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Herbal antibiofilm effects against phenotypically and genotypically detected clinical Methicillin-resistant staphylococcus aureus isolates

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Abstract: This study was conducted to detect the effect of some medicinal plant extracts on the biofilm formation of Methicillin-resistant *staphylococcus aureus* (MRSA) isolated from patients in Mansoura University Hospitals.

Results: Fifty samples (Blood, Wound swab, nasal swab and urine) from patients revealed the total number of isolates were methicillin-resistant *staphylococcus aureus* (MRSA). Molecular analysis revealed that mec Agene was detected in all isolates. then MRSA isolates were positive for biofilm genes specific (icaA and icaD). Among all herbal plant extracts, *Camellia sinensis* (green tea) extract was the greater inhibitor of the biofilm formation of MRSA isolates. In this connection, this effect showed the lysis of MRSA cell wall through Transmission Electron Microscope (TEM).

Conclusions: *Camellia sinensis* leaf extracts could be valuable in fighting evolving drug- resistance caused by MRSA.

keywords: Methicillin,Resistant,Phenotypic, Genotypic, Staphylococcus aureus.

1.Introduction

A significant problem in the fight against infectious diseases is the bacterial development of antibiotic resistance. The most commonly found multi-drug resistance bacteria, particularly through nosocomial infections are methicillin-resistant *staphylococcus aureus* (MRSA)**[1].**

MRSA is still a significant issue in infection control around the world. MRSA is a strain of *staphylococcus aureus* that is characterized by its resistance to methicillin. MRSA is a major threat to human health because it is resistant to β –lactams and variety of antibacterial agent including penicillin, oxacillin, amoxicillin, methicillin, cephalosporins and chloramphenicol [2,3].

MRSA can cause a variety of disease from crust and forgiving skin impurities to grim intrusive corruptions such as pneumonia, bacteremia, endocarditis and osteomyelitis [4].

MRSA contaminations are serious due to the occurrence of multi-drug resistance strains and also the existence of isolates that can form a resilient biofilm, which is considered an important virulence factor, it is most frequently associated with the synthesis of polysaccharide intracellular adhesion (PLA) encoded by ica operon, This synthesis is mediated by fibronectin-binding protein sclumping factors and biofilm-binding proteins **[5,6].**

Substitute antimicrobial causes are wanted to be advanced and employed to control multi-drug resilient bacteria. To answer this task, there have been increasing benefits to finding antimicrobial mixtures from therapeutic plant extracts as another method to discover new antimicrobial compounds. The antimicrobial doings of some herbal drugs against diverse pathogens **[7,8].**

Plant extracts have been used to reduce antibiotic use, prevent the worsening of antibiotic resistance and decrease MRSA biofilm formation which produces many biologically active substances, providing defense against environmental microbes **[9].**

These natural medicines are harmless, inexpensive and affordable. Herbal plants include *scamellia sinensis* (green tea),

cinnamomum zelyanicum (cinnamon) and *Allium sativum* (garlic) **[10].** The aim of this study was planned to investigate the phenotypic and genotypic methods of MRSA isolates to detect the antibacterial activity of the herbal plant extracts against clinical isolates

2 .Materials and methods

Bacterial strains

Fifty MRSA isolates were inaccessible from patients who admitted to different Mansoura university hospitals from august 2021 to December 2021. Samples were still by sterile cotton wipes and impassive to the microbiology and immunology Department for further studies.

Isolation and Identification of MRSA

1) Phenotypic identification:

All samples were injected into blood agar and mannitol salt agar dishes and then gestated at 37°C for 24 hrs. Identification of the isolated *S. aureus* colonies were done according to the typical bacteriological performance **[11].**

S. aureus isolated performed as yellow clusters on mannitol salt agar and produce β-haemolysis on blood agar. Under microscopic examination. *S. aureus* isolates appear as gram-positive cocci (violet color) arranged in grape-like groups. In biological reaction. *S. aureus* isolates were coagulase and catalase tested positive **[12].**

All insulates recognized as *S. aureus* were added and divided for methicillin resistance by disc diffusion method using cefoxitin (Fox 30 μg). The zones on inhibition $\leq$ 25 were measured to be MRSA according to CLSI guidelines **[13].**

2) Genotypic identification:

The molecular characterization of MRSA depends on the detection of mecA gene. The presence of mecA gene was detected by polymerase chain reaction (PCR) using forward and reverse primer**[14]**as recorded in **Table 1** The magnification database for mecA gene involved in opening denaturation was done at 94°C for 5 min followed by 10 cycles of denaturation 94°C for 45 sec, Annealing steps 65°C for 45 sec and an extension step 72°C for 1.5min followed by 25 cycles with final extension step was done at 72°C for 10 min **[15].**

Table 1: Sequences of the primer sets designed in this study for the amplification of mecA gene.

