مجلة الدراسات التربوية والعلمية - كلية التربية - الجامعة العراقية
العدد العشرون - المجلد الأول - علوم الحياة - تشرين أول 2022 م
doi.org/10.52866/esj.2022.01.20.16

In-Vitro Antibacterial, and Antibiofilm Effects of *Astragalus hamosus* Phenolic Roots Extract.

Aseel Abdulsattar Jabori Alakiali 1, Zubadia A. Lateef Ismaeel 1 , Ayyad W. Al-Shahwany*
Al-Iraqia University / College of Education 1
* University of Baghdad / College of Science
* Corresponding Author: a61_bio@yahoo.com

Abstract:

Because bacteria acquire resistance to antibacterial drugs, there is always a need for new drugs to inhibit many human bacterial pathogens. Novel studies were carried out to assess the antibacterial effect of *Astragalus hamosus* 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*. 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."

KEY WORDS: Antibacterial, antibiofilm, *Astragalus hamosus, klebsiella pneumoniae*.

تأثير التضادي البكتيري والغشاء الحيوي لمستخلص الفينولي نبات *Astragalus hamosus* خارج الجسم الحي

أسيل عبد الستار جبوري العقيلي ، زبيدة عبد اللطيف إسماعيل ، اياد وجيه الشهواني
الجامعة العراقية / كلية التربية جامعة بغداد / كلية العلوم

الخلاصة:

نظرًا لمقاومة البكتريا للمضادات الحيوية، فقد دعت الحاجة الى مضادات جديدة تعمل على تثبيط العديد من مسببات المرضية البكتيرية. تم اجراء دراسة حديثة لتقييم فعالية المستخلص الفينولي لجذور نبات *Astragalus hamosus* في تثبيط النمو وتثبيط تكوين الغشاء الحيوي لأربعة عزلات بكتيرية ممرضة لكل من لـ (*Klebsiella pneumoniae*) و (*Staphylococcus aureus*) من خلال قياس اقل تركيز تثبيطي لهم وتحديد الحساسية هذه العزلات لخمسة مضادات حيوية خارج جسم الكائن الحي . تضمنت التخافيف المستخدمة للمستخلص الفينولي (50، 25، 12.5، 6.25، 3.125، 1.625 ملي غرام / مل)، وقد أظهرت نتيجة اختبار الحساسية مقاومة العزلات البكتيرية لمضادات الميكروبات المختلفة وخاصة أموكسيسيلين وسيفالوثين وميثيسيلين. كما تراوحت التراكيز المثبطة لـ ٪50 من المستعمرات البكتيرية بين 0.017 إلى 0.650 ملغرام / مل للعزلات *K. pneumoniae* و *S. aureus* . علاوة على ذلك، فقد أظهرت نتائج ان أعلى فعالية لتراكيز المستخلص الفينولي في تثبيط تكوين الغشاء الحيوي للعزلات البكتيرية هو 1.3 ملغ / مل مقارنة بباقي التراكيز المستخدمة في الدراسة. يتبين مما سبق انه، يمكن اعتبار المستخلص الفينولي لجذور نبات *A. hamosus* مضاد بكتيري وذلك لقدرته على تثبيط نمو وإيقاف تكوين الغشاء الحيوي لبعض الأنواع البكتيرية الممرضة وبتراكيز منخفضة نسبيًا.

الكلمات الدالة: مضاد بكتري، مضاد الغشاء الحيوي، *Astragalus hamosus , klebsiella pneumoniae*. *Staphylococcus aureus*،

Introduction

Astragalus L. (Leguminosae) is a genus found primarily in Europe, Asia, and North America, and is extensively dispersed throughout the temperate world. Also, the Astragalus genus is distributed in Mediterranean climatic regions in Europe and North Africa. There are over 2000 species described, with 372 in North America, and 114 species have been found in the IRAQ [1].

Astragalus species have been shown to increase reticuloendothelial phagocytosis, stimulate pituitary-adrenal cortical activity, and restore deficient red blood cell production in bone marrow. Its antibacterial, antiperspirant, anti-inflammatory, diuretic, and tonic properties are also well-known [2]. The pharmacological effects of several Astragalus plants are well documented, including hepatoprotective, immunostimulant, and antiviral properties [3].

