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Screening for bioactive secondary metabolites in Actinomycetes isolated from soils around old buckthorn and palm trees

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ABSTRACT

Antibiotic resistance is one of the biggest public health threats increased globally in this century. This study is aimed to determine the compounds with antimicrobial activity exhibited in *Actinobacteria* as a source for new antibiotic to treat highly-resistant bacteria. The *Actinobacteria* isolated from the soil around buckthorn and palm old trees using IPS4 agar media for first screening. ISP2 agar and broth media were used for *Actinobacteria* identification and fermentation and secondary metabolites production respectively. Secondary metabolites screening was done using extracellular crude extract against extended spectrum beta-lactamases (ESBLs) producing *Escherichia coli, Klebsiella pneumonia* and *Pseudomonas aeruginosa* and beta lactam resistant *Staphylococcus aureus* by well diffusion method. The total number of *Actinobacterial* isolates was 10, most of them showed good antimicrobial activity appeared by a particularly good zone of inhibition around resistant bacteria. This finding gives a promise that *Streptomyces* in soil around buckthorn and palm old trees considered as a source for new antibiotics.

Keywords: Streptomyces, *ESBLs*, Multidrug resistance, Bioactive compounds.
Article type: Research Article.

INTRODUCTION

Streptomyces is a Gram-positive bacterium belongs to Actinomycete family. It is considered the important bacteria within micro-flora community in soil constituting 10 to 50% (Oluwaseyi & Olubukola 2019; Pallavi *et al*. 2013). Species of the genus *Streptomyces* are particularly that grow near plant and colonize plant roots associated with high production of secondary metabolites in the form of antibiotics, enzymes with antibacterial or antifungal activity, antitumor and biodegradation compounds during their screening as biocontrol agent (Jog *et al*. 2016). Actinomycetes produces two thirds of natural form of antibiotics, 75% out of them from *Streptomyces* containing about 7600 bioactive compounds (Franco-Correa *et al*. 2010). The *Streptomyces* is the greatest genus of bacteria used in pharmaceutical industries for drug production and discovery of new bioactive compounds (Kumari *et al*. 2017). Recently, the bacterial resistance become alarming health problem worldwide (Dehbandi *et al.* 2019; Tonekabony *et al.* 2021; Sharafkhah *et al.* 2022), so it is very necessary to discover new antibiotics to treat threatening infections by such pathogens especially in immune-deficient patients to prevent the evolution of new diseases and to discover new antimicrobial products from *Streptomyces* instead of the currently-used ineffective and toxic antimicrobial compounds (Al-Rubaye 2016; Al-Rubaye *et al*. 2018).

MATERIALS AND METHODS

Collection of samples

The total number of soil samples was 23 which collected from different areas around old buckthorn and palm trees after removing approximately 3 cm of the soil surface and taken up from a depth of 15 cm. By using sterile

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plastic bags, the samples transported to the laboratory and kept at refrigerator (at 4-6 °C) prior to analysis. Samples should be treated by drying at 70 ºC for 1-1.5 h in oven to remove other unwanted microorganisms (Korn-Wendisch 1992; Al-Rubaye *et al*. 2018; AL-Samarraie *et al.* 2019).

Isolation and identification *Streptomyces* spp.

First step was carried out by adding one gram of dried soil sample to 99 mL sterile distilled water to make a stock suspension. Stock solution shacked perfectly at 120 rpm for 30 minutes then serial dilutions were made (10^{-1} to 10^{-3}) and left for 10 minutes at room temperature.

From each dilution, 0.1 mL was pipetted and spread by sterile swab on ISP4 agar media supplemented with Tetracycline (50 mg L^{-1}) and Nystatin (50 mg L^{-1}) for first screening. ISP2 agar media were used for identification and characterization by sub-culturing from first screening. The plates were examined for growth after incubation at 28 °C for 7 to 14 days. ISP2 broth media were used for flask fermentation and the crude was extracted after 3-4 days of incubation at 28 °C (Al-Rubaye *et al*. 2020; AL-Samarraie *et al.* 2014).

