



EFFECTS OF GREEN TEA ON PROTEASE IV GENE EXPRESSION BY *PSEUDOMONAS AERUGINOSA* ISOLATED FROM EYE INFECTION

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Abstract

The current research conducted to evaluate the antibacterial effect of green tea extracts against *Pseudomonas aeruginosa* isolated from eye infection. The parameters which were used in this study, including determination the Minimum inhibitory concentration (MIC) of green tea extracts and Gentamicin against *P. aeruginosa* and detecting their effect on the gene expression of Protease IV from bacterial cell suspensions by the using quantitative real time reverse transcription PCR (RT-qPCR) technique. The agar-well diffusion method was used for the concentrations 100, 200 and 300 mg/ml respectively. Results showed that the green tea alcohol extract has antibacterial activity against *P. aeruginosa* and the minimum inhibitory concentration (MIC) was 100mg/ml with inhibition zone of 18 mm, on the other hand, MIC value of Gentamicin was $\geq 10 \mu\text{g/ml}$. The results obtained from the gene expression of Protease IV by *P. aeruginosa*, showed there was significant induction of the expression in the groups treated with gentamicin in compare with green tea and the highest induction in expression of Protease IV gene was at Sub MIC concentration of gentamicin (5 mg/ml).

Key words: Green Tea, *P. aeruginosa*, Gentamycin-resistant, Protease IV

Introduction

A number of researchers have successfully used various extracts from plants against various pathogens (Visintini *et al.*, 2011). Phenolic compounds extracted from plants are commonly used productively due to their antioxidant functions and positive effects on production (Dorri *et al.*, 2012).

Green tea have flavonoids, tannin, vitamins, fluoride and other mineral salts. Some of antioxidant and antimicrobial agents of green tea could increase the life and efficiency of teeth. Tannins are biosynthetic materials which have a effective antibacterial effect (Tariq *et al.*, 2010).

Using of green tea as mouth wash down against plaques on teeth, that was stress the routine consumption of green tea in humans might have reduced the intensity of teeth caries, green tea showed a good reduction in bacterial colonies (Moghbel *et al.*, 2012).

Pathogenes is in *P. aeruginosa* is arbitrated by multiple bacterial virulence factors that help adhesion and/or disrupt host cell signaling pathways while targeting the extracellular matrix. *P. aeruginosa* stands out as a

unique and threatening organism as it is capable of causing strict invasive disease and of evading immune defenses causing persisting infections that are nearly impossible to eradicate (Pier & Ramphal, 2005).

Protease IV is important in the pathogenesis of *P. aeruginosa* induced microbial keratitis, and in relationship with other proteases has a major role in corneal virulence (Wilderman *et al.*, 2001). The virulence of protease IV in ocular infection has been credited to the devastation of host proteins, including fibrinogen and components of the immune system (Engel *et al.*, 1998).

Latest studies have indicated that *P. aeruginosa* adapts to the hostile environment of the CF lung by progressively undergoing phenotypic or genotypic modifying that down-regulate virulence factors required for acute infection and up-regulate factors required for bio-film formation (Smith *et al.*, 2006).

The Aim of this study was to evaluate the antibacterial effect of green tea and its effect on Protase IV gene expression by *P. aeruginosa* isolated from eye infection.

Materials and Methods

Green Tea (*Camellia sinensis*) Extraction

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Ahmad brand green tea leaves were obtained from a local retail market in Baghdad and then ground to powder using a coffee blender. Three serial dilution 100, 200, 300 mg/ml of *greentea* leaves were geared up by suspending 1, 2 and 3 gm respectively in 10 ml of 95% ethanol. Each concentration was mixed then filtered through whatman (No. 1) and kept in sterile test tube at 4°C until used (Kumar *et al.*, 2012).

Bacterial Isolation and Identification

P. aeruginosa isolate was obtained from patients suffering from eye infection in Baghdad city. Diagnosis of all these isolates were depended on the cultural and biochemical tests, then the diagnosis was confirmed by using API 20 system kit. (Junkins, 2010).

