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Efficacy of green tea extracts against multi drug resistant *Pseudomonas aeruginosa* isolates from burn wounds

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Abstract

Burn is one of the most common and devastating forms of trauma which is caused by severe thermal injury to skin and soft tissue. It has been estimated that about 75% of the mortality associated with burn injuries is related to sepsis especially in developing countries. Multi Drug Resistant (MDR) *Pseudomonas aeruginosa* is the most commonly isolated pathogen from burn wounds. The present study was designed to isolate and characterize the MDR isolates of *Pseudomonas aeruginosa* followed by evaluating the antibacterial effect of green tea extracts against the isolated pathogens. A total of 65 samples from burn wound patients were collected for isolation of *Pseudomonas aeruginosa* using Pseudomonas agar. The samples were confirmed using biochemical tests. The antibiotic sensitivity testing and antimicrobial potential of green tea extracts (aqueous and n-hexane) against isolated *P. aeruginosa* was determined by using Kirby-Bauer disc diffusion method. *Pseudomonas aeruginosa* was isolated from 23 (35.4%) samples and among these 10 (44%) isolates were multi drug resistant. The *Pseudomonas aeruginosa* isolates were found 100% resistant to ceftriazone (CRO), tobramycin (TOB) and piperacillin-tazobactam (TZP) while majority of the isolates were intermediately resistant to other commonly used antibiotics. Ciprofloxacin was found to be the most effective drug which was sensitive to 50% of isolates. The n-hexane extract of green tea showed good antibacterial activity having mean zone of inhibition (ZOI) 21.5±0.84mm and 21.8±0.63mm at 100mg/ml and 200mg/ml concentrations as compared to aqueous extracts. This study showed the high frequency of multidrug resistant *Pseudomonas aeruginosa* in burn wounds and revealed that n- hexane extract of green tea is a good substitute to antibiotics in the management of MDR *P. aeruginosa* infections in future.

Keywords: Green tea, MDR, *Pseudomonas aeruginosa*, Antibiotic Sensitivity Testing, Burn wound

Introduction

Pseudomonas aeruginosa is a Gram-negative, motile bacterium and important opportunistic human pathogen that causes acute respiratory, urinary and chronic infections in immunocompromised and burn patients (Kang et al., 2003). *P. aeruginosa* is found to be the most common bacterial cause of nosocomial infections with reported incidence 17–26% of burn wound infections (Shahzad et al., 2012). The use of antibiotics has been beneficial when prescribed and taken by patient correctly. These drugs have been used so widely against the infectious organisms that the organisms are adapted to them, making them less effective which we called as antibiotic resistance. Some microorganisms develop resistance to single agent/class, while others develop resistance to several antimicrobial agents or classes. Such types of organisms are known as Multidrug resistant (MDR) strains (Nascimento, 2000). The main risk for public health is that resistance genes are transferred from environmental bacteria to human pathogens (Kruse et al., 1999). Resistance either acquired or intrinsic towards commonly used antibiotics makes the pathogen a superbug: which posed a challenge to eradicate it (Breidenstein et al., 2011). *Pseudomonas aeruginosa* is considered as superbug and hence the selection of appropriate drugs for treatment of *pseudomonas* infections has become more problematic (Hosseinidoust et al., 2013).

Now a days, herbal medicines are gaining importance throughout the world and they are reviving again due to their easy availability, long-lasting curative effects, natural way of healing and less side-effects (MacIntosh, 1999). Green tea has been used as a beverage and a medicine in most of Asian countries as a tool to control bleeding, heal wounds, regulation of body temperature and blood sugar (Anderson et al., 2005). Green tea is a gift of nature to mankind and efficiently utilized drink in the world after water (Gomikawa et al., 2008). Tea polyphenols are well known for their antioxidant and antimicrobial properties (Chan et al., 2010; Reygaert, 2014). The most energetic and plentiful catechin in green tea is epigallocatechin-3-gallate (EGCG) (Wu and Yu, 2006). Studies have shown that the strong antioxidant properties of green tea are attributed to EGCG and EGC (Farhoosh et al., 2007). Green tea poly-phenolic catechins especially

