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ANTIBACTERIAL POTENTIALS OF SOME PLANTS EXTRACTS AND THEIR COMBINATIONS AND THEIR SYNERGISTIC ACTIVITIES AGAINST MULTIDRUG RESISTANT BACTERIA

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The antimicrobial activity and synergistic effect of four plant aqueous extracts their 1:1couple were evaluated against drug resistant strains of Escherichia coli and Pseudomonas aeruginosa. The MIC of the aqueous extracts of Green tea, Laura's nobilis, Rosmarinus officinalis and Ammi visnaga determined using resazurin microdilution method. Only green tea and Rosmarinus officinalis aqueous extracts and their combinations have antibacterial potency for both microorganisms with variable minimal inhibitory concentration (MIC). The results revealed a synergistic effect between both plants aqueous extracts with cefotaxime in both microorganisms. Also, a synergistic effect was found between nitrofurantoin and both plants extracts and between norfloxacin and rosemary extract for Pseudomonas aeruginosa. PCR confirmed the presence The pslA, exoS, qnrA, blaTEM genes on P. aeruginosa and stx2, iss, Mcr1, floR genes on E.coli . Using relative quantification RT-PCR revealed that Sub-MIC green tea extract highly reduced the relative genes expression of pslA, exoS, qnrA, blaTEM genes of P. aeruginosa , and stx2, iss, Mcr1, floR genes of E. coli compared to endogenous genes (16s rRNA). The results revealed the possibility of use of antimicrobial drugs and plant extracts in combination in treating infectious diseases caused by MDR microorganisms. Also, more research is required to investigate the interaction between the effects of plant extracts on the activity of antimicrobial agents and the effect of plants extracts on virulence and antimicrobial resistance genes expression of pathogenic bacteria

Keywords: *plant extracts, P.aeruginosa,. resistance and virulence genes, RT-PCR*

INTRODUCTION

Medicinal plants have been used as sources of medicine in virtually all cultures. In last decade, the use of traditional medicine has expanded globally. People use herbal remedies due to their efficacy, tradition and their low cost. However, they often do not inform their physicians about their use of medicinal plants[1]**.** Medicinal plants are important elements of indigenous medical systems worldwide as well as in developing countries.

In recent years multiple antibiotic resistance (MDR) microorganism particularly pathogen bacteria has dramatically increased in human and animal. Because of indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases, resistance microorganism emergence of new bacterial strains that are multi-resistant was posed and resulted in increased morbidity and mortality and creates enormous health problems. However, the situation is alarming specially in developing countries due to indiscriminate use of antibiotics[2-3] Due to these resistance properties of microorganism, researchers started looking for alternative way for treatment or for preventing diseases. Nowadays, ingredients obtained from plants can be used as

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alternatives to antibiotics. Also, a strategy employed to overcome this resistance is the use of combination of drugs. The secondary metabolites from plant are good sources for combination therapy. There are a wide range of phytochemicals which act as multidrug resistance modifiers depicted. [4]

Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a blue dye that can be irreversibly reduced by oxidoreductase in active bacteria to a pink and highly red fluorescent substance called resorufin, which can be further reduced to hydroresorufin, giving a direct quantifiable measure of bacterial metabolic activity used to determine the MIC of substances. The resazurin assay utilising microtitre-plate, described by[5] , has been modified to achieve more accuracy in the determination of the minimum inhibitory concentration (MIC) values of natural products, including crude extracts, chromatographic fractions or purified compounds against various bacterial strains. This modified resazurin method is simple, sensitive, rapid, robust and reliable, and could be used successfully to assess antibacterial properties of natural products**.** [6]

In the present study, we have selected 4 medicinal plants Green tea, Laura's nobilis (bay leaf), Rosmarinus officinalis (rosemary) and Ammi visnaga to assess their antibacterial and synergistic effect with antibiotic drugs against multi-drug resistant *Escherichia coli* strain, representing gastrointestinal pathogens and *Pseudomonas aeruginosa*, as an environmental pathogen.

MATERIAL AND METHODS

Prepararion of aqueous plants exracts

In this study, the plant materials were acquired from Egyptian supermarkets and then they were fine grinned. Boiling 2 g of fine ground plant material in 20 ml distilled water for 20 minutes in a flask. Then The flask was taken off the fire and set aside to cool to get clean extract, the contents of the flask were filtered by filter paper and residue discarded. [7]

Examined bacteria

This study was approved by the Institutional Animals Care and Use Committee, Research Ethics Board, Faculty of Veterinary Medicine, Benha University (No. BUFVTM 34-10-22) following animal welfare guidelines. Clinical bacterial isolates which was formerly, identified biochemically and serologically as *E.coli* and *Pseudomonas aeruginosa* were gained from the bacteriology unit of the poultry diseases department, Animal Health Research Institute, Egypt. *Pseudomonas aeruginosa* strain was employed as an environmental pathogen, whereas *Escherichia coli* strain represented bacteria that cause gastroenteritis. Using a sterile wire loop, select a colony of relevant bacteria and suspend it in a 5 ml brain heart infusion broth medium suspended in a 5cc sterile Brain heart infusion liquid medium and incubated for 24 hours at 37 °C. Each active culture's optical density was adjusted to 0.1 at 625 nm for the purpose of standardising each inoculum using fresh broth. This resulted in a standard inoculum that contained 10^6 colony forming units (CFU) per milliliter. [8]

