Activity of Olive Leaf (*Olea europea* L.) Extract Against Human Pathogenic Bacteria in Erbil- Kurdistan Region

A Research Project

Submitted to the Council of the College of Education-Shaqlawa, Salahaddin University – Erbil in Partial Fulfillment of the Requirements for the Degree of Bachelor in Biology

By

Paywast Rasul Hasan

Israa Sadraddin Hussein

Cheman Star Ibrahim

Supervised by

Asst. Lect. Hiwa Hussein Hasan

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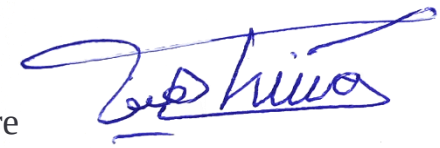
CERTIFICATE

This research project has been written under my supervision and has been submitted for the award of the **BSc.** degree in **Biology** with my approval as a supervisor.

Signature 
Name: Assistant lecturer. Hiwa H. Hasan

Date: 6/4/2023

DEDICATION

I dedicate my work to my Parents whom always have encouraged me during all the steps of the study. Also, I dedicate it to my sisters and my brothers whom have been helped me in every way possible to finish the study. My love for you all can never be quantified.

Paywast Rasul Hasan

Israa Sadraddin Hussein

Cheman Star Ibrahim

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Finally, I'm grateful to whoever helped me in conducting this study.

ABSTRACT

Olive leaf extract OLE (*Olea europaea* L.) has been used traditionally as an herbal supplement since it contains polyphenolic compounds with beneficial properties ranging from increasing energy level, lowering blood pressure, and supporting the cardiovascular and immune system. In addition, to the beneficial effects on human health, OLE also has antimicrobial properties. The main objective of this research was to investigate the antimicrobial effects of olive leaf aqueous and Dichloromethane (DCM) extract against 15 foodborne pathogens. The antimicrobial activities were evaluated by using disc diffusion and microdilution methods. Our results demonstrated that the diameters of inhibition zones of the olive leaf DCM extract were observed 6.1 mm against *Enterobacter aerogenes* and 20.1 mm against *Enterococcus sp*. whereas the olive leaf aqueous extract were observed 5.1 mm against *Enterobacter aerogenes* and 19.2 mm against *Enterococcus sp*. While Minimum Inhibitory Concentration MIC of olive leaf DCM extract against all strains was ≥32 mg mL^{-1} except *Proteus spp*. and *Staphylococcus aureus* was ≥64 mg mL^{-1} and *Enterobacter aerogenes* was ≥16 mg mL^{-1}. In contrast, MIC of olive leaf aqueous extract against (*Enterococcus sp., K. oxytoca, E. aerogenes, B. cepaci, Pseudomonas sp., A. salmonicida,* and *M. wisconsens*) were ≥32 mg mL^{-1} and the others that used in this study were ≥64 mg mL^{-1}. It's considered that some other works should be conducted about using olive leaf extract in food industry as a natural antimicrobial food additive as well as medicine and pharmaceutical industry.

Keywords: olive leaves, antimicrobial activity, *Oleaeur opaea* L., Minimum Inhibitory Concentration, and Disc Diffusion.

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1. INTRODUCTION

Antibiotics are important biochemical produced by microorganisms and widely employed in current medical use for a long time in semi-synthetic forms. Unfortunately, uncontrolled use of antibiotics, caused from either patients or prescriptions made without cell cultures analyses, increased resistance of bacteria. Some bacteria are multi-drug resistant, ie, they are resistant to three or more classes of antibiotics. These features limit the choices of antibiotics, therefor, there is an urgent need to search new compounds characterized by having effective arsenal against the resistance of bacteria.

Over the past decades, olive has been the symbol of peace, wisdom, glory, fertility, power and pureness, there are many benefits from olive and extraction of it, such as Vitamins, olive oil, and using it for medicine as against bacteria (Boskou, 2015). Since the earliest days of microbiology, the biological nature and relationships of the bacteria have been subjects of perennial discussion and Resistance is a means whereby a naturally susceptible microorganism acquires ways of not being affected by the drug. Understanding the mechanisms of resistance is important in order to define better ways to keep existing agents useful for a little longer but also to help in the design of better antimicrobial agents that are not affected by the currently known, predicted, or unknown mechanisms of resistance (Munita and Arias, 2016).

