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Study on antimicrobial effects of *Olea europaea* (Olive) and olive leaf extracts on pathogenic bacteria using Taguchi methodology

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ABSTRACT

Antibacterial activity has recently been demonstrated in *Olea europaea* products. This was correlated with the presence of different substances such as oleuropein. In this study antibacterial activity of different olive products was examined and their oleuropein content were determined. Also, the effect of different factors on antimicrobial activity was assayed by Taguchi methodology. Three varieties of fruit, leaves, and unrefined olive oil were purchased from Roodbar in Guilan province and ethanolic extract was examined. Antimicrobial activity of olive leaf extracts was performed by disk and well diffusion methods, and *pour plate method*. Also, effect of different factors such as incubation time, bacterial suspension concentration, type of bacteria, and type of extracts on antibacterial activity was evaluated using Taguchi methodology. HPLC was also performed to measure the oleuropein content. Data analysis was done by using Minitab software. Leaves, fruits, and oil olive extract had significant impact on *Escherichia coli*, *Staphylococcus aureus, Pseudomonas aeruginoa*. The results showed that exposure time and type of extracts had a significant antimicrobial effect on pathogenic bacteria. Also, fruit extract had greater antimicrobial activity than olive oil and *S. aureus* was more (susceptible) than *E. coli* and *P. aeruginosa* to olive extracts. In current study, HPLC confirmed oleuropein as main antimicrobial ingredient of mari leaf extracts at 2.63ppm. Different examined extracts of olive have potential for preparation herbal medicines.

1. Introduction

Over the years, therapeutic effects of herbal extracts in preventing pathogenic agents from attacking viruses, bacteria, fungi and parasites were known (Hosainzadegan et al., 2010). Many advantages of these extracts are the absence of hazardous side effects and the extent of their performances. Olive products have been used for centuries as food, natural preservatives and in traditional medicine.

In addition, over the course of the 19th century, the extract of cooked olive leaves served as a treatment for patients with malaria (Medina et al., 2013). Olive leaf extract is also used today for microbial diseases treatment

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(Aliabadi et al., 2012). It has been reported that the antimicrobial activities of olive oil and leaf extract have been associated with phenolic compounds, including oleuropein, hydroxytyrosol, Oleanolic acid and among others (Brahmi et al., 2012; Kiritsakis et al., 2010; Mihriban Korukluoglu et al., 2010).

Meanwhile, in another study has been demonstrated that the antimicrobial activities against food-borne pathogens such as *Salmonella enteritidis* and *Staphylococcus aureus* have also been related to oleuropein. Moreover, oleuropine can be as an antimicrobial effect against these bacteria pathogens in vitro (M Korukluoglu et al., 2008). Antibiotic resistant bacteria are one of the most problems that have been observed in numerous other microorganisms. *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* are important worldwide health problem (Moradi, 2016).

Due to excessive and arbitrary consumption of pharmaceutical products, including antibiotics, the medical community faces numerous problems in the treatment of resistant bacteria. Various studies have shown that different plants in Iran have an effective antibacterial effect (Dosari et al., 2016).

Antimicrobial activity of different olive products been limited and none of the studies have evaluated the effects of different variables on antimicrobial properties. The aim of this study was to determine the effects of incubation time, suspension concentration, the extract and bacteria type on the antimicrobial properties of fruits, leaves and olive oil against pathogenic bacteria using Taguchi methodology.

2. Materials and methods

2.1. Collection of samples

Leaf, fruit and olive oil were obtained from Roodbar city in Guilan province from tree. 3 varieties of black leaf, marigold leaves and yellow leaves were collected. Fruit samples also included three varieties of black fruit, marijuana fruit and yellow fruit. Of course, these specimens were purely salt water and no other material was added to these olives.

2.2. Preparation of fruit and olive leaves extracts

Leaves and fruits were shade-dried and minced into small pieces for extraction. The phenolic compounds from leaves and fruits were extracted by 80% ethanol. Then, solvent was evaporated by vacuum evaporator at 40°C for 24 hours. Then, leaf and fruite extracts without solvent was filtered through Whatman filter paper (0.45 microns) and was dried by vacuum freeze dryer. All extract samples were stored at 20°C.

