114 *Egypt. J. Chem.* **Vol. 65,** No. 2 pp. 413 - 419 (2022)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Evaluation of local *Origanum vulgare* aqueous extract for eradication of biofilm production bacteria

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Abstract

*Origanum vulgare*leaves are collected from a local garden, Baghdad, Iraq. The hot and cold aqueous extract was prepared to perform an In vitro antibacterial and antibiofilm actions .Antibacterial activities on various antibiotic resistant bacteria (*Pseudomonas aeruginosa, Klebsiella pneumoniae, Echerichia coli, Staphylococcus aurous* and *staphylococcus epidermidis*) were tested by well diffusion method. The biofilm-forming ability of the tested microorganisms was determined by the Microtiter-plate method. Fresh hot extract showed the highest effect and the dried cold extract had the lowest effect against gram-negative and gram-positive bacteria. fresh hot extract decrease significantly the biofilm formation and showed good ability as an antibiofilm to all strains that tested.,Antimicrobial activity of the extract seemed to be related to the aqueous extract preparation method and that appeared by the difference between fresh and dried leave extract activity against pathogens and it can be used as potential antibiofilm agent against the biofilm forming strains.

Keywords: *Origanum vulgare*, antibacterial, antibiofilm, fresh hot extract. Microtiter-plate method

Introduction

Biofilm phenomena is considered as one of the virulence factors of bacteria to overcome the antibiotic therapy and immune system [as explained by 1], it could be the cause of complicated infections in over 60% of microbial infections [2] and that make the biofilm formation one of the most common problem in hospitals and health center through contaminants of instruments and grafts that used in medical centers especially in the indwelling catheters which used for long time [as depicted by 3, and 4]. Various bacteria have the ability to form the biofilm by adhering irreversibly to a various solid surface. These bacteria are usually produce an extracellular polymeric matrix that was the bacteria buried their self [5].

Resistance of biofilm to conventional antibiotic treatments may be attributed to many reasons as limiting the diffusion of antibiotics into the biofilm matrix, type IV secretion systems, expression of multidrug efflux pumps, reduced the penetrability, and the action of antibiotic-modifying enzymes [as explained by 6], and due to the high tolerance toward conventional antibiotics treatment the biofilm formation is difficult to treat and may expose the patient to the risk of recurrence infection [as mentioned by 7].

The increase of bacterial resistance to a broad spectrum antibiotics and biofilm resistance to different treatments regime give rise to develop new control strategies [8]. Researches on medicinal plants has increased over the last few decades due to the emergence of MDR strains of important pathogens like *S. aureus*, *P. aeruginosa*, and *E. coli*. Many studies reported that plants considered as a rich reservoir of compounds that have numerous biological activities including mainly antimicrobial properties [as explained by 9], thereby becoming a good resource to explore usefulness and novel antimicrobial products.Many plants have a pharmaceutical importance duetheir content of several therapeutics’ agents such as flavonoids, alkaloids, tannins, and terpenoids [8], and the row extracts of the different parts (root, stem, twigs, flower and fruit) of these medicinal plants which possess antibacterial, antioxidant, anti-cancer, antifungals, analgesics, insecticides, anticoccidial activity and also may acts as a growth promoter [as mentioned by 10] are now used to treats some human disorders (Khan et al. 2013). During the latest years

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Receive Date: 26 July 2021, Revise Date: 03 August 2021, Accept Date: 18 August 2021
DOI: 10.21608/ejchem.2021.87568.4226
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attention are directed to the Oregano biological activities by researchers and industry, as well as consumers [as explained by 11 and 12].
In this study we test the fresh and dried aqueous extracts of oregano leaves in vitro as a potential anti-biofilm and antibacterial agents.

Materials and Methods

Microorganisms

A total of 20 pathogenic strains of (*Pseudomonas aeruginosa, Klebsiella pneumoniae, Echerichia coli, Staphylococcus aurous and staphylococcus epidermidis*) were provided from AL-Yarmok hospital laboratory Baghdad/Iraq which identified depending on morphological characteristics and Vitek-2 system (Bio-Merieux, France) according to the manufacturer's instructions.

