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RESEARCH ARTICLE

Anti-biofilm Activity of Purified Compounds E1 and E2 Isolated from *Streptomyces griseus* Against Pathogenic Bacteria

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ABSTRACT

Streptomyces griseus is capable of producing novel bioactive compounds, as well as investigating their biological effect on biofilm pre-production by aerobic bacterial isolates from skin wounds and burns when examined. The cultural characteristics the morphology of arial mycelium colors, Pale yellow on ISP2 media at 28°C for 7–14 days and the biochemical tests. The purified bioactive compounds from *S. griseus* (E1 and E2) gave their effects to overcame the biofilm formation by Gram-positive and Gram-negative bacteria when treated with concentration 500 µg\mL of E2 the formation of biofilm reduced than E1. The noticeable effect of purified active substance (E1 and E2) can reduction the biofilm formation by Gram-negative bacteria more than Gram-positive.

Keywords: Bioactive compound, biofilm formation, *Streptomyces griseus*

INTRODUCTION

The genus *Streptomyces* belongs to order Actinomycetes. These bacteria are filamentous, aerobic, and Gram-positive and spread mainly in soil and considered as a good source for more than half of all antibiotic, also, it is known to produce many other products like extracellular enzymes and inhibitors.[1-10] *Streptomyces griseus* is a specie of bacteria which belongs to the genus *Streptomyces* commonly found in soil. Very few strains have also been reportedly sourced from deep-sea sediments. It is a Gram-positive bacterium with high GC content. Along with most other members of the group.[1]

S. griseus strains are recognized producers of antibiotics and other such commercially significant secondary metabolites. These strains are known to produce of more than 70% of the known natural antibiotics produced.[11] Streptomycin which is the first antibiotic ever reported from a bacterium comes from strains of *S. griseus*. Long before now, it is having been stated that the whole genome sequence of one of its strains had been completed.[11]

Fredericamycin is a novel antibiotic produced by a soil isolate of *S. griseus* with strain named as (FCRC-48). *In vitro*, fredericamycin exhibits antibacterial, antifungal, and cytotoxic activities. *In vivo*, fredericamycin exhibits very good antitumor activity.[12,13] The formation of microbial biofilms is an important reason for failure of antimicrobial therapy. Biofilm-associated infections represent one of the major threats of modern medicine.[14] The present study was aimed to inhibition of biofilm pre-production that form by aerobic bacterial isolated from skin wounds and burns after treated with purified active substance (E1 and E2).

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MATERIALS AND METHODS

Samples Collection

One hundred samples were collected, samples were exposed to the air for a week, also they pretreated with $CaCO_3$ with a ratio of (10:1 soil: $CaCO_3$) and kept at ambient temperature for a week.[15]

Isolation of *S. griseus*

1 g of dried and treated soil samples were suspended in distilled water to get a concentration 1%W/V. The samples were shacking in a shaker incubator at 120 rpm for 30 min at room temperature. Put on supplemented malt extract yeast extract agar (ISP2), using dextrose as source of Carbone, with addition of Nystatin and Streptomycin 50 μg/ml each, then the inoculum was spread by a sterile swab to make a uniform distribution of the suspension on the surface of the media. The inoculated plates were incubated at 28°C for 7–14 days.[16]

Identification of *S. griseus*

Suspected bacterial isolates of *S. griseus* were primarily identified by microscopic and cultural examinations, then by the biochemical tests for Czapeck medium, Citrate Utilization, Cellulase, Urease, Catalase, and Melanin, final identification.

Specimens Collection from Skin Infections, Wounds and Burns

Specimens in Table 1 were collected from 200 patients of different ages and genders suffering from skin infections and from patients suffering from acute and chronic wounds with purulent discharge or painful spreading erythema around wounds and those severing burns with red blistering skin that looks like a burn or scald. Specimens were taken from different skin infections using sterile disposable cotton swabs and kept into test tubes containing non-nutritional medium which maintains the viability of organisms without significant multiplication (Stuart transport medium).

