THE ANTIMICROBIAL ACTIVITY OF COMBINATIONS WITH OLIVE OIL (Olea europaea L.) OF FATTY ACIDS OF SOME MEDICINAL PLANTS AGAINST SOME PATHOGENS

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ABSTRACT

In this study, the combinations with *Olea europaea* (olive) oil of fatty acids of *Punica granatum*, *Calendula officinalis* and *Helianthus annuus* seeds to detect interactions on *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Candida albicans* and *Candida parapsilosis* were determined by antimicrobial checkerboard method. It was revealed that linoleic, oleic, palmitic and stearic acid were to be main component of *O. europaea*, *C. officinalis* and *H. Annua*. By contrast, *P. granatum* seed oil contained more than 70% conjugated linolenic acids (CLnA), followed by linoleic acid, oleic acid, stearic and palmitic by using GC-FID. Exposed microorganisms to combinations with *O. europaea* oil of fatty acids of *P. granatum*, *C. officinalis*, *H. annua* overnight, according to FICI (Fractional Inhibitory Concentration Index) interpretation model, remarkable InDE was observed as 71.43% against *E.coli* and 76.19% against *S. aureus*, *B.subtilis*, *Calibicans* while lower AntE noted as 28.57% against *E.coli* and 23.81% against *S.aureus*, *B.subtilis*, *Calibicans*. 

Keywords: Checkerboard method, *Olea europaea*, *Punica granatum*, *Calendula officinalis*, *Helianthus annuus*

INTRODUCTION

Plant-derived antimicrobials like secondary metabolites are very important sources of novel therapeutics and used in traditional medicine for years. The well-known examples of those are terpenes, phenols and fatty acids. In spite of the fact that secound metabolites are high antimicrobial potentials, their interactions with each other may decrease or increase this effect. Several studies have reported that various components of plant oil interact with antibiotics to changes antimicrobial performance and may lead to new approaches for treating infectious diseases (Delaquis et al., 2002; Hemaiswarya et al., 2008; Hammer et al., 2012; Kon and Rai, 2012; Lv et al., 2011). However, there are not any studies with the interaction of the olive oil with the other oils from medicinal plants such as safflower, sunflower and pomegranate. Olive (*Olea europaea*), sunflower (*Helianthus annuus* L.), safflower (*Calendula officinalis* and pomegranate (*Punica granatum*)) are important medicinal plants used in food, cosmetic and pharmacology areas (Flagella et al., 2002; Ashwlayan et al., 2018; Pardo et al., 2011; Meerst et al., 2000). Olive (*Olea europaea*), known as “Mediterranean diet”, is the most important traditional food because of its beneficial functions on human health, preventive effects on some cardiovascular and cancer diseases. It has a rich biochemical content and includes terpenic alcohols, steroids, hydrocarbon derivatives, fatty acids, essential oils and antioxidants (Artajo et al., 2006; Tuck and Hayball, 2002). Olive oil has different ratios of fatty acid ratios such as oleic (C18:1), palmitic (C16:0), linoleic (C18:2), palmitoleic (C16:1), stearic (C18:0), linolenic (C18:3) and the main component is always oleic acid. These compounds have antimicrobial properties on many pathogenic microorganisms such as *Escherichia coli*, *Klebsiella pneumoniae* and *Bacillus cereus* (Aziz et al., 1998, Trichopoulou et al., 2003). *P. granatum* is an important medicinal plant used to treat various diseases such as cardiovascular diseases, cancer, aphthia, diarrhea, diabetes, infantile brain ischemia, gastritis, Alzheimer and ulcer due to its secondary metabolites in pharmaceutical industry (*Yilmaz and Usta, 2010; Jurenka, 2008*). Pomegranate, which is seen in Mediterranean shrub formation, is frequently consumed in our country and pomegranate seeds is constitute the waste of this plant. Pomegranate oil extracted from its seeds contain important fatty acids such as linoleic, oleic, palmitic acid, punicic acid. Especially, the seeds, which contain a high proportion of conjugated linolenic acid (CLnA) isomers, make the fruit more valuable (*Firestone, 2005*). *Calendula officinalis*, is a member of the family Asteraceae, grown especially in parks and gardens due to its attractive appearance. It was reported that the fatty acids extracted from its seed has bioactive components such as triterpene saponosites, triterpene esters, phenolic compounds, carotenoids and volatile components. (Ozgüll-Yücel 2005; Gruenenwald et al., 2000). In addition, sunflower (*Helianthus annuus* L.) is one of the most important oil plants produced in the world and Turkey. It grows in almost every region of our country (Turkey) and meets 50% of vegetable oil consumption. The oil obtained from the seeds regulates metabolism and has vital benefits in the reproductive and digestive system. But, it has been associated with obesity in recent years. Sunflower oil contains high amounts of unsaturated fatty acids (82.75%) and were reported to constitute of mostly oleic acid, linoleic acid, linolenic acid (*Kirbaşlar et al.*, 2012). 