The principal active ingredients of Astragalus include saponins, flavonoids, and polysaccharides [4]. Anthraquinones, alkaloids, amino acids, -sitosterol, and metallic metals are among the components. The antibacterial, antioxidant, and anti-inflammatory effects of the Astragalus plants have been reported in many studies [5].

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, *K. pneumoniae* is the second most common cause of gram-negative bacteremia after *Escherichia coli*. bacteremia causes significant morbidity and mortality in general populations [6,7].

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 become 10-1000 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 [8].

The members of the genus Astragalus have great interest as traditional drugs in several folk systems including Iraq. However, two species of pharmacological effects Astraga-

lus grown in Iraq, *Astragalus hamosus* and *Astragalus tribuloides*. These species are perennial plants, about 16 to 36 inches tall, that is native to the northern and eastern parts of IRAQ, as well as some aria of turkey and Iran. It has hairy stems with leaves made up of 12 to 18 pairs of leaflets. The root is the medicinal part of the plant, and is usually harvested from 2-year-old plants [9].

The aim of this study was evaluated the antibacterial effect of *A. hamosus* phenolic root extracts (APR) and on the development of biofilm formation of bacterial species (*Klebsiella pneumoniae*) and (*Staphylococcus aureus*) using four strains.

Method and materials

Materials and Methods

Plant material and extract preparation

Plant materials were collected from various parts of Mosul-Iraq. Authentication of plant root was carried out at the herbarium of the Department of Biology, College of Sciences/ Baghdad University/Iraq. The plant materials were rinsed thoroughly with tap water to remove extraneous contaminants and 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 [10].

Crude phenols extract

Phenolic Compounds Separation:

The separation of the phenolic component was performed according to Harborn [10]. Approximately 200g of herb powder has been shaken with 1L of 80 percent 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 plant extract. Then different concentrations of plant extract were prepared (0.3, 0.5 and 1.3 $mg.ml^{-1}$), by mixing known volume from the stock solution with using distal water the following equation:

$C1V1 = C2V2$.

C1 = Concentration of stock solution.

V1 = Volume that obtained from stock solution.

C2 = Final concentration.

V2 = Final volume.

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 onto 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. [11].

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 [12]. 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 [13].

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 [14]. 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 [14].

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 [15] 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 37°C. After incubation, supernatants part was removed from each well and the plate were gently washed twice with normal saline, then dried

and fixed at 65°C 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) [15] .

Statistical Analysis

Complete Randomized Design (CRD) was used as experimental design. Data were analyzed using statistical analysis system- SAS (2003) 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≤ 0.05 [17].

Results and discussion

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 [18]. 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 [19]. 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.

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 [20].

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 bacteria 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.

Table 3. Antibacterial activity of *A. hamosus* root phenolic (APR) extracts against some of *S. aureus* and *K. pneumoniae* strains.

strains	Concentrations						Average			
	mg.ml^{-1} 0.3		mg.ml^{-1} 0.5		mg.ml^{-1} 1.3					
	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

