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Antimicrobial and antibiofilm activities of alcoholic extract of olive leaves *(olea europaea)* against pathogenic bacteria

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A long time ago the importance of olive leaf extract was proven in the treatment of many cases that can be caused by viruses, bacteria, fungi and others organisms. And in this study results showed that, all bacteria were resisted to almost antibiotics which used in this study. The antibacterial activity of alcoholic olive leaf extract (OLE) was confirmed with agar well diffusion method and the OLE was had a good antibacterial activity with low concentrations against pathogenic bacteria were used in this study. And MIC, MBC concentrations were showed that OLE had higher concentrations of MIC 30 mg/ml and MBC of 60 mg/ml respectively against *Pseudomonas aeruginosa*. And lower concentrations of MBC *Staphylococcus aureus* (MRSA) isolate was sensitive to (OLE) in 15 mg/ml, while MIC was 7.5 mg/ml. The results were showed that all bacteria were used in this study were strong biofilm production, and (OLE) can inhibit biofilm formation in pathogenic bacteria were selected in this study, ($p \leq 0.05$) .except *Escherichia coli* the OLE did not have activity against biofilm which produced by this bacteria, p value = (0.2).

Keywords: Olive leaves extract, antimicrobial activity, biofilm formation.

INTRODUCTION

The olive tree known as *Olea europaea*, and its herbal product famous as olive leaf extract. It was shown a good activity against many diseases (Huang et al., 2003).Much research was mentioned there is an antimicrobial activity of olive oil, and was tested against phytopathogenic microorganisms. It was found that several salt-free solutions from olive oil production processes are rich in antimicrobial compounds, and they have bactericidal and antifungal activity against these microorganisms; and diseases causing them like: influenza, Epstein-Barr virus (EBV), HIV/ARC/AIDS, chronic fatigue, hepatitis B, Candida infections, meningitis, pneumonia, tuberculosis, gonorrhea, malaria, dengue, severe diarrhea, and dental, ear, and urinary tract infections (Eduardo et al., 2013).Polyphenols of olive leaf, especially oleuropein, have interesting effects on the human body such as antioxidant capability, antihypertensive, hypoglycemic, hypocholesterolemic (Patrícia et al., 2015).

Antibiotics are important biochemical produced by microorganisms and widely used. Unfortunately, wrong use of antibiotics from either patients or physicians increased resistance of bacteria; therefore caused an increasing interest in antimicrobial plant extracts (Freidman, 2007). Antimicrobial effect of OLE was improved against food borne pathogens, like *Listeria*

monocytogenes, Escherichia coli O157:H7, and Salmonella Enteritidis at a concentration 62.5 mg/ml, OLE was improved inhibit the growth of these pathogenic bacteria (Yanhong et al., 2017). On the other hand (Aliabadi M. A. et al., 2012) they proved the antibacterial activity of Olive leaf aqueous extracts, and were tested their activity against pathogenic bacteria such as: *(Staphylococcus aureus*, *Salmonella typhimurium*, and *Escherichia coli*, *Klebsiella pneumonia*e, *Bacillus cereus*). While the biofilm formation is the most important factor in the development of chronic infection and allows for immune invasion as well as resistance to antimicrobial agents (Francolini, and Donelli, 2010).

The ultimate aims were: determine if OLE were a potential antimicrobial activity for use in therapy of UTIs, and their activity to inhibit the biofilm formation of these selected pathogens isolated from UTI.

MATERIALS AND METHODS

Isolation of bacterial strains:

Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, as well as *Staphylococcus aureus* (MRSA) were collected from Al-yarmuk hospital from patients with urinary tract infections (UTIs).

Identification of bacteria:

The isolated bacteria were identified with biochemical tests according to (Collee et al., 1996), and the identification was confirmed with VITEK compact 2 systems. MacConkey agar (Himedia/India) was used for identification of *P. aeruginosa, K. pneumoniae* and *E. coli* isolates, and indole test with cimmon citrate, also oxidase test was used in diagnosis of *P. aeruginosa* that was used. Blood agar was used for the detection of hemolytic activity and the kind of hemolysis. Mannitol salt agar (Himedia /India) was also used for identification and isolation of *S. aureus* (MRSA).