Gene	Primer Sequence	size (bp)
***mecA*F:**	5′TGGCTCAGGTAC TGCTATCCAC 3′	1080bp
***mecA*R:**	3′AGTTCTGCAGTA CCGGATTTGC 5′	

Detection of biofilm formation

1) Phenotypic detection:

Phenotypic biofilm formation of MRSA was detected by Congo red agar (CRA) way. MRSA insulates were cultured on brain heart infusion agar with 0.08 % congo red addition with 30 % sucrose. The strains were injected in streaks and incubated at 37°C for 24hrs. The Biofilm appeared as black colonies, while the non-biofilm appear as white or red colonies **[16].**

2) Genotypic detection:

The company of biofilm-linked genes icaA and icaD were examined by conformist PCR on the beforehand removed DNA of *s. aureus* isolates using specific primers for each **[17].** The classification of textbooks for the tested genetic factor was listed in **Table 2**

Table 2: Sequences of the primer sets designed in this study for the amplification of icaA and icaD genes.

Gene	Primer Sequence	size(bp)
***ica A*F:**	5ACACTTGCTGGC GCAGTCAA-3	198bp
***ica A*R:**	5TCTGGAACCAAC ATCCAACA-3	
***ica D*F:**	5ATGGTCAAGCCC AGACAGAG-3	198 bp
***ica D*R:**	5AGTATTTTCAAT GTTTAAAGC-3	

The amplification program for icaA and icaD genes consisted of initial denaturation done at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 45 sec, an annealing step at 60°C for 45 sec, an extension step at 72°C for 1 min with final extension step was done at 72°C for 7 min **[18].**

Antimicrobial susceptibility test

Detection of antimicrobial susceptibility in clinical isolates of MRSA was done by disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI)

guidelines **[19].** The following antibiotic discs from MAST Categories Ltd., Merseyside, UK, were used: Penicillin (P, 10µg), Amikacin (AK, 30µg), Oxacillin (OX, 1µg), Azithromycin (AZM, 15µg), Cefoxitin (FOX, 30µg), Ofloxacin (OFX, 5µg), Ciprofloxacin (CIP, 5µg), Vancomycin (VA, 30µg), Amoxicillin (AM, 30µg) and Tetracycline (TE, 30µg). MRSA was a control strain used for all antibiotics discs.

Plant extracts preparation

The three herbal plants *camellia sinensis* (green tea), *cinnamomum zelyanicum* (cinnamon), and *Allium sativum* (garlic) were dried and pulverized into fine powder.The powdered material was stored in air-tight sterile containers and protected from sunlight until required according to Whatman, USA. and Handa *et al*,.**[20].**

Effect of medicinal plant extracts on biofilm formation of MRSA

A) By using the agar well diffusion method the strains were inoculated by streaking the sterile cotton swab in three directions over the entire surface of the agar plates to obtain a uniform inoculum **[21].** Then sterile cork borer was used to make wells of 6mm in diameter in an agar plate. 150µL of each plant extract was poured into each well using a sterile Pasteur pipette. Then plates were incubated at 37°C for 24hrs.

B) By Micro-titration plate method according to the method of O.Toole**[22].**

C) By Transmission Electron Microscope (TEM) technique according to the method of William and barry**[23].**

3 .Results

Bacterial isolates

In this study, fifty clinical MRSA isolates were obtained from male and female patients admitted to different Mansoura university hospitals. Each sample was cultured on a specific medium. Results recorded in **Table (3)** shows that wound swab samples were the commonest samples giving positive MRSA growth while throat gave the lowest number of MRSA isolates.

