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Anatomical study with Antibacterial and Antibiofilm Effects of Erodium cicutarium (L.) L'H Phenolic Roots Extract

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ABSTRACT

Bacteria acquire resistance to antibacterial drugs,so there is always a need for new drugs to inhibit many human bacterial pathogens. Novel studies were carried out to study the internal structures as well as the antibacterial effect of Erodium cicutarium phenolic root extracts (APR) on the development of biofilm formation of bacterial species (Klebsiella pneumoniae) and (Staphylococcus aureus) using four strains from both clinical and environmental samples In-Vitro. Anatomy of all the vegetative parts showed a presence of tannin filled cells in their structures. Also, the experiments were conducted to study the antibacterial effect by determining the minimum inhibitory concentrations (MICs), and susceptibility test of these strains for five antibiotics. Although the APR concentrations (50, 25, 12.5, 6.25, 3.125, and 1.625 µg/ml) (W/V) were used through antibiotic and biofilm inhibition assay. The result of sensitivity test showed more resistant to various antimicrobial especially Amoxicillin, Cephalothin and Methicillin for both bacteria strains. Also, among the MICs tested, showed that the ranged value was in concentration between 0.017 to 0.650 mg/ml for both K. pneumoniae and S. aureus strains. Furthermore, biofilm reduction assay results for treatments showed that the highest activity was obtained with phenolic root extracted, biofilm eradication at 1.3 mg/ml. In conclusion, the results showed that relatively low concentrations of phenolic root extracts displayed promising antibacterial and antibiofilm capabilities making them attractive for additional studies as "novel therapeutic agents."

Keywords: *ALP, Hypercolestrolemia ,Antioxadinat*

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INTRODUCTION

Geraniaceae is Cosmopolitan family of mostly temperate and subtropical annual and perennial herbs and a few small shrubs, comprising about 750 species belong to 11 genera. There are 74 species belonging to the genus Erodium, which can be found in many regions, especially in the Mediterranean basin (1). The most common of these species is E. cicutarium. It was annual and naturally self-growing five-leaved herbaceous plant with pink blooms in spring (2,3). Although this species is consumed as fresh and dried vegetable in the regions where it grows, it is widely used for traditional medicine (4). It is used as a traditional medicine in many diseases, especially in diseases such as dysentery, uterine bleeding, constipation and diabetes (5,6,7,8,9,10,11,12). The fresh form of the plant is mostly used as a vegetable, while; the dry form of the plant is used as a traditional medicine. The principal active ingredients of Erodium include Tannin, catechins, gallic and elagic acids, sugars (glucose, galactose, fructose), amino acids (glycine,alanine, proline, histidine, tryptophan, tyrosine,glutamic acid), vitamins K and C (13). Plant anatomy, the study of the internal structure of plants, had been a source of fascination and field of scientific inquiry since the time of earliest microscopists. It plays an important role in the understanding of plant biology. A realists interpretation of morphology, physiology and phylogeny must be based on a thorough knowledge of cells structure and tissue (14).

Among the factors contributing to microbial resistance is the ability of the microbes to exist in biofilm forms that allow them to withstand harsh environmental conditions and antimicrobial agents. Staphylococcus aureus is the most common infectious agent involved in the development of Skin infections that are associated with antibiotic resistance, such as burn wounds. Also, Klebsiella pneumoniae is the second most common cause of gram-negative bacteremia after Escherichia coli. Bacteremia causes significant morbidity and mortality in general populations (15,16). Biofilms can be very problematic in various aspects of our lives ranging from medical to industrial areas. In addition to their increased resistance to antimicrobial agents, biofilms can form on many medical implants such as catheters, artificial hips, and contact lenses. The most worrisome fact is that cells existing in a biofilm can become10-1000 times more resistant to antimicrobial agents, mainly through the production of extracellular polymeric substance matrix that hinders the access of antibiotics to the bacterial cells. These infections can often only be treated by removal of the implant, thus increasing the trauma to the patient and the cost of the treatment. It has been estimated that biofilms are associated with 65% of nosocomial infections and that treatment of these biofilm-based infections costs >$1 billion annually (17).

The aim of this study was to Identify anatomical characters of root, stem, petiole and leaf blades of E.cicutarium in addition to evaluated the antibacterial effect phenolic root extracts (APR) and on the development of biofilm formation of bacterial species (Klebsiella pneumoniae) and (Staphylococcus aureus) by using four strains.

