

Synergistic Effects between *Mentha Piperita*, *Mentha Longifolia* and *Ocimum Basilicum* on Different Bacterial Strains

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Abstract

The present study aimed to quantitatively evaluate antimicrobial activity of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum* from Lamiaceae family, compare between them and to evaluate the type of interaction between them by microbroth dilution method and calculation of fractional inhibitory concentration. Dried leaf of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum* were extracted using same extraction method and solvent the highest ratio yielded by *Mentha longifolia* 9% and lowest ratio yielded by *Ocimum basilicum* 6%. *Mentha piperita* exhibit the lowest minimum inhibitory concentration value ranged between 1.5-<0.1 mg/ml, followed by *Mentha longifolia* and *Ocimum basilicum* ranged between 3-<0.1mg/ml with minimum bactericidal concentration ranged between 6-0.1875. On combination of leaf extract *Mentha piperita* with *Mentha longifolia* exhibit the lowest minimum inhibitory concentration value ranged between 0.1875-<0.05 mg/ml, while combination of *Mentha longifolia* with *Ocimum basilicum* revealed minimum inhibitory concentration ranged between 0.75-<0.05. Among tested bacterial strain *Streptococcus mitis* showed high sensitivity against all tested leaf extract alone and in combination form. The combinations of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum* showed synergistic effects against most of bacteria. *Mentha longifolia* and *Ocimum basilicum* revealed antagonistic effect against *Staphylococcus aureus*. As a result three Lamiaceae species showed broad spectrum antimicrobial activity against isolated bacterial strain and their activity in combination form more pronounced than alone.

Keywords: Lamiaceae species, MIC, checkerboard method, herbal-herbal interaction

1. Introduction

Infectious diseases are consider second-major cause of death throughout the world in children and young adults and kill about 50,000 people every day (World Health Organization [WHO], 1999; WHO, 2002). An increase of multi-drug resistance in gram positive and gram negative bacteria is threatening world population and require immediate efforts to identify antimicrobial drugs with broad-spectrum activity from natural products (Sanches et al., 1998). Secondary plant metabolites are most important structurally diverse bioactive compounds with antimicrobial activity achieved from plant to protect it from wide range of microorganisms such as environmental organisms and plant pathogens. The bioactive compounds to combat infection work by different mechanism may be combined to enhance their individual activity the phenomena known as synergism (Hemaiswarya et al., 2008), or known as antagonism if the combination results in worsening effect and known as indifference if effect is less than synergistic but not antagonistic (Rani et al., 2009). *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum* belonging to family Lamiaceae. This family distribute from temperate to tropical regions but is found primarily in the Mediterranean basin (De Judicibus, 2011). Different part of those plant such as leaf, flower, stem and seeds used widely in traditional medicine for treatment of infectious disease, coughs, colds, fever, influenza, diarrhea, wounds, kidney disorders, swollen glands also as carminative, expectorant, stimulant and diaphoretic (Mossa et al., 2000; Van Wyk et al., 1997). The essential oil of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum* showed antimicrobial activity against gram negative and gram-positive bacteria, yeasts and molds (Suppakul et al., 2003; Mimica-Dukic et al., 2003; Singh et al., 2015) such as pulegone, neomenthol, menthone, isomenthone, menthyl acetate, menthol, 1,8-cineole, borneol, menthofuran, and piperitenone oxide in *Mentha piperita* and *Mentha longifolia* (Mkaddem et al., 2009; Rodrigues et al., 2004), thymol, chavicol methyl ether, linalool, eugenol, copaene, p-menth-2-en-1-ol, , bornyl acetate, himachalene, rosifoliol and cubebene in *Ocimum basilicum* (Unnithan et al., 2013). However, alkaloids and tannins isolated from *Mentha* spp. and *Ocimum basilicum* have pronounced effects against growth of

gram-negative and gram positive bacteria (Al-Younis & Argushy, 2009; Edeoga et al., 2005). Phenolic compounds of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum* such as caftaric acid, gentisic acid, caffeic acid, chlorogenic acid, p-coumaric acid, ferulic acid, isoquercitrin, rutin, rosmarinic acid, quercitrin, quercetin, luteolin, kaempferol and apigenin also responsible on antimicrobial activity (Akroum et al., 2009; Vlase et al., 2014). The study aimed to estimate quantitatively antimicrobial activity of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum* on gram positive and gram negative bacterial strains, compare between them and to evaluate the type of interaction between leave extract by determination of fractional inhibitory concentration index.

2. Methods

2.1 Collection of Plant

The leave of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum* were collected during July 2015, from the garden in Erbil city. The plants were authenticated and identified in the department of pharmacognosy, Hawler Medical University, Kurdistan region/Iraq.

