Antibacterial, Antifungal and Synergistic Effect of *Lawsonia inermis*, *Punica granatum* and *Hibiscus sabdariffa*.

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Abstract
The increased prevalence of antibiotic resistance, as a result of extensive antibiotic use, may render the current antimicrobial agents insufficient to control, at least, some bacterial infections. The concept of this research is based on the Sunnah of Prophet Mohammad "peace be upon him" of using Henna as a medication for wounds. The aim of this study is to investigate the antimicrobial and antifungal activity of *Lawsonia inermis*, *Punica granatum*, *and Hibiscus sabdariffa* and to examine the synergistic effect of mixing plant extracts with antibiotic. By extraction 30g of grounded plant extract with 500ml from the solvent by soxhlet apparatus (soaked the grounded plant in solvent 72h). The antibacterial and antifungal activities were investigated by using sensitivity test on Mueller Hinton agar and Potato Dextrose agar, respectively. The methanolic extract of *Punica granatum* showed the highest antibacterial activity especially against *Staphylococcus aureus,* while*, Klebsiella pneumonia and Escherichia coli* showed the least sensitivity to the same extract. Association of antibiotics and plant extract showed synergistic antibacterial activity especially with Ciprofloxacin and Erythromycin on *Pseudomonas aeruginosa,* and *Staphylococcus aureus*, respectively. The activity of Ketoconazole and Fluconazole drugs was highly increased after mixing with the aqueous *extract of Hibiscus sabdariffa*. The Nystatin antifungal showed higher activity when mixed with plant extract. In conclusion, all tested plants extracts showed potential antimicrobial activity against the tested pathogens. The synergistic effect against fungi was more clear and higher from the effect against bacteria.
Keywords: Antimicrobial activity, medicinal plants, *Lawsonia inermis*, *Punica granatum*, *Hibiscus sabdariffa*, Synergistic effect, Gaza, Palestine.

التأثير المضاد للبكتيريا والفطريات والاثر التآزري لكل من الحناء, الرمان والكركديه.

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الملخص

إن زيادة مقاومة الميكروبات للمضادات الحيوية الناجم عن الاستخدام المفرط جعل هذه المضادات غير كافيه لعلاج بعض الانتانات البكتيرية .هدف هذه الدراسة الى التحقق من نشاط كل من نبات الحناء, الرمان والكركديه كمضاد للبكتيريا والفطريات بالاضافة الى تاثيرها التازري عند خلط مستخلصات هذه النباتات مع المضادات الحيوية .أظهرت النتائج فعالية عالية ضد البكتيريا لمستخلص الرمان بالميثانول خصوصاً ضد *Staphylococcus aureus* بينما كانت كل من *Klebsiella pneumonia* و *Escherichia coli* أقل حساسية تجاه نفس المستخلص .أظهرت أيضاً نتائج خلط مستخلصات النباتات المفحوصة مع المضادات الحيوية نشاط تآزري مضاد للبكتيريا خصوصاً مع السبروفوكساسين ضد *Pseudomonas aeruginosa* ومع الايرثروميسين ضد *Staphylococcus aureus*.لوحظ إزدياد النشاط المضاد للفطريات لكل من الكيتوكونازول والفلوكونازول عند خلطها مع مستخلص الكركديه .كذلك الامربالنسبة للنستاتين والذي أظهر زياة في النشاط المضاد للفطريات عند خلطه مع كل المستخلصات المستخدمة .وخلصت الدراسة الى ان كل المستخلصات المفحوصة أظهرت نشاط مضاد للميكروبات المفحوصة وكان التأثير التآزري للمستخلصات أكثر فعالية ضد الفطريات منه ضد البكتيريا.

كلمات مفتاحية :النشاط المضاد للميكروبات ,نباتات طبية, الحناء, الرمان, التأثير التآزري ,فلسطين.

Introduction:

Multiple microbial resistance is a growing problem and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, it is necessary to take measures to reduce microbial resistance and to explore alternative antimicrobial sources [1]. Products are used in their natural forms in traditional herbal medicines or in their purified form in pharmaceutical industry [2].