MATERIALS AND METHODS

Collection and extract preparation of the studied plant

Plant materials were collected from various parts of Mosul-Iraq during the flowering period in 2022. Authentication of the plant was carried out at the herbarium of the Department of Biology, College of Science/ Baghdad University/Iraq. After that, the plant materials were rinsed thoroughly with tap water to remove extraneous contaminants. The wanted parts for the anatomical study were slit into small pieces and fixed in formalin acetic acid solution . After 24 hrs, the plant parts were washed several times with Ethyl Alcohol solution with concentration of 70% then save in it even use (18). The method of (19) and (20) were used with some modifications. Some sectionings stained by safranin and glycerine mixture 1:10. For extract preparation, roots were cut into small pieces, oven-dried at 50°C until stability of dry weight was observed, and then grounded into powder with an electric-grinder to prepare it for extraction (21).

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Crude phenols extract

Phenolic Compounds Separation

The separation of the phenolic component was performed according to (21). Approximately 200g of root powder has been shaken with 1L of 80% ethanol for 72 hrs in cool and dark location. The extract, then filtered and dried at a temperature of 30-40 °C via a rotary evaporator. For acid hydrolysis 10 percent concentration HCl was used for 10- 30 min in a water bath. This step resulted in the hydrolyse the glycosidic linkage to get aglyconic part, cooled and filtrated. Finally, the mixture was extracted using chloroform 1:1 percent in three times separation fennel. The polyphenolic fraction (chloroform layer) was collected and submitted to rotary evaporator to remove the solvent form. The ferric chloride test was applied to detect phenol compounds in the roo extract. Then different concentrations of the extract were prepared (0.3, 0.5 and 1.3 mg.ml-1).

Microorganisms

The bacteria K. pneumoniae and S. aureus strains were obtained from microbiology laboratory in the College of Sciences - University of Baghdad. The strains were sub-cultured into cetrimide agar plates and incubated at 37°C for 24 h. Following incubation, single colonies were transferred from the plates and inoculated into tubes containing brain heart broth. The cultures were incubated at 37°C for 24 h before used (22).

Antibiotic Susceptibility Test

From an overnight culture plate, 4-5 colonies of K. pneumoniae and S. aureus strains were picked up and suspended in 5ml of sterile normal saline until the turbidity is a proximately equivalent to that of the McFarland No. 0.5 turbidity standard. Ten minutes later, by a sterile forceps the antimicrobial disc Amoxicillin, Ampicillin, Cefotaxime, Cephalothin and Methicillin were picked up and placed on the surface of Mueller Hinton plates (23). The plates were incubated at 37°C for 18-24 hours. After incubation, the plates were examined for the presence of inhibition zone of bacterial growth around the antimicrobial discs. The diameter of the zone of inhibition was measured by millimeters using a metric ruler and compared to standard inhibition zone according to Clinical and Laboratory Standards Institute (24).

Determination of minimum inhibitory concentration (MIC)

MIC of plant extracts was determined by microdilution method in sterile 96-wells microliter plates according to the protocol described previously (25). Different plant extracts concentrations (50, 25, 12.5, 6.25, 3.125 and 1.625 µg/ml) (W/V) were prepared containing bacterial cells comparable to McFarland standard no. 0.5 in a final volume of 200 µl. Sterile distilled water, broth and plant extracts was used as a negative control while sterile distilled water, broth and bacteria was used as positive control. After 24 h at 37°C, the MIC of each sample was determined. The MIC is considered the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after 24 h incubation .

Effect on adherence and biofilm formation

The effect of different concentrations of plant extracts and on adherence and biofilm-forming ability was tested on polystyrene flat-bottomed microtiter plates as described by (26) with some modifications. overnight cultures were diluted (1:100) with trypton soya broth supplemented with 1% (w/v) glucose. (200µl) from the culture were then transferred to the wells of a 96-well polystyrene microliter plate and incubated overnight at 37ºC. After incubation, supernatants part was removed from each well and the plate were gently washed twice with normal saline, then dried and fixed at 65ºC for 1 hr. therefore all the plates were stained with 0.1% (w/v) crystal violet for 10 min, gently washed and the quantitative analysis of biofilm was performed by adding 200 µl of 95% ethanol for 10 min. Finally, the biofilm was measured at 630 nm by microplate reader in the present of the methylene blue in the de-staining solution (ethanol) .

Statistical Analysis

Complete Randomized Design (CRD) was used as experimental design. Data were analyzed using statistical analysis system- SAS to study the effect of different plant extracts and the nanoparticles on some bacterial isolates. Least significant difference (LSD) was used to compare the significant difference between means at difference were considered significant when $P \leq 0.05$ (27).

