THE EFFECT OF ANTIBIOTIC , BACTERIOPHAGE , LAWSONIA INERMIS (HENNA) AND NIGELLA SATIVA EXTRACTS ON STAPHYLOCOCCUS AUREUS AND THEIR EFFECT ON VIRULENCE GENE EXPRESSION.

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Abstract

The rise and spread of antibiotic resistant, just as the advancement of new strains of sickness causing agents, are of incredible worry to the worldwide wellbeing local area. Compelling treatment of an infection involves the advancement of new drugs or some expected wellspring of novel medications. The over use of antibiotics created a multidrug resistant bacteria which make researches to find another way to control on multidrug resistant bacteria . Bacteriophage is considered one of the most newly way used as a treatment against multidrug resistant bacteria . Also utilized therapeutic plants of our local area could be an amazing source of medications to block this issue. This study is focused around controlling of Methicillin resistant staphylococcus aureus bacteria which isolated from nose patient’s by using both of bacteriophage and plant extract. The antimicrobial ability of two different plant extracts was screened against twenty pathogenic microorganisms**.** Our study designed to demonstrate the antimicrobial activity of an aqueous Henna (Lawsonia inermis) and Nigella sativa extracts against Methicillin – resistant staphylococcus aureus .The antibacterial potential of both herbs was checked by agar diffusion method and minimum inhibitory concentration (MIC) assay. Four isolates of staphylococcus aureus (S.aureus) were inhibited by both extracts. The MIC values of Henna extract ranges (0.5 – 8 µg/ml) less than MIC for Nigella sativa (1 – 8 µg/ml) against MRSA . PCR examination demonstrated that all S.aureus carried the hlg (hemolysin) and icaA (intracellular adhesion) genes . Real – time Rt-PCR assays were measured to impact on the harmfulness qualities. Henna(Lawsonia inermis) and Nigella sativa concentrates can be utilized as antimicrobial specialists against S.aureus .

Keywords : Antibiotic resistance , staphylococcus aureus , Henna (Lawsonia inermis), Nigella sativa , Gene expression .

Introduction

The increasing appearance of multi-drug resistant (MDR) microorganisms in clinics is a rising serious threat to human health (1) . Most recently, an estimated 2.5 million people acquire antibiotic-resistant infections every year in Europe and USA leading to approximately 50,000 deaths . Antimicrobial resistance in pathogenic bacteria is an urgent health problem that must be addressed by governments and health authorities worldwide. Recent estimates based on clinical data indicate that approximately 25,000 deaths per year in the European Union and 700,000 globally are due to infections caused by antibiotic-resistant pathogens. One of the most problematic bacterial pathogens at present is Staphylococcus aureus.(**2**) Staphylococcus aureus is a common commensal bacterium which is an opportunistic pathogen capable of causing a variety of diseases like endocarditis, pneumonia, osteomyelitis and toxic shock syndrome. It is also a leading cause of infections associated with catheters or devices(3) . Strains of this bacterium have been gradually acquiring antimicrobial resistance determinants throughout the antibiotic era(4) . Methicillin-resistant Staphylococcus aureus (MRSA) is an MDR (multi-drug resistant) organism that is resistant to every antibiotic except for Vancomycin. It was initially detected during early 1960's in United Kingdom and is now regarded as a major hospital acquired pathogen throughout the world(5) . Also, community-acquired MRSA infections have increasingly been identified within the past two decades(6) . This microorganism has been declared an international concern by the World Health Organization. Methicillin resistance in S. aureus is due to the presence of the mecA (or mecC) gene, which encodes the penicillin-binding protein PBP2a, which has low affinity for semisynthetic penicillins (7) . Methicillin-resistant Staphylococcus aureus (MRSA) is a major threat to human health. It is considered to be a Multi-