Plant material

Fresh oregano leaves featuring vivacious green color and a firm stem were obtained from privet local house garden in Baghdad during September 2019 (Fig 1), later these plants leaves were wash under tap water, then aired to remove excess water. The leaves are separated for two groups, first one is fresh leaves that will be used in the fresh extraction (hot and cold) directly, while the second group left for slowly drying in room temperature (25 - 30 °C) until completely dried for about (3- 4) days .The dried leaves were crushed to a fine powder by an electrical grinder and put in tightly sealed glass container, stored in refrigerator till used for dried leaves extraction (hot and cold).

Figure 1: Oregano in local house garden

Preparation of crude aqueous extract

An aqueous hot extraction of the Oregano leaves was carried out by using boiled distilled water which is considered as a very effective way for extracting of active ingredients according to method [as described by 13]. A twenty grams of dried (powder) plant leaves was added to flask contain 200 ml of boiled distilled water, the flask was hold over the burner with continuous stirring for 15 min, then it mixed for (15-20) minutes away from burner by a magnetic stirrer . The yielded solution was filtered firstly through sterile double layer gauze to remove the large particles that did not grinded well, the second step of filtration was through a Whatman filter paper (twice) (Fig 2). After that, the extract was centrifuged at 5000 rpm for 10 minutes. The supernatant dispensed in to Petri dishes and incubated at (37-38 °C) to facilitate evaporation of extract solution until completely dried. The cold leave extract was prepared by putting 20 g of plant leaves in sterile flask contain 200 ml of distilled water for 24h at room temperature with frequent shaking, the filtration then done as mention above. The final output for fresh and dried leaves was kept in dark sealed lid containers and stored in refrigerator. The same method was used to prepared cold and hot extract from the fresh leaves of Oregano after grand 20 gm. of fresh leaves with 200 ml D.W. by electric mixer until the contents blended well.

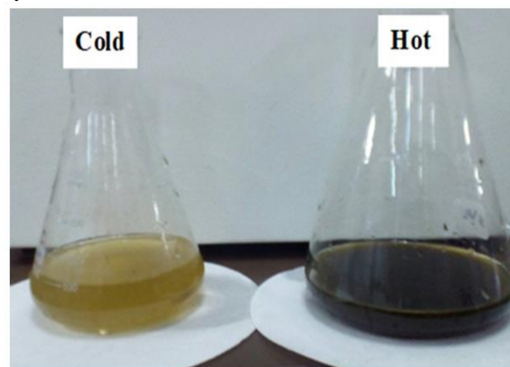

Figure 2: Hot and cold Oregano leaves extract

Stock solution of plants extracts

Stock solution of aqueous extract was prepared by dissolving of 1 gm. of plant extract powder in 10 ml of D. W. from each extract (hot & cold) and then passed through 0.45 µm syringe Millipore filter for sterilization and stored in -20°C until be used.

Antimicrobial effect

The antimicrobial effect was tested against 20 pathogenic strains of 5 different species by using the classical agar-diffusion method [explained by 14] that intended for fast-growing under aerobic conditions microorganisms on Mueller Hinton agar (pH 7.2 - 7.4) with a layer thickness of 4 mm. . The inoculums were adjusted in sterile saline solution to the final density of 0.5 McFarland standard (~1.5 x 108 CFU/ml) as suggested by National Committee for Clinical Laboratory Standards (2015). It was performed immediately before the introduction of the herbal extracts and the control in five wells. The prepared aqueous extract was administered by instillation of 15 µl in each well, in parallel; a sterilized physiological saline was used in the control well as the same manner for tested extract. The plates are left for 2 hours at room temperature for settle down to insure the complete diffusion of the

aqueous components in the agar. Incubation was conducted at 37 º C for 18 - 24 hours. Next day we observed the inhibition zones and we measured the diameters of zones by digital vernier caliperin millimeters.

