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Evaluation of the antibacterial effects of *Eucalyptus camaldulensis* L., *Glycyrrhiza glabra* L. and *Morus nigra* L. extracts against some pathogenic bacteria *in vitro*

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Abstract

This study aimed to test the effect of using different concentrations of three different plants extracts to inhibit the growth of gram negative and gram positive bacteria by two technics. *Eucalyptus camaldulensis* bark, *Glycyrrhiza glabra* rhizomes and *Morus nigra* leaves ethanolic extract at (0,20,30,40 and 50 mg/ml) were used. The antimicrobial activity and the biofilm inhibition assay used with these extracts showed positive effect in inhibiting the growth of bacteria. *E.amaldulensis* extract showed the higher effect than *G. glabra* and *M.nigra* extracts in antimicrobial activity assay, while the effect of *E. camaldulensis* extract in biofilm inhibition assay was higher than *G. glabra* that was higher than *M. nigra* extracts for both gram negative and positive bacteria. These results confirmed the effect of *E. camaldulensis, G. glabra* and *M. nigra* extracts on retarding the growth of bacteria.

Keywords: Antibacterial, *Eucalyptus camaldulensis* L., *Glycyrrhiza glabra* L., *Morus nigra* L.

قياس التأثيرات المضادة للبكتريا لمستخلصات اليوكالبتوس و السوس و التوت الأسود ضد بعض انواع البكتريا الممرضة مختبريا

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الخلاصة

هدفت هذه الدراسة الى اختبار تأثير استخدام تراكيز مختلفة من ثلاثة مستخلصات نباتية لتثبيط نمو البكتريا السالبة لصبغة كرام و البكتريا الموجبة لكرام بطريقتين . تم استخدام المسخلص الكحولي لقلف اليوكالبتوس و رايزومات السوس و اوراق التوت الأسود بالتراكيز (0 و 20 و 30 و 40 و 50 ملغرام لكل مللتر). اظهرت النتائج باستخدام قياس الفعالية البايولوجية و تكوين الغشاء الحيوي وجود تأثير ايجابي لهذه المستخلصات في تثبيط نمو البكتريا. كان لمستخلص اليوكالبتوس التأثير الاعلى من السوس و التوت الاسود في قياس الفعاللية البايولوجية، في حين اظهر مستخلص اليوكالبتوس في قياس تثبيط تكوين الغشاء الحيوي تأثيرا اكبر من السوس و بدوره كان اكثر من التوت الاسود لكل من البكتريا السالبة و الموجبة لصبغة كرام. اثبتت هذه النتائج التأثير الفعال لمستخلصات اليوكالبتوس و عرق السوس و التوت الاسود في تثبيط نمو البكتريا.

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Introduction

Plants are important sources of potentially useful structures for the development of new chemotherapeutic agents. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids and flavonoids that have improved *in vitro* to have antimicrobial properties. The first step towards this goal is the *in vitro* antimicrobial activity assay [1, 2]. Currently, research and development of new drugs from natural resources in a systematic and strategic manner has become the global trend. Natural product derived medicines are widely used and account for more than 30% of therapeutic agents presently prescribed in clinics [3].The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants [4].

Blue gum (*Eucalyptus camaldulensis* L.) family Myrtacea, Eucalyptus tree is evergreen with a height 25-60 meters, it is a fast growing tree and it is native to Australia. The leaves are grayish green color, the bark is light brown colored, this plant first began to be used in 18th century, it has been considered one of the best plant species for the treatment of respiratory system and skin because its antimicrobial properties of the essential oils that contains and widely used in cosmetics industry due to the eucalyptol which is an aromatic component [5]. The leaves contain flavonic compounds such as eucalyptin, quercetin and rutin, also contain phenolic compounds such as gallic, ferulic, caffeic acids and a complex of phenolic heteroside known calyptoside [6].

