nanoparticles
Source: [25]

Nucleation begins when the metallic salt is reduced due to the action of a suitable reducing agent, generating zero-valent metal atoms. These metal atoms, along with metallic ions, trigger the formation of a metallic core, which is referred to as a seed [25].

The growth of the nuclei occurs through diffusion and the mass transfer of metallic ions to the surface of these nuclei. During this stage, optimal control of both temperature and concentration is necessary, as these parameters significantly influence this phase. Stabilization depends on the use of a stabilizing agent, which acts to prevent agglomeration. This effect is caused by the repulsion between particles, which is due to the double electric layer formed by ions adsorbed on the surface of the nanoparticles [20].

1.9. Toxicity of Silver Nanoparticles

Given the constant growth in the applications of silver nanoparticles, human exposure, as well as the release of these nanoparticles into the environment, makes it necessary to analyze their potential toxicological impact.

Although the use of silver nanoparticles (AgNPs) has been reported for several years, evidence of their toxicity remains unclear. Nonetheless, several accredited organizations, including the FDA (Food and Drug Administration) and the EPA (Environmental Protection Agency), have approved the use of these nanoparticles in various industries such as textiles, cosmetics, and food, among others. The European Commission for Nanotechnology is also among these organizations, stating that the toxicity of these nanoparticles is very low. As a result, some products intended for human consumption release small amounts of silver, which do not have significant health consequences[26].

Regarding environmental concerns, it is established that the use of silver at the nanoscale constitutes a small percentage compared to the total amount of silver (Ag) present in the environment. However, it is worth noting that certain species, particularly aquatic organisms, could absorb this silver easily, although the probability of this occurring is minimal [26].

Research on the interaction of these nanoparticles with human tissues and the main exposure pathways has shown that silver nanoparticles can exhibit cytotoxicity through interaction with proteins and enzymes at the intracellular level.

Nevertheless, no cases of side effects related to the use of silver nanoparticles have been reported to date. Research continues to determine whether these nanoparticles are indeed harmless to the body, as well as to the environmen [20]

2. Materials and Methods

Data collection is the result of a wide variety of techniques and tools used to develop the research, including interviews and observation. This research consists of an investigative format or prototype based on the application of modern research techniques. Models, which may be discriminatory and questionable, will define and systematize the interviews to be used, providing flexibility in data collection.

In this investigation, the primary foundation comprises scientific texts, doctoral theses, and data obtained from experimental trials conducted in the chemical engineering laboratories at the University of Guayaquil, regarding the synthesis of silver nanoparticles and their effect on E. coli, S. aureus, and B. subtilis bacteria.

From another perspective, secondary sources of inquiry include recent articles on the synthesis of various types of nanomaterials, such as gold and silver nanoparticles, surface modification methods, pathogenic microorganisms, and the antimicrobial effect of nanoparticles.
Table 1 shows a list of all the equipment used in this research, along with important laboratory instruments. Most of these are located in the chemical engineering department at the University of Guayaquil. The Scanning Electron Microscope (SEM) is located in the microscopy laboratory at the National Institute of Public Health Research "Dr. Leopoldo Izquieta Perez." The analyses of terpenes and total phenols were conducted at the "UBA

Analytical Laboratory," where the quantity of terpenes and total phenols present in rosemary extract was determined.

Table 1.
Materials and equipment used

Laboratory Equipment and Tools	Brand / Model	Usage
Magnetic stirrer with heating	Elicrom 4.8x22mm	Used for the preparation of cilantro extract and the synthesis of nanoparticles.
pH strips	Merck Millipore	pH control of the extract and nanoparticles.
Analytical balance	Elicrom	Used to weigh solids, such as silver nitrate (AgNO3).
Test tube rack	Fisher, Isolab, Bel-Ar	Support for test tubes.
Filtration equipment		Used for the filtration of the extract.
Filter paper Whatman No.1	Whatman	Paper used to retain solids or particles in the rosemary extract.
Amber containers	Green	Containers used to store silver nanoparticles and protect them from sunlight.
Watch glass	Pyrex	Glass utensil used to weigh small amounts of silver nitrate.
Pipettes	Pyrex	Volumetric tool used to measure exact amounts of reagents in phytochemical assays.
Oven	Memmert	Used for drying instruments and glassware.
Asbestos wire mesh	ASEMED	Used to maintain temperature balance during heating.
Electric burner	Corning PC-400D and Mtx	Heating equipment used to increase the temperature for rosemary extract preparation and nanoparticle synthesis.
Incubator	VWR	Used for the development and maintenance of bacterial cultures.
Scanning Electron Microscope (SEM)	JEOL JSM 5310	Determines the shape and size of silver nanoparticles.
Spectrophotometer	Genesys 10-uv	Measures the absorbance of a sample in ultraviolet and visible light spectrums from 200 to 700 nm.
Digital thermometer	Multi-thermometer	Temperature controller in various processes.
Mortar		Used to crush rosemary.
Laboratory refrigerator	Labomersa & Mabe	Used for low-temperature preservation of rosemary extract, nanoparticle samples, prepared agars, and inhibition disks.
Stopwatch	SmartWatch, Casio HS-3V-1	Time controller for the synthesis of silver nanoparticles (AgNPs).

