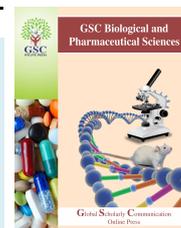


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(RESEARCH ARTICLE)



## Antibacterial effect of the leaves of *Eucalyptus globulus* against clinical bacterial isolates

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### Abstract

Antibiotic resistance being a major threat to public health, instigated the search for new antimicrobial agents especially in the recognized medicinal plants. In this study the antibacterial effect of the leaves of the plant: *Eucalyptus globulus*, used in herbal medicine, against clinical Gram positive and Gram negative clinical bacterial isolates was studied. This antibacterial effect was determined using the well agar diffusion and agar dilution methods. Using the well agar diffusion method, the aqueous extract of *E. globulus* exhibited a weak inhibitory effect evident at a volume of 200  $\mu$ l against the methicillin resistant and methicillin sensitive *Staphylococcus aureus* isolates and 2 of the 3 *Enterococcus faecalis* isolates and at a volume of 300  $\mu$ l for the *Acinetobacter baumannii* isolates. On the other hand, the methanolic extract of the plant showed a notable inhibitory activity against methicillin-resistant and methicillin sensitive *S. aureus*, *A. baumannii*, *Streptococcus pyogenes* and 2 of the 3 *Enterococcus faecalis* isolates. The *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli* isolates were resistant to the volumes used of both extracts and were only inhibited at higher concentrations using the agar dilution method. At a ratio of methanolic extract to MHA equal to 0.15, the *P. aeruginosa* isolates exhibited moderate growth while the *K. pneumoniae* and *E. coli* isolates showed weak growth. At a ratio of methanolic extract to MHA of 0.2, the three Gram negative bacteria were completely inhibited. The results of this study highlight the possibility of extracting an efficient broad-spectrum antibacterial agent from the leaves of *Eucalyptus globulus*.

**Keywords:** Antibiotic resistance; Antibacterial effect; Ethnomedicine; *Eucalyptus globulus*; Herbal medicine; Methanol extracts.

### 1. Introduction

The rapid rise and spread of bacterial resistance to antibacterial agents has become a global problem demanding urgent attention. The World Health Organization declared that antibiotic resistance is currently one of the top three threats to public health [1]. Many factors contributed to magnifying the problem including the lack of patients' compliance with proper usage of antibiotics and the improper prescription of antibiotics in as much as in 30% to 50% of medical cases [2, 3].

The urgency of the antibiotic resistance crisis demanded immediate remedies, among which was the search for new antimicrobial agents. From nature, which harbors a vast variety of plants, many have, over time, been used for medicinal purposes. The search for new antibacterial drugs, led scientists to include these medicinal plants in their pursuit. One group of such plants was the members of the genus *Eucalyptus*. *Eucalyptus* is a diverse genus of flowering trees and shrubs in the *Myrtaceae* (myrtle family). There are approximately 900 species of *Eucalyptus* mostly native to Australia, with only 15 species occurring outside. These species of *Eucalyptus* are cultivated throughout the tropics and subtropics including the Americas, England, Africa, the Mediterranean basin, the Middle East, China and the Indian subcontinent

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[4, 5]. Members of the *Eucalyptus* were used traditionally, by different ethnic groups, for their antibacterial, antimalarial, antioxidant, antifungal, antiviral, antihistaminic, anti-inflammatory, anticancer, and antiseptic properties [6]. Specifically, the medicinal uses of *Eucalyptus globulus* (*E. globulus*) have been investigated in many studies. The antioxidant effect of its leaves, marking the plant's neuroprotective ability against oxidative stress in H<sub>2</sub>O<sub>2</sub> - induced stress experiments, was reported by Burgos et al. [6].