The objective of this study was to assess the susceptibility of some pathogen microorganisms to single and combinations with *O. europaea* oil of fatty acids of *P. granatum*, *C. officinalis*, *H. annua* to detect synergistic effect (SynE) or indifferent effect (IndE) or antagonistic effect (AntE), using checkerboard method. 

MATERIAL AND METHODS

Plant Materials and Chemical Analysis

Seeds of *P. granatum*, *C. officinalis*, *H. annua*, *O. europaea* were purchased from Mecidifendi firm., and the fresh oil of their seeds were provided by cold pressing machine. The chemical characterization of the oils were carried out in the Mersin University Advanced Technology Education Research and Application Center (MEU-METAM) by GC-FID analysis. The oils were stored 4°C in the dark before their employment in the following tests.

GC-FID Analysis

The oils were converted into corresponding fatty acid (FA) methyl esters according to Jennings and Akoh’s work (2001). The GC-FID analysis was carried out on an Agilent gas chromatograph equipped with a flame ionization detector (FID) and a data handling processor. The separation was achieved using an HP Innowax capillary column (30 m*0.25 mm*0.25 μm film thickness), helium as carrier gas (20 cm/sec). Column temperature: 40°C with 5 min initial hold, and then to 140°C at 10°C/min, and then to 250°C after 15 min; injection mode 1 μL, 280°C, split 100:1, Injector and detector temperatures were 280°C
and 330°C, respectively. The results were compared with CLnA, and FAME (Fatty acid methyl esters) standards (Sigma-Aldrich-supelec).

Antimicrobial Screening (MIC, FIC and FICI)

The combined antimicrobial activity of *O. europaea*, *P. granatum*, *C. officinalis*, *H. annuus* were researched on several pathogens, namely *Escherichia coli* (ATCC 25293), *Enterococcus faecalis*, *Staphylococcus aureus* (ATCC 29252), *Bacillus subtilis* (ATCC 6633), *Candida albicans* and *Candida parapsilosis* using modified spectrophotometric checkerboard microdilution technique. The inoculums of microorganisms were prepared in 4 ml Triptic Soy Broth for bacteria, 4 ml Sabouraud Dextrose Broth for yeasts and incubated at 37°C, overnight. After 24 hours, the culture suspensions were adjusted to 0.5 McFarland Standard Turbidity (~ 10^8 for bacteria, (~ 10^7 for yeasts) and stored at +4°C until use (*McFarland, 1987*). Cold-pressed oil of the plants were dissolved at 50 µl/ml (6.8 mg *O. europaea*, 42.5 mg *P. granatum*, 3.5 mg *C. officinalis*, 40.8 mg *H. annuus*) with dimethyl sulfoxide (10% DMSO). The experiment were performed on 96-well microtiter plates and firstly 50 µl of Mueller Hinton Broth (MHB) medium were added into all wells. Two-fold serial dilutions of 25 µl solution of *P. granatum* or *C. officinalis* or *H. annuus* oil was made (A1-H1) on the y-axis along of chequerboard plate. Two-fold serial dilutions of (50 µl) *O. europaea* was made x-axis along from 2nd to 10th columns and *P. granatum* or *C. officinalis* or *H. annuus* solution (single concentration) was added to each well to make fraction and obtain the FIC final concentrations. Columns 11 and 12 were used as negative (medium) and positive (ampicillin for bacteria and fluconazole for yeast) controls, respectively. These standard antibiotics inhibited microorganisms at all concentration more than 16 µg/mL. Finally, 10 µL culture of microorganisms was inoculated on all wells except negative control.