2.1. Preparation of the Extract

The preparation of the extract followed the methodology described by [27]. n this process, Rosmarinus officinalis (rosemary) leaves were used to obtain a natural extract that promotes the reduction of silver nitrate and the formation of nanoparticles, without the need for chemical substances that could generate secondary toxic substances. The preparation process of the extract involves several stages listed below:

- Plant collection
- Selection and washing of the leaves
- Size reduction using a mortar

- 100 g of well-washed leaves were boiled in a beaker with 500 ml of distilled water for a period of 20 to 25 minutes until boiling
- A heating plate with a magnetic stirrer was used to adjust the temperature from 50°C to 200°C, maintaining continuous stirring at 300 rpm
- The resulting liquid was filtered, and 100 g of additional leaves were added, boiling it again under the same conditions to concentrate the solution based on the rosemary content
- The concentrated extract was then heated to reduce its volume by evaporation to 50 ml, further concentrating the liquid
- Finally, the final extract was filtered, left to cool at room temperature, and then stored in a sterilized glass container (flask). To preserve the extract, it was kept refrigerated at a temperature of 4°C.

Fig. 5. Rosemary leaf extract with different solvents

For the determination of secondary metabolites, three types of extracts were used, each with different solvents: distilled water, ethanol, and acidulated water; with the goal of using the extract that presents the highest compound content and chemical properties for its intended use in the production of nanoparticles.

2.2. Preparation of Aqueous Silver Nitrate (AgNO3)

To prepare the silver nitrate solution at a concentration of 1 [mM], 0.034 grams of AgNO3 were dissolved in 200 ml of distilled water in a sealed glass container until its intended use. [28]

$$x = 1[mM]\, x\, \frac{0.001[M]}{1[mM]} = 0.001\ [M] \qquad (1)$$

$$m_{(g)} = PM_{(\frac{g}{mol})}\, x\, V_{(L)}\, x\, molaridad_{(M)} \qquad (2)$$

$$m_{(g)} = 169.87\ g/mol\ x\ 0{,}2\ L\ x\ 0.001\ M \qquad (3)$$

$$m_{(g)} = 169.87\ g/mol\ x\ 0{,}2\ L\ x\ 0.001\ mol/L \qquad (4)$$

$$m_{(g)} = 0.034\ g \qquad (5)$$

2.3. Synthesis of Silver Nanoparticles

For the synthesis of silver nanoparticles, the methodology described by Camacho Polo, a scientific study conducted at the University of Cartagena, was followed, making several experimental modifications to the variables to achieve a quantitatively better efficacy in the results.

The preparation of silver nanoparticles was carried out by varying some factors such as the days of the extract, pH, and the volume of the extract. The following table describes the modified variables in the experimentation throughout the process.

Table 2.
Modified Variables in the Production Process of (AgNPs)

Rosemary Extract									
Day 1					Day 3				
Quantity of rosemary extract (ml)					Quantity of rosemary extract (ml)				
5	10	20	30	40	5	10	20	30	40
pH					pH				
8		9		10	8		9		10

The process began using chemically pure silver nitrate ($AgNO_3$) as the precursor agent and preparing an aqueous solution of one millimolar (mM).

It is important to note that the same concentration of silver nitrate will be used throughout the process, varying the volume of the rosemary extract to determine through experimentation which concentration is most suitable. Specifically, 5 ml of the silver nitrate solution (1 mM) was taken and added to the rosemary extract at different volumes (5, 10, 20, 30, 40 ml), titrating with sodium hydroxide (NaOH) at 0.1N in order to adjust the pH to basic. This reaction was carried out with constant stirring at a reaction temperature of 65°C, with variations in pH values (8, 9, and 10).

