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### Bactericidal Effect of Aqueous Extracts of the Bark of the Pomegranate (*Punica granatum* L.) on Bacteria

<sup>1</sup>Rabah Chadli  
<sup>1</sup>Aman Bouzid  
<sup>2</sup>Khadidja Bouzid  
<sup>1</sup>Hamida Nader

<sup>1</sup> University Abdelhamid Ibn Badis, Algeria  
Faculty of Natural Sciences and Life, Mostaganem, 27000  
<sup>2</sup> Djillali Liabes University, Algeria  
Laboratory eco-development areas  
Faculty of Natural Sciences and life, Sidi Bel Abbes, 22000  
**Corresponding author:** Chadli Rabah  
E-mail: [chadli\\_rabah@yahoo.fr](mailto:chadli_rabah@yahoo.fr)

#### Abstract

This research concerns the study of antibacterial properties of different aqueous extracts of the bark of the pomegranate (*Punica granatum* L.). Three bacterial strains were used in this test: *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Salmonella*. Very interesting bactericidal properties of aqueous extracts of the bark of the pomegranate were found on bacteria. The inhibition zones have a very large diameter up to 20 mm and the MIC and MBC are low, of the order of 0.78 mg/ml. This work has shown antibacterial activity against three bacteria may contribute to the fight against infectious diseases, and possibly offer the possibility of using pomegranate peel pharmaceutical and food industries.

**Keywords:** *Punica granatum* L.; bark; antibacterial activity; aqueous extracts; inhibition.

#### Introduction

The use of medicinal plants for therapeutic purposes is an ancient practice. The pomegranate (*Punica granatum* L.) has fascinated all civilizations by its beauty and juiciness of the fruit. Used empirically in traditional medicines, to treat gastrointestinal diseases and parasitic diseases, pomegranate has anti-oxidant properties.

The bark of the pomegranate fruit is the hard part of the fruit. It is generally used dried in the form of brownish pieces. These fragments are leathery consistency. They are formed of parenchyma cells with thin walls, in the middle of which there are groups of stone cells and vascular bundles. The flavor of pomegranate rind is bitter and astringent. The crust is an anthelmintic and astringent and useful in the treatment of diarrhea, dysentery and stomach pains [1]. For centuries, bark, leaves, flowers and fruits of *Punica granatum* L. (Punicaceae), known as the grenade name, were used to treat many diseases [2]. Bacterial and fungal infections due to multidrug-resistant microorganisms are a major concern for both health problem in developing countries where they are the main cause of high mortality rates in industrialized countries where

resistance to existing antibiotics grow alarmingly [3]. This situation creates a growing need to find new compounds; identifying new sources of antibiotic natural products and the expansion of the chemical diversity of antibiotics provide leads for new chemical drugs. Medicinal plants, particularly those used traditionally, are a potential source of such compounds. One strategy for this research is to explore the plants used in traditional medicine to treat many infectious origin pathologies. Capable of colonizing a wide variety of tissue causing, among others, respiratory, intestinal, skin or urinary [4]. They have enormous therapeutic potential to cure many infectious diseases [5]. Thus, for two decades studies have been conducted on the development of new applications and the exploitation of natural properties of essential oils in different areas. The use of essential oils is relevant today. As part of our effort to achieve this goal, we evaluated the antibacterial activity of the plant used traditional medicine Pomegranate (*Punica granatum* L.) belongs to the family Punicaceae. It is a shrub native to Asia and has been cultivated since ancient times throughout the Mediterranean region of Africa and parts of Europe.

## **Materials and methods**

### **1. Biological material**

#### **1. 1. Harvesting of grenade and preparation of the powder**

Fresh fruit of the grenade were collected during the month of October 2013 from a grenadier field. The barks were cleaned, peeled and dried in the shade for two weeks and then crushed in a traditional mortar then they were ground to a fine powder using an electric coffee grinder. The powder was stored in clean vials before proceeding with the extraction.

#### **1. 2. Method of study**

**Extraction processes.** Three different extracts (chloroform, acetone, ethanol) were prepared from the powdered bark of the grenade.