Table 1: Local hospitals and laboratory where specimen's collection

Number	Area of isolation	Site of isolation	
		Wound	Burn
1	Al Yarmouk hospital	50	40
2	Medicinal city	25	40
3	Al Fallujah hospital	15	15
4	Private laboratory in Baghdad	10	5

Identification of Aerobic Bacterial Isolates by VITEK 2 System

The VITEK 2 which is installed at the Al Yarmouk hospital/Ministry of Health is an automated microbiological system utilizing growth-based technology. The reagent cards have 64 wells that each can contain an individual test substrate. Substrates measure various metabolic activities such as acidification, alkalization, enzyme hydrolysis, and growth in the presence of inhibitory substances. An optically clear film present on both sides of the card allows for the appropriate level of oxygen transmission while maintaining a sealed vessel that prevents contact with the organism-substrate admixtures. Each card has a pre-inserted transfer tube used for inoculation (described below). Cards have bar codes that contain information on product type, lot number, expiration date, and a unique identifier that can be linked to the sample either before or after loading the card onto the system. There are currently four reagent cards available for the identification of different organism classes as indicated in Table 2 and as follows:

- GN: Gram-negative fermenting and non-fermenting bacilli.
- GP: Gram-positive cocci and non-spore-forming bacilli.
- YST: yeasts and yeast-like organisms.
- BCL: Gram-positive spore-forming bacilli.

Table 2: Suspension turbidities 0.5 used in the VITEK 2 reagent cards inoculation

Product
G+ve
G−ve
Yeast
G+ve spore forming *Bacilli*

A sterile swab sample used to transfer a sufficient number of colonies of a pure culture and to suspend them into 3 ml of normal saline (NaCl = 0.85–0.9%, pH = 5–7) in a 12 × 75 mm clear plastic (polystyrene) test tube. The turbidity is adjusted and measured.[17]

Fermentation Condition

Secondary screening for the production of antimicrobial metabolites was done by the inoculation of 1.5 mL of prepared stock suspension cultures into 150 mL of ISP_2 medium using glucose as carbon source with $CaCO_3$ and incubated at 28 ± 1°C, 150 rpm for 7 days in a shaking incubator.[18,19]

Extraction of the Bioactive Compound from Isolated *S. griseus*

The extraction method to obtain the bioactive compounds from the isolated *S. griseus* was employed according to Pandey *et al.*, 1981.[20] method with some modification, Briefly the whole broth from different fermentation media run with about 500 mL ISP2 medium was used to get *S. griseus* extra and intracellular production. The pH of the broth was adjusted to 2.0 with dilute sulfuric acid and left at 4°C for 96 h. The lysate was filtered through Whatman filter paper and the filtrate was transferred to reparatory fennel for extract the secondary metabolites yields with ethyl acetate. The process was repeated many times. All acetate layers were collected and washed with distilled water to get rid of any access acid residues. The aqueous phase was discarded and the combined ethyl acetate layers were evaporated to dryness using rotary evaporator apparatus at 45°C. The residue that yielded was as a dark red powder.

Purification of Bioactive Compounds

Purification technique for the yielded bioactive compounds by preparative thin layer chromatography (TLC) was involved. The best solvent system gave separated clear spot was the solvent ethyl acetate: methanol: water (10:1.5:1). Application 2 ml of crude extract in concentration of 100 mg\1 ml methanol on the reparative TLC glass plate was done then scrapping for each separated band to be subjected to further analysis. Two major compounds were obtained named as (E1 and E2) which represented the purified components get from the isolated local strain.

Detection of Antimicrobial Efficacy of Purified Substances against Biofilm Production by Micro Titer Plate Assay

The potential of the purified substances to prevent initial cell attachment (inhibition of biofilm formation) was investigated through the biofilm inhibition assay. Briefly, a 100 µL pathogenic bacterial isolates was added into individual flat-bottomed 96-well microtiter plates and incubated at 37°C for 4 h without shaking. Then, the plates were removed from the incubator and (1 mg/mL). Purified substances were added in eight replicates into the wells of 96-well microtiter plates to give a final concentration of 7.81 mg/mL and then incubated further at 37°C for 24 h without agitation. DMSO served as positive control while Muller Hinton media as negative controls. The biomass was quantified using the modified crystal violet staining method.[21] The assay was done briefly, the 96-well microtiter plates were washed 5 times with sterile distilled water, air dried and then dried for 45 min. The wells were then stained with 100 µL of 1% crystal violet and incubated at room temperature for 15 min after which the plates were washed thrice with sterile distilled water to remove unabsorbed stain. At this point, biofilms were observed as purple rings at the side of the wells. The semi-quantitative assessment of biofilm formation was done by adding 125 µL of ethanol to de-stain the wells. A 100 µL aliquot of the de-staining solution was transferred to a new sterile plate and the absorbance was measured at 590 nm using a microplate reader. The mean absorbance of the samples was determined, and percentage inhibition of biofilm was determined using the equation below:

Percentage (%) inhibition = OD Negative control – OD Experimental × 100

Although it was not done in the procedure we followed, it would have been interesting to determine if the cells in the biofilm were still viable using the crystal violet assay.

