

## Effect of Partially Purified Flavonoids from *Albizia lebbek* (L) Benth Leaves against Antibiotic-Resistant *Streptococcus* Isolates

Rawaa A. Khalaf<sup>1</sup>

Mohammed A. Fayidh<sup>2\*</sup>

Affiliation: <sup>1</sup>Biotechnology Research Center /Al-Nahrain University /Iraq

<sup>2</sup>Department of Biology/ College of Education for Pure Science Ibn-Al-Haitham/  
University of Baghdad/ Iraq

\*Correspondence: [mohammedcbt66@gmail.com](mailto:mohammedcbt66@gmail.com)

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### Abstract

**Background:** Plant flavonoids have strong biological activity, one of which is their antimicrobial activity. The design of this study, the content of flavonoids was determined in the leaves of the *Albizia lebbek* plant, which is considered one of the medicinal plants. **Materils and Methods:** Extraction of flavonoids from the leaves of *Albizia lebbek* .Qualitative determination of flavonoids using thin-layer chromatography (TLC) of *Albizia lebbek* leaf extract and quantitative analysis using high-performance liquid chromatography (HPLC) . **Results:** showed the appearance of spots of flavonoid groups. Kaempferol, while quantitative analysis using high-performance liquid chromatography (HPLC) showed the presence of Catechin, Gallic acid Epicatechin, Chlorogenic acid as two isomers a & b, Caffeic acid, Luteolin, Apigenin and Quercetin. Partially purified flavonoid extract from *Albizia* leaves showed a clear inhibition activity against 4 types of *Streptococcus* bacteria resistant to antibiotics: ampicillin, tetracycline, clarithromycin, clindamycin, and rifampin. **Conclusion:** These results encourage the possibility of using the active compounds of this plant as safe alternatives against pathogenic isolates of the genus *Streptococcus*. Other bacterial genera.

**Keywords:** flavonoids, *Albizia lebbek*, antibacterial activity, *Streptococcus*.

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## Introduction

Flavonoids are the largest group of phenolic compounds that are produced naturally in various parts of the plant either in free form or as glycosides. Many flavonoids have recorded different biological activities, including their antimicrobial activity, as the flavonoid compounds apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcone have proven strong anti-bacterial activity, as well as some of them, have anti-ulcer, anti-arthritic activities, anti-cancer, and others (1). Flavonoids are formed from two benzene rings separated by a propane unit, and flavonoids and flavonols are the most prevalent among all phenolic compounds (2). It also has antioxidant activity and provides protection against certain types of cancer. Bioflavonoids also have pharmacological effects such as the ability to inhibit histamine secretion, and platelet adhesion, block the inflammatory effects of hepatotoxicity, and act as a heart tonic (3) (4).

*Albizia lebeck* (L) Benth belongs to the Fabaceae family, Leguminosae, (5). *A.lebeck* is a fast-growing deciduous tree, which may reach an average height of 30 meters in rainforests, but usually, in maturity, it grows 15-20 meters in length and in diameter 2 until to more than 5 meters. With spreading and densely growing branches. Leaves pinnate compound peduncle 10-5 cm long, bearing 11-3 asymmetric pairs of leaflets. The entire inflorescence is "fluffy" in appearance, 60 mm in diameter, and yellow-green with a pleasant aroma. The tolerance of the *Albizia* tree to temperatures ranges between 5-50 °C. The time of flowering is between the months of July and August, while the fruits are collected during the winter season (6) (7).

Recent studies indicate that the leaves of the *Albizia* plant have antiseptic activity, anti-tuberculosis, relieving cough, and are useful for colds and chest and lung infections, (8). The leaves are used in traditional Indian medicine in the treatment of many diseases, including allergies, asthma, and bronchitis. And allergic rhinitis, gingivitis, toothache, and leprosy, all of which are caused by bacterial infections, confirm their effectiveness against various pathogens (9).

Despite the spread of the plant in many gardens, sidewalks, and residential complexes, and despite the huge amount of its plant parts. In Iraq, it did not receive much attention from researchers, as it contains biologically effective components that can be extracted throughout the year, so this study focused on investigating its effective components with the possibility of its use as a suggested alternative against antibiotic-resistant bacterial isolates, including *Streptococcus* Isolates, isolated from the respiratory tract.

