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Option : Biochimie appliquée

Thème

Antibacterial Activity of Limonium spathulatum and it's application

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(Page sans bordure externe) Résumé (1) langue du mémoire + mots clés

This study examined the potential therapeutic properties of the medicinal plant Limonium spathulatum. The study evaluated the yield, antibacterial activity, and nature of the antibacterial activity of the plant's methanolic extract against bacterial strains Klebsiella pneumoniae, Enterococcus faecalis, and Bacillus subtilis. The extract exhibited antibacterial activity against all three strains, with bacteriostatic activity determined for all. The study also explored the association between the extract and the antibiotic gectapen, finding a synergistic effect in reducing the MIC values of the antibiotic. Additionally, the extract's inhibitory effect on the growth of B. subtilis in yogurt was evaluated, showing a significant reduction in bacterial colonies and making the extract a potential natural preservative in food products to prevent spoilage or pathogenic bacteria.

Key Words : *limonium spathulatum,* methanolic extract, antibacterial activity, yogurt,

General Introduction

For a long time, traditional medicine has been the primary recourse for people to treat their health problems, with around 80% of the population relying on it for primary care (Ladoh et al., 2014). Simultaneously, humans have used various ingredients to preserve and enhance the quality of their food. Salt has been used to preserve meat or fish, while saffron has been used for coloring dishes since the Middle Ages. Advances in knowledge have allowed us to better understand these ingredients and isolate their specific compounds, leading to the regulation of food additives as small quantities of ingredients with technological roles **(Ahamed, 2010)**. The 19th century witnessed the industrial development of the food industry, accompanied by advancements in chemistry and microbiology, which gave rise to new molecules known as "food additives." Strict usage standards set by national and international bodies are in place to protect consumers, with specific classifications defining the role of each food additive **(Diezi, 2011).**

The Limonium genus, belonging to the Plumbaginaceae family, comprises approximately 370 species of perennial plants and shrubs that are halophytes. These plants have the ability to secrete salt through salt bladders and salt glands as an adaptation to high saline conditions **(Yuan et al., 2016).** Limonium species, also known as sea lavenders, are predominantly found in the Mediterranean region, particularly in the northern and southern states (Brullo and Erben, 2016). In North Africa alone, 107 species have been discovered **(Dobignard et al., 2013).** Some species within the genus have significant ornamental value, while others are ethno-pharmacologically used to treat various illnesses, particularly cardiovascular and inflammatory conditions **(González-Orenga et al., 2021).**

Currently, extensive research is focused on controlling bacterial resistance and infections due to their implications in the development of multiple pathologies, which pose significant public health challenges. This research involves exploring and utilizing new natural antimicrobial agents, as well as developing plant-based food additives that offer ecological safety, health benefits, and environmental protection.

In our present study, our primary objective is to evaluate the phenolic compounds present in the methanolic extract of the endemic plant Limonium spathulatum. Phenolic compounds are known for their antioxidant and antimicrobial properties, which make them potentially valuable for various applications, including food preservation.

To achieve this, we will employ different analytical techniques to identify and quantify the phenolic compounds present in the extract. This will provide us with valuable information about the chemical composition and potential bioactive components of the extract.

In addition to phenolic compound analysis, we will also assess the antimicrobial activity of the methanolic extract against various foodborne pathogens. This will involve conducting antimicrobial tests, such as the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), to evaluate the effectiveness of the extract in inhibiting the growth of bacteria.

Furthermore, we are interested in investigating the preservative effect of the methanolic extract of Limonium spathulatum on food production. We will conduct experiments to evaluate the extract's ability to extend the shelf life of food products by inhibiting microbial growth and preventing spoilage. This assessment will involve monitoring the microbial load, pH, and sensory attributes of the treated food samples over a specified storage period.

Moreover, we will explore the potential synergistic effects of the Limonium spathulatum extract with antibiotics. This combination approach aims to enhance the antimicrobial activity and potentially overcome antibiotic resistance in foodborne pathogens. By combining the extract with antibiotics, we can assess any synergistic effects that may result in improved antimicrobial efficacy.

Overall, our study aims to contribute to the understanding of the phenolic composition, antimicrobial properties, and preservative effects of the methanolic extract of Limonium spathulatum in the context of food production. The findings from this research may have implications for developing natural and eco-friendly strategies to improve food safety and preservation.

In order to present our work and share the results, we have organized this dissertation using the IMRAD (Introduction, Methods, Results, and Discussion) method. The structure of our dissertation is as follows:

- General Introduction: In this section, we provide a comprehensive introduction to the topic of our research. We present the background information, highlight the importance of the study, state the research objectives, and outline the scope of our work. This section sets the context for the rest of the dissertation.

- Materials and Methods: In the Materials and Methods section, we describe in detail the experimental procedures and methodologies employed in our research. We provide information on the materials, samples, and equipment used. We explain the data collection process, including any extraction or analysis techniques. This section aims to provide a clear understanding of the experimental setup and methodology used in our study.
- Results: In the Results section, we present the findings of our research. We present the collected data, experimental observations, and any statistical analyses conducted. This section is typically presented using tables, graphs, figures, or other visual aids to effectively present the results obtained. The results are organized in a logical manner, allowing the readers to understand and interpret the outcomes of our study.
- Discussion: In the Discussion section, we interpret and analyze the results obtained in light of the research objectives. We compare our findings with existing literature and theories, discuss any limitations or challenges encountered during the research process, and propose possible explanations or hypotheses. We highlight the significance and implications of our results and offer insights into the broader context of our study.
- Conclusion: In the General Conclusion section, we summarize the main findings and contributions of our research. We restate the research objectives and discuss how they have been achieved. We provide a concise summary of the key results and their implications. We also discuss any potential areas for future research or recommendations based on our findings

Material And Methods

In this section, we will provide a detailed overview of the methods used in our research to accomplish our objectives. We will describe the experimental procedures and techniques employed in our study. Additionally, we will offer a comprehensive understanding of the concepts and definitions that are pertinent to our research topic.

I- Vegetal material:

I-1- Presentation of vegetal material -*Limonium spathulatum*-:

Limonium spathulatum, commonly known as Spoonleaf sea lavender, is a species of flowering plant in the family Plumbaginaceae. It is native to coastal regions of the Mediterranean and is known for its unique spathulate (spoon-shaped) leaves. The plant thrives in saline or alkaline habitats such as salt marshes, coastal dunes, and sandy beaches, showcasing its ability to tolerate high levels of salinity and drought.

Limonium spathulatum exhibits tall, erect stems with branched inflorescences. The small flowers, often in shades of purple or pink, add vibrant colors to the coastal landscape. Beyond its aesthetic appeal, this species plays a crucial ecological role. It aids in stabilizing sandy soils, preventing erosion, and providing habitat and food sources for various organisms.

The botanical taxonomy of *Limonium spathulatum* is as follows **(Tropicos, 2021)**:

Kingdom: Plantae (Plants)

Division: Magnoliophyta (Angiosperms)

Class: Eudicots

Order: Caryophyllales

Family: Plumbaginaceae

Genus: *Limonium*

Species: *Limonium spathulatum*

Figure 1: *Limonium spathulatum* **(Photos taken by Tarek Hamel, 2023)**

I-2- Collect of vegetal material:

The aerial parts of *Limonium spathulatum* were collected from the region of Ras el Hadid (Skikda, northeastern Algeria) on January 28, 2023.

After the plant was harvested, it was naturally dried at room temperature (approximately 24°C) in a dark and dry environment. To facilitate extraction, the plant material was finely ground into a powder using an electric mill to increase the surface area for better interaction between the solid and the solvent. Subsequently, the powder was stored in a flask for further use.