Microorganisms used to test antimicrobial activity

ESBLs producer Gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumonia*) and Gram positive Beta lactam resistant and sensitive isolates (*Staphylococcus aureus*) were included in this study to determine the antimicrobial activity of *Streptomyces* isolates against them. These microorganisms were obtained from Biotechnology Department, University of Baghdad, Iraq. Double check for these isolates was carried out by activation in Nutrient Broth and incubated at 37 °C for 24 h, followed by sub-culture in selective media (MacConkey, Mannitol salt agar and Cetramide agar), Gram staining, microscopic examination and biochemical test analyses (Imvic, Oxidase, Catalase and Coagulase). Double disc synergy method (Livermore 2002; Abdullah *et al.* 2015) was done using Cefotaxime; Ceftazidime and Ceftriaxone disk around Amoxicillin-clavulanate disk in center (Bioanalyse, Turkey) to detect ESBLs producers *K. pneumoniae, E. coli* and *P. aeruginosa.* The same antibiotics used to check *S. aureus* susceptibility.

Three to five colonies were selected and transferred into normal saline (3 mL) in a test tube and the density adjusted to 0.5 McFarland standards. About 0.1 mL of each adjusted bacterial growth was distributed across the surface of Mueller-Hinton agar using a sterile spreader and the antibiotic discs were applied to the surface of the inoculated agar. Finally, the plates were incubated overnight at 37 °C for 18-24 h. The resistance and sensitivity of each antibiotic was performed after measuring the diameter of zone of inhibition and compared to the Standard chart of National Committee for Clinical Laboratory Standard institute (NCCLS 2010).

Preparation of *Streptomyces* isolates for bioactive compound production

Extracellular crude Extraction from *Streptomyces* isolates

The extracellular crude was extracted from 10 *Streptomyces* isolates (flask fermentation) by taking 5 mL of the fermented broth from each isolate and transferred into a test tube, spun at 6000 rpm for 5 minutes and the supernatants containing the extracellular crude were filtered through Whatman No.1 **(**Khan & Patel 2011; Pallavi *et al*. 2013).

Screening bioactive compounds by agar well diffusion method

The supernatants filtrate of 10 *Streptomyces* isolates which containing the extracellular crude was checked for their bioactivity by agar well diffusion method. The tested microbial pathogens were spread on the surface of Mueller Hinton agar plate. Wells were formed on the seeded plates a sterilized pasture pipette, then 100 µL of extracellular crude extracts were loaded in each well and plates incubated at 37 °C for 24 h. The zones of inhibition were observed by naked eyes after incubation and determined using a metric ruler (Khan & Patel 2011; Pallavi *et al*. 2013).

RESULTS AND DISCUSSION

Biochemical Tests and culture results for tested pathogenic bacteria

The results of biochemical tests are depicted in Table 1. It is represented that IMVIC test differentiates members of Enterobacteriaceae; *E. coli* showed positive indole and methyl-red and negative for Voges-Proskauer and citrate utilization results, while *K. pneumonia* showed the opposite result, both of them are lactose fermenter on

MacConkey agar. The crucial result for *P. aeruginosa* was Oxidase positive and catalase tests which grow on Cetrimide agar with pyocyanin pigment production. *S. aureus* was characterized by their ability to grow on NaCl-containing media (mannitol salt agar) with yellow pigment colonies, also they are positive for Coagulase and Catalase and negative for Oxidase tests (Buchanan & Gibbns 1974).

Table 1. Biochemical Tests results for tested pathogenic bacteria.

Test	*E. coli*	*K. pneumonia*	*P. aeruginosa*	*S. aureus*
Indole	+	-	-	
Methyl red	+	-	-	
Voges-Proskauer	-	+	-	
Citrate	-	+	+	
Oxidase	-	-	+	-
Catalase	+	+	+	+
Coagulase				+

Isolation and characterization of *Streptomyces* spp.

Fig. 1 shows the first step for Actinomycetes isolation and characterization. As represented, many colonies (Actinomycetes, yeast, bacteria and fungus) differed in size, consistency and colour after culturing the stock solution. The Actinomycetes-suspected colonies were selected according to their colour which varies from grey to creamy or white, diameters ranged from 1 to 10 mm with smooth surface then changed to become powdery, soft and granular (arrows). Al-rubaye *et al*. (2018) and Arifuzzaman *et al*. (2010) isolated the *Streptomyces* spp. from river sediments and soil samples respectively and showed the same colony morphology during the first screening. Single suspected colonies from the first screening sub-cultured in ISP2 media showed *Streptomyces* spp. characteristic colonies according to the morphology of colonies (the aerial and substrate mycelium), presence of soluble pigment (Fig. 2), and microscopic features like spore chain arrangement in accordance with the series established in the Bergey's manual of determinative bacteriology by Buchanan and Gibbons (Buchanan & Gibbns 1974). The number of characterized and identified *Streptomyces* spp. among 23 soil samples was 10 isolates which processed for antimicrobial activity screening (Fig. 2).