Microtiter-plate (MTP) method for biofilm detection

All the 20 isolates were subjected to biofilm production**.** Bacterial isolates were cultured in Brain Heart Infusion broth(BHI) and incubated at 37 ºC for 24hr. Bacterial culture was diluted in BHI broth and adjusted in comparison to MacFarland tube no. 0.5. 96-well plates are inoculated with 200 µl per well, incubated for 24 hr. at 37 ºC. After incubation period, all wells were washed out with normal saline, 200 µl of 1% crystal violet was added to each well with shaking the plates to help the dye to reach the bottom of the wells. Plates were kept at room temperature for (15min). Finally, the wells were washed out with 200µl of sterile saline (three times). The crystal violet that bounded to the formed biofilm was extracted with 200µl of ethyl alcohol, and then absorbency was determined at 540nm in an ELISA reader. Controls were also performed with crystal violet binding to the wells exposed only to the culture medium without bacteria. All the assays were performed in triplicates. Analysis of test based upon the ODs of formed bacterial films. Optical density cut-off value (ODc) for the 96 microtiter-plate which defined [according to 15] as average OD of negative control + 3x standard deviation (SD) of negative control categorized as a fellow

≤ ODc / ODc< ~ ≤ 2x ODc: **Non / Weak(0)**/(+), 2x ODc< ~ ≤ 4x ODc: **Moderate** (++),> 4x ODc:**Strong(+++)**

Assessments of plant extracts on biofilm formation

Adopting modified method [explained by 16] a spectrophotometric assay was used to evaluate the biofilm inhibition in 96 wall plates. Cell suspension of each isolate were added into 96 well microtiter plate at 100µl /well, then 100µl of plant extracts was added, the plates were incubated at 37ºC for 24hr. after that, the liquid suspension was removed and 100µl of 1% w/v solution of crystal violet was added, leave at room temperature for 30 min, the dye was removed and the wells were washed, then, 95% ethanol was added and the reaction mixture was read at 540 nm.

Statistical analyses

All statistical analyzes were performed using Minitab 19.1. Analysis of variance (ANOVA) was performed to assess the significant difference between the different study groups.

Results

Antibacterial Inhibitory effect of aqueous extracts

The testing of antibacterial activity of crude aqueous extract of Oregano leaves against the five different bacterial isolates revealed the effectiveness of both methods of extraction (Hot and Cold) for the dried and fresh leaves. The fresh hot extract exhibit the highest inhibition zone against *Staphylococcus aureus* isolates(Mean ± SD = 26.8 ± 0.3) and that all so applies for the fresh cold extract which exhibit approximating inhibition zone against same bacterial isolates (Mean ± SD = 21.4 ± 0.5). Meanwhile, the dried cold extract showing the lowest effect against *E. coli* isolates (Mean ± SD = 7.9 ± 0.3) while the fresh cold extract showing little more effectiveness against *E. coli* isolates (Mean ± SD = 8.6 ± 0.5) (Table 1)(Figure 3).

The statistical analysis revealed that the fresh extract is more effective against the five bacterial isolates that used in this study as anti-bacterial agent than the dried extract by hot methods; even so the fresh cold extract was more effective from dried cold extract. These results indicate that the fresh extract is more efficient as antibacterial agent than the dried one, also can consider the hot extraction methods was more efficient than cold methods (Table 2)

Biofilm forming and antibiofilm action of the oregano extract

We assayed the 20 isolates that used in this study for the production of biofilm, under the same conditions, each strain shows a different potential ability to form biofilm. The results showed that the highest biofilm production is shown in the *E. Coli* isolates (0.390 ± 0.131) and the lowest biofilm production were shown by an isolate of *Staph. aureus* (0.302 ± 0.016) and the rest of bacterial isolates ranged between that (Table 3). The fresh hot extract was chosen to test the antibiofilm activity due to its highest antibacterial activity.