2.2 Preparation of Plant Extracts

Fifty g of dried powdered leaves were extracted with 100 ml (80%) ethanol for 1 hr using ordinary reflux extractor that yielded an extract, which was after drying dissolved in 20 ml (20%) HCl and refluxed for 30 min, followed by liquid-liquid fractionation using chloroform (10 x 3 ml) resulted an chloroform fraction on drying in vacuum that used for evaluation of antimicrobial activity.

2.3 Bacterial Strain

The bacterial strain used in this study such as *Staphylococcus aureus*, *Staphylococcus auricularis*, *Streptococcus mitis*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Escherichia coli*. The bacterial strain obtained from the department of Biology, Collage of Education, Salahadin University. The bacterial strains grown on nutrient agar at 37 °C for 24h, then suspended in a saline solution (0.9 %, w/v) NaCl and adjusted to a turbidity of 0.5 Mac Farland standard (10^8 CFU/ml).

2.4 Quantitative Estimation of Antimicrobial Activity by Microbroth Dilution Method

The minimum inhibitory concentrations (MICs) of leave extract determined in duplicate by micro broth dilution method (Kaya & Ozbilge, 2012) in sterile flat-bottom 96-well polystyrene plates. The concentrations used for each plant extract ranged between 0.0122-50 mg/ml prepared by serial dilution in well containing 100 µl nutrient broth. For determination of interaction effect of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum* same quantity from each plant extract mixed with each other, followed by addition 10 µl of bacterial suspension. Well containing bacterial suspensions and nutrient broth were used as positive control and the well with only nutrient broth used as negative control. The plates covered and incubated aerobically at 37 °C for 24h. The MIC was defined as the lowest dilution that showed no growth in the nutrient broth as indicated by the absence of turbidity.

2.5 Minimal Bactericidal Concentration (MBC)

MBC values were determined by subculturing 100 µl of medium from each well of MIC test demonstrating no visible growth and inoculating nutrient agar plates. Plates were incubated at 37 °C for a total period of 48h. MBC was recorded as a lowest extract concentration have ability to kill 99.9 % of the bacterial inocula after 48h incubation at 37 °C. Each experiment was repeated at least three times (Rabe et al., 2002).

2.6 Checkerboard Method

The interactions between different plant extracts were determined using checkerboard method (Petersen et al., 2006). The range of different leave extract concentration used in the checkerboard assay was such that the dilution range encompassed the MIC for each plant used in the analysis. The Fractional inhibitory concentration (FIC) was derived from the lowest concentration of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum* combination permitting no visible growth of the test organisms on the nutrient broth after incubation for 24 h at 37 °C.

FIC indices were calculated using the formula: FIC index = (MIC of extract a in combination / MIC of extract a alone) + (MIC of extract b in combination / MIC of extract b alone).

Combinations were classified as synergistic, if the FIC indices were ≤ 1 , indifferent if the FIC indices were between 1 and 2 and antagonistic if the FIC indices were ≥ 2 (Jarrar et al., 2010).

3. Results

The results of table 1 showed that percentage yield of leave extract of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum* varied by use same extraction method and solvent, the highest percentage produced by *Mentha longifolia* (9%), then *Mentha piperita* (7%) and lowest percentage by *Ocimum basilicum* (6%).

Table 1. The percentage yield of chloroform extracts of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum*.

Plant	Yield (%)	Color	Consistency
<i>Mentha piperita</i>	7	Dark olive	Greasy
<i>Mentha longifolia</i>	9	Dark olive	Greasy
<i>Ocimum basilicum</i>	6	Dark olive	Greasy

The present study on quantitative estimation antimicrobial activity by microbroth dilution method revealed activity of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum* alone and in combination with each other against four gram positive and two gram negative bacteria but with various degree according to the microorganism as shown in table 2 & table 3. Among the tested leave extract against bacterial strain *Mentha piperita* alone showed the lowest MIC value ranged between 1.5-<0.1mg/ml with MBC value 3-0.1875 mg/ml, followed by *Mentha longifolia* and *Ocimum basilicum* ranged between 3-<0.1mg/ml with MBC value 6-0.1875 mg/ml. On combination of leave extract *Mentha piperita* with *Mentha longifolia* exhibit the lowest MIC value ranged between 0.1875-<0.05 mg/ml with MBC value 0.375-0.097 mg/ml, then *Mentha piperita* with *Ocimum basilicum* ranged between 0.375-<0.05 mg/ml with MBC value 0.75-0.097 mg/ml, while *Mentha longifolia* with *Ocimum basilicum* 0.75-<0.05 mg/ml and three leave extract together exhibit MIC value ranged between 0.75-<0.03 mg/ml with MBC value 1.5-0.048 mg/ml.