Antibiotic susceptibility testing

The effect of eight conventional antibiotic discs (Oxoid®) was measured using the disk diffusion technique against the identified bacteria. [9] Pure colonies of each microbe were suspended in sterile saline until they reached a turbidity of 0.5 (1.5 *08 CFU/ ml) in McFarland tube number 0.5. Each adjusted organism was swabbed onto Muller Hinton agar in a loop (Difco Laboratories). Commercial antimicrobial medicines such as norfloxacin (10 g), ampicillin (10 g), (30 lg), Gentamicin (10 g), enrofloxacin (10 g), cefotaxime (30 g), colistin sulphate (25 g), nitrofurantoin (300 g), and sulfamethoxazole/trimethoprime (25 g) were sterile dispensed onto The inoculated agar plate's surface.were then incubated at 37 degrees Celsius for 24 hours. The widths of the zones of inhibition were measured in millimeters and categorized as resistant, moderate, or sensitive to the tested microorganisms, according to recommendations established by the Clinical Laboratory Standards Institute. [10]

Resazurin microtiter test for measuring antibiotic activity and MIC (REMA)

REMA method was carried out as described by Martin and Palomino [11]**.** Resazurin was prepared at 0.015 % by dissolving 0.015 g, vortexed and filter sterilised

(0.22 lm filters) and stored at 4 Celsius degrees for a maximum of 2 weeks after preparation. 100 µl MHB broth was dispensed in each well of the 96-well plate. The first and second columns were kept as negative and controls for the corresponding bacterial growth column. 100 µl of stock solution of each aqueous extracts and their couple combinations in ratio 1:1 was added, and serial two-fold dilutions were done in columns on the plate. resulted concentrations ranging from 100 to 0.781 mg/ml for each extract. A 100 µl of each bacterial inoculums of concentration $1\text{-}3\times10^5$ CFU/ml was added in each well. The plates were incubated at 37 Celsius degrees for 24 hrs. After incubation, 30 µl of resazurin solution was added to each well and the plates were re-incubated for 1-2 hours. The change in color from blue to pink indicates reduction of resazurin and bacterial growth. A change in color from blue to pink indicated the growth of bacteria and the MIC was defined as the lowest concentration of the drug that prevented this change in color.

Evaluation of the synergistic action between antibiotics and plant extracts

For determination of the synergytic effect between the selected antibiotics and two selected plant extracts, each antibiotic filter paper disk was placed on the surface of infected and categorized MHA plates, soaked with 50 µl of known concentration extracts (100 mg/ml), and incubated for 24 hours at 37 Celsius degrees. The dimension of the cleared zones was measured and compared with that of antibiotic alone.[12] All of the exams were conducted in in triplicate.

Molecular analysis PCR and RT-PCR

PCR

For PCR analysis , DNA was extracted and was checked for the organization of *P. aeruginosaz* pslA, exoS, qnrA, and blaTEM genes, and *E.coli* stx2 , iss, Mcr1, and floR genes. DNA extraction was doing using QIAamp DNA mini Kit(Qiagen Germany-GmbH) . Oligonucleotide primers were supplied from Metabion (Germany). 200 µl of specimen suspension were treated at 56°C to 10 min. with 200 µl of lysis buffer and 10 µl from proteinase K . The lysate was then given 200 µl of 100% ethanol. With the assistance of 100 µl of elution buffer. The samples were centrifuged and washed.

Tables (1 and 2) below lists the primers that were given by Metabion (Germany). A 25µl reaction including 6 µl of DNA template, 1 µl of each primer at a concentration of 20 pmol,12.5 µl of Emerald AMP Max PCR master mix (Takara, Japan), and 4.5 µl of water was utilized to employ the primers. The product of PCR was separated via gel electrophoresis using 1.5% agarose gel (Applichem, Germany, (GmbH) stained with Ethidium bromide and photographed with a gel documentation system under ultraviolet light.

RNA extraction

To prevent RNA degradation, a double size (1 ml) of the RNA protect Bacteria Reagent (Qiagen, Germany, GmbH) one volume was mixed with (0.5 ml) of the harvested culture's broth. The mixture was then vortexed, allowed to sit at room temperature for (5) minutes, then centrifuged for (10) minutes at(8000) rpm. They decanted the supernatant. Subsequently, the pellet was mixed with 200 µl of TE buffer that included 1 mg/ml of Lysozyme (Biochemica, Applichem). Additionally,10 µl of β-mercaptoethanol per milliliter with 700 µl of RLT buffer was added, 10 µl of β-mercaptoethanol per milliliter. After adding 500 µl of 100% ethanol, the procedure was finished in accordance with the QIAamp RNeasy Mini kit's Enzymatic Lysis of Bacteria protocol (Qiagen, Germany, GmbH).