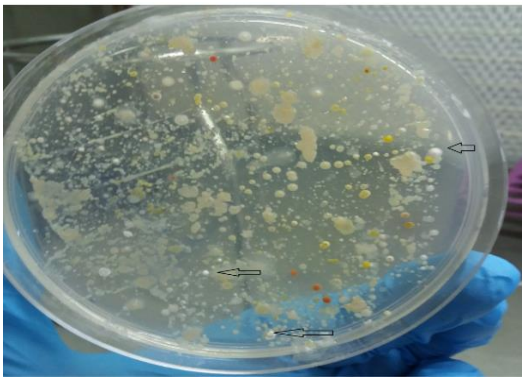


Fig. 1. First screening of Actinomycetes from soil samples in casein salt starch agar (dilution 10^{-2}) at 28 °C for 10-14 days cultured in ISP4 agar media. Arrows: white and powdery single colony of Actinomycetes.

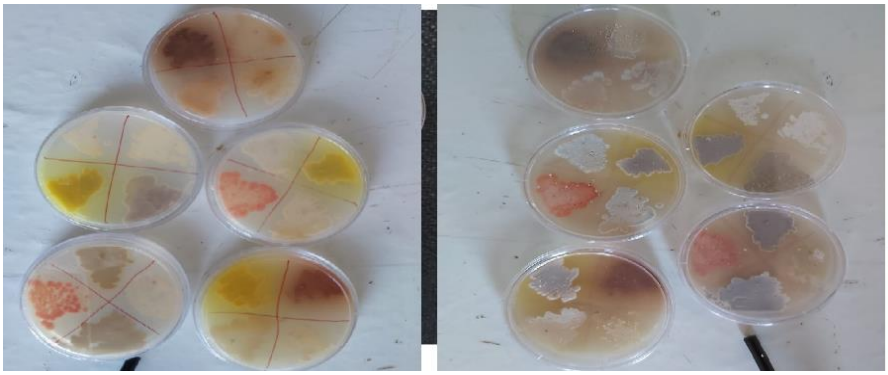


Fig. 2. Different species of the *Streptomyces* isolates with and without pigment using ISP2 agar media.

Screening S*treptomyces* isolates for bioactive compounds production

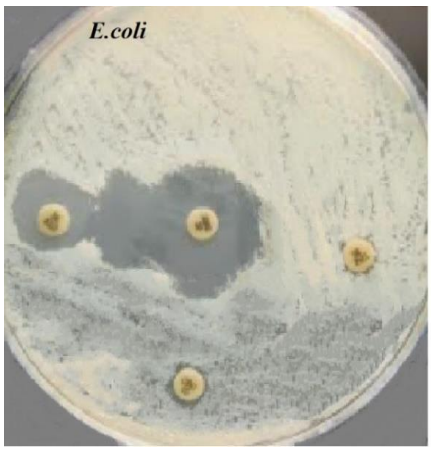
Flask's fermentation

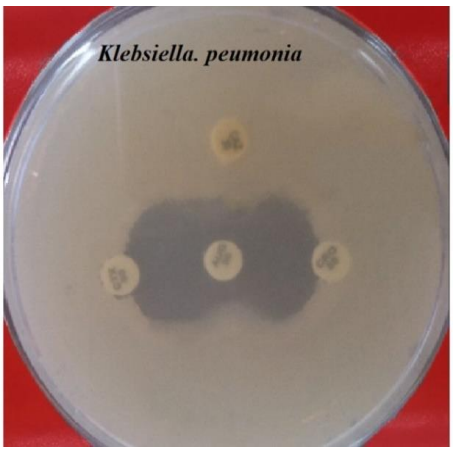
Fig. 3 shows flask's fermentation of different *Streptomyces* spp. cultured in ISP2 broth with different colours. The supernatant filtrate from these isolates were exposed to extracellular crude extraction and screened for bioactive compounds against the ESBLs producing *E. coli*, *Klebsiella* and *Pseudomonas* and beta lactam resistant *S. aureus* screened by plate well method (Fig. 5) which exhibited a good zone of inhibition of the supernatant against ESBLs producing *E. coli* and beta lactam resistant *S. aureus.*

