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The Combination Effect of Essential Oils and Ciprofloxacin on *Staphylococcus aureus* and *Escherichia coli* O157:H7 Isolated from Urinary Tract Infections

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Abstract

Antibiotics misused lead to the emergence of resistant isolates, making most drugs less effective. Therefore, several alternative antimicrobial compounds have been explored, such as essential oils from medicinal plants. Essential oils of two medicinal plants, *Cupressus sempervirens* and *Eucalyptus*, were used to evaluate their antibacterial effects with Ciprofloxacin against *Staphylococcus aureus,* as a Gram-positive bacterium, and *Escherichia coli* O157:H7, as a Gram-negative bacterium, that were obtained from an M.Sc. student in the biology department who isolated them from urinary tract infections. Identification of isolates was confirmed by some biochemical tests and the VITEK-2 Compact system. The toxicity effect of the essential oil of *Cupressus sempervirens* appears to have lower toxicity at 2500 ppm; its hemolysis per cent was 2.6%, while *Eucalyptus* essential oil was 100% at the same concentration. *Cupressus sempervirens* essential oil showed a higher antibacterial effect against *E. coli* with MIC=5000 ppm than *Eucalyptus* essential oil, which gave MIC=20000 ppm. All combinations of essential oils with Ciprofloxacin showed a synergistic effect, killing bacteria in all prepared concentrations. The essential oil of *Cupressus sempervirens* showed slightly lower activity against *S. aureus*, with the MIC of (10000 ppm), while *Eucalyptus* essential oil showed the same effect against *E. coli.* Generally, combinations showed slightly lower effects than those against *E. coli.*

Keywords: *Escherichia coli* O157:H7, *Staphylococcus aureus, Cupressus sempervirens, Eucalyptus,* Essential oils.

التأثير المشترك للزيوت الاساسية والسيبروفلوكساسين على المكورات العنقودية الذهبية والإشريكية القولونية *O157:H7* المعزولة من التهابات المسالك البولية

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الخلاصة

الاستخدام الخاطئ للمضادات أدى الظهور سلالات مقاومة والتي جعلت معظم العلاجات اقل فعالية. ولذلك استعملت العديد من المواد البديلة ذات الفعالية الضد مايكروبية مثل الزيوت الأساسية لبعض النباتات

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الطبية. الزيوت الأساسية لاثنين من النباتات الطبية وهما السرو و اليوكاليبتوس قد استعملت لتقييم فعاليتهم الضد مايكروبية مع مضاد السيبروفلوكساسين ضد البكتريا الموجبة لصبغة غرام (المكورات العنقودية الذهبية) و البكتريا السالبة لصبغة غرام (المشرشبة القولونية) والتي تم الحصول عليها من طلبة الماجستير في قسم علوم الحياة والمعزولة من إصابات القناة البولية. اكد تشخيص العزلات بوساطة بعض الاختبارات البايوكيميائية ونظام الفايتك−2. بالنسبة لبكتريا المشرشبة القولونية أظهرت الزيوت الأساسية لنبات السرو سمية اقل عند تركيز 2500 جزء من المليون نسبة 2.6 % تحلل دموي بينما أظهرت الزيوت الأساسية لنبات اليوكليبتوس نسبة تحلل 100 % عند تركيز 2500 جزء من المليون. كما أظهرت الزيوت الأساسية لنبات السرو فعالية ضد مايكروبية اذ كان التركيز المثبط الأدنى 5000 جزء من المليون مقارنة بالزيوت الأساسية لنبات اليوكاليبتوس و التي كان التركيز المثبط الأدنى 20000 جزء من المليون. كل عمليات الدمج بين الزيوت الأساسية والمضاد السيبروفلوكساسين أظهرت فعلا تأزربا والتي قتلت البكتريا في كل التراكيز المحضرة. اما بالنسبة لبكتريا المكورات العنقودية الذهبيةفقد اظهر الزيت الأساسي لنبات السرو فعلبية اقل قليلا حيث كان التركيز المثبط الأدنى 10000 جزء من المليون. اما الزيت الأساسي لليوكاليبتوس فقد اظهر نفس الفعل المذكورة تجاه البكتريا المشرشبة القولونية وكذلك عمليات الدمج بين الزيت الأساسي والمضاد السيبروفلوكساسين أظهرت تاثير اقل قليلا مما ظهر تجاه البكتريا المشرشبة القولونبة.