Liquorice is the common name of *Glycyrrhiza glabra* L, family Leguminoceae from which a somewhat sweet flavor can be extracted. The liquorice plant is a legume that is native to southern Europe, India and parts of Asia. It is a herbaceous perennial growing to one meter in height with pinnate leaves about 7-15 cm long. The roots are stoloniferrous and its scent comes from a complex and variable combination of compounds of which anethol is the most minor component. Much of the sweetness in liquorices comes from glycyrrhizin which has a sweet taste 30-50 times the sweetness of sugar. The compound glycyrrhizin found in liquorices is an antiviral and antimicrobial compound [7].The isoflavene glabrene and the isoflavene glabridine, found in the roots of liquorices, are phytoaestrogens [8].

Black Mulberry (*Morus nigra* L.) family Moraceae is widely distributed in Asia, Europe, North and South America and Africa. It is a deciduous tree growing to 13 meters and has a dense head of branches, springing from a rough trunk. Leaves are thick, blunt toothed and lobed with short, stiff hairs on the upper surface. It is an economically important plant used for sericulture, as a feed for the domesticated silkworm *Bombyx mori* L. [9]. The decoction of leaves possesses blood purifying properties, reduces fever and is diuretic. The bark of Morus nigra L. was reputed to be used to expel tap worm and its extracts have been reported to have antibacterial and antifungal activity. The extracts of fruit were reported to have a protective action against peroxidative damage to biomembranes and biomolecules [10].

Biofilm-adherent colonies of bacteria surrounded by a matrix of extracellular polymer substance (EPS) are the microbial lifestyle in the environment [11]. The role of the biofilm is to adhere to a biotic surfaces, the epithelia of multicellular organisms and the interfaces such as that between air and water [12]. Surface adhesion of bacteria is an essential step for bacteria to arrange themselves favorably in the environment [13].

Some bacterial biofilms have been reported to have positive effects on food chains, sewage treatment plants, to eliminate petroleum oil / spillage from the oceans [14]. Now the biofilm is considered as a major goal for the pharmacological development of drugs. A biofilm serves to promote bacteria persistence by resisting antibiotic treatment and host immune responses [15].

Materials and Methods

Plant materials

Fresh and healthy bark of *Eucaylptus camaldulensis*, rhizomes of *Glycyrrhiza glabra* L. and leaves of *Morus nigra* L. plants were collected from different regions of Baghdad. These parts of plants were washed by tap water followed by succession washing in distilled water then left to dry in shade by air at room temperature for five days. The dried parts of plants were transferred to the blender to be grind to its powder.

Crude extractions of plants

Dried plant materials was extracted by taking (50g) powder of each plant separately with (250ml) of ethanol (99 %) in 500ml conical flask, then put in thermoshaker at 150 rpm for 24 hours at 25^{o}C,

then filtered through Whatman filter paper No.1 and concentrated by a rotary vacuum evaporator. Stock solutions was made of 20,30,40,50 mg/ml for each sample and stored at 20^{o}C until use [16].

Microbial studies

Bacterial isolates

Bacterial strains were obtained from different sources, *Escherichia coli* obtained from Urinary tract infection (UTI) patients of Yarmook hospital in Baghdad–Iraq, *Klebsiella pneumoniae*, *Salmonella* spp, *Staphylococcus aureus* and *Staphylococcus epidermidis* were obtained from department of Biology/college of science (University of Baghdad), while *Streptococcus mutans* obtained from Microbiology laboratory/college of Dentist (University of Baghdad).

Microbial activity

Petri dishes prepared with Muller-Hinton Agar, the bacterial isolates were cultured on the media by streaking with sterile swab, then four wells of 6 mm diameters were punched into the medium and filled with the gradient concentrations for each plant, while the well made in the center contained the control (absolute ethanol). Gradient concentrations of plant extracts were added with 100µl in each well using sterile micropipette. The plates were incubated at 37^{o}C for 24hrs., inhibition zones were measured with millimeters [17].

Biofilm formation in the presence of plant extracts All studied bacteria that isolated from fresh brain heart infusion agar were inoculated in 10 ml of trypton soya broth tube, then incubated over night at 37^{o}C. The tubes after incubation were diluted 1:100 with fresh broth, and inoculated the wells of sterile flat bottom microtiter plate with 200 µl of cultured broth as a positive control. Negative control were made by using sterile broth without culture, then the effect of plant extracts were showed on biofilm.