2.3. Preparation of microbial suspension

Microbial strains including: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC: 25923) were prepared from the collections of IROST. To obtain microbial suspension one or more colonies of microorganism were added to a sterile test tube containing 5 ml of distilled water, and then, using a spectrophotometer, the opacity of the suspension was measured at 625 nm, which is equivalent to $1/5\times10^8$ CFU/ml was created.

2.4. Disk diffusion method

For determination of antimicrobial activity of olive extract, 30 µl extract of leaf and fruit samples (including Mari, yellow and black) were inoculated into Blank disks. The disks were then dried in sterile air for an hour. Finally, the discs of plant extracts and activity were assayed with standard CLSI guild line.

2.5. HPLC method

Measurement of Oleuropein was assayed through HPLC device (VARIAN, USA) of Guilan Science and Technology Park. A C18 column was used to isolate and quantitatively measure the amount of oleuropein in the

extracts. Generally, distilled water and organic solvents including acetonitrile (70:30) and methanol (20:80) were used for fruit and leaf respectively. It also has a sample loop of about 20 µl and a 325 prosthetic UV detector.

2.6. Taguchi experimental design

The design of the experiment was arranged using Taguchi statistical analysis (Table 1). Four different factors each one in two levels were assayed (Table 2).

Table 1. Variables tested their levels and their values in the Taguchi method.

Factor		Level 1	Level 2	Level 3
Extracts	Fruit	Yellow Fruit	Black Fruit	Mari Fruit
	Leaves	Yellow leaves	Black leaves	Mari leaves
Time (hr)		2	4	6
Concentration (µl)		20	40	

Table 2. Taguchi design of four variables in codes.

Number of tests	A	B	C	Time (hr)
1	*s. aureus*	1	1	1
2	*s. aureus*	1	2	2
3	*s. aureus*	1	3	3
4	*E. coli*	1	1	1
5	*E. coli*	1	2	2
6	*E. coli*	1	3	3
7	*P. aeruginosa*	1	1	2
8	*P. aeruginosa*	1	2	3
9	*P. aeruginosa*	1	3	1
10	*s. aureus*	2	1	3
11	*s. aureus*	2	2	1
12	*s. aureus*	2	3	2
13	*E. coli*	2	1	2
14	*E. coli*	2	2	3
15	*E. coli*	2	3	1
16	*P. aeruginosa*	2	1	3
17	*P. aeruginosa*	2	2	1
18	*P. aeruginosa*	2	3	2

3. Results

The inhibition zone of olive leaf including yellow leaf, black leaf and marl leaf on *Staphylococcus aureus* were 18, 28 and 16 mm respectively, resulting in the most antimicrobial activity belong to black leaf and mari leaf extract. Also, antimicrobial effect of leaf on *E. coli* 10, 8 and 9 mm, and black leaf had the most and the best antibacterial effect respectively. Also, the diameter of the inhibition zone against *Pseudomonas aeruginosa* was measured for three varieties of yellow leaf, black leaf and mari leaf of 14, 12 and 10 mm.

Yellow leaf extract has the highest antimicrobial activity and marigold extract with 10 mm had minimum inhibition zone. According to the results, it can be concluded that *E. coli* showed more resistance in compare to *Staphylococcus aureus* and *Pseudomonas* against olive leaf extract.

The results of disc diffusion method for three varieties of yellow, mari and black extracts on *Staphylococcus aureus* and *Escherichia coli* and *Pseudomonas aeruginosa* showed that the most antimicrobial activity was found in Poraqueiba paraensis Ducke, known as "mari", extract on all three pathogenic bacteria (Table 3).

Table 3. The zone inhibition diameters for different olive fruit types

Zone of inhibition (mm) / Type of Bacteria	Mari	Black	Yellow
Staphylococcus aureus	12	10	11
Escherichia coli	10	7	7
P. aeruginosa	9	7	8

Table 4. Statistical Analysis of Olive leaf extract

Factor	P-value of leaf extract	P-value of fruit extract
Concentration	0.096	0.097
Time	0.001	0.027
Type of bacteria	0.001	0.037
Type of Olive	0.014	0.048

The results of well diffusion experiments for olive oil showed that the diameter of inhibition zone against three *Staphylococcus aureus* and *Escherichia coli* and *Pseudomonas aeruginosa*, respectively with 10, 9 and 8 mm is thus the highest and lowest antimicrobial activity were observed against *Staphylococcus aureus* and the lowest property Antimicrobial against *pseudomonas aeruginosa*.