Detection of Methicillin-resistant staphylococcus aureus (MRSA)

The discovery of MRSA was ended by using cefoxitin disc. The results showed that all isolated strains were cefoxitin-resistant.The mecA gene was noticed in all (100%) MRSA isolates shown in **figure [1].**

Detection of biofilm formation

All isolates were verified for their aptitude to form biofilm using the congo red agar method which optimistic result appears as black groups as shown in **figure [2].** All isolates were verified for the company of biofilm-linked genes: icaA and icaD by straight PCR. The primers used in the experiment showed specificity with a single band as shown in **figure[3].**

Table (3): Distribution of MRSA strains among different clinical samples.

Sample	Number of MRSATotal = 50	Percentage (%)
Wound	26	52%
Blood	12	24%
Urine	5	10%
Sputum	3	6%
Nose	3	6%
Throat	1	2%
Total	50	100%

Among all clinical MRSA isolates, 22 isolates were from females (44%) and 28 isolates from males (56%) as shown in **Table (4)**

Table (4): Percentage of clinical samples collected from different genders.

Samples	Gender				Total	
	Female		Male			
	No.	%	No.	%	No.	%
Wound	11	22%	15	30%	26	**52%**
Blood	5	10%	7	14%	12	**24%**
Urine	3	6%	2	4%	5	**10%**
Sputum	1	2%	2	4%	3	**6%**
Nose	1	2%	2	4%	3	**6%**
Throat	1	2%	0	0%	1	**2%**
Total	**22**	**44%**	**28**	**56%**	**40**	**100%**

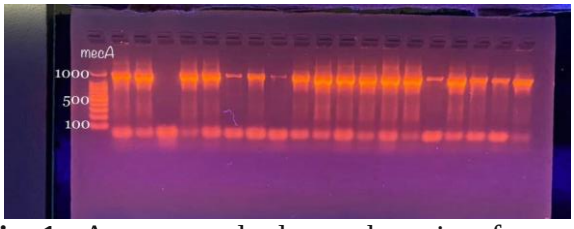


Fig 1: Agarose gel electrophoresis of mecA gene

Fig 2: A: Biofilm formation (Black colonies)& B: Non-biofilm formation (Red colonies)

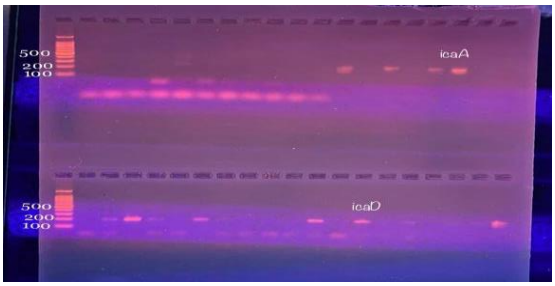


Fig 3: Agarose gel electrophoresis of icaA&icaD genes

Antimicrobial susceptibility

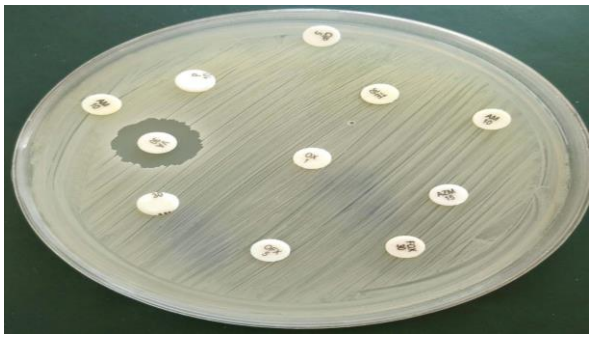


Fig 4: Resistance of 9 antibiotics as P, OX, FOX, AZM, CIP, TE, AM, OFX, AK. Sensitive to 1 antibiotic is VA.

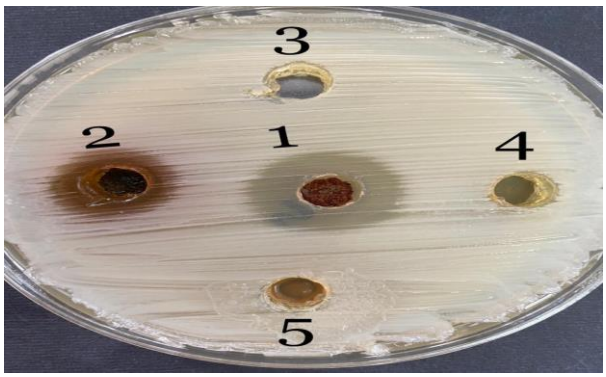


Fig 5: activity of different herbal plant extracts against MRSA isolates. 1: Green tea, 2: Cinnamon, 3: Garlic, 4: Ginger, 5: Demso act as a control

Comparison between the activity of 5 antibiotics and different 3 plant extracts against clinical MRSA isolates

It is interesting to notice that the crude extracts of tested herbal plants showed good activity against clinical MRSA isolates while the antibacterial treatment had limited effect as shown in Table (6).