##### ***Chloroform extraction***

A mixture of 100 g of bark powder *Punica granatum* L. 1Litre with chloroform and placed in a Soxhlet apparatus and then the mixture was heated at 70 °C for one hour. After cooling, the homogenate was filtered under vacuum on Whatman paper (n°3), then the filtrate was concentrated under reduced pressure with a rotary evaporator [6].

##### ***Acetone extraction***

100 g of the powder was mixed with 1L of distilled water in an Erlenmeyer flask, then the mixture was stirred at laboratory temperature (25 °C) for 24 hours [7]. After 24 hours, filtered the mixture under vacuum Whatman paper (n°3) and then the filtrate concentrated under reduced pressure with a rotary evaporator [6].

##### ***Ethanol extraction***

For the ethanol extract, 100 g of the powder was mixed with 1L of ethanol and the mixture was placed under stirring at room temperature (25 °C) for 24 hours [7]. The mixture was filtered on Whatman paper, then the filtrate was concentrated under reduced pressure with a rotary evaporator [6].

## **2. Microbiological study**

### **2.1. Study of the antimicrobial activity**

The antimicrobial study consists in determining the antibacterial parameters (MIC) of our extracts of pomegranate, for it was used the agar diffusion method [8].

### **2.2. Preparation of inoculum**

**The bacterial inoculum.** Using a handle previously outbreak then cooled, was taken a colony of *S. aureus*, *P. aeruginosa*, then were seeded on a nutrient agar slope and incubated the tube at 37 °C for 24 hours.

### **2.3. Preparing extracts dilutions**

After their concentration by rotary evaporation the extracts taken a pasty form prior to use they were solubilized in DMSO [9, 10] to give a stock solution of 100 mg / ml. From this solution, dilutions up to 6.25 % were achieved.

### **2. 4. Agar diffusion**

Microbial suspensions were prepared in physiological saline and then the optical densities were adjusted from 0.08 to from 0.1 to a wavelength of 600 nm for the bacterial strains. Seeding was done by flooding on Petri dishes containing 10 ml of Mueller-Hinton agar in a thickness of

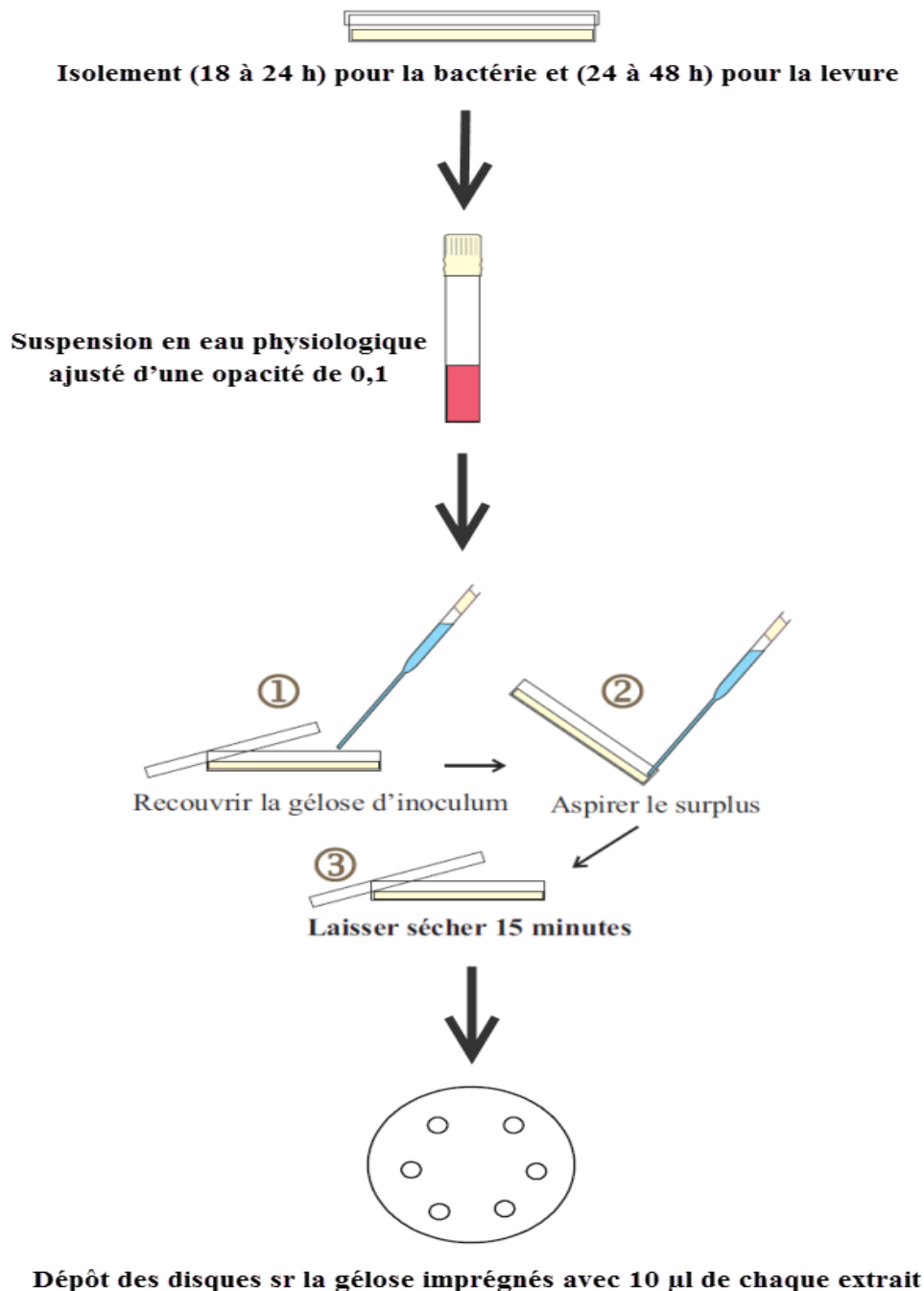
4 mm [11, 12] then the boxes were left open to dry aseptically [9]. Sterile disks of 06 mm diameter were placed on the agar using sterile forceps [13], then pressed to ensure their application then they were impregnated with 10 µl of each extract at different concentrations using a micropipette.