Preparation of Olive Leaves Extract (OLE):

Olive leaves used in this study were collected from olive trees in Baghdad ´s university gardens. They were collected in spring (March) and properly prepared for drying process in the day they were collected. Leaves were washed with water, and then dried in an air oven for 3 days at 380 C. The air dried plant materials were ground in a blender. Each 10 g of plant powder was extracted with 200 ml of 80% (v/v) aqueous ethanol by Soxhlet apparatus use for 8 hrs, then the extracts were dried in oven, and the powder of extracted plant was kept in a closed glass tubes in refrigerator until to use (ALTAF et al.,2014).

Antibiotics sensitivity test:

Many antibiotics were used in this study from (Turkey/Bioanalyse). Susceptibility test was done by Kirby-Bauer disc diffusion method (Kirby et al.,1966), and organisms were grown on Mueller Hinton agar (MHA) plates by sterile spreader after dilution to (1×10^8 CFU /ml) and then antibiotics discs were placed on media and incubated overnight at 37ºC. And in this study were used eight antibiotics discs: (Cefoxitin (30µg), Augmentin (30 µg), Gentamicin (10µg), Amikacin (30 µg), Cefixim (10µg), Amoxicillin (10µg), Ciprofloxacin (5µg), Imipenem (10µg). The results were comparing with CLIS data in (2016).

Agar well diffusion test:

Agar well diffusion method by (Mounyr et al., 2016) was used to detect antimicrobial activities of olive leaf ethanol extract. The plates of Mueller Hinton Agar inoculated with pathogenic strains (*Staphylococcus aureus, Pseudomonas aeruginosa, and Escherichia coli, Klebsiella pneumoniae*) were adjusted to a density of 1×10^8 CFU/ml .Wells of 7 mm in diameter were picked holes in these agar plates and dried extract were dissolved in Dimethyl Sulfoxide (DMSO) at different concentrations (5, 10, 15, 20, 25, 30, 35,40,45,55 and 60 mg/ml), placed into each well. Then plates were incubated at 37°C for overnight, and antibacterial activity was identified with determine the diameter of the inhibitory zone and measured in millimeter by ruler. Statistically the Excel program was used to determine mean ±SD.

Determine the Minimum Inhibitory Bactericidal Concentrations of OLE:

The dilution susceptibility test was used to determine the MIC and MBC values. The MIC and MBC of ethanol extracts from the OLE were determined against the four clinical selected isolates.MIC was determined by the macro broth dilution assay. In this method, standard bacterial suspension (0.1 ml) and 1 ml of different concentrations of extract (0.93, 1.87, 3.75, 7.5, 15, 30, 60 mg/ml) were added to tubes containing 1 ml MHB. These tubes were incubated at 37 °C over night. The first tube without growth (clear) was the MIC value. MBC value was measured by culturing of the tubes which apparent no growth on MHA, and then incubated at 37 °C for

overnight. The lower concentration that without appears any growth of colony on agar was considered as MBC (Berghe and Vlietinck, 1991).

Biofilm Formation Assay of bacteria:

The method described by (Steven and Timothy , 2010) was followed as standard test for the detection of biofilm formation. A single colony of selected bacteria that used in this study were inoculated into brain heart infusion broth (BHI), and incubated at 37◦C overnight. Diluted bacterial cultures (200 µL) only, were added to a micro titer plate previously rinsed with 80% ethanol. Then added 200 µL of BHI was considered as a negative control. The micro titer plate was incubated at 37◦C for 18-24hrs. After that the medium was removed, and the plate washed five times with sterile distilled water and dried for 15 min by air. The plate was stained with 0.1% Crystal Violet for 15 min, and washed five times with sterile distilled water. Then, 200µl of 95% methanol was added to each well for 10 minutes. The amount of crystal violet extracted by the ethanol in each well can be directly quantified spectrophotometrically by measuring the OD580nm using a specific micro plate reader.

