Efficacy of methanolic extract of green and black teas against extended-spectrum β-Lactamase-producing *Pseudomonas aeruginosa*

Arezou Taherpour[1], Ali Hashemi*[2], Soroor Erfanimanesh[2] and Elahe Taki[2]
[1]Microbiology Department, Kurdistan University of Medical Sciences, Sanandaj, Iran
[2]Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Abstract: *Pseudomonas aeruginosa* is one of the major bacteria causing acute infections. β-Lactamase production is the principal defense mechanism in gram-negative bacteria. The aim of our study was to evaluate the antibacterial activity of Methanolic Extracts of Green and Black Teas on *P. aeruginosa* Extended Spectrum-β-Lactamases (ESBLs) production. This research was carried out on burn wounds of 245 hospitalized patients in Kerman, Iran. *P. aeruginosa* ESBLs and MBL producing strains were detected by Combination Disk Diffusion Test (CDDT) and Epsilometer test (E-test) strips, respectively. Minimum inhibitory concentration (MIC) was measured for Ceftazidime, Meropenem, Imipenem, Aztreonam, Cefotaxime and methanollic extracts of *Camellia Sinensis* (Green Tea). From 245 patients in the burn ward, 120 cases were infected with *P. aeruginosa*. 41 isolates contained ESBL while MBL was not detected. *P. aeruginosa* were resistant to Cefotaxime, Aztreonam, Ceftazidime, Meropenem and Imipenem, 72 (60%), 50 (41.66%), 79 (65.83%), 33 (27.5%) and 24 (20%), respectively. Green tea extract had the highest anti-bacterial effect on standard *and P. aeruginosa* strains in 1.25mg/ml concentration. This study determined that the methanolic extract of green tea has a higher effect against ESBL producing *P. aeruginosa* than Cefotaxime, Aztreonam and Ceftazidime.

Keywords: *Pseudomonas aeruginosa,* Beta-lactamases*,* Antibiotic Resistance, *Camellia Sinensis*

INTRODUCTION

Burn infections are a major problem of thermal injury in that, patients are at risk for acquiring infection with bacteria especially *P. aeruginosa*. Damaged body skin which is the primary defense barrier, prolonged hospitalization time, poor care condition and advised antibiotics are other contributing factors that help to establish an infection in a burn wound (Tawfik *et al*., 2012). *P. aeruginosa* as an opportunist agent cause infections such as septicemia, pneumonia, urinary tract infection, endocarditis, skin, ears and eye infections (Tredget *et al*., 2004). Also, it can cause nosocomial infection and it is a major agent of death among cystic fibrosis, neutropenic, burn and AIDS patients (Misaghi and Basti, 2007). *P. aeruginosa* is a serious threat for hospitalized patients throughout the world (Villegas *et al*., 2006).Class A and class B beta-lactamases are found in some *P. aeruginosa* strains that are known as Extended-Spectrum-beta-lactamase (ESBLs) and metallo-beta-lactamases (MBLs), respectively (Pasteran *et al*., 2005). ESBLs -frequently plasmid encoded- are enzymes that cause resistance to cephalosporins and is commonly found in *Klebsiella pneumoniae*, *Escherichia coli* and *P. aeruginosa* (Jiang *et al*., 2006; Dhillon and Clark, 2012; Villegas *et al*., 2004). ESBLs in *P. aeruginasa* is mostly associated with chromosomal Amp-C or mechanisms such as antibiotic efflux pumps or outer membrane impermeability (Poole *et al*., 2011) or plasmid acquired Ambler class A ESBLs such as Pseudomonas extended resistance (PER), Vietnam extended-spectrum (VEB), Guyana ESBLs (GES), Temoneira (TEM) and sulfhydryl variable (SHV) type (Drawz and Bonomo, 2010).Furthermore, MBLs can hydrolysis carbapenems and almost all beta-lactam antibiotics With a broad spectrum of activity (Altoparlak *et al*., 2005). Several of MBLs have been reported in *P. aeruginosa*, including Imipenemase (IMP), São Paolo metallo β-lactamase (SPM), Verona imipenemase (VIM), Seoul imipenemase (SIM), Japan, Kyorin University Hospital Imipenemase (KHM), German imipenemase (GIM), New-Delhi metallo-beta-lactamase-1 (NDM-1) and Australian Imipenemase (AIM). The genes of both IMP (Imipenemase) and VIM-type (Verona integron-encoded metallo-β-lactamases)in clinical strains of *P. aeruginosa* are often encoded on mobile element inserted into class1 integrons (Khosravi *et al*., 2011).The integrons are located on transposons or plasmids, the distribution of which contributes to the wide spread of this resistance mechanism (Viedma *et al*., 2012). The emergence