### 2.5. Antibiotics (control)

They are discs of 06 mm diameter were placed on the agar with sterile forceps, and then pressed to make their application. It has 04 deferent antibiotics (Penicillin P, PG, Streptomycin, Netroxolin).

### 2.6. Minimum inhibitory concentration (MIC)

The evaluation of the minimum inhibitory concentration is to determine the lowest concentration of an antimicrobial agent required to inhibit any visible culture. It is determined by using a dilution series of the antimicrobial agent, added to a series of tubes of a liquid culture medium [14, 15].



### **Extraction and preparation of plants**

A local variety of grenadier, *Punica granatum* L., was chosen as plant material. Bark fruits were dried in ambient air and then reduced in the form of powder. Grenadier extracts were extracted in 80 % hot ethanol. After centrifugation, the supernatant was filtered and dried and stored in eppendorf at 4°C. The tested extracts were resuspended in dimethyl sulfoxide (DMSO) to a final concentration of 100 mg / ml. After dissolving in the water bath, the mixture was centrifuged at 3000 RPM for 15 minutes at room temperature. The supernatant was then separated and stored at -20°C. And the frozen extracts were thawed at 37°C and diluted to the required concentration in the culture medium before each experiment in order to obtain a final concentration of 0.1 to 0.2 % DMSO.

### **Results**

#### **1. 1. Extraction**

The yields of different extracts of *Punica granatum* bark obtained are:

- 34,27 g dark brown for the ethanol extract.
- 29,68 g dark brown for the acetone extract.
- 26,47 g light brown color to the chloroform extract.

#### **1. 2 Microbiological study**

**Diameters of inhibition zones.** The acetone extract exhibited a considerable antimicrobial activity against three bacterial strains tested against two other extracts (ethanol and chloroform), with diameters of the inhibition zones s 20 mm for *Salmonella* by a dose of 75 mg, 17 and 18 mm (*S. aureus*, *P. aeruginosa*) 50 mg, respectively.

