

SYNERGISTIC EFFECT OF DIFFERENT PLANT EXTRACTS AND ANTIBIOTICS ON SOME PATHOGENIC BACTERIA

Suzan A. Shareef ^{a,*}, Abdulilah S. Ismaeil ^b, Akhter A. Ahmad ^b

^a Dept. of General Sciences, College of Basic Education, Salahaddin University, Kurdistan Region, Iraq – (suzan.sharif@su.edu.krd)

^b Dept. of Biology, College of Science, Salahaddin University, Kurdistan Region, Iraq – (abdulilah.ismaeil; akhter.ahmed)@su.edu.krd

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ABSTRACT:

In this study, the antibacterial activity of methanol extract of henna (*Lawsonia inermis*) leaves, ethanol extract of pomegranate (*Punica granatum*) peel, volatile oil of sesame (*Sesamum indicum*) and peanut (*Arachis hypogaea*) were investigated against some Gram-positive and Gram-negative bacteria including *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Acinetobacter sp.* Henna extract was most effective substrate against all tested bacteria followed by pomegranate and peanut while sesame was less effective. All extracts were screened for their antibacterial activity in combination with commonly used antibiotics, including ciprofloxacin and erythromycin to evaluate synergistic effects using Minimum inhibitory concentrations (MIC) method which determined by microbroth dilution assays. Different interactions (synergistic and indifference) were observed between plant extracts and used antibiotics. The fractional inhibitory concentration (FIC) index ranged from 0.01 to 1.25 for *B. cereus*, 0.5 to 1 for *P. aeruginosa*, 0.01 to 0.3 for *S. aureus* and 0.06 to 0.25 for *A. baumannii*. The best synergistic capacity appeared between erythromycin and sesame. In vitro interaction between antimicrobial agents in combination with tested plant extracts showed synergistic effects. The MICs of each antibiotic was decreased to half when it is used in combination with tested plant extracts. This decreasing in MICs was observed in all plant extracts against tested bacteria as well as the extracts exhibited weak antibacterial activity alone.

KEYWORDS: Antibacterial activity; Combination; Minimum inhibitory concentration (MIC); Plant extract; Synergistic.

1. INTRODUCTION

Infectious diseases caused by bacteria and fungi affect millions of people worldwide. Throughout the history of mankind, infectious diseases have remained a significant cause of death and disability; it accounts for one-third of all deaths in the world (Usha *et al.*, 2010). The discovery of antibiotics was an essential part of combating bacterial infections that once ravaged humankind (Usha *et al.*, 2010). Antibiotics are critical weapons in combating bacterial infections and can be beneficial for human health (Berge and Wierup, 2012). However, overtime, the effect of antibiotics that routinely used have decreased against certain infections due to production of toxic reactions and the development of resistant strains of bacteria (DiMasi *et al.*, 2016). The rapid development of drug-resistant bacteria is an important health problem that occurred worldwide (Rouveix, 2007 and Ahmed, 2013). The alarming growth of the number of antibiotic resistant bacteria and difficulties in treatment of infections, besides sometimes antibiotics use may cause opposite effects, such as allergic reactions, immune destruction, and hypersensitivity have initiated a search for new antibacterial compounds and develop new alternative strategies in combating bacterial infections (Agrawal *et al.*, 1996). Medicinal plants, with their long history of use in folk medicine for the treatment of infectious diseases, have become a promising innovative antimicrobial substances by extraction of phytochemicals, which are active to prevent infections (Abiramasundari *et al.*, 2011 and Agrawal *et al.*, 1996). Plant-derived compounds could exhibit a direct

antibacterial activity and/or an indirect activity as antibiotic resistance modifying compounds, which, combined with antibiotics, increase their effectiveness (Haroun and Al-Kayali, 2016).