The result showed that the *Oreganium* extract has the aptitude to inhibit / prevent the biofilm formation for all bacterial isolates that tested in this study. The extract was having the ability to prevent successfully and significantly the biofilm formation of the highest biofilm former isolates in this study (*E. Coli*) (F (1, 6) = 16.40, p = 0.007) as well as the lowest biofilm former isolates (*Staph. aureus*) (F (1, 6) = 403.70 , P = 0.0001), meanwhile the extract effect significantly on the *klebsiella pneumoniae* ability to form biofilm (F (1, 6) = 20.67, p = 0.004) but not to reach the complete prevention (The rest of others isolate are summarized in (table 4)

Table 1: Mean ± SD inhibitory effect of dried and fresh Oregano leaves (hot and cold) aqueous extracts against the five bacterial isolates type as anti-bacterial agent

Bacteria	Dried Hot Extract	Mean ± SD	Fresh Hot Extract	Mean ± SD	Dried Cold Extract	Mean ± SD	Fresh Cold Extract	Mean ± SD
staphylococcus aureus	24.75	24.2 ± 0.4	27	26.8 ± 0.3	12.6	12.3 ± 0.3	22	21.4 ± 0.5
staphylococcus aureus	24.2		26.4		12.3		20.75	
staphylococcus aureus	24.1		26.7		12		21.5	
staphylococcus aureus	23.75		26.9		12.2		21.5	
staphylococcus epidermidis	21	20.9± 0.4	23.8	23.3 ± 0.4	9.7	10.1 ± 0.4	12.1	12.1± 0.2
staphylococcus epidermidis	20.75		23		9.9		12.3	
staphylococcus epidermidis	21.3		23.4		10.6		12.25	
staphylococcus epidermidis	20.4		23		10		11.75	
klebsiella pneumoniae	17	17.0± 0.2	19.5	19.6 ± 0.4	13.25	12.8± 0.5	14.2	13.9 ± 0.3
klebsiella pneumoniae	16.9		19.7		12.7		13.8	
klebsiella pneumoniae	16.8		19.1		12.2		14	
klebsiella pneumoniae	17.3		20		13		13.4	
pseudomonas aeruginosa	17.5	17.1± 0.3	19.2	19.2 ± 0.3	16	15.8± 0.4	14	16.5 ± 1.7
pseudomonas aeruginosa	17.1		19		15.7		17.4	
pseudomonas aeruginosa	16.8		18.8		15.3		17.8	
pseudomonas aeruginosa	17		19.6		16.2		16.9	
E. coli	10	10.0± 0.3	10.7	11.1 ± 0.4	8	7.9± 0.3	9.1	8.6 ± 0.5
E. coli	9.8		11.3		7.7		8.8	
E. coli	10.4		11.5		7.5		8.5	
E. coli	9.9		11		8.2		8	