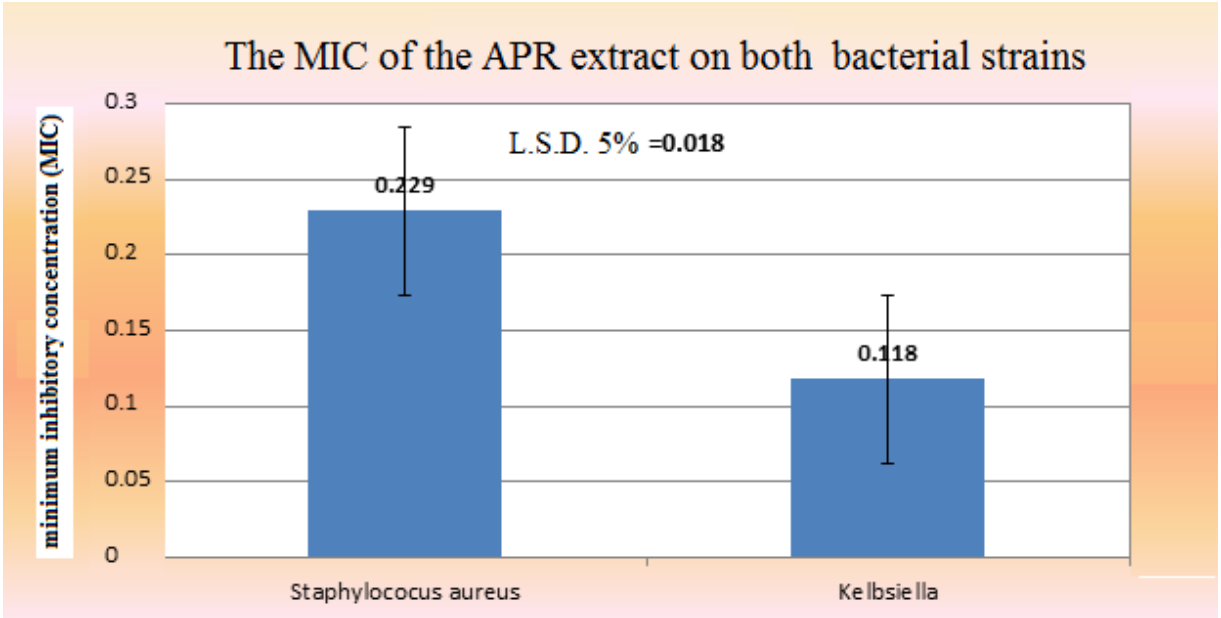

Figure 1. Minimum inhibitory concentration (MIC) value for *S. aureus* and *K. pneumoniae*

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.

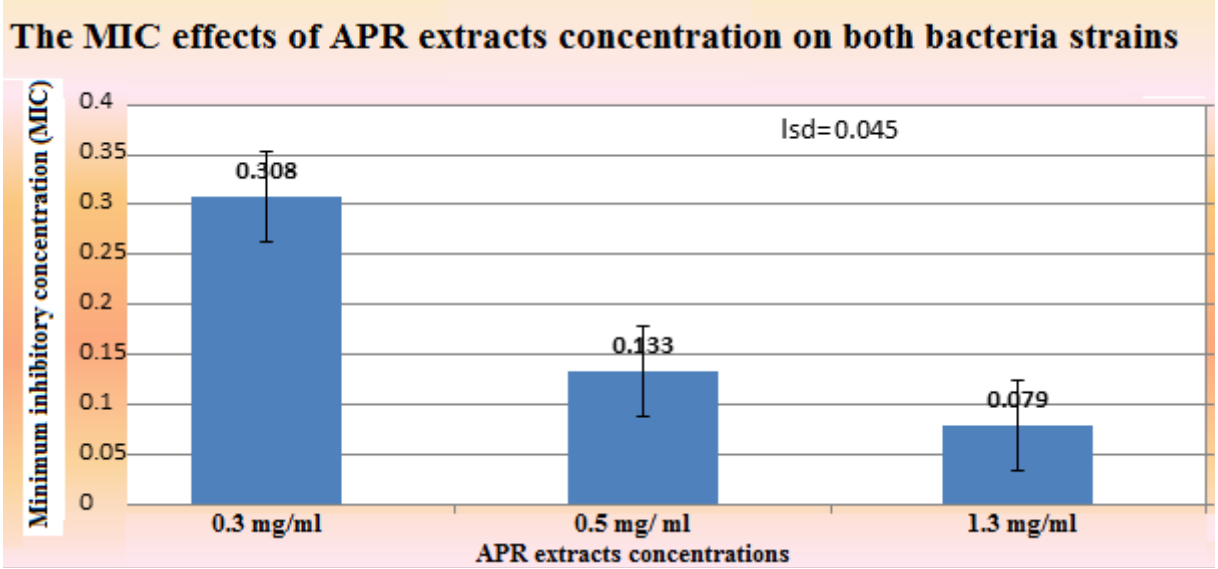

Figure 2. Minimum inhibitory concentration (MIC) value for APR extracts concentration.

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.

