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Research Article

Evaluation of the inhibition activity of some plant extracts against Staphylococcus aureus and Klebsiella pneumoniae isolated from patients with tonsillitis.

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Article Info

Article history:

Received 12-4-2023

Received in revised form 31-5-2023

Accepted 21-6-2023

Available online 23 -7 -2023

Keywords: Camellia sinensis, Syzygium aromaticum, Glubara glycyrrhizin, Cinnamomum verum, Staphylococcus aureus, Klebsiella pneumoniae.

Abstract :

The Inhibition activity of hot aqueous extract of *Camellia sinensis*, *Syzygium aromaticum*, *glubara Glycyrrhizin*, and *Cinnamomum verum* at concentrations (100, 80, 60, 40, 20, 10) mg/ml was investigated against 6 isolates of *Staphylococcus aureus* and 7 isolates of *Klebsiella pneumonia* previously isolated from patients with tonsillitis in the holy city of Kerbala, these isolates are characterized by multidrug resistance (MDR) and have a strong ability to build biofilm.

The results showed that the *Camellia sinensis* extract had a higher inhibition efficiency, with an inhibition diameter of 19.67 mm at a concentration of 100 mg/ml of the extract against *S. aureus*, while the highest inhibition diameter was 14.5 mm against *K. pnuemoniae* at a concentration of 100 mg/ml of the extract compared to the rest. The remaining plant extracts and the rest of the extracts did not show any inhibition activity against the isolates of *K.pnuemoniae* bacteria isolates.

The Minimum Inhibitory Concentration (MIC) value for green tea extract was determined at a concentration of 8 mg/ml against *S. aureus* and 36 mg/ml against *K.pneumoniae*.

The cytotoxicity of green tea extract was also studied, as the percentage of toxicity was 8%, and it was found that the results were good, and there was no hemolytic activity against human red blood cells.

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Peer review under responsibility of Iraqi Academic Scientific Journal and University of Kerbala.

1. Introduction

Tonsillitis is an inflammation of the tonsils. It is a common disease of the upper respiratory tract and affects all the age groups, especially children and adults. It is a common clinical condition caused by a bacterial or viral infection. The tonsils play an important role in the immune system as a first line of defense against microbes that enter through the nose and mouth, the most common symptoms of tonsillitis are sore throat, red, swollen tonsils, pain when swallowing, fever, cough, headache, fatigue, chills, enlarged lymph nodes in the neck, pain in the ears or neck, change in voice and bad breath. The most important pathogenic bacterial causes of the upper respiratory tract are *Streptococcus pneumoniae*, *Haemophilus influenza*, gonorrhea, chlamydia, *Klebsiella pneumoniae* and *Staphylococcus aureus* [1].

Medicinal plants have aroused great interest in the modern era through their use to treat some diseases, and they have become a means of health in many developed countries. The green tea plant is one of the most important plants, as green tea contains many antioxidants, which play a major role in protecting the respiratory system and reducing inflammation in the lungs and improving lung health, and helps to inhibit tumor development and inhibit the rate of cell multiplication, and it also has an anti-microbial effect as it has a clear effectiveness in inhibiting the growth of microorganisms [2].

The study aimed to find out the inhibitory effect of aqueous extracts of Green Tea (*Camellia sinensis*), Clove (*Syzygium aromaticum*), Liquorice (*Glubara Glycyrrhizin*), and Cinnamon (*Cinnamomum verum*), and compare it with the effect of antibiotics on bacteria in an attempt to find therapeutic alternatives to reduce the excessive use of antibiotics. Confirm the diagnosis of these isolates using the VITEK 2 compact system.

2. Methodology:

The bacteria were isolated and diagnosed from previous research (6) isolates of *S. aureus* and (7) isolates of *K. pnuemoniae* were selected, all of which were characterized as multidrug resistance (MDR) and have a strong ability to build a biofilm.

2.1 Preparation of plant extracts:

Four types of plant extracts (licorice roots, green tea leaves, dracaena bark and clove seeds) were prepared using the hot water extract method [3].