Epicatechin gallate (ECG) and Epigallocatechin gallate (EGCG) can hinder the growth activity of a wide range of bacterial species (Peter et al., 2005). Green tea has been claimed to have antimicrobial activity against a mixture of microorganisms including *Staphylococcus aureus*, *Salmonella* sp., *Escherichia coli*, *Candida albicans*, *Enterococcus* sp., Herpes simplex virus, HIV and influenza virus (Jigisha et al., 2012). Due to the following benefits and qualities of green tea (*Camellia sinensis)*, the present study was conducted to evaluate the antibacterial activity of green tea extracts against multi drug resistant *P. aeruginosa* isolated from burn wounds.

Materials and Methods

Isolation and Characterization of Isolates

A total of 65 burn wound samples were collected using sterile cotton swabs from patients of Allied Hospital Faisalabad, Pakistan. Samples were inoculated on pseudomonas agar (Difco, USA) to isolate *P. aeruginosa*. Specific biochemical tests such as oxidase, catalase, indole and methyl red tests were performed for the confirmation of bacterial isolates.

Selection of Multi drug resistant *Pseudomonas aeruginosa*

The selection of multi drug resistant *P. aeruginosa* was done by evaluating its resistance pattern against commonly used antibiotics (Table 1).

Collection of Plant Material

The fresh leaves of green tea (*Camellia sinensis*) were collected from the local market and identified from Department of Botany, Government College University Faisalabad, Pakistan. The leaves were washed with water to remove dust and dirt particles, sun dried for seven days and ground to powder by using mixer grinder.

Preparation of aqueous extract of green tea

A total of 10 gm grounded green tea powder was soaked for two days (maceration) in 100 ml of autoclaved distilled water in a 250 ml sterile conical flask, with intermittent shaking at room temperature. The extract was then filtered through a sterile muslin cloth for coarse residue and finally filtered through sterile Whatman No.1 filter paper. The filtrates were then concentrated by using rotary vacuum evaporator and stored in an air tight container at 4ºC until use. Different concentrations (100 mg/ml and 200 mg/ml) of aqueous extracts were prepared (Archana and Abraham, 2011).

Preparation of n- Hexane extract of Green tea

A total of 1000 gm green tea power was soaked thrice in methanol at room temperature for 7 days and the material was filtered each time by using sterile Whatman No.1 filter paper. The filtrate was concentrated and evaporated to dryness with the help of vacuum evaporator to obtain a crude methanolic extract of green tea. The (125gm) crude extract was suspended in distilled water (100ml). Partitioning was done in n-hexane (3x100ml) by modified Kupchan method and the yield was 100gm. Two different concentrations of n-hexane fractions were prepared by dissolving the appropriate amount of residue in the solvent (Mukherjee and Das, 2012).

Antibacterial Susceptibility Testing

Antibacterial susceptibility of *P. aeruginosa* isolated strains was carried out by using disc-diffusion method according to CLSI guidelines. Antibacterial susceptibility test of the isolated strains was performed with commonly used antibiotic disks (Oxoid, USA) such as cefepime (30µg), ceftazidime (30 µg), ceftriaxone (30 µg), tobramycin (10 µg), ciprofloxacin (10 µg), piperacillin-tazobacam (110 µg), amikacin (30 µg) and piperacillin (100 µg). Zones of inhibition were measured and compared with CLSI scale (Beige et al., 2015).

Screening of Antimicrobial Activity

Antibacterial activity of the extracts was determined by agar diffusion assay according to CLSI guidelines. The 0.5 McFarland standard of bacterial suspension was inoculated on Pre-warmed Mueller-Hinton agar (Oxoid) plates. A 20 µl of green tea extracts were dispensed onto sterile paper discs (6 mm diameter, Oxoid) and were hygienically placed on the surface of inoculated agar plates. The central disc was kept as negative control and was soaked with (50 µl) of sterile distilled water and n- hexane. Ciprofloxacin (10 µg) was used as antibacterial positive control. The concentrations of extracts employed were 100mg/ml and 200mg/ml. Zone of inhibition was measured after 24 hours incubation at 37ºC (Radji et al., 2013).