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Drug Resistant (MDR) pathogen capable of causing a variety of diseases (8) . MRSA is also one of the most important nosocomial pathogens in burn infection. As a result, therapeutic strategies to counter clinical infectious caused by these bacteria has become limited(9) . Therefore, it is critical that alternative antibacterial agents and programs are developed . Moreover, due to rapid acquisition of resistance to the new antibiotics and rising production costs, there has been little incentive to develop new antibacterial agents(10). A strategy against MRSA infections and to provide a new solution against the threat of MDR infections, phage therapy and plant extract . phage therapy has attracted a great deal of attention(11) . This approach uses bacterial viruses (phages) which can specifically attack pathogenic bacteria and kill them . Phage therapy has the potential of being highly specific against only the species and even strain responsible for an infection(12) . Moreover, their remarkable specificity prevents them from affecting human cells, microbial composition of body microbiota and inducing antimicrobial resistance in different bacterial species(13) . plants have had the option to give another mixtures of incredible advantage to humanity . Many methodologies have been expected to discover natural biological standards in plants(14) .An illustration of those resources is public medication as a correct screening of them may lead to the discovery of another successful antibacterial components (15) . Numerous plants have been utilized for a long time as a conventional plant medication. They have achieved the situation with the normal wellspring of new and strong antimicrobial specialists(16) . About 20% of the plants, which were found on the planet had been submitted to drug. Many studies on Lawsonia inermis leaves extractions showed that it had antibacterial activity against Gram positive bacteria(17) . Lawsonia inermis regularly known as Henna' is a sharb as often as possible belongs to family (Lynthraceae) developed in the Middle East, along the African bank of the Mediterranean Sea and Indi(18) .Different medical plants are utilized for the treatment of diseases that are resistant to present day medicines. One of such therapeutic plants is Nigella sativa (19). A herbaceous plant , N. sativa . (Dark cumin) has been utilized for quite a long time for the treatment of different ailments, including infectious illnesses(20). The seeds are reported to have several medicinal properties, which are regularly utilized in Asian and Mediterranean recipes(21) . The Nigella sativa (scientific name: Nigella sativa Linn) belongs to the Ranunculaceae family. It is known by a few names , for example, dark seed, and dark cumin, and it began in southeast Asia, utilized in antiquated Egypt, Greece, the Middle East, and Africa(22) . The lowest antimicrobial value of antimicrobial concentration which inhibit the growth of microorganism is called (MIC) , where the value of an antimicrobial concentration that below one able to inhibit the detectable growth and replication of microorganism known as (SIC)(23) . (MBC) is the least concentration value of antimicrobial agent needed to kill microorganism(24) .

Materials and Methods

This prospective work was carried out in the microbiology laboratory of six October university , faculty of pharmacy . a period of one year July 2019 till March 2020 following the ethical guidelines. clinical specimens from patient' s nose .

Bacterial isolates

A total of one hundred nasal samples were isolated by sterile swabs moistened with sterile saline solution .The tips were inserted into the patient's nose about 1 – 2 cm and rotating of both nostrils for six times. Then , The swabs were immediately inserted into a sterilized tubes containing sterile solution ,The nasal swab specimens were inoculated into Mannitol salt agar (Oxiod , Hampshire , England) , All inoculated plates were incubated at 37 ˚C for 24 hours under aerobic conditions , The default suspected colonies of S.aureus from media were taken and confirmed by Gram staining , catalyse and coagulase tests following standard procedures (25) .

Antimicrobial susceptibility testing

Inoculum was prepared from the colonies grown on Mannitol salt agar plates and antibiotic susceptibility testing was carried out on Mueller Hinton Agar after adjusting the turbidity to 0.5 McFarland standard. Modified Kirby Bauer disc diffusion method was followed for antibiotic susceptibility testing using antibiotic discs like Penicillin (10 μg) , Methicillin (5 μg) , Vancomycin (30 μg) , Clindamycin (2 μg) and Linezolid (30 μg) procured from Hi-media (26) . Zone of inhibition was measured and results were interpreted as Susceptible, Intermediate and Resistant following the recommendations of CLSI guidelines . Screening for MRSA was done using Methicillin discs (5 µg) on Mueller Hinton agar and zone of inhibition zero mm indicates methicillin resistance and was reported as MRSA (27) .

DNA extraction and PCR amplification

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DNA extraction

DNA extraction from samples was performed by utilizing the QIAamp DNA Mini unit (Qiagen, Germany, GmbH) with alterations from the producer's suggestions. Briefly, 200 µl of the example suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56OC for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acids was eluted with 100 µl of elution buffer gave in the pack. The characteristics of all used primers, as well as amplicons length and cycling conditions, are summarized by Ciftci et al., Kumar et al.,and Huehn et al. (28)

PCR amplification

Primers were utilized in a 25µl reaction containing 12.5 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific), 1 µl of each primer of 20 pmol concentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler(29) .