Another wells were filled with 100 µl of cultured broth and 100 µl of plant extract at 6.25mg/ml , because the other concentrations (50,40,30,20mg/ml) gave high false reading due to the interference of the pigments of each extract with the stain. The plate is incubated at 37^{o}C for 24 hours and the floating bacteria were removed by gentle tabing then washed with distilled water, the plate was stained with 0.1 % crystal violet for 10 minuets at 25^{o}C, then the plate was washed with 200 µl 96 % ethanol for 10 minuets to prepare the plate for reading with micro plate reader (ELISA) at 630 nm [18]. Inhibition mediated reduction of biofilm formation was calculated by the following formula [19]:

$$\% \text{ of biofilm inhibition} = \frac{\text{O.D. of control \_ O.D. of treatment}}{\text{O.D. of control}} \times 100$$

Results and discussion

The results of microbial activity revealed that all the plants extracts at all cocentrations had inhibiting effects on the growth of different strains of bacteria in different ways.

E.camaldulensis bark extract inhibited the growth of gram negative bacteria *Salmonella* spp. and *klebsiella pneumoniae* more than *Escherichia coli*,it was obvious at 50 mg/ml treatment and 40,50mg/ml for *Salmonella* spp., while the inhibiting effect on gram positive bacteria *Streptococcus mutans* was higher at 50mg/ml treatment more than *Staphylococcus aureus* and *Staphylococcus epidermidis* (Table-1, Figures- (1, 2).

The effect of this plant may be due to its content of essential oils that were considered as natural antibiotics for several infectious diseases caused by germs [20], or to its content of phenols, flavonoids and tannins that had an antibacterial effect [21].

Table 1- Inhibiton zones (mm) caused by antimicrobial activity of *E. camaldulensis* bark extract.

Bacterial strains	*E. camaldulensis* L. ethanolic extract concentrations (mg/ml)				
	0	20	30	40	50
Escherichia coli	0	12	15	15	16
Klebsiella pneumoniae	0	13	13	16	20
Salmonella spp.	0	11	12	20	20
Staphylococcus aureus	0	13	15	18	20
Staphylococcus epidermidis	0	15	15	18	20
Streptococcus mutans	0	11	16	18	22

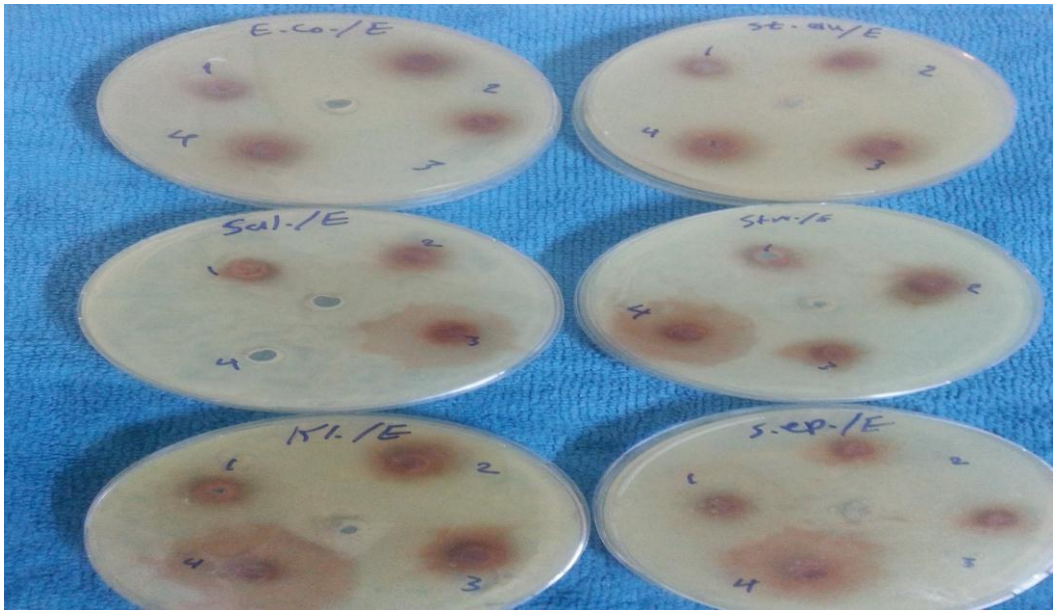

Figure 1- The antimicrobial activity of *E. camaldulensis* bark extract at the concentrations : 0 in the center, 1(20), 2(30), 3(40), 4(50mg/ml) against different strains of bacteria.