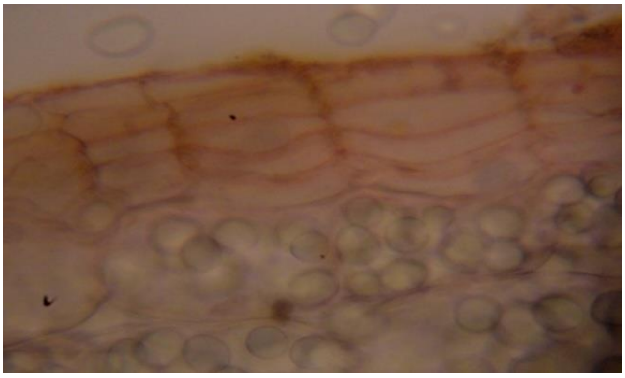
RESULTS AND DISCUSSION

Anatomical study

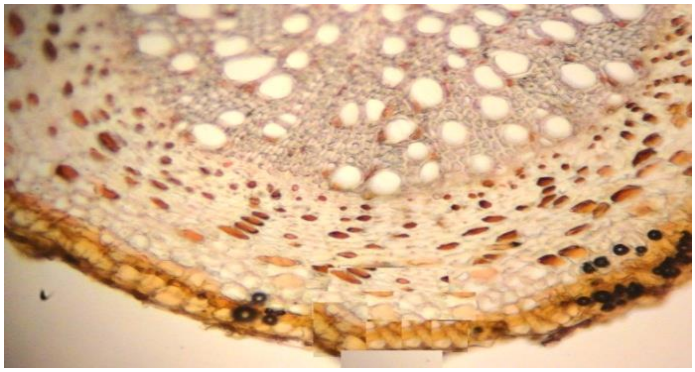
In transverse section of E. cicutarium roots, the periderm layer on the outermost surface was rectangular to cuboidal cells arranged in 4-6 rows with mean thickness 58.02μm. The multilayered parenchymatous cortex composed of numerous of storage parenchyma cells and characterized by presence of tannin filled cells in the tissue. Mean thickness of the cortex layer was 121.66μm. The phloem seems as a conical masses of cells attached from the base as a continuous ring with mean thickness 21.36μm. Xylem was regular diffused porous wood with mean half diameter 413.33μm (Plate1. A & B). In transverse sections of the hairy stem of the studied species, shape of the stem was circular. Epidermis is single layered covered with thin layer of minute projections cuticle with mean thickness 5.93μm. Epidermal cells were ovate to oblong ovate, thickened vary from 13.6μm to 21.76μm. Below the epidermis, cortex represented by 13-16 rows of ovate to oblong ovate parenchymatous cells with mean thickness 168.41μm containing starch grains and tannin filled cells in highly amounts. Pericycle contains a well defined continuous one ring of sclerenchyma, which comprised of unite of the arcs of interfascicular sclerenchyma with the fiber caps of the bundles, so as to form a continuous one ring. The caps of the pericyclic fibers are smaller in diameter than those of the interfacicular sclerenchyma arcs. Central cylinder composed of 13-15 collateral vascular bundles separated by medullary rays. Wood has crescent shape and the tracheary elements arranged as groups. Pith consists of circular to sub circular parenchymatous cells. These cells stored highly amounts of starch in addition to tannic acid filled cells as clusters in scattered regions. (Plate1. C&D). Transverse sections of the hairy leave showed that, it was covered with one layer of epidermis with polygonal or rectangular ordinary epidermal cells with thick 24.48μm to 27.74 μm. The outer walls of the epidermal cells have well developed cuticle of 3.98 μm thick. The palisade region of the leaf consists of 3 – 4 rows of elongate – prismatic shaped cells, then spongy layer beneath the palisade layer comprised of 3 – 4 rows of nearly isodiametric cells. One central circular –ovate vascular bundle observed in leaf centre. Tracheary elements of the bundle were arranged as group. In the midrib of the leaf, large amount of tannin filled cells were observed (Plate 1. E). Cross sections of petioles have crescent shape comprised of the outermost layer; cuticle, with mean thickness 6.8 μm covering the uniseriate epidermis which composed of oblong – ovate to sub-rectangular cells with mean thickness 19.22 μm. Cortex was parenchymatous tissue type with the outer rows specialized as chlorenchyma. Petiole has five conical vascular bundles with tracheary elements arranged in form of group. Phloem groups bounded externally by caps of fibers. petioles tissue.have tannin filled cells, storage parenchyma cells and druses crystals (Plate 1. F&G). Tannin filled cells were observed in all the studied parts. This result show a parallelism with the record of (28,29,30).