**Table 1:** The diameters of the inhibition zones for different antibiotics

Antibiotics Bacterial strains	Penicillin P diameter in mm	Penicillin G diameter in mm	Streptomycin diameter in mm	Nitroxolin diameter in mm
<i>Salmonella</i>	0	11	13	21
<i>Staphylococcus aureus</i>	10	14	15	11
<i>Pseudomonas aeruginosa</i>	12	14	18	12

**Table 2:** The diameters of the inhibition zones for different extracts by a dose of 100 mg

Bacterial strains	Extracts	Wells method, mm	Disk method, mm
<i>Staphylococcus Aureus</i>	Acetone	16	13
	Ethanol	12	10
	Chloroform	0	0
<i>Salmonella</i>	Acetone	17	14
	Ethanol	16	15
	Chloroforme	5	6
<i>Pseudomonas Aeruginosa</i>	Acetone	15	10
	Ethanol	15	14
	Chloroform	0	0

**Table 3:** The diameters of the inhibition zones for different extracts

Extracts		Wells method, mm	
		Dose of 75 mg	Dose of 50 mg
Bacterial strains			
	Staphylococcus <i>Aureus</i>	Acetone Ethanol	16 15
<i>Salmonella</i>	Acetone Ethanol	20 18	18 15
	<i>Pseudomonas</i> <i>Aeruginosa</i>	Acetone Ethanol	17 16

**MIC**

In the procedure of the determination of the minimum inhibitory concentration of the ethanolic extract of the biological assay was used to graded concentrations of 50,75 and 100 mg/ml.

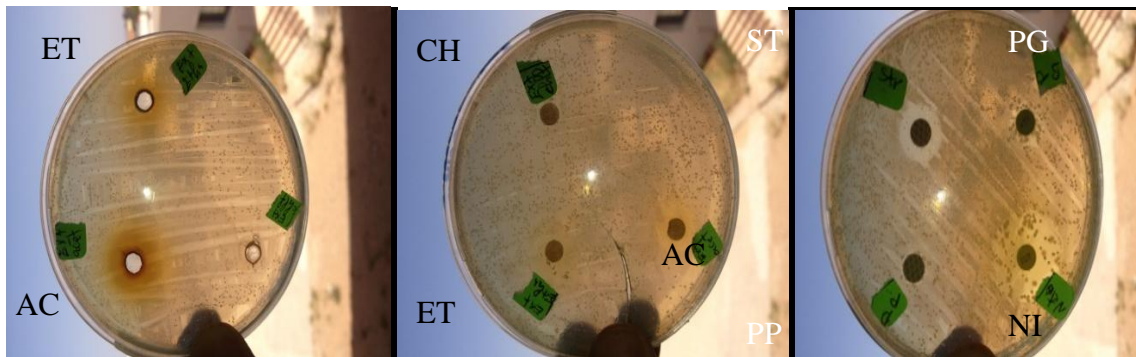
**Discussions**

**1. Extraction**

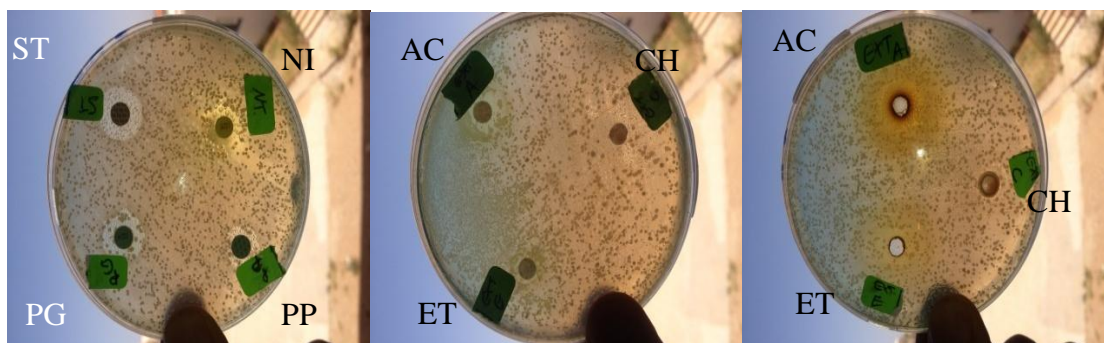
Extraction processes have resulted in different yields depending on the nature of the solvent [7], the method and the conditions under which the extraction was carried out [16].

This result could be explained by the difference in solubility of the components of the crude extracts which vary depending on the solvent used and the way in which they are prepared.