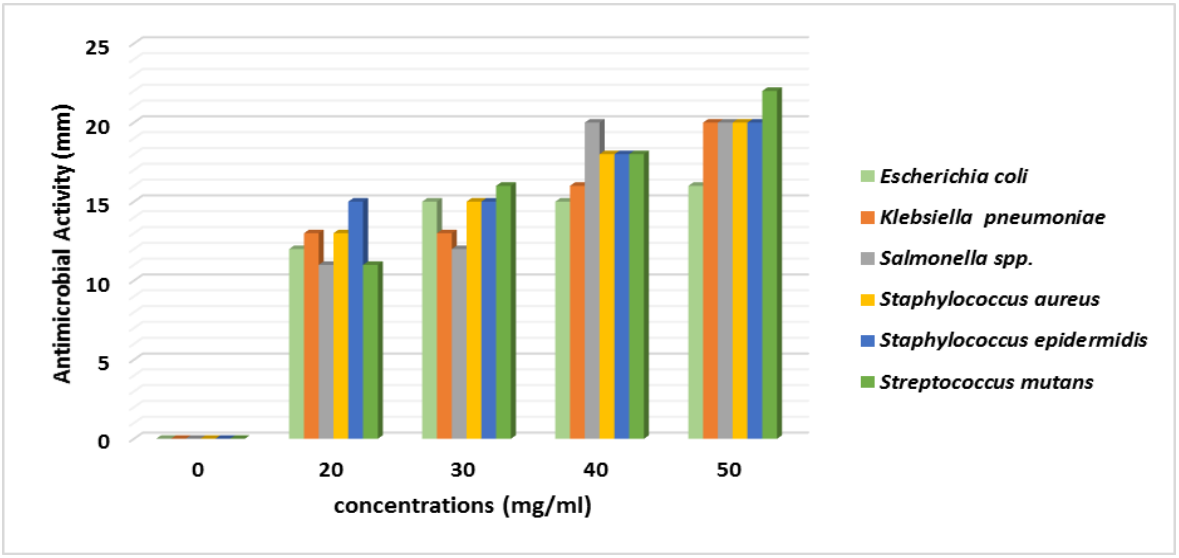

Figure 2- The antimicrobial activity of *E. camaldulensis* bark extract against different strains of bacteria.

G. glabra rhizomes extract inhibited the growth of gram negative bacteria *Salmonella* spp. more than *Klebsiella pneumoniae* that was more than *Escherichia coli* at 50mg/ml then 40 then 30 then 20mg/ml, while the inhibiting effect on gram positive bacteria *Streptococcus mutans* was more than *Staphylococcus aureus* and *Staphylococcus epidermidis*, it was obvious at 50mg/ml then 40mg/ml then 30mg/ml then 20mg/ml (Table- 2, Figure- (3, 4). *G. glabra* has been used for centuries as a herbal therapeutic substance for its wide ranging therapeutic properties. The plant has been clinically employed for its antibacterial, antifungal and antioxidant effect [22, 23].

Table 2- Inhibition zones (mm) caused by antimicrobial activity of *G. glabra* L. rhizomes extract.