The mixtures were then stored in amber bottles of 30 and 100 ml capacity, maintaining them at a temperature of 4°C. The aforementioned procedure was performed by varying the time concerning the preparation time of the extract on days 1 and 3, allowing it to rest in refrigeration. Subsequently, it was observed in the spectrophotometer which day presented better absorbance. A duration control was performed on the obtained samples, lasting from 1 to 7 days.

3. Results

As a result, an aqueous extract of rosemary leaves was obtained for the production of silver nanoparticles; the extract, when characterized by qualitative assays, presented four of the metabolites found in the chemical composition of rosemary, and through quantitative analysis, excellent results were obtained in the aqueous extract, reporting the presence of phenols and terpenes at 24%. This indicates that the extract of rosemary leaves produces its own secondary metabolites with reducing capacity, in this case, acting as a reducing agent for silver.

3.1. Phytochemical Analysis

Table 3.
Phytochemical Analysis - Contents in the Aqueous Extract of Rosmarinus officinalis Leaves

Phytochemical Tests (Secondary Metabolites)	Aqueous Extract	Ethanol Extract	Acidulated Extract
Saponins	Negative	Positive	Negative
Reducing Compounds (Fehling's Reagent)	Positive	Positive	Positive
Phenolic Compounds	Positive	Positive	Negative
Flavonoids	Positive	Positive	Negative
Alkaloids (Dragendorff Test)	(+++)	(++)	(+++)
Resins	Negative	Negative	Negative
Terpenoids	Positive	Positive	Positive

As seen in Table 3, there was no presence of saponins as no foams were observed in the sample, nor of resins from the aqueous extract. However, the expected compounds, such as phenolic compounds and terpenoids, along with reducing sugars, flavonoids, and alkaloids, were present.

Through the phenolic compound assay, the presence of a red coloration with a slight green tint was confirmed, qualitatively indicating the presence of phenolic acids and possibly small amounts of tannins.

In Table 4, we can quantitatively observe the amounts of phenols and terpenes contained in the aqueous rosemary extract, which are the compounds with the highest chemical properties.

Table 4.
Quantitative Determination of Main Secondary Metabolites

Secondary Metabolite	Method	Results	Units
Phenols	Singleton and Rosy	24.0	%
Terpenes		2844.0	mg/kg

Source: Laboratorios UBA
Prepared by: (Arias & Palma, 2018)

3.2. Synthesis of Silver Nanoparticles

In the synthesis process of nanoparticles, a color change was observed from a yellow hue produced by the extract to a dark brown color. Various authors, such as [29], [30], and [31], state that this coloration is a result of the growth and development of (AgNPs) when using natural plant-derived solvents, such as cilantro, matico, Sideroxylon capiri, Vitis vinifera, prickly pear, aloe vera, etc.

At the end of the process, the samples of nanoparticles with pH 10 were stored in amber bottles protected from light and kept at temperatures of 4°C. The (AgNPs) samples were allowed to rest for 1 to 3 days, where they were seeded in culture media on day 0 and day 3, aiming to verify the best inhibitory effect.

Fig. 6. Solution of (AgNPS) synthesized from rosemary leaf extract as a reducing agent for silver (Ag)

3.3. Characterization of Silver Nanoparticles (AgNPs)

The nanoparticles were characterized using the Genesys 10-UV spectrophotometer.

Table 5.
Analysis of (AgNPs) with different volumes and pH 8 by UV-VIS Spectroscopy

Volume Ratio	Day	Wavelength (λ)	Absorbance	T (%)	Absorption Factor (L/mol.cm)	Concentration (mg/l)
5-5			2.318			33.25
	1	365	2.315	0.5	24.28	33.22
			2.317			33.28
			2.394			33.92
	3	298	2.396	0.4	18.25	33.91
			2.396			33.93
5-10			2.354			33.50
	1	325	2.354	0.7	21.53	33.55
			2.353			33.54
			2.380			33.84
	3	312	2.382	0.3	14.36	33.85
			2.384			33.84
5-20			2.339			33.35
	1	345	2.337	0.2	12.78	33.38
			2.334			33.35
			2.308			33.16
	3	382	2.307	0.6	14.40	33.14
			2.306			33.14
5-30			2.343			33.45
	1	335	2.342	0.3	16.14	33.43
			2.341			33.44
			2.014			33.14
	3	369	2.015	0.4	20.94	33.16
			2.017			34.12
5-40			2.372			33.76
	1	318	2.374	0.4	10.06	33.77
			2.372			33.74
			2.265			33.65
	3	324	2.267	0.6	14.22	33.64
			2.268			33.67