Bacteria, not humans or animals, become antibiotic-resistant. These bacteria may infect humans and animals, and the infections they cause are harder to treat than those caused by non-resistant bacteria. The world urgently needs to change the way it prescribes and uses antibiotics. Even if new medicines are developed, without behaviour change, antibiotic resistance will remain a major threat. Behavior changes must also include actions to reduce the spread of infections through vaccination, hand washing, practising safer sex, and good food hygiene (Aslam et al., 2018). Olive (*Olea*

europaea) belongs to family Oleaceae, have been used widely in folk medicine in European Mediterranean area, Arabia peninsula, India and other tropical and subtropical regions, as diuretic, hypotensive, emollient and for urinary and bladder infections (Omar, 2008).

Olive leaf extract (OLE) can be considered a plant antimicrobial with both antimicrobial and antioxidant activities (Lee and Lee, 2010). OLE also has health benefits such as increasing energy levels, lowering blood pressure, and supporting the cardiovascular and immune systems (Khayyal et al., 2002, Visioli et al., 2002, Covas, 2007, El and Karakaya, 2009). OLE has been shown to have antimicrobial activities against foodborne pathogens such as Staphylococcus aureus, E. coli, Salmonella spp., and L. monocytogenes (Techathuvanan et al., 2014). For example, OLE has been used to reduce bacteria in shrimp and organic leafy greens (Moore et al., 2011, Ahmed et al., 2014). In addition, OLE has been shown to enhance the quality and shelf-life of meat products (Hayes et al., 2010a, Hayes et al., 2010b). Despite the broad spectra of antimicrobial activities of OLE, the mode of its action on foodborne pathogens is still unclear.

The aim of this research has been to investigate the antimicrobial activity of olive leaf extract by using agar disc diffusion and microdilution broth methods against a wide range of food spoilage bacteria. The ultimate goal was to determine if OLE is a potential antimicrobial for use in the food industry, as either a food additive or sanitizing material for the processing plants. And also getting the results from other Articles and thesis because of curfews we could not reach the results that we have planned.

2. METHODOLOGY AND RESEARCH DESIGN

2.1. Olive Leaf collection:

The fresh olive leaves (*Olea europaea* L.) were collected in winter 2023 from Arbil-Kurdistan Region – Iraq.

2.2. Preparation of olive leaf extraction:

2.2.1 Dichloromethane (DCM) extraction:

Extraction of olive leaf was prepared according to the method of (Zahkok et al., 2016) with slight modifications. Thus, fresh olive leaf was washed with (Distil Water) to remove impurities such as dust and then dried at room temperature for 5 days and crushed to a moderately-coarse powder. Then 500 grams of resulting powder was suspended in (1L) Dimethyl Sulfoxide (DMSO) $(CH_3)_2SO$ for one week. The supernatant was filtered, and solvents were evaporated at 45˚C using oven for 24 hours. The dry extracts obtained were kept away from the light (at room temperature) in amber-colored glass bottles until further analysis (Figure 1).

2.2.2 Aqueous Extraction:

Leaves were washed with (Distil Water) to remove impurities such as dust and then dried at room temperature for 5 days and crushed to a moderately-coarse powder. One-liter boiled distil water was added to 500 grams powder obtained from leaves and put on the shaker to be solved thoroughly. Finally, after 2 hours obtained solution was passed through filter and put on petri dish and dried in oven to become powder (Mobasher et al., 2006).

2.3 Microorganisms collection:

The bacterial samples used in this study were *(Proteus spp., Escherichia coli, Enterococcus sp., Klebsiella oxytoca, Serratia marcescens, Enterobacter aerogenes, Burkholderia cepacia, Streptococcus vestibularis, pseudomonas sp., Neisseria sp., Staphylococcus aureus, Aeromonas salmonicida, Moellerella wisconsensis, Aeromonas sobria, Staphylococcus epidermis*) obtained from laboratory of (Bio lab) and Microbiology lab in biology department– college of science – Cihan University.