Antimicrobial capabilities, attributed to different essential oils in many *Eucalyptus* species, were also reported [7, 8]. These reports also pointed out that these oils had a greater effect on Gram positive than on Gram negative bacteria. Specifically, Salari et al. [9], reported that the *E. globulus* leaves demonstrated an antibacterial effect against bacteria responsible for respiratory tract infections such as *Staphylococcus aureus* (*S. aureus*), *Streptococcus pyogenes* (*S. pyogenes*), and *Hemophilus influenzae* (*H. influenzae*). The main constituent of the leaves of *E. globulus* was reported to be 1,8-cineole or eucalyptol (63.81%) [10]. In a study by Mergheni et al. [11], 1, 8-cineole showed an antibiofilm and anti-quorum sensing effect against methicillin-resistant *S. aureus*, highlighting a major therapeutic effect. In addition, the possible use of the leaves of *E. globulus* as a disinfectant and antiseptic was recommended, after a study demonstrated their ability to inhibit both *Escherichia coli* (*E. coli*), and *S. aureus* [12]. A synergistic effect between antibiotics and the *E. globulus* leaf extract was also observed against isolates of *Pseudomonas aeruginosa* (*P. aeruginosa*), responsible for respiratory tract infections with a reported 55% increase in that effect [13]. Moreover, data compiled by Barbosa et al. [14] also showed that *S. aureus* was very sensitive to the essential oils extracted from *Eucalyptus* leaves while *P. aeruginosa* was very resistant to these oils.

Traditionally, in Lebanese villages, boiled *E. globulus* leaves were used for their ability to relieve asthma, cough, and chest pain. Accordingly, this study was designed to determine any antibacterial effect of the aqueous and methanolic extracts of *E. globulus*, grown in Lebanon, against a variety of local clinical isolates of important Gram positive and Gram negative bacterial pathogens.

## 2. Material and methods

### 2.1. Bacterial isolates

The bacterial isolates used in this study were clinical bacterial isolates courteously provided by the clinical microbiology laboratory of the Lebanese American University Medical Center - Rizk Hospital (LAUMC- RH). Namely, the isolates were: methicillin resistant and methicillin sensitive *S. aureus* (3 each), *S. pyogenes* (1), *Enterococcus faecalis* (*E. faecalis*) (3), *E. coli* (2), *Klebsiella pneumoniae* (*K. pneumoniae*) (2), *Acinetobacter baumannii* (*A. baumannii*) (3) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (2).

### 2.2. Definitive identification of the bacterial isolates

The identity of the isolates, included in this study, was reconfirmed using standard tests [15]. The identification of members of the family *Enterobacteriaceae* was also done using the API 20E kits (Biomerieux), while the identification of the non-fermentative bacteria was also done using the RapID NF Plus kits (Thermo Fisher Scientific).

### 2.3. Preparation of the plant leaves

The leaves of *E. globulus* were freshly picked from the Deir El Zahrani area, in Southern Lebanon, and immediately sent to the microbiology laboratory. After the confirmation of their identity by our botanist, they were washed and dried in preparation for the following steps.

### 2.4. Aqueous extraction

Thirty grams of fresh *E. globulus* leaves were weighed and boiled in 150 ml of sterile deionized water for 10 minutes. The solution obtained was filtered using vacuum filtration. Subsequently, the aqueous extract was stored in a sterile bottle for later use.

### 2.5. Methanol extraction

After 30 g of fresh *E. globulus* leaves were weighed, they were chopped and blended with 150 ml of pure methanol. The extraction process was done by keeping the solution in a shaking incubator for 7 days at a temperature of 42 °C, while shaking at 80 rpm. The solution obtained was then filtered using vacuum filtration and stored in a sterile bottle for later use.

## 2.6. Standardization of the bacterial isolates

A suspension of each of the bacterial isolates, to be used in the tests, was adjusted to a turbidity matching that of a 0.5 McFarland standard, equivalent to  $1.5 \times 10^8$  CFU/ml. [16].