1. Introduction

Plant components, such as volatile oils and some oils collectively named essential oils, are active biological compounds against several species of bacteria, including Staphylococci and colon bacteria. The most common Gram-negative bacterium, *Escherichia coli*, has developed different resistance mechanisms against various antimicrobial components, including antibiotics, especially those that inhibit the gyrase and topoisomerase enzymes, such as Ciprofloxacin, and those that inhibit cell wall synthesis, carbapenems, which are often used in emergency cases. The ability of bacteria to acquire resistance genes, leading to the formation of resistance isolates, and transfer them through plasmids and other processes of gene transformation [1]. *Staphylococcus aureus*, especially Methicillin-resistant *Staphylococcus aureus* (MRSA), has become a big problem due to its resistance to antibiotics such as methicillin and other groups of antibiotics, such as β-lactam antibiotics [2]. Urinary tract infections (UTIs) are a common health issue in Iraq and the world, which can be caused by different microbial agents. However, *E. coli* is the main responsible agent for UTIs, followed by other agents such as *Klebsiella pneumoniae, Enterococcus faecalis*, *Proteus mirabilis*, *Staphylococcus saprophyticus*, *Pseudomonas aeruginosa,* and others[3-5].

Staphylococcus aureus causes several diseases, including infections of the dermis, sepsis, and pneumonia; however, treatment can be complicated due to antibiotic resistance. Strains of bacteria that exhibit high resistance to antibiotics may lead to epidemic waves as a result of methicillin resistance [6]. *E. coli* is a member of Enterobacteriaceae that colonizes the colon as normal flora, but some strains, especially *E. coli* O157:H7[7], cause pain in the stomach, diarrhea, vomiting, and nausea in persons with a normal immune system and who are taking immunosuppressant drugs. Misuse and overuse of drugs (antibiotics), including not completing or a full course of antibiotic treatment and inappropriate prescriptions, accelerate and prepare conditions for the emergence of drug-resistant isolates or strains that have resistance to antimicrobial compounds in both pathogenic and commensal strains of bacteria. The distribution of bacterial isolates with high resistance traits leads to an emergence situation for the health of communities, which makes illnesses more complicated, increases drug costs, and death rates. Food pathogenic strains, especially during food manufacturing, also contribute to this issue, posing a threat to public health [8,9].

Plant oils, especially essential oils, have been used for several years as protective agents against the pathogenic effects of bacteria. Essential plant oils possess several characteristics

that render them beneficial against bacteria, including antimicrobial effects and high antioxidant content [10]. Moreover, they are appropriate for creating antimicrobial compounds without side effects [11]. Researchers try different ways to examine their antimicrobial effect or anti-inflammatory properties. Some essential oils have traits that make them safe and effective antioxidant extracts [12]. Several benefits have been obtained from using natural alternative compounds, such as essential oils, in the medical field, which can help cure some cases and alleviate pain [13]. Antibiotics, essential oils, and some other compounds are represented as secondary metabolites and are produced during the stationary phase, and play a role in the defense and competition[14].

Essential oils represent only a tiny amount of the total mass of a plant, but they have crucial roles in the distinctive properties of the plant's aromatic traits. They are composed of many substances that have biological activity. The most important compounds were sesquiterpenes and monoterpenes, while ketones, phenols, and aldehydes represented oxygenated compounds. Esters and ethers can also be found in large quantities. All these compounds enhance the essential oils' odor and taste. They are considered available, safe substances with antimicrobial activity, compounds with fewer side effects. They have shown higher effects and efficacy than bench-used antimicrobial substances[15].

Cupressus is a plant (evergreen) that contains some oils (essential oils) in its aroma. It is most used to treat diabetes, laryngitis, toothache, and inflammation. *Cupressus* has been used extensively to treat flu, colds, and other diseases. Several parts of the *Cupressus* plant body have beneficial effects in the medical field, for example, the leaves for stomach pain and diabetes, dried fruit for laryngitis, and the branches of *Cupressus* used as an antiseptic [16].
Another aromatic plant widely used worldwide is the *Eucalyptus* species, which are distributed in Australia, and have very important roles in paper and wood production [6]. Plantations of *Eucalyptus* occupy approximately 5.7 million hectares of Brazil, the largest country in South America [7], and play a vital role in the country's economic situation [17]. Some substances with therapeutic effects are found in the *Eucalyptus* plant, including essential oils and limonene [8]. *Eucalyptus* products (essential oils) have been noticed to have antibacterial action against *S. aureus* and *E. coli* bacteria, indicating that the *Eucalyptus* essential oil can be prepared as an alternative antibiotic for several diseases caused by these two bacteria [18]. Researchers have noted that 1,8-cineole, which is represented as a primary compound of *Eucalyptus*, may be responsible for therapeutic action [19].