The extracts of *Lawsonia inermis* (Henna) and *Punica granatum* (Pomegranate) were shown to have promising antibacterial properties against *Staphylococcus aureus* and *Pseudomonas aeruginos*a [3]. The naturally available *Lawsonia inermis* could be a potential alternative to antimicrobials that become less effective or ineffective against certain pathogenic microorganisms [4]. *Punica granatum* peel is traditionally used to treat genital infections, mastitis, acne, folliculitis, piles, allergic dermatitis, tympanitis, scalds and also as an antioxidant [5]. The constituents of *Punica granatum* include gallocatechins, delphinidin, cyanidin, gallic acid, ellagic acid, pelargonidin and sitosterol, which have therapeutic properties [6]. *Hibiscus sabdariffa* (Roselle), a herb used in foods and beverages by local communities in Africa and other parts of the world, therefore, is of potential benefits. The preliminary phytochemical screening of the ethanolic seed extract of *Hibiscus sabdariffa* demonstrated the presence of alkaloids, saponins, cardenolides, deoxy sugar, tannins, steroidal rings, cardiac glycosides flavonoids and anthraquinones [7].

The plant commonly known as Henna or Mhendi is abundantly available in tropical and subtropical areas. Ancient history of India describes its diverse uses and also plays appreciable role in Ayurvedic or natural herbal medicines [8]. Its use became popular in India because of its cooling effect in the hot Indian summers. Henna leaves, flowers, seeds, stem bark and roots are used in traditional medicine to treat a variety of ailments such as rheumatoid arthritis, headache, ulcers, diarrhea, leprosy, fever, leucorrhoea, diabetes, cardiac disease, hepatoprotective and as a coloring agent [9, 10].

The objectives of this study are to investigate the antimicrobial and antifungal activity of *Lawsonia inermis*, *Punica granatum*, *Hibiscus sabdariffa* on some gram positive and gram negative bacteria and fungi and to determine the synergistic effect of mixing *Lawsonia inermis*, *Punica granatum*, *Hibiscus sabdariffa* with antibiotic.

This research was inspired by the Sunnah of Prophet Mohammad (Peace be upon him) particularly for the henna plant. "The Prophet never suffered from a wound or a thorn without putting Henna on it" – Hadith, narrated by Umm Salamah (RadiAllahu ^anha) [at-Tirmidhi, al-Bayhaqi].

Material and Methods:

Plant Materials*:* Air dried plants (*L. inermis*, *H. sabdariffa*, peel of *P. granatum*) were identified and purchased from the local market by Mr. Ashraf Al-Shafay, Biology Department, faculty of science, Islamic University of Gaza.

Microorganism: *Bacillus* species, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Proteus* species, *Escherichia coli*, and *Pseudomonas aeruginosa* were used as test organisms. The microbiology department at El-Shifa hospital in Gaza supplied the bacterial isolates and Mr. Nahed Abd El-Lateef head of microbiology department at AL-Remal clinic identified and provided *Candida albicans* and *Microsporum* species. The identity of all tested microorganisms was confirmed at the Medical Technology

Department, Islamic University-Gaza by the authors.

Plant Extraction: The air dried and powdered materials of *L. inermis*, *H. sabdariffa* and peel of *P. granatum* (30 g) were extracted with water and methanol (500L, 72h) on soxhlet apparatus. The solvents were then removed by air drying using oven over 3 days at 40°C to obtain a crude extract, which were stored at 4°C in dark vials [2].

Antibacterial, Antifungal assays:

Agar-Well Diffusion Methods: Muller Hinton Agar (MHA) (HiMedia, India) was used to determine the diameter of inhibition zone (ID) by the well diffusion methods. To standardize the incoulum density for a susceptibility test; a $BaSO_4$ turbidity standard, equivalent to a (0.5) McFarland standard was used**.** Mueller Hinton agar plates were inoculated with the standardized suspension of the test organism. Using a 5 mm diameter sterile glass Pasture pipet, several holes in the inoculated plates were made. *Microsporum* was subcultured and tested for sensitivity test on Potato Dextrose Agar (PDA) where it was inoculated into the media before it solidified, 0.5 g from each aqueous crude extract was dissolved in sterile distilled water (1:3 w/v, final concentration 250mg/ml), and methanolic crude extract was dissolved in Dimethyl sulfoxide (DMSO) (1:3 w/v, final concentration 250mg/ml). A 25µl from each extract was introduced into the appropriate well in the inoculated media plate using automatic pipette. Sterile water and DMSO were used as negative controls. Then the prepared plates were incubated at 37°C for 24 h for bacteria and *C. albicans*. The *Microsporum* plates were incubated at room temperature for 5 day. At the end of incubation period, each plate was examined. The diameter of the zone of complete inhibition, including the diameter of the well was measured in millimeter (mm) [11].

The Synergistic Effect: Commercially available antimicrobial disks (HiMedia, India) were applied on the surface of inoculated MHA by pressing slightly, and then 25µl from the extracts was carefully and slowly dispensed on the antibiotic disk. The plates were incubated at 37ºC for 24 h. At the end of the period, the inhibition zone formed on the media was measured with a transparent ruler in mm.