## 2.7. Well agar diffusion method

Mueller-Hinton agar (MHA) plates were used as recommended [16]. The plates were seeded with the standardized inoculum. Using a sterile cork-borer, 13 mm wells were burrowed in the middle of each plate. Into the wells of different plates, different volumes (100  $\mu$ l, 200  $\mu$ l, 300  $\mu$ l) of the aqueous extract, methanolic extract, and methanol (control) were added. The plates were then incubated at 35 °C for 18-24 hours. The reported results were the average diameters of 3 diameter measurements for each zone of inhibition of growth of each plate.

## 2.8. Agar dilution method

In two separate preparations of Mueller-Hinton agar (MHA), amounts of the methanolic extract of *E. globulus* was added in ratios of 0.15 and 0.2 of extract to agar volumes respectively. The solutions were then put in a water bath at 70 °C for 15min in order to evaporate the methanol. After the plates were dispensed and left to solidify, they were seeded, using the standardized suspension, with 2 isolates of each of: *P. aeruginosa*, *K. pneumoniae* and *E. coli*. The inoculated plates were then incubated at 35 °C for 24 hours, after which they were checked for bacterial growth.

## 3. Results

Using the well agar diffusion method, the aqueous and methanolic extracts of *Eucalyptus* were tested for their antibacterial activity against the different clinical isolates. The initial volume of the extracts used was 100  $\mu$ l. Gradually, it was increased to 200  $\mu$ l and then to 300  $\mu$ l. The aqueous extract showed an antibacterial effect with the varying volumes tested against some of the bacteria isolates tested. Table 1 shows that the methicillin-resistant *S. aureus* (MRSA), methicillin-sensitive *S. aureus* (MSSA) and 2 of the 3 *E. faecalis* isolates were inhibited using a volume of 200  $\mu$ l of the aqueous extract, with an obvious stronger inhibitory effect using the higher volume of extract (300  $\mu$ l). For *A. baumannii*, an antibacterial effect was demonstrated on all isolates but only at a volume of 300 $\mu$ l. The aqueous extract, however, did not show an antibacterial effect with all the volumes tested, against all the *S. pyogenes*, *P. aeruginosa*, *E. coli* and *K. pneumoniae* isolates.

The methanolic extract, on the other hand, demonstrated a clear inhibitory effect against the methicillin-resistant *S. aureus*, methicillin-sensitive *S. aureus*, *S. pyogenes*, *A. baumannii*, and 2 of the 3 *E. faecalis* isolates by all the volumes of extract tested, even when the lowest volume of extract of 100  $\mu$ l was used. It is obvious from Table 1 that this inhibitory effect increased with increasing the volume of extract added, as shown by the obvious increase in the diameters of the zones of inhibition of growth. The same isolate of *E. faecalis* which was not inhibited by the aqueous *E. globulus* extract, was also not affected by its methanolic extract.

Since the tested isolates of *P. aeruginosa*, *E. coli* and *K. pneumoniae* were not inhibited by the volumes used in the agar well diffusion method of the aqueous and methanolic extracts of *E. globulus*, and as the inhibitory effect of the methanolic extracts proved to be more inhibitory to the other tested organisms, It was attempted to check the inhibitory ability of the methanolic extract at higher concentrations than those tested earlier, by adding that extract to the culture medium, using the standard agar dilution method. The results are shown in Tables 2 and 3 for growth of the above organisms on media containing a ratio of methanolic extract to MHA of 0.15 and 0.2 respectively. When a ratio of methanolic extract to MHA of 0.15 was used, both *P. aeruginosa* isolates exhibited moderate growth, the *E. coli* isolates showed slight growth, and while one of the *K. pneumoniae* isolates also showed slight growth, the other isolate was inhibited (Table 2). When the ratio of methanolic extract to MHA was increased to 0.2, all the bacterial isolates tested were inhibited and showed no growth on any of the plates (Table 3).

**Table 1** Antibacterial effect of the aqueous and methanolic *E. globulus* extracts, as tested by the agar diffusion method, reported as diameters of the zones of inhibition of growth in millimeters.