Biofilm Formation Assay of bacteria with OLE:

The diluted bacterial cultures (150 µL) and 50 µL of OLE at final concentration (7.5mg/ml)(this concentration selected because, it's the minimal concentration of OLE) for each bacterium were added to a micro titer plate previously rinsed with 80% ethanol. 200 µL of BHI was used as a negative control. The micro titer plate was incubated at 30◦C in a humidified container for 48 h. After that the medium was removed, then plate was washed five times with distilled water and dried for 45 min by air. The plate was stained with 0.1% Crystal Violet for 45 min, and washed five times with distilled water. After 30 min was stained with 200 µL of 95% ethanol, the absorbance at O.D.595 nm. Three replicates were done for each bacterium.Where p value (≤ 0.05) was considered significant results (Steven and Timothy , 2010).

RESULTS AND DISCUSSION:

Isolation of bacterial strains:

The bacteria from urine patients with UTIs were isolated from Al-yarmouk hospital/Baghdad. Four clinical isolates were used in this study; three of them were gram negative bacteria: *Pseudomonas aeruginosa, K. peumoniae* and *E. coli*. And one only was gram positive bacteria: *Staphylococcus aureus* (MRSA). The *Pseudomonas aeruginosa*, *K. peumoniae* and *E. coli* isolates were diagnosed primarily by growing on MacConkey agar. *E. coli* and *K. peumoniae* colonies appeared pink colonies because they ferment lactose and indole positive for *E. coli* while cimmon citrate positive for *K. peumoniae* .The *P. aeruginosa* colonies appeared pale on MacConkey because it does not ferment lactose. The *P. aeruginosa* colonies were tested for their ability to produced oxidase enzyme by using oxidase test, the isolate gave positive result. *Staphylococcus aureus* (MRSA) isolate was diagnosed primarily on Blood agar, their colonies gave β-hemolyic and on Mannitol salt agar they grew and gave yellow colonies because of their ability to ferment Mannitol, then the diagnosis was confirmed by VITEK 2 compact system for all isolates.

Antibiotics sensitivity test:

The results were showed in table (1) that *E. coli* isolate was sensitive only to two antibiotics and resistant to the other antibiotics (Imipenem, Cefixim, Amoxicillin, Amikacin, Ciprofloxacin, Gentamycin). While *P. aeruginosa* isolate was resisted to (Cefotaxim,Ciprofloxacin, Cefixim, Augmenten, while sensitive to (Amikacin, Imipenem). And *S. aureus* (MRSA) isolate was sensitive to (Amikacin, Ciprofloxacin, Cefotaxim), while it was resisted to (Cefixim, Augmenten, Amoxicillin, Gentamicin). But *K. pneumoniae* isolate was resisted to (Amoxicillin, Augmenten, Gentamicin, Cefotaxim, Cefixim, Imipenem), and sensitive to (Ciprofloxacin, Amikacin). Therefore the bacteria in this study were multi drugs resistant to antibiotics. And these results agree with other results that the bacteria isolated from UTI were resistant to almost antibiotics used by (Richards et al., 1999). another study was mentioned that *K. pneumoniae* isolated from UTI, were 100% of isolates were sensitive to Norfloxacin,Cefotaxime, Imipenem/ Cilactin, and Sparfloxacin, and all isolates resistance to Cefoxitin, Amoxicillin, Ticarcillin/Clavulanic Acid, Cefitrixon, Trimthoprim, and Cefradine (Sweedan E. Ghassan,2018).while in another study, the sensitivity of *P. aeruginosa* to the antibiotics was tested, the results showed these isolates were resistant to the used antibiotics except Chloramphenicol and Ciprofloxacin.(Flayyih et al.,2012)

Agar well diffusion test:

The anti-bacterial activity of olive leaf

alcoholic extract measured by the agar diffusion method against selected pathogenic bacteria. The olive leaf extract showed good inhibitory effects on pathogenic bacteria showed in table (2).

