



Antimicrobial activity of olive leaves extracts and application of leaves powder in meat preservation

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ABSTRACT :

The applications of natural antimicrobial compounds from plants in food becomes very frequent. Olive leaves extracts were assessed as antimicrobial agents. Petroleum ether, ethyl acetate, methanol, and ethanol 50% were used for extraction of phytochemicals and disk diffusion method was applied against tested bacterial strains. Ethanol 50% revealed the greater extraction yield 22.5% followed by methanol 8.2%. Ethyl acetate, methanol, and ethanol 50% extracts showed good antibacterial activity against Gram- positive strains at concentration 10 mg/disk whereas *Listeria monocytogenes* was the most susceptible strain followed by *Staphylococcus aureus*. In agreement with the current trend of raising the value to natural resources, the direct addition of natural compounds to food is the most common method of application. Olive leaves powder was added to minced meat as a preservative at concentrations of crude extract percent of ethanol 50% equivalent to dried olive leaves powder: 16 and 32 mg crude extract per gram of meat. Results indicated that minced meat treated with olive leaves powder showed a decrease in total viable count as well as total coliform count in comparison with control along storage period at 4 : 6°C. *Staphylococcus aureus* and *Escherichia coli* were killed by the effect of olive leaves powder addition in meat samples. Organoleptic evaluation showed that olive leaves powder at concentration 16 mg/g enhanced the taste of minced meat with no significant differences in comparison with control sample in other sensory attributes.

KEYWORDS: Olive leaves, antimicrobial, disk diffusion method, minced meat, organoleptic evaluation.

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1. INTRODUCTION:

Olive (*Olea europaea* L., Oleaceae family) which is an evergreen and small tree is native to Mediterranean region and some parts of Asia. Its by-products (leaf, bark, seed etc.) are used for their nutritional value and health benefits to treat various ailments (Güzelmeriç et al., 2020). The extracts from these byproducts can consist of hydroxytyrosol, tyrosol, caffeic acid, *p*-coumaric acid, vanillic acid, vanillin, oleuropein, luteolin, diosmetin, rutin, verbascoside, luteolin-7-glucoside, apigenin-7-glucoside, and diosmetin-7-glucoside. Compounds found in olive extracts such as 4-hydroxytyrosol are known to have antioxidant, anticancer, anti-cholesterol, anti-aging, antidiabetic, and antimicrobial properties as well as protect against bone loss, heart disease, oxidative injury of kidney cells, and suppress oxidative stress (Cordery et al., 2018). There are numerous trials conducted on the antioxidant and antimicrobial properties of a variety of plants used in minced meat as natural preservatives to inhibit microbial spoilage and to slow down the oxidative reactions. Many plants have been reported to contain some antimicrobial and antioxidant properties due to the presence of bioactive compounds. This bioactivity is mainly

2.2. Microbial strains

Microorganisms used in this study including: Three Gram positive bacterial strains: *Bacillus cereus* ATCC 33018, *Listeria monocytogenes* ATCC 19115 and *Staphylococcus aureus* ATCC 20231. Three Gram negative bacterial strains: *Escherichia coli* 0157:H7 ATCC 6933, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella typhimurium* ATCC 14028. All strains were obtained from Microbiological Resource Center (MIRCEN), Faculty of Agriculture, Ain Shams University.

attributed to a variety of phenolic compounds which have the ability to inhibit or prevent the growth of spoilage and pathogenic microorganisms within the food system (Beya et al., 2021). Toxicity studies showed that olive leaves extracts are generally reliable and do not show any toxic effects even at high doses (Acar-Tek and Ağagündüz, 2020).

2. MATERIALS AND METHODS:

2.1. Preparation of extracts

The dried ground olive leaves were extracted with 100 ml of each solvent individually; petroleum ether, ethyl acetate, methanol, and ethanol 50% (A portion of 10 grams for each solvent) by soaking for 48 hours (Yusof and Saat, 2017). Samples were filtered and the filtrates were evaporated at temperature below 50°C. The residues (crude extract) were weighed, and then reconstituted to prepare a stock solution with known concentrations for the following procedure. Extraction yield was calculated proportional to the dry weight of each plant material as follows:

$$\text{Yield\%} = (W1 / W0) \times 100$$

Where; W1= weight of crude extract after solvent removal,

W0= weight of dried olive leaves powder (10 g).