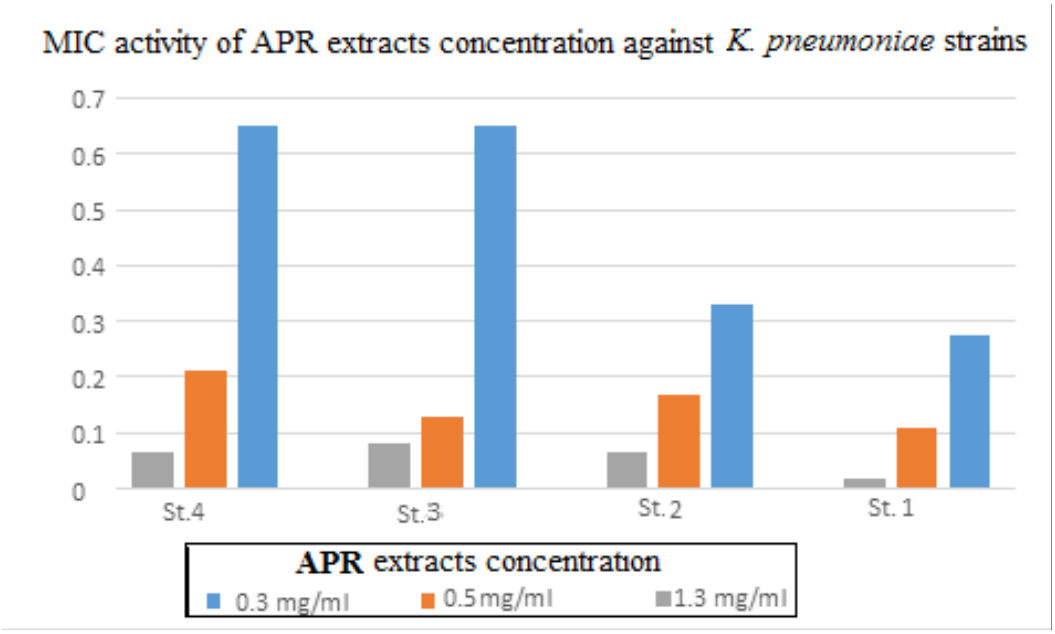

Figure3. the interaction effects between Minimum inhibitory concentration (MIC) value for *K. pneumoniae*. and the APR extracts concentrations.

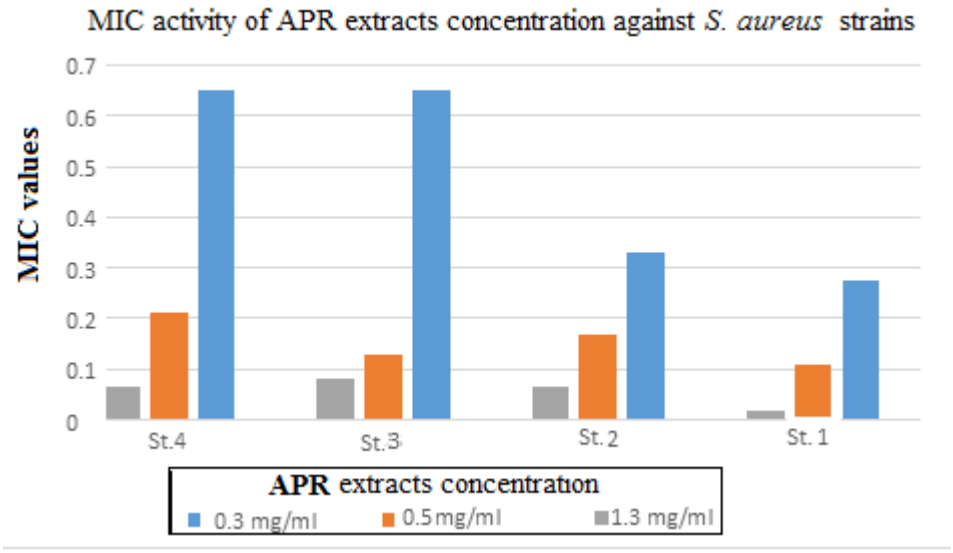

Figure 4. the interaction effects between Minimum inhibitory concentration (MIC) value for *S. aureus*. And the APR extracts concentrations.

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 [21]. 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 [22]. Al-Shahwany *et. al.,* [20] 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).