Upon observing the table of collected data, the spectrophotometer yields values ranging from 296 to 418 nm, indicating the presence of nanoparticles. Certain ranges, specifically from 296 to 325 nm, were discarded as they do not indicate good stability in the NPs. This is due to the surface plasmon resonance in the nanometric scale of the spectrophotometer, which absorbs visible light in the ranges of 400 to 800 nm. In relation to the electronic sweep, it can be observed that the values do not exceed 400 nm, and the light transmitted will be projected considering that, according to the wavelength value, the absorbed color of the sample will be reflected. In this table, since lower ranges are presented, and as observed in Table 5, there is no coloration reflected in these ranges; therefore, they were discarded.

Table 6.
Analysis of (AgNPs) with different volumes and pH 9 by UV-VIS Spectroscopy

Volume Ratio	Day	Wavelength (λ)	Absorbance	T (%)	Absorption Factor (L/mol.cm)	Concentration (mg/l)
5-5			2.403			34.02
	1	402	2.405	0.5	16.10	34.05
			2.405			34.04
			2.435			34.30
	3	390	2.432	0.3	14.12	34.32
			2.430			34.31
5-10			2.412			34.15
	1	396	2.414	0.4	18.02	34.16
			2.414			34.19
			2.478			34.87
	3	342	2.476	0.5	12.11	34.87
			2.478			34.88

5-20	1	376	2.419 2.420 2.422	0.8	19.24	34.78 34.79 34.81
	3	369	2.424 2.422 2.423	0.6	17.05	34.81 34.84 34.84
5-30	1	395	2.416 2.416 2.418	0.4	13.72	34.21 34.23 34.24
	3	382	2.438 2.438 2.440	0.3	11.23	34.52 34.54 34.55
5-40	1	388	2.426 2.426 2.428	0.6	17.99	34.48 34.46 34.46
	3	309	2.396 2.396 2.398	0.4	14.42	34.90 34.92 34.94

It was observed that in the 5-5 proportion, a wavelength of 402 nm was recorded on day 1, followed by the 5-10 proportion with 396 nm on the same day; then 5-5 was recorded again on day 3. These values show that on day 1, longer wavelengths are present, although their amounts are very close. This table was discarded since most of the values showed low ranges; however, the two values that presented the best results in this section were taken as a reference, namely those from the proportions of 5-5 and 5-10.

Table 7.
Analysis of (AgNPs) with different volumes and pH 10 by UV-VIS Spectroscopy

Volume Ratio	Day	Wavelength (λ)	Absorbance	T (%)	Absorption Factor (L/mol.cm)	Concentration (mg/l)
5-5	1	418	2.328 2.326 2.329	0.4	16.98	34.21 34.27 34.25
	3	405	2.362 2.363 2.362	0.2	14.12	34.72 34.75 34.76
5-10	1	409	2.374 2.373 2.376	0.4	12.32	34.61 34.67 34.65
	3	412	2.312 2.317 2.318	0.5	14.54	34.50 34.52 34.52
5-20	1	404	2.368 2.367 2.367	0.4	16.38	34.78 34.77 34.77
	3	399	2.416 2.417 2.418	0.3	15.03	34.84 34.84 34.83
5-30	1	401	2.392 2.395 2.394	0.3	12.14	34.80 34.81 34.80
	3	410	2.364 2.363 2.361	0.4	14.74	34.66 34.66 34.67
5-40	1	389	2.425 2.427 2.423	0.4	13.37	34.96 34.97 34.95
	3	400	2.396 2.398 2.397	0.5	15.02	34.90 34.92 34.94

In Table 7, it is considered that the data are mostly the best in comparison to those in Tables 5 and 6. This is because the molecules in basic pH disperse and interact more effectively, allowing for a more uniform union between the Ag molecule and that of the extract. According to the results, the values obtained are very close, indicating that their variations are small, but the best results were observed in the 5-5 ratio on day 1, followed by the 5-10 ratio.