2.1.1 Hot aqueous extract:

The following extraction method was adopted to reach the truth about the efficiency of treatment with plant extracts (tea herbs) The aqueous extracts of the plants used in the study were prepared (which were grinded several times to obtain a fine powder from each plant part in an electric mill) by mixing 15 g of the plant powder and placing it in a glass beaker and adding 500 ml of boiled sterilized distilled water to it. shaker stirrer for 10 minutes to increase the extraction rate and to disintegrate and tear the plant cell walls, then the mixture was filtered through layers of sterile medical gauze pieces and then filtered again using filter paper with a diameter of 0.22 micrometers and was collected and placed in sterile test tubes and centrifuged at a speed of 3000 cycles / minutes for 10 minutes, after which the extract was placed in large trays until the water evaporated completely, and thus the dried samples were scraped off and placed in dark glass bottles tightly closed and in moisture-free conditions until use, and the process was repeated several times to obtain a sufficient amount of the extract [4].

2.1.2 Preparation of concentrations of solutions of plant extracts to test their inhibitory effectiveness against bacteria:

The stock solution was prepared and sterilized by dissolving 1.5 mg / ml of the plant extract powder separately in 15 ml of distilled water. The concentration of 100 mg / ml was also considered as treatment. Concentrations (80, 60, 40, 20, 10) mg / ml were reported as follows [3]:

- Prepare 5 ml with a concentration of 10 mg / ml by taking 0.5 ml of the storage solution and adding 4.5 ml of sterile distilled water
- Prepare 5 ml with a concentration of 20 mg / ml by taking 1 ml of the storage solution and adding 4 ml of sterile distilled water
- Prepare 5 ml with a concentration of 40 mg / ml by taking 2 ml of the storage solution and adding 3 ml of sterile distilled water
- Prepare 5 ml with a concentration of 60 mg / ml by taking 3 ml of the storage solution and adding 2 ml of sterile distilled water
- Prepare 5 ml with a concentration of 80 mg / ml by taking 4 ml of the storage solution and adding 1 ml of sterile distilled water

2.1.3 Well Diffusion Method

The diffusion method was used to detect the inhibition activity of the selected plant extracts in this study against the bacteria under study according to (Farjana *et al.*, 2014) [5].

1. The isolates kept on previously prepared culture media, such as *Staphylococcus aureus* bacteria on Mannitol salt agar medium and *Klebsiella pneumoniae* bacteria on MacConkey Agar medium, were activated and incubated at a temperature of 37 °C for a period of (24-48) hours.
2. Preparing the bacterial sludge by taking 2-3 colonies for *Klebsiella pneumoniae* and 3-4 colonies for *Staphylococcus aureus* and transferring them to a tube containing 3 ml of physiological solution and comparing it with the turbidity of the standard MacFarland tube in the laboratory which is (1.5×10^8) cells/ml.
3. 100 microliters of the bacterial suspension were spread onto container plates on Muller Hinton Agar (MHA) medium by an L-Shape diffuser and left for 30 minutes.
4. Making holes on the surface of the agar using a corkscrew (6 mm in diameter), then placing 50 microliters of each concentration of the plant extracts under study. A negative control well was left by adding 50 microliters of sterile distilled water.
5. The dishes were incubated in the incubator at 37 °C for 24 hours. After incubation, the diameters of the bacterial growth inhibition zone were measured in millimeters (mm) using a ruler [6].

2.2 Detection of the minimum inhibitory concentration (MIC) by using the Microdilution method:

The microplate dilution method was used according to (Wijesundara *et al*., 2019) [3]. The absorbance was read by an ELISA device.

2.3 Determination of toxicity of green tea plant extract:

The toxicity of green tea plant extract was determined by a hemolysis method that inactivates red blood cells (RBCs) in the laboratory. Therefore, healthy, non-smoking human blood was used in this test as described in (Bataineh *et al.*, 2021) [7]:

1- The blood was centrifuged 5000 cycles for 5 minutes and suspended in 2% saline phosphate buffer solution.