## Materials and Working Methods

### Plant Material Collection

The leaves of *A. lebeck* (L.) Benth were collected at the beginning of September 2021 from the gardens of Baghdad University in Jadiriya. And it was classified (10) by Dr. Sakina Abbas Aliwi in the Department of Life Sciences/College of Science/ Mashhab University of Baghdad.

### Preparing Plant Extracts

The leaves were washed from the materials and dust attached to them using distilled water, then they were brushed and spread in a place with good ventilation and away from direct light, taking into account the constant stirring. After making sure that the plants were completely dry, they were ground using an Electric Blender, then filled into a clean, sterile dry glass container, the name of the plant was written on the container and kept in a dry, dark place until use (11).

### **Preparation of Partially-Purified Flavonoid Extract**

#### **Extraction of Flavonoids:**

Weigh 100 g of Albizia leaf powder and put it in a (1) liter glass beaker, then add (1000) ml distilled water acidified with HCL at 10% V/V. Reflex extraction was performed for 8 continuous hours to ensure that the glycosidic bond was broken of flavonoids and obtained the non-glycan fraction), then the extract was filtered and the filtrate was cooled. The non-sugar fraction, which is the active part of the flavonoids, was extracted by an organic solvent from ethyl acetate by adding 50 ml of it for every 50 ml of the extract. The process was repeated three times using a separating funnel, then the acetate layer was collected in the separating funnel again and an equal amount of distilled water was added to it to remove the residual acid HCL used in the extraction. Finally, the acetate layer was dried using a rotary evaporator at 45°C. A final weight of 2.5 gm of flavonoid extract was obtained and kept in an airtight container at 4°C until use (12).

#### **Qualitative Determination of Flavonoids Using Thin Layer Chromatography (TLC):**

Samples of the standard flavonoids solution were prepared and included Rutin, Quercetin, Luteolin and Kaempferol, then samples of them were loaded on aluminum plate by thin layer chromatography (TLC), covered with 0.1 mm thick material of silica 60, representing the stationary phase separation process stained. As for the mobile phase, a solution of ethyl acetate: formic acid was used at a ratio of 90:10 (13).

#### **Determination of Flavonoids using High Performance Liquid Chromatography (HPLC):**

HPLC high-performance liquid chromatography was used to determine the quality, percentage, and concentration of flavonoids in the leaves of the Albizia plant through their retention time per minute. Standard solutions were used for each of Rutin, Quercetin, Coumarin, Coumarin, Apigenin, Kaempferol, Myricetin, and Catechin, in addition to the flavonoid extract, and the analysis conditions were adopted according to (14).

Sample migration was carried out using an HPLC device (supplied by Shimadzu/ Japan) at a wavelength of 340 nm. The deportation conditions were according to the following criteria: Mobile phase = A: acetonitrile (CAN) 0.5% Formic acid (%70,30%), B: CAN: 0.5% Formic acid (70 %, 30%), Column = Reverse phase ODS C18 (150 × 4.6 id) mm, 6 AM particle size, Flow rate = 0.7ml/min.

### **Bacterial Isolates Used in the Study**

Twenty-three isolates of Streptococcus bacteria were diagnosed with VITEK 2 Compact system, isolated from patients suffering from respiratory tract infections. Sixteen isolates that are resistant to antibiotics were selected for the tests of this study.

### **Preparation of Bacterial Inoculate**

Brain heart infusion broth medium was used for the purpose of preparing the isolates for testing. The medium was distributed in test tubes of 9 ml per tube and sterilized by autoclave. The inoculum was prepared for each bacterial isolate by transferring part of each of the pure and grown isolates on blood agar medium to the tubes designated for them, and the tubes were incubated at a temperature of 37 ° C for 24 hours.