SYBR green Rt-PCR

First, 0.5 µl of each primer with a concentration of 20 pmol was used on a 25 µl reaction that also included 0.25 µl of RevertAid Reverse Transcriptase (200 U/µL) (Thermo Fisher), 12.5 µl of the 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 3 µl of RNA template, and 8.25 µl of water. the reaction was carried out Using a Stratagene MX3005P real-time PCR equipment, as seen in **Table (3)** .

Table 1: Primers used for the amplification and Sequences of different genes in *P. aeruginosa* and *E.coli.*

Bacteria	Target gene	Primers sequences	Amplified segment (bp)
P. aeruginosa	*pslA*	TCCCTACCTCAGCAGCAAGC TGTTGTAGCCGTAGCGTTTCTG	656 bp
	exoS	GCGAGGTCAGCAGAGTATCG TTCGGCGTCACTGTGGATGC	118 bp
	qnrA	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA	516 bp
	blaTEM	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516 bp
E. coli	*Stx2*	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779 bp
	Iss	ATGTTATTTTCTGCCGCTCTG CTATTGTGAGCAATATACCC	266 bp
	floR	TTTGGWCCGCTMTCRGAC SGAGAARAAGACGAAGAAG	494 bp
	Mcr1	CGGT CAGTCCGTTTGTTC CTTGGTCGGTCTGTAGGG	308 bp

Table 2: Cycling conditions used for the amplification of different genes in *P. aeruginosa* and *E.coli.*

Bacteria	Target gene	Primary Denaturation	Amplification (35 cycles)			Final extension	Ref.
			Secondary denaturation	Annealing	Extension		
P. aeruginosa	*pslA*	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**12**
	exoS			55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**13**
	qnrA			55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**14**
	blaTEM			54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**15**
E. coli	*Stx2*			58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**16**
	Iss			54˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**17**
	floR			50˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**18**
	Mcr1			60˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**19**

Table 3: Primers sequences, target genes and cycling conditions for SYBR green rt-PCR.

Bacteria	Target gene	Primers sequences	Reverse transcription	Primary Denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)			Ref
					Secondary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	Annealing	Final denaturation	
P. aeruginosa	*pslA*	TTGTTGTAGCCGTAGCGTTTC TGCCCTACCTCAGCAGCAAG C	50°C 30 min.	94°C 15 min.	94°C 15 sec.	60°C30 sec.	72°C 30 sec.	94°C 1 min.	60°C 1 min.	94°c 1 min.	
	exoS	GCGAGGTCAGCAGAGTATCG TTCGGCGTCACTGTGGATGC				55°C30 sec.			55°C 1 min.		
	qnrA	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA				55°C30 sec.			55°C 1 min.		
	b;laTEM	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC				54°C30 sec.			54°C 1 min.		
	16S rRNA	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA				50°C30 sec.			50°C 1 min.		**21**
E. coli	*Stx2*	CCATGACAACGGACAGCAGT T CCTGTCAACTGAGCAGCACT TT				58°C30 sec.			58°C 1 min.		
	Iss	ATGTTATTTTCTGCCGCTCTG CTATTGTGAGCAATATACCC				54°C30 sec.			54°C 1 min.	54°C 1 min.	
	floR	TTTGGWCCGCTMTCRGAC SGAGAARAAGACGAAGAAG				50°C30 sec.			50°C 1 min.	50°C 1 min.	
	Mcr1	CGGT CAGTCCGTTTGTTC CTTGGTCGGTCTGTAGGG				60°C30 sec.					
	16S rRNA	GCTGACGAGTGGCGGACGGG TAGGAGTCTGGACCGTGTCT				55°C 30 sec.					22

Data Analysis

The SYBR green rt-PCR analysis. Software from stratagene MX3005P was used to obtain ct values and amplification curves. Using the ratio (2-DDct) to compare each specimen's CT with that of the positive control group, the "ΔΔCt" approach [20] was utilized to estimate the variance in gene expression on the RNA of the various samples. Whereas ΔΔCt = ΔCtreference - ΔCttarget.ΔCt reference = Δ control - Δ treatment,. ΔCt target.

RESULTS AND DISCUSSION

Results

The results of antibiotic susceptibility tests of the used bacterial strains showed that both *Pseudomonas aeruginosa* and *Escherichia coli* was resistant to more than four antibiotics which revealed that both of them was multidrug resistant. *Escherichia coli* was resistant to Norfloxacin, Ampicillin, Gentamycin, Cefotaxime, Enrofloxacin and Sulphamethoxazole/trimethoprim while *Pseudomonas Aeruginosa* strain was found to be resistant to Norfloxacin, Ampicillin, Gentamycin, Colistin sulphate ,Nitrofurantoin and Sulphamethoxazole/trimethoprim.