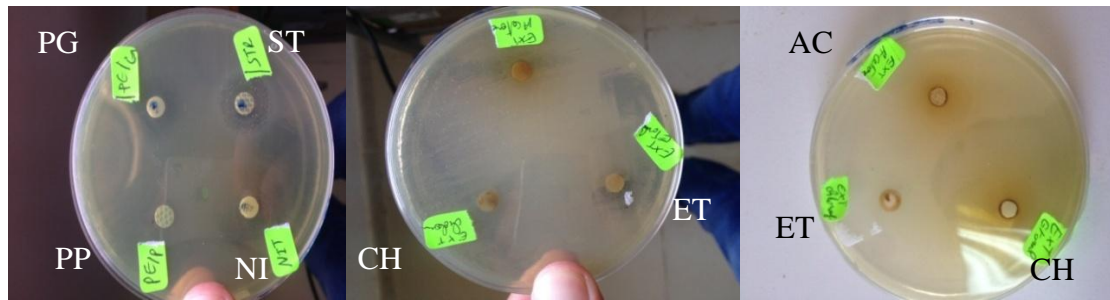
**Susceptibility testing.** In this study, the antibacterial activity was evaluated the ethanol extract, acetone and chloroform *Punica granatum* pericarp of the growth of three strains of bacteria (*Salmonella*, *S aureus* and *P aeruginosa*) by the agar diffusion method in measuring the diameters of inhibition of bacterial growth areas (Figure 1 - 6).



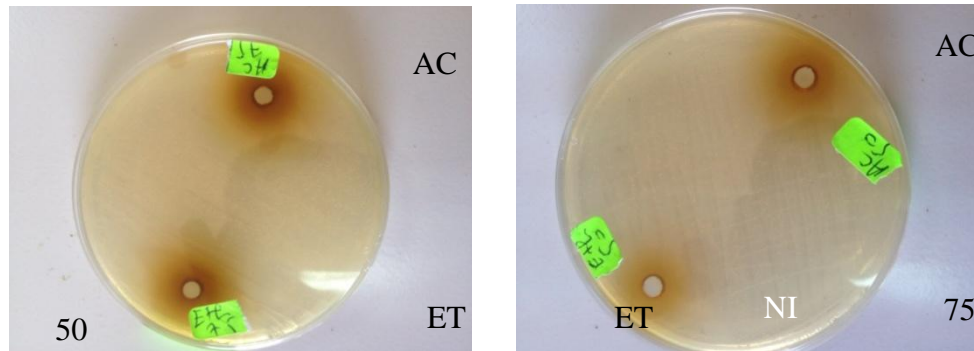
**Figure 1.** Sensitivity of *S. aureus* to the different extracts (100 mg) and antibiotics.



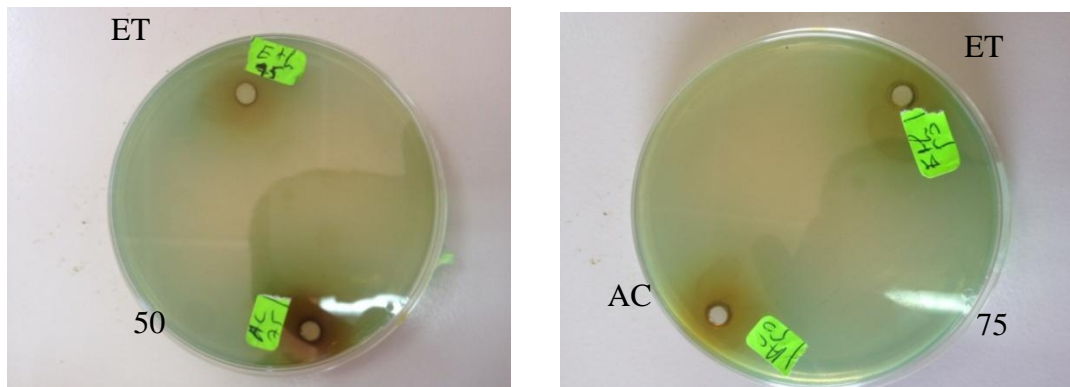
**Figure 2.** Sensitivity of *Salmonella* to the different extracts (100 mg) and antibiotics.