### **Bacterial Sensitivity to Antibiotics**

The Kirby-Bauer Disk Method was used to test the efficacy of the extracts against the study isolates. The isolates were cultured on a nutrient agar blood medium and incubated for 24 hours under 10% CO<sub>2</sub> condition. The suspension was prepared by inoculating a tube containing physiological saline with a pure colony of test isolates (the growth density of which is equal to that of tube No. 5 ( $1.5 \times 10^8$ ) from previously prepared McFarland tubes. 0.1 ml of the suspension growth was spread over Muller-Hinton solid medium. The plates were allowed to dry at the laboratory temperature, then distributed the antibiotics discs which are shown in (Table 3) on the surface of the solid culture media with equal dimensions (30 mm apart). The plates were incubated anaerobically at 37 ° C for 24 hours. The results were recorded by measuring the area of inhibition around the discs. The results were compared with the tables Clinical and Laboratory Standards Institute—CLSI (15).

### Inhibitory Activity of Extracts against Streptococcus SP Isolates

The inhibitory activity of the prepared extracts was tested against four isolated bacteria of the genus Streptococcus SP, with concentrations ranging from 6.25, 12.5, 25 ,50 ,100% of the crude extract. The diffusion method by drilling of agar was used with a medium of Mueller-Hinton agar

Bacteria were diluted for each bacterial isolate compared to McFarland tube no. (5) To obtain the appropriate dilution for each bacterial isolate. 0.1 µL of the dilution of each bacterial isolate was transferred to dishes containing 20 mL of Muller-Hinton agar solid medium, spread with an L-Shape diffuser, and the dishes were left for 1 hour in the Hood culture cabinet. Drills were made on the surface of the acre using a 5 mm diameter cork borer with equal distances between one hole and another. Graded concentrations of (25, 50,100) % of the prepared extracts were prepared. 100 µl of each concentration of the extracts under study were placed in each hole, left for one hour in the Hood, and then incubated in the incubator at 37 °C for 24 hours. The diameters of bacterial growth inhibition (mm) were measured using a ruler after the incubation period was completed (16).

### Results and Discussion

#### Qualitative Determination of Flavonoids Using Thin Layer Chromatography (TLC):

The types of flavonoids extracted from Albizia leaves were investigated by thin-layer chromatography (TLC). Spots of different dimensions were obtained when the plate was exposed to UV rays at a wavelength of 254 nm. The Spots represent samples of standard flavonoids and the partially purified extract of Albizia leaves.

In Figure (1), each spot on the TLC plate represents a standard flavonoid compound (A, L, Q, R), in addition to the partially purified flavonoid compound in the leaf extract (F) and the aqueous extract (W).The retention factor (Rf value) for each flavonoid as well as the flavonoid compounds in the leaf extract was calculated in Table (2). By comparing the Rf values of the standard flavonoids with the flavonoids diagnosed in the leaves of the plant, measured under the same conditions, the presence of compounds similar to the standard flavonoids of Quercetin and Luteolin, Apigenin, was observed in the leaf extract and it was devoid of Rutin. While other spots appeared in the aqueous extract (W) not compatible with the approved standard compounds, non-flavonoids may have similar properties when examined under the same migration conditions on TLC.

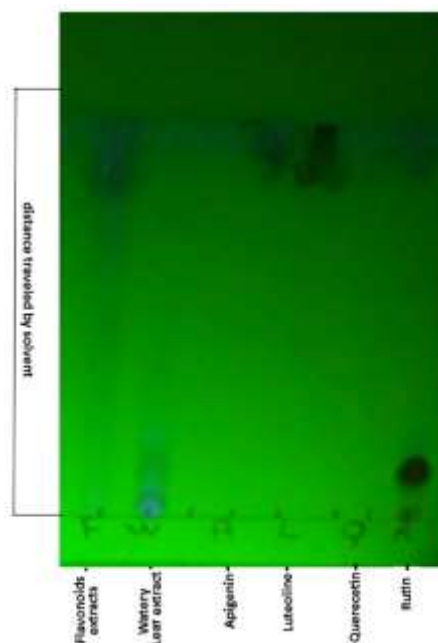


Figure (1): Mobile phase of thin layer chromatography for separating standard compounds (A, L, Q, R) and separated flavonoids from Albizia Leaves.