Bacterial susceptibility to antibiotics

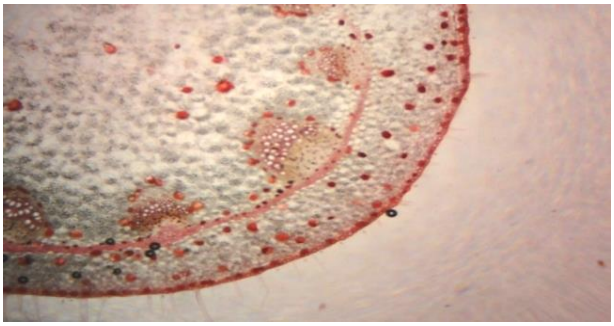
In this study, the bacterial strains were chosen because the importance of these strains in the hospital environment and their outbreak in the community. Susceptibility tests were summarized in Table 1 and 2 for the S. aureus and K. pneumoniae strains to five different antibiotics by disc diffusion method recommended by CLSI (31). The result of the antibiotic susceptibility tests showed that the bacteria were more resistant to various antimicrobial treatments. These antibiotics were used in this study due to their mode of action inhibiting cell wall synthesis which cause the release of the bacterial cell DNA into the surroundings (32). Table 1 and Table 2 indicate that all strains were resistant to Ampicillin, Amoxicillin. Besides, all S. aureus strains were ranging from intermediate and sensitive to Cefotaxime antibiotic, While the K. pneumoniae strains were intermediately resistant to Cefotaxime antibiotic with more resistance to other antimicrobial drugs.



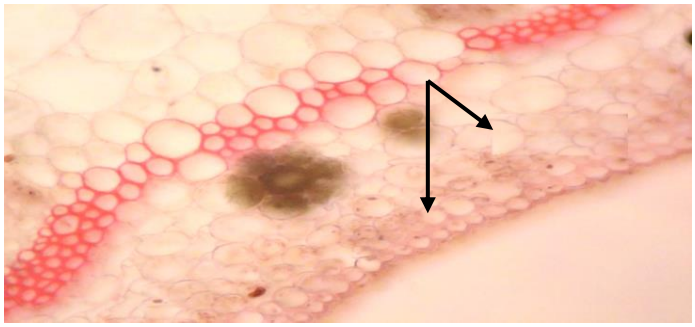
(A) C.S in root (200 X)



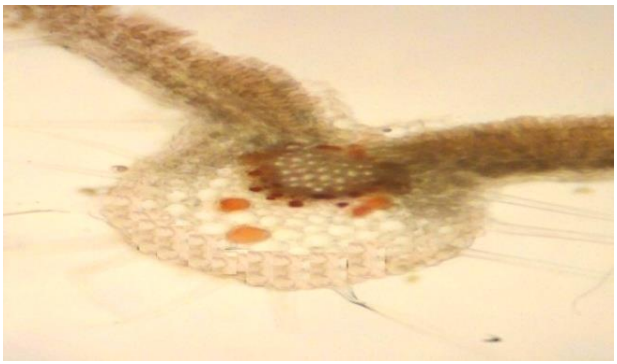
(B) C.S in root (2000 X)



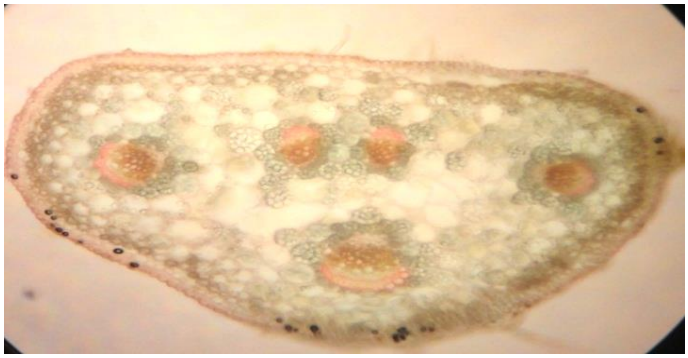
(C) C.S in stem (200 X)



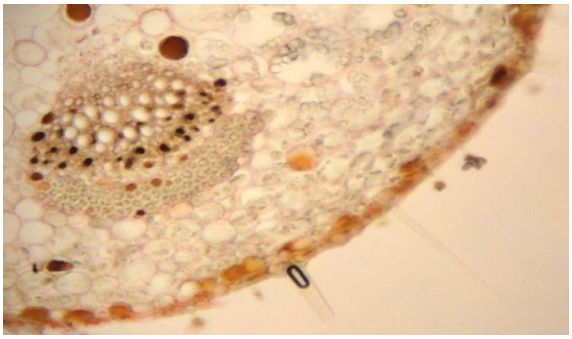
(D) C.S in stem, tannin filled cells (500 X)