All the plates were incubated at 37°C for 24 hours, the growth (turbidity) was measured at 600 nm for bacteria, 415 nm for yeasts. For MIC analysis, the optical density was read both before, T0 and after 24 hours-incubation, T24. For each plate, MIC were calculated using the following formula: The OD for each replicate at T0 was subtracted from the OD for each replicate at T24. The Percent growth = (ODtest/OD contro)x100. Percent Inhibition = 1-(OD test well/OD of corresponding control well)x100 for each row of the 96-well plate. The dose–response curves obtained from plotting the line of the concentration of the oils against the percent resulting inhibition of microbial growth were obtained with the regression analysis, giving an R^2 value. MIC (the lowest concentration of test material which results in 99.9% inhibition of growth) were calculated using the R^2 formula on inhibition curve. For each plate, FIC and FICI (Fractional Inhibitory Concentration Index) were calculated using the following formula: all wells of the microtiter plates that corresponded to an MIC, the sum of the FICs (ΣFIC) was calculated for each well with the equation:

$$\Sigma FIC = FIC_o + FIC_p, FIC_o = (MIC_o + D), FIC_p, FIC_p = (MIC_p + D),$$

where MICo and MICp, MICo are the MICs of *O. europaea* and the oils of *P. granatum* or *C. officinalis* or *H. annuus* alone, in all wells corresponding to an MIC (isoeffective combinations) (*Erdoğdu Eliziz and Scek, 2018*; *Stergiopoulos et al., 2008*). In present study, FICI values were interpreted following the conventional model suggested by Odds’s work; According to that, a synergistic effect (SynE) is observed when FICI value ≤0.5; an indifferent effect (IndE) when 0.5< FICI≤4 and an antagonistic effect (AntE) when FICI value≥4 (Odds 2003).

**Statistical Analysis**

Statistical analyses and significance were measured by Tukey or Dunnet (2-sided) test in one-way analysis of variance for MICs using SPSS 25. The experiment was repeated at least 3 times. Differences were considered significant at p ≤ 0.05.

**RESULTS AND DISCUSSION**

**Chemical Composition**

The components of fatty acid extracted from *O. europaea*, *P. granatum*, *C. officinalis* and *H. annuus* seeds with their retention time (Rt) and area (%) were listed in Table 1. The *O. europaea* oil was abundant in oleic acid (73.65%), palmitic acid (12.86%), linoleic acid (7.37%), stearic acid (3.12%), palmitoleic acid (1.05%), and trace amounts less than 1% with aracidic acid (0.12%), lauric acid (0.1%), in *H. annuus* oil were linolenic (50.25%), oleic (36.97%), palmitic acid (6.74%), stearic acid (4.62%), and trace amounts less than 1% with aracidic acid (0.45%), palmitic acid (0.22%), n9-cis-11-eicosenoic acid (0.2%), palmitoleic acid (0.15%), behenic acid (0.06%), the major FAs in *C. officinalis* oil were linolenic (78.82%), oleic (10.67%), palmitic acid (6.49%) acids, stearic acid (1.82%), and trace amounts less than 1% with aracidic acid (0.22%), n9-cis-11-eicosenoic acid (0.07%), while in *P. granatum* oil were remarkably conjugated linolenic acids (CLnAa) (62.08%), oleic acid (8.35%), linolenic acid (7.34%), palmitic acid (4.11%) acids, stearic acid (2.90), n9-cis-11-eicosenoic acid (1.01%), and trace amounts less than 1% with aracidic acid (0.7%).

The results about chemical composition of the plants in this report compared with previous reports in literature and found that they are essentially similar. It was revealed that linoleic, oleic, palmitic and stearic were to be main component of *O. europaea* (*Andjelkovic et al., 2009*), *C. officinalis* (Ashwlayan et al., 2018) and *H. annuus* (Flagella et al., 2002). By contrast, pomegranate seed oil contained more than 70% conjugated linolenic acids (CLnAa), followed by linoleic acid, oleic acid, stearic and palmitic (Ö zgül-Yücel, 2005; Fadavi et al., 2006).