2.4 Culture media preparation:

2.4.1 Mueller Hinton Agar (MHA) (Merck; Germany):

Suspend 0.95 gm of the medium in 25 ml of distilled water and heat with frequent agitation and boil for one minute to completely dissolve the medium. Then autoclave at 121°C for 15 minutes. After that it cool to room temperature. Finally, pour cooled Mueller Hinton Agar into sterile petri dishes on a level, horizontal surface to give uniform depth and allow cooling to room temperature (Figure 2).

2.4.2 Brain Heart Infusion Broth (BHIB) (Sigma–Aldrich Inc., St. Louis, MO, USA):

Suspend 9.27 grams of the medium in 25 ml of distilled water, then mix well and dissolve by heating with frequent agitation. Boil for one minute until complete dissolution. Dispense into appropriate containers and sterilize at 121°C for 15 minutes. The prepared medium should be stored at 2-8°C. The color is amber. For best results, the medium should be used on the same day or, if not, heated in a boiling water bed to expel the dissolved oxygen and left to cool before using (Figure 2).

2.4.3 Tryptone Soy Broth TSB with agar (Merck; Germany):

Suspend 0.75 gm of the TSB medium in 25 ml of distilled water and add 1.5% of agar in your TSB while preparing and warm slightly to dissolve completely. Dispense into

appropriate containers and sterilize at 121°C for 15 minutes. This media may be stored at 2-30°C. Protect from freezing and/or overheating (Figure 2).

2.5 Test assays for antibacterial activity:

2.5.1 Disc Diffusion Method:

Antimicrobial activity of olive leaf extract (*Olea europaea*) was researched by disc diffusion method using the Kirby-Bauer technique on Mueller-Hilton agar (Merck; Germany). Combined phenolics in the olive leaf extract were dissolved in %1 Dimethyl Sulfoxide (DMSO) and sterilized by filtration through 0.45 μm millipore filters. All assays were carried out under aseptic conditions and performed twice to check the results. Suspension of the tested microorganisms (106 CFU/μL) was spread on the solid media plates. Then the 6-mm diameter paper discs (Oxoid CT0998B) were impregnated (800 μg/disc) with 10 μL of the olive leaf extract and placed on the inoculated agar and they were incubated at 37°C for 24 h. As control Gentamicin 10 μg (Oxoid CT0024B) and Ciprofloxacin 5 μg (Oxoid CT0425B) were used. The antimicrobial activities were evaluated by measuring the zones of inhibition against the test organisms (Bauer et al., 1966).

2.5.2 Minimum Inhibitory Concentration MIC:

Determination of MIC was carried out by using broth microdilution assay according to Hsueh et al. (2010). Six different concentration were tested in the microdilution method. OLE was tested in doubling dilutions ranging from 256 to 8 mg mL^{-1} (512 mg mL^{-1} for olive leaf extract) for broth assays and prepared, inoculated at 1% (v/v) with an inoculum of 107 CFU/mL and incubated for 24 h at 37°C. The MIC was determined by observing the lowest concentration of extract that inhibited visual bacterial growth. Sterile water was used as negative control and chloramphenicol (Sigma-Aldrich, USA) 100 μg/mL as positive control.

2.6. Statistical Analysis:

Statistical Package for the Social Sciences (SPSS) versions 26 and 27 was used for statistical analyses. Data are indicated as a mean of triplicate replicates (n = 3). For antioxidants, the mean values were subjected to Tukey significant difference (HSD) post hoc test at $p \leq 0.05$ (Cronie et al., 2020)

3. RESULTS

Antibacterial activities of olive leaf tree from Erbil, Kurdistan Region - Iraq against 15 bacteria were analyzed. Antibacterial activities were determined by using minimum inhibitory concentration, agar disc diffusion, and microdilution method.

3.1 Disc Diffusion Method:

The diameters of inhibition zones of the olive leaf aqueous and DCM extract of was observed 5.1 mm and 6.1 mm against *E. aerogenes* and 19.2 mm and 20.1 mm against *Enterococcus sp.* respectively. There was a similarity between the inhibition zones of olive leaf aqueous extract and Gentamicin discs against *E. coli, Pseudomonas sp., S. aureus*, and *A. salmonicida*. The diameters of inhibition zones of Ciprofloxacin discs are larger than the diameters of inhibition zones of olive leaf extracts and Gentamicin discs (Table 1) and (Figure 3).