The findings showed that with increasing exposure time of the extract, the number of germs decreased. The study was performed at 3, 2, 4 and 6 hours, with the highest antimicrobial activity in 6 hours. Also, the antibacterial effect of olive leaf extract on *Staphylococcus aureus* was than *Pseudomonas* and *Escherichia coli*. The concentration and antimicrobial activity of olive leaf and fruit extract was not observed (Table 4).

According to data analysis, there was a significant relationship between fruit extract and incubation time, bacterial and fruit type ($p<0.05$). But there was no significant relationship between the antimicrobial concentration and antimicrobial properties of olive fruit extract (Figures 1 and 2). The most antimicrobial activity of the fruit extract was observed after 6 h incubation time. Table 4 shows the statistical details of the fruit extracts.

HPLC analysis confirmed the presence of oleuropein in all samples. Exit time of Oleuropein using standard HPLC apparatus for standard leaves, yellow leaves, black leaves and Mari leaves was 2.97, 2.78, 2.92, and 2.77 min, respectively (Figures 3 to 6). Also, the amount of Oleuropein yellow leaf, black leaf and Mari leaf samples were evaluated as 1.46 ppm, 0.75 ppm and 2.63 ppm respectively.

The time of departure of oleuropein by using the HPLC device for standard fruit samples, yellow fruit, black fruit and mari fruit was 2.43, 2.40, 2.51, and 2.33 minutes, respectively (Fig. 7 to 9). Also, the amount of oleuropein in yellow fruit, black fruit and mari fruit was estimated at 0.354 ppm, 0.15 ppm and 0.510 ppm.

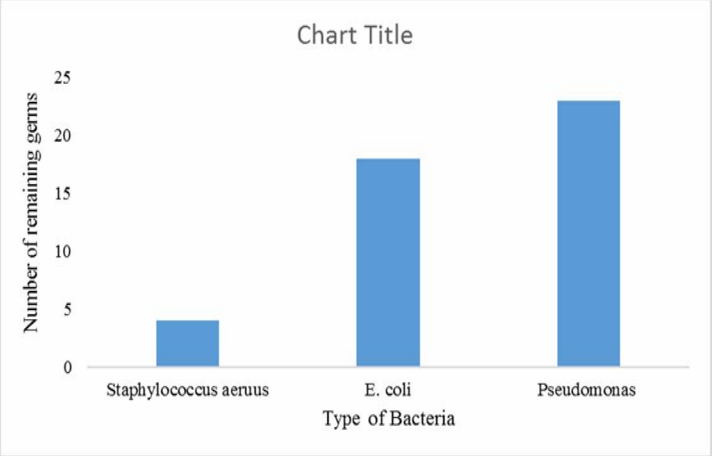

Figure 1. The antimicrobial effect of olive leaf extract on different pathogenic bacteria

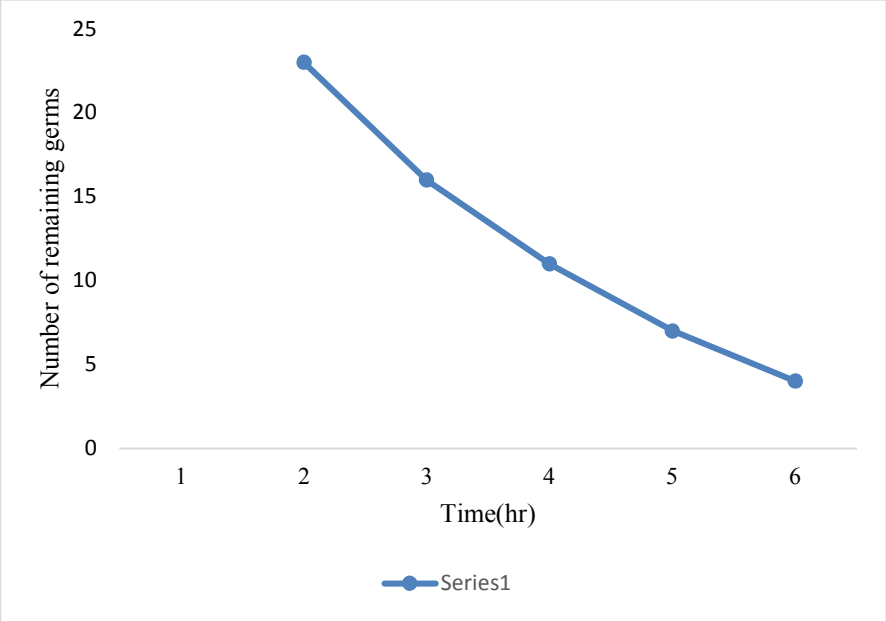

Figure 2. The relationship between incubation time and bacterial count in leaf extract