It is said that each plant contains its own chemical properties and that each one can react differently, either quickly or slowly. In this case, the reducing agent from rosemary (rosmarinic acid) reacts quickly on day 1, maintaining its values within the range until day 3.

Therefore, the nanoparticles obtained as a result at pH 10 with a 5-5 ratio on day 1 exhibited a higher reaction rate, reaching the highest peak according to the graph and consequently yielding a wavelength value of 418 nm.

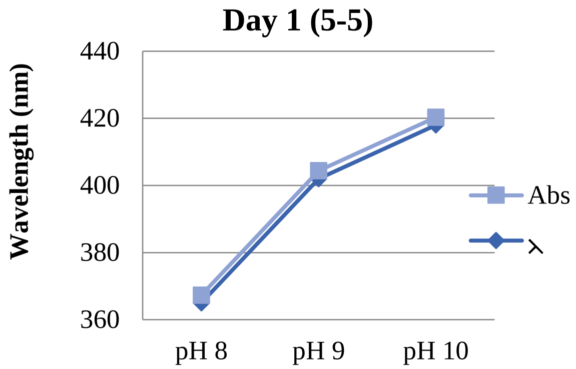

Fig. 7. Representation of the data obtained on the spectrophotometer Day 1 (5-5)

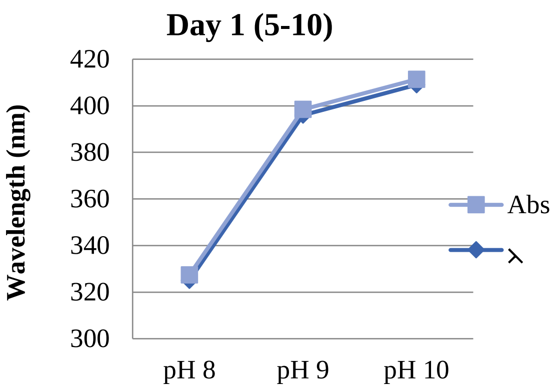

Fig. 8. Representation of the data obtained on the spectrophotometer Day 1 (5-10)

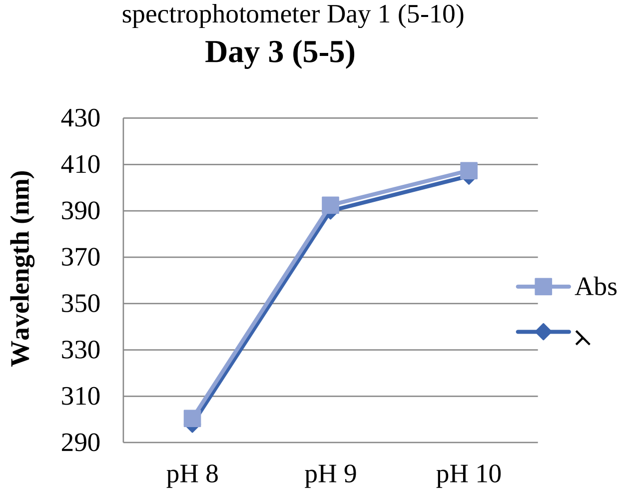

Fig. 9. Representation of the data obtained from the spectrophotometer on Day 3 (5-5)

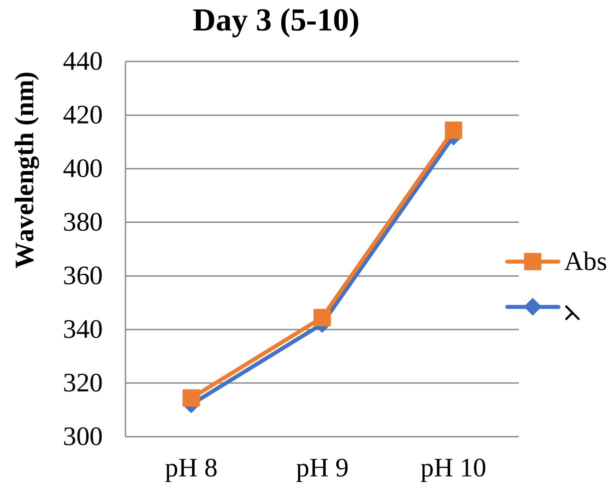

Fig. 10. Representation of the data obtained from the spectrophotometer on Day 3 (5-10)