Table 1. Inhibition diameter zones (mm) on the tested bacteria of olive leaf aqueous and DCM extract (*Olea europea*; OLE).

Bacteria	**Inhibition zone in (mm)**			
	OLE 10µg aqueous extract	**OLE 15µg DCM extract**	**Ciprofloxacin 5µg**	**Gentamicin 10µg**
Proteus spp.	17.33 ± 0.37	18.9 ± 0.14	28.5 ± 0.32	14.5 ± 0.13
E. coli	13.5 ± 0.45	13.6 ± 0.32	27.0 ± 0.55	13.5 ± 1.05
Enterococcus sp.	19.2 ± 0.75	20.1 ± 0.72	26.5 ± 0.54	13.5 ± 1.12
K. oxytoca	11.7 ± 0.12	11.9 ± 0.16	27.2 ± 0.85	14.9 ± 0.05
S. marcescens	5.6 ± 0.67	7.2 ± 0.29	28.0 ± 0.74	15.2 ± 0.42
E. aerogenes	5.1 ± 0.25	6.1 ± 0.83	26.5 ± 0.23	16.0 ± 0.17

B. cepacia	12.4 ± 1.23	14.2 ± 0.24	27.3 ± 0.19	14.8 ± 0.76
S. vestibularis	10.2 ± 1.75	8.1 ± 0.87	28.9 ± 1.37	13.8 ± 0.27
Pseudomonas sp.	13.3 ± 0.14	12.5 ± 1.37	29.1 ± 1.15	13.3 ± 0.62
Neisseria sp.	8.7 ± 0.99	8.7 ± 1.02	27.8 ± 0.19	14.9 ± 0.99
S. aureus	15.7 ± 0.36	10.2 ± 0.91	26.7 ± 0.67	15.7 ± 1.55
A. salmonicida	13.4 ± 0.24	14.2 ± 0.61	26.2 ± 0.34	13.4 ± 1.29
M. wisconsensis	14.6 ± 0.55	11.5 ± 0.49	28.4 ± 0.53	15.4 ± 0.45
A. sobria	13.9 ± 0.85	14.8 ± 0.80	27.6 ± 0.44	16.3 ± 0.16
S. epidermis	16.3 ± 0.29	16.1 ± 0.13	28.1 ± 0.68	14.9 ± 0.59

Values presented are Means ± standard deviation

3.2 Minimum Inhibitory Concentration MIC:

Minimum Inhibitory Concentrations MIC of olive leaf extract against some bacteria were presented in (Table 2). While MIC of olive leaf aqueous and DCM extract against *Proteus spp.* and *S. aureus* was ≥64 mg mL^{-1}, the MIC of OLE aqueous extract against *E. coli*, *S. marcescen, S. vestibularis*, *Neisseria sp.*, *A. sobria*, and *S. epidermis* was ≥64 mg mL^{-1}, while the other bacteria was ≥32 mg mL^{-1}. In addition, the MIC concentration of OLE DCM extract which inhibit bacteria was ≥32 mg mL^{-1} except *Proteus spp.* and *S. aureus*. However, *E. aerogenes* was the more sensitive bacteria which inhibit in ≥16 mg mL^{-1} MIC concentration of OLE DCM Extract.

Table 2. Minimum Inhibitory Concentrations MIC of olive leaf extract against some bacteria.

Olive Leaves Extract concentrations (mg/mL)

	OLE Aqueous Extract / OLE DCM Extract											
Bacteria	256		128		64		32		16		8	
Proteus spp.,	–	–	–	–	–	–	+	+	+	+	+	+
E. coli	–	–	–	–	–	–	+	-	+	+	+	+
Enterococcus sp.	–	–	–	–	–	–	–	–	+	+	+	+
K. oxytoca	–	–	–	–	–	–	–	–	+	+	+	+
S. marcescens	–	–	–	–	–	–	+	–	+	+	+	+
E. aerogenes	–	–	–	–	–	–	-	-	+	-	+	+
B. cepacia	–	–	–	–	–	–	–	–	+	+	+	+
S. vestibularis	–	–	–	–	–	–	+	–	+	+	+	+
Pseudomonas sp.	–	–	–	–	–	–	–	–	+	+	+	+
Neisseria sp.	–	–	–	–	–	–	+	–	+	+	+	+
S. aureus	–	–	–	–	–	–	+	+	+	+	+	+
A. salmonicida	–	–	–	–	–	–	–	–	+	+	+	+
M. wisconsensis	–	–	–	–	–	–	–	–	+	+	+	+
A. sobria	–	–	–	–	–	–	+	–	+	+	+	+
S. epidermis	–	–	–	–	–	–	+	–	+	+	+	+

+, Growth observed; -, no growth observed, the black and red color represent aqueous and DCM extract respectively.