Table (1): The diameter inhibition zones of antibiotics discs against selected bacteria:

The antibiotics discs	*K. pneumoniae*		*E. coli*		*P. aeruginosa*		*S. aureus*	
Cefotaxim	10mm	R	26mm	S	8mm	R	28mm	S
Ciprofloxacin	30mm	S	11mm	R	4mm	R	26mm	S
Cefixim	12mm	R	10mm	R	8mm	R	0mm	R
Augmenten	9mm	R	27mm	S	10mm	R	7mm	R
Amoxicillin	5mm	R	0mm	R	0mm	R	0mm	R
Amikacin	26mm	S	10mm	R	27mm	S	25mm	S
Imipenem	8mm	R	5mm	R	20mm	S	0mm	R
Gentamicin	10mm	R	2mm	R	6mm	R	2mm	R

R: Resist, S: Sensitive

Table (2): Diameter of inhibition zones of OLE against pathogenic bacteria:

Concentrations of (OLE) mg/ml	Diametr of inhibition zone in mm			
	E.coli	*K. pneumoniae*	*S. aureus*	*P. aeruginosa*
5 mg/ml	**-**	**-**	**-**	**-**
10 mg/ml	-	-	-	-
15 mg/ml	-	-	5	
20 mg/ml	6	8	10	10
25 mg/ml	10	11	16	15
30 mg/ml	13	14	20	18
35 mg/ml	15	15	22	20
40 mg/ml	16	17	25	21
45 mg/ml	17	18	27	23
50 mg/ml	19	20	30	25
55 mg/ml	21	25	33	26
60 mg/ml	22	27	35	28

All gram negative bacteria were used in this study were showed sensitivity to olive leaves extract in concentrations (20,25,30,35,40,45,50,and 60 mg/ml) and in (5,10,15 mg/ml) concentrations were resisted to (OLE),and if increased the concentration of (OLE) the effect of its will be increased against bacteria .only *S. aureus* (MRSA)isolate was sensitive to (OLE) in 15 mg/ml ,and this agree that gram positive bacteria more sensitive than gram negative bacteria may be because the cell wall structure. Many studies confirm positive role of olive leaf extract to inhibit pathogenic bacteria, one of them by (Zahra et al., 2016). Which was investigated Olive leaf extract was not broad spectrum in action against all microorganisms but could be a useful source of antibacterial agent against special bacteria. But the antimicrobial effect of the extract varied according to the solvent used .OLE has antiviral activity, and also against fungi .These results indicate that OLE can be considered as a good natural antimicrobial alternative limited study is available on pharmacokinetics of OLE ingredients but may be loss its antimicrobial activity in vivo. Another study their results showed that the hot aqueous extract of olive oil (0.5, 1, 3, and 6) % had inhibition activity against the pathogenic *Staphylococcus aureus* bacteria and *Streptococcus mutans* (Buthina et al., 2018).

Determine the Minimum Inhibitory and Bactericidal Concentrations of OLE:

Results of this investigation revealed that most of the assayed (OLE) have antibacterial activity, though to varying degrees as indicated by the inhibition of the growth pattern of the isolates fig. (1). The results of the minimum inhibitory showed that OLE had better MIC and MBC of 15 mg/ml and 30 mg/ml respectively on *K. pneumoniae*, and *E. coli,* the same MIC and MBC respectively. The higher MIC was 30 mg/ml and MBC 60 mg/ml respectively against *P. aeruginosa*. This means that (OLE) is needed in higher concentrations to kill or inhibit the growth of this bacterium. (OLE) had inhibitory activities against *S. aureus* (MRSA) at lower concentrations than other bacteria were used in this work, the MIC value was 7.5 mg/ml and MBC 15 mg/ml .It was proved that antibacterial of (OLE) have bacteristatic and bactericidal activity and can inhibit or kill of bacteria isolated from UTIs. Although this is appropriate with the fact that Gram negative bacteria are less sensitive to polyphenols than Gram positive bacteria (Seow et al., 2014). The Gram-positive bacteria are more sensitive to polyphenols because their membrane interacts with hydrophobic compounds of the polyphenols. Also, Gram-negative bacteria are more resistant to polyphenols because their cell wall possesses hydrophilic components Calo et al., 2015).). OLE was shown an antimicrobial activity against food borne bacteria such as: *Staphylococcus aureus, E. coli, Salmonella spp.,* and *Lesteria monocytogenes* (Techathuvanan et al., 2014).

A study of (Zahra et al., 2016), about the antimicrobial activity of olive leaves was recorded that the minimal inhibitory concentrations values obtained by them were higher than the values in the present study.