Bacterial strains	*G. glabra* L. ethanolic extract concentrations (mg/ml)				
	0	20	30	40	50
Escherichia coli	0	9	9	10	10
Klebsiella pneumoniae	0	9	10	11	14
Salmonella spp.	0	10	11	15	15
Staphylococcus aureus	0	10	10	12	12
Staphylococcus epidermidis	0	10	10	11	12
Streptococcus mutans	0	14	15	15	16

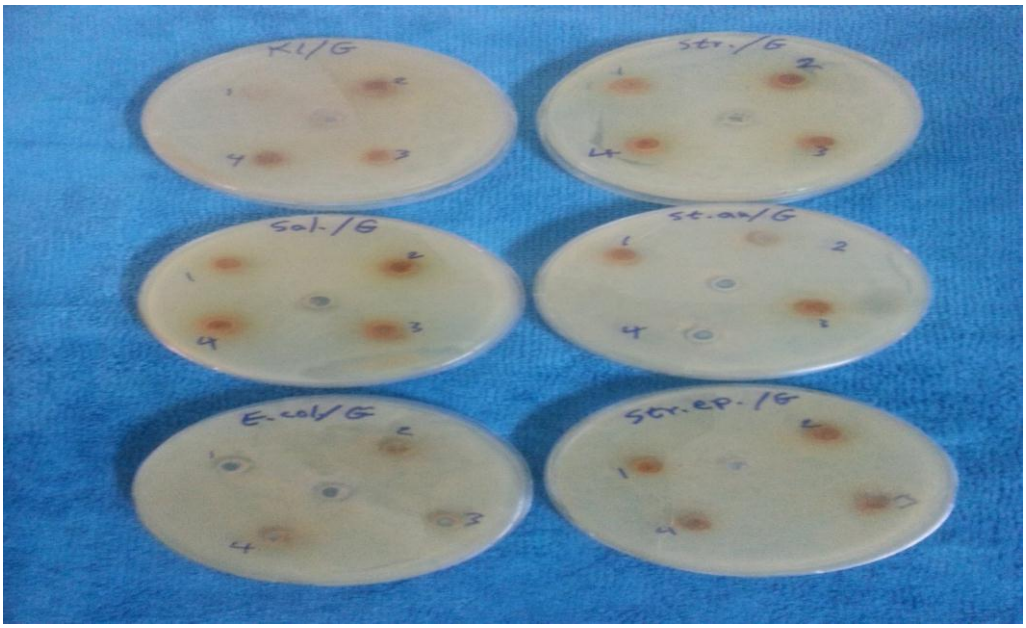

Figure 3- The antimicrobial activity of *G. glabra* rhizomes extract at the concentrations : 0 in the center, 1(20), 2(30), 3(40), 4(50mg/ml) against different strains of bacteria.

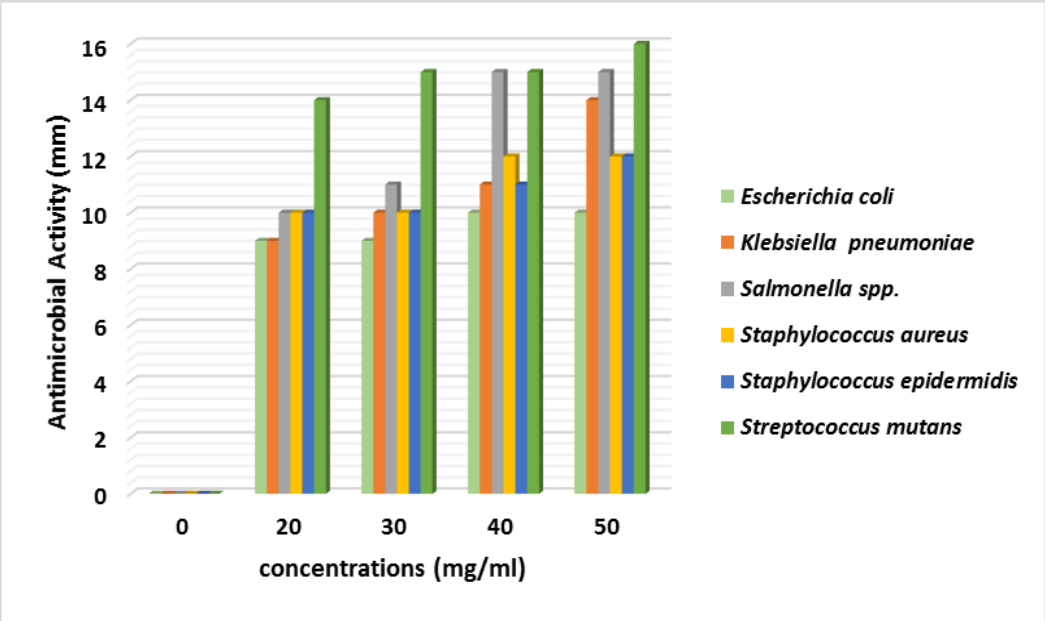

Figure 4-The antimicrobial activity of *G. glabra* rhizomes extract against different srains of bacteria.