4. DISSCUSION

Its reported by some researchers that the oleuropein which is included in these products has a lot of pharmacological properties including antioxidant, antimicrobial, anti-inflammatory, antiatherogenic anticarcinogenic and antiviral activities (Casas-Sanchez et al., 2007).

Inhibition zones with diameter less than 12 mm were considered as having low antibacterial activity. Diameters between 12 and 16 mm were considered as moderately active and these with >16mm were considered as highly active (Indu et al., 2006). According to this the olive leaf aqueous and DCM extracts were highly active against *Proteus spp.*, *Enterococcus sp*., and *S. epidermis*, and low antibacterial activity against *K. oxytoca, S. marcescens, E. aerogenes, S. vestibularis,* and *Neisseria sp.* however, the others are moderately active.

Many studies confirm positive role of olive leaf in inhibitory pathogenic bacteria. Markin et al. (2003), also reported that water extract of olive leaf with a concentration of 0.6% (w/v killed *E. coli, P. aeruginosa, S. aureus* and *K. pneumonia* in 3h exposure. *B. subtilis* on the other hand was inhibited only when the concentration was increased to 20% (w/v) possibly due to spore forming ability of this species. In another study, Korukluoglu et al. (2010), investigated the effect of the extraction solvent on the antimicrobial efficiency of *S. aureus, E. coli, S. enteritidis*, *S. thypimurium* and some others. They reported that solvent type affected the phenolic distribution and concentration in extracts, and antimicrobial activity against tested bacteria.

As regard MIC, the olive leaf aqueous and DCM extract values against the tested microorganisms, ranged from 16-64 mg mL−1. By considering the results as reported in (Table 2), one Gram-positive bacteria *Staphylococcus aureus* and one Gram-negative bacteria *Proteus spp*. were the most resistant tested microorganisms.

According to this investigation the inhibition of bacteria with DCM extract of olive leaves in both disc diffusion and MIC by microdilution were most affective than aqueous extract. Which is incompatible with the finding of (Pereira et al., 2007, Nora et al., 2012), the antibacterial activity noted in present study might be due to successful inhibition of bacterial respiratory system after the treatment with olive leave extracts.

The difference between present study and above referred study is obviously due to different extraction solvent system. In addition, the origin of olive trees and the time of leave collection and experimental conditions must be taken into consideration.

5. CONCLUSIONS AND RECOMMENDATIONS

According to study:

1. The olive leaf extract presented the highest antibacterial activity against *E. aerogenes* and the lowest antibacterial activity against *S. aureus* and *Proteus ssp.* (Table 1 and 2).
2. Gram negative and gram-positive strains used in this study was sensitive to olive leaf extract.
3. Our finding shows that the olive leaf DCM extract was more powerful than olive leaf aqueous extract against studied microorganisms in compression.
4. Its considered that some other works should be conducted about using olive leaf extract in food industry as a natural antimicrobial food additive as well as medicine and pharmaceutical industry.