Table 8.
Measurements of the halos formed with respect to the analyzed samples

Analyzed Samples	E. coli	S. aureus	B. subtilis
Extract	2.929	2.043	1.972
	2.912	2.200	2.252
	2.828	2.127	1.803
Average	2.889	2.123	2.009
Silver Nitrate Solution (AgNO3)	1.803	1.296	1.093
	1.418	1.313	9.31
	1.430	1.319	1.133
Average	1.550	1.309	1.052
Silver Nanoparticles (AgNPs)	3.255	2.082	2.329
	3.218	2.269	2.341
	3.159	2.197	2.168
Average	3.211	2.182	2.279

Source: Laboratorio LAZO

The results obtained demonstrate that the 1 mM AgNO3 solution and the rosemary extract possess antimicrobial activity. However, the synthesized silver nanoparticles showed a better response regarding inhibition.

The nanoparticles inhibited three types of bacteria, showing greater sensitivity to the E. coli bacteria. This is due to the fact that it is a gram-negative bacterium, which has a triple layer in its cell wall: a membrane composed of phospholipids and lipopolysaccharides, a cytoplasmic membrane, and a thin layer of peptidoglycan. In contrast, gram-positive bacteria have a much thicker layer of peptidoglycan and their cytoplasmic membrane. [32]

Therefore, it can be stated that the best results were observed in the gram-negative bacterium (E. coli). This is because they have a thinner layer of peptidoglycan compared to gram-positive bacteria (S. aureus and B. subtilis), which have a much thicker layer that impedes the synthesized nanoparticles from easily entering the membrane.

The inhibition caused by the AgNPs resulted in halos ranging from 2.1 cm to 3.2 cm, with the latter being the largest diameter.

Thus, it can be said that the silver nanoparticles synthesized with rosemary extract exhibit antimicrobial activity against the bacteria E. coli, S. aureus, and B. subtilis, being most effective against E. coli.