**Table 1 Chemical composition of *O. europaea*, *P. granatum*, *C. officinalis* and *H. annuus* seed oil.**

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th><em>O. europaea</em></th>
<th><em>C. officinalis</em></th>
<th><em>H. annuus</em></th>
<th><em>P. granatum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ra</td>
<td>Rt</td>
<td>Ra</td>
<td>Rt</td>
</tr>
<tr>
<td>C12:0 lauric acid</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>13.6</td>
</tr>
<tr>
<td>C16:1 palmitoleic acid</td>
<td>1.05</td>
<td>12.581</td>
<td>0.15</td>
<td>12.561</td>
</tr>
<tr>
<td>C18:2 linoleic acid</td>
<td>7.37</td>
<td>15.199</td>
<td>78.82</td>
<td>15.287</td>
</tr>
<tr>
<td>C20:0 arachidic acid</td>
<td>0.47</td>
<td>16.371</td>
<td>0.22</td>
<td>16.364</td>
</tr>
<tr>
<td>C20:1 n9 cis-11-eicosenoic acid</td>
<td>0.31</td>
<td>16.592</td>
<td>0.2</td>
<td>16.586</td>
</tr>
<tr>
<td>CLnA (Conjugated linolenic acid)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Total** 98.99% 98.59% 99.61% 86.49%

Legend: Ra: Relative abundance (%); Rt: Retention time

**Antimicrobial Activity and Evaluation of FICI Values**

The antimicrobial activity of *E. coli*, *E. faecalis*, *S. aureus*, *B. subtilis*, *C. albicans* and *C. parapsilosis* to single and combinations with *O. europaea* oil of fatty acids of *P. granatum*, *C. officinalis*, *H. annuus* to detect synergistic effect (SynE) or indifferent effect (IndE) or antagonistic effect (AntE) were showed on Table 2. Strong antimicrobial activity alone were noticed for the oils of *O. europaea* (26-158.4 µg/mL), *C. officinalis* (23.4-44.8 µg/mL), *H. annuus* (15.7-211.6 µg/mL), *P. granatum* (48.2-389.25 µg/mL).
The MIC results of the oils of safflower, sunflower and pomegranate alone were 54.0 ± 0.2 µg/mL, 39.07 ± 0.2 µg/mL, 36.82 ± 0.2 µg/mL for E. coli, 149.7 ± 0.2 µg/mL, 15.9 ± 0.2 µg/mL for C. albicans, respectively. The highest MIC was 15.9 ± 0.2 µg/mL for C. albicans while the highest MIC was 277.4 ± 12.5 µg/mL for P. dumerilii, and the lowest MIC was 0.2 ± 12.5 µg/mL for P. dumerilii against A. hydrophila. For the yeast, the MIC values were reported as 16.8 µg/mL in the fraction with Od+P, while the highest MIC was 277.4 ± 12.5 µg/mL in Od+H2 against A. hydrophila. For the fungus, the MIC values were reported as 16.8 µg/mL in the fraction with Od+P, while the highest MIC was 277.4 ± 12.5 µg/mL in Od+H2 against A. hydrophila.

When the antifungal activities of combinations with O. europaea oil of fatty acids of P. granatum, C. officinalis, H. annuus were examined, the values of the MICs in all fractions with oil of safflower, sunflower and pomegranate were found to be statistically different (p<0.05) from the MICs determined from the plant’s alone against pathogen microorganisms in this study. The lowest MIC value were reported as 31.7 µg/mL in the fraction with Od+C12,5 while the highest MIC was 389.25 µg/mL in Pd against E. coli. While the lowest MIC value were reported as 29.9 µg/mL in the fraction with Od+P, the highest MIC was 1550.0 µg/mL in Od+C12,5 against E. coli. The lowest MIC value were reported as 25.6 µg/mL in the fraction with Od+H2, while the highest MIC was 378.1 µg/mL in Od+P against S. aureus, while the lowest MIC value were reported as 20.9 µg/mL in the fraction with Od+C12,5, the highest MIC was 635.9 µg/mL in Od+C12,5 against B. subtilis. For the yeast, the lowest MIC value were reported as 16.8 µg/mL in the fraction with Od+P, while the highest MIC was 277.4 µg/mL in Od+H2 against A. hydrophila.