Results:

Antibacterial Effect of Plant Extracts*:* The results of the effects of aqueous and methanolic extracts of the plants using 25µl from the extracts (250 mg/ml crude extract) against the tested microorganism are presented in table (1). All Plant extracts showed considerable antimicrobial activity almost on all of the tested microorganisms with the exception of *L. inermis* aqueous extract which showed the least effect on most bacterial samples tested. The zone of inhibition exhibited by the effect of *L. inermis* on *S. aureus* is illustrated in figure (1).

Table 1: The effect of aqueous and methanolic plant extracts on test bacteria**.**

Microorganism	**Zone of inhibition (mm, diameter) of plant extract**					
	LI-A	LI-M	PG-A	PG-M	HS-A	HS-M
S. aureus	11	19	15	22	15	10
Bacillus spp.	8	15	0	20	11	22
K. pneumonia	0	11	0	0	9	10
Proteus spp.	14	20	13	21	15	15
E. coli	0	10	0	12	6	11
P. aeruginosa	0	15	14	17	15	16
Enterococcus spp.	0	14	9	16	13	17
A= Aqueous; M=Methanol; LI=*L. inermis;* PG=*P. granatum and* HS= *H. sabdariffa.*						

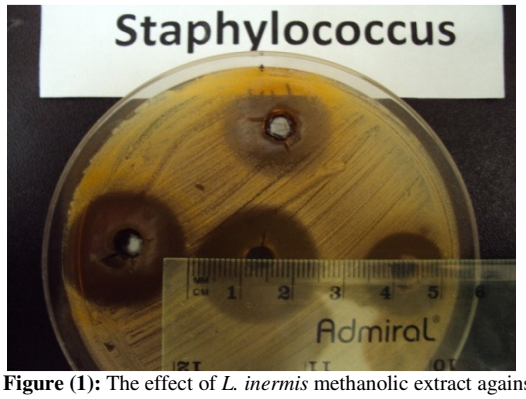

Figure (1): The effect of *L. inermis* methanolic extract against *S. aureus*.

Antifungal Effects of Plant Extracts: The effect of aqueous and methanolic extract of the plant using 25 µl of the extracts against *C. albicans* and *Microsporum* are presented in table (2). The aqueous extract of *H. sabdariffa* has the highest activity against *C. albicans*. There was no effect for any of the extracts against *Microsporum* spp.

Table 2: The effect of aqueous and methanolic plant extracts on tested fungi.

Plant extracts / **Fungi**	**Zone of inhibition (mm, diameter) of plant extract**					
	LI-A	LI-M	PG-A	PG-M	HS-A	HS-M
C. albicans	15	14	15	15	21	0
Microsporum spp.	0	0	0	0	0	0

A= Aqueous; M=Methanol; LI=*L. inermis;* PG=*P. granatum and* HS= *H. sabdariffa.*

The Synergistic Effect: The synergistic effect of plant extracts and antibiotics against bacteria was not observed, especially against gram negative bacteria; however, there was a considerable synergistic effect against *S. aureus*. But mixing plant extract with antifungal drug showed highly increased activity against fungi specially *H. sabdariffa* extract with fluconazole. The results are presented in table 3, 4 and 5.

Table 3: Effects of different concentration of Antifungal drug on both *C. albicans* and *Microsporum* spp.

Antifungal / Fungi	Zone of inhibition is expressed in terms of mm								
	Ketoconazole			Nystatin			Fluconazole		
	1/1	1/10	1/100	1/1	1/10	1/100	1/1	1/10	1/100
C. albicans	0	0	0	14	0	0	0	0	0
Microsporum spp.	18	0	0	0	0	0	0	0	0

Table 4: Synergistic effects of antifungal drug when mixed with aqueous plant extract on *C. albicans* and *Microsporum* spp.

Antifungal / Fungi	Zone of inhibition is expressed in terms of mm								
	Ketoconazole			Nystatin			Fluconazole		
	LI-A	PG-A	HS-A	LI-A	PG-A	HS-A	LI-A	PG-A	HS-A
C. albicans	0	20	26	14	20	0	20	22	30
Microsporum spp.	0	0	0	11	11	14	0	0	32

A= Aqueous; LI=*L. inermis;* PG=*P. granatum and* HS= *H. sabdariffa*

Table 5: Synergistic effects of antifungal drug mixed with methanolic extraction of the plant extract on *C. albicans* and *Microsporum* spp.