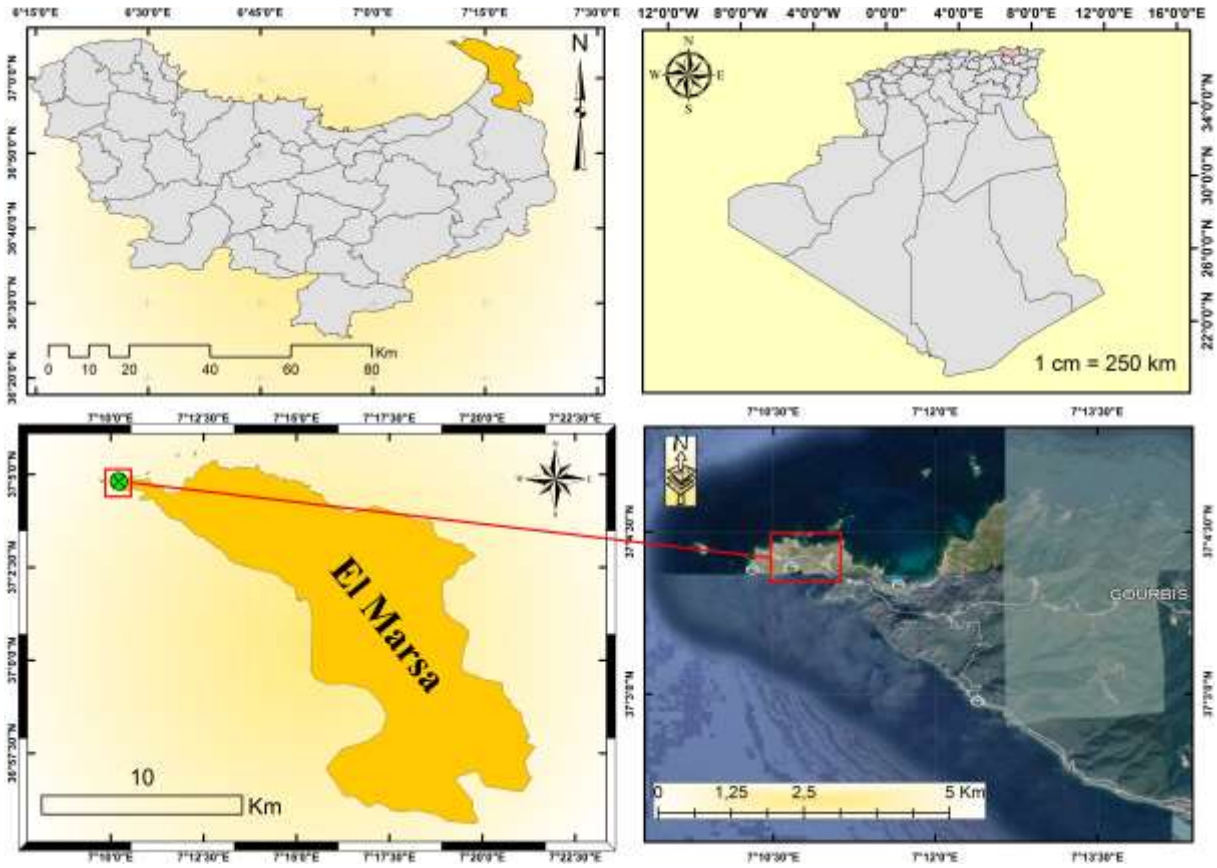


Figure 2: Geographical localization of Rass El Hdid (skikda)

II- Extraction of bioactives molecules:

Extraction of bioactive molecules is a crucial step in various fields, including pharmaceuticals, nutraceuticals, and natural product research. It involves the isolation and purification of biologically active compounds from natural sources such as plants, microorganisms, and marine organisms. These bioactive molecules possess potential therapeutic, antioxidant, antimicrobial, anti-inflammatory, and other beneficial properties.

The extraction process aims to obtain a concentrated and pure form of the desired bioactive compounds from the complex matrix of the source material. Several extraction techniques are employed, including solvent extraction, steam distillation, supercritical fluid extraction, and solid-phase extraction. Each method has its advantages and limitations, and the choice of extraction technique depends on factors such as the nature of the target compounds, solubility, stability, and economic feasibility.

In recent years, there has been growing interest in the extraction of bioactive molecules due to their potential applications in various industries. These include the development of new

drugs, functional foods, dietary supplements, and natural-based products for cosmetics and personal care.

In our research, we focused on the extraction of phenolic substances using the maceration method with an organic solvent. Before delving into the details of the extraction method, we present a brief literature review on phenolic substances.

II- 1- Phenolic substances review:

Polyphenols are a family of bioactive compounds. They are aromatic secondary metabolites that are ubiquitous in the plant kingdom **(Lucci et al., 2016)**, comprising more than 10,000 substances with highly diverse structures **(Mojzer et al., 2016).**

Polyphenols constitute a vast and heterogeneous group of phytochemical substances containing phenolic cycles **(Kim et al., 2016).** They can be classified into different groups based on the number of phenolic cycles they contain and the structural elements that link these cycles **(Li et al., 2014)**. The most widely adopted classification involves dividing phenolic compounds into two main groups: flavonoids and non-flavonoids, and this classification has been commonly used in the literature **(Durrazo et al., 2019).**

Polyphenols have been found in numerous plants and foods, such as fruits, vegetables, tea, cereals, medicinal plants, microalgae, and edible and wild flowers. They have demonstrated various biological activities and health benefits **(Thabti et al., 2012; Li et al., 2014).** In plants, polyphenols are generally involved in defense against different types of stress. They provide protection against reactive oxygen and nitrogen species, UV radiation, pathogens, parasites, and plant predators. Additionally, they substantially contribute to the organoleptic properties of plants, foods, and cosmetics **(Mojzer et al., 2016).**

For human health, it has been established that polyphenols reduce morbidity and/or slow the progression of cardiovascular, neurodegenerative, and cancerous diseases. The mechanism of action of polyphenols is strongly linked to their antioxidant activity. Polyphenols are known to decrease the level of reactive oxygen species in the human body. Furthermore, the beneficial health properties of plant polyphenols include anti-inflammatory, antiallergic, anti-atherogenic, antithrombotic, and antimutagenic effects **(Rasouli et al., 2017; Gorzynik-Debicka et al., 2018; Staszowska-Karkut and Materska, 2020; Yan et al., 2020; Baky et al., 2022;**

Clodoveo et al., 2022; Islam et al., 2022; Rana et al., 2022; Wang et al., 2022; Zhang et al., 2022).

II- 2- Maceration method with organic solvents:

II-2-1- Principle:

Maceration is a widely used method for the extraction of polyphenols using organic solvents. It involves the soaking of plant material in a solvent to allow the transfer of bioactive compounds, including polyphenols, into the liquid phase.

During maceration, the plant material is typically finely ground to increase the surface area and enhance the extraction efficiency. The solvent is then added to the plant material, and the mixture is allowed to stand for a specified period, often at room temperature or with mild agitation. This enables the polyphenols to dissolve in the solvent, resulting in an extract rich in these bioactive compounds.

Organic solvents commonly employed in maceration include ethanol, methanol, and acetone, among others. These solvents have the ability to selectively dissolve polyphenols from the plant matrix, extracting a wide range of phenolic compounds with diverse chemical structures.

II-2-2- Experimental protocol:

A test sample was prepared by combining 150 g of finely powdered Limonium spathulatum plant material with 300 ml of petroleum ether. The mixture was allowed to macerate at room temperature for 24 hours. Afterward, the mixture was filtered, and polar solvents (300 ml of chloroform and 300 ml of methanol) were added successively for extraction at room temperature for 72 hours. The resulting solutions were filtered using Whatman paper, and the solvents were removed under pressure using a rotary evaporator at 65°C to obtain a dry extract. The methanol extract was then placed in a petri dish to dry, and the yield was subsequently determined **(Dall'Acqua et *al.*, 2010).**

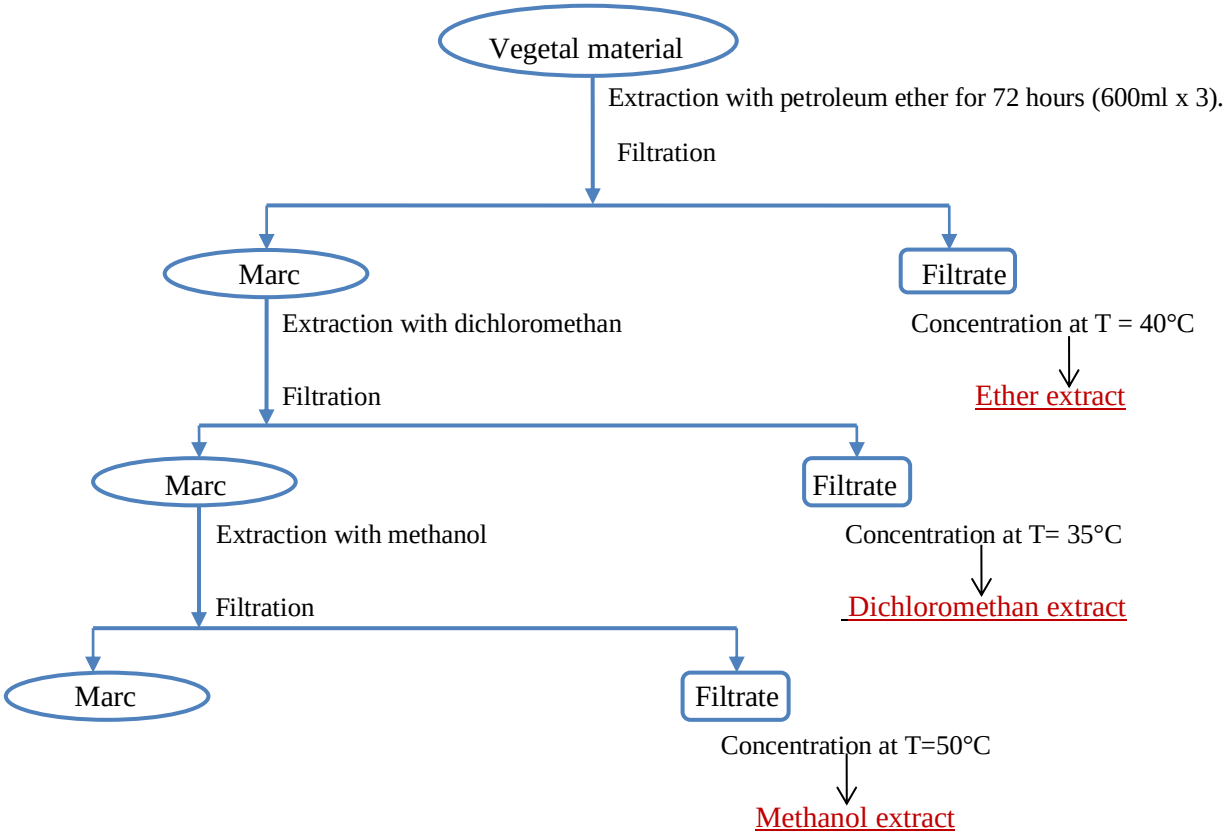


Figure 5: Extraction protocol

III- Ultraviolet analysis:

Ultraviolet (UV) spectroscopy is a commonly used technique for the qualitative analysis of polyphenols. It allows for the identification and characterization of polyphenolic compounds based on their specific UV absorption patterns.