On the other hand, the determination of antibacterial activity and the MIC of different plant extracts against *E. coli* and *Pseudomonas aeruginosa* by Resazurin microtiter assay (REMA) revealed antibacterial activity of both Rosmarinus officinalis (rosemary) and green tea and their mixtures with MIC values ranges from (100 to 25) mg /ml concentration of the crude extract. On contrast, there was no effect of both Laura's nobilis (bay leaf), and Ammi visnaga aqueous extracts on the examined microorganisms **Fig.(1 and 2).** The MIC value for green tea was (25 mg /ml) while for Rosemary was (50 mg /ml) while the MIC for the combination of green tea and rosemary was 50 mg /ml. On the other hand, MIC for the green tea extracts in combination with laurua nobilis or Ammi visnaga were 100 and 50 mg/ml respectively. The MIC for the combinations between rosemary and laurua nobilis or Ammi visnaga was 100 mg/ml for both.

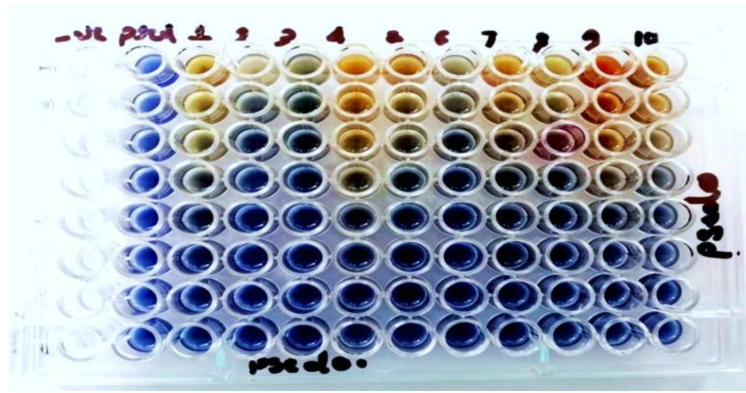


Fig. 1 : Antimicrobial and MIC values to activity antibacterial plant extracts against *Pseudomonas aeruginosa.*

-ve= negative Control Pseud= Pseudomonas Control ,1= rosemary extract , 2= LauraExtract 3= Ammi Extract 4= Green tea extract 5= rosemar extract+ Laura Extract , 6= Laura Extract+ Ammi Extract , 7= Laura Extract+ Green tea extract , 8= rosemary extract+ Ammi Extract , 9= rosemary extract+ Green tea extract 10= Ammi Extract+Green tea extract.

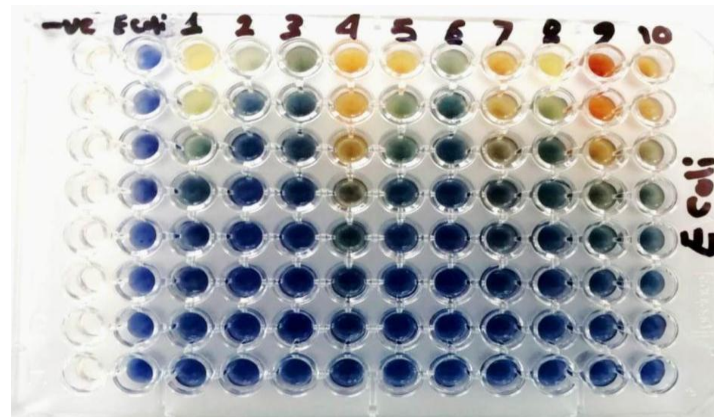


Fig. 2: MIC values to activity antibacterial plant extracts against *E.coli*.

-ve= negative Control , E.coli = E.coli Control ,1= rosemary extract , 2= LauraExtract , 3= Ammi Extract 4= Green tea extract , 5= rosemary extract+ Laura Extract, 6= Laura Extract+ Ammi Extract, 7= Laura Extract+ Green tea extract , 8= rosemary extract+ Ammi Extract , 9= rosemary extract+ Green tea extract, 10= Ammi Extract+ Green tea extract.

Synergistic antibacterial efficacy of two plant extracts and different antibiotics against *Pseudomonas and E.coli* strains was determined by comparing the zones of inhibition of each antibiotic against the examined strain with the same zones with the antibiotics and each of rosemary and green tea plant extracts **Table(4).** As described by [23] proposed a method for determining additivity's end point, with interactions that are higher or lower than additivity being classed as synergism and , respectively. The results revealed a synergistic effect between the both rosemary and green tea extract and cefotaxime for both examined microorganisms. Also, rosemary showed a synergestic effect with both norfloxacin and nitrofurantion against the *Pseudomonas* strain while had antagonistic effect on colistin sulphate, norfloxacin and nitrofurantion against *E.coli*. On the other hand, the green tea extract had antagonistic effect on norfloxacine against *Pseudomonas aerugenosa and* antagonistic effect on colistin sulphate, enrofloxacin, norfloxacin and nitrofurantion against *E.coli.*

Fig.(3 and 4) show the width of the zone of inhibition for two plant extract combinations .Combinations of extracts and antibiotics have either synergistic or antagonistic effects on bacteria in some circumstances. A negative interaction is indicated by lower inhibition zones.