**Table (1): Rf values of the standard flavonoids identified in the flavonoid extract of Albizia leaves.**

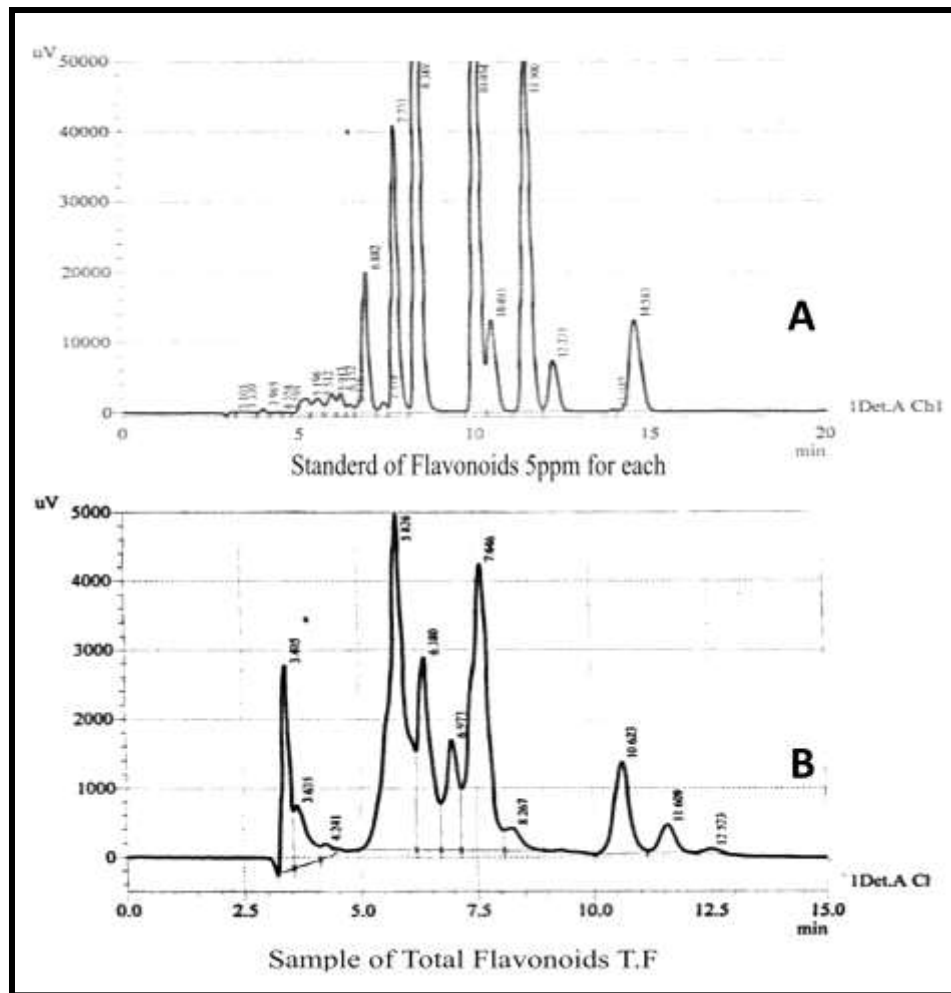
Standard flavonoids	Total flavonoids extract	Relative flow rate for standard materials ( <i>R<sub>f</sub></i> )
1-Apigenin (Ap)	0.91	0.91
2-Luetolin	0.84	0.84
3-Querecetin(Q)	0.82	0.82
4-Rutin (R)	-	0.143

**Determination of Flavonoids Using High Performance Liquid Chromatography (HPLC):**

The results of the standard analysis of flavonoids using high-performance liquid-phase chromatography (HPLC) (Fig. 2-A), showed that the flavonoid extract of the plant leaves contained many active compounds, including Rutin, Myrecetin Catechin, Coumarin, Apigenin, Kaempferol, Isorhamnetin, and Quarecitin. The retention time for each of them was minutes in (0.01, 4, 8, 10, 15, 20) respectively (Fig. 2-B), as the retention time *R<sub>t</sub>* for these compounds coincides with the retention time for the standard compounds and as fixed in Table (2) (14).