RESULTS AND DISCUSSION

Samples Collection

From 100 samples, only 85 samples gave bacterial growth, these isolates were further purified and subjected to a full identification according to their morphological and cultural characteristics and biochemical tests. From 85 soil sources, have 45 (52.9%) isolates are a species of *Streptomyces*. Table 3 showed that from the local *Streptomyces* spp., only 15 isolates (17.6%) were suspected to contain *S. griseus*.

Isolation of *Streptomyces*

From 100 soil sources, have 45 isolates were suspected to contain *Streptomyces* species, isolated on the basis of forming colonies with inhibitory or clear zone around them and developed to an aerial mycelium that appeared as granular, powdery and soft, and grey with cream color as described by Stackebrandt *et al.*[22] and Ramazani *et al.*[23] Furthermore, it possesses earthy odors.[24] Figure 1 Shows that the suspected colonies of *Streptomyces* were selected which are characterized as small, white, pin-point, rough, chalky, and a clear zone of inhibition around them, to obtain a pure growth of *Streptomyces* spp.

Isolation of *S. griseus*

As shown in Figures 2 and 3, only 15 soil sources which represented 15% of total collected samples were suspected to be the *Streptomyces griseus* with a different morph. Types and the color of colonies characterized by color ranged from pale yellow to grey yellow, grown on a malt extract yeast extract agar (ISP2), as recommended by[16,25] and this was in agreement with that described by Saadoun *et al.*[24]

Table 3: *Streptomyces griseus* isolated from soil of local regions at Baghdad city

Number	Locally region	Number of samples collected	Number of isolates give growth	Percentage of growing isolate	Suspected *Streptomyces* sp.	Suspected *Streptomyces griseus*
1	HA	15	10	11.7	7	4
2	JA	25	23	27.2	10	2
3	QA	30	27	31.7	13	2
4	KA	30	25	29.4	15	7
Total		100	85	100	45 (52.9%)	15 (17.6%)

HA: Harthyia, JA: Jadreia, QA: ALQadesia, KA: Karada

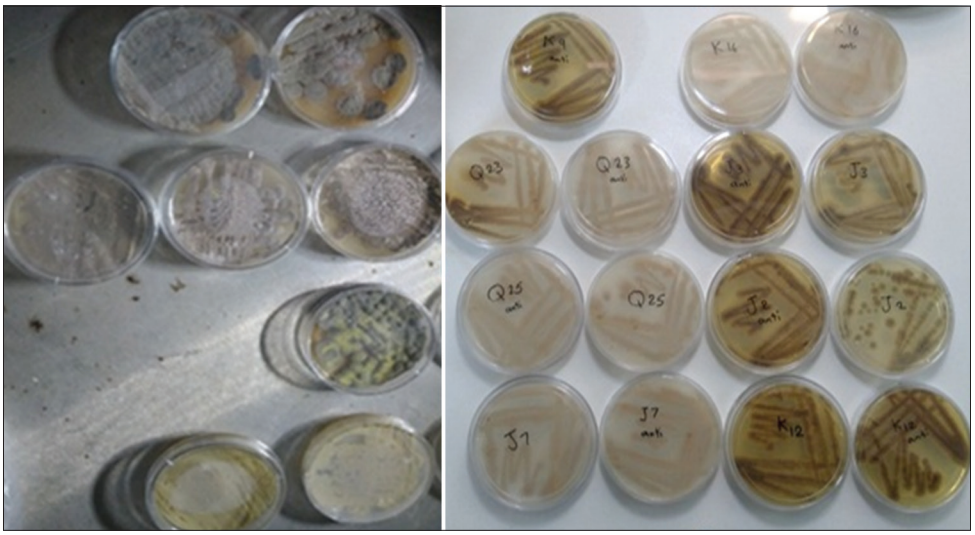


Figure 1: Local *Streptomyces* spp. Isolate grow on ISP2 agar at 28°C after 7–14 days