2.3. Antimicrobial effect of olive leaves extracts by disk diffusion method

Disks were impregnated with 10 mg/disk of each extract of olive leaves. Mueller Hinton agar MHA medium was used with bacterial strains. Incubation for bacterial strains was at 37°C for 24 hours. Inoculation of plates was made by swabbing agar surface with fresh pure cultures of tested bacterial strains (Elbanna et al., 2014 and Owayss et al., 2020). Disks of examined plant extracts

were directly put down in each plate of bacterial strain using clean and aseptic forceps. Clear zones of inhibition around disks were measured in mm after incubation.

2.4. Application of olive leaves in minced meat preservation

Olive leaves powder was applied as antimicrobial preservative in a food system. Olive leaves powder was thoroughly and separately mixed with minced meat at concentrations; 16 and 32 mg crude extract per g of the final weight of meat, as described by **Aouidi et al., 2017** who studied the effect of olive leaves extract

2.5. Microbiological analysis

The impact on microbial load along storage period through 0, 3, 6 and 9 days in refrigerated condition 4-6 °C was estimated. Plant powder was mixed thoroughly with minced meat at concentrations 16 and 32 mg/g of the final weight of minced meat. Microbial counts were expressed as means of two replicates.

The total viable count (TVC) of minced meat samples were determined using nutrient agar medium. The plates were incubated at 37°C for 24 h (**Difco & BBL, 2009**).

Total fungi count of minced meat samples were determined on PDA where the plates were incubated at 25 - 30°C for 3 - 5 days (**Difco & BBL, 2009**).

Total coliform count of minced meat samples determined on MacConkey agar medium, the plates were incubated at 37°C for 24 h (**Bridson, 2006**).

Escherichia coli count of minced meat samples was detected on eosin methylene blue agar medium EMB, the plates were incubated at 37°C for 24 h (**Bridson, 2006**).

and powder on the quality and stability against oxidation of minced meat at concentrations of total phenolic content equivalent to dried olive leaves powder per gram of meat.

The amount of plant powder to be added was calculated depending upon the extraction yield of ethanol 50% as this solvent is the most polar and the best among studied solvents. Serial decimal dilution technique was performed, and microbiological analysis was carried out to examine the effect of the plant power in a food system.

Staphylococcus aureus count of minced meat samples was detected on mannitol salt agar medium, the plates were incubated at 37°C for 24 h (**Bridson, 2006**).

2.6. Organoleptic properties

Meat samples were cooked to evaluate the organoleptic properties by 10 panelists from students and members of Microbiology Dept., Faculty of Agriculture, Fayoum University. Minced meat was evaluated according to **Jayawardana et al., 2015** whereas sensory characteristics including color, texture, odor, flavor and overall acceptability was determined by using a five points hedonic scale. The score card sheet with 5 points for taste, 5 points for color, 5 points for texture, 5 points for odor and 5 points for overall acceptability was applied.

2.7. Statistical analysis

Data were analyzed using General Linear Models (GLM) procedure of Statistical Package for the Social Sciences (SPSS, version 21.0). Duncan's multiple range test was used to compare between the means (**Duncan, 1955**).

3. RESULTS AND DISCUSSION:

3.1. Extraction yield

The most polar solvent ethanol 50% resulted in the most yield due to the appropriate polarity with existed phytochemicals in olive leaves. Generally, as polarity increased the extraction yield

increased. *Olea europaea* leaves revealed the highest extraction yield percent 22.5% with ethanol 50%, 6.3% with ethyl acetate and 8.2% with methanol, while was 1.2% with petroleum ether, the nonpolar solvent.