**Table (2): The main flavonoid compounds shown by HPLC chromatographic analysis, showing the concentration of each of them with their retention time**

Flavonoid	Concentration ( $\mu\text{g/ml}$ )	<i>R<sub>t</sub></i> (min)	Area
Rutin	0.62	6.973	30275
Myrecetin	1.03	7.646	106348
Catechin	0.03	8.267	7580
Coumarin	undetected	undetected	Undetected
Quarecitin	0.70	10.623	30166
Apigenin	0.05	11.609	9426
Kaempferol	0.06	12.523	1649
Isorhamnetin	undetected	undetected	undetected



**Figure (2) A- Chromatogram of analysis using HPLC for standard samples of compounds expected to appear in Albizia leaf extract. B - Total flavonoid compounds in leaf extract and their retention time using HPLC**

HPLC technology showed the presence of 6 flavonoids in the leaves of the Albizia plant: Myrecetin and Catechin, Quarecitin, Apigenin, Kaempferol, and Rutin. These compounds are considered effective against a wide range of organisms, and this was confirmed by numerous studies on plant extracts rich in flavonoids for different types of plants (17).

#### **Antibiotic Susceptibility Patterns of *Streptococcus***

The sensitivity of 22 disease isolates obtained during this study, belonging to six species of the genus *Streptococcus*, including *S. viridans*, *S. pyogenes*, *S. agalactiae*, *S. pneumonia*, *S. parasangainia*, and *S. sverars*, Table (2), these isolates were tested with Selection of nine antibiotic tablets that are approved as a treatment for infection with these bacterial isolates. The antibiotics are shown in Table (3) including Ceftriaxone, Doxycycline, Ampicillin, Tetracycline, Clarithromycin, Levofloxacin, vancomycin, Rifampin, and clindamycin which are shown in Table (3).

The isolates of *S. parasangainia* and *S. salivarius* showed high sensitivity to all antibiotics with a percentage of 100%. While the other four species of the genus *Streptococcus* showed varying rates of resistance to the antibiotics under study. *S. agalactiae* showed high resistance of 100% to ampicillin, tetracycline, clarithromycin, vancomycin, rifampin, and clindamycin, while *S. pneumonia* showed 100% resistance to four antibiotics, doxycycline, tetracycline, rifampin and doxycycline, and resistance in lower proportions to the rest of the antibiotics (18). As for the two isolates, *S. viridans* and *S. pyogenes*, their sensitivity to the tested antigens was distributed between more than 50% to less than that, according to the severity and type of the antigen (19) Table (3) shows the details of the tested isolates against the antibiotics used in the study and the sensitivity or resistance ratios for each antibiotic.

**Table (3): Results of the antibiotic susceptibility test against *Streptococcus* isolates**

Bacterial isolates (No. of isolates)		Antibiotic								
		CRO	DO	AM	TE	CLR	LEV	VA	RA	DA
<i>S. viridans</i> (8) isolates	S	3(37.5%)	5(62.5%)	3(37.5%)	3(37.5%)	3(37.5%)	6(75%)	5(62.5%)	2(25%)	4(50%)
	R	5(62.5%)	3(37.5%)	5(62.5%)	5(62.5%)	5(62.5%)	2(25%)	3(37.5%)	6(75%)	4(50%)
<i>S. pyogenes</i> (5) isolates	S	3(60%)	4(80%)	1(20%)	2(40%)	1(20%)	4(80%)	5(100%)	4(80%)	3(60%)
	R	2(40%)	1(20%)	4(80%)	3(60%)	4(80%)	1(20%)	0(0%)	1(20%)	2(40%)
<i>S. agalactiae</i> (2) isolates	S	1(50%)	2(100%)	0(0%)	0(0%)	0(0%)	2(100%)	0(0%)	0(0%)	0(0%)
	R	1(50%)	0(0%)	2(100%)	2(100%)	2(100%)	0(0%)	2(100%)	2(100%)	2(100%)
<i>S. pneumonia</i> (5) isolates	S	3(60%)	0(0%)	1(20%)	0(0%)	2(40%)	5(100%)	1(20%)	0(0%)	0(0%)
	R	2(40%)	5(100%)	4(80%)	5(100%)	3(60%)	0(0%)	4(80%)	5(100%)	5(100%)
<i>S. parasangainia</i> (1) isolate	S	1(100%)	1(100%)	1(100%)	1(100%)	0(0%)	1(100%)	1(100%)	1(100%)	1(100%)
	R	0(0%)	0(0%)	0(0%)	0(0%)	1(100%)	0(0%)	0(0%)	0(0%)	0(0%)
<i>S. salivarius</i> (1) isolate	S	1(100%)	1(100%)	1(100%)	1(100%)	0(0%)	1(100%)	1(100%)	0(0%)	0(0%)
	R	0(0%)	0(0%)	0(0%)	0(0%)	1(100%)	0(0%)	0(0%)	1(100%)	1(100%)