M. nigra extract inhibited the growth of gram negative bacteria *Escherichia coli* that was more than *Klebsiella pneumoniae* that was more than *Salmonella* spp. at 40mg/ml and 50mg/ml, while the inhibiting effect on gram positive bacteria *Streptococcus mutans* and *Staphylococcus aureus* was more than *Staphylococcus epidermidis* at 50mg/ml, there was almost a same inhibiting effect on *Streptococcus mutans* and *Staphylococcus epidermidis* at 20mg/ml, 30mg/ml and 40mg/ml treatment (Table- 3, Figure-(5, 6). There are many phenolic compounds that have antioxidant and antibacterial properties found in all parts of this plant [24, 25].

Table 3- Inhibition zones (mm) caused by antimicrobial activity of *M. nigra* L. leaves extract.

Bacterial strains	*M. nigra* L. ethanolic extract concentrations (mg/ml)				
	0	20	30	40	50
Escherichia coli	0	12	12	23	20
Klebsiella pneumoniae	0	12	13	15	15
Salmonella spp.	0	11	12	12	13
Staphylococcus aureus	0	13	12	10	18
Staphylococcus epidermidis	0	15	16	16	17
Streptococcus mutans	0	15	15	16	18

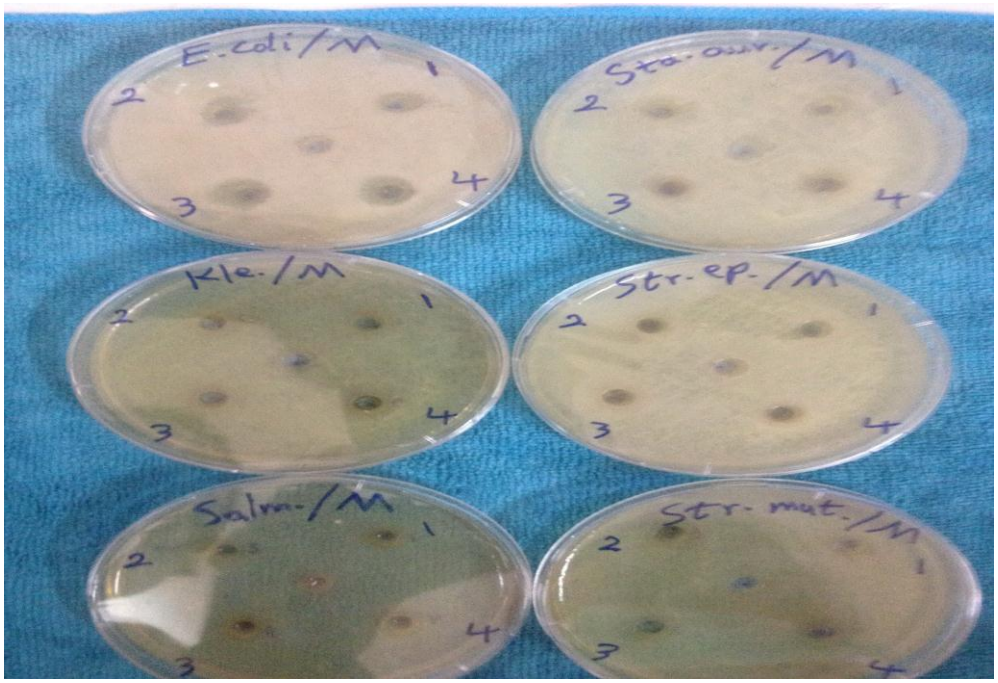

Figure 5- The antimicrobial activity of *M. nigra* leaves extract at the concentrations : 0 in the center, 1(20), 2(30), 3(40), 4(50mg/ml) against different strains of bacteria.