2. Materials and Methods

Test Isolates

Two isolates from urinary tract infection cases were used as test isolates, obtained from the Department of Biology, College of Science, University of Baghdad. The isolates belonged to the Staphylococcaceae family, Gram-positive member, *S. aureus*, and the Enterobacteriaceae family, the Gram-negative member, *E. coli* O157:H7.

The isolates were reidentified by being cultivated on 4-methylumbelliferyl-β-D-glucuronide nutrient agar (MUG), MacConkey Agar, Eosin Methylene Blue (EMB) agar, and selective media for *Staphylococcus* (Mannitol salt agar), and then incubated for 24 hours at 37°C. After that, bacteria were identified using the Gram stain method and microscopic examination. Biochemical tests were conducted to continue the identification process, and catalase, indole, methyl red, and lactose fermentation tests were performed. Moreover, isolate identification was confirmed by the VITEK 2 compact system.

Antibiotic resistance test

The VITEK-2 compact system performed antibiotic resistance tests on isolates to confirm that they were multidrug-resistant. The Antibiotic Susceptibility Card Types used AST—P515 for *Staphylococcus aureus*, while AST—N010 was used for Gram-negative bacteria (*E. coli* O157:H7).

Collection of plant samples

Plant samples included the aerial parts of *Cupressus sempervirens*, purchased from the local market, and leaves of Eucalyptus, collected from the gardens of Baghdad University during March and April. Cleaning of plants was the first step, then washed with water (tap water), dried at 25°C (RT), and kept in appropriate conditions that were ready for later experiments.

Preparation of Essential Oil of Cupressus sempervirens and Eucalyptus

For the extraction of essential oils, 200 grams of dried *Eucalyptus* leaves and the petals of *Cupressus sempervirens* were used, employing the steam distillation method (Cleavinger). The leaves of *Eucalyptus* and the petals of *Cupressus sempervirens* were mixed with D.W (1.2 L) and boiled for three hours; the essential oil was stored at 4°C until use [20,21]. The extraction procedure is replicated twice to obtain the required quantity for the subsequent tests.

Hemolysis assay

Fresh human blood was obtained from a blood bottle, then red blood cells (RBCs) were separated using a heparin tube, and the RBCs were washed three times with normal saline (0.9% NaCl) using a centrifuge (3000 rpm for 15 min). Then the RBC was prepared as a stock solution by using normal saline. 100 µL of the stock solution was mixed with 900 µL of normal saline containing *Eucalyptus* or *Cupressus sempervirens* essential oil in different concentrations (20000, 10000, 5000, 2500, 1250, and 625 ppm), and incubated for three hours at 37 °C. The different mixtures were centrifuged for 16 min at 10000 rpm. The supernatant was isolated from precipitated RBC, and its absorbance (AS) was determined at 540 nm. Distilled water is used as a positive control (AP) while normal saline is used as a negative control (AN). The rate of hemolysis (HR %) was calculated using the following equation [22]:

$$\text{Hemolysis (\%)} = (AS(\text{sample}) - AN(\text{saline}))/(AP(\text{D.W.}) - AN(\text{saline})) \times 100\%$$

AS = sample absorbance

AN = negative control absorbance

AP = positive control absorbance

Antimicrobial effect of essential oils alone and in combination with antibiotics

Stock solution of essential oil

Microdilution assay: One hundred µl of bacterial suspension standardized according to the MacFerland tube (No. 0.5) was inoculated in double-strength nutrient broth with different concentrations of essential oil starting from the concentration 40000 ppm (v/v) by adding forty microliters of the absolute extract of *Eucalyptus* or *Cupressus sempervirens* essential oil to 950 microliters of nutrient broth and 10 µl of Tween 80 as an emulsifier, then diluting to 20000, 10000, 5000, 2500, 1250, and 625 ppm (v/v). Negative control tubes contained broth only, while positive control tubes contained bacteria and broth. All concentrations and control tubes were incubated in the dark at 37 °C for 24 hours.