Table 4: Synergistic effect of two plant extracts and several antibiotics against *P.aerugenosa* and *E coli.*

Antibiotic	Disc conc. (µg)	inhibition zone (mm)					
		Pseudomonas aerugenosa			*E .coli*		
		AB+G	AB+R	AB	AB+G	AB+R	AB
Ampicillin(AP)	10	0	0	0	0	0	0
Colistin sulphate(CS)	25	0	0	0	30	18	18
Cefotaxime(CTX)	30	24	30	30	20	30	30
Enrofloxacine(ENR)	10	16	0	0	16	16	0
Gentamycine(GM)	10	12	12	12	8	8	8
Norfloxacine(NOR)	10	10	15	10	16	14	0
Nitrofurantoin(NI)	300	8	14	18	30	16	18
Sulphamethoxazole/ trimethoprim(TS)	25	0	0	0	0	0	0

AB: antibiotic. R: Rosemarinus extract. G: green tea extract.

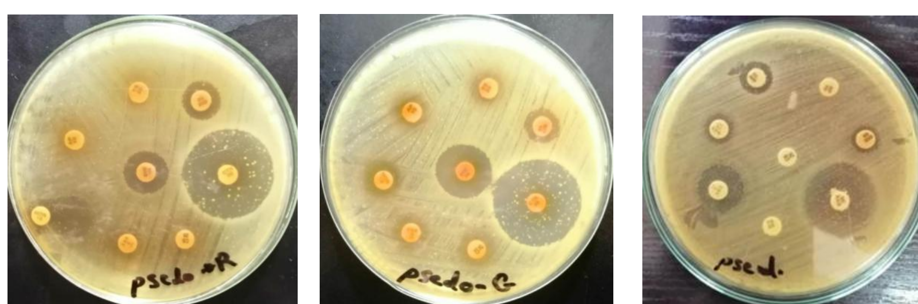


Fig. 3: The Synergistic Impact of Two Plant Extracts and Several Antibiotics Against *P.aerugenosa.*

Fig. 4: The Synergistic Impact of Two Plant Extracts and Several Antibiotics Against *E coli.*

PCR and RT-PCR

PCR technique is detecting resistant genes and virulence. PCR confirmed the presence of *P. aeruginosa* bacteria pslA, exoS, qnrA, blaTEM genes and *E.coli* bacteria stx2 , iss, Mcr1 , floR genes explained **Table (5 and 6) , Fig.(5).**

Gene expression of virulence and resistance genes before and after treatment with green tea extract using The relative threshold cycle (CT) method revealed that The green tea extract Sub-MIC (25 µg/ml) highly reduced the relative expression of both virulence gene (pslA and exoS genes) and resistant genes (qnrA and blaTEM genes) of *P. aeruginosa* with a fold change of 0.2017 , 0.1081, 0.1638 and 0.0848 respectively compared to control **Table (7).**

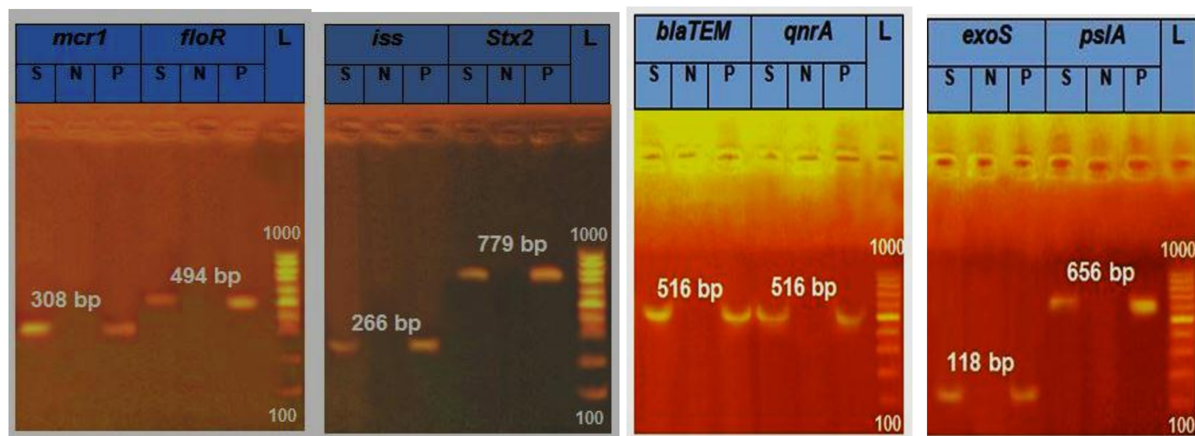
On the other hand, gene expression of virulence and resistance genes before and after treatment with green tea extract using The relative threshold cycle (CT) method revealed that green tea extract Sub-MIC (25 µg/ml) highly reduced the relative expression of both virulence gene (Stx2 and iss genes) and resistance genes (floR and Mcr genes) of *E.coli* with a fold change of 0.2736, 0.2483, 0.4763 and 0.1340 respectively compared to control **Table (8), Fig.(5).**

Table 5: Resistance and virulence genes of *P. aeruginosa.*

Sample	pslA	exoS	qnrA	blaTEM
P. aeruginosa	+	+	+	+

Table 6: Resistance and virulence genes of *E.coli* .