Add 1.5 ml of the selected plant extract concentrate prepared previously and 5.1 ml of RBCs in a test tube, and a test tube was prepared to prepare the negative control

consisting of 5.1 RBCs and 5.1 of normal saline, while for the positive control it was prepared from 5.1 ml. RBCs1 and 5.1 from Triton x-100, then incubated at 37 °C for 1 hour, then centrifuged at 1500 cycles for 10 minutes, then the clear extract was taken for reading using a spectrophotometer at 540 nm wavelength according to the following equation :

$$\% \text{ hemolysis} = \frac{\text{At} - \text{An}}{\text{Ac} - \text{An}} \times 100\%$$

Where:
At: absorbance of test sample
An: absorbance of the negative control (normal saline)
Ac: absorbance of the positive control (Triton X-100)

3. Results and discussion:

3.1 Evaluation of the inhibitory effect of *green tea, licorice, cinnamon and cloves*:

The results of the hot aqueous extract of green tea against the bacterial growth of 6 isolates of *S. aureus* bacteria were compared with gentamicin antibiotic as control. It is clear from Table No. (1) that the aqueous extract of green tea leaves has inhibitory activity against *S.aureus* bacteria, and this study showed significant differences with a probability level of 0.05, as the effectiveness was at its best at a concentration of 100 mg / ml, with an inhibition diameter of 19.67 mm, which was statistically superior to the control treatment 17.83 mm. We conclude from this that the higher the concentration of the aqueous extract of green tea leaves, the greater the diameter of the inhibition zone, and this indicates the effective direct effect of the aqueous extract of green tea leaves as an inhibitor for the bacteria isolates under study.
The isolates of *S. aureus* bacteria differed statistically in their sensitivity to the aqueous extract of green tea, as the isolates S.a.101 and S.a.33 were more sensitive to the hot aqueous extract of green tea and recorded the highest diameter of inhibition with an average of (17.35, 16.7) mm, respectively, compared to the other isolates. The interaction between concentration and isolates had a significant effect on the sensitivity of the isolates under study, as the highest inhibition diameter of S.a.101 was 22.5 mm at a concentration of 100 mg / ml, while the isolate S.a10 was more resistant as it recorded an inhibition diameter of 9 mm at a concentration of 10 mg / ml. For the plant extract of green tea.

Table (1): Inhibition Rates (IZD) of Aqueous Extract of Green Tea Leaves Against *S.aureus* by Diffusion Method

Concentrations (mg/ml)								
Retarding diameters (mm)	Control. gentamycin (10 µg)	100	80	60	40	20	10	Isolation symbol
17.35	17	22.5	18.5	17.5	15.5	15	15.5	$S.a_{101}$
16.07	18.5	21.5	18	16.5	15.5	12.5	10	$S.a_{89}$
15.9	17.5	19	17.5	17.5	15	14.5	10.5	$S.a_{67}$
17	17	20.5	19.5	17.5	16	14.5	14	$S.a_{50}$
16.7	18	21	18	18.5	15.5	14	12	$S.a_{33}$
12	19	13.5	11.5	11.5	9.5	10	9	$S.a_{10}$
0.357							0.946	L.S.D (0.05)
15.8	17.8	19.67	17	16.5	14.5	13	11.8	Retarding diameters (mm)
							0.386	L.S.D (0.05)

As for *K .pnuemoniae* bacteria, the results of the hot water extract of green tea showed 7 bacterial isolates and compared those results with Azithromycin antibiotic as control. This study showed significant differences at the level of probability 0.05, and the effectiveness was at its best at a concentration of 100 mg / ml. The average diameter of inhibition was 14.5 mm compared to the control treatment of 16.64 mm. We conclude from this that the greater the concentration of the water extract of green tea leaves, the larger the diameter of the inhibition zone, and these results It agrees with the results of (Kadhim *et al.*, 2012), as it showed that it obtained the highest inhibition diameter at a rate of 15 mm [8].

Klebsilla .pnuemoniae isolates differed statistically in their sensitivity to the aqueous extract of green tea, as the isolate K.p.110 and k.p17 were more sensitive to the hot aqueous extract of green tea and recorded the highest diameter of inhibition with an average of (11.92, 11.35) mm, respectively, compared to other isolates.