Results

The results of present study revealed that out of 65 wound samples from burn patients 23 (35.5%) were found positive for *P. aeruginosa.* The entire positive (n=23) isolates were tested for their resistance against commonly used antibiotics and found that 10 (44%) isolates showed resistance against majority of the antibiotics and nominated as multidrug resistant *P. aeruginosa* (MDRPA1-MDRPA 10) as shown in (Table 1).

All the isolated strains of *P. aeruginosa* were found resistant to ceftriazone (CRO), tobramycin (TOB) and piperacillin-tazobactam (TZP) and intermediate resistant to other used antibiotics. Ciprofloxacin (CIP) was found to be most effective drug as 50% isolates were sensitive followed by amikacine (AK), ceftazidime (CAZ) and piperacillin being effective against 30% isolates (Figure 1). The zone of inhibition of green tea extracts against different MDR *P. aeruginosa* isolates are shown in (Table 2). Green tea extract in n-hexane showed good antibacterial activity against

all the isolates having mean zone of inhibition (ZOI) 21.5±0.84mm and 21.8±0.63mm at 100mg/ml and 200mg/ml concentrations respectively. Aqueous extracts showed moderately good results against all the isolates with exception to MDRPA 3 and MDRPA 7. The mean ZOI of aqueous green tea extracts were 19.8± 2.20mm and 20.6± 1.15mm at 100mg/ml and 200mg/ml concentrations respectively.

Discussion

The emergence of multi drug resistant (MDR) strains of pathogenic bacteria is a challenging issue for the scientists currently to control of infectious diseases in communities, in hospital settings and in developing as well as in developed countries. Multi drug resistant *P. aeruginosa* play a vital role in the colonization and infection of burns and hospitalized patients. These bacteria are leading cause of nosocomial infections, bacteraemia and urinary tract infections (UTIs). It is an uphill task for clinicians to treat the burn wounds infections due to frequent emergence of MDR bacteria to a wide range of antibiotics. Sooner or later these burn wounds infections will become untreatable as no more alternatives will be left with clinicians. To combat this challenge it is the need of a day to explore new antimicrobial compounds of herbal origin which could be incorporated as an alternative therapy in near future (Mbuthia et al., 2014).

P. aeruginosa is the most commonly isolated bacteria with an ability to colonize in burn wounds. The results of present study showed 35.5% frequency of *P. aeruginosa* from burn wounds, which were near to some previous research findings of (Singh et al., 2003) with 31% and (Rezaei et al., 2011) 26.7% incidence. In contrast a much higher incidence was also recorded (57%) of *P. aeruginosa* in burn wounds (Estahbanati et al., 2002). Similarly 44% of the isolated strains of *P. aeruginosa* were multi drug resistant and this is in agreement with recent findings of 22.7% and 30% MDR *P. aeruginosa* strains as mentioned by (Gill et al., 2011; Khan et al., 2014) respectively. This alarming pattern is increasing day by due to excessive and uncontrolled use of antibiotics which lead towards multi drug resistance in pathogenic bacteria.

The present study showed that (100%) isolates of *P. aeruginosa* was found highly resistant to toberamycin, piperacillin- tazobactam and ceftriaxone. Study conducted by (Khan et al., 2014) in Karachi during 2014 showed the similar resistance pattern against these antibiotics. Noticeable resistance against amino glycosides and cephalosporin were detected in the studies conducted by (Shah et al., 2015; Bhatt et al., 2015). Fluroquinolone compounds are one of the important antimicrobial agents that have been used for variety of infections. Present study illustrated 50% sensitivity against ciprofloxacin followed by amikacin (30%), ceftazidime and piperacillin. Similar results were reported by other scientists that (50%) isolates were sensitive to ciprofloxacin, (30%) to ceftazidime and about 20% to Amikacin (Gill et al., 2011; Shah et al., 2015; Bhatt et al., 2015). Amikacin is used sparingly only in severe forms of the disease due to high treatment costs and the intravenous nature of administration, which is one of the reason behind its slow emergence of resistance. Quinolones are preferred mainly because of the easy availability in oral forms and reasonable cost (Drago et al., 2011). However, the rising quinolone resistant strains of *P. aeruginosa* have surfaced owing to the changes in target enzymes of the bacteria and active efflux pumps generated to prevent the entry of the drugs (Strateva and Yordanov, 2009).