(E) V.S in leaf (500 X)



(F) C.S in petiole (200 X)



(G)C.S in petiole (500 X)

PLATE 1 : Anatomical sectioning of root, stem, leaf and petiole of *E. cicutarium*

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TABLE 1. Antibiotic susceptibility of S. aureus strains according to CLSI

S. aureus Strains	Amoxicillin	Ampicillin	Cefotaxime	Cephalothin	Methicillin
1	R	R	S	R	R
2	R	R	I	R	R
3	R	R	S	R	R
4	R	R	S	R	R

R=Resistant, I=Intermediate, S=Sensitive

TABLE 2. Antibiotic susceptibility of K. pneumoniae strains according to CLSI

K. pneumoniae Sterns	Amoxicillin	Ampicillin	Cefotaxime	Cephalothin	Methicillin
1	R	S	I	R	R
2	R	S	I	R	R
3	R	R	I	R	R
4	R	R	I	R	S

R=Resistant, I=Intermediate, S=Sensitive

Multidrug resistance has been recognized as a virulence factor of great scale in clinical infections. Because of the increase in the intricacy of the majority of microbial infections and the resistance to straight treatment, researchers have been prompted to identify alternatives for the action of infections. Plant extracts biologically active compounds isolated from plants have gained extensive attention in this look as they have been known to cure diseases and sickness since very old times. Silver nanoparticles are also able to assess human health through a variety of commercial products. Studies have shown that silver nanoparticles cause toxicity to germline stem cells through a reduction in mitochondrial function and induction of membrane leakage and apoptosis (33).

Evaluation of MIC values

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) value is important to determine efficacy of antibacterial agent. Low MIC value may be an indication of high efficacy or that microorganism has no potential to develop resistance towards the bioactive compound. The result in table 3 and figures 1, 2, 3 and 4 showed that the bacterial strains behaved significant differences in their sensitivity to the different extracts added to their growth medium. Obviously, MIC ranged value was in concentration between 0.017 to 0.650 mg/ml for both K. pneumoniae and S. aureus strains. Moreover, figure (1) results indicated that the K. pneumoniae strains were intermediate effects than S. aureus strains which were sensitive to APR extracts concentrations. Also, the result in figure 2 showed that the lowest MIC was (0.079) with a high APR concentration of 1.3 mg/ml and the highest MIC was (0.308) after treatments with a lower APR concentration of 0.3 mg/ml for both bacteria strains.

TABLE 3. Antibacterial activity of E.cicutarium root phenolic (APR)extracts against some of S. aureus and K. pneumoniae strains.

strains	Concentrations						Average	
	0.3 mg.ml^{-1}		0.5 mg.ml^{-1}		1.3 mg.ml^{-1}			
	Bact. 1	**Bact. 2**	**Bact. 1**	**Bact. 2**	**Bact. 1**	**Bact. 2**	**Bact. 1**	**Bact. 2**
St. 1	0.133	0.273	0.107	0.107	0.120	0.017	0.120	0.132
St. 2	0.133	0.330	0.130	0.170	0.150	0.067	0.138	0.189
St. 3	0.133	0.650	0.107	0.130	0.067	0.080	0.102	0.287
St. 4	0.160	0.650	0.109	0.210	0.068	0.067	0.111	0.309
Average	0.140	0.476	0.113	0.154	0.101	0.058	0.118	0.229
L.S.D 5%	0.018							0.026
	0.037							