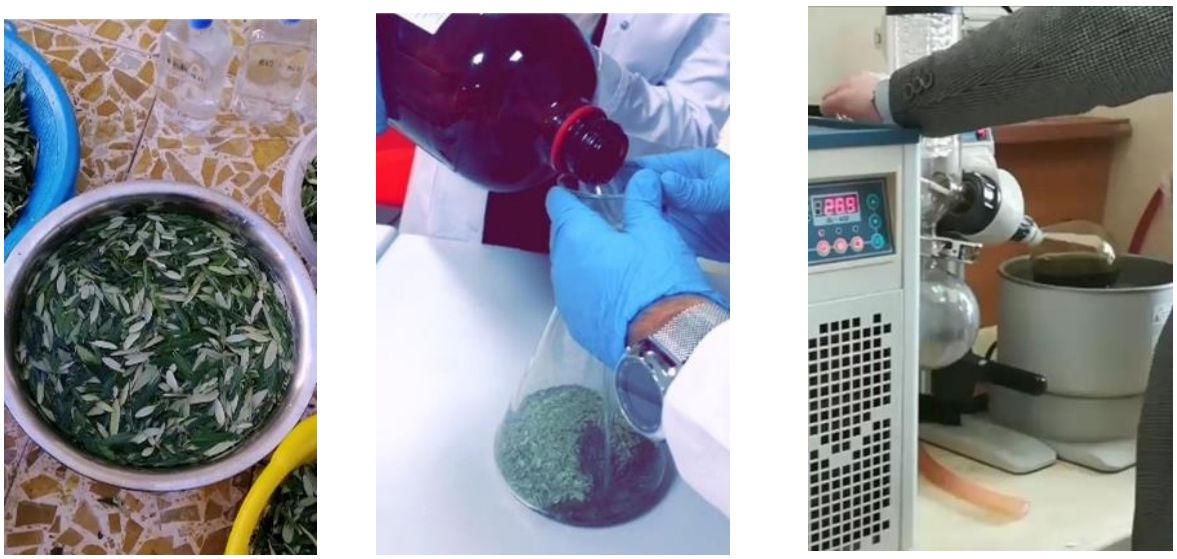

Figure 1. Dichloromethane (DCM) extraction of olive leaf (*Olea europaea*).

Figure 2. Culture media extraction MHA, BHIB, and TSB.

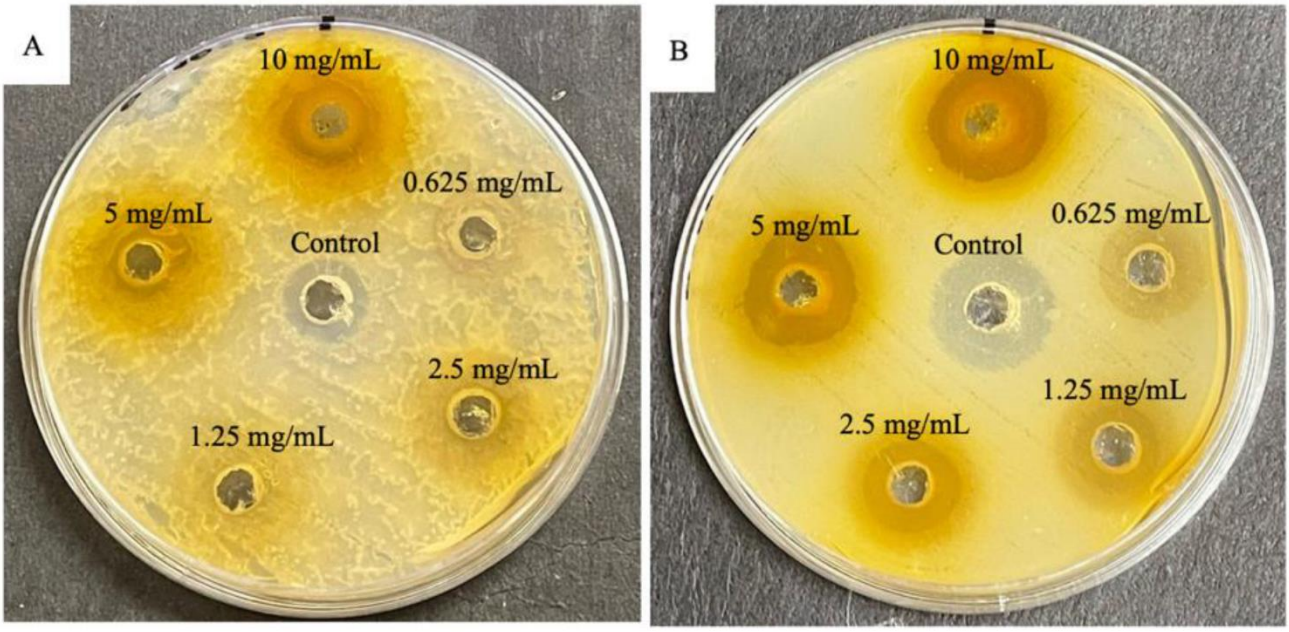

Figure 3: Plates displaying antibacterial activity of DCM crude extract from the leaves of *Olea europea* L. **(A)** Gram-positive *Staphylococcus aureus* bacteria against control ciprofloxacin; **(B)** Gram-negative *Escherichia coli* bacteria against control gentamycin.