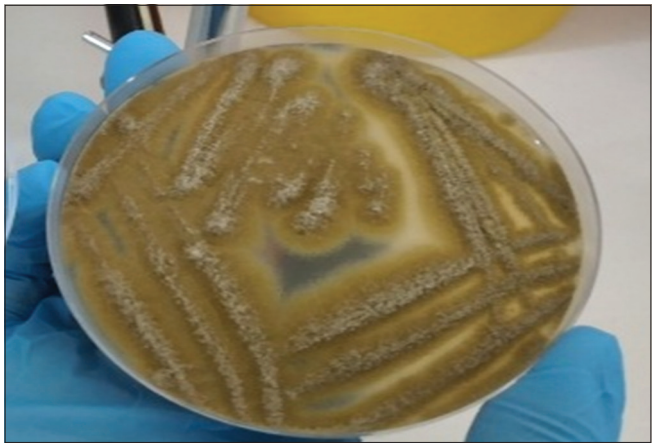


Figure 2: Local isolate *Streptomyces griseus* distinguished by color on ISP2 at 28°C after 7–14 days

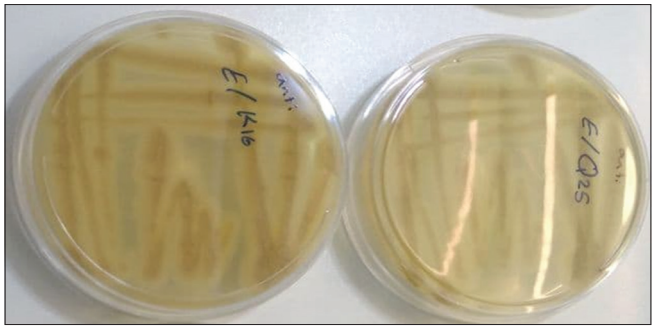


Figure 3: Mycelium surface of four isolate of *Streptomyces griseus* distinguished by color on ISP2 at 28°C after 7–14 days

Identification of *S. griseus*

The following identification results were obtained for identifying *S.griseus* isolate

Cultural Characteristics

S. griseus isolates produced diffusible pigment in the surrounding media in accordance with the aerial mycelium color. Soluble pigment was also observed in some isolates. Table 4 showed distinctive color established, in the Bergey's manual of systemic bacteriology.[26]

Figure 4 showed that the local isolates after gram staining method revealed that local *Streptomyces griseus* was a Gram-positive stained and rod in shape, possessing a branched mycelium in their cell morphology.[27,28]

The Biochemical Results of *S. griseus*

Table 5 showed the resulting biochemical tests of the isolates from Iraqi's soil.

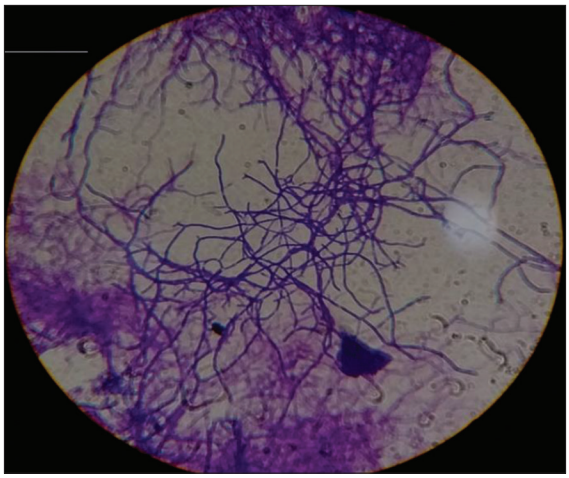


Figure 4: *Streptomyces griseus* hyphae, grown on ISP2 agar. Branching filaments, abundant aerial mycelia, and long chains of small spores are visible, all of which are characteristic of *S. griseus* ×100

Table 4: Cultural characteristics of *Streptomyces griseus* isolates from soils grown in ISP2 media at 28°C for 7–14 days