The interaction between the concentration and the isolates had a significant effect on the sensitivity of the isolates under study, as the highest inhibition diameter of the isolate, k.p91, was 15.5 mm at a concentration of 100 mg / ml, while the isolate K.P.17 was more resistant, as it recorded an inhibition diameter of 5 mm at a concentration of 10 mg / ml. of green tea botanical extract. These results coincided with what was indicated by (Anita *et al.*, 2014)[9] about the effectiveness of green tea extract against many microorganisms and its ability to inhibit them because it contains flavonoids and (Catechin) that resist infections caused by microorganisms

Table (2): Inhibition rates of diameters of the aqueous extract of green tea leaves against *K.pnuemoniae* by diffusion method.

Retarding diameters (mm)	Concentrations (mg/ml)							Isolation symbol
	Cont. AZM(15µg)	100	80	60	40	20	10	
8.14	15	13.5	9.5	10.5	8.5	0	0	$k.p_{112}$
11.92	18	14.5	12	13.5	13	7	5.5	$k.p_{110}$
10.85	16	15.5	12	12.5	10.5	5.5	4	$k.p_{91}$
10.71	18	13	12.5	11.5	11	5	4	$k.p_{83}$
11.14	17.5	14.5	13.5	11.5	10	6	5	$k.p_{76}$
11	15	15.5	15	11	9.5	6.5	4.5	$k.p_{22}$
11.35	17	15	14	12.5	9	7	5	$k.p_{17}$
0.240							0.634	L.S.D (0.05)
14.56	16.64	14.5	12.64	11.85	10.21	5.28	4	Retarding diameters (mm)
							0.259	L.S.D (0.05)

And between (Taguri *et al*., 2006) the efficiency of the aqueous extract of green tea leaves inhibition of this bacteria is due to the green tea containing several effective compounds, including alkaloids, saponins, caffeine, pectin, fiber, starch, catechin, epigallocatechin, epigallocatechingallate and also contains carotene And riboflavin, nicotinic acid, pantothenic acid, and ascorbic acid are what give the high activity of this extract in inhibition.[10]

3.2 Effect of hot aqueous extract of licorice plant against *S.aureus* and *K.pnuemoniae*:

The results confirmed that the hot aqueous extract of licorice plant did not have any inhibitory effect against *K. pnuemoniae* bacteria and for all concentrations of the aqueous extract used in this study.

The results showed that the hot aqueous extract of licorice plant had a high inhibitory activity against *S. aureus* bacteria, as shown in Table (3). Inhibition at a concentration of 100 mg / ml 13 mm compared to the control treatment 17.83 mm.

The isolates of *S. aureus* bacteria differed statistically in their sensitivity to the hot aqueous extract of licorice plant, as the isolate S.a.50 was more sensitive to the hot aqueous extract of licorice and recorded the highest inhibition diameter with an average of 13 mm compared to the other isolates.

The interaction between concentration and isolates had a significant effect on the sensitivity of the isolates under study, as the highest inhibition diameter of the isolate was 14.5 S.a101. mm at a concentration of 100 mg / ml, while the isolate S.a10 was more resistant, as it recorded an inhibition diameter of 7 mm at a concentration of 10 mg / ml of the plant extract of licorice roots.

The results of our study agreed with the findings of the researcher (Chakraborty *et al.*, 2011), as it was shown that the hot aqueous extract of licorice plant has inhibitory activity against *S.aureus* bacteria. Glycosides and saponins, have an important role in inhibiting the growth of bacteria, as they work to inhibit the enzymes responsible for basic metabolic reactions through their non-specialized interference with proteins, which leads to protein denaturation, which causes the death of bacteria [11].

Table (3) Inhibition rates of diameters of the aqueous extract of licorice roots against *S.aureus* by diffusion method.

Concentrations (mg/ml)								
Retarding diameters (mm)	Control. gentamycin (10 µg)	100	80	60	40	20	10	Isolation symbol
12	17	14.5	14	12.5	12	8	7.5	$S.a_{101}$
12	18.5	13	13	11.5	12	11	7	$S.a_{89}$
10.5	17.5	10.5	12	10	7.5	8.5	8	$S.a_{67}$
13	17	14	13	13.5	12.5	11.5	9.5	$S.a_{50}$
12	18	13	13.5	11.5	11	9.5	7.5	$S.a_{33}$
11.9	19	14	11.5	11.5	11	9.5	7	$S.a_{10}$
0.284							0.752	L.S.D (0.05)
12	17.8	13	12.8	11.75	11	9.6	7.75	Retarding diameters (mm)
							0.307	L.S.D (0.05)

3.3 The effect of the hot aqueous extract of the *Cinnamomum verum* plant against *S.aureus* and *K.pnuemoniae* bacteria:

The results showed that the hot aqueous extract of the *Cinnamomum verum* plant did not have an inhibitory effect against *K.pnuemoniae* bacteria.