Table (5) recorded the data of antimicrobial susceptibility of 50 MRSAisolates against 10 agents. **Figures [4&5]** shows the difference between the effects of the antibacterial and the medicinal plant on the MRSA isolates. It is obvious that herbal plants, especially Green tea extract more effective than the antibacterial.

Table (5) Susceptibility of MRSA isolates to 10 antibiotics

Antimicrobial agents (Antibiotics)	**Potency (µg/disc)**	**MRSA inducibility**
Pencillin(10µg)	P	R
Tetracyclin(30µg)	TE	R
Vancomycin(30µg)	VA	S
Oxacillin(1µg)	O	R
Azithromycin(15µg)	AZM	R
Cefoxitin(30µg)	FOX	R
Ofloxacin(5µg)	OFX	R
Amoxixillin(30µg)	AM	R
Ciprofloxacin(5µg)	CIP	R
Amikacin(30µg)	AK	R

R: resistant , S: sensitive

Effect of medicinal plant extracts on biofilm formation of MRSA

The most effective herbal plant extract were green tea and cinnamon extracts against MRSA isolate values of MIC were determined by using the microtitration plate method shown inFigure [6] and Table (7) & (8). MRSA was resistant to cinnamon extract, so used a higher concentration of cinnamon extract than green tea extract to inhibit the biofilm formation of MRSA.

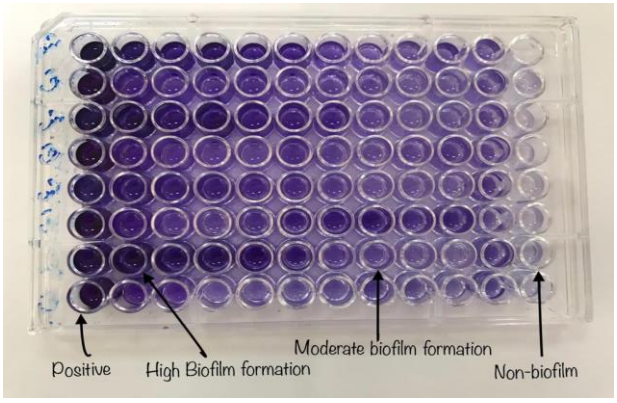


Fig 6: indicated that inhibition of biofilm formation of MRSA with the increase of plant extracts concentration and vice versa.

The results in **Table (6)** Comparison between the activity of TE,VA,DA,AK,E antibiotics and different plant extracts against clinical MRSA isolates.

MRSA isolates No.	Diameter of inhibition zone (mm)							
	TE30 µg S15≥	**VA30 µg** S ≥12	**DA2µg** S ≥14	**AK30µg** S ≥18	**E15 µg** S≥23	**Green tea**	**Garlic**	**Cinnamon**
1	0	0	0	0	0	28	0	14
2	0	0	0	0	0	10	0	8
3	0	17	0	6	0	14	0	0
4	0	0	0	0	0	21	0	10
5	0	10	0	0	2	16	0	20
6	0	14	0	0	0	20	0	13
7	0	20	3	0	0	22	0	12
8	0	0	0	0	0	11	0	0
9	0	0	0	0	0	13	0	6
10	0	9	0	0	0	18	0	22
11	0	18	7	0	0	25	0	17
12	0	5	0	0	0	9	0	3
13	0	0	0	0	0	21	0	11
14	0	0	0	0	0	21	0	25
15	0	12	0	9	0	27	0	16
16	0	0	0	0	0	15	0	16
17	0	0	0	0	0	10	0	5
18	0	7	0	0	0	16	0	20
19	0	0	0	0	0	23	0	13
20	0	0	0	0	0	19	0	24

TE: Tetracyclin , VA: Vancomycin , DA: Clindomycin , AK: Amoxicillin , E: Erythromycin