**CRO: Ceftriaxone, DO: Doxycycline, AM: Ampicillin, TE: Tetracycline, CLR: Clarithromycin, LEV: Levofloxacin**

**VA: Vancomycin, RA: Rifampin, DA: Clindamycin. S- sensitive, R-resistant.**

The conclusion of the results of this test is consistent with what was found by (20) of the high rates of resistance against beta-lactams, which are Ampicillin and Ceftriaxone, as *S. pneumoniae* bacteria were resistant to 80% of Ampicillin.

The presence of high resistance to beta-lactam antagonists by these bacteria may be attributed to beta-lactamase enzymes that analyze the amide bond in the quaternary beta-lactam ring. The results of the current study were also in agreement with what was found by (21).

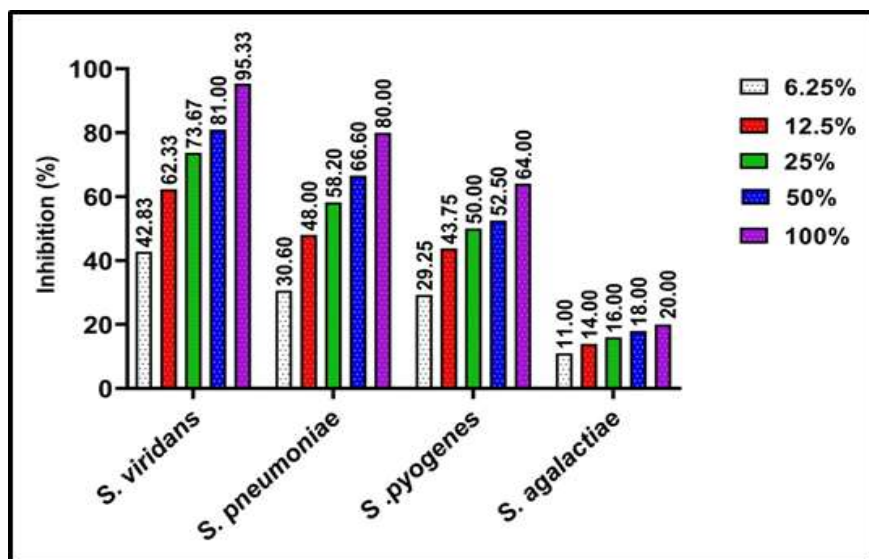
The results of this test are also consistent with the findings of (22), which indicated that *S. galactiae* isolated from a hospital in Brazil were sensitive to Ceftriaxone and Levofloxacin by 100% and 99.3%, respectively, while the Levofloxacin inhibited all isolates of *S. pyogenes*.

**Antibacterial Activity of Flavonoid Extract against Bacterial Isolates in this Study**

When testing the effect of partially purified flavonoid extract from Albizia leaves against most antibiotic-resistant isolates in previous experiments, namely, *S. viridans*, *S. pyogenes*, *S. galactiae*, and *S. pneumoniae*.