Sample	Stx2	Iss	Mcr1	floR
E. coli	+	+	+	+



E.coli vir gene *E.coli* AB gene *P.aeruginosa* AB gene *P.aeruginosa* vir gene

Fig.5: Results of PCR analysis of identification of resistance and virulence genes of *P.aeruginosa* and *E.coli.*

Table 7: Effect of green tea exctract on the resistance and virulance genes expression of *P. aeruginosa*.

Sample No	Treatment	P. aeruginosa								
		16S rRNA	***pslA***		***exoS***		***qnrA***		***blaTEM***	
		CT	**CT**	**Fold change**	**CT**	**Fold change**	**CT**	**Fold change**	**CT**	**Fold change**
P1	-	22.13	19.78	**-**	20.57	**-**	21.46	**-**	20.08	**-**
P2	Green tea	22.25	22.21	**0.2017**	23.90	**0.1081**	24.19	**0.1638**	23.76	**0.0848**

Table 8: Effect of green tea exctract on the resistance and virulance genes expression of *E.coli.*

Sample No	Treatment	E.coli								
		16S rRNA	***Stx2***		***Iss***		***floR***		***Mcr1***	
		CT	**CT**	**Fold change**	**CT**	**Fold change**	**CT**	**Fold change**	**CT**	**Fold change**
E1	-	18.43	22.61	**-**	23.33	**-**	20.78	**-**	21.90	**-**
E2	Green tea	19.23	25.28	**0.2736**	26.25	**0.2483**	22.65	**0.4763**	25.60	**0.1340**

Discussion

Antibiotic resistance is becoming a serious global problem, and herbal constituents may provide a valuable alternative for disease prevention or treatment. Gram-negative bacteria are more resistant to antibiotics than the Gram-positive bacteria due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism[24] the *E. coli* and *P. aeruginosa* strains used in the current study was selected to be highly resistant isolate against 6 different antibiotics. Recently, the emergence of *E. coli* strains that

shown to be highly resistant to different types of antibiotics usually used by clinicians and veterinarians to treat Gram-negative bacteria infections.[25] Also, *P. aeruginosa* is known to be resistant to high concentrations of salts and dyes, weak antiseptics, and many commonly used antibiotics making susceptibility testing essential . [26, 27]

Despite the development of a number of new antimicrobial drugs in recent years, microorganism resistance to drugs has increased rapidly. In therapeutic treatment, the empoly of plant extracts with known antimicrobial properties can be very useful .Several study had been conducted in recent years to confirm the activity of plant extracts against bacterial infections. [28-32]

Many plants has been used due to compounds synthesized in the plant secondary metabolism that have antimicrobial properties. Green tea, Laura's nobilis (bay leaf), Rosmarinus officinalis (rosemary), and Ammi visnaga were investigated for antimicrobial activity and MIC against MDR *E. coli* and *P. aeruginosa* strains using the Resazurin microtiter assay (REMA). The REMA method has been endorsed by the World Health Organization [33] and has been proven to be a reliable method for revelation of antibacterial effectiveness recent experience. Resazurin was very cheap, reducing the cost of testing. Results were easily determined visually by reading the change to a stable color from blue to pink which allows the MIC value for biosurfactants to be determined with a high level of accuracy and reproducibility. Also, no special equipment was required to perform REMA, giving the opportunity for its widespread application and Problems with poor solubility of the compound under test have largely been overcome by the incorporation of resazurin into the test since the test now includes a measure of bacterial activity[34] The MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism by overnight incubation, usually reported as mg/L.[35] The results revealed antibacterial activity of the aquoues extracts of both Rosmarinus officinalis (rosemary) and green tea and their mixtures with MIC values ranged from 100 to 25 mg /ml. The MIC value for green tea was (25 mg /ml) while for Rosemary was (50 mg /ml) while the MIC for the combination of green tea and rosemary was 50 mg /ml. On the other hand, MIC for the green tea extracts in combination with laurua nobilis or Ammi visnaga were 100 and 50 mg/ml respectively. The MIC for the combinations between rosemary and laurua nobilis or Ammi visnaga was 100 mg/ml for both.