Here is a general outline of the qualitative analysis process using UV spectroscopy:

- ✓ Sample preparation: The sample, typically a polyphenol-containing extract or solution, is prepared using appropriate extraction methods. The sample may need to be purified or concentrated to ensure accurate UV analysis.
- ✓ UV spectrum recording: The prepared sample is placed in a UV-transparent cuvette, and its UV spectrum is recorded using a UV-visible spectrophotometer. The spectrum is usually scanned over a specific wavelength range, typically in the UV region (200-400 nm).
- ✓ Absorption peaks identification: Polyphenols exhibit characteristic absorption peaks or bands in the UV spectrum due to their conjugated double bond systems. These peaks can help identify the presence of specific polyphenolic compounds. Comparing the obtained UV spectrum with reference spectra or published data aids in the identification process.

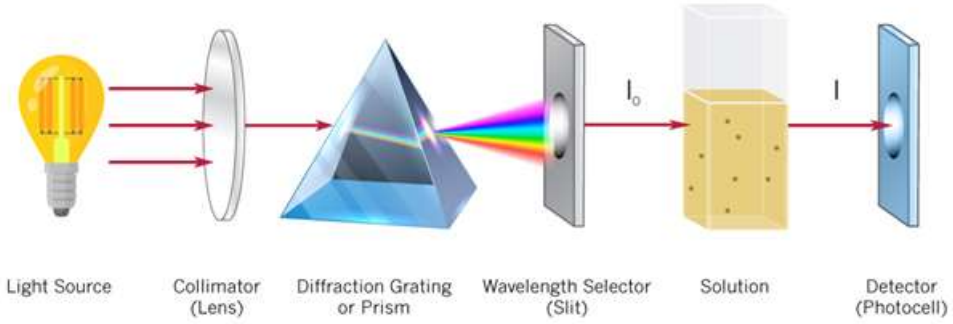


Figure 4: Ultraviolet spectroscopy principle

IV- Evaluation of antibacterial activity and it's applications:

The antibacterial activity of plant extracts is attributed to the presence of secondary metabolites such as phenolic compounds, alkaloids, terpenoids, flavonoids, and essential oils. These compounds possess antimicrobial properties and can disrupt the growth and survival of bacteria by various mechanisms, including inhibiting cell wall synthesis, damaging cell membranes, interfering with bacterial enzymes, and disrupting essential cellular processes.

The applications of plant extracts with antibacterial activity are extensive. They have been utilized in the pharmaceutical industry for the development of new antimicrobial drugs or as alternative treatments to combat bacterial infections. Plant extracts can also be incorporated into personal care products, such as soaps, lotions, and oral care products, to provide natural antibacterial effects. Furthermore, these extracts have shown potential in the preservation of food products, as natural antimicrobial agents to inhibit the growth of spoilage and pathogenic bacteria.

The use of plant extracts as antibacterial agents offers several advantages, including their natural origin, potential broad-spectrum activity, and the possibility of targeting antibiotic-resistant bacteria. However, it is important to evaluate the safety and efficacy of these extracts before their widespread application.

In our research, we were evaluated the antibacterial potential of *Limonium spathulatum*, and their applications in food conservation and as a synergical with an antibiotic.

IV- 1- Determination of the minimal inhibitory concentration (MIC):

The determination of the minimal inhibitory concentration (MIC) is based on the principle of assessing the lowest concentration of a substance that inhibits the visible growth of microorganisms. The MIC is an important parameter used to evaluate the antimicrobial activity of compounds.

There are several methods available for determining the MIC, including the broth dilution method, agar dilution method, and the microdilution method. Each method has its advantages and limitations, and the choice of method depends on various factors such as the type of microorganism, the nature of the test substance, and the specific objectives of the study **(Clinical and Laboratory Standards Institute, 2018).**

IV- 1- 1- Resazurin-microplate method:

In our work, we determined the minimal inhibitory concentration (MIC) using the resazurin-microplate method. The resazurin-microplate method is a colorimetric assay that allows for rapid and quantitative determination of MIC values.

To perform the assay, a series of dilutions of the test substance were prepared in a growth medium containing the microorganisms. Resazurin, a blue dye, was added to the wells containing the test samples. The microplates were then incubated for a specific period of time, allowing the microorganisms to grow.

After incubation, the color change of resazurin was observed. The blue color of resazurin is reduced to a pink color in the presence of metabolically active microorganisms. The lowest concentration of the test substance that does not show a color change (indicating no microbial growth) is considered as the MIC.

The resazurin-microplate method offers several advantages, including its simplicity, rapidity, and cost-effectiveness. It is a widely used method for determining MIC values in various research areas **(Bala et *al*., 2016)**.

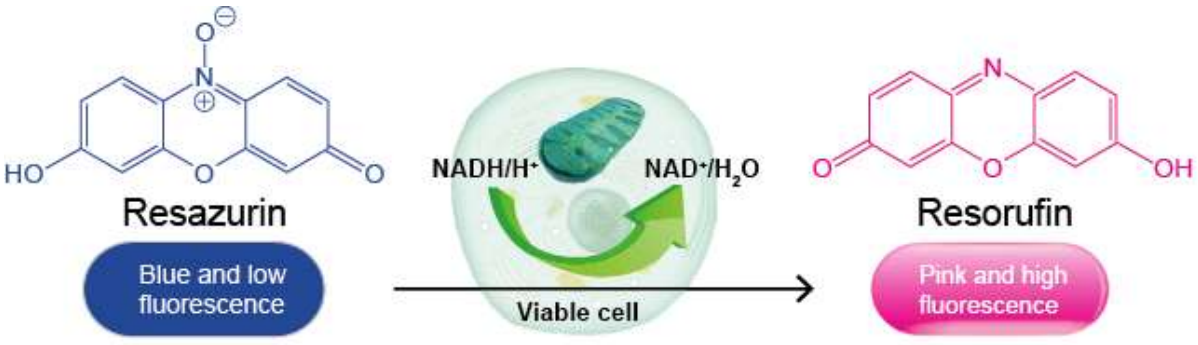


Figure 5: The principle of the resazurin reaction

IV- 1- 1- 1-Experimental protocol:

IV-1-1-1-1- Bacterial strains:

- ***Klebsiella pneumoniae*** (Kp; K. pneumoniae) is an important nosocomial and community-acquired pathogen known for causing various infections such as pneumonia, urinary tract infections, bloodstream-related infections, meningitis, and pyogenic liver abscesses. Carbapenems have been widely used as the primary treatment for Enterobacteriaceae infections. However, the emergence of carbapenem-resistant Klebsiella pneumoniae (CRKP) has become a global concern due to the increasing prevalence of antibiotic resistance (**Meijing et *al*., 2023).**

- ***Enterococcus faecalis*** is a Gram-positive facultative anaerobic bacterium and an opportunistic pathogen commonly found in the human oral cavity and gastrointestinal tract. It is frequently isolated from root canals that have failed endodontic treatment, making it one of the most common pathogenic microorganisms in RAP root canals. Enterococcus faecalis is also a leading cause of nosocomial infections worldwide and has been associated with various infections such as pelvic infection, intra-abdominal abscess, postoperative infection, bacteremia, endocarditis, and urinary tract infection. It possesses inherent cold tolerance, allowing it to survive in hospital settings and resist host defenses. Moreover, Enterococci are known for their ability to acquire antibiotic resistance and spread these resistance elements **(Zilong et al., 2023; Shonna et al., 2009).**

- ***Bacillus subtilis*** is a ubiquitous Gram-positive bacterium with remarkable adaptive potential, enabling it to thrive in diverse environments. It is non-pathogenic and exhibits substantial genetic diversity even among closely related strains. The Bacillus subtilis group comprises four species, namely Bacillus subtilis, Bacillus pumilus, Bacillus licheniformis, and Bacillus amyloliquefaciens. Over time, new species and subspecies have been identified based on molecular evolution, physiology, and chemotaxonomic characteristics. Bacillus subtilis serves as a model organism for studying various cellular processes, including cell motility, biofilm formation, protein secretion, cell division, secondary metabolite biosynthesis, root and fungal hyphae attachment, cytoplasmic transport through nanotubes, and kinship recognition. In biotechnology, Bacillus subtilis is widely used for the biosynthesis of natural products, ranging from enzymes to bioactive compounds. Its genetic engineering capabilities and well-established gene expression systems make it attractive for diverse applications. Additionally, it has gained attention as a biocontrol agent in agronomy for combating plant pathogens and promoting plant growth **(Iqbal et *al*., 2023)**

Klebsiella pneumoniae

Enterococcus faecalis

Bacillus subtilis

Figure 6: Bacterial strains

Table 1: References and origin of the microbial strains used

Bacterial strains	ATCC	Gram
Klebsiella pneumoniae	13883	-
Enterococcus faecalis	19433	+
Bacillus subtilis	0486	+

IV-1-1-1-2- Culture media and solvents:

The culture media and solvents utilized in the antibacterial tests consisted of the following components:

- Nutrient agar
- Mueller-Hinton broth

Chemical products and solvents:

- Sterile distilled water was used to prepare dilutions of the extract.
- DMSO (Dimethyl sulfoxide)
- Resazurin.