In vitro study

Antibacterial Activity of the green tea extract

Antibacterial activity of the green tea extract was tested on the selected organism by Agar well diffusion method (Archana *et al.*, 2011). In this test, (0.1) ml of a 24h broth culture of bacteria adjusted to 10⁸CFU/ml (0.5 McFarland) was aseptically introduced and evenly spread using sterile "L" rod on the surface of sterile Mueller Hinton agarplates. Four wells of about 8 mm diameter were aseptically cut on agar-plate using a sterile corkborer. Fixed volumes (0.6 ml) of each concentration 100, 200 and 300 mg/ml were then introduced into each well with the help of a micropipette. A control well was made in the centre with the extracting solvent. The plates were incubated over night at 37°C and the diameter of any resulting zones of inhibition was measured in (millimeters). This was repeated three times. The minimum inhibitory concentration is defined as the lowest concentration of extract that gives limited bacterial growth.

Minimum Inhibitory Concentration Determination (MIC) for Gentamicin Against *P. aeruginosa*

The MIC was determined by broth macro-dilution assay. A set of test tubes with different concentrations gentamicin with the same volume were prepared. Tubes were inoculated with the test microorganism of log⁸CFU/ml (0.5McFarland standard). After incubation of *P. aeruginosa*, susceptibility test was done for *P. aeruginosa*, tubes were examined for changes in turbidity as an indicator of growth. The first test tube that appeared clear was considered as MIC. This test was achieved according to Morello *et al.*, (2006) as the following:

- 1- Sterile tubes of Mueller-Hinton broth, each tube contained 2ml of sterile Mueller-Hinton broth.
- 2- A serial of two-fold dilutions of antibiotics were prepared by adding of 2ml of antibiotic stock solution

(80µg/ml) to the first tube of Mueller-Hinton broth, mixed the contents, then 2ml transferred from this tube into a second tube, mixed the contents of the second tube and transferred of 2ml to a third tube. The dilution process was continued until reach the last tube. After the contents of the last tube mixed well, discarded 2ml of broth, so that the final volume in all tubes was 2ml.

- 3- From the Nutrient agar plate culture of bacterial isolate the suspension of organism was prepared in 5ml of normal saline that equivalent to McFarland 0.5 (10⁸ CFU/ml) standard.
- 4- With a sterile pipette, 0.1ml of the bacterial suspension was transferred to the each of the serial of antibiotic broth tubes.
- 5- Each tube was shaken gently to mix the tube contents and placed in the incubator at 35°C for 18-24 hours.
- 6- The experiment was included the following control tubes:
 - A tube contained sterile broth (Sterility control).
 - A tube contained broth and bacterial isolate (Growth control).
 - A tube contained antibiotic and sterile broth.
- 7- After the incubation the tubes were examined for the presence or absence of turbidity, the lowest concentration that inhibits the visible growth of bacteria was determined as MIC.

Primers

The primers were used in this study including β -*actin* gene primer used as Housekeeping gene and Protease IV gene primers that were used as target genes table 1. These primers were designed using NCBI- Gene Bank data base. The primers were used in quantification of gene expression using RT-qPCR techniques based SYBR Green DNA binding dye and provided from (Bioneer, Korea) company.

Effects of Green Tea on Protease IV gene expression

This experiment was done to detect the effect of green tea in virulence factor of *P. aeruginosa*. The

Table 1: Details of primers that were used in the study.

Primer		Sequence	Amplicon
² - <i>actin</i>	F	GGGTGGAGCCAAACGGGTC	530bp
	R	GGAGTTGCTGTTGAAGTCGCA	
Protease IV	F	TCCGCAGG TAG CACT CAGTTC	353bp
	R	AAGCCGGATT CATAGGTGGTG	

Table 2: Morphological and biochemical tests of *P. aeruginosa*.

Bacteria spp.	Morphological examination		Biochemical tests	
	<i>P. aeruginosa</i>	Gram stain	–	Indol test
	Blood agar culture	- ² hemolysis	Motility test	+
	MacConky agar culture	pale color colonies as it is non-lactose fermenting	Catalase test	+
	Brain heart agar	colorless colonies	Oxidase test	+
	Cetramide Agar	Yellow to Green Colonies	TSI	Alkaline (K)/ Alkaline (K) gas (-), H ₂ s (-)

quantitative real-time PCR was used to determine the mRNA expression of the Protease IV from bacterial cell suspensions (Lehmann *et al.*, 2001). The experiment include five groups:

- Group 1 (G1):** (Control negative) bacterial suspension.
- Group 2 (G2):** bacterial suspension with green tea 100 mg/ml.
- Group 3 (G3):** Bacterial suspension with green tea 50 mg/ml.
- Group 4 (G4):** Bacterial suspension with Gentamicin 10 µg/ml.
- Group 5 (G5):** Bacterial suspension with Gentamicin 5 µg/ml.