Green tea contains polyphenols, which may account for up to 30% of the dry weight. Most of the green tea polyphenols (GTPs) are flavonols, commonly known as catechins.[36] Several epidemiological studies and clinical trials showed that green tea may reduce the risk of many chronic diseases[37] Also, the effectiveness of green tea in treating any type of diarrhea and typhoid has been known in Asia since ancient times[38, 39] This beneficial effect has been attributed to the presence of high amounts of polyphenols (**Table 9**) which are potent antioxidants. Phenolic compounds initially, affect cell membrane, as high correlation between toxicity and hydrophobicity of different phenolic compounds, changing the permeability and causing the leakage of cellular content or interfere with membrane proteins resulting in structure disrupting[40-42]

Green tea has been demonstrated to decrease the risk of a variety of chronic illnesses in several epidemiological studies and clinical trials[36] Green tea's efficacy in treating any type of diarrhea or typhoid fever has been known in Asia since ancient times[37, 38] The presence of high levels of polyphenols, which are powerful antioxidants, is thought to be responsible for this beneficial effect. Phenolic chemicals influence cell membrane permeability, causing cellular content leakage, or interact with membrane proteins, causing structural disruption, as a result of the significant correlation between toxicity and hydrophobicity of diverse phenolic compounds[39-42]

Table 9: Chemical Composition Of Green Tea and Rosmarinus officinalis extracts according to[43, 44].

.GREEN TEA EXTRACT	Rosmarinus officinalis extracts
Polyphenols: Catechins includes catechin (C), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin Gallate (EGCG) **Flavonoids** :myricetin glycosides, quercetin glycosides, and behenyl glycosides , Anthocyanins are a class of water-soluble pigments monosaccharides, such as glucose, galactose, rhamnose, arabinose, etc., and disaccharides or trisaccharides **Phenolic Acids :** gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ellagic acid, quinic acid, and tea gallate alkaloids :(caffeine 2-5%) amino acids: L-theanine ,γ-aminobutyric acid, glutamic acid, arginine, serine, and aspartic acid, chlorophyll and carotinoids minerals : potassium, zinc, manganese, copper and selenium	**Polyphenols: including phenolic acids, flavonoids, and terpenoids : luteolin-7-O-glucoside, rosmarinic acid, carnosic acid, carnosol, ursolic acid, s** lipophilic compounds (carnosic acid and carnosol). **alvianolic acid A &B** volatile compounds Oleanolic acid Ursolic acid Vitamins C, E, Alpha-pinene (9.0-26 %), 1,8-cineole (15-55 %), borneol (1.5-5.0 %), camphor (5.0-21 %), camphene (2.5-12 %), β-pinene (2.0-9.0 %) and limonene (1.5-5.0 %)

moreover, our results showed that *R. officinalis*, extract had stronge antibacterial activities which consistent with previous reports by[45-48] Plant extract had been shown to had capacity to act on biofilms adhered to a surface . These products can thus prevent planktonic cell adhesion, prevent biofilm formation and as a result, prevent microbial colonization.[49] phyto compounds and Plant extracts can stop microbial colonization by reduction adhesion ability, resulting in a biofilm of adhered cells that is facilety removed.[50] The interaction of plant compounds with sticky proteins on the microbial surface is another well-established approach, which inhibits the adhesion of new bacteria and decreases the cohesiveness of attached microorganisms.[49] The bacterial lipid bilayer could be a potential interaction target. The phospholipids of the bacterial cytoplasmic membrane are chemically attracted to carvacrol and thymol, causing loss of cellular substance such as genetic material, ions and adenosine triphosphate (ATP) and the membrane breakdown[51, 52] Laura's nobilis (bay leaf) and Ammi visnaga aqueous extracts, on the other hand, had no effect on the microorganisms tested. It's possible that the low readings for some plant extracts are related to the fact that they're in crude form and hence contain relatively few bioactive chemicals. In contrast to another solvents like hexane , ethanol or water, previous studies found that methanol was the best solvent for consistently extracting antimicrobial substances from medicinal plants[53] Also,[54] and [55] reported that extraction with organic solvents is the best way to extract

antioxidant according to the polarity as solvents seemed to be preferable to either nonpolar or polar solvents So the extraction using methanol 80% with ultrasonic-assisted was better solvents for extracting phenolic compounds owing to their higher polarity and good solubility.

Synergistic interaction between two agents, in which one agent enhances the effect of the other and together they act more efficiently than as individual agents, motivated many scientists to examine and assess the significance of synergistic acting of plant-derived compounds and traditional antibiotics[56, 4]

The results revealed a synergistic effect between both rosemary and green tea extract and cefotaxime for both examined microorganisms. Many authors detected a synergistic interaction between plant extracts and cefotaxim for both gram negative and gram positive bacteria[57-60] found that green tea aqueous extract showed antibacterial and synergistic activity with cefotaxime against ESBL producing *E.coli* gastrointestinal infections. Also, rosemary showed a synergestic effect with both norfloxacin and nitrofurantion against the *Pseudomonas* strain while had antagonistic effect on colistin sulphate, norfloxacin and nitrofurantion against *E.coli*. On the other hand, the green tea extract had antagonistic effect on norfloxacine against *Pseudomonas aerugenosa and* antagonistic effect on colistin sulphate, enrofloxacin, norfloxacin and nitrofurantion against *E.coli.* More research is required to investigate the interaction between the activity of plant extracts and antimicrobial agents' activity.