3.2. Antimicrobial activity by disk diffusion method

Table 1. Inhibition zone diameter (mm) of olive leaves extracts against tested bacterial strains.

Olive leaves extract	Inhibition zone diameter (mm)					
	<i>B.c</i>	<i>E.c</i>	<i>L.m</i>	<i>P.a</i>	<i>Sa.t</i>	<i>St.a</i>
Petroleum ether	0	0	0	0	0	0
Ethyl acetate	0	0	22	0	0	13
Methanol	11	0	29	0	0	20
Ethanol 50%	13	0	28	0	0	13

B.c=*Bacillus cereus*, *E.c*=*Escherichia coli*, *L.m*=*Listeria monocytogenes*, *P.a*=*Pseudomonas aeruginosa*, *Sa.t*=*Salmonella typhimurium*, *St.a*=*Staphylococcus aureus*

Data in Table (1) revealed that ethyl acetate extract of olive leaves inhibited *L. monocytogenes* and *Staph. aureus* with 22 mm and 13 mm, respectively. Methanolic extract exhibited a good antimicrobial effect with inhibition zones 11, 29 and 20 mm against *B. cereus*, *L. monocytogenes* and *Staph. aureus*, respectively. Ethanol 50% extract showed strong influence on *L. monocytogenes* with 28 mm inhibition zone while moderate influence with 13 mm on both *B. cereus* and *Staph. aureus*. Gram-negative bacterial strains resist all extracts of olive leaves at concentration 10 mg/disk. Petroleum ether extract did not reveal any antimicrobial activity. These results are in accordance with **Hussain et al., 2014** who reported that *L. monocytogenes* and *Staph. aureus* were affected by ethyl acetate extract of olive leaves with inhibition zone diameter 12.4 mm for both, while *B. cereus* and *S. typhimurium* showed no inhibition

zones. Similar results were obtained by **Ghanem et al., 2019** who showed that ethyl acetate extract exhibited antimicrobial effect against *Staph. aureus* and *B. subtilis* with inhibition zone diameter 8.3 and 12.4 mm, respectively. They added that ethyl acetate extract showed inhibitory effect against *Salmonella* spp. with 9 mm inhibition zone, but no effect was observed against *E. coli* and tested fungal strains *Candida albicans* and *Aspergillus fumigatus*. Also, they mentioned that total phenolic compounds in olive leaves were found to be high in the 80% methanol extract and the lowest in water and ethyl acetate fractions. They concluded that olive leaves could be considered as a potential inexpensive source for food supplements for human health. In addition to **Gökmen et al., 2014** who studied the antimicrobial activity of olive leaf extracts against five Gram

positive and five Gram negative bacterial strains by using disk diffusion and microdilution methods. The diameters of inhibition zones were 13.33 mm against *S. typhimurium* and 21.67 mm against *B. cereus*.

3.3. Microbiological analysis of minced meat treated with olive leaves powder

Olive leaves powder was selected with high potent principles to investigate the impact on microbial load along storage period through 0, 3, 6 and 9 days in refrigerated condition 4-6 °C.

3.3.1. Total viable count (TVC)

Table 2. Effect of olive leaves powder on total viable count (log cfu/g) of minced meat during storage period at 4-6 °C.

Treatments	Storage time (days)			
	0	3	6	9
Control	5.675 ^{ab}	6.460 ^c	7.665 ^c	7.980 ^c
<i>O. europaea</i> (16mg/g)	5.610 ^a	5.915 ^b	7.010 ^b	7.285 ^b
<i>O. europaea</i> (32mg/g)	5.630 ^a	5.790 ^a	6.915 ^a	7.200 ^a

*Means in each column with different superscript letters are significantly different at ($p < 0.05$)

Total viable count is an indicator of microbial contamination in meat products. The data in table (2) indicated that the samples treated with olive leaves powder at 16mg/g and 32mg/g revealed a decrease in aerobic plate count microorganisms in comparison with control. This result is agreed with **Shalaby et al., 2018** who reported that olive leaves extract possesses antimicrobial activity against total bacterial count in minced beef.