The use of plant extracts can be highly significant in therapeutic treatments and there are many different medicinal plants used in traditional medicine by the traditional herbalists to treat varieties of human ailments (Aali *et al.*, 2018). Numbers of different plants have been used due to their antimicrobial activity as a result of their active substances while others by a combination of their common phytochemicals with antibiotics (Ahmed *et al.*, 2010). For this reason, the antibacterial activities of plant extract alone and when it combined with different antibiotics have been studied in many parts of the world by a number of researchers. Farooqui *et al.* (2015) investigated synergistic antimicrobial activity of *Camellia sinensis* and *Juglans regia* with nalidixic acid against 350 Gram-positive and Gram-negative strains belonging to 10 different bacterial species. While Liu *et al.* (2017) studied synergistic antimicrobial effect of lipopeptides and tea polyphenols against *V. parahaemolyticus* and their result showed that the combination of lipopeptides and tea polyphenols displayed strong synergistic antibacterial effect against *V. parahaemolyticus* with a fractional inhibitory concentration index of 0.19. In another study by Ennacerie *et al.* (2017) also tested the antibacterial potency and evaluate the possible synergistic effect between both aqueous and ethanolic extracts, flower buds and fruits of *Capparis spinosa*, and antibiotics against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and their results showed a synergistic effect with ICIF ranging from 0.02 to 0.24. From the points of view, the present study

* Corresponding author

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conducted to investigate whether the combination of some plant extracts with commonly used antibiotics has any synergistic effects on some clinically isolated bacteria or not.

2. MATERIALS AND METHODS

2.1. Plant materials and extraction

Henna (*Lawsonia inermis*) leaves, pomegranate (*Punica granatum*) peel, Peanut (*Arachis hypogaea*) and Sesame (*Sesamum indicum*) were selected as medicinal plants for extraction.

The plant materials were processed by the methods described previously by (Harborne, 1998). Briefly, pulverized plant material (after cleaning and shade drying) was extracted using normal hexane, methanol, and ethanol as solvents. To remove the solvents from the extracts vacuum evaporator was used to attain the crude extract of each fraction. The extracts stored at -20 °C and freshly dissolved in dimethyl sulfoxide (DMSO, Merk Germany) before use.

2.1.1. Extraction of henna leaf: Fifty grams of henna leaves were dried under shade, milled and extracted with n-hexane by the use of Soxhlet extractor apparatus for about six hours. Extraction was carried out the color of the solvent in the last siphoning time became colorless. Then, the solvent evaporated under reduced pressure by the use of a rotary evaporator apparatus. The marc obtained from henna was shade dried and re-extracted with methanol using the above method.

2.1.2. Extraction of Pomegranate Peel: Fifty grams of Pomegranate dry powder was mixed with ethanol, sonicated, and filtered by Whatman paper no.1. The supernatant was dried by rotary evaporator.

2.1.3. Volatile oil of sesame and peanut: One hundred grams of both sesame and peanut weighted were put in 2000 ml rounded bottom capacity flask, separately. Both Clevenger receiver and condenser were attached to the top of the flask, 1000 ml of distilled water was added. The system was heated to 100°C for about 4h until the oil volume at the upper part of the receiver fixed. The oil was pipetted and dried over sodium sulfate anhydrous. Then, it was stored in a dark container in the refrigerator till used (Harborne, 1998).

2.2. Preparing of oil concentrations

Each plants extract was dissolved in dimethyl sulfoxide (DMSO) (50% of the final volume) and was then diluted with Tryptic Soy Broth medium (TSB; Oxoid) to concentrations (2, 4, 8, 16, 32, 64, 128) mg/ml.

2.3. Antimicrobials

Pure antimicrobial powders of Ciprofloxacin and Erythromycin were selected as antimicrobials in this study. The stock solution was prepared by dissolving the powder in TSB in different concentrations (2, 4, 8, 16, 32, 64, 128) mg/ml (Lalitha, 2004).