Bact. 1: K. pneumoniae, Bact. 2: S. aureus, St.: strains

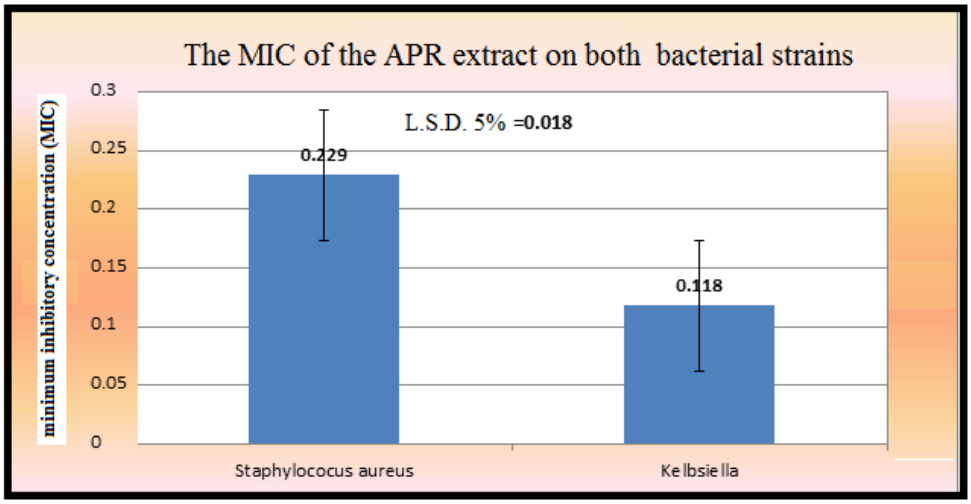

FIGURE 1. Minimum inhibitory concentration (MIC) value for S. aureus and K. pneumoniae

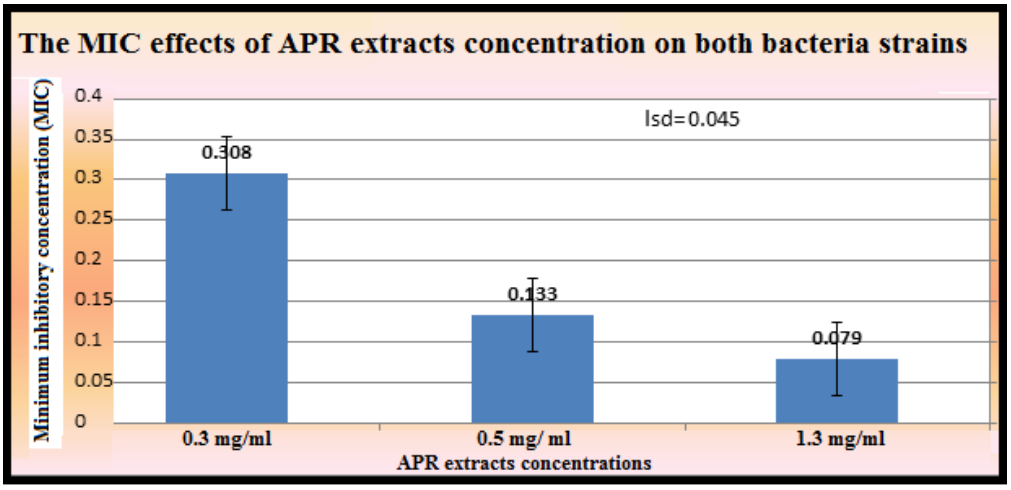

FIGURE 2. Minimum inhibitory concentration (MIC) value for APR extracts concentration.

Moreover, the evaluation of the inhibition potential of plant extracts against S. aureus and K. pneumoniae strains (Figure 3 and 4) showed that APR extracts present a greater effect on bacteria strain No. 3 and 4 than 1 and 2 for both kinds, especially S. aureus strains.

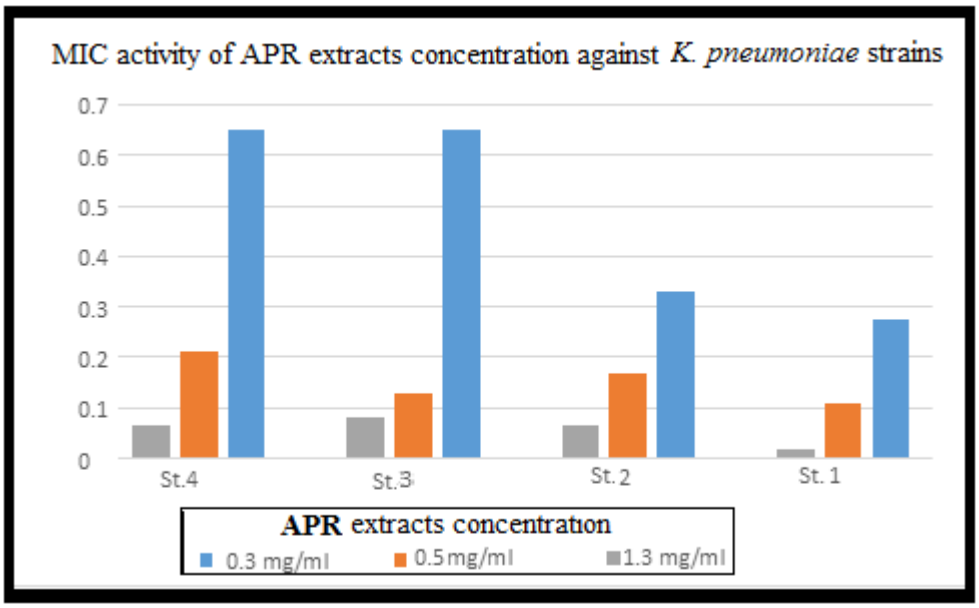

FIGURE 3. the interaction effects between Minimum inhibitory concentration (MIC) value for K. pneumoniae. and the APR extracts concentrations.