However, olive leaves powder decreased the microbial counts in minced meat samples compared to control through storage period. **Chibane et al., 2019** stated that the antimicrobial efficacy similar to that in vitro cultures could be achieved by adding higher amounts of plant extract to foods up to two-fold, ten-fold and more. This variance can result from the interactions that occur in food systems between hydrophobic bioactive constituents of plant extracts and major

food ingredients such as fat and proteins. In food matrices rich in fat, a lipid coating that wraps the microorganisms and protects them from antimicrobials can form. The known interaction of many polyphenols with proteins might result in polyphenol-protein complexation and thus limit the antimicrobial action. Additionally, **Hafez and Abdelrahman, 2015** mentioned that the application of plant antimicrobial compounds in food system could be influenced by proteins and fat composition, which are known to bind and/or solubilized phenolic compounds, reducing their availability. Furthermore, it has been reported that antimicrobial activity of spice is lower in food systems than in microbiological media.

Control meat samples started to decompose at day 6, while decomposition within the treated samples with olive leaves powder was delayed until day 9 of storage.

3.3.2. Total coliform count (TCC)

Table 3. Effect of olive leaves powder on TCC (log cfu/g) of minced meat during storage period at 4-6 °C.

Treatments	Storage time (days)			
	0	3	6	9
Control	3.460 ^a	4.330 ^b	4.590 ^c	5.035 ^c
<i>O. europaea</i> (16mg/g)	3.430 ^a	3.760 ^a	4.020 ^b	4.075 ^b
<i>O. europaea</i> (32mg/g)	3.450 ^a	3.725 ^a	3.880 ^a	3.925 ^a

*Means in each column with different superscript letters are significantly different at ($p < 0.05$)

The obtained results in Table (3) showed that the coliform count in control samples were 3.460, 4.330, 4.590 and 5.035 log cfu/g after 0, 3, 6 and 9 days of storage, respectively. Treated meat samples with 16mg/g and 32 mg/g of olive leaves powder did not decompose until the 9th day of storage. These results showed the positive preservative effects of olive leaves powder on minced meat samples, where it decreased the TCC compared with the control and prolonged shelf life of chilled meat to the ninth day of storage. Similar results were obtained by Saleh et al., 2020

3.3.3. Total fungal count (TFC)

Table 4. Effect of olive leaves powder on total fungal count (log cfu/g) of minced meat during storage period at 4-6 °C.

Treatments	Storage time (days)			
	0	3	6	9
Control	3.455 ^b	4.165 ^c	5.025 ^c	5.465 ^b
<i>O. europaea</i> (16mg/g)	3.350 ^a	3.585 ^b	4.505 ^b	5.330 ^a
<i>O. europaea</i> (32mg/g)	3.450 ^b	3.455 ^a	4.350 ^a	5.300 ^a

*Means in each column with different superscript letters are significantly different at ($p < 0.05$)

With regard to Table (4) which showed that the total fungal count include mold and yeast of the control meat samples were increased along storage period more than samples treated with plant powder as the count was 4.165 log cfu/g for control sample at day 3 while the count at the same time was 3.585 and 3.455 log cfu/g with olive leaves powder at 16mg/g and 32mg/g, respectively. These findings showed that

who reported that olive leaves extract significantly decreased the total Enterobacteriaceae count in the treated poultry meat at concentrations; 0.25, 0.5 and 1% and delayed the decomposition within the treated samples with 0.25 and 0.5% of olive leaves extract to the 9th and 12th days of storage, respectively while 1% treatment did not decompose until the 15th day of storage.

the samples treated with olive leaves powder were decreased in total mold and yeast counts compared to the control ones. Özcan and Matthäus, 2017 demonstrated that olive leaves extract exhibited antifungal activity especially against *Candida albicans*. Additionally, Shalaby et al., 2018 also reported that olive leaves extract decreased total yeast and mold counts in minced beef.