Statistical Analysis

Data were investigated by using SAS (Statistical Analysis System - version 9.1). One way ANOVA, Two-way ANOVA and Least significant differences (LSD) post hoc test were performed to assess significant difference among means. $P < 0.05$ was considered statistically significant (SAS, 2010).

Results and Discussion

Identification of *P. aeruginosa*

The *P. aeruginosa* showed as a large flat colonies, Gram-negative, single rod, usually motile with peritrichous flagella (Junkins, 2010). As well that produced zones of beta-haemolysis with a grape like odor on blood agar and the colorless colonies on MacConkey agar. The capacity to produce green-blue/yellow pigments when cultured on

Table 3: *In-vitro* antibacterial activity of different concentrations of green tea extract against *P. aeruginosa* growth.

Concentration mg/ml of green tea .	<i>P. aeruginosa</i> (inhibition zone-mm) (Mean ±SE)
100	18±0.33
200	20±0.57
300	23 ±0.66
95% ethanol	0.00±0.00 D

Values represent mean ±S.E

cetramide agar because it is a selective differential medium used for the identification of *P. aeruginosa* (Aslanzadeh, 2006).

A number of biochemical tests were performed for identification of bacteria listed in table 2. The mentioned tests; along with the API 20E System, confirmed that the isolates belonged to *P. aeruginosa*.

Anti-bacterial effects of green tea extract

The results of agar-well diffusion method demonstrated that the inhibition zones of green tea extract against *P. aeruginosa* were (18, 20, 23) mm, due to the three concentrations of alcoholic extract 100, 200, 300 mg/ml respectively as in table 3. Different capital letters mean significant ($P < 0.05$) results between different concentrations. The MIC of green tea against *P. aeruginosa* was 100mg/ml.

The results of the study showed that the leaves extract of green tea show the presence of potent antibacterial activity, which confirms its use against infection. The assessment of antimicrobial activity was based on measurement of inhibition zones formed around the discs. These observations may be attributed to green tea catechin compounds and polyphenols. These compounds have been found to possess antibacterial action (Saikia *et al.*, 2006), these observation in agreement with Radji *et al.*, (2013) who found that antimicrobial activities of tea extracts are very selective. This difference in their activity depends upon the concentration and type of the extracts. Flayyih *et al.*, (2013) showed that black tea (*Camellia sinensis*) significant had impact on virulence factors produced by *P. aeruginosa* and on its resistance to some antibiotics. Ponnaniakamideen *et al.*, (2014) who found that ethanolic and methanolic extracts of all the tea samples were inhibitors of the growth of *Streptococcus* and *Staphylococcus*, due to the organic solvents exhibit the stronger efficiency in extraction of antimicrobial compounds as compared to other methods. As well as Some medical plant extracts which have antibacterial activity with higher minimum inhibitory concentration values have established anti-QS activity even at lower concentrations (Jeon *et al.*, 2014).

In addition, these results might be due to the direct effect of the green tea polyphenols as well as bactericidal effect against *P. aeruginosa*, these effects may be also show a discrepancy depending on the bacterial species so that they may be either growth inhibitory or stimulatory (Tiwari *et al.*, 2005), these evidence supported by Kumar *et al.*, (2012) who reported that the green tea produce highest zones of inhibition against *Bacillus subtilis* and *Enterococcus* spp. which damages bacterial cell membrane.

Tea constituents may contribute to human health including the prevention of cancer and cardiovascular diseases, the anti-inflammatory, anti-arthritic, antibacterial, anti-angiogenic, anti-oxidative, antiviral, neuroprotective, and cholesterol-lowering effects. However, the biologically active compounds of plants extracts are measured as antimicrobial agents, because of their ability to bind with adhesions and to disturb the availability of inhibitory effects of aqueous, methanolic and ethanolic extracts of tea plant (Chacko *et al.*, 2010).