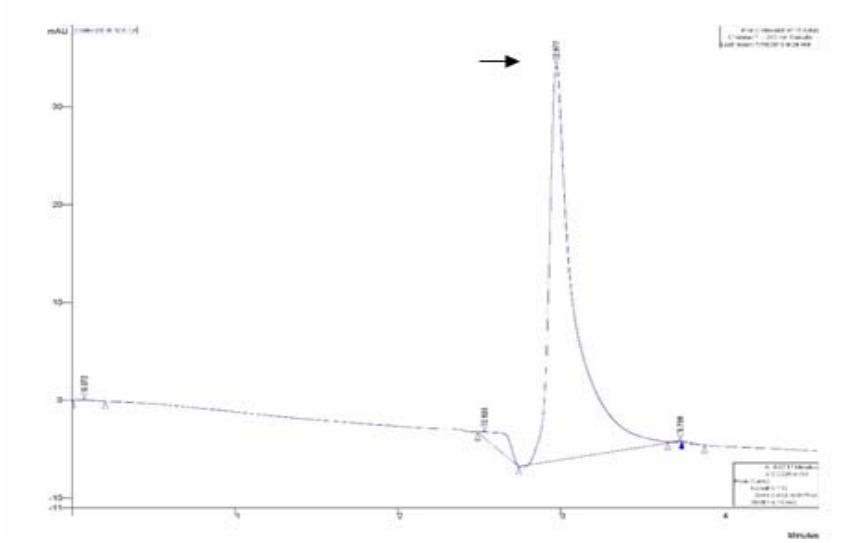

Figure 3. HPLC standard curve for Oleuropein

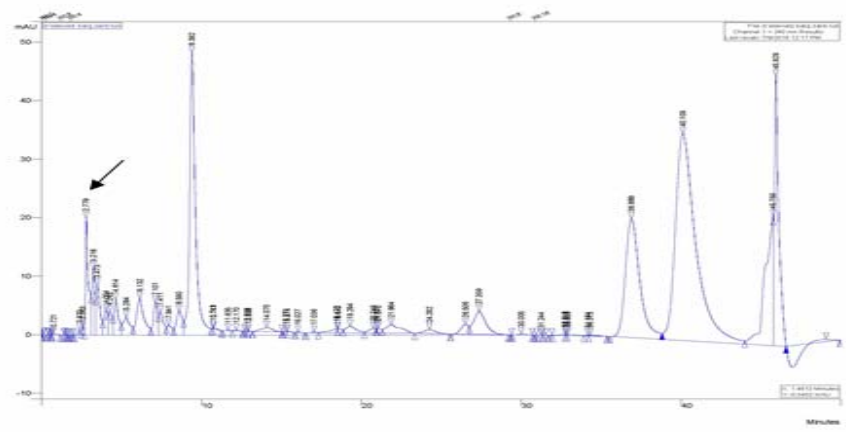

Figure 4. HPLC curve of yellow leaf extract

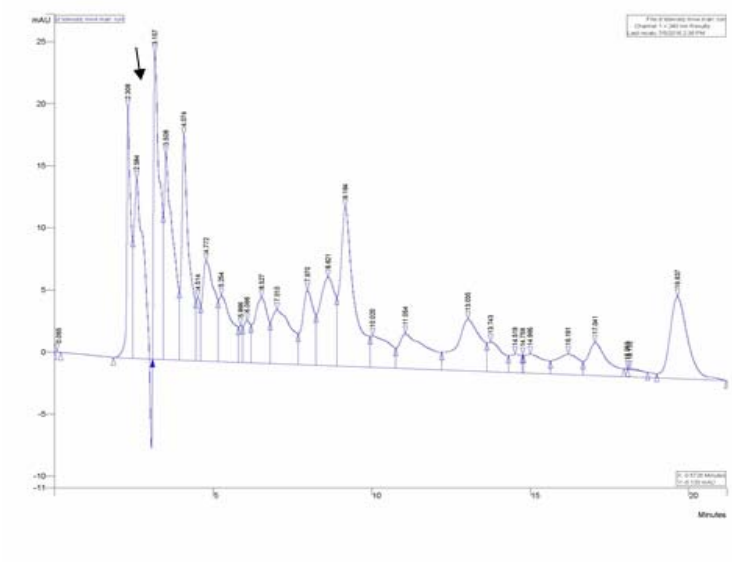

Figure 5. HPLC curve of mari leaf extract.