2.4. Bacterial isolates

Clinical isolates investigated in this study were; *Acinetobacter baumannii* and *Pseudomonas aeruginosa* as Gram-negative, *Bacillus cereus* and *Staphylococcus aureus* as Gram-positive (The isolates were obtained from Bacteriology laboratory of West Emergency Hospital in Erbil City. Vitek II automated system (bioMérieux Marcy l'Étoile, France) (Vitek Systems Version: 06.01) was used to identify the isolates.

2.5. Minimum inhibitory concentration (MIC) determination

Broth microdilution assay was performed to determine the minimum inhibitory concentration for the galls extracts against the identified isolates (Roberts *et al.*, 2012). Ten µl of bacterial cells (equilibrated to OD550 0.5) inoculated into 100µL TSB containing a range of extracts or antimicrobials concentrations beginning (1-128 mg ml⁻¹) in the polystyrene microtiter plate (MTP) wells. The MTPs have incubated overnight at 37 °C. The lowest concentration that did not show any obvious growth was considered as minimum inhibitory concentration. To determine the minimum bactericidal concentration, 100 µl from MTP wells that did not show any obvious growth was streaked on sterile plates of nutrient agar (NA; Oxoid). Nutrient agar (NA) plates were incubated overnight at 37 °C. Concentrations that have no growth on NA plates were considered as minimum bactericidal concentration (MBC). The level below the MICs was considered as subinhibitory concentrations (SICs) which then used to evaluate the synergistic effect of the extracts with antimicrobials. Three replicates were considered on distinct occasions.

2.6. Checkerboard technique to determine the synergistic antimicrobial activity

To investigate the effect of the combination of each extract with the selected antimicrobials 150 µL of TSB medium containing a mixture of SIC of each plant extract and each of the tested antimicrobials were added to 96-well microtiter plates, TSB with no extract and antimicrobials was used as a control. The wells were inoculated with 10 µL of a bacterial suspension. All experiments were achieved in triplicate, and the MTP was incubated overnight at 37°C. To determine the synergistic effect of the extracts and the antimicrobials the broth in the wells were sub-cultured on NA plates.

The combination between the extracts and the antibiotics were evaluated by the checkerboard method as described by Petersen *et al* (2006). The fractional inhibitory concentration (FIC) was derived from the lowest concentrations of the extract in tryptic soy broth tube after overnight incubation at 37 °C. FICs were calculated using the following formula:

$$\sum \text{FIC (FIC index)} = \frac{\text{MIC of plant extract in combination}}{\text{MIC of plant extract alone}}$$

$$\sum \text{FIC (FIC index)} = \frac{\text{MIC of plant extract in combination}}{\text{MIC of plant extract alone}} +$$

$$\frac{\text{MIC of antibiotics in combination}}{\text{MIC of antibiotics alone}} \frac{\text{MIC of antibiotics in combination}}{\text{MIC of antibiotics alone}}$$

When antibiotic combined with a plant extract, synergy is happened when FIC index ≤ 0.5, additivity when 5 < index FIC ≤ 1, indifference when 1 < FIC index ≤ 4 and antagonism when FIC index > 4 as described by Petersen *et al.* (2006). While Kamatou *et al* (2006) defined synergy that happened when FIC index < 1.0, additivity when FIC index = 1.0 and antagonism when FIC index > 1.0. Hence, due to checkerboard assay, Olajuyigbe and Afolayan (2012) indicated that synergy is determined when FIC is less than or equal to 0.5 or is less than or equal to 1.

2.7. Statistical data analysis

All the data were analyzed by Statistical Package Social Science (SPSS) version 21.0. The experimental results were expressed as mean ± standard error of the mean (SEM). Groups were compared by analysis of variance using Two-way ANOVA and Dunnett's multiple comparisons test. Less than 0.05 of *p*-value was regarded as statistically significant. Significance was defined as *p* < 0.05, *p* < 0.01.