Then, combinations with antibiotics were prepared at the same different concentrations. Then, the combination of all three substances (*Cupressus sempervirens, Eucalyptus* essential oils, and Ciprofloxacin) was prepared by mixing equivalent concentrations.

Results and Discussions

Confirmation of Escherichia coli and Staphylococcus aureus identification

Confirmatory tests were used for *E. coli* obtained from a Master student in the biology department, and the identification of the isolates was based on the cultural characteristics of three different selective and differential media for *E. coli*. Isolates appeared on MacConkey agar as pink colonies, on EMB agar as green metallic sheen (Figure 1), and on MUG nutrient agar as fluorescence colonies under the UV illuminator system.

The preliminary culturing results for the isolates agreed with the identification result obtained using the VITEK 2 compact system.

Staphylococcus aureus appears as golden colonies on the Mannitol salt agar medium, which differentiates it from other *Staphylococcus* species. Identification of isolates was confirmed by the VITEK 2 compact system (Figure 1).

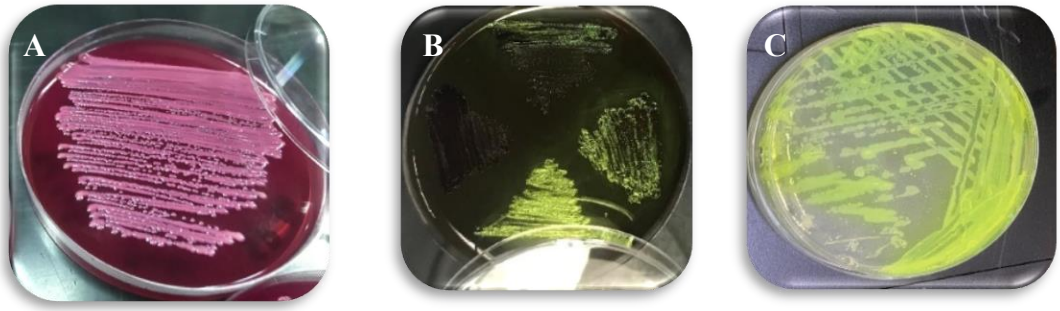

Figure 1: Preliminary identification of test isolates: A: *E. coli* on MacConkey agar. B: *E. coli* on EMB agar. C: *S. aureus* on nutrient agar

Toxicity of essential oil

The different essential oil concentrations exhibited a gradual effect on RBC (Table 1), particularly with *Cupressus sempervirens*, which still had a low impact at 5000 ppm. In comparison, *Eucalyptus* caused complete RBC lysis at a concentration of 2500 ppm (Figure 2). Alnaji *et al*. showed that when people swallow a small amount of *Eucalyptus* essential oil, their hematological parameters increase: white blood cells increase, while RBCs, hemoglobin, and blood platelets decrease [23]. Bushra *et al.* reported that *Eucalyptus* essential oil caused 68.77% RBC lysis at a concentration of 500 mg/ml [24].

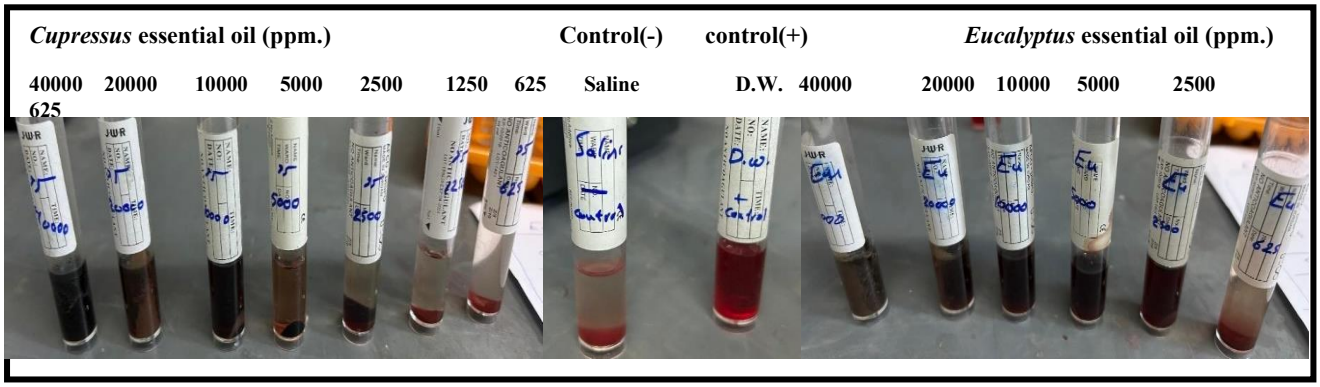

Figure 2: Hemolysis by essential oils of *Eucalyptus* and *Cupressus*.