Analysis of PCR products

The results of PCR were isolated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature utilizing gradients of 5V/cm. For gel examination, 20 µl of the PCR items were loaded in each gel opening. Gelpilot 100 bp in addition to DNA Ladder (Qiagen, Germany, GmbH) and Generuler 100 bp ladder (Fermentas, Thermo Scientific, Germany) were utilized to decide the part measures. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was investigated through computer software (30) .

Quantitative analysis of gene expression

The virulence gene expression was analysed by quantitative real - time PCR (qRT-PCR) and the 16S rRNA housekeeping gene filled in as an inner control to standardize the expressional levels between tests. Preliminaries were used in a 25-µl response containing 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of RevertAid Turn around Transcriptase (200 U/µL) (Thermo Fisher), 0.5 µl of every preliminary of 20 pmol fixation, 8.25 µl of water, and 3 µl of RNA template. The response was performed in a Stratagene MX3005P real-time PCR with specific conditions mentioned in the **(Table 1)** (31) . Amplification curves and Ct values were determined by the Stratagene MX3005P software. To estimate the variety of gene expression on the RNA of the various examples, the Ct of each example was compared and that of the positive control group according to the "ΔΔCt" technique expressed by **Yuan et al.,(2006)** (32).

Bacteriophage isolation

Six sources of swage samples were collected from different places as Zagazig university hospital , main source of pipe from Bahr elbaqr – Alsharkeya Government , General 6th October Hospital and sewage 6th October station . samples were centrifuged and the supernatants were filtered through through 0.45 µm sterile filter. 10 ml of supernatants and 100 ml of liquid broth were mixed with 5 ml of overnight culture clinical MRSA , and then incubated at shaker incubated deice for 24 hours at 37°C . After incubation the mixture were taken for centrifugation , then the supernatants filtered by 0.45µm and the filtrate (Phage stock) was taken to check for the presence of phages by spot and plaque assay test (33) .

Spot test

This test applied according to (**Nakamura, T et al** , **2020**) (34) , which indicates there is no phage in an isolated suspension .

Extracts preparation

Aqueous Henna and Nigella sativa Extracts were prepared according to **Liaqat et al., (35)**

Determination of antibacterial activity of herbal extracts

Agar diffusion assay

Agar well diffusion technique was utilized for showing antimicrobial impact of plant extract and vancomycin against S. aureus . The tested isolates were inoculated into 10 ml sterile nutrient broth for 18 hours incubation . By using sterile swabs , the cultures were swabbed into nutrient agar media .Agar wells were set up with assistance of sanitized plug drill with 10 mm distance across . utilizing micropipette , 100 µl of 95% ethanolic and 500 mg/ml vancomycin focus from each concentrate . The plates were incubated at 37˚C for 24 hours . The restraint zones diameter was estimated in mm and the results were recorded. The inhibition zones diameter of under 12 mm were considered as having no antibacterial activity (36) .

Determination of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

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In order to estimate the antimicrobial susceptibility, the broth microdilution method was used with 96-well plates (TPP, Switzerland). The antibiotics were diluted twofold in LB broth (Acumedia, Michigan, USA), and the wells were inoculated with 1×106 CFU of bacteria (in a 0.2 ml final volume). The incubation period was 24 h at 37°C. The MIC testing was performed according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI). The range of the concentrations assayed for each antibiotic was 1 to 512 µg/ml. The absorbance was determined at 590 nm (Thermo-Lab Systems Reader, Finland). The positive control was done without adding any herbal plants or antibiotics, the last concentration able to be used as bactericide was adopted as MBC . The MIC value is the lowest antimicrobial concentration which inhibits the growth of microorganism where subinhibitory concentration (SIC) value is an antimicrobial concentration that below one able to inhibit the detectable growth and replication of a microorganism. The minimum bactericidal concentration value (MBC) value was determined according to **Owuama, (37) .**

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Table (1) Primers sequences, target genes, amplicon sizes and cycling conditions of S. aureus virulence genes.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
icaA	CCT AAC TAA CGA AAG GTA G	1315	94˚C 5 min.	94˚C 30 sec.	49˚C 1 min.	72˚C 1 min.	72˚C 12 min.	(Ciftci et al. 2009)
	AAG ATA TAG CGA TAA GTG C							
Hlg	GCCAATCCGTTATT AGAAAATGC	937	94˚C 5 min.	94˚C 30 sec.	55˚C 45 sec.	72˚C 50 sec.	72˚C 10 min.	(Kumar et al. 2009)
	CCATAGACGTAGCA ACGGAT							