Green tea (*Camellia sinensis*) has been considered and reported to have modest activity against a range of pathogenic bacteria including MDR *Pseudomonas aeruginosa*. It is clearly evident from the results of our study that the n-hexane extracts of green tea has a potent antibacterial activity at both concentrations (100mg/ml and 200 mg/ml) against MDR *P. aeruginosa*. In present study n-hexane extracts of green tea showed mean zone of inhibitions with diameter of 21.5±0.84 mm and 21.8±0.63 mm at 100mg/ml and 200 mg/ml while aqueous extracts showed 19.8± 2.20mm and 20.6± 1.15mm at 100mg/ml and 200mg/ml concentrations respectively. These results are better than the previous work done by other scientists showing 15.66 ±3.20mm and 17.66±3.26mm at both concentrations (100mg/ml and 200 mg/ml) of n-hexane extract and also 17.670 ± 0.398mm zones of aqueous extract of green tea (Rajdi et al., 2013; Dubay and Abhishek, 2016). The extracts of n-hexane of the tested plant formed greater suppression zones against the isolated bacteria as compared to aqueous extract. High inhibitory effect of n-hexane extracts may correlate with its capability to extract more active ingredients from green tea than aqueous extract. The antibacterial property of green tea is mainly due to the presence of polyphenolic components including Epicatechin (EC), Epicatechin gallate (ECG), Epigallocatechin (EGC) and Epigallocatechin gallate (EGCG) (Jazani et al., 2007; Fanaki et al., 2008). The direct antibacterial activity of tea catechins is the result of its damage to bacterial cell membrane. This inhibits the ability of the bacterial cell to bind to host cells and also to bind to each other to from bio films (Koech et al., 2013; Radji et al., 2013).

Conclusion

It is concluded from the present study that frequency of MDR *Pseudomonas aeruginosa* is

increasing day by day in burn wound patients. Ciprofloxacin is the most sensitive drug to be used against the isolated MDR *P. aeruginosa*. The n-hexane extracts of green tea were more effective as compared to aqueous extract against MDR isolates. In future, the combined use of green tea extracts and antibiotics could also be useful in fighting emerging drug-resistant problems.