Day *et al*. found that *Eucalyptus* essential oil was one of the causative agents that correlated with poisoning incidence in the hospitals among children under five years old in the USA. Several cases were noted to involve vaporiser solutions, as well as many products that contain *Eucalyptus* essential oil preparations and other *Eucalyptus*-essential oil-containing medicinal products. About *Cupressus sempervirens* oil, some researchers revealed that it had the lowest toxicity, compared to the other *Cupressus sempervirens* plant parts extract, especially methanolic extracts, which had the highest toxic effect detected at 10000 µg/mL against Caco2 cell lines [25].
Galovičová *et al* mentioned that cell viability testing of *Cupressus sempervirens* essential oil has no effects on the MRC-5 cell line. [26]

Table 1: Hemolysis % of *Eucalyptus* and *Cupressus* essential oils

Concentrations(ppm)	Hemolysis %	
	***Eucalyptus* essential oil**	***Cupressus sempervirens* essential oil.**
40000	**43.95**	**75.4**
20000	**75.9**	**72.5**
10000	**94**	**67.3**
5000	**98.8**	**13.8**
2500	**100**	**2.6**
1250	**36**	**2.4**
625	**4.7**	**2.3**

Antimicrobial effect of Cupressus sempervirens, Eucalyptus essential oil, and Ciprofloxacin combinations against E. coli O157:H7
Eucalyptus essential oil alone has not shown a strong effect, as it kills bacteria only at a high concentration of 20,000 ppm, while lower concentrations have no effect. *Cupressus* essential oil showed a higher effect, killing bacteria at a concentration of 5000 ppm. Ciprofloxacin showed an MIC of 1250 ppm. All combinations (*Cupressus sempervirens* essential oil with Ciprofloxacin, *Eucalyptus* essential oil with Ciprofloxacin and *Cupressus sempervirens, Eucalyptus* essential oil, and Ciprofloxacin) showed the synergetic effect that kills at all concentrations (Table 2).

Dhaouadi *et al.,* mentioned that the effect of *Cupressus sempervirens* essential oil on *E. coli* ranged from 170 to 515 µg/ml, while the effect of *Eucalyptus* essential oil ranged from 53.33 to 512 µg/ml. The researcher also demonstrated an increasing synergistic effect between *Cupressus* essential oil and Colistin against isolates that represent colistin-resistant, such as *E. coli*, with a decrease in concentration ranging from fractional inhibitory concentration index (FICI) 3 to 32-fold lower than the MICs when the compounds were used individually [27].

Cupressus sempervirens essential oil showed a low MIC (50) against *Yersinia enterocolitica* and *Salmonella enterica*. At the same time, intermediate MIC values were observed for *Candida glabrata* and the bacteria that produced biofilm, such as *Salmonella enterica*. High MIC values were observed for *Bacillus subtilis, Pseudomonas aeruginosa*, *Enterococcus faecalis, Staphylococcus aureus*, and *Candida albicans*[26].

Table 2: Effect of *Eucalyptus, Cupressus sempervirens* essential oil, and Ciprofloxacin on *E. coli* O157:H7

ppm(v/v)	*Eucalyptus*	*Eucalyptus and* Ciprofloxacin	Ciprofloxacin	*Cupressus and* Ciprofloxacin	*Cupressus*	*Eucalyptus, Cupressus,* and Ciprofloxacin
20000	-	-	-	-	-	-
10000	+	-	-	-	-	-
5000	+	-	-	-	-	-
2500	+	-	-	-	+	-
1250	+	-	-	-	+	-
625	+	-	+	-	+	-
Control -ve	-	-	-	-	-	-
+ : there is growth - : there is no growth						
Control +ve	+	+	+	+	+	+

Antimicrobial effect of Cupressus sempervirens, Eucalyptus essential oil, and Ciprofloxacin combinations against Staphylococcus aureus