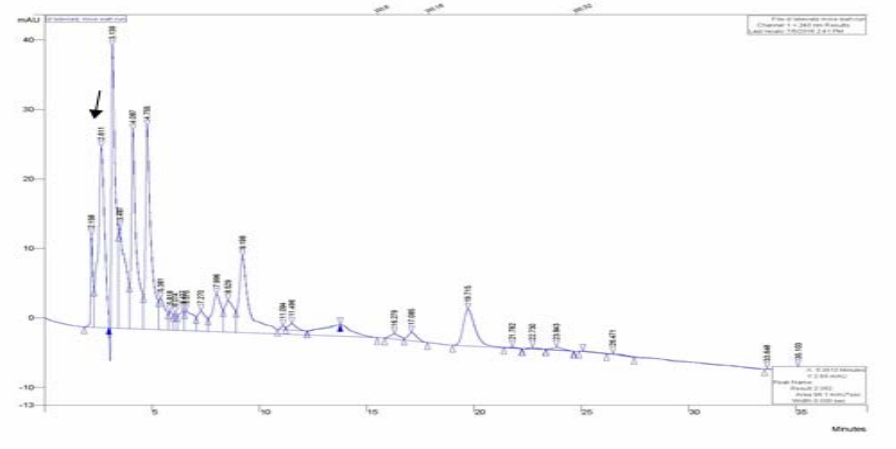

Figure 6. HPLC curve of black leaf extract

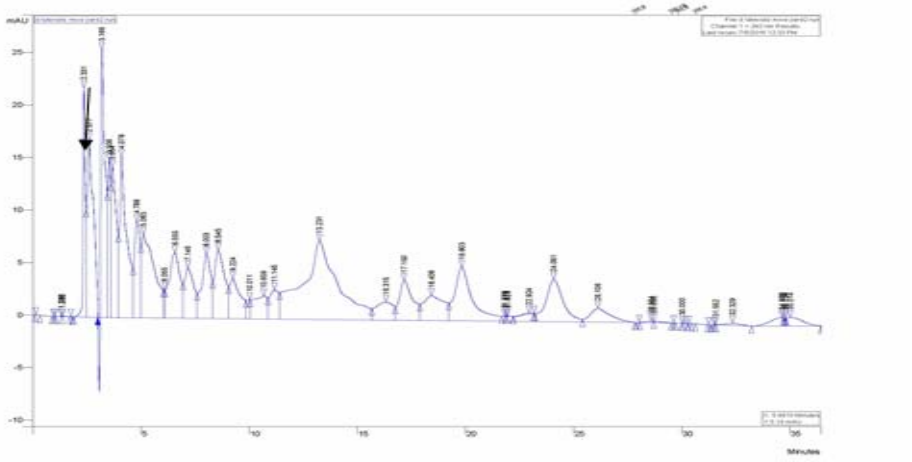

Figure 7. HPLC curve of yellow fruit extract

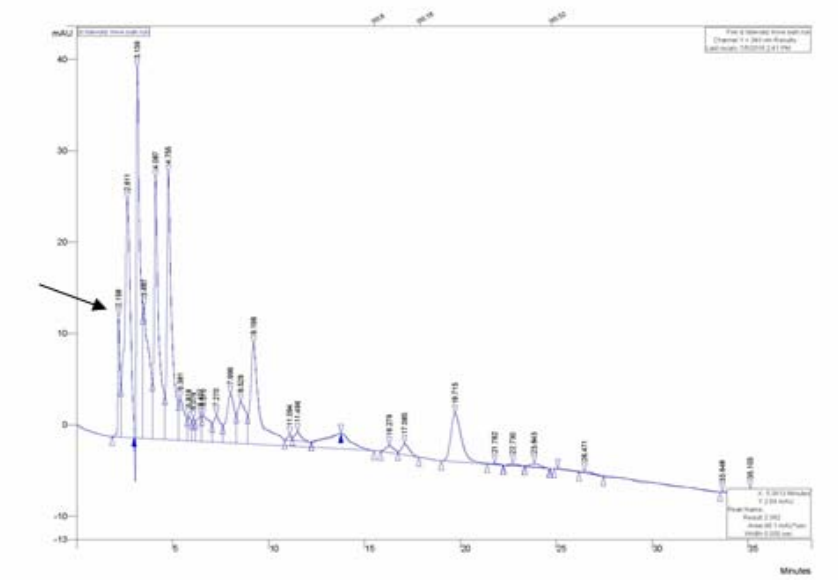

Figure 8. HPLC curve of black fruit extract

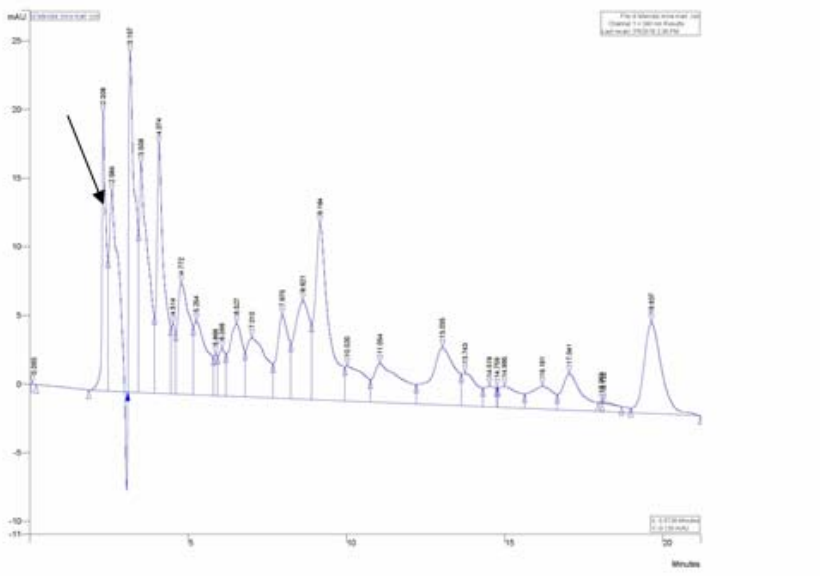

Figure 9. HPLC curve of mari fruit extract

4. Discussion

In this study, leaf extract, fruit and olive oil had a significant effect on *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*. Data analysis showed that increasing the concentration did not have a significant effect on bacterial reduction, but the exposure time and the type of extracts had a significant effect on bacterial reduction. Also, the fruit extract had more antimicrobial effect than olive oil.

Sudjana *et al* found that olive leaf extract had antimicrobial property on *Staphylococcus aureus*, *Campylobacter jejuni* and *Helicobacter pylori*. In a study Özkan et al. found olive fruit

extract had an antibacterial effect on *Staphylococcus aureus* (Özkan et al., 2015), which is consistent with the results of the present study.

Also, other studies in Iran and other countries have examined the effects of different variables such as incubation time, bacterial suspension concentration and bacterial type on the antimicrobial activity of olive extract, that correspond with the results of this study. Sousa et al., leaf extract had antimicrobial activity against some pathogenic bacteria.

Aliabadi et al., also reported antimicrobial activity of olive leaf extract upon intestinal pathogenic bacteria including *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Bacillus cereus* in different concentrations. Bafna et al., founded that the olive fruit extract had an antibacterial effect on *Streptococcus mutans* (Bafna et al., 2015).

Janakat et al. reported the antimicrobial effect of olive oil on gram positive and gram negative bacteria. They found that the antimicrobial effect of olive oil on gram-positive bacteria is approximately 2 times that of gram-negative bacteria, this is probably due to the cell wall of the gram-positive bacteria, which is consistent with the results obtained from the study.

Conclusion

Due to the excessive use of antibiotics, the emergence of *Pesudomonas*, *Escherichia coli*, and *Staphylococcus aureus* bacteria with a widespread antibiotic resistance has led to many problems in treating and transmittable resistant infections among bacteria. In this study, the antimicrobial activity of the different olive products against the *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* showed that the use of policies for the use of them rather than antibiotics is very important. It is also possible to study the effect of conjugate synergism on olive extract with antibiotics or nanoparticles against pathogenic bacteria.

Refereces

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