Friedman (2006) has stated that the ocular pathogenic bacteria infect the eyes produce high quantity of gelatinases. (epigallocatechingallate) ECGC present in black tea inhibited gelatinase activity produced by numerous strains of ocular pathogens. The inhibition can delay the invasive spread of the bacteria in the eyes that thrive on a gelatin substrate.

The current finding showed the MIC of gentamicin against *Paeruginosa* was 10 µg/ml indicating that this isolate was gentamicin resistance.

The *P. aeruginosa* is known for its multidrug resistance. Due to the efflux pump, *P. aeruginosa* can be resistant to antibiotics such as penicillin, cephalosporin, tetracycline and more, even lacking the R plasmid that is usually accountable for antibiotic resistance among bacteria. Resistance in *Paeruginosa* is caused by the

outer membrane of the bacterium, as it is not very permeable. The efflux pump is situated in the cell membrane. The pump transports the antibiotics to the outer membrane of the bacterial cell (Hamud-Socoro, 2004).

As well as the infections caused by *P. aeruginosa* are usually resistant to treatment by many antibiotics and can lead to severe and persistent infections (Bonomo and Szabo, 2006; Doshi *et al.*, 2011).

The mechanisms producing resistance to antibacterial drugs include production of enzymes by bacteria which destroy or inactivate the drug and reduction of bacterial cell permeability, bacteria may also develop alternative metabolic pathways to these inhibited by the drug (Quinn *et al.*, 2006).

Detection of Gene expression of Protease IV by using RT- qPCR

The effect of green tea in compare with gentamicin on Protease IV gene expression by *P. aeruginosa* was determined, the results Fig. 1 showed that there was significant induction ($P < 0.05$) of the expression in the groups treated with gentamicin in compare with green tea, on the other hand, the highest induction in expression of Protease IVmRNA gene of *P. aeruginosa* was at Sub MIC concentration of gentamicin (5 mg/ml).

The results of protease IV gene expression of G1 showed that there were significantly ($P < 0.05$) lower fold change than G2 and G3, these result could be due to the phenomenon which termed quorum sensing, the generically termed 'quorum sensing', bacterial cell-to-cell communication, enables a bacterial population to trigger a unified response that is beneficial to its survival. This response improves access to complex nutrients or environmental niches and develops collective defence mechanisms against alternative competitive microorganisms. Furthermore, it also optimizes the population's survival by differentiation into morphological forms better adapted to combating environmental threats (Bagge *et al.*, 2003).

Thus, the term 'quorum-sensing' has been associated to describing the bacterial ability to monitor cell density prior to expressing a phenotype. A strategy that uses QS adversary to block the native signal from interacting with the receptor has also been exposed in vitro to control biofilm development, to inhibit virulence factor expression, to reduce biofilm toxicity and remove the protective shield to PMNs and to create the biofilm more sensitive to antibiotic treatment (Hentzer *et al.*, 2002).

G1 : control negative

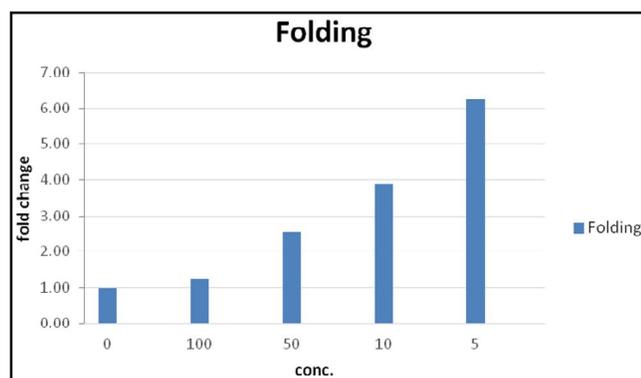


Fig. 1: The relative proteaseIV gene expression treated group and non treated. Data are shown as the fold change in mRNA level in G1, G2, G3, G4 and G5 by Q RT-PCR.