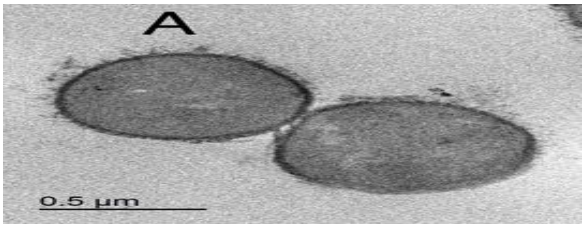
Table (7): Inhibition of biofilm formation by MRSA under the action of Green tea &cinnamon extracts

Serial Conc. of green tea& cinnamon extracts (mg/ml)	**Optical density readings of MRS at 450-550nm**							
	MRSA isolates							
	3		**15**		**22**		**24**	
	Green tea	cinnamon	Green tea	cinnamon	Green tea	cinnamon	Green tea	cinnamon
Negative control	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
50	0.11	0.15	0.1	0.12	0.12	0.23	0.11	0.18
25	0.15	0.18	0.13	0.14	0.16	0.29	0.11	0.23
12.5	0.19	0.19	0.17	0.17	0.19	0.38	0.16	0.27
6.25	0.22	0.27	0.17	0.20	0.22	0.40	0.18	0.33
3.1	0.35	0.42	0.23	0.23	0.27	0.44	0.20	0.45
1.56	0.41	0.55	0.28	0.37	0.33	0.59	0.25	0.54
0.78	0.49	0.69	0.30	0.44	0.33	0.66	0.36	0.56
0.37	0.58	0.73	0.45	0.63	0.47	0.80	0.47	0.73
0.2	0.67	0.84	0.59	0.96	0.55	0.99	0.61	0.89
Positive MRSA	1.05	1.05	1.05	1.05	1.05	1.05	1.05	1.05

Electron microscopic examination of plant-susceptible MRSA isolates

The most effective medicinal plant extract against MRSA isolates was green tea. The isolates were examined under the transport electron microscope (TEM) before and after treatment with plant extract. Result in **figure 7**

(A&B) cell deformation, membrane and cell wall rupture of isolate



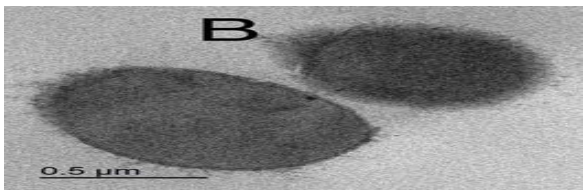


Fig 7 A: Before treatment & B: After treatment by green tea extractes

Discussion

MRSA, also known as methicillin-resistant *staphylococcus aureus,* is the main basis of nosocomial infections all over the world. It reasons minor to simple taints which are occasionally hard to luxury due to its fight against multiple antibiotics and carriage of manifold virulence features **[24].**

Ofloxacin and Ciprofloxacinare endorsed for serious infections associated with *staphylococci*, But Vancomycinstill remains the drug of choice for most MRSA-related illnesses **[25].**

Additionally, the capacity of MRSA strains to form biofilms and their frequently present multidrug resistance profile increase, which cause resistance to many antimicrobial agents, so searching for natural plants other to commonly used antimicrobial agent is increasing **[26].**

In the current study, we inspected the result of herbal plant of three plant species (Green tea-Cinnamon and Garlic) on MRSA isolates. Discovery new antibacterial drugs is very vital, the gotten results have shown that Green tea extract had an inhibitory and bacteriocidal effect on isolates at low concentrations 0.2 mg/ml of the studied extracts **[27].**

But the cinnamon extract were affected on growth of MRSA but at a high concentration of 25 mg/ml , so MRSA more resistant to cinnamon extract than green tea extract to inhibit biofilm formation of MRSA **[28].**

The possessions of green tea abstract which inhibit MRSA growth are largely linked to their polyphenolic components counting epicatechin, epicatechingallate, epigallocatechin and epigallocatechingallate in contradiction of MRSA isolates **[29].**

Green tea was also informed to have a synergistic effect with β –lactam antibiotics alongside MRSA, it was also testified that the main component of tea polyphenols, Epigallocatechingallate (EPGG) can reverse methicillin-resistance of MRSA by inhibiting the synthesis of PBP2, which also increase non β–lactam cell wall biosynthesis inhibitors **[30, 31].**

Conclusion

Based on the result obtained here, green tea could be used as a substitute treatment to treat infectious illnesses produced by multi-drug-resistant MRSA.

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