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پوختە

دەرهاویشتەی گەڵای زەیتون (Olea europaea L.) OLE بە شێوەیەکی نەریتی وەک تەواوکەرێکی گیایی بەکاردەهێنرێت چونکە پێکهاتەی پۆلیفینۆلیکی تێدایە کە تایبەتمەندی سودبەخشی هەیە لە زیادکردنی ئاستی وزە، دابەزاندنی پەستانی خوێن، و پشتگیریکردنی سیستەمی دڵ و خوێنبەرەکان و بەرگری لەش. جگە لەوەش، بۆ کاریگەرییە سودبەخشەکانی لەسەر تەندروستی مرۆڤ، OLE تایبەتمەندی دژە میکرۆبییشی هەیە. ئامانجی سەرەکی ئەم توێژینەوەیە لێکۆڵینەوە بوو لە کاریگەرییە دژە میکرۆبییەکانی ئاوی گەڵای زەیتون و دەرهاویشتەی دیکلۆرۆمیتان ((DCMدژی 15 ماددە نەخۆشخوازەکانی خۆراک. چالاکییە دژە میکرۆبییەکان بە بەکارهێنانی ڕێگاکانی بڵاوبوونەوەی دیسک و مایکرۆدیلۆشن هەڵسەنگێندرا. ئەنجامەکانمان دەریانخست کە تیرەی ناوچەکانی ڕێگریکردن لە دەرهاویشتەی DCMی گەڵای زەیتون 6.1 ملم لە دژی *Enterobacter aerogenes* و 20.1 ملم لە دژی *Enterococcus sp.* لە کاتێکدا دەرهاویشتەی ئاوی گەڵای زەیتون 5.1 ملم لە دژی ئێنتەرۆباکتێر ئایرۆجین و 19.2 ملم لە دژی ئێنتەرۆکۆکۆس سپ. لە کاتێکدا کەمترین چڕیی ڕێگریکەر MICی دەرهاویشتەی DCMی گەڵای زەیتون لە دژی هەموو جۆرەکان ≤32 میلیگرام مل-1 بوو جگە لە جۆرەکانی .Proteusو ستافیلۆکۆکۆسی ئاوریۆس ≤64 میلیگرام میلی لیتر-1 بوو و ئایرۆجینی ئینتێرۆباکتێر ≤16 میلیگرام میلی لیتر-1 بوو. لە بەرامبەردا، MIC ی دەرهاویشتەی ئاوی گەڵای زەیتون لە دژی (.Enterococcus sp، K. oxytoca، E. aerogenes، B. cepaci، Pseudomonas sp.، A. salmonicida، و ≥32 (M. wisconsens میلیگرام mL-1 و ئەوانی تر بوون کە لەم لێکۆڵینەوەیەدا بەکارهێنراون ≤64 میلیگرام mL-1 بوون. وا دادەنرێت کە پێویستە هەندێک کاری تر ئەنجام بدرێت سەبارەت بە بەکارهێنانی دەرهاویشتەی گەڵای زەیتون لە پیشەسازی خۆراکدا وەک زیادکەرێکی سروشتی دژە میکرۆبی خۆراک و هەروەها پیشەسازی دەرمان و دەرمانسازی.

چالاکی دەرهاویشتەی گەڵای زەیتون (*Olea europea* L.) دژی بەکتریا نەخۆشخوازەکانی مرۆڤ لە هەولێر- هەرێمی کوردستان

پرۆژەی دەرچوونە

پێشکەش بە بەشی بایۆلۆژی کراوە، وەک بەشێک لە پێداویستیەکانی بەدەستهێنانی بروانامەی بەکالۆریۆس لە زانستی بایۆلۆژی

ئامادەکراوە لەلایەن:

ناوی قوتابی

پەیوەست رسول حسن

اسرا صدرالدین حسین

چیمەن ستار ابراهیم

ناوی سەرپەرشتیار

م.ی. هیوا حسین حسن

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