Volume added	Diameter of the zone of Inhibition of growth (mm)								
	100 $\mu$ l			200 $\mu$ l			300 $\mu$ l		
Isolate (s)	Aqueous extract	Methanol extract	C	Aqueous extract	Methanol extract	C	Aqueous extract	Methanol extract	C
<i>Staphylococcus aureus</i> (MR) 1	0	22.5	0	19.5	23	0	20.5	25	0
2	0	20	0	17.5	21	0	20	22	0
3	0	22.5	0	17	24	0	22.5	25	0
<i>Staphylococcus aureus</i> (MS) 1	0	19	0	17	20	0	22	23	0
2	0	20.5	0	17.5	22	0	21.5	24	0
3	0	21.5	0	19	23	0	23	24	0
<i>Streptococcus pyogenes</i>	0	17.5	0	0	20	0	0	22	0
<i>Enterococcus faecalis</i> 1	0	20	0	17	21	0	20	22	0
2	0	0	0	0	0	0	0	0	0
3	0	22	0	20	23	0	21	24.5	0
<i>Acinetobacter baumannii</i> 1	0	21.5	0	0	22.4	0	19	23	0
2	0	19	0	0	22.5	0	17.5	24.5	0
3	0	18.45	0	0	23.5	0	17	24	0
<i>Pseudomonas aeruginosa</i> 1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
<i>Escherichia coli</i> 1 (ESBL)	0	0	0	0	0	0	0	0	0
2 (non-ESBL)	0	0	0	0	0	0	0	0	0
<i>Klebsiella pneumoniae</i> 1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0

0: no zone of inhibition of growth; C: Control

**Table 2** Growth of bacteria on plates with and without the methanolic extract of *E. globulus* with a ratio of extract to MHA of 0.15, using the agar dilution method.

Microorganism	MHA with Methanol Extract	Control without Methanol Extract
<i>Pseudomonas aeruginosa</i> isolate 1	++	+++
<i>Pseudomonas aeruginosa</i> isolate 2	++	+++
<i>Klebsiella pneumoniae</i> isolate 1	-	+++
<i>Klebsiella pneumoniae</i> isolate 2	+	+++
<i>Escherichia coli</i> isolate 1 (ESBL)	+	+++
<i>Escherichia coli</i> isolate 2 (Non ESBL)	+	+++

+++ : confluent growth; ++ : moderate growth; + : slight growth; - : no growth.

**Table 3** Growth of bacteria on plates with and without the methanolic extract of *E. globulus* with a ratio of extract to MHA of 0.2, using the agar dilution method.

Microorganism	MHA with Methanol Extract	Control without Methanol Extract
<i>Pseudomonas aeruginosa</i> isolate 1	-	+++
<i>Pseudomonas aeruginosa</i> isolate 2	-	+++
<i>Klebsiella pneumoniae</i> isolate 1	-	+++
<i>Klebsiella pneumoniae</i> isolate 2	-	+++
<i>Escherichia coli</i> isolate 1 (ESBL)	-	+++
<i>Escherichia coli</i> isolate 2 (Non ESBL)	-	+++

+++ : confluent growth; ++ : moderate growth; + : slight growth; - : no growth

#### 4. Discussion

Traditionally, the leaves of *Eucalyptus* were boiled in water and used to treat respiratory tract infections and the effects of cold [17]. The results of this study support, such practices and even go on to show that *E. globulus* leaves can be used to treat more serious infections if the proper extract was used.

The evaluation of the antibacterial effect of the extracts of the leaves of *E. globulus*, showed that the methanolic extract exhibited a greater inhibitory effect than the aqueous extract. Although in this research, the major compounds making the *Eucalyptus* leaves were not studied for their antibacterial effect, yet it would be a logical to conclude that some of these ingredients may have been deactivated by the boiling temperature utilized in the preparation of the aqueous extract of the leaves explaining its weak antibacterial effect as compared to the methanolic extract. This result can also be attributed to the fact that essential oils of plants are generally hydrophobic and only soluble in alcohol or non-polar solvents [14]. This result is, none the less, consistent with previous studies that also concluded that the alcoholic extracts of plants was more inhibiting to bacterial growth than their aqueous extracts [13, 18].