Fig. 3. Shake flask fermentation for most active *Streptomyces* isolates, was carried out in flasks containing 80 mL of production medium (ISP2) broth, shaken at 170 rpm for 3-4 days.

Drug sensitivity testing

The ESBLs producer isolates which identified by DDST, showed an inhibition zone between the Clavulanic acid central disc and any of the Cephalosporin discs, forming a ghost like appearance inhibition zone due to blocking the beta lactamase activities around the central disk (zone of inhibition larger than that toward cephalosporins (Figs. 4-5). Tested bacteria (*S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *E. coli*) received as a nice gift from Biotechnology Department. Negative results represented when resistance to Cephalosporins is not due to ESBL production as observed by Teklu *et al.* (2019). They found high percentage of ESBLs and MDR isolates and most blood and urine isolates was ESBLs. The highest ESBLs production was observed among *K. pneumoniae,* so the continuous monitoring systems and effective infection control measures are essential. Regarding *S. aureus* which resist to β-lactam-β-lactamase inhibitor combinations and cephalosporins, including cefoxitin, cefotetan, ceftriaxone and cefotaxime may be related to AmpC β-lactamases production like CMY-2, DHA-1, ACT-1 and P99 which encoded by *bla* genes on the bacterial chromosome. The causes for high resistance toward clavulanate and sulbactam may be due the presence of AmpC β-lactamase production among *Enterobacteriaceae* members like *Citrobacter* spp. and *Enterobacter* spp. (Buchanan & Gibbns 1974). Another to overproduction of TEM-1, TEM-2 or SHV-1 β-lactamases as reported by Pérez-Moreno *et al.* (2004).





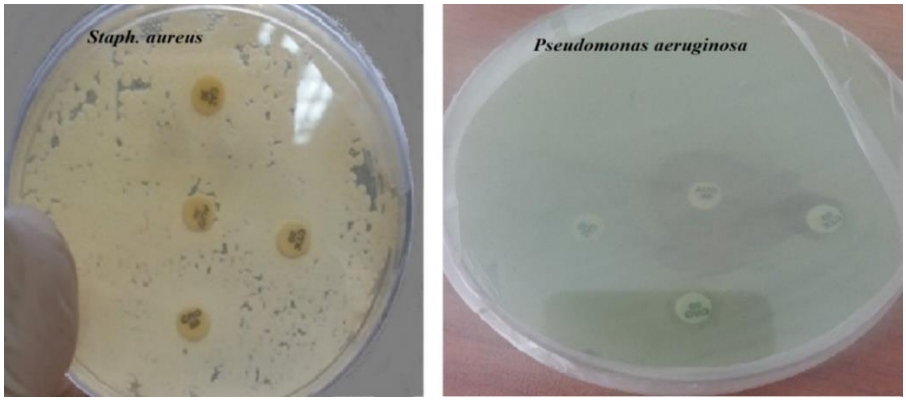


Fig. 4. Detection of ESBLs producing *K. pneumoniae*, *E. coli* and *Pseudomonas aeruginosa* by Double disc synergy method: Cefotaxime disk (CTX); Ceftazidime disk (CAZ) and Ceftriaxone (CRO) disk around Amoxicillin-clavulanate (AMC) disk in centre. Note the synergy between the AMC and CRO in the left and the AMC and CTX in the right. Also, *S. aureus* showed a beta-lactam resistance by using the same antibiotics.