IV-1-1-1-3- Resazurin-microplate test:

The microdilution technique, with slight modifications, was employed to determine the minimum inhibitory concentration (MIC) in 96-well microplates, as described by **Rodi et *al.* (2019)**. The methanolic extract was initially serially diluted in DMSO, serving as an emulsifier. From a stock solution of the methanolic extract, a series of dilutions (including a positive control with "gectapen antibiotic") was prepared in Muller-Hinton medium, resulting in a final volume of 50 µL for each concentration. Subsequently, 50 µL of bacterial inoculum, at a final concentration of 106 CFU·ml^{-1}, was added to each well, followed by the addition of 10 µL of resazurin as a bacterial growth indicator.

The microplates were then incubated at 37 °C for 18-20 hours. Bacterial growth was indicated by a change in resazurin color from purple to pink. The MIC value was determined as the lowest concentration that prevented a color change in resazurin. The 12th well was designated as the growth control. All experiments were conducted in triplicate.

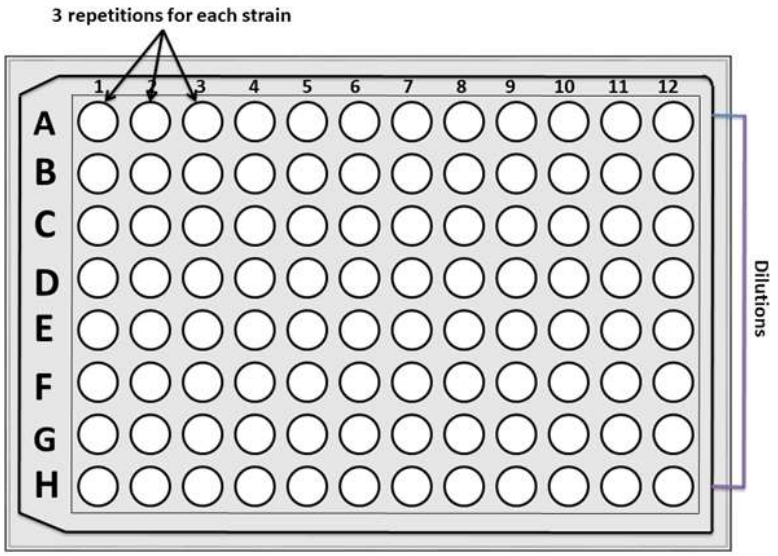


Figure 7: 96-well microplate work surface**.**

IV- 2- Determination of the minimal bactericidal concentration (MBC):

The principle of determining the minimal bactericidal concentration (MBC) is to identify the lowest concentration of an antimicrobial agent that can kill the microorganisms, resulting in no visible growth upon subculture. The MBC test is usually performed following the minimal inhibitory concentration (MIC) determination.

To determine the MBC, the microbial suspension from the wells showing no visible growth in the MIC assay is subcultured onto solid agar plates without the antimicrobial agent. The plates are then incubated at the appropriate temperature for a sufficient period, typically 24-48 hours.

After incubation, the MBC is determined as the lowest concentration of the antimicrobial agent that completely inhibits the visible growth of the microorganisms on the subcultured plates. This concentration indicates the bactericidal activity of the tested compound.

The MBC is typically expressed as the same unit of concentration as the MIC (e.g., µg/mL, mg/L) and is often reported as a range to account for any variability in results. The MBC is crucial in assessing the bactericidal potential of an antimicrobial agent and understanding its effectiveness in eradicating the target microorganisms.

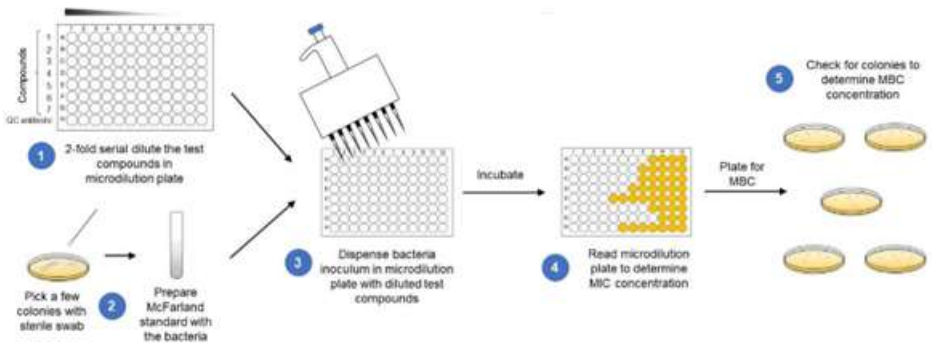


Figure 8: MIC and MBC assays

IV- 3- Evaluation of food preservation potential of Limonium spathulatum:

In our research, we evaluated the potential of *Limonium spathulatum* for yogurt preservation purposes. Yogurt is a popular dairy product that is susceptible to spoilage caused by various microorganisms, including bacteria and fungi.

IV- 3-1- Yogurt preparation:

Yogurt is a fermented dairy product that is made by the bacterial fermentation of milk. The process involves the use of specific starter cultures containing lactic acid bacteria, which convert lactose (milk sugar) into lactic acid through fermentation. This gives yogurt its characteristic tangy flavor and thick texture.

To prepare yogurt, we followed the following steps:

- Milk Selection: Start by selecting fresh, high-quality milk. You can use various types of milk, such as cow's milk, goat's milk, or plant-based alternatives like soy milk or almond milk.
- Heating: Heat the milk to a specific temperature to kill any potential harmful bacteria and to promote the denaturation of milk proteins. This step is called pasteurization. The temperature may vary depending on the type of milk used, but it is typically around 85-90°C (185-195°F).
- Cooling: Allow the milk to cool down to a suitable fermentation temperature, usually around 40-45°C (104-113°F). It is essential to maintain this temperature range to facilitate the growth of the starter cultures.
- Fermentation: Introduce the starter culture " lactic acid bacteria: *Lactobacillus bulgaricus* and *Streptococcus thermophilus*". These bacteria ferment lactose into lactic acid and produce the characteristic texture and flavor of yogurt. The recommended amount of starter culture may vary, so follow the instructions provided by the manufacturer.
- Incubation: Transfer the milk and starter culture mixture into individual containers or a large container. Cover the containers and place them in a warm environment to allow the fermentation process to occur. The ideal temperature for fermentation is typically around 40-45°C (104-113°F). You can use a yogurt maker, an oven with the pilot light on, or even a thermos to maintain the desired temperature. The fermentation time may vary but is usually between 4 to 8 hours, depending on the desired thickness and tanginess of the yogurt.
- Refrigeration: Once the desired fermentation time is reached, remove the containers from the warm environment and refrigerate them. Cooling the yogurt will halt the fermentation process and help set the texture

After the preparation of yogurt, it was inoculated with a known concentration of the bacterial strain Bacillus subtilis. Following that, a series of concentrations of Limonium spathulatum extract were added to assess its preservation effect on yogurt. Potassium sorbate was used as a positive control for comparison purposes.

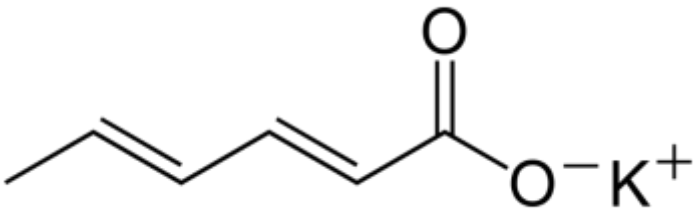


Figure 9: Potassium sorbate structure

IV- 3- Synergistic effect of *L. spathulatum* with an antibiotic:

The synergistic effect of *L. spathulatum* with an antibiotic was evaluated in our study. We combined different concentrations of *L. spathulatum* extract with a specific antibiotic (Gectapen) to assess their combined antibacterial activity. The purpose was to determine if the simultaneous use of *L. spathulatum* extract and the antibiotic could enhance the overall antibacterial efficacy compared to the individual use of each component.