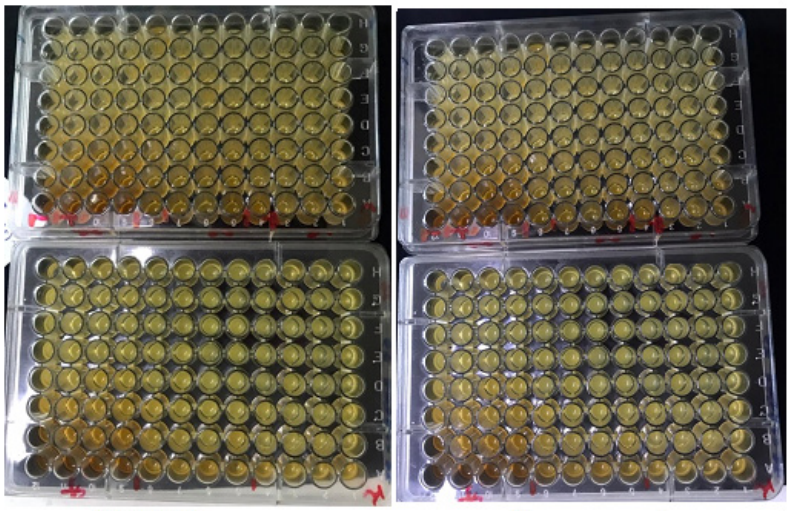

Figure (5): Biofilm of *S. aureus* and *K. pneumoniae* formation tests

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 [23].

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. m^{l-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**

Discussion

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 chose a Iraqi endemic species of Astragalus which is A. hamosus in an effort to find novel agents that could aid in fighting bacterial infections and their biofilms. In the present work, we assessed and tested for the first time the antibacterial and antibiofilm activities of phenolic root extracts from the plant portions of A. hamosus against two types bacterial with five strains for each of them. 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. It is worthy to note that the most successful extract contained fair amounts of several secondary metabolites mainly flavonoids, coumarins, and volatile oils. 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 [24]. 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[25], on the antimicrobial synergism within plant extract combinations from three South African medicinal bulbs [25]. 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.

References

1) Al-Snafi, A. E. (2018) Chemical constituents and pharmacological effects of *Astragalus hamosus* and *Astragalus tribuloides* growth in Iraq. Asian Journal of Pharmaceutical Science & Technology. Vol 5, Issue 4:321-328.
2) Al-Mothafar, N. A., & Al-Shahwany, A. W. (2022). Phenolic compounds from Thymus vulgaris, Artemisia annua extracts and pure Thymol were tested against twenty Pseudomonas spp. strains for antibacterial and anti-biofilm activities. International Journal of Health Sciences, 6(S1), 204-217.
3) Linnek, J., Mitaine-Offer, A. C., Miyamoto, T., Tanaka, C., Paululat, T., Avunduk, S., ... & Lacaille-Dubois, M. A. (2011). Cycloartane glycosides from three species of Astragalus (Fabaceae). Helvetica Chimica Acta, 94(2), 230-237.
4) Ibrahim, L. F., Marzouk, M. M., Hussein, S. R., Kawashty, S. A., Mahmoud, K., & Saleh, N. A. (2013). Flavonoid constituents and biological screening of *Astragalus bombycinus* Boiss. Natural product research, 27(4-5), 386-393.
5) Jaradat, N. A., Zaid, A. N., Abuzant, A., Khalaf, S., & Abu-Hassan, N. (2017). Phytochemical and biological properties of four Astragalus species commonly used in traditional Palestinian medicine. *European Journal of Integrative Medicine, 9, 1-8*.
6) Lee JH. (2003). Methicillin (Oxacillin) -Resistant *Staphylococcus aureus* Strains Isolated from Major Food Animals and Their Potential Transmission to Humans. Appl. Environ. Microbiol. 69 (11): 6489-6494.
7) Tsay RW, Siu LK, Fung CP, Chang FY. (2002). Characteristics of bacteremia between community-acquired and nosocomial *Klebsiella pneumoniaee* infection: risk factor for mortality and the impact of capsular serotypes as a herald for community- acquired infection. Arch Intern Med. 162: 1021-7.
8) Cox PA. ,Balick MJ (1994). The ethnobotanical approach to drug discovery. Sci Am. 270(6): 82-7.
9) Khansaa R. M. (2016). Biosystematics study of certain species of the genus Astragalus L. from the family Leguminosae in Iraq. Ph.D. thesis. Department of Biology, College of Education for Pure sciences- Ibn Al- Haitham, Baghdad University.
10) Harborne, J.B. (1984). *Phytochem-*

ical methods. Chapman and Hall. New York 2nd ed. Pp: 288.