Antifungal / Fungi	Zone of inhibition is expressed in terms of mm								
	Ketoconazole			Nystatin			Fluconazole		
	LI-M	PG-M	HS-M	LI-M	PG-M	HS-M	LI-M	PG-M	HS-M
C. albicans	12	14	22	19	12	17	24	26	30
Microsporum	0	12	0	13	14	15	0	0	19

M=Methanol; LI=*L. inermis;* PG=*P. granatum and* HS= *H. sabdariffa.*

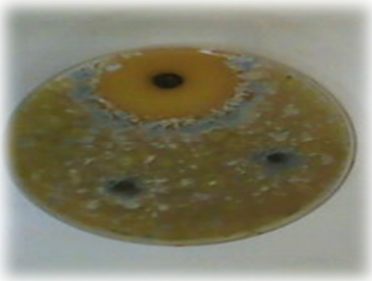

Figure 2: Synergistic effect of mixing aqueous extract of *H. sabdariffa* with Fluconazole.

The Effect of the Plant Extracts against Multi-resistance Bacteria: Data presented in table (6) and (7) suggest that the plant extracts has high activity against multi-drug resistant bacteria.

Table 6: Effects of antibiotic against multi drug resistant bacteria (zone of inhibition in mm).

Microorganism	Cefuroxime	Ciprofloxacin	Cefotaxime	Ceftazidime	Gentamicin	Doxycyclin	Amikacin	Cotrimoxazole	Meropenem	Gentamicin
E. coli	0	0	0	0	0	0	0	0	0	0
Pseudomonas aeruginosa	0	0	0	0	0	0	0	0	0	0
Acinetobacter (isolate 1)	0	0	0	0	0	0	0	0	10	0
Acinetobacter (isolate 2)	0	0	0	0	0	0	0	0	0	0
Acinetobacter (isolate 3)	0	0	0	0	0	0	0	0	0	0

Table 7: Effects of aqueous and methanol extracts of the plant against multi drug resistant bacteria.

Microorganism	**Zone of inhibition (mm, diameter) Plant extract**					
	LI-A	LI-M	PG-A	PG-M	HS-A	HS-M
Escherichia coli	15	20	15	19	33	20
Pseudomonas aeruginosa	17	23	13	21	31	31
Acinetobacter isolate 1	17	20	15	22	31	30
Acinetobacter isolate 2	12	20	15	23	25	27
Acinetobacter isolate 3	16	20	18	22	28	28

A= Aqueous; M=Methanol; LI=*L. inermis;* PG=*P. granatum and* HS= *H. sabdariffa.*

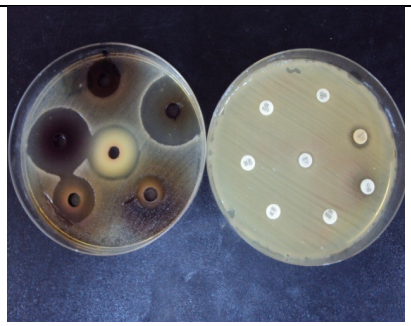

Figure 3: Effect of plant extracts against multi-resist bacteria.

Discussion and Conclusions:

The main objective of the present study was to evaluate the ability of extracts from three plants to inhibit the growth of pathogenic bacteria and fungi. The aim is to explore possible future use of these extracts as alternatives to common antibiotics and to determine their ability to enhance activity of antibiotics. As a general rule, a plant extract is considered active against both fungi and bacteria when the zone of inhibition is greater than 6 mm [2]. In this study, almost all tested plant extracts showed antimicrobial activity against both bacteria and fungi. In general, methanolic extracts showed

higher activity than those obtained by aqueous extraction. Some studies suggested that Henna has a wide spectrum of antimicrobial activity including antibacterial, antiviral, antimycotic and antiparasitic activities. With the ever increasing resistant strains of microorganisms to the already available and synthesized antibiotics, the naturally available *L. inermis* (Henna) could be a potential alternative [4]. Many reports cite the inhibitory activity of Henna against gram negative and gram positive organisms [2, 12, 13, 14, 15]. The extracts of tested plants showed a great activity in inhibiting the growth of bacteria and fungi, probably due to the presence of active ingredients that inhibit bacterial and fungal growth. Henna contains Lawsone in about 0.5 to 1.5% of its ingredients. Lawsone (2-hydroxynapthoquinone) is the principal constituent responsible for the dyeing properties of the plant. However, Henna also contains mannite, tannic acid, mucilage and gallic acid [16].