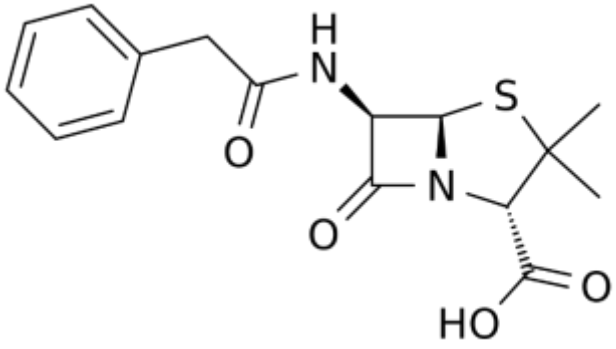


Figure 10: Gectapen structure

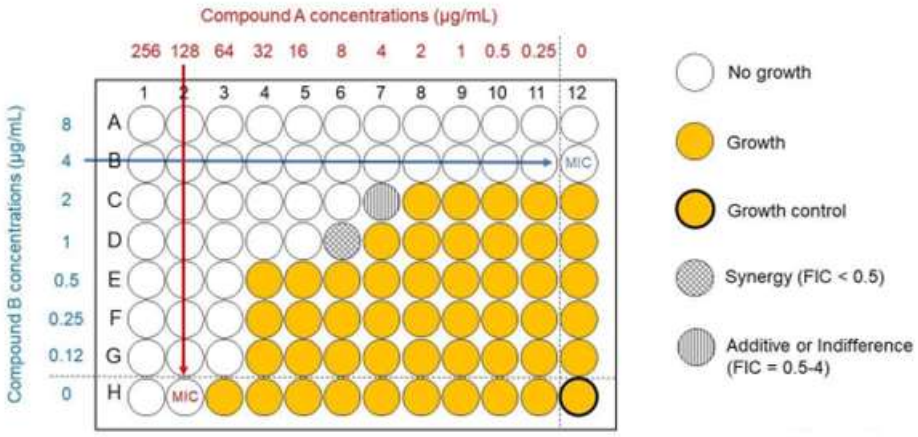


Figure 11: Synergy checkerboard assay

I- Yield results:

The first step in investigating the biological features of plants is to extract chemical compounds from plant material. This process is essential for extracting the active components and enhancing the yield. We utilized a solvent extraction method using petroleum ether, chloroform, and methanol to obtain the methanolic extract of *Limonium spathulatum*. The results are illustrated in the figure below:

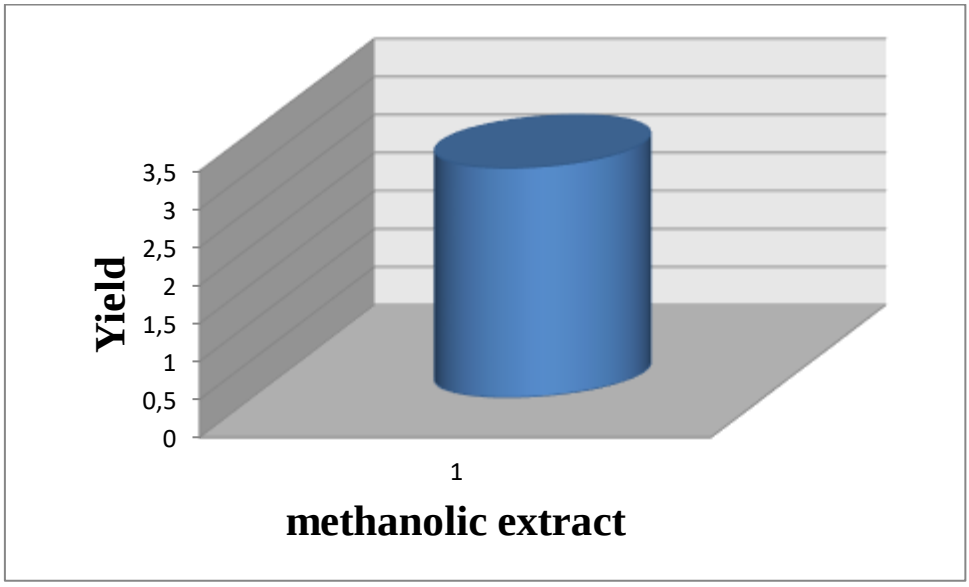


Figure 12: extraction yield

II-Antibacterial activity: determining the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was determined using the micro-dilution method on 96-well microplates. The methanolic extract of *Limonium spathulatum* was tested against three strains: *Klebsiella pneumonia*, *Enterococcus faecalis*, and *Bacillus subtilis*. Its effects were compared with those of the antibiotic. The results are depicted in the figure below:

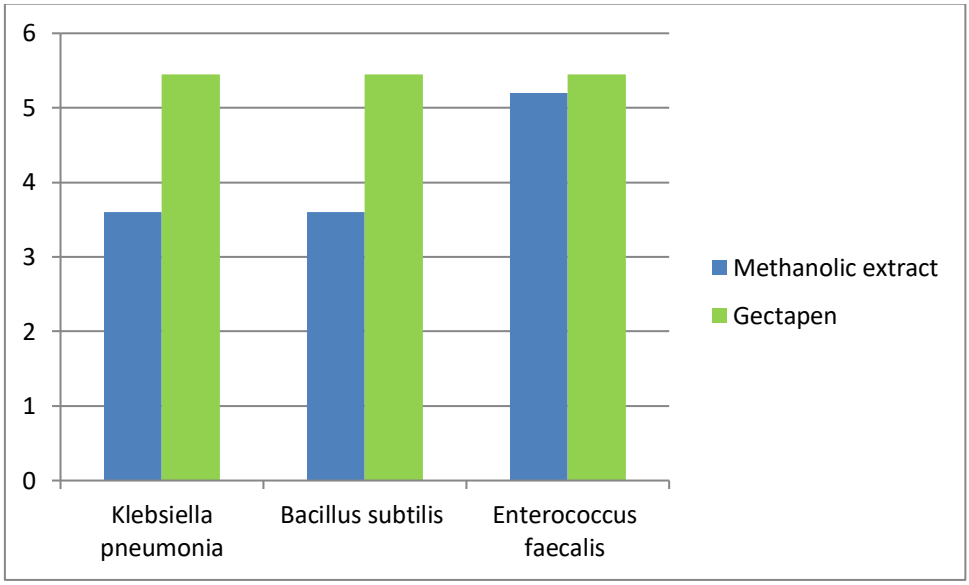


Figure 13: MIC values of the methanolic extract and the antibiotic gectapen

The results indicated that our methanolic extract exhibited activity against all three tested strains. When comparing the MIC values of *Limonium spathulatum* M.E with those of Gectapen, our M.E demonstrated a similar effect to the antibiotic against *Enterococcus faecalis* (MIC ±5.2mg/ml and MIC ± 5.45 mg/ml), while it displayed a higher level of activity against *Klebsiella pneumonia and Bacillus subtilis (*MIC ±3.2 mg/ml and MIC ± 5.42mg/ml).

An illustrative example of the obtained results is presented in the figure below:

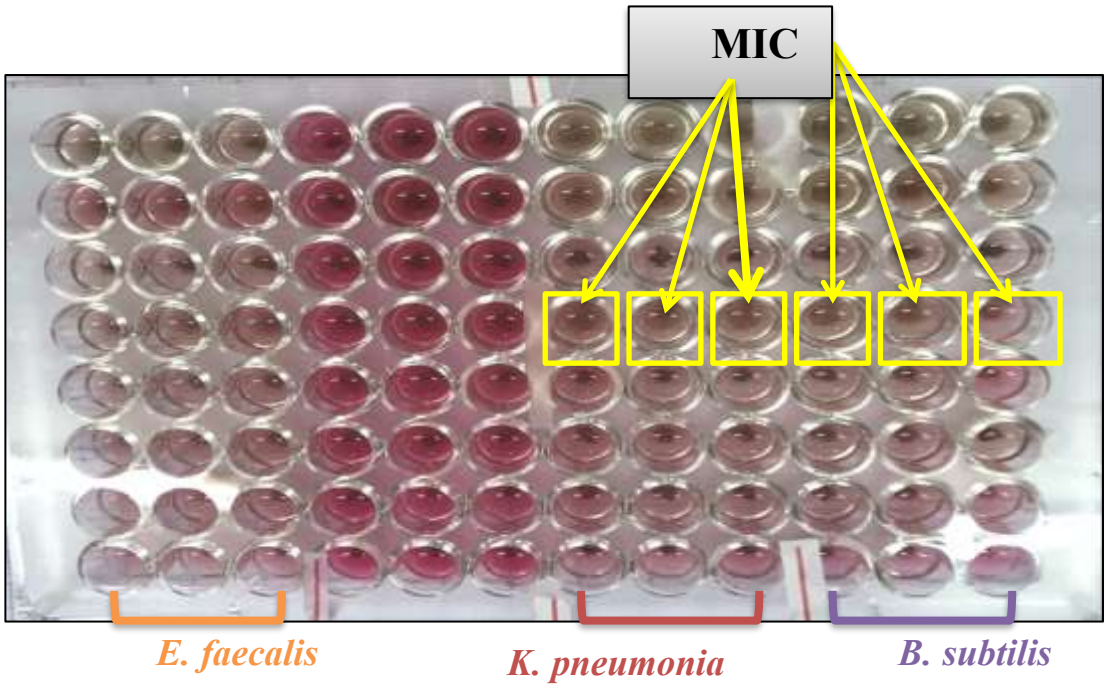


Figure 14: color change in the microplate wells in the presence of bacterial activity under the effect of resazurin