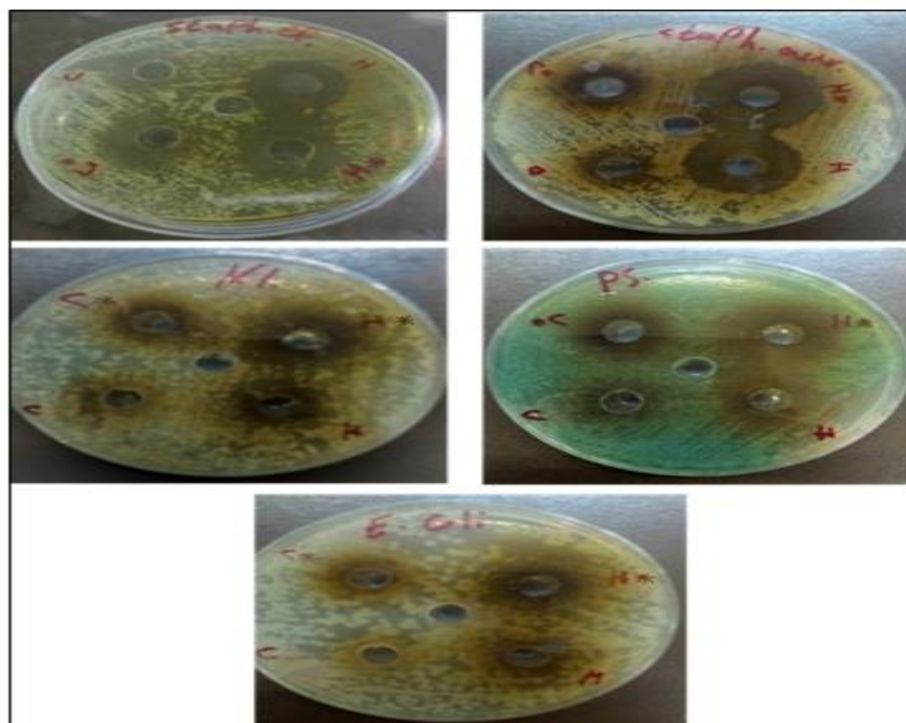

Figure 3: Antibiogram effect of aqueous extracts (Hot and cold) of Oregano against different bacterial isolates

Table 2: Anova of dried and fresh Oregano leaves (hot and cold aqueous extracts) against the five bacterial isolates type as anti-bacterial agent

Extract	***staphylococcus aureus***	***staphylococcus epidermidis***	***klebsiella pneumonia***	***pseudomonas aeruginosa***	***E. coli***
Fresh Hot ExtractDried Hot Extract (FH * DH)	$F(1, 6) = 2429.14$, $p = 0.0001$	$F(1, 6) = 81.31$, $p = 0.0001$	$F(1, 6) = 140.21$, $p = 0.0001$	$F(1, 6) = 82.67$, $p = 0.0001$	$F(1, 6) = 25.25$, $p = 0.002$
Fresh Cold Extract Dried Cold Extract (FC * DC)	$F(1, 6) = 336.36$, $p = 0.0001$	$F(1, 6) = 79.42$, $p = 0.0001$	$F(1, 6) = 14.08$, $p = 0.009$	$F(1, 6) = 0.67$, $p = 0.443$	$F(1, 6) = 7.11$, $p = 0.037$
Dried Hot Extract Dried Cold Extract (DH * DC)	$F(1, 6) = 69.81$, $p = 0.0001$	$F(1, 6)$ 1581.87, $p = 0.0001$	$F(1, 6) = 283.21$, $p = 0.0001$	$F(1, 6) = 28.17$, $p = 0.002$	$F(1, 6) = 114.11$, $p = 0.0001$
Fresh Hot Extract Fresh Cold Extract (FH * FC)	$F(1, 6) = 6325.30$, $p = 0.0001$	$F(1, 6) = 2408.45$, $p = 0.0001$	$F(1, 6) = 505.86$, $p = 0.0001$	$F(1, 6) = 8.93$, $p = 0.024$	$F(1, 6) = 74.46$, $p = 0.0001$

Table3: Biofilm formation(OD) and the effect of use Fresh Hot extract as antibiofilm

Bacteria	(OD) Before treatment	Mean ± SD	Biofilm production before treatment	(OD) After treatment	Mean ± SD	Biofilm production after treatment
staphylococcus aureus	0.317	0.302 ± 0.016	++	0.112	0.124 ± 0.008	0
Staphylococcus aureus	0.282			0.131		
Staphylococcus aureus	0.298			0.128		
Staphylococcus aureus	0.312			0.123		
staphylococcus epidermidis	0.490	0.354 ± 0.097	++	0.136	0.125 ± 0.008	0
staphylococcus epidermidis	0.346			0.124		
staphylococcus epidermidis	0.315			0.118		
staphylococcus epidermidis	0.265			0.123		
klebsiella pneumoniae	0.246	0.311 ± 0.069	++	0.175	0.146 ± 0.024	+
klebsiella pneumoniae	0.352			0.155		
klebsiella pneumoniae	0.260			0.121		
klebsiella pneumoniae	0.386			0.131		
pseudomonas aeruginosa	0.382	0.322 ± 0.059	++	0.128	0.125 ± 0.015	0
pseudomonas aeruginosa	0.320			0.139		
pseudomonas aeruginosa	0.241			0.103		
pseudomonas aeruginosa	0.343			0.130		
E.coli	0.363	0.390 ± 0.131	++	0.138	0.124 ± 0.010	0
E.coli	0.559			0.117		
E.coli	0.398			0.116		
E.coli	0.241			0.125		