3. RESULTS

The Minimum inhibitory concentration (MIC) of plant extracts alone, and antibiotics alone were examined against tested bacteria, as shown in Table (1), Figure (1).

Ciprofloxacin showed inhibited active on bacterial species at (4 mg/ml) against all tested bacteria while erythromycin was more active and inhibited tested bacteria at 1 mg/ml except *S. aureus* (2 mg/ml).

The antibacterial activities of the plant extract varied in relation to the tested organisms. Henna extract was the most effective against all tested bacteria. The most active concentration was 4 mg/ml concentration that inhibited the growth of *Staphylococcus aureus*, *Acinetobacter baumannii* and *Bacillus cereus* utterly, while *Pseudomonas aeruginosa* was inhibited by 8 mg/ml concentration of henna extract.

Pomegranate also showed antibacterial activity with different concentrations against all tested bacteria. Most effective concentration was 4 mg/ml against *P. aeruginosa*, 16 mg/ml against *Acinetobacter* while *S. aureus* and *B. cereus* were inhibited at a minimum inhibitory concentration of 32 mg/ml. Peanut showed similar effects to that of henna against *P. aeruginosa* at MIC of 8 mg/ml and *A. baumannii* at MIC of 4 mg/ml, while it was less effective against *B. cereus* at MIC of 32 mg/ml.

Table 1. Minimum inhibitory concentration (MIC) of plant extracts alone and antibiotics alone against tested bacteria

Antibiotic and Plant extract	MIC (mg/ml)			
	<i>S. aureus</i>	<i>B. cereus</i>	<i>P. aeruginosa</i>	<i>A. baumannii</i>
Henna (<i>Lawsonia inermis</i>)	4	4	8	4
Pomegranate (<i>Punica granatum</i>)	32	32	4	16
Peanut (<i>Arachis hypogaea</i>)	64	32	8	4
Sesame (<i>Sesamum indicum</i>)	128	64	64	32
Ciprofloxacin	4	4	4	4
Erythromycin	2	1	1	1

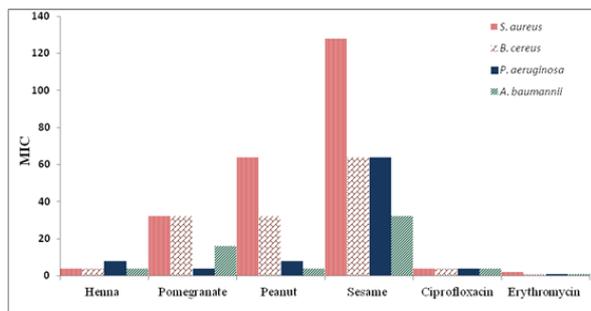


Figure 1. Minimum inhibitory concentration (MIC) of plant extracts alone and antibiotics alone against tested

In vitro interaction between antimicrobials and tested plant extracts by microdilution method showed a reduction of MIC of the antimicrobials when combined with plant extracts. The minimum inhibitory concentration of antibiotics in combination with plant extracts against pathogenic bacteria tested by microdilution method is shown in Table [2].

Table 2. Minimum inhibitory concentration of antibiotics in combination with plant extracts against tested bacteria using the microdilution method

Antibiotic and Plant extract combination	MIC (mg/ml)			
	<i>S. aureus</i>	<i>B. cereus</i>	<i>P. aeruginosa</i>	<i>A. baumannii</i>
Cip + Sesame	2:64	N	N	N
Cip + Henna	N	2:2	2:4	2:2

Cip + Pomegranate	N	N	2:2	2:8
Cip + Peanut	N	2:16	N	N
Ery + Sesame	1:64	0.5:32	N	N
Ery + Henna	N	N	N	N
Ery + Pomegranate	N	N	N	N
Ery + Peanut	N	0.5:16	N	N