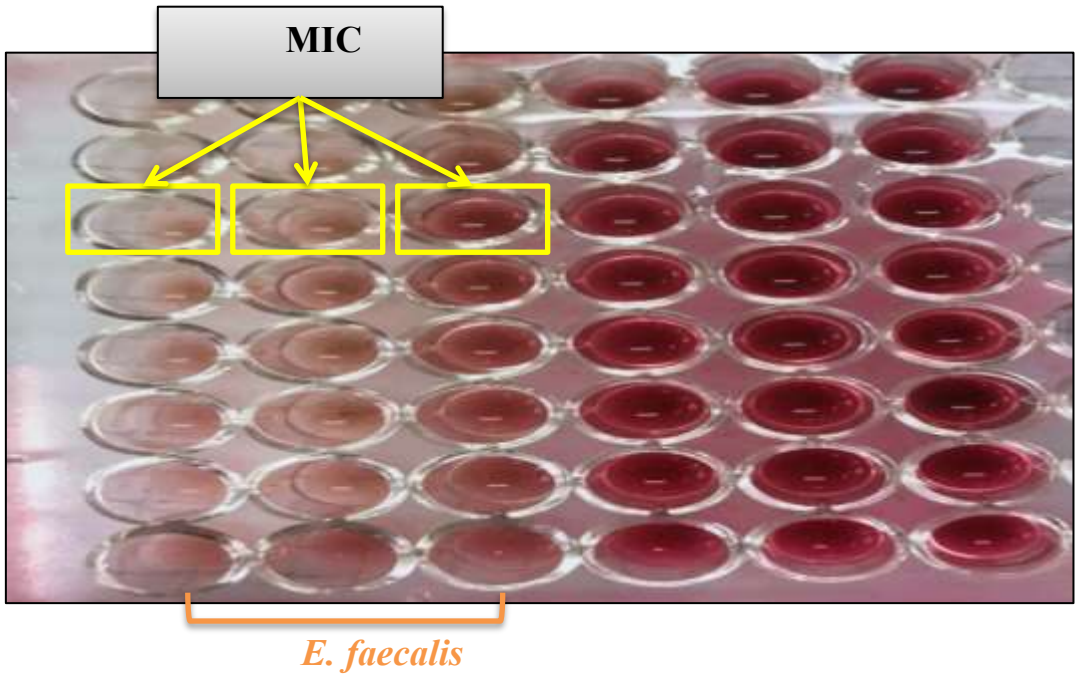


Figure 15: MIC determination for the bacterial strain *E. faecalis* ATCC 19433 on microplate**.**

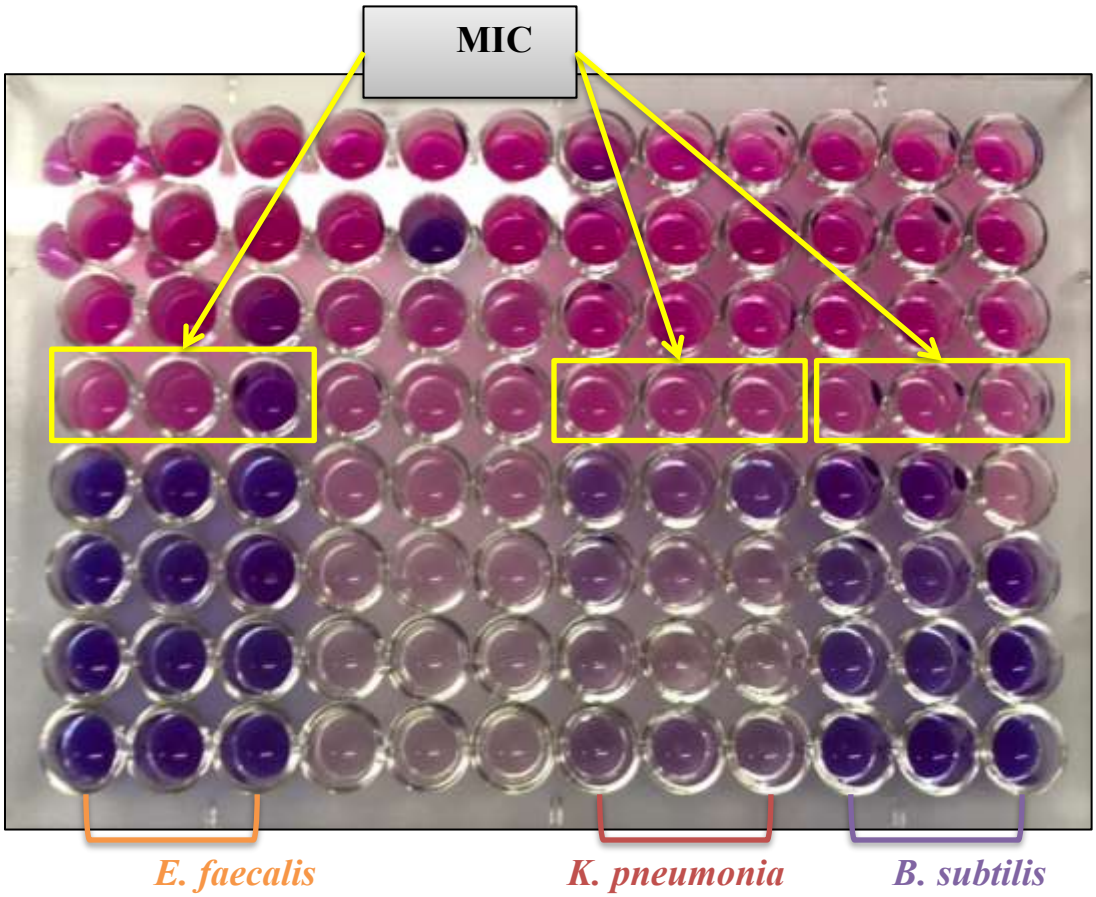


Figure 16: MIC determination for the three bacterial strains of gectapen

The figures illustrate how the growth of various bacterial strains was impacted by the concentrations of the methanolic extract and the antibiotic (Gectapen) that were tested. Wells with no visible deposits and a clear appearance indicate the complete inhibition of bacterial growth. Conversely, wells exhibiting microbial growth (indicated by a pink color) suggest that the methanolic extract and Gectapen had no inhibitory effect on bacterial growth.

Utilizing the microplate method, we were able to determine the minimum inhibitory concentration and bactericidal concentration values by observing disruptions in bacterial growth. The detailed results can be found in Tables 5 and 6, as well as in the accompanying figures.

Table 5: CMI and CMB of methanolic extract tested on the three bacterial strains studied expressed in mg/ml

Strains	MIC	MBC
K. pneumoniae ATCC 13883	3.6	> 4.6
B. subtilis ATCC 0486	3.6	> 4.6
E. faecalis ATCC 19433	5.2	> 6.4

Minimum inhibitory concentrations values for the methanolic extract were determined over a wide concentration range from [2.9 - 6.4 mg/ml] , while minimum bactericidal concentration values were > 4.6 mg/ml.

The results obtained from the determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of the methanolic extract of limonium spathulatum showed that the extract had antibacterial activity against the tested strains. The MIC values obtained for K. pneumoniae ATCC 13883 and B. subtilis ATCC 0486 were the lowest at 3.6 mg/mL whereas the MIC value obtained for E. faecalis ATCC 19433 was slightly higher at 5.2 mg/mL. These results indicate that the extract has relatively high efficacy against gram-negative and gram-positive bacteria.

Furthermore, it was observed that there was a difference in sensitivity to the extract at different concentrations. The bacteria *K. pneumoniae* and *B. subtilis* were found to be the most sensitive to the extract tested, with the lowest MIC value obtained at 3.6 mg/mL. On the other hand, *E. faecalis* was observed to be less sensitive to the extract, with a slightly higher MIC value of 5.2 mg/mL.

The different MIC values obtained show that antibacterial activity is a function of the bacteria and the different concentrations of methanolic extract

It's worth noting that this study's results are consistent with those of other studies on different Limonium species. For example, a study published in the Journal of Ethnopharmacology in 2014 found that a methanol extract of Limonium aureum also had significant antibacterial activity against several bacterial strains, including K. pneumoniae and B. subtilis. Similarly, a study published in the Journal of Natural Medicines in 2018 found that a methanol extract of

Limonium sinense had antibacterial activity against several bacterial strains, including E. faecalis, with MIC values ranging from 32 to 256 μg/ml.

Another study published in the Saudi Pharmaceutical Journal in 2019 evaluated the antibacterial activity of a methanol extract of Limonium brasiliense against several bacterial strains, including K. pneumoniae and E. faecalis. The study found that the extract had significant antibacterial activity against all strains tested, with MIC values ranging from 0.25 to 2 mg/ml.

Overall, these studies suggest that several Limonium species possess antibacterial activity, with variations in activity observed between species and bacterial strains. It's worth noting that the bioactive compounds responsible for this activity may vary between species, and more research is needed to determine the specific compounds responsible for the observed antibacterial effects.

In conclusion, Limonium species are a promising source of natural antibacterial compounds. Further research is needed to explore the specific compounds responsible for the observed activity, as well as the potential for Limonium extracts to be used as antibacterial agents in clinical settings.

Table 6: CMI and CMB of gectapen antibiotic tested on the three bacterial strains studied expressed in mg/ml

Strains	MIC	MBC
K. pneumoniae ATCC 13883	5.45	> 6.36
B. subtilis ATCC 0486	5.45	> 6.36
E. faecalis ATCC 19433	5.45	> 6.36

Minimum inhibitory concentrations values for the gectapen antibiotic were determined over a wide concentration range from [4.7 – 6.36 mg/ml], while minimum bactericidal concentration values were > 6.36 mg/ml.

From Table 6, we find that the antibiotic Gectapen is active against the tested strains, with the same MIC value, which is: 5.45 mg/ml

These findings suggest that Gectapen has a bacteriostatic effect on these bacterial strains at the tested concentrations, inhibiting their growth but not completely killing them. The lack of bactericidal effect may indicate that higher concentrations of Gectapen may be required to achieve a complete eradication of the bacteria.

It is interesting to note that while the MIC value was the same for all three strains, there might be differences in their sensitivity to different concentrations of the gectapen antibiotic. This implies that the bacterial species tested may respond differently to varying concentrations of the antibiotic, even though they all exhibited the same MIC value.