11)

C. Kaya, D. Higgs, M. Ashraf, M.N. Alyemeni, P. Ahmad (2020). Integrative roles of nitric oxide and hydrogen sulfide in melatonin-induced tolerance of pepper (*Capsicum annuum* L.) plants to iron deficiency and salt stress alone or in combination. Physiologia plantarum, 168 (2020), pp. 256-277.

12) OECD, (2001). Guidelines for Testing of Chemicals. Acute Oral Toxicities up and down Procedure. 425: 1-26.

13) OECD, (2008). Test no. 425: Acute oral toxicity: Up-and-down procedure. OECD Publishing.

14) McBain, A. J., Ledder, R. G., Srinivasan, P. and Gilbert, P. (2004). Selection for high-level resistance by chronic triclosan exposure is not universal, Journal of Antimicrobial Chemotherapy, 53 (5), p: 772-777.

15) Saising J, Dube L, Ziebandt AK, Voravuthikunchai SP, Nega M, Götz F. (2012). Activity of Gallidermin on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. Antimicrob Agents Chemother.;56(11):5804-10.

16) Amaral, M. M., Coelho, L. R., Flores, R. P., Souza, R. R., Silva-carvalho, M. C., Teixeira, L. A., Ferreira-carvalho, B. T. and Figueiredo, A. M. S.(2005). The predominant variant of the Brazilian epidemic clonal complex of methicillin-resistant S. aureus has an enhanced ability to produce biofilm and adhere to invade airway epithelial cells. J Infect. Dis. 192:801-810.

17) Mason, R. L., Gunst, R. F. and Hess, J.L. (2003). Statistical design and analysis of experiments. Welly-Interscience, New Jersey.

18) CLSI (2013) . Clinical and Laboratory Standards Institute. http://clsi.org/membership/current/my-clsi/.

19) Seguin, JC, Walker RD, Caron JP, Kloos WE, George CG, Hollis R.J, Jones RN, Pfaller MA. (1999).Methicillin-Resistant Staphylococcus aureus Outbreak in a Veterinary Teaching Hospital: Potential Human-to-Animal Transmission. J. Clin. Microbiol.. 37(5): 1459-1463.

20) AL Shahwany, Ayyad W., Heba K. Tawfeeq, Shahad E. Hamed (2016). Antibacterial and Anti-biofilm Activity of Three Phenolic Plant Extracts and Silver Nanoparticles on *Staphylococcus aureus* and *Klebsiella pneumoniae*. Biomedicine and Biotechnology, Vol. 4, No. 1, 12-18.

21) Djenane D, Yangüela J, Derriche F, Bouarab L, Roncales P.(2012) Utilisation des composés de feuilles d'olivier comme agents antimicrobiens; application pour la conservation de la viande fraîche de dinde. Nat Technol.; 7:53-61.

22) Abirami P, Gomathinayagam M, Panneerselvam R. (2012). PreliminaryStudy on the antimicrobial activity of Enicostemmalittorale using different solvents. Asian Pacific J Trop Med.;5(7):552-555.

23) Lazar, V.(2011). Quorum sensing in

biofilms–How to destroy the bacterial citadels or their cohesion/ power? Anaerobe,. 17, 280-285.

24) Alakiali, A. Abdulsattar, Ayyad W. Al-Shahwany, Zubadia A. Lateef Ismell.(2022). The effectiveness of the Astragalus root phenolic extracts on mice blood profile. Journal of herbal medicine, Vol. 35. October.

25) Ncube B, Finnie JF, Van Staden J.(2012). *In vitro* antimicrobial synergism within plant extract combinations from three South African medicinal bulbs. *J Ethnopharmacol*. ;139:81-9.