N= No effect

Antibacterial combination considered as synergism in $\sum FIC \leq 1.0$, indifference in $1.0 < \sum FIC \leq 4$ and antagonism in $\sum FIC > 4$ [Table 3]. According to the standard evaluation measures of Kamatou et al. (2006) and Grytten et al. (1988). In combination between ciprofloxacin and henna leaf extract, the MIC of ciprofloxacin was reduced from 4 mg/ml to 2 mg/ml and showed synergistic interaction against *P. aeruginosa* and *Acinetobacter* ($\sum FIC$ is less than or equal to 1.0), while it was indifference ($1.0 < \sum FIC \leq 4$) against *B. cereus*. The MIC of henna leaf extract was decreased from 4, 8, 4 mg/ml to 2, 4, 2 mg/ml against *B. cereus*, *P. aeruginosa* and *Acinetobacter* respectively. The combination interaction between ciprofloxacin with pomegranate (against *P. aeruginosa* and *Acinetobacter*) and peanut (against *B. cereus*) showed a significant reduction of MIC for both antibiotic and plant extract and the combination was classified as synergy ($\sum FIC \leq 1.0$). Erythromycin and sesame activity against *S. aureus* and *B. cereus* also were more effective when combined together and reduced the MIC against these bacteria while no synergistic effect of them was produced against the other tested bacteria. No significant reduction of MIC occurred in combination between erythromycin and both henna and pomegranate, while there was a synergistic effect of the combination of erythromycin and peanut and the MICs of both of them decreased against *B. cereus*.

Table 3. Fractional inhibitory concentrations (FIC) of different combination of the extracts and the antibiotics.

Antimicrobials and Plant extract combination	Fractional Inhibitory Concentration		
	Tested bacteria	FIC index	Remarks
Cip + Henna	<i>B. cereus</i>	1.25	Indifference
Cip + Henna	<i>P. aeruginosa</i>	0.5625	Synergy
Cip + Henna	<i>A. baumannii</i>	0.0625	Synergy
Cip + Peanut	<i>B. cereus</i>	0.12890625	Synergy
Cip+ Pomegranate	<i>P. aeruginosa</i>	1	Indifference
Cip+ Pomegranate	<i>A. baumannii</i>	0.25	Synergy
Cip + Sesame	<i>S. aureus</i>	0.3149414	Synergy
Ery + Peanut	<i>B. cereus</i>	0.0317383	Synergy
Ery + Sesame	<i>S. aureus</i>	0.015625	Synergy
Ery + Sesame	<i>B. cereus</i>	0.015625	Synergy

Cip= Ciprofloxacin, Ery = Erythromycin

4. DISCUSSION

Many studies have been reported, assuring the antimicrobial activities of an individual or combined extracts of medicinal plants. In this study, henna extract was more effective against all tested bacteria than the other plant extracts. Henna has many traditional and commercial uses, the most common being as a dye for hair, skin and fingernails because it contains Lawsone (2-hydroxynaphthoquinone) which is one of the component with (0.5-1.5%) responsible for dyeing. It also contains tannic acid, mucilage, gallic acid and mannite (Kelmanson *et al.*, 2000). The antimicrobial effect of henna may be according to numerous free hydroxyls which are able to combine with the bacterial cell wall structures including carbohydrates and proteins as suggested by Harborne and Baxter (1995) and they attributed that to their attachment to enzyme site rendering them inactive.