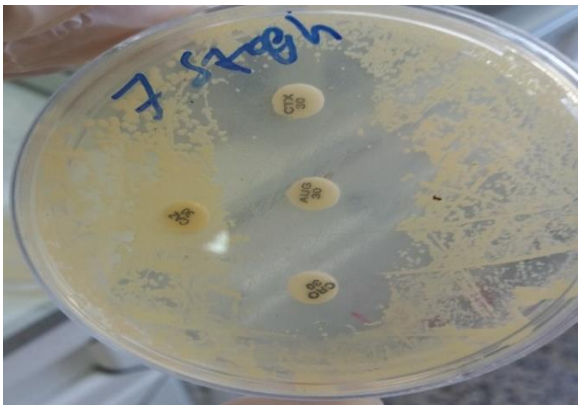


Fig. 5. Sensitive *S. aureus* isolate to Augmentin and Cephalosporin's antibiotic.

Secondary screening of *Streptomyces* isolates for bioactive compounds production

The filtrate of the flask's fermentation of ten *Streptomyces* isolates were screened and subjected to antibacterial activities against the pathogenic Gram positive (*S. aureus*) and Gram negative (*E. coli*, *P. aeruginosa* and *K. pneumonia*) using plate well. These isolates received from Biotechnology Department, College of Science as a nice gift. The antibacterial activity of the supernatant was incredibly good as represented in Figs. 7-8. A particularly good zone of inhibition was observed in 6 isolates, however only 3 isolates exhibited the highest antibacterial activities. Regarding *E. coli* and *S. aureus* (Fig. 6), as it represented that the same isolates (1, 3, 8, 9 and 10) displayed the highest antimicrobial activities against both. This explained either by the presence of many bioactive compounds that act together with synergistic effects or due to new antibiotics. Al-rubaye (2018) reported the same reaction of *Streptomyces* isolated from river sediments against *E. coli* and *S. aureus*. Fig. 7 showed a high antibacterial activity of most of the *Streptomyces's* supernatant samples against *S. aureus* which were sensitive to Augmentin, Cefotaxime, Ceftazidime and Ceftriaxone. This may be due to that samples 2, 4, 5, 6 and 7 contain only the antibiotics that *E. coli* and *S. aureus* resist to them.

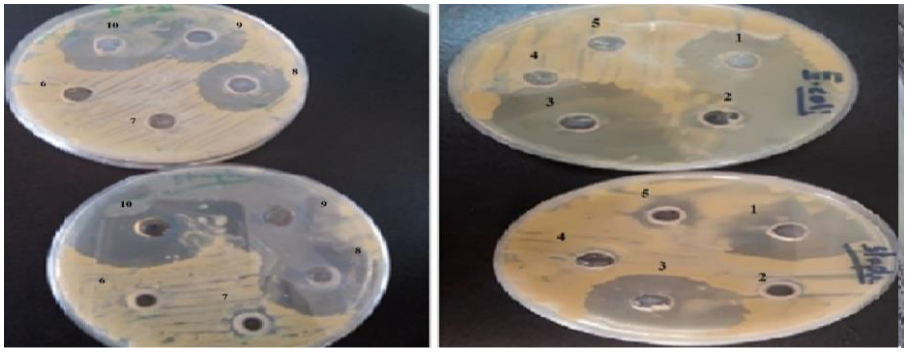


Fig. 6. Antibacterial activity crude extract of *Streptomyces* against against *S. aureus* and *E. coli.*

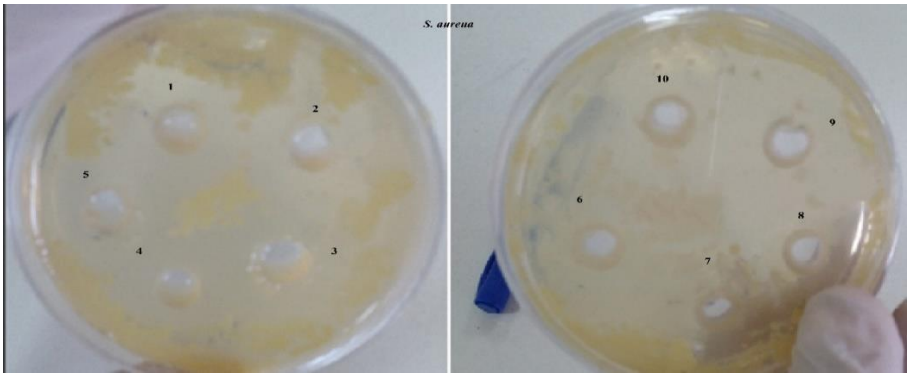


Fig. 7. Antibacterial activity of supernatant crude of *Streptomyces* against Augmentin, ceftazidime and ceftriaxone sensitive *S. aureus.*