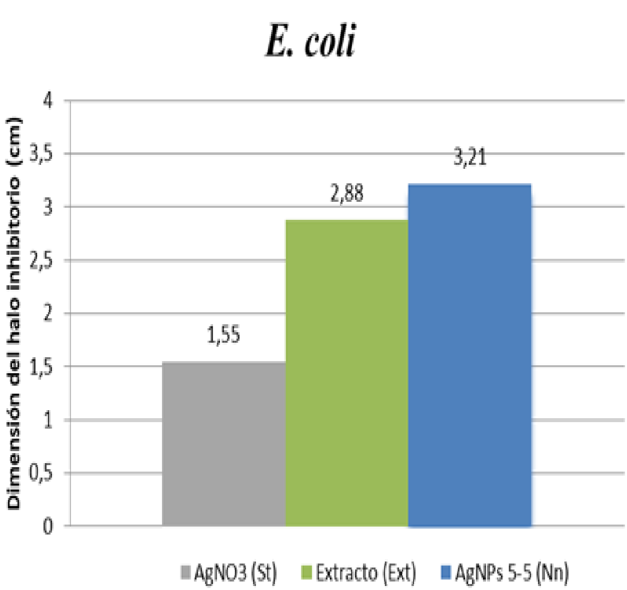

Fig. 11. Antimicrobial activity of the samples used against E. coli

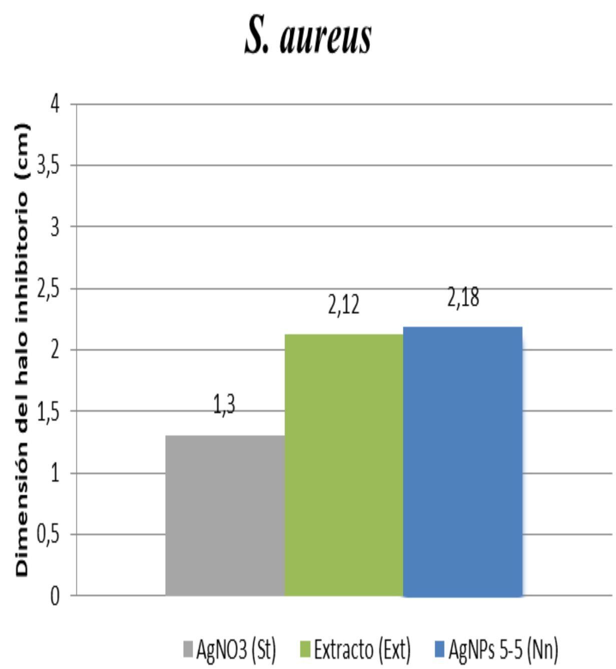

Fig. 12. Antimicrobial activity of the samples used against S. aureu

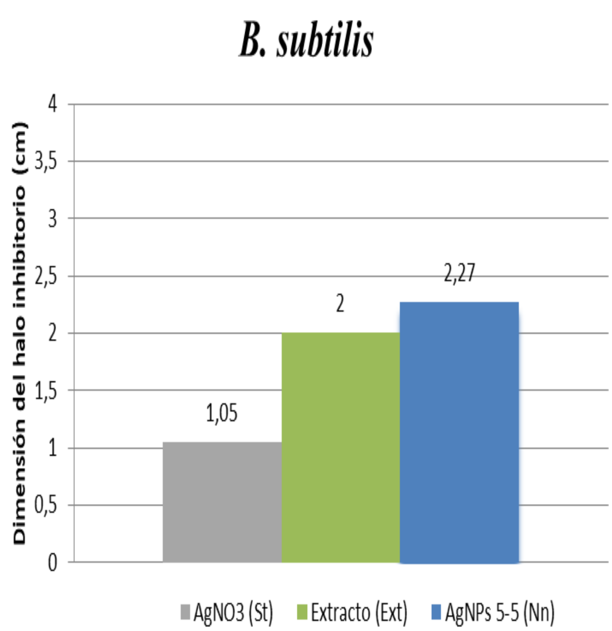

Fig. 13. Antimicrobial activity of the samples used against B. subtilis

4. Conclusions

Silver nanoparticles were synthesized using an extract from rosemary leaves, as well as their study of antimicrobial activity tested on strains of E. coli, S. aureus, and B. subtilis. To achieve this type of action, it was necessary to investigate the reducing chemical properties of rosemary, as well as each reducing mechanism of silver ions and the potential microbiological interactions that can be generated.

Regarding the phytochemical assays performed on the aqueous extract of rosemary leaves, it can be stated that it presents the most important secondary metabolites, including phenolic acids, flavonoids, terpenoids, and reducing compounds. The generation of polyphenols indicates that this plant can develop an excellent interaction with the molecules of Ag nitrate, although it is estimated that the reaction occurs rapidly.

The results obtained in Table 4 show the quantitative analysis performed by spectrophotometry, where it is evident that the results obtained for phenolic acids are considerable since the reducing agent is naturally present in the same plant, making it feasible for the synthesis of silver nanoparticles.

The silver nanoparticles (AgNPs) were characterized by UV-Vis spectrophotometry, as shown in Tables 5, 6, and 7; the best results were presented in the pH 10 table on day 1 with a v/v ratio. This is due to the detection of the best sample at 418 nm, representing the highest peak concerning the graphs. Additionally, the scanning electron microscope (SEM) allowed for the visualization of the AgNPs, revealing a spherical morphology with a size of 10 nm, which indicates that they fall within the nanometric range, considered an excellent dimension necessary for effectively inhibiting pathogens.

The AgNPs used in this study generated antimicrobial action against the three types of analyzed bacteria: E. coli, S. aureus, and B. subtilis. To test the inhibition or determine which type of bacteria showed greater sensitivity to the synthesized silver nanoparticles, the Bauer-Kirby method was employed, where halos ranging from 1 to 3 cm in diameter were observed, demonstrating high efficiency. This indicates that the AgNPs exhibited positive results in response to antimicrobial action.

As mentioned in the previous paragraph, inhibition was observed in all three types of bacteria; however, the largest halos occurred in the Gram-negative bacteria (E. coli) due to its thinner membrane layer compared to Gram-positive bacteria. E. coli displayed the highest sensitivity to the synthesized silver nanoparticles (AgNPs), followed by the Gram-positive bacteria (S. aureus and B. subtilis). It is important to note that the best results, according to the cultures performed, were at time zero, maintaining greater stability and interaction with the bacteria.

The antimicrobial effect was demonstrated on red apples, where nanoparticles and beeswax were used as a preservative. This compound delayed the ripening process, maintained the final weight compared to the initial weight, and, most importantly, prevented any type of microorganism from entering the interior of the fruit. Therefore, it can be concluded that this work is highly efficient for preserving fruits naturally without altering their organoleptic properties.

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