Concerning gene expression of some virulence and resistance genes before and after treatment with green tea extract using The relative threshold cycle (CT) method revealed that The green tea extract Sub-MIC (25 µg/ml) highly reduced the relative expression of both virulence gene (pslA and exoS genes) and resistant genes (qnrA and blaTEM genes) of *P. aeruginosa* with a fold change of 0.2017, 0.1081, 0.1638 and 0.0848 respectively compared to control.

On the other hand, gene expression of virulence and resistance genes before and after treatment with green tea extract using The relative threshold cycle (CT) method revealed that green tea extract Sub-MIC (25 µg/ml) highly reduced the relative expression of both virulence gene (Stx2 and iss genes) and resistance genes (floR and Mcr genes) of *E.coli* with a fold change of 0.2736, 0.2483, 0.4763 and 0.1340 respectively compared to control **Table 8, Fig.(5).** And [60] investigate the effects of Plantago ovata plant extract on the expression of beta-lactamase-producing genes in multidrug-resistant (MDR) K. pneumoniae isolates and found that the extract significantly downregulated OXA-48 and OXA-181 genes ($p<0.005$, CI=95%). they concluded that Downregulation of beta- lactamase enzyme-producing genes can be considered as the possible mechanism action of antibacterial effects of the plant. [62] tested the inhibitory effect of cinnamon oil containing 60% trans-cinnamaldehyde against Escherichia coli O157:H7 Shiga toxin (Stx) production. Cinnamon oil significantly reduced Stx2 production and the expression of stx2 gene, and it was confirmed by a Vero cell cytotoxicity assay,[63] reviewed the results the effect of The essential oils (EOs) extracted from plants extracted from several plant species on bacterial gene expression .[64] revealed that the nano-emulsion extract of laurus nobilis Sub-MIC highly reduced the relative expression of both virulence gene coA gene) and antibiotic resistant genes (mecA and blaZ genes) of *S. aureus* and the relative expression of both virulence gene (Stx2 andissgenes) and antibiotic resistant genes (floR and Mcr1 genes) of *E.coli.*

The effect of thyme oil perfume on resistance genes and virulence genes was high; after treatment of Salmonella enteritidis bacteria and Staphylococcus aureus bacteria which was the effect of the absent mecA and co-agulase genes in Staphylococcus aureus and absent qacA gene in Salmonella enteritidis when examined with PCR technology. [65, 66]

Conclusions

The plant extracts have a significant effect in reducing the effect of both virulence and antibiotic resistance genes. Therefore, it can be used as an alternative to or with antibiotics to control bacterial pathogen infection.

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نشرة العلوم الصيدليـــة
جامعة أسيوط

الإمكانات المضادة للبكتيريا لبعض مستخلصات النباتات ومركباتها ونشاطاتها التآزرية ضد البكتيريا المقاومة للأدوية المتعددة

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تم تقييم النشاط المضاد للميكروبات والتأثير التآزري لأربعة مستخلصات مائية نباتية بنسبة ١:١ ضد سلالات مقاومة للأدوية من Escherichia coli و Pseudomonas aeruginosa. تم تحديد MIC للمستخلصات المائية للشاي الأخضر و Laura's nobilis و Rosmarinus officinalis و Ammi visnaga باستخدام طريقة التخفيف الدقيق للريزازورين. فقط مستخلصات الشاي الأخضر و Rosmarinus officinalis المائية ومجموعاتهما لها قوة مضادة للبكتيريا لكلا الكائنات الحية الدقيقة بتركيز مثبط أدنى متغير (MIC). كشفت النتائج عن وجود تأثير تآزري بين المستخلصات المائية لكلا النباتين مع السيفوتاكسيم في كل من الكائنات الحية الدقيقة. كما وجد تأثير تآزري بين النيتروفورانتوين ومستخلصات كلا النباتين وبين النورفلوكساسين ومستخلص إكليل الجبل لـ Pseudomonas aeruginosa. وقد أكد تفاعل البوليميراز المتسلسل وجود الجينات pslA و exoS و qnrA و blaTEM على P. aeruginosa والجينات stx2 و iss و Mcr1 و floR على E. coli. وباستخدام القياس النسبي كشف تفاعل البوليميراز المتسلسل العكسي أن مستخلص الشاي الأخضر Sub-MIC قلل بشكل كبير من التعبير النسبي للجينات pslA و exoS و qnrA و blaTEM من P. aeruginosa والجينات stx2 و iss و Mcr1 و floR من E. coli مقارنة بالجينات الذاتية (١٦s rRNA). وكشفت النتائج عن إمكانية استخدام الأدوية المضادة للميكروبات ومستخلصات النباتات معًا في علاج الأمراض المعدية التي تسببها الكائنات الحية الدقيقة المقاومة للأدوية المتعددة. كما أن هناك حاجة إلى مزيد من البحث للتحقيق في التفاعل بين تأثيرات المستخلصات النباتية على نشاط العوامل المضادة للميكروبات وتأثير المستخلصات النباتية على التعبير عن جينات الضراوة ومقاومة مضادات الميكروبات للبكتيريا المسببة للأمراض.