These results provide valuable insights for understanding the efficacy and potential clinical applications of the gectapen antibiotic against these specific bacterial strains.

II-1-1- Nature of antibacterial activity of methanolic extract studied

Determination of the nature of the antibacterial activity of the M.E of limonium spathulatum against the three microbial strains studied was carried out using the dilution boxes > 4.6 mg/ml showing visible growth inhibition. The results are shown in table and figure

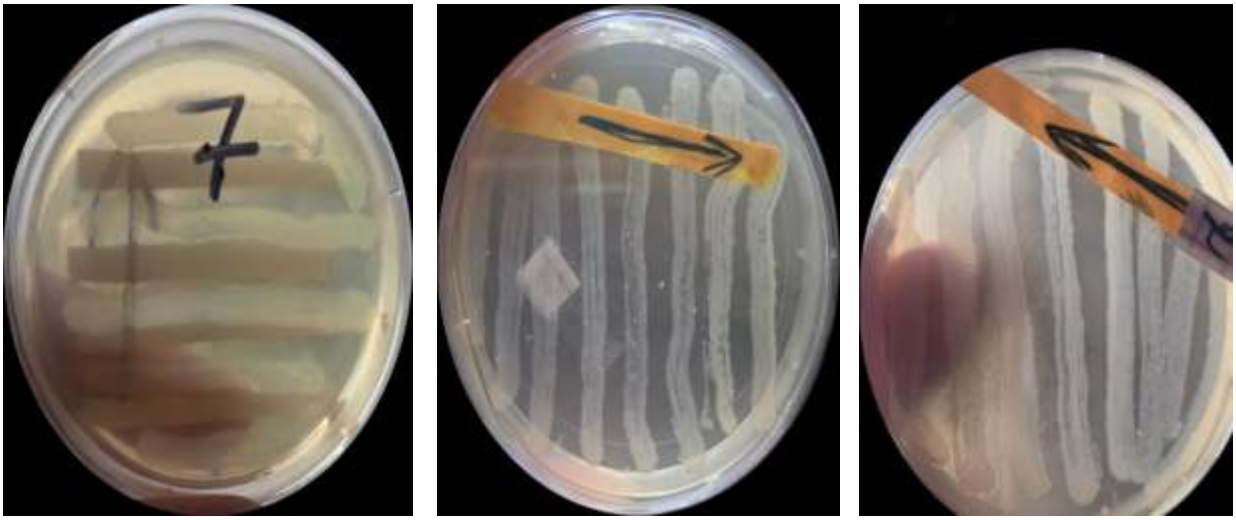


Figure: Determination of the MBC of the M.E on the bacterial strains studied

These results are summarized in the following table:

Table 7: nature of the antibacterial activity of the M.E of limonium spathulatum against the three microbial strains studied

Microbial strains	Type of activity
K. pneumoniae ATCC 13883	Bacteriostatic
E. faecalis ATCC 19433	Bacteriostatic
B. subtilis ATCC 0486	Bacteriostatic

Our results show that the antibacterial activity of limonium spathulatum methanolic extract is bacteriostatic for the following strains: *K. pneumoniae (ATCC* 13883), *E. faecalis (ATCC* 19433) and *B. subtilis (ATCC* 0486).

Overall, these findings indicate that the M.E has activity against the tested bacterial strains, but it may require higher concentrations to achieve bactericidal effect

Nature of antibacterial activity of gectapen antibiotic studied

Determination of the nature of the antibacterial activity of the G.A against the three microbial strains studied was carried out using the dilution boxes > 6.36 mg/ml showing visible growth inhibition. The results are shown in table and figure

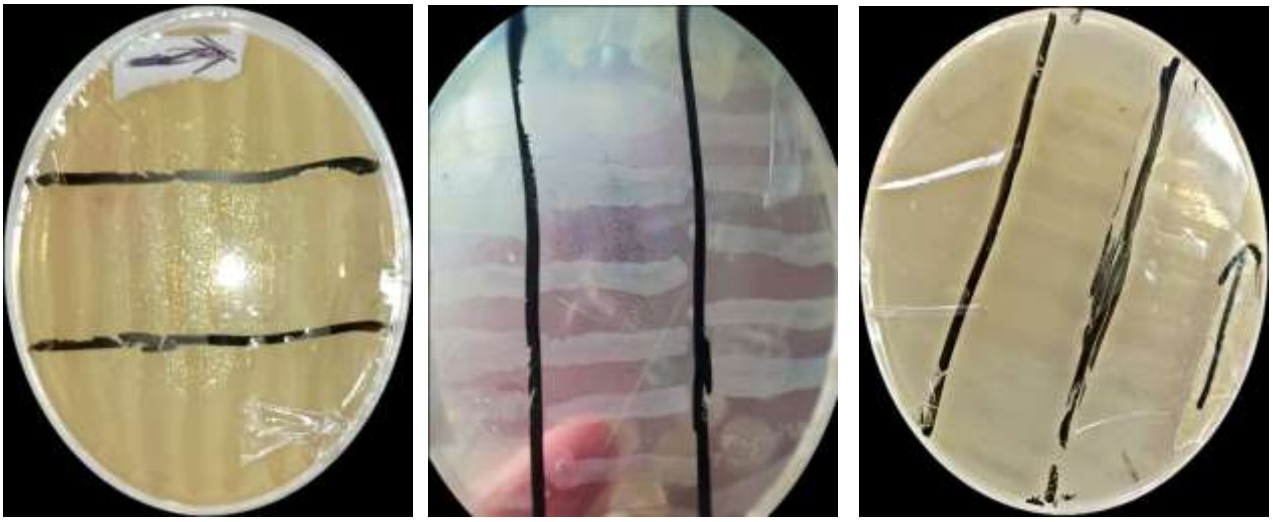


Figure: Determination of the MBC of the G.A on the bacterial strains studied

These results are summarized in the following table:

Table 8: nature of the antibacterial activity of the G.A of against the three microbial strains studied

Microbial strains	Type of activity
K. pneumoniae ATCC 13883	Bacteriostatic
E. faecalis ATCC 19433	Bacteriostatic
B. subtilis ATCC 0486	Bacteriostatic

Our results show that the antibacterial activity of G.A is bacteriostatic for the following strains: *K. pneumoniae (ATCC* 13883), *E. faecalis (ATCC* 19433) and *B. subtilis (ATCC* 0486).

These findings suggest that Gectapen has a bacteriostatic effect on these bacterial strains at the tested concentrations, inhibiting their growth but not completely killing them. The lack of bactericidal effect may indicate that higher concentrations of Gectapen may be required to achieve a complete eradication of the bacteria.

III-Study of the association "methanolic extract / antibiotic" by the method of microdilution

Synergistic effect of methanolic extract in combination with antibiotic evaluated using the microplate technique. This synergy was detected in the *K. pneumoniae (ATCC* 13883)and *E. faecalis (ATCC* 19433) strains

The results are shown in the following figures:

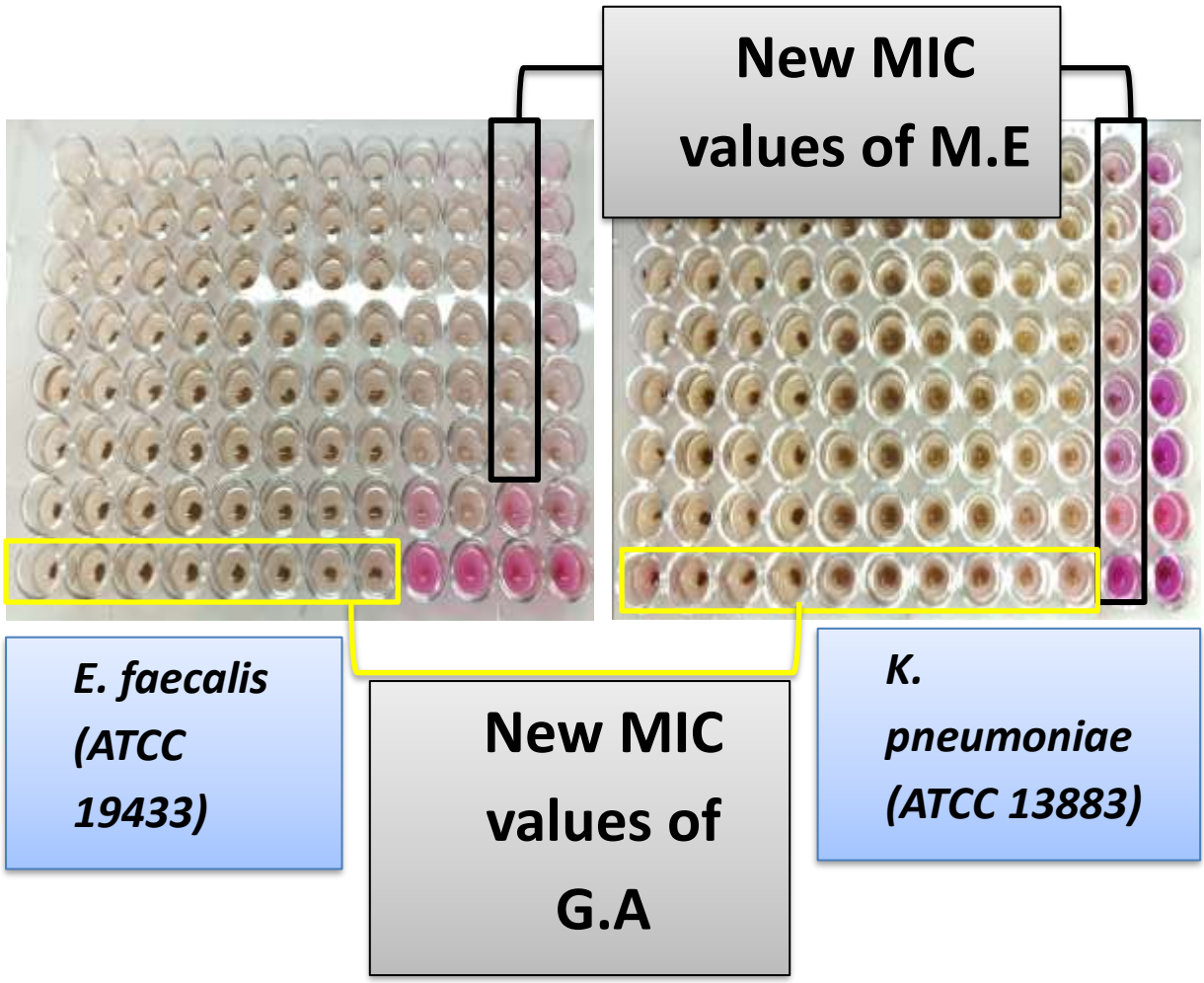


Figure: Synergy test between methanol extract and antibiotic in both strains *E. faecalis (ATCC* 19433) *K. pneumoniae (ATCC* 13883)

The results obtained indicate that the methanolic extract of limonium spathulatum possesses antibacterial activity and has a synergistic effect with the antibiotic gectapen, reducing the MICs of the latter. This reduction varies from strain to strain.

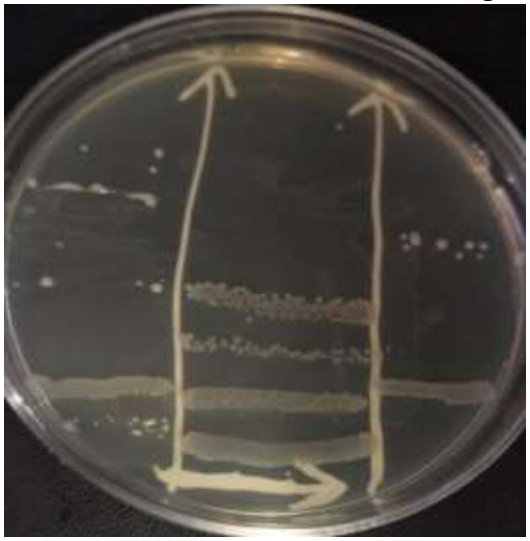
Table: Synergy results for the association between methanolic extract and antibiotic

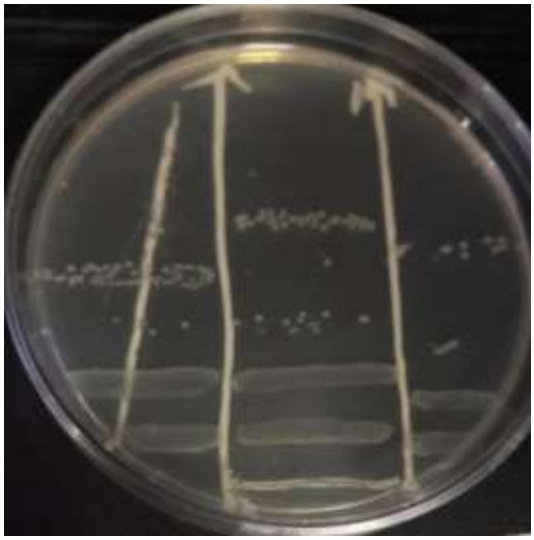
Straines	**MIC of methanolic.E (mg/ml)**	**MIC of Antibiotic (.mg/ml)**	**MIC of the association (mg/ml)**	**Synergic effect**
K. pneumoniae ATCC 13883				**present**
E. faecalis ATCC 19433				**present**

Determination of Minimal Bactericidal Concentration (MBC) of the association between methanolic extract and antibiotic

After obtaining the MIC results for the combination of methanolic extract and antibiotic, the contents are sampled and transferred to GN medium using the same concentration range used for MIC evaluation.

The results are shown in the following images:



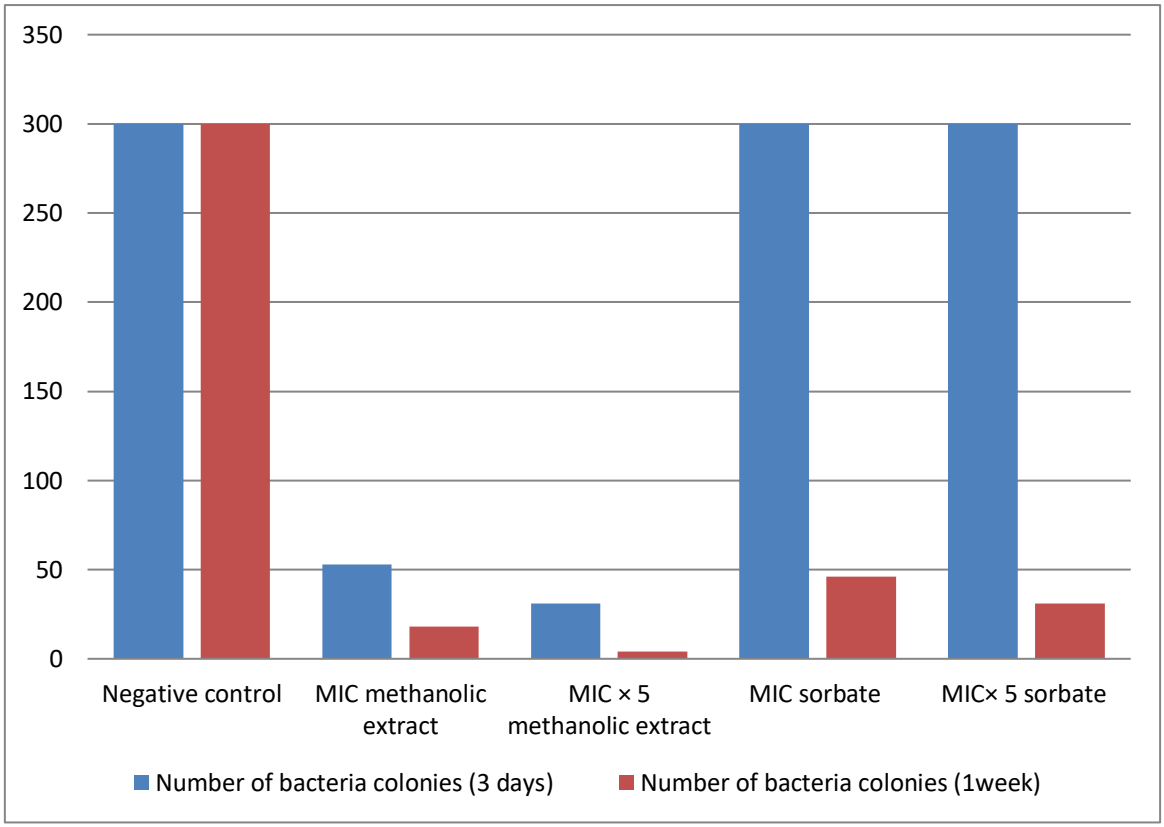


E. faecalis ***K. pneumoniae***

Our results show that the antibacterial activity of the association of *limonium spathulatum* methanolic extract and gectapen antibiotic is bactericide for both strains: *K. pneumoniae (ATCC* 13883), *E. faecalis (ATCC* 19433).

IV- The inhibitory effect of methanolic extract against pathogen bacteria B. subtilis in yoghurt

The results are shown in this histogram and the table:



The effect of methanolic extract on the development of the number of colonies of Bacillus subtilis during 3 days and within a week.

This table shows the inhibitory effect of methanolic extract on the pathogen bacteria B. subtilis in yogurt. The results demonstrate that the methanolic extract has a significant inhibitory effect on the growth of B. subtilis in yogurt, as evidenced by the reduction in the number of bacterial colonies.

At the 72-hour mark, the methanolic extract at the MIC concentration showed a significant inhibitory effect on B. subtilis growth in yogurt, reducing the number of colonies to 53. Increasing the concentration five-fold further decreased the colonies to 31, indicating a dose-dependent effect. In contrast, the positive control using the MIC of sorbate food preservative did not exhibit substantial inhibition, as the colony count remained at 300. The negative control showed uncontrolled bacterial growth, with colonies reaching 300 as well.

At the 168-hour mark, the methanolic extract continued to demonstrate its inhibitory potential. The MIC concentration resulted in a further reduction of colonies to 18, while the five-fold concentration yielded only 4 colonies. In comparison, the positive control with sorbate food preservative displayed 46 colonies, and the higher concentration had 31 colonies. The negative control maintained unimpeded growth with 300 colonies.

Overall, these results indicate that the methanolic extract possesses strong inhibitory properties against Bacillus subtilis in yogurt. Its effectiveness exceeded that of the sorbate food preservative, suggesting its potential as a natural alternative for enhancing the safety and shelf life of yogurt.