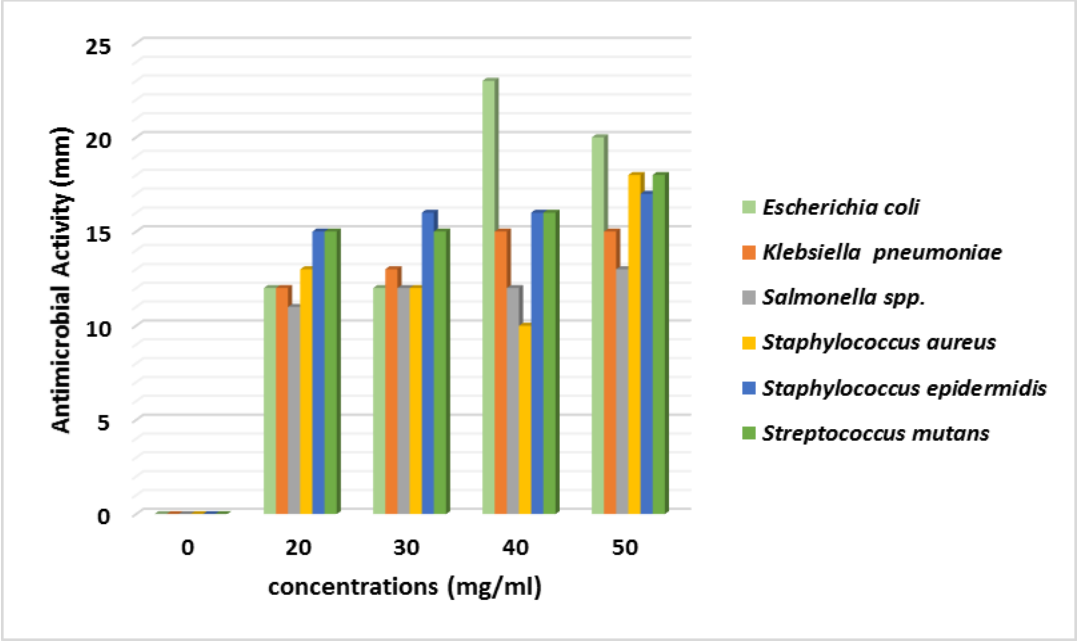

Figure 6-The antimicrobial activity of *M. nigra* leaves extract against different srains of bacteria.

Increased antimicrobial activity accompanied with increasing the concentrations of plant extract, and the *E. camaldulens* bark had the most inhibiting effect on bacterial growth than *G.glabra* rhizomes and *M. nigra* leaves.

The effect of plant extracts on biofilm formation Tables-(4,5) Figure-7 showed that *E. camaldulensis* bark extract had the highest inhibiting effect on all bacterial strains, it was more inhibiting on *Salmonella* spp. then *Klebsiella pneumoniae* then *Escherichia coli* (gram negative), while it was more inhibiting on *Streotococcus mutans* then *Staphylococcus epidermidis* then *Staphylococcus aureus* (gram positive). Many studies support the antimbiofilm formation of bacteria by *E. camaldulensis* extract, this was contributed to the role of essential oils in retarding biofilm formation [26, 27].

The effect of *G. glabra* rhizomes extract on biofilm formation had the highest inhibiting effect on *Salmonella* spp. then *Klebsiella pneumoniae* then *Escherichia coli* (gram negative), while it was more inhibiting on *Streptococcus mutans* then *Staphylococcus aureus* then *Staphylococcus epidermidis* (gram positive). This effect was related in many studies to glycyrrhizinic acid and phenolic compounds that are exist in licorice [28, 29].

The effect of *M. nigra* leaves extract on biofilm formation had the highest effect on *Salmonella* spp. then *Klebsiella pneumoniae* then *Escherichia coli* (gram negative), while it was more inhibiting on *Streptococcus mutans* then *Staphylococcus aureus* then *Staphylococcus epidermidis* (gram positive). The effect of retarding biofilm formation may be due to the phenolic acids, alkaloids, flavonoids and terpinoids that exist in this plant [30, 31].