Table 2. MIC values of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum* alone and in combination form against gram positive and gram negative bacteria

Bacteria	Chloroform extract (mg/ml)						
	Mp	MI	Ob	Mp & MI	Mp & Ob	MI & Ob	Mp,MI &Ob
<i>Staphylococcus aureus</i>	1.5	0.75	0.1875	0.1875	0.1875	0.75	0.1875
<i>Staphylococcus auricularis</i>	0.1875	0.1875	0.1875	<0.05	<0.05	<0.05	<0.03
<i>Streptococcus mitis</i>	<0.1	<0.1	<0.1	<0.05	<0.05	<0.05	<0.03
<i>Streptococcus pneumoniae</i>	1.5	3	3	<0.05	0.375	0.75	0.75
<i>Klebseilla pneumoniae</i>	1.5	1.5	3	<0.05	<0.05	<0.05	<0.03
<i>Escherichia coli</i>	0.375	0.1875	<0.1	<0.05	<0.05	<0.05	<0.03

Mp. *Mentha piperita*; MI. *Mentha longifolia*; Ob. *Ocimum basilicum*

Table 3. MBC values of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum* alone and in combination form against gram positive and gram negative bacteria

Bacteria	Chloroform extract (mg/ml)						
	Mp	MI	Ob	Mp & MI	Mp & Ob	MI & Ob	Mp,MI &Ob
<i>Staphylococcus aureus</i>	3	1.5	0.375	0.375	0.375	1.5	0.375
<i>Staphylococcus auricularis</i>	0.375	0.375	0.375	0.097	0.097	0.097	0.048
<i>Streptococcus mitis</i>	0.1875	0.1875	0.1875	0.097	0.097	0.097	0.048
<i>Streptococcus pneumoniae</i>	3	6	6	0.097	0.75	1.5	0.75
<i>Klebseilla pneumoniae</i>	3	3	6	0.097	0.097	0.097	0.048
<i>Escherichia coli</i>	0.75	0.375	0.1875	0.097	0.097	0.097	0.048

Mp. *Mentha piperita*; MI. *Mentha longifolia*; Ob. *Ocimum basilicum*

FIC index which is an indicator of various degree of interaction between leave extract against bacterial strains. The result of determination type of interaction between leave extract as shown in table 4 & table 5 revealed synergistic effect between different leave extract against most bacterial stain with FIC index ranged between 0.037-1.

Table 4. FIC index values determination of different combination chloroform extract of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum*.

Bacteria	FIC		FIC index	Interaction	FIC		FIC index	Interaction
	Mp	MI	Mp & MI		Mp	Ob	Mp & Ob	
<i>Staphylococcus aureus</i>	0.125	0.25	0.375	Synergy	0.125	1	1.125	Indifference
<i>Staphylococcus auricularis</i>	0.48	0.4	0.88	Synergy	0.24	0.24	0.48	Synergy
<i>Streptococcus mitis</i>	0.5	0.5	1	Synergy	0.5	0.5	1	Synergy
<i>Streptococcus pneumoniae</i>	0.03	0.015	0.045	Synergy	0.25	0.125	0.375	Synergy
<i>Klebseilla pneumoniae</i>	0.03	0.03	0.06	Synergy	0.03	0.03	0.06	Synergy
<i>Escherichia coli</i>	0.12	0.24	0.36	Synergy	0.12	0.24	0.36	Synergy

Mp. *Mentha piperita*; MI. *Mentha longifolia*; Ob. *Ocimum basilicum*

Table 5. FIC index values determination of different combination chloroform extract of *Mentha longifolia*, *Ocimum basilicum* and *Mentha piperita*

Bacteria	FIC		FIC index	Interaction	FIC		FIC index	Interaction	
	MI	Ob	MI & Ob		Mp	MI	Ob		Mp, MI & Ob
<i>Staphylococcus aureus</i>	1	4	5	Antagonistic	0.125	0.25	1	1.375	Indifference
<i>Staphylococcus auricularis</i>	0.24	0.24	0.48	Synergy	0.12	0.12	0.12	0.36	Synergy
<i>Streptococcus mitis</i>	0.5	0.5	1	Synergy	0.24	0.24	0.24	0.72	Synergy
<i>Streptococcus pneumoniae</i>	0.25	0.25	0.5	Synergy	0.5	0.25	0.25	1	Synergy
<i>Klebseilla pneumoniae</i>	0.03	0.015	0.045	Synergy	0.015	0.015	0.007	0.037	Synergy
<i>Escherichia coli</i>	0.24	0.5	0.74	Synergy	0.06	0.12	0.24	0.42	Synergy