Regarding *P. aeruginosa* (Fig. 8), supernatant's samples showed good (sample 9) to moderate (samples 1, 2 and 3) activity. Screening and characterization of the highest producing isolate (9) by advanced methods like HPLCs, GC-MS is important to discover such antibiotic in this case which helps in resolution many resistance problems. The supernatant activity against *Pseudomonas* is not much like that against *E. coli* and *S. aureus*. This may be related to the prominent mechanisms for resistant *Pseudomonas* spp., which are biofilm formation and efflux pump activity among other mechanisms as reported by Ugwuanyi *et al*. (2021). Heydari & Eftekhar (2015) suggested that the strong or weak biofilm are associated with β-lactamase phenotype.

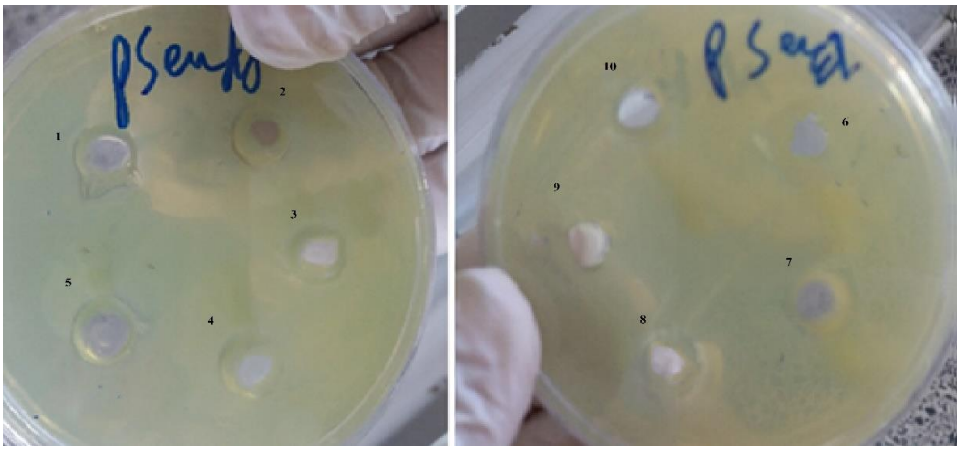


Fig. 8. Antibacterial activity of supernatant crude of *Streptomyces* against ESBLs producing *Pseudomonas aeruginosa.*

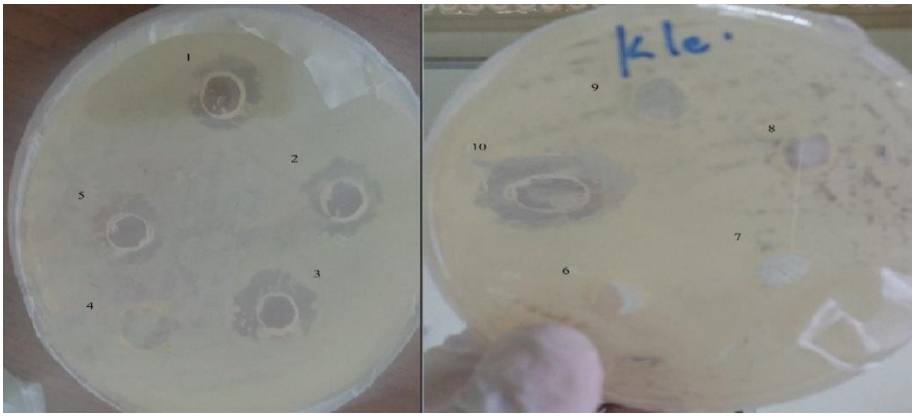


Fig. 9. Antibacterial activity of supernatant crude of *Streptomyces* against ESBLs producing *K. pneumonia.*

In the case of Fig. 9, the supernatant shows a particularly good zone of inhibition against ESBLs producing *K. pneumonia* less than that observed in *E. coli* and *S. aureus*. This may be due to the presence of capsule polysaccharide in *K. pneumonia*, which mediates resistance to antimicrobial agents by preventing the interaction of the agents with membrane targets as explained by Campos *et al*. (2004).

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