Table 4-Effect of plant extracts on biofilm formation of some bacterial strains.

Bacterial strains / O.D.(630nm)	*E. coli*	*K. pneumoniae*	*S.* spp.	*S. aureus*	*S. epidermidis*	*S. mutans*
before treatment	0.247	0.258	0.441	0.240	0.220	0.390
E. camaldulensis extract treatment	0.180	0.178	0.177	0.197	0.148	0.187
G.glabra extract *treatment*	0.224	0.221	0.151	0.194	0.178	0.232
M. nigra extract treatment	0.245	0.234	0.233	0.226	0.199	0.226

Table 5- Effect of plant extracts on Biofilm inhibition (%).

Plant extracts	Bacterial strains					
	E.coli	*K. pneumoniae*	*S.* spp.	*S. aureus*	*S. epidermidis*	*S. mutans*
E. camaldulensis	27.125 %	31.007 %	59.863 %	17.916 %	32.727 %	52.051 %
G. glabra	9.311 %	14.341 %	65.759 %	19.166 %	19.090 %	40.512 %
M. nigra	0.809 %	9.302 %	47.165 %	5.833 %	9.545 %	42.051 %

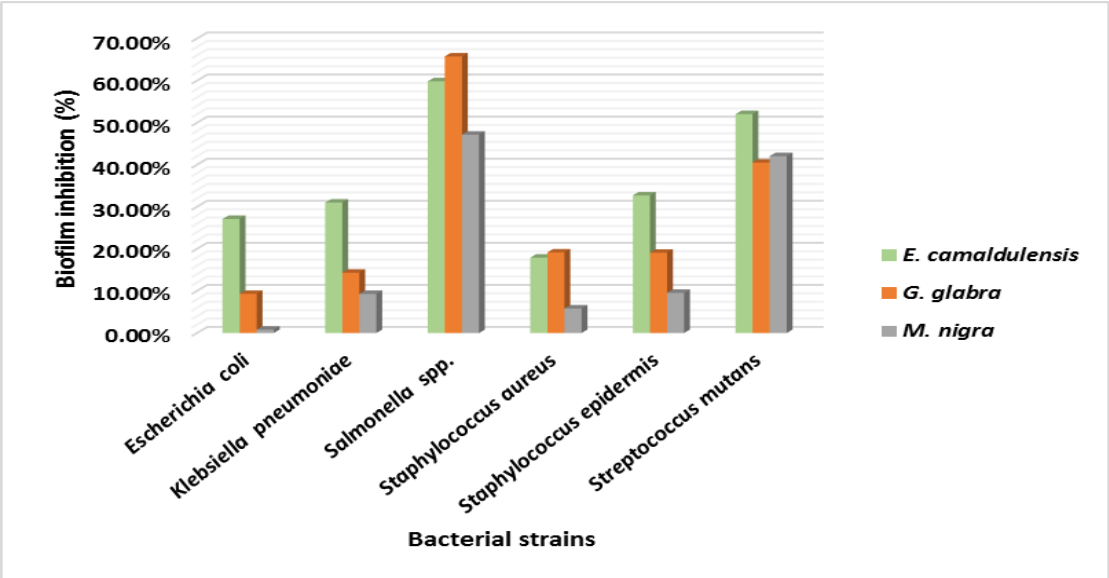

Figure 7- Effect of plant extracts on Biofilm inhibition (%).

The highest inhibiting effect on biofilm formation was by *E. camaldulensis* on most bacterial strains then *G. glabra* then *M. nigra*. These results are encouraging because there is a strong relation between access of biofilm formation and antibiotics resistance, this resistance causes technical and medical problems, so the alternative treatments by using plant extracts are interesting in this regard [32, 33].

Conclusion

Natural resources as *E. camaldulensis, G, glabra* and *M. Nigra* extracts that have certain secondary metabolites are good alternative medicines specially with the drug resistance in these days.

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