Colony morphology	Arial mycelium	Mycelium surface	Soluble pigment	Spore surface
Irregular edge	Pale yellow	Yellowish	Yellow	Hairy

Table 5: Biochemical tests results of *Streptomyces griseus* isolates

Tests	Reaction	Results
Czapeck medium	Growth	Positive
Citrate utilization	Deep blue color	Positive
Cellulase	Clear zone	Positive
Urease	No change	Negative
Catalase	No bubbles	Negative
Melanin	Black to brown	Negative

Extraction of the Bioactive Antimicrobial Metabolites from *S. griseus* Isolate

Each 500 mL broth yielded 1.8 g crude complex as a dark red powder which represented the bioactive compounds produced by *S. griseus* isolate extra and intracellularly. The extraction method for obtain the bioactive compounds preform on *S. griseus* isolate could be intracellular compounds that might be degraded the cell well of the bacteria to achieve high amount the bioactive compounds present inside it.[29]

It was suggested that the secondary metabolite production began at 4th day after seed build-up and also at the production level. The pH rose to a maximum of 7.2 and gradually decreased to

pH = 6.2 as the secondary metabolite production peaked. At 36 h, the dissolved oxygen dropped to zero and the rate of glucose consumption became 0.1%/h. As in shake flasks, production closely followed glucose utilization. The rate of a production increased.[18] Figure 5 showed the band of resulting compound using the solvent composed of ethyl acetate: methanol:water(10:1.5:1).

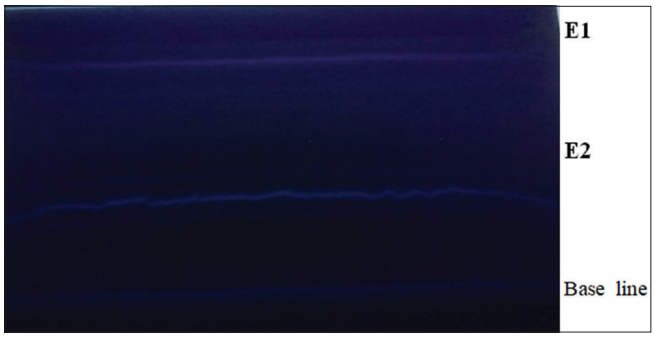
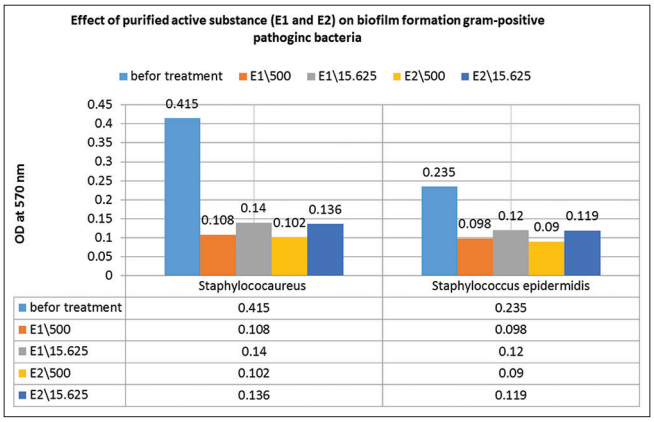


Figure 5: Preparative thin layer chromatography from *Streptomyces griseus* crude extract show two layers represented as two compounds obtained