3.3.4. *Staphylococcus aureus* count

Table 5. Effect of olive leaves powder on *Staphylococcus aureus* count (log cfu/g) of minced meat during storage period at 4-6 °C.

Treatments	Storage time (days)			
	0	3	6	9
Control	3.000	3.505	3.730	4.010
<i>O. europaea</i> (16mg/g)	0.0	0.0	0.0	0.0
<i>O. europaea</i> (32mg/g)	0.0	0.0	0.0	0.0

Results in Table (5) showed that *staph. aureus* count of the control samples was increased along storage period while disappeared with both olive leaves powder treatments. These findings revealed that olive leaves powder had an important role in controlling *Staph. aureus*. **Owen et al., 2003** reported that the phenolic compounds present in olive leaves extract indeed exhibited antimicrobial activity against various microorganisms, especially *Staph. aureus*. Moreover, **Markin et al., 2003** documented that aqueous olive leaves extract at 0.6% (w/v) concentration killed *Staph. aureus* within three hours of exposure. Also, **Hafez and Abdelrahman, 2015** mentioned that lower concentrations of olive leaves extract were sufficient to kill *Staph. aureus* and most pathogenic bacteria. **Saadony et al., 2019** also reported that olive leaves extract was found to be effective against some pathogenic bacteria, such as *E. coli*, *Staph. aureus* and *S. typhimurium*, although being more effective against Gram-positive than Gram-negative bacteria. Additionally, **Chibane et**

al., 2019 reported that the mechanism of action of phenolic compounds the essence of plant extracts including rupture of the cytoplasmic membrane which induces loss of cellular content, hydrogen bonding of the phenolic compounds to enzymes through their OH groups that cause changes in various intracellular functions, and modification of fungal morphology (cell wall rigidity and integrity losses) induced by different interactions with cell membranes. They added that flavonoids are considered able to promote complex formation by linking with soluble proteins located outside the cells and within the cell walls of bacteria. Furthermore, some flavonoids may act by inhibiting both energy metabolism and DNA synthesis. Moreover, **Saleh et al., 2020** reported that the partial hydrophobic nature of phenolic compounds may degrade the cell wall, disrupt the cytoplasmic membrane, damage membrane proteins, and interfere with membrane-integrated enzymes, which may eventually lead to bacterial cell death.

3.3.5. *Escherichia coli* count

Table 6. Effect of olive leaves powder on *E. coli* count (log cfu/g) of minced meat during storage period at 4-6 °C.

Treatments	Storage time (days)			
	0	3	6	9
Control	3.460	3.780	4.325	4.655
<i>O. europaea</i> (16mg/g)	0.0	0.0	0.0	0.0
<i>O. europaea</i> (32mg/g)	0.0	0.0	0.0	0.0

Data in Table (6) indicated that *E. coli* was only found in control samples and absent in treatments. This result in accordance with Aytul, 2010 who reported that the use of 2% and 3% olive leaves extract could affect the microbial load, the total viable and coliform counts. Also, Saadony et al., 2019 who reported that the olive leaves extract possesses inhibitory effects against Gram-negative bacteria, especially those from Enterobacteriaceae, including *E. coli* and *Salmonella*.

3.4. Organoleptic evaluation

Table 7. Organoleptic evaluation of cooked minced meat treated with olive leaves powder.

Treatments	Taste	Color	Texture	Odor	Overall acceptability
Control	4.15 ^b	4.60 ^a	4.55 ^a	4.45 ^a	4.40 ^a
<i>O. europaea</i> 16 mg/g	4.65 ^a	4.55 ^a	4.50 ^a	4.75 ^a	4.65 ^a
<i>O. europaea</i> 32 mg/g	3.00 ^c	2.80 ^b	2.55 ^b	3.15 ^b	3.00 ^b

*Means in each column with different superscript letters are significantly different at ($p < 0.05$), each value is a mean of 10 replicates

Results in Table (7) showed that olive leaves powder at concentration 16 mg/g did not affect overall acceptability since no significant differences were observed in comparison with control sample in all sensory attributes except taste whereas olive leaves powder enhanced the taste of minced meat with no negative effects. These findings in accordance with Aouidi