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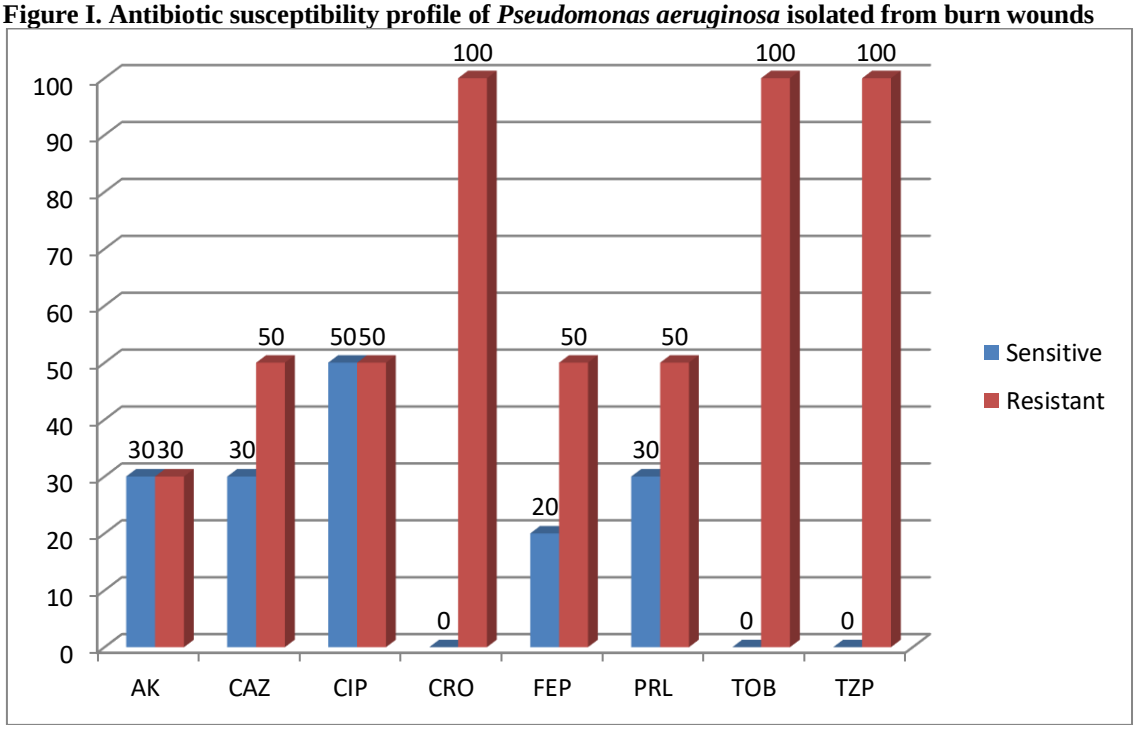
Figure I. Antibiotic susceptibility profile of *Pseudomonas aeruginosa* isolated from burn wounds



FEP, cefepime; CAZ, ceftazidime; CRO, ceftriazone; TOB, tobramycin; CIP, ciprofloxacin; TZP, piperacillin-tazobactam; AK, amikacin; PRL, piperacillin.

Table I. Resistance Pattern of *Pseudomonas aeruginosa* isolates against different antimicrobial drugs

Strain #	FEP (mm)	CAZ (mm)	CRO (mm)	TOB (mm)	CIP (mm)	TZP (mm)	AK (mm)	PRL (mm)
MDRPA1	R	R	R	R	26	R	R	22
MDRPA 2	IR	18	R	R	R	R	19	R
MDRPA 3	IR	IR	R	R	R	R	IR	R
MDRPA 4	19	R	R	R	24	R	18	R
MDRPA 5	R	R	R	R	26	R	IR	IR
MDRPA 6	R	IR	R	R	R	R	R	R
MDRPA 7	20	R	R	R	R	R	IR	IR
MDRPA 8	R	R	R	R	25	R	R	22
MDRPA 9	IR	19	R	R	25	R	IR	R
MDRPA 10	R	18	R	R	R	R	19	IR

FEP, cefepime; CAZ, ceftazidime; CRO, ceftriazone; TOB, tobramycin; CIP, ciprofloxacin; TZP, piperacillin-tazobactam; AK, amikacin; PRL, piperacilin.

Table II. Antibacterial activity of green tea extracts against MDR *Pseudomonas aeruginosa* isolates

Isolate No	Aqueous Extract (100mg/ml) ZOI (mm)	Aqueous Extract (200mg/ml) ZOI (mm)	n-hexane Extract (100mg/ml) ZOI (mm)	n-hexane Extract (200mg/ml) ZOI (mm)	n-hexane Control group
MDRPA1	22	23	23	23	00
MDRPA 2	21	21	21	22	00
MDRPA 3	16	19	20	21	00
MDRPA 4	19	20	21	22	00
MDRPA 5	21	22	22	22	00
MDRPA 6	20	20	21	21	00
MDRPA 7	16	20	21	22	00
MDRPA 8	20	21	22	22	00
MDRPA 9	21	20	22	22	00
MDRPA 10	22	20	22	21	00
Mean ± SD	19.8± 2.20	20.6± 1.15	21.5±0.84	21.8±0.63	0.00