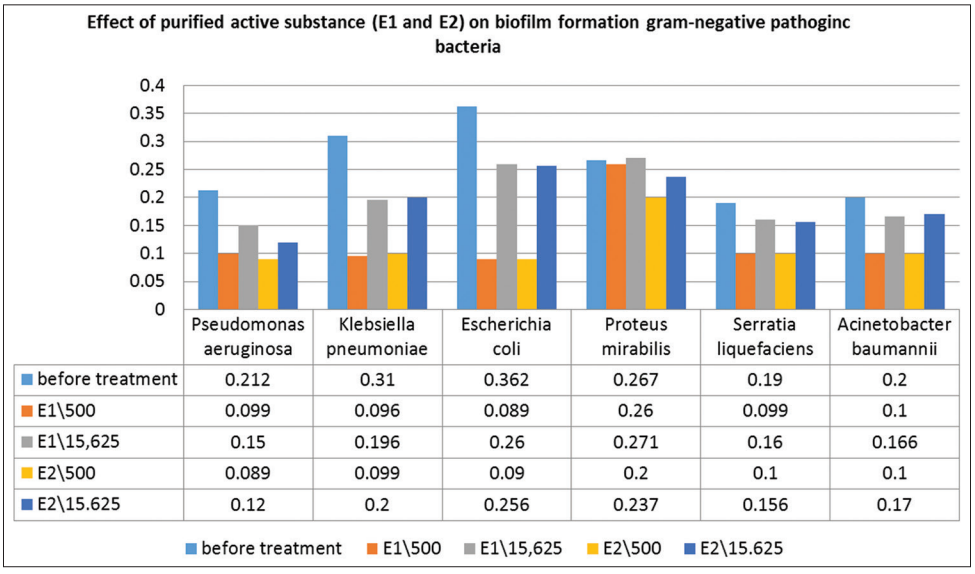


	Staphylococaureus	Staphylococcus epidermidis
befor treatment	0.415	0.235
E1\500	0.108	0.098
E1\15.625	0.14	0.12
E2\500	0.102	0.09
E2\15.625	0.136	0.119

Figure 6: Chart show the detection of antimicrobial efficacy of purified active substance (E1 and E2) in microtiter plate against biofilm formation by Gram-positive bacteriaisolates isolated from different skin infections before and after the treatment

Detection of the Purified Compounds (E1 and E2) Anti-biofilm Efficiency against Pathogenic Bacteria Isolated from Skin Infections by Microtiter Plate Assay

Figure 6 showed the biofilm formation in microtiter plate by the pathogenic bacteria isolated from different skin infections. The purified bioactive compounds from *S. griseus* (E1 and E2) gave their effects to overcame the biofilm formation by Gram-positive bacteria are shown in Figure 7. *Staphylococcus epidermidis* biofilm formation inhibition. The O.D. of negative control was (0.070) represented the lack of biofilm production (medium only), where the positive value indicated biofilm production. For *Staphylococcus aureus* O.D. was 0.415, when treated with concentration of 500µg\ml E1, the formation O.D. got down to (0.108), and (0.140) when the pathogen treated with 15.625 µg/mL of E1. Less biofilm inhibition effect was shown with E2 compound toward the same pathogen and at both concentrations (500 and 15.625 µg/mL) as shown in Figure 7. Similar effects were obtained against.



	Pseudomonas aeruginosa	Klebsiella pneumoniae	Escherichia coli	Proteus mirabilis	Serratia liquefaciens	Acinetobacter baumannii
before treatment	0.212	0.31	0.362	0.267	0.19	0.2
E1\500	0.099	0.096	0.089	0.26	0.099	0.1
E1\15,625	0.15	0.196	0.26	0.271	0.16	0.166
E2\500	0.089	0.099	0.09	0.2	0.1	0.1
E2\15.625	0.12	0.2	0.256	0.237	0.156	0.17

Figure 7: Chart shows the detection of antimicrobial efficacy of purified active substance (E1 and E2) in microtiter plate against biofilm formation by Gram-negative bacteria isolates isolated from different skin infections before and after the treatment

On the other hand, the Gram-negative bacteria treated with the purified bioactive compounds from *S. griseus* (E1 and E2) shown in Figure 7. Biofilms formation play an important role in the pathogenicity, persistence, and eventual treatment of disease. Biofilm production by bacteria and other microorganisms was genetically controlled property, which represent one of the most important virulence factors in productive organism.[30] The results showed in Figure 7 the high biofilm formation by Gram-negative bacteria with OD (0.362) was by *Escherichia coli*. When treated with concentration 500µg/mL of E1 the formation of biofilm reduced to give OD (0.089), and when treated with concentration 15.625 µg/mL of E1 show the less of reduction in the formation of biofilm to give OD (0.260). *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Serratia liquefaciens,* and *Acinetobacter baumannii* show that results when treated with E1 and E2. The noticeable effect was gotten when treated with E1 and E2.

CONCLUSION

Streptomyces griseus is capable of producing novel bioactive compounds. The purified bioactive compounds from *S. griseus* (E1 and E2) gave their effects to overcame the biofilm formation by Gram-positive and Gram-negative bacteria when treated with concentration 500 µg/mL of E2 the formation of biofilm reduced than E1.

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