Antimicrobial activity may be due to numerous free hydroxyl ions that have the capability to combine with the carbohydrates and proteins in the bacterial cell wall. They may get attached to enzyme sites rendering them inactive [17]. In our study, the plant extracts showed high activity against most microorganisms tested except for the aqueous *L. inermis* which showed lowest antimicrobial effect on most bacteria tested. Our results are in agreement with the results of a previous study which demonstrated that for the antibacterial activity of Henna extracts, alcoholic and oily extracts were more effective than the water based extract [18]. This may be due to the lack of the solvent properties which plays an important role in antibacterial efficacy [19]. Among the tested microorganisms, the methanolic extract of *L. inermis* was more active against *S. aureus* and *Proteus* spp. than others. These results are similar to that obtained from a recent study [20]. Other investigations showed that Henna is effective against different microorganisms especially against *P. aeruginosa* [21]. This difference in antimicrobial effects may be due bacterial strains differences used in that study. Our study showed that the methanolic extract of *H. sabdariffa* have antibacterial activity against *Bacillus* spp., *S. aureus*, *K. pneumonia*, *Proteus* spp., *Escherichia coli*, and *P. aeruginosa*. This result is in agreement with another study, which showed that the methanolic extract of *H. sabdariffa* had antibacterial effect against *S. aureus, Bacillus stearothermophilus, Micrococcus luteus, Serratia mascences, Clostridium sporogenes, E. coli, K. pneumoniae, Bacillus cereus, Pseudomonas fluorescence* [22].

Data from antibacterial activity of the extracts against the multi-drug resistant isolates, it has been observed that *S. aureus* is more susceptible to the employed plant extracts than *P. aeruginosa*. This finding is in agreement with earlier reports where the antibacterial activity of the phytoconstituents of *L. inermis* were active against gram positive bacteria such as *Staphylococcus aureus*, *Enterococcus faecium* and *Bacillus subtilis*, but were less active against gram negative bacteria [14]. Our data showed that *H. sabdariffa* did not have any effect against *Candida albicans*, this was also reported by Tolulope *et al.,* (2007) who concluded that this plant extract could not be used to treat fungal disease [22]. The aqueous extract of *H. sabdariffa* showed antimicrobial effect against all tested bacteria including *Pseudomonas aeruginosa***.** This group of gram negative bacteria (especially *P. aeuroginosa*); are known for being notorious for their ability to survive in the environment, particularly in moist conditions. It may contaminate medicines, surgical equipment, clothing, and dressing with the ability to cause serious infections in immunocompromized patients [23]. Therefore, the aqueous

extract of *H. sabdariffa* has a very promising potential uses against this particular pathogen.

A preliminary phytochemical screening of the ethanolic seed extract of *H. sabdariffa* detected the presence of alkaloids, saponins, Cardenolides, Deoxy sugar, tannins, steroidal rings, cardiac glycosides flavonoids and anthraquinones [7]. Some of these constituents may be responsible for the antimicrobial effect exhibited by the palnt. Santhamari *et al.,* (2011) evaluated the antibacterial activity of the extracts of two plants mainly *P. granatum* and *L. inermis* against *P. aeruginosa* and *S. aureus*. The highest antimicrobial potency was observed for the extracts of *P. granatum* which inhibited 75% of resistant isolates of *P. aeruginosa* and for *L. inermis* which inhibited 68.75% of resistant isolates of *S. aureus* [24]. Prashanth *et al.,* (2001) have tested the antibacterial activity of petroleum ether, chloroform, methanol and water extracts of pomegranate rinds, and reported that the methanol extract was the most effective against the tested organisms such as *S. aureus*, *E. coli, K. pneumoniae, Proteus vulgaris, Bacillus subtilis* and *Salmonella typhi* [25]. This is in agreement with results obtained in this study wherein the methanolic extract of *P. granatum* demonstrated promising antibacterial activity against both gram positive and gram negative bacteria. Contrary to our results, *E. coli and P. aeruginosa* were shown in a similar to study to be particularly suscptible to the methanolic extract of *P. granatum* [26]. The constituents of *P. granatum* include gallocatechins, delphinidin, cyanidin, gallic acid, ellagic acid, pelargonidin and sitosterol, which are very well known for their therapeutic properties [6].

In conclusion, *L. inermis*, *P. granatum* and *H. sabdariffa* extracts have antibacterial and antifungal activities and exhibited synergistic effects when used with commercial antimicrobials. Therefore, our data clearly demonstrate the importance of plant extracts in the control of resistant bacteria, which are becoming a threat to human health.

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