Mp. *Mentha piperita*; MI. *Mentha longifolia*; Ob. *Ocimum basilicum*

4. Discussion

Because of bacterial resistance against antibiotics and single treatment, using two or more alternative natural product could be interested (Rybak & McGrath, 1996). In the present study all leave extract alone and in combination with each other were revealed antimicrobial activity against all tested bacterial strain, but combination form exhibit more pronounced activity than extract alone. In comparison between activity of leave extract alone and in combination form *Mentha piperita* showed the lowest MIC value alone and in combination with *Mentha longifolia* reduced from 1.5-<0.1 to 0.1875-<0.05 mg/ml. *Streptococcus mitis* showed high sensitivity against all tested leave extract alone and in combination form. while *Streptococcus pneumoniae* and *Klebseilla pneumoniae* showed low sensitivity against leave extract alone but there were observed decreasing in MIC value in combination form. *Ocimum basilicum* not exhibit any decreasing in MIC value in combination with *Mentha longifolia* against *Staphylococcus aureus* (Table 2). Antimicrobial activity of *Mentha piperita* recorded by other workers against *Staphylococcus aureus* and *Escherichia coli* with MIC value 3.67 mg/ml and 31.25 mg/ml respectively (Probst et al., 2011). Several studies reported that *Mentha longifolia* possessed a broad spectrum antimicrobial activity with MIC value for ethyl acetate extract 1.25 mg/ml against *Staphylococcus aureus*, *Klebseilla pneumoniae* (Saeidi et al., 2014) and methanolic extract 34.512 µg/ml against *Escherichia coli* (Razavi et al., 2012). *Ocimum basilicum* showed antimicrobial activity with MIC value 250 µg/ml against *Escherichia coli*, *Staphylococcus aureus* (Zel et al., 2005) and chloroform extract 0.312 mg/ml against *Escherichia coli* (Balamurugan, 2013). The antimicrobial activity of different leave extract combinations were further assessed on the basis of the FIC index. The number of synergistic interaction between leave extract combination with each other against different bacterial strain is in the following order: *Mentha piperita* with *Mentha longifolia* was synergistic against all tested bacteria, while *Mentha piperita* with *Ocimum basilicum*, *Mentha longifolia* with *Ocimum basilicum* and *Mentha piperita* with *Mentha longifolia*, *Ocimum basilicum* were synergistic against five tested bacteria (Table 4 & table 5). Combination therapy with synergistic effect can provide broader-spectrum activity, increase efficacy, delay emergence of resistance, and reduce toxicity (Marr et al., 2004). *Mentha piperita* with *Ocimum basilicum* and *Mentha piperita* with *Mentha longifolia*, *Ocimum basilicum* had indifferent effect only against *Staphylococcus aureus*. *Mentha longifolia* with *Ocimum basilicum* showed antagonistic effect against *Staphylococcus aureus*. Indifferent and antagonistic interactions were due to the increased MIC values obtained from the low sensitivity and accepting resistance of those bacteria to different leave extract. Similarly the combinations of ethanolic extracts of propolis with ginger and mint essential oils, and cinnamon with ginger and clove essential oils, showed synergistic effects against *Staphylococcus aureus* and *Escherichia coli* (Probst et al., 2011). Al-Bayati (2008) reported that combinations of *Thymus vulgaris* and

Pimpinella anisum essential oils showed inhibitory activity against pathogenic bacteria and these essential oils were more efficient than the drug maxipime in the assays. Bioactive constituents which are found in plants act by different mechanism each one potentiate the action of other in each plant. Different chemical compounds were inhibit growth of microorganism at the same time, thus increasing the antimicrobial activity of the products. For example some constituents act on the plasma membrane of bacteria, affecting the efflux pump (Kristianse & Amaral, 1997). Essential oils act as membrane permeabilizers, enhancing the intake of other constituents (Helander et al., 1998), flavonoids disrupt microbial membranes (Tsuchiya et al., 1996), saponins having detergent properties serve as lytic agents (Abukakar et al., 2008), alkaloids in extracts interchelate with DNA (Phillipson & O'Neill 1987), tannins precipitate microbial protein (Prasad et al., 2008).

5. Conclusion

As a result concluded that leave extract of *Mentha piperita*, *Mentha longifolia* and *Ocimum basilicum* alone and in combination with each other were revealed antimicrobial activity against six tested bacterial strain. *Mentha piperita* showed highest activity followed by *Mentha longifolia* and *Ocimum basilicum*. Combination of leave extract produced more pronounced activity than extract alone and cause observed reduction in MIC value. Also combined leave extract of different Lamiaceae species shown to delay the emergency of bacteria resistance and produce desirable synergistic effects in the treatment of bacterial infection.

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Conflict of Interest: Authors have declared that no competing interests exist.

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