The OD of control: Mean ± SD = 0.117 ± 0.003

Table 4: Anova of biofilm formation (OD) before (B) and after (AF) usingFresh hot extract as antibiofilm on five bacterial isolates

	staphylococcus aureus	*staphylococcus epidermidis*	*klebsiella pneumoniae*	*pseudomonas aeruginosa*	*E. coli*
(OD) B. * (OD) AF	$F(1, 6) = 403.70$, $p = 0.0001$	$F(1, 6) = 22.28$, $p = 0.003$	$F(1, 6) = 20.67$, $p = 0.004$	$F(1, 6) = 40.94$, $p = 0.001$	$F(1, 6) = 16.40$, $p = 0.007$

Discussion

The use of plants as a medication is a very old concept due to their various advantages, safety, efficacy and availability throughout the world [as discussed by 17]. Plants are considered as a substantial reservoir for many compounds that have numerous biological activities including antimicrobial properties [as mentioned by 18].

Although there are many reports on the antimicrobial and antibiofilm properties of O*riganum vulgare* aqueous extracts, The present study used the fresh and dried O*riganum* leaves for hot and cold extraction to determine the best .The hot extracts from each (fresh and dried) exhibit antimicrobial activity in vitro, this agreed with [the results of 12] who found that the hot water extract had the strongest antioxidant properties and the highest phenolic content. In addition, the phenols are known to damage the membrane of microorganisms first, making the cells more sensitive [as mentioned by 19 and 20], they found that the different chemical composition of the same extract may be related to many reasons like the part of the plant used in extraction, drying, stage of the plant while collected, growth factors like type of soil, temperature as well as the extraction process. Bacterial isolates affected differently to the action of extracts where the results showed that the fresh extract had the strongest antibacterial action, the antimicrobial activity seemed to depend on the extracting ingredients and Gram positive bacteria were more susceptible than Gram negative bacteria. This gram negative resistance may be due to the double membrane- cell envelope in compared with the single membrane of Gram-positive bacteria which contain 90-95% lipids that does not provide the appropriate medium to reaction with extracts, This results agreed with [the results in 21, and 22].

Furthermore, this research focused on the development of a potential anti-biofilm medication that are nontoxic and will not leads to drug resistance in the future. Biofilm Production by bacteria makes it unaffected to antibiotic treatment and host immunity, once established, a biofilm becomes difficult to eliminate [as discussed by 23]. The fresh hot aqueous extract of O*riganum* used as antibiofilm agent. It's important to note that the extract influence all strains in different degree under the same conditions of experimentation, this agreed with the study of [24] which indicated that the plant extract affects the biofilm formation by caused modifications in the bacterial surface structures responsible for binding to the biotic surface . The activity of plant extract significantly inhibited biofilm formation , *E. coli* rods can be clarified by the presence of Flavonoids such as quercetin, kaempherol, naringenin and apigenin that decrease biofilm synthesis because they can suppress autoinducer-2 activity which is responsible for cell-to-cell communication [as explained by 25, and 26]. The less sensitive bacterial isolate was *K. pneumoniae* which may be due to the negatively charged alginate rich matrix that inhibits the penetration of the extract [as results by 27].

Conclusion

- Aqueous extracts of the herboreganom which prepared through hot and cold extraction of active substances, exhibit antimicrobial and antibiofilm activity in vitro.
- The fresh hot extract had the highest effecton the tested Gram-positive and gram negative bacterial isolates.
- Both extract type (fresh and dried) are exhibiting the most pronounced inhibitory effect on the *Staphlococcus* isolates.
- The results appeared that Fresh hot extract had the ability to inhibited the formation of biofilm, therefore ,it could be used as a source of herbal medicine to treat the multidrug resistant bacteria isolates.

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