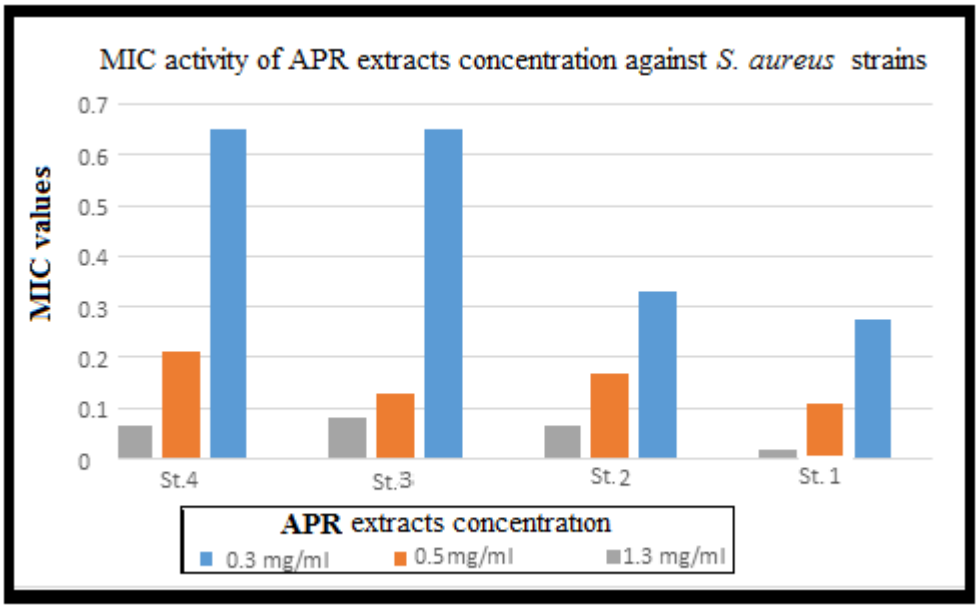

FIGURE 4. the interaction effects between Minimum inhibitory concentration (MIC) value for S. aureus. And the APR extracts concentrations.

Analysis of the experimental data revealed that organic extracts were more effective against Gram-positive than Gram-negative bacteria. Gram-negative bacteria are highly resistant, and this resistance is likely related to the nature of their outer membranes, which are impervious to lipophilic compounds (34). Gram positive bacteria are more sensitive and less protected against polyphenolic agents because they only have an outer layer of peptidoglycans, which can only prevent the diffusion of molecules whose molar mass is greater than 50 000 D (35). (36) obtained similar results to those of the present study, and supporting the hypothesis that Gram-positive bacteria are more sensitive to plant extracts. In a recent study, reported that the antimicrobial activity of an extract is likely due to the presence of synergy among various phenolic components.

Antibiofilm activity in a 96-well microplate

Table (4) (5) and figure (5) shown significant differences between two bacteria types and plant extract concentrations, the results of in vitro biofilms were presented in table 4 and table 5. Furthermore, biofilm reduction assay results showed that APR at 0.347mg/ml could inhibit 100% of K. pneumoniae biofilm and at 0.053 decrease 50 % of biofilm formation (table 4).