It is clear from Table No. (4) that the hot aqueous extract of *Cinnamomum verum* had high inhibitory effectiveness against *S.aureus* bacteria, and these results showed significant differences with a probability level of 0.05, as the effectiveness was at its best at a concentration of 100 mg/ml, and the highest inhibition drop rate at a concentration of 100 mg/ml was 21.7 mm, which was statistically superior to the control treatment of 17.8 mm.

The isolates of *S. aureus* differed statistically in their sensitivity to the hot water extract of *Cinnamomum verum*, as the isolates S.a33 and S.a101 were more sensitive to the hot water extract of the two scholars and recorded the highest inhibition diameter with a mean of (14.2, 13.7) mm, respectively, compared to the other isolates.

The interaction between concentration and isolates had a significant effect on the sensitivity of the isolates under study, as the highest inhibition diameter of S.a101 was 22.5 mm at a concentration of 100 mg / ml, while the isolate S.a10 was more resistant as it recorded an inhibition diameter of 7.5 mm at a concentration of 20 mg / ml. For the plant extract of *Cinnamomum verum*.

Table (4): Inhibition diameters of the aqueous extract of *Cinnamomum verum* against *S.aureus* by diffusion method .

Retarding diameters (mm)	Concentrations (mg/ml) Control. gentamycin (10 µg)	100	80	60	40	20	10	Isolation symbol
13.7	17	22.5	17.5	16.5	15	7.5	0	$S.a_{101}$
12.8	18.5	21.5	17	17.5	15	0	0	$S.a_{89}$
12.5	17.5	20	18.5	16	15.5	0	0	$S.a_{67}$
13.1	17	23	18	18	16	0	0	$S.a_{50}$
14.2	18	20.5	19	17.5	14.5	10	0	$S.a_{33}$
12.9	19	22.5	17.5	17	14	0	0	$S.a_{10}$
0.310							0.821	L.S.D (0.05)
13.2	17.8	21.7	17.9	17.1	15.0	2.9	0.0	Retarding diameters (mm)

Sharifi-Rad et al., (2017) indicated that the inhibition activity is due to the fact that the extract contains flavones, glycosides, alkaloids, tannins, resins, saponins, coumarins, phenols, terpenes and steroids, which in turn are antioxidants, and that these effective groups made hot aqueous extracts are highly effective against microorganisms [12].

3.4 Effect of hot aqueous extract of cloves against *S.aureus* and *K.pnuemoniae*:

The results confirmed that the hot aqueous extract of the clove plant did not have an inhibitory effect against *K. pnuemoniae* bacteria and for all the concentrations of the extract used in this study.

The results showed that the effectiveness of the hot aqueous extract of the clove plant varied towards the growth of both types of bacteria under study. It is clear from Table No. (5) that the aqueous extract of cloves against *S.aureus* bacteria had high inhibitory activity against the isolates. This study showed significant differences with a probability level of 0.05, although the effectiveness was at its best at a concentration of 100 mg/ml, and the highest inhibition diameter was at a concentration of 100 mg/ml, 19 mm, which was statistically superior to the control treatment, 17.8 mm.

The isolates of *S.aureus* bacteria differed statistically in their sensitivity to the hot aqueous extract of clove plants, as the isolates, S.a10, S.a33, S.a50 were more sensitive to the hot aqueous extract of cloves and recorded the highest inhibition diameter with a mean of (13.6, 13.8, 13.8) mm, respectively. compared to other isolates.

The interaction between concentration and isolates had a significant effect on the sensitivity of the isolates under study, as the highest inhibition diameter of S.a89 was 18.5 mm at a concentration of 100 mg / ml, while the isolate S.a50 was more resistant as it recorded an inhibition diameter of 7 mm at a concentration of 10 mg / ml. For the plant extract of cloves.