The MIC results of the oils of safflower, sunflower and pomegranate alone were 54.0 ± 0.2 µg/mL, 39.07 ± 0.2 µg/mL, 36.82 ± 0.2 µg/mL for E. coli, 149.7 ± 0.2 µg/mL, 15.9 ± 0.2 µg/mL for C. albicans, respectively. The highest MIC was 15.9 ± 0.2 µg/mL for C. albicans while the highest MIC was 277.4 ± 12.5 µg/mL for P. dumerilii, and the lowest MIC was 0.2 ± 12.5 µg/mL for P. dumerilii against A. hydrophila. For the yeast, the MIC values were reported as 16.8 µg/mL in the fraction with Od+P, while the highest MIC was 277.4 ± 12.5 µg/mL in Od+H2 against A. hydrophila. For the fungus, the MIC values were reported as 16.8 µg/mL in the fraction with Od+P, while the highest MIC was 277.4 ± 12.5 µg/mL in Od+H2 against A. hydrophila.
this technique, researches were done about combinations of drugs occurred strong antimicrobial effect on the some microorganisms species. Singh et al., (2015) were determined that the combinations of ethanolic and fruits (Calendula officinalis: Carissa: Mentha 1:2:1) showed strong antimicrobial activity against B. cereus and S. aureus. In the study which antibacterial activities of combination of oleic acid and gentamicin against Staphylococcus aureus (including meticillin-resistant MRSA), synergistic effect were reported (Atashbeyk et al., 2014). The linoleic and oleic acid extracted from Helichrysum pedunculatum combinations were inactive against the Gram-negative species, while synergistic effect was observed against Staphylococcus aureus and Micrococcus kristinae (Dilika et al., 2000).

According to the findings of this results, the interaction data in the form of the fractional inhibitory concentration indices (FICIs) were listed in Table 2. According to the current study, exposing microorganisms to olive oil and safflower or sunflower or pomegranate combinations overnight, mostly AntE or IndE were occurred between 23% and 77% for all the pathogens. Remarkable IndE was observed as 71.43% against E.coli and 76.19% against S. aureus, B.subtilis, Calibics while lower AntE noted as 28.57% against E.coli and 23.81% against S.aureus, B.subtilis, Calibics (FICI range of 0.6 to 23.4). AntE and IndE were noted 52.38% and 47.62% for E.fecalis (FICI range of 0.67 to 85.7), respectively. Interestingly, low level of SynE (4.76%), equally level of AntE and IndE (47.62%) were reported against C.parapsilosis (FICI range of 0.01 to 116.4) in this study (Figure 1).

It is claimed that fatty acids have more antagonistic effects on Gram-positive than Gram-negative bacteria (Braun et al., 1980; Russell, 1991). In present study, remarkable IndE was observed as 71.43% against E.coli and 76.19% against S. aureus, B.subtilis, Calibics while lower AntE noted as 28.57% against E.coli and 23.81% against S.aureus, B.subtilis, Calibics (FICI range of 0.6 to 23.4). AntE and IndE were noted 52.38% and 47.62% for E.fecalis (FICI range of 0.67 to 85.7), respectively. Interestingly, low level of SynE (4.76%), equally level of AntE and IndE (47.62%) were reported against C.parapsilosis (FICI range of 0.01 to 116.4). A synergistic effect was only observed in C. parapsilosis. According to the obtained results, the combination of O. europaea with C. officinalis, H. annuus, P. granatum oils one by one showed mostly indifferent or antagonistic effect against six tested microorganisms.

CONCLUSION

This study indicates that the combination of fatty acid of olive and the three medicinal plants (safflower, sunflower and pomegranate) has significant potential for the development of new antimicrobial treatment, which will permit to find the treatment of many diseases caused by microorganisms. From the results obtained, the fatty acids of olive oil acts in mostly indifferent with the tested other medicinal plants oil. This interaction could lead to new approaches in ethnomedical treatment of infectious diseases. Hence, there is a strong possibility that studies on the biochemical of the antagonist, indifferent and synergistic interactions between medicinal plants will improve in the long term.

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REFERENCES


Figure 1 Percentage of FICI values of O. europaea alone or in combinations with P. Granatum, C. officinalis, H. annuus and FICI values of the combination of them with E. coli, E. faecalis, S. aureus, B. subtilis, C. albicans and C. parapsilosis by 24 hours of incubation. Antagonistic: Ant, synergistic: Syn, Indifferent: Ind Effect.