Biofilm Formation Assay of bacteria:

This study suppose that OLE had anti-biofilm activity against pathogenic bacteria were isolated from UTI .The results were showed all bacteria used in this study were strong biofilm production and (OLE) have a good activity against biofilm production in pathogenic bacteria were selected in this study except *E. coli* results in table (3). In bacteria *P. aeruginosa* with OLE was affected biofilm production by decrease biofilm production ($p< 0.05$).While *K. pneumoniae* was showed significant differences ($p< 0.05$) with OLE. But *S. aureus* (MRSA) was showed significant differences ($P< 0.05$) with OLE. Except *E. coli* which was not showed significant differences (p value 0.2) and this result disagree with results by (Carraro et al., 2014), this results may be because the bacteria in this study *E. coli* was isolated from UTIs, while in the other study were isolated from patients with diarrhea therefore, our results not match with other results because this is the first time in our study showed the antibiofilm activity of (OLE) against multi drugs resistant bacteria isolated from UTIs.

Table (3): The results of biofilm formation of selective bacteria:

Concentration of (OLE)	*E. coli*		*K. pneumoniae*		*P. aeruginosa*		*S. aureus*	
	Without OLE	With OLE	Without OLE	With OLE	Without OLE	With OLE	Without OLE	With OLE
7.5 mg/ml	0.0665± 0.7169	0.061±0.6015	0.251±0.6012	0.2855±0.0031*	0.236±0.0820	0.161±0.0007*	0.3555±0.1060	0.0815±0.0141*

Mean ± SD, * P value ≤ 0.05, except E. coli (P value 0. 2)

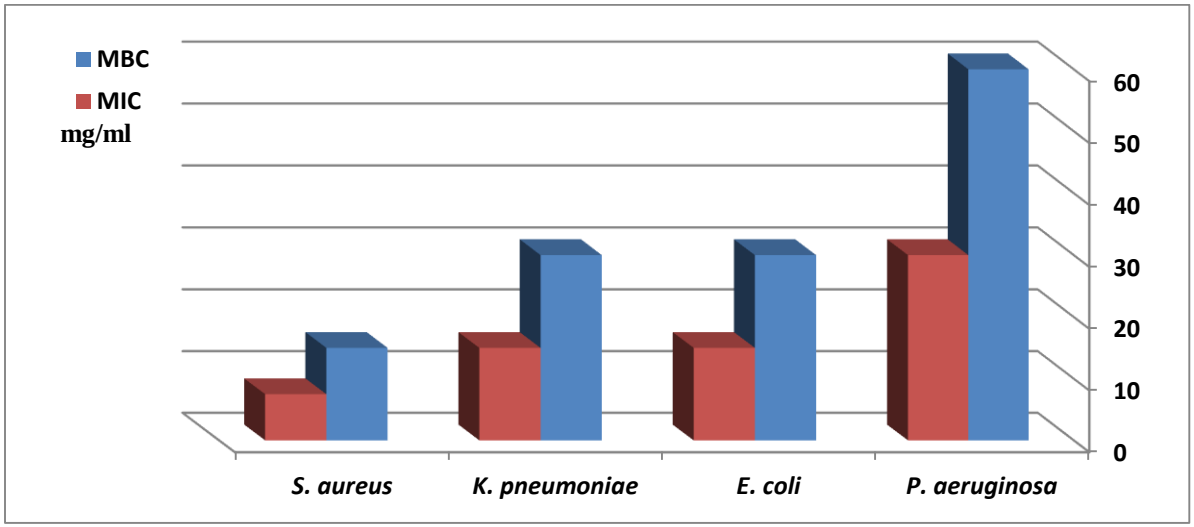

Figure (1): MIC and MBC concentrations (mg/ml) of Alcoholic extract of olive leaves

CONCLUSION

The OLE was have a good antibacterial activity against all selected pathogenic bacteria used in this study and anti-biofilm formation activity against *P. aeruginosa*, *K. pneumoniae*, and *S. aureus* (MRSA) ,except *E. coli*. And the results were showed that OLE can use for therapy of UTIs because its ability to inhibit growth of bacteria isolated from UTIs and was reduced their biofilm formation**.**

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed to the design of the experiments EGS designed and performed the experiments and reviewed the manuscript and also wrote the manuscript, and data analysis. AMD, AMN and MA performed the experiments and data analysis. All authors read and approved the final version.

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