Results of the present study showed that pomegranate peel extract was effective against tested bacteria. In particular, among plants, *Punica granatum* used in traditional medicine, is known for its pharmacological properties that have been evaluated due to antiparasitic, antibacterial, antifungal, antiproliferative, apoptotic, and anticancer effects (Jurenka, 2008). Literature data reported that extracts of *Punica granatum* peel in different concentrations were effective against different bacterial species such as *S. aureus*, *E. coli*, *Salmonella enterica*, *Shigella sonnei*, *Enterococcus faecalis*, and *Bacillus subtilis* (Pagliarulo *et al.*, 2016; Subramaniam *et al.*, 2012; Rosas-Burgos *et al.*, 2017 and Dey *et al.*, 2015). Pomegranate beverage contains several compounds that are responsible for the antimicrobial activity, depending on their abundance such as tannins which are considered to be toxic to microorganisms (Viuda-Martos *et al.*, 2010). Their hydrophilic site cooperates with the polar region of the bacterial cell membrane, while the hydrophobic site is immersed in the non-polar region of the bacterial membrane, this affects transporting of substances into the cell (Cristani *et al.*, 2007). Likewise, Naz *et al.* (2007) suggested a phenolic toxicity through reactions with sulfhydryl groups or through more non-specific interactions with proteins leading to loss of function.

Sometimes the use of antibiotic alone does not give the desired inhibitory effects, this can be defeated by a combination of drugs which appears their synergistic effect, and this is more significant than their effects alone (Kamatou *et al.*, 2006). Synergism is defined as a positive interaction created when two agents are combined and together they exert an inhibitory effect (on the targeted organisms) that is greater than the sum of their individual effects (Levinson and Jawetz, 2002). Consequently, mixing plant extracts with antibiotics enhanced and synergized their effect and decreased their MICs and this fact has clearly emerged in this study. The synergistic effect could be related to the formation of complex chemical products that can be greatly effective to inhibit many species of microorganisms by preventing cell wall to synthesize or may lead to lyses and finally, it dies (Chanda and Rakholiya, 2011). There was a significant synergistic effect of combination between both ciprofloxacin and erythromycin with henna, sesame, pomegranate, and peanut and the MIC of both of them was decreased against tested bacteria and this could be referred to that these crude extracts have many different phytochemicals which might inhibit bacteria by different mechanisms (Duke *et al.*, 2003). This result reveals that plant extracts were potentiating the effects of the ciprofloxacin and erythromycin. This double attack of both agents on different target sites of the bacteria could theoretically lead to either an additive or a synergistic effect (Esimone *et al.* 2006). In a previous similar study performed by Sato *et al.* (2004), they were

combined between methanolic extract of pomegranate and antibiotics and found that the antimicrobial activity of flavonoids and polyphenols when they combined with antibiotics could alter the bacterial resistant properties to be more effective. Cushnie *et al.* (2005) in different study indicated synergism between antibiotics and flavonoids. Tsuchiya *et al.* (1996) reported that flavonoids disrupt bacterial cell membranes while Prasad *et al.* (2008) found that tannins precipitate bacterial protein. Yang *et al.* (2005) and Aqil *et al.* (2005) reported in a previous in vitro studies, significant decreasing in the MICs and synergistic effects of the antibiotics when combined with number of plant extracts against *Staphylococcus aureus*.

Ciprofloxacin is a second generation fluoroquinolone, interrupts DNA replication by inhibiting both topoisomerase II (an enzyme that reduces the amount of supercoiling of the DNA double-stranded helix during the replication process) and IV thus preventing cell division (Grohe *et al.*, 1987). By the way, Liu *et al.* (2011) stated that flavonoids are exist in many types of our food such as vegetables, and fruits and they are able to combine with fluoroquinolone antibiotics to exhibit an effective antimicrobial agent.

5. CONCLUSION

Obtained results confirm the antibacterial activity of henna, pomegranate, peanut and sesame extract and shows their potential use as agents which enhance antibiotic activity. Sometimes mixing plant extracts with different antimicrobials enhanced and increase their antibacterial activity and the antimicrobials that produce side effects can be used by reducing dose concentration exploiting their synergy with the medicinal plants. Mixing plant extracts with antimicrobials also increases the spectrum of antibiotic, avoids the development of resistance and decreases toxicity so exhibiting antibacterial activity better than that estimated from each antibiotic alone. Further studies are required to determine the specific substrates that have synergistic effect and approved with in vivo studies.

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