The finding that Gram negative organisms were less susceptible to the action of essential oils extracted from plants, than Gram positives is perhaps to be expected, since they possess an outer membrane as part of their cell wall [19], which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering [20, 21]. Gram positive bacteria do not have such an outer lipid membrane in their cell wall, which allows entry of hydrophobic extracts, that might have an antibacterial action, more easily. Therefore, the hydrophobic components found in the leaves, such as eucalyptol, which is the most abundant ingredient in *E. globulus* leaves, could describe the varying inhibitory activity observed, as it can injure the Gram positive cell membrane causing leakage of constituents and eventually cell death [21]. These results are in conformity with many previous reports that showed that Gram positive bacteria were more susceptible to the essential oils, found in plant extracts, than Gram negative bacteria [7, 8]. Other studies, however, obtained an opposite result [22].

In this study, methicillin-resistant *S. aureus* was the most sensitive bacterial isolate to the *E. globulus* methanolic extract. Different strains of MRSA are known to be serious pathogens that caused an array of hospital associated and community acquired infections in the past years [23]. This reaffirms the importance of the use of plant sources, such as *E. globulus* leaves, to fight off antibiotic multi-resistant bacteria. *A. baumannii*, another very serious nosocomial and multi-resistant pathogen, was noted to be the only Gram negative bacterium tested that showed sensitivity to the *E. globulus* methanolic extract, at low concentrations, using the well diffusion method.

On the other hand, the Gram negative bacteria, *P. aeruginosa*, *K. pneumoniae* and *E. coli*, were not affected by any of the volumes used of the aqueous and methanolic extracts by the well agar diffusion method. When tested using the agar dilution method, where a higher concentration of the methanolic extract was used, the growth of these organisms was inhibited. The antibacterial effect, thus, increased with an increase in volume of the methanolic extract added to the agar. The finding that *P. aeruginosa* was the most resistant bacterium to the plant extract was consistent with previous results [14]. The results also indicated that a higher volume of the methanolic extract was required to inhibit the growth of *P. aeruginosa*, *K. pneumoniae* and *E. coli* as compared to *A. baumannii* and the Gram positive bacteria tested. It is worth noting, however, that one *E. faecalis* isolate was resistant to all the volumes of aqueous and methanolic extracts used in this study. This and other variations in the sensitivity of different isolates of even the same species are known to be due

to different genetic profiles conferring varying resistance patterns. The genetic plasticity of bacteria allows them to overcome the threat of antibiotics so that only the fittest will survive and adapt to newer environments [24]. Hence, the advent of new antibiotics is constantly confronted by the uncanny ability of bacteria to constantly change and adapt to new situations. It must be emphasized that there is a dire need to uncover and understand the mechanisms of these genetic variations to be able to introduce proper antibacterial compounds that will be able to withstand such a challenge.

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## 5. Conclusion

The *E. globulus* methanolic extract proved to be more efficient than the aqueous extract in inhibiting the clinical bacterial isolates in this study. The Gram positive isolates were more sensitive to the methanolic extract than the Gram negative isolates which required a higher volume of the extract to be inhibited. The results showed that the leaves of *E. globulus* can be used to treat a wide variety of diseases and are not only restricted to respiratory tract infections as traditionally used. Further research is needed to determine the best extraction method for the *E. globulus* active compound(s) responsible for the antibacterial effect in the methanolic extract, as these may be valuable for confronting the multi-resistant bacterial strains.

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## Compliance with ethical standards

### Disclosure of conflict of interest

The authors have not declared any conflict of interests.

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