Organoleptic test was performed by ten panelists on minced meat treated with olive leaves powder to assess the impact on sensory quality. minced meat samples treated with concentrations of plant powders corresponding to 16 and 32 mg extract per gram of meat were cooked after salt addition. The sensory evaluation was conducted by the 5 points hedonic scale whereas;

0.5 : 1.5= very poor; 1.5 : 2.5= poor; 2.5 : 3.5= common; 3.5 : 4.5= good and 4.5 : 5= very good.

et al., 2017 who studied the effect of olive leaves extract and powder on the quality and stability against oxidation of minced meat, they found that meat containing olive leaves may be consumed without any problem regarding sensory quality including taste which was more agreeable than the taste of the control sample. The improvement of the taste of cooked minced

meat by the addition of olive leaves could be explained by the fact that this natural supplement impedes off-flavor or inhibits its development in meat. Similarly, **Shalaby et al., 2018** reported that using olive leaves extract as a natural preservative on minced meat enhanced its quality attributes, in addition to **Saleh et al., 2020** who confirmed that olive leaves extract maintained overall acceptability of treated poultry meat. With regard to the current study, the concentration 32 mg/g of olive leaves powder affect the sensory attributes whereas decreasing score values were obtained. **Chibane et al., 2019** stated that the dose of phenolic compounds

applied for food preservation should be set with sensory considerations and not based solely on in situ antimicrobial efficacy. They added that phenolic-rich plant extracts should not strongly impart their typical color and flavor to foods. to be accepted as food preservatives. Furthermore, **Beya et al., 2021** mentioned that two aspects are critical for the application of natural preservatives in food systems; the change of sensory attributes of food such as color, smell, and texture when the natural preservative is added, and the interactions of the natural preservative with other food ingredients in the system.

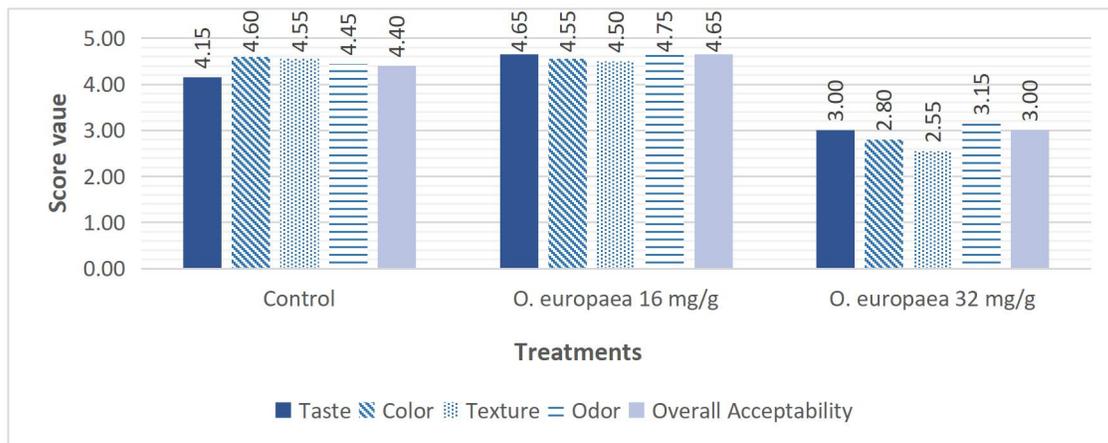


Fig 1. Organoleptic characteristics of cooked minced meat treated with olive leaves powder.

CONCLUSION:

Olive leaves extracts showed antibacterial activity against tested Gram-positive bacterial strains at low concentrations. Olive leaves powder was applied as minced meat preservative and reduced total viable count and prevented *Staphylococcus aureus*

and *E. coli* growth along storage period until 9 days under cooling. Meat samples treated with olive leaves powder showed an improvement in flavor as well as overall acceptability in comparison with control samples.

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