The results showed that the hot aqueous extract of clove seeds has high effectiveness against *S. aureus* bacteria, and this is consistent with what was indicated by (Cortés-Rojasf *et al.*, 2014), as he confirmed that this result is considered good as a treatment for this bacteria, as it is one of the plants that contain Basic substances such as carbohydrates and proteins, in addition to that clove oil has anti-inflammatory properties, as it contains Eugenol, which is considered an antibiotic [13].

Table (5): Inhibition diameters of clove aqueous extract against *S.aureus* bacteria by diffusion method .

Retarding diameters (mm)	Concentrations (mg/ml)							Isolation symbol
	Control. gentamycin(10 µg)	100	80	60	40	20	10	
9	17	19	17.5	9.5	0	0	0	$S.a_{101}$
8.4	18.5	22.5	18	0	0	0	0	$S.a_{89}$
8.5	17.5	13.5	15	13.5	0	0	0	$S.a_{67}$
13.8	17	21	17.5	14.5	11.5	8.5	7	$S.a_{50}$
13.8	18	20	18.5	12.5	10.5	10	7.5	$S.a_{33}$
13.6	19	18	15.5	14	11	10.5	7.5	$S.a_{10}$
0.292	0.772							L.S.D (0.05)
11.2	17.8	19	17	10.6	5.5	4.8	3.6	Retarding diameters (mm)

3.5 Detection of the minimum inhibitory concentration (MIC) using the microdilution method:

The results of the current study showed that the minimum inhibitory concentration (MIC) of green tea leaf extract against two types of bacteria, one of which is Gram-positive and the other negative, and its value was determined to be 8 mg/ml against *S. aureus* and 36 mg/ml against Gram-positive bacteria. The bacteria positive for Gram stain were more sensitive than bacteria negative for Gram stain to the aqueous extract of green tea. Biofilm production and its ability to permeate plant extracts, but it does not contain the outer membrane, as it acts as a barrier that reduces or prevents the penetration of many antimicrobials and is therefore responsible for the resistance of Gram-negative bacteria to a wide range of antibiotics, or the difference may be due to the inhibitory effect between positive bacteria And negative for several factors affecting the MIC value, including bacterial production of some enzymes (Ghai, & Ghai , 2018) [14].

The obtained MIC value matched the results of (Cui *et al.*, 2012) study, where the MIC value for *S.aureus* was 6 mg/ml and for *K.pnuemoniae* it was 38 mg/ml.[15]

3.6 Cytotoxicity of green tea extract *in vitro*:

The cytotoxicity of green tea aqueous extract was determined *in vitro* using a hemolysis method, in which human blood erythrocytes (RBCs) of healthy, non-smokers were used.

The results confirmed the absence of hemolysis cytotoxicity of green tea extract due to its insignificant effect on red blood cells disorder, as the toxicity percentage was 8%, and these results are consistent with the study (Zhao et al., 2022) as green tea leaves consumed naturally and in doses ranging from one to two cups per day is safe and does not result in any toxicity or harmful effects, and there is no hemolytic activity against human red blood cells. Also, drinking high doses of green tea may cause an accumulation of substances in the teeth and their pigmentation, insomnia, anxiety, nausea, headache, upset stomach, heart disorders, and tremors [16].

Our results agreed with (Bataineh et al., 2021) [7], as the percentage of cytotoxicity was 10%, as well as the ability of phenolic compounds present in green tea to protect red blood cells from dissolution.

4. Conclusion :

1- *Staphylococcus aureus* and *Klebsiella pneumoniae* are among the main pathogens of tonsillitis for people under study.
2- There is a significant relationship between the ability of bacterial isolates to build a biofilm and their resistance to antibiotics, which indicates the main role of the biofilm as a virulence factor against antibiotics.
3- Efficiency of the aqueous extract of green tea leaves in its inhibitory activity against the isolates under study compared with the rest of the plant extracts (cloves, dracaena, licorice) whose inhibitory activity was weak with *Staphylococcus* isolates and completely nonexistent with *K.pnuemoniae* isolates.

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