Partially purified flavonoid extract at 100% concentration showed antibacterial activity for the growth of these isolates. The concentration of 100% of flavonoid extract gave the highest inhibition for *S. viridans*, with a percentage of 95.33%, followed by *S. pneumoniae* with 80%, then *S. pyogenes* with 64%, while the inhibition percentage was 20% for *S. galactiae*. When testing the effect of other extract concentrations (50%, 25%, 12.5%, and 6.25%), it is noted that the inhibition rates for the same isolates decrease with a decrease in the concentration, as shown in Figure (3),(23).

It is known that flavonoids are synthesized by plants in response to microbial infection, so the obvious bacterial inhibition in the experiments of this study can be attributed to the flavonoids purified from the leaves of the Albizia plant. (5).

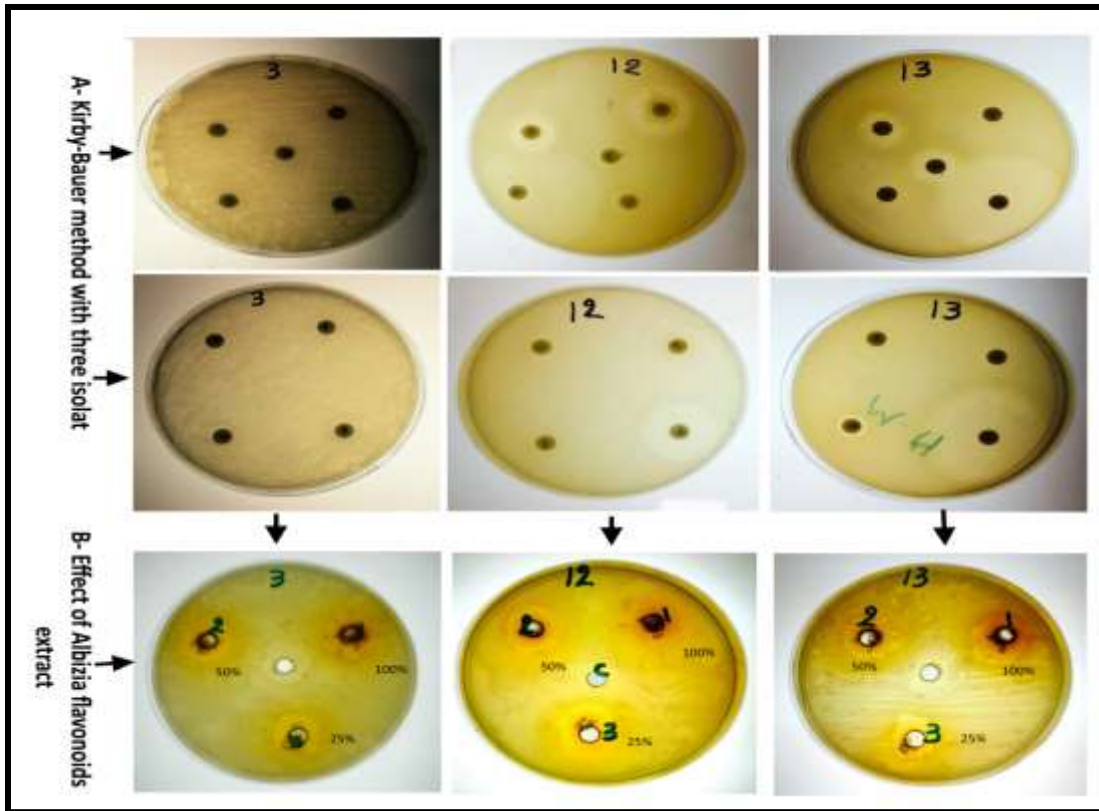


**Figure (3): shows the inhibition percentage of partially purified flavonoid extract with four types of Streptococcus bacteria**

Figure (4) shows the sensitivity of three bacterial isolates of the study, which were tested by the sensitivity tablets and shown in part (A3), compared with the effect of the purified leaf extract of flavonoids on the same bacterial isolates (3B), which showed high resistance to the antibiotics used in this study. Isolate number 3 represents *S. viridans*, isolate number 12 refers to *S. pyogenes*, and isolate number 13 represents *S. galactiae*.