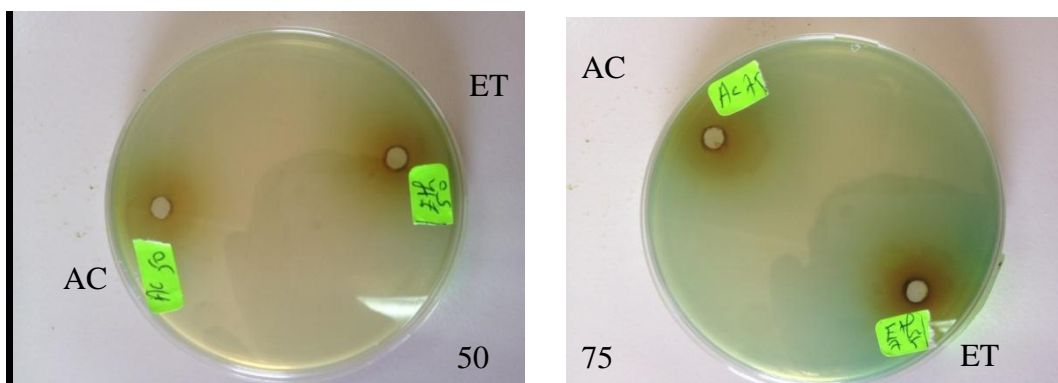
**Figure 3.** Sensitivity of *P. aeruginosa* to the different extracts (100 mg) and antibiotics



**Figure 4.** Sensibility of *S. aureus* to the different extracts (50–75 mg)



**Figure 5.** Sensibility of *Salmonella* to the different extracts (50-75mg)



**Figure 6.** Sensibility of *P. aeruginosa* to the to the different extracts (50–75 mg)

Subsequently, our choice fell on the ethanol extract, which showed better antimicrobial activity. Then we proceeded to the determination of the MIC values of the three strains tested.

Salmonella strain has shown the most sensitive to the extracts studied with diameters of 20 mm for the acetone extract, 18 mm for the ethanol extract and 05 mm for the chloformique extract.

For bacterial strain *S. aureus* showed a sensitivity screw overlooked the acetone extract with a diameter of 17 mm and a diameter of 16 mm for the ethanol extract and for *P. aeruginosa*, has the same sensitivity of 18 mm diameter towards the two extracts (acetone, ethanolic).

*Punica granatum* (pomegranate) contains tannins. Tannins are known to precipitate the protein and might be involved in the extracts inhibitory mechanism, however, the exact mechanism of inhibition is not known [18].

The data obtained on the three bacteria are similar to those carried out by Vasconcelos et al. [19] and Beckman et al. [17], where the pomegranate extract showed significant inhibition. Melendez and Capriles [20] and Senhaji et al. [13] also reported that pomegranate fruit extracts have antibacterial activity in vitro against many bacteria tested, including our bacterial strains, confirming our results.

In general, the acetone extract has a high antimicrobial activity, it can be linked to the power of acetone to dissolve one or more compounds contained in the homogenate of the pericarp [13]. The efficiency of an extract depends on its concentration, the sole of which it arose and strain tested [21].

## Results

The development of drug resistance in human pathogens against commonly used antibiotics has necessitated the search for new antimicrobial substances from other sources, including plants and microbes [22]. This antibacterial activity may be indicative of the presence of metabolic toxins or compounds with broad spectrum antibiotics. This is consistent with previous reports of several researchers [1, 23, 24]. Methanol extracts showed a higher degree of antimicrobial activity against extracted with acetone. Voravuthikunchai et al. [24] reported that *P. granatum* contains large quantities of tannin (25 %) and the antibacterial activity can be indicative of the presence of certain secondary metabolites. The ethanol extract of *P. granatum* showed antibacterial activity against *E. coli* [24] and *S. aureus* [23].

Due to the increasing development of drug resistance and adverse effects caused by existing antifungal agents, search for new antimicrobial compounds has been the subject of a number of studies [25]. Interest in natural products has increased over the years, and medicinal plants have been identified as sources of bioactive compounds. These data support the observations of some research such as those of [12, 18, 26–31], demonstrated that pomegranate extract inhibits affects tannins, the ability to inhibit the growth of the *Candida* yeast species due to their action in the cell, particularly in the cell membrane, protein precipitation.

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