Eucalyptus essential oil alone has not shown a strong effect, as it kills bacteria at a concentration of 20,000 ppm only, while lower concentrations have no effect. *Cupressus sempervirens* essential oil showed a higher effect, killing bacteria at a concentration of 10000 ppm. Ciprofloxacin showed an MIC of 5000 ppm. All combinations (*Cupressus sempervirens* essential oil with Ciprofloxacin, *Eucalyptus* essential oil with Ciprofloxacin, and *Cupressus sempervirens*, *Eucalyptus* essential oil, and Ciprofloxacin) showed the synergetic effect that kills at lower concentrations (Table 3). But it can be noticed that the three compounds together have a slightly lower impact than other combinations. It showed MIC at 2500 ppm. While other combinations showed MIC at 1250 ppm, generally, there are no combinations of compounds that affect *S. aureus* at 625 ppm. In contrast to *E. coli*, which was killed at 625 ppm with the same compounds or combinations

Table 3: Effect of *Eucalyptus, Cupressus sempervirens* essential oil, and Ciprofloxacin on *Staphylococcus aureus*

ppm(v/v)	*Eucalyptus*	*Eucalyptus and* Ciprofloxacin	Ciprofloxacin	*Cupressus* and Ciprofloxacin	*Cupressus*	*Eucalyptus, Eucalyptus,* and Ciprofloxacin
20000	-	-	-	-	-	-
10000	+	-	-	-	-	-
5000	+	-	-	-	+	-
2500	+	-	+	-	+	-
1250	+	-	+	-	+	+
625	+	+	+	+	+	+
Control -ve	-	-	-	-	-	-
Control +ve	+	+	+	+	+	+

Pseudomonas aeruginosa, S. aureus, and *E. faecalis* appeared as resistant to the antimicrobial activity of *Cupressus* essential oil (zone of inhibition of 4.33 ± 1.15 mm, 2.67 ± 0.58 mm, and 1.00 ± 0.00 mm, respectively), the agar microdilution method was more efficient against biofilm-producing bacteria and Gram-positive bacteria compared to the disk diffusion method[26].

Cui *et al.,* mentioned that the MIC (v/v%) of *Eucalyptus* essential oils the same (0.313) against *E. coli* and *Staphylococcus*, while the optimum concentration was 12.50 against *E. coli* and 1.56 against *S. aureus, Cupressus* essential oil showed the same effect with the same concentration against *E. coli*, but it exhibited the effect at 1.250 against *S. aureus*, optimum concentrations were 6.25 against *E. coli* and 25 against *Staphylococcus*. Combinations of essential oils from *Eucalyptus* and *Cupressus*, prepared with different antibiotics, exhibited varying effects; however, most of them increased the inhibition zone [27, 28].

Farsi and Alaidaroos revealed that *Eucalyptus* essential oil had antibacterial activity against different bacteria, such as MRSA, with diameters of inhibition zones of 11.33±0.66 mm, and it showed antibacterial effect against *E. coli* with the diameter inhibition zone of 6.83±0.16 mm. The highest activity was found against MRSA, whilst the least antibacterial activity appeared with *E. coli* [29].

Akermi and coworkers used *Cupressus sempervirens* essential oil to screen for its antibacterial activity against four bacterial strains (*S. aureus, Listeria monocytogenes, Salmonella typhimurium*, and *E. coli*). *Cupressus sempervirens* essential oil showed antibacterial activity against the four tested bacteria, with inhibition zones of 21 mm against *S. aureus* and *Listeria monocytogenes*, while it exhibited *a* 15mm inhibition zone against *Salmonella typhimurium* and *E. coli*. The obtained MIC values indicated that *Cupressus sempervirens* essential oil was most effective against Gram-positive bacteria (MICs = 6.25 _µg/mL) than Gram-negative bacteria [30].

Conclusions

Interesting results were obtained that should prompt us to consider the side effects of natural compounds, such as essential oils, particularly in terms of their safety in use and the sensitivity of RBCs. Essential oils increase activity against pathogenic bacteria, but this should be accompanied by selecting the safe and appropriate concentrations. This approach will decrease the dependence on antibiotics alone and will provide, as an alternative, natural compounds that can be used for therapeutic applications with lower side effects. The current findings pave the way for several combinations between the essential oils themselves and antibiotics.

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Conflict of interest

None

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