G2 : bacterial suspension with green tea 100 mg/ml

G3 : bacterial suspension with green tea 50 mg/ml

G4: bacterial suspension with Gentamicin 10 µg/ml

G5: bacterial suspension with Gentamicin 5 µg/ml

Protease IV, a 26 kDa serine endoprotease (Engel *et al.*, 1998), is secreted by most *P. aeruginosa* isolates causing microbial keratitis and in relationship with other proteases has a major role in corneal virulence (Wilderman *et al.*, 2001). The virulence of protease IV in ocular infection has been credited to the destruction of host proteins, including fibrinogen and components of the immune system (Engel *et al.*, 1998). Protease IV also degrades structural proteins such as elastin, facilitating bacterial adhesion and infection. Limited suggest that protease IV may contribute to acute lung injury induced by *P. aeruginosa* through loss of surfactant function. Protease IV has been revealed to be the iron-regulated protein PrpL, which suggests that its expression is differentially regulated compared with the other *P. aeruginosa* extracellular enzymes, protease IV has been concerned as an important virulence factor that supply to the pathogenesis of *Pseudomonas* keratitis. (Malloy *et al.*, 2005).

Additionally, a fourth inter-cellular communication signal has been showing to be capable of integrating environmental stress cues with the quorum sensing network (Lee *et al.*, 2013). PQS is also essential for full virulence towards plants (Cao *et al.*, 2001).

Low phosphate levels also raise IQS production in wild type *P. aeruginosa* (Lee *et al.*, 2013). These result show up the importance of environmental factors in modulating the bacterial QS systems and the plasticity of the QS networks in accommodation and development of environmental changes for the benefit of bacterial pathogens. On the other hands, the results of protease IV gene expression of G2 and G3 (green tea extract) showed that there were significantly ($P < 0.05$) lower fold change than G4 and G5 (bacterial suspension with Gentamicin), these indicate that the antibacterial effect of Green tea (100 and 50 mg/ml), these result due to that green tea act as antimicrobial by interaction with the negatively charge microbial cell surface, ultimately resulting in impairment of bacterial activities by decreasing virulence production of the protease IV. In addition that green tea polyphenols act as potent free radical scavengers due to the hydroxyl groups in their chemical structure. The hydroxyl groups can form complexes with free radicals and neutralize them, preventing the progression of the disease process and the inflammation reactions (Tiwari *et al.*, 2005). Also the green tea

polyphenols have demonstrated significant anti-oxidant, anti-carcinogenic, anti-inflammatory, thermogenic, probiotic and anti-microbial properties in numerous human, animals and in vitro studies (Kakuda, 2002), the result were in agreement with Subhashini *et al.*, (2010) who demonstrated that green tea which have a flavonoids, tannins, alkaloids, saponins that, potent antibacterial effect.

Drug resistance and side effects run into the use of synthetic drugs has led to the surge for novel and safe alternatives. Since ancient times, plants have proved to be an archetypal source of medicine. Green tea being the non-fermented type possess more Catechin than the other types (Subramaniam *et al.*, 2012). The main catechins present in green tea polyphenols are epicatechin (EG), epigallocatechin (EGC), EGC gallate (EGCG), EGCG can cause cell membrane disruption and prevent DNA super coiling eventually leading to bacterial destruction. EGC interacts with proteins and distorts their tertiary structure. Since cariogenic bacteria have the ability to produce lactic acid that erode the tooth surface and can encourage the plaque adherence by producing sticky dextran from sucrose. GFT is a key enzyme, which metabolizes dietary sucrose (Goenka *et al.*, 2013).

Tabas *et al.*, (2013) also reported that polyphenol act as immunomodulator enhance the innate immune system to initiate responses to LPS of Gram-negative bacteria through binding of LPS to TLR4 which leads to the activation of NF- κ B, and the action of polyphenol effect lead to stimulation of immune response and inhibit activation of NF- κ B through the modulation of inflammatory pathways.

The results of treated group with green tea indicated that green tea are potent antibacterial against *P. aeruginosa* which superiority than gentamycin. Many research investigations have demonstrated that the polyphenols possess bioactivity that have been shown to scavenge oxygen and nitrogen derived free radicals, modulating antioxidant enzymes and cellular redox transcription factors (Watson *et al.*, 2014).

Conclusion

This study has shown that Green tea has antibacterial activity against *P. aeruginosa* and that superior than gentamicin in eye infection. As well as has effects on expression of virulence gene expression (protease IV), suggesting it as alternatives to control *P. aeruginosa*.

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