Table (2): Primers sequences, target genes and cycling conditions for SYBR green rt-PCR

Target gene	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)			Reference
			Secondary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	Annealing	Final denaturation	
Staphylococcus 16S rRNA	50˚C 30 min.	94˚C 15 min.	94˚C 15 sec.	55˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	55˚C 1 min.	94˚C 1 min.	**(Mason et al. 2001)**
Hlg				55˚C 30 sec.			55˚C 1 min.		**Kumar et al., 2009**
icaA				49˚C 30 sec.			49˚C 1 min.		**Ciftci et al., 2009**

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Results

A total of twenty isolates

In the present study one hundred nasal samples were isolated from hospitalized patient's. Among one hundred samples , twenty strains (20 %) were identified for S. aureus by different biochemical testes . Gram staining , catalase , coagulase and phenotypic identification markers of S . aureus for four isolates which controlled by plant extract .

Antimicrobial activity

Ten tested isolates were inhibited by both plant extracts and vancomycin .The maximum inhibition zone for four isolates was observed after 24 hours incubation , we can find a difference in the inhibition zone size of S .aureus . The maximum inhibition zone diameter of S . aureus against plant extracts and vancomycin ranges from (14 – 30 mm) .

Table (3) : SIC , MIC and MBC of Henna , Nigella sativa and Vancomycin against S . aureus isolates .

Isolates	Concentration of SIC, MIC,MBC(µg/ml)								
	Henna			Nigella sativa			Vancomycin		
	SIC	MIC	MBC	SIC	MIC	MBC	SIC	MIC	MBC
1	4	8	16	1	2	4	0.5	1	2
2	0.5	1	2	2	4	8	1	2	4
3	2	4	8	4	8	16	2	4	8
4	0.25	0.5	1	0.5	1	2	0.125	0.25	0.5

Nigella sativa extract more effective antibacterial agents (MBCs 2 - 8 µg/ml) than Henna extract (1-16 µg/ml) . The MIC values of Henna (0.5 – 8 µg/ml) is lower than those of Nigella sativa (1- 8 µg/ml) . Vancomycin shows MBC values ranges (0.5 – 8 µg/ml) and MIC values ranges (0.25 – 4 µg/ml) .

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Detection of some virulence genes in S.aureus by conventional PCR

Tested S. aureus possess virulence genes which have characteristic bands icaA gene at 1315 bp and hlg at 937 bp .

Spot test

The isolated sources of bacteriophage give a negative result against isolated S. aureus that indicate there is no bacteriophage found affected on isolated S. aureus .

Quantitave assessment effect of Henna and Nigella sativa on some virulence genes in S.aureus using the qRT-PCR

Comparing the amount of examining virulence gene products (CDNA) by RT-PCR before and after treatment with a sub-inhibitory concentration of Henna and Nigella sativa.

Table (4) : Results of RT-PCR showing expression of hlg , icaA genes in S.aureus isolates before and after treatment of Henna , Nigella sativa and Vancomycin.

Genes	Isolates No.	Fold Change		
		Henna	Nigella sativa	Vancomycin
Hlg	1	0.12940	0.35355	0.05872
	2	0.05671	0.10013	0.03419
	3	0.05440	0.41179	0.05006
	4	0.02875	0.24485	0.00670
icaA	1	0.1476	0.7737	0.3634
	2	0.0947	0.3660	0.0151
	3	0.1303	0.6241	0.0583
	4	0.0174	0.0785	0.0113

Results showed that the amount of examining gene products was relatively increased in untreated samples with both extracts than those treated, that leads to high threshold cycle (Ct) value in treated than untreated. Interestingly, we found that Henna and Nigella sativa extract were more effective in significantly reducing the expression of S. aureus virulence .