From The figure shows a clear effect of the flavonoid extract partially purified from the leaves of Albizia plant, especially with the concentration of 100% of the extract, compared to the antibiotic tablets that the same isolates showed resistance to. This gives evidence of the possibility of adopting Albizia extracts, including flavonoid extracts, as alternatives to antibiotics, to which resistance is increasing with time (24).





**Figure (4)-A: Results of susceptibility testing for three Streptococcus isolates using the Kirby-Bauer method. B: Image showing the inhibition of partially purified flavonoid extract of the same antibiotic-resistant bacterial isolates.**

**3-S. viridans, 12 - S. pyogenes, 13- S. agalactia.**

Flavonoids can act by several mechanisms against bacteria, as they can interfere with the lipid bilayers by causing bacterial membrane rupture and inhibiting many processes such as biofilm formation, cell envelope synthesis, DNA synthesis, electron chain transfer, and ATP synthesis (25). For example, catechin, epicatechin, and epigallocatechin gallate, work to kill bacteria because of their high toxicity (26). Quercetin may inhibit the biofilm formation of bacterial cells producing it, while myricetin and quercetin inhibit DNA replication (25).

**Conclusions**

The results of the qualitative and quantitative detections of the flavonoid extract in the leaves of Albizia by TLC and HPLC analysis showed that it contains clinically important active compounds such as Rutin, Myrecetin, Catechin, Quarecitin, Apigenin, Epicatechin, Catechin, Gallic acid, Chaferogenic acid, Caffaic two a&b, Luetolin, Apigenin, Quercetin and in varying proportions. On the other hand, the extract showed an inhibitory effect of bacterial growth depending on the concentration ratios. It can be concluded from the current study that flavonoids purified from the leaves of the Albizia plant have an anti-bacterial effect; it can be a suitable alternative for many bacterial isolates that show resistance to some antibiotics.

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تأثير مركبات الفلافونويد المنقاة جزئياً من أوراق *Albizia lebbeck (L) Benth* ضد عزلات *Streptococcus*  
المقاومة للمضادات الحيوية

رواء عدنان خلف محمد عباس فياض

مركز بحوث التقنيات الاحيائية / جامعة النهرين -العراق  
قسم علوم الحياة/ كلية التربية للعلوم الصرفة ابن الهيثم / جامعة بغداد -العراق  
\*Correspondence: mohammedcbt66@gmail.com

الخلاصة

**خلفية البحث :** تمتلك الفلافونيدات النباتية نشاطاً بيولوجياً قوياً، ومنها فعاليتها المضادة للحياة المجهرية. المواد وطرائق العمل : في هذا البحث، تم تحديد محتوى الفلافونيدات في اوراق نبات *Albizia lebbeck* الذي يعتبر من النباتات الطبية ودراسة تأثيرها على بكتريا *Streptococcus*.  
**النتائج :** اظهر التقدير النوعي للفلافونيدات باستخدام كروموتوغرافيا الطبقة الرقيقة (TLC) لمستخلص اوراق نبات *Albizia lebbeck* ظهور بقع لمجاميع الفلافونويد انها تمثل مركبات Kaempferol, Apigenin, Quercetin, Catechin, and Rutin Myrecetin , بينما أظهر التحليل الكمي باستخدام تقنية كروموتوغرافيا السائل عالي الاداء (HPLC) وجود مركبات Catechin, Gallic acid Epicatechin, Chlorogenic acid , Luteolin, Apigenin and Quercetin as two isomers a & b, Caffeic acid, Luteolin, Apigenin and Quercetin الالبيزيا فعالية تثبيط واضحة ضد 4 انواع مرضية من بكتريا *Streptococcus* من المقاومة للمضادات الحيوية tetracycline , ampicillin ، rifampin·clindamycin·clarithromycine . **الاستنتاج:** هذه النتائج تشجع على امكانية استخدام المركبات الفعالة لهذا النبات كبديل آمن ضد العزلات المرضية لجنس *Streptococcus* او الاجناس البكتيرية الاخرى.

**الكلمات المفتاحية:** مركبات الفلافونويد ، البيزيا لبيك , نشاط مضاد للبكتريا، *Streptococcus*.