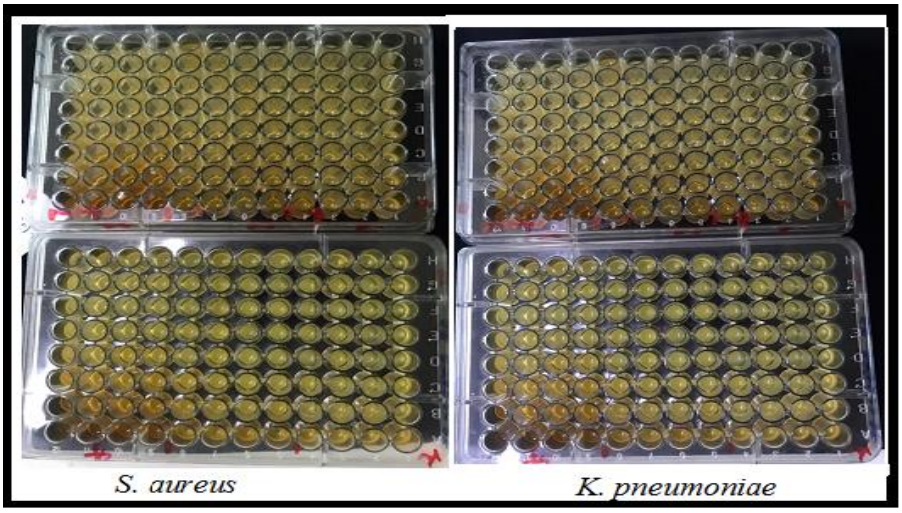

FIGURE 5: Biofilm of S. aureus and K. pneumoniae formation tests

Furthermore, biofilm reduction assay results showed that the average treatments which decrease significantly biofilm formation for K. pneumoniae strains No.1,2,3 and 4 were 0.332, 0.214, 0.154 and 0.192 mg/ml, respectively. While the average treatments which decrease biofilm formation for S. aureus strains No. 1,2,3 and 4 were 0.169, 0.285, 0.404, 0.331 mg/ml, respectively. It is noteworthy that significantly the best plant extracts effective concentrations were 1.3 mg/ml which able to inhibit biofilm formation by average treatments 0.221 and 0.136 mg/ml of all S. aureus and K. pneumoniae strains, respectively (tables 4 and 5).

The significantly difference in biofilm thickness results from different reasons such as differences in strains capacity to form biofilm, perhaps the primary number of cells that succeeded in adherence and the differences of quality and quantity of autoinducers (Quorum sensing signaling molecules) that produced from each strain and play an essential as well as important role in biofilm formation (36).

TABLE 4: Effects of APR extract on K. pneumoniae biofilms formation at 24 h.

strains	Treatments				Average treatments
	Before	Conc. 0.3 $mg.ml^{-1}$	Conc. 0.5 $mg.ml^{-1}$	Conc. 1.3 $mg.ml^{-1}$	
Iso 1	0.505	0.284	0.344	0.194	0.332
Iso 2	0.367	0.209	0.162	0.119	0.214
Iso 3	0.261	0.086	0.168	0.101	0.154
Iso 4	0.253	0.188	0.198	0.130	0.192
Average treatments	0.347	0.192	0.218	0.136	0.223
L.S.D 5%	strains		Treatments		Interaction
	0.008**		0.008**		0.016**

TABLE 5: Effects of APR on S. aureus biofilms formation at 24 h.

Strains	Treatments				Average treatments
	Before	Conc. 0.3	Conc. 0.5	Conc. 1.3	
Iso 1	**0.252**	**0.233**	**0.063**	**0.126**	**0.169**
Iso 2	**0.394**	**0.232**	**0.291**	**0.223**	**0.285**
Iso 3	**0.680**	**0.346**	**0.306**	**0.283**	**0.404**
Iso 4	**0.472**	**0.225**	**0.376**	**0.250**	**0.331**
Average treatments	**0.449**	**0.259**	**0.259**	**0.221**	0.297
L.S.D 5%	strains		Treatments		Interaction
	0.007**		**0.007****		**0.013****

CONCLUSION

Increasing drug resistance to traditional treatments observed in several bacterial strains, in addition to harmful APR extracts and substantial cost of treatment shifted the pace toward finding novel therapeutic agents, natural products such as plants and plant-derived compounds were the primary target due to their efficacy, safety, and lower cost. In this study, we choose E. cicutarium in an effort to find novel agents that could aid in fighting bacterial infections and their biofilms. MIC values indicate that not all concentrations exhibited an activity. Regarding the MIC assay, only 1.3 mg/ml APR extract concentration was able to inhibit bacterial growth, among which was most significant where it achieved the lowest MIC and had an effect against multiple strains. We also noticed that one of our tested strains, K. pneumoniae No.1 was insensitive to all treatments used, that is, independently of the strain being a Gram-positive one but rather probably related to the strain itself or the nature of our extract concentration.. Furthermore, it was evident that most of the active extracts were thymol, which are characterized by containing flavonoids and phenols that are known antimicrobial agents (37). This could be due to a potential synergistic effect between the different phytochemical constituents found in the whole plant. The synergism effect in plant extracts was also highlighted in literature as in the study of (38) on the antimicrobial synergism within plant extract combinations from three South African medicinal bulbs. The plants extract compound (phenolic compound) may provide a safe and highly effective alternative to commonly used antibiotics, which are ineffective towards the antibiotic-resistant S. aureus and K. pneumoniae. Thus, further studies are required to test their activity against other pathogenic bacteria and fungi and study the possibility of using these active components by drugs companies. Finally, further studies using in vivo models are needed to study the impact of APR on health.

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