Discussion

Staphylococcus aureus is both a commensal bacterium and a human pathogen. Approximately 30% of the human population is colonized with S. aureus (38). Staphylococcus aureus, a major human pathogen, has a collection of virulence factors and the ability to acquire resistance to most antibiotics. Clinical use of methicillin has led to the appearance of methicillin-resistant S. aureus (MRSA). The past few decades have witnessed the existence of new MRSA clones (39) . In the present study, isolation of Staphylococcus aureus and methicillin - resistant Staphylococcus aureus from patients nose(40). Detection of four isolates affected by plant extract Lawsonia inermis and Nigella sativa isolated from patients nose .In recent years, the use of natural compounds has gained attention due to increasing concerns over the safety of synthetic chemicals and emerging antibiotic resistance in bacteria (41) . Due to the resistance of antibiotics and isolated bacteriophage gave a negative result in our isolated Staphylococcus aureus . for this reasons , plant extracts, because of their established antimicrobial activities as well as their relatively lower toxicity and reduced number of side effects, may be potentially useful replacements for chemical preservatives . Also, plant extracts have a multi-component nature, so it is more difficult for bacteria to develop resistance than many commonly used antibiotics, which have a single target site (42) . The results of this study indicates that the Lawsonia inermis and Nigella sativa Extracts have a high antibacterial activity on isolated S.aureus (43). Bacteriophages (phages), as natural predators of bacteria , can evade the resistance developed to antibiotics through their different mechanisms of action(44) .In our study we tried to isolate an effective bacteriophage can effect against isolated S.aureus , but unfortunately give a negative result which made us to use plant extracts against isolated S.aureus (45). plant extracts, because of their established antimicrobial activities as well as their relatively lower toxicity and reduced number of side effects, may be potentially useful replacements for chemical preservatives (46). Also, plant extracts have a multi-component nature, so it is more difficult for bacteria to develop

resistance than many commonly used antibiotics, which have a single target site , Tested S. aureus possess virulence genes which have characteristic bands icaA gene at 1315 bp and hlg at 937 bp which indicate the isolated bacteria is virulence (47) . Of interest, all four tested S. aureus was inhibited by aqueous Lawsonia inermis and Nigella sativa extracts(48) .The antibacterial action of Lawsonia inermis may be due to numerous free hydroxyls 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 (49) . The results of this study indicate that the aqueous Lawsonia inermis and Nigella sativa have a high antibacterial activity for than gram positives S. aureus (48) with MICs ranges from (8 - 0.5 µg/ml) for Lawsonia inermis and MICs ranges (2-1 µg/ml) for Nigella sativa , these compounds act by interfering with the cell wall .Gram – positive bacteria have a thick cell wall , high in peptidoglycan and teichonic acid(50). The pathogenicity of S. aureus is to a great extent, upon the secretion of a variety of extracellular and intracellular virulence factors (51).PCR profile revealed that all obtained S. aureus isolates (100%) were carrying genes involving in producing bio-film formation (icaA) and gamma-hemolysin (hlg). It has been reported that the dose of antibiotics needed to kill biofilm bacteria was up to 1000-fold greater than the dose needed to kill planktonic bacteria (47).Therefore, we propose that our extracts are a cost effective antimicrobial agent for the treatment of biofilm infection. Both extracts used for the treatment of S. aureus infections not only depends on the respective bacteriostatic or bactericidal effects but also on the ability to prevent the release of virulence factors by dying or stressed bacteria (52). To address this, a real-time RT-PCR assay was used to assess the impact of Lawsonia inermis and Nigella sativa extracts on the expression level of the examined virulence gene. Based on our findings, these extracts at sub-inhibitory concentrations are capable of decreasing biofilm formation and hemolysin production by S. aureus via significantly down-regulating icaA and hlg genes. These suppressing effects were more remarkable for Lawsonia inermis extract in comparison with Nigella sativa extract. Several studies have shown that some herbal extracts have inhibitory effects on virulence expression of Gram-positive. In conclusion, our study supports the prospects for the use of Lawsonia inermis and Nigella sativa extracts as an antipathogenic remedy in combined therapy with antibiotics against resistant bacteria.

Abbreviations:

MIC: minimum inhibitory concentration ; MDR : multi-drug resistant; MRSA: Methicillin-resistant Staphylococcus aureus ;

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Conflicting Interest (If present, give more details): No Conflict of Interest

No financial disclosure

-Acknowledgements

Not applicable

Declarations

-Ethics approval and consent to participate

Written informed consent was obtained from all patients and the study was approved by the research ethical committee of Faculty of Science , Zagazig University (International review board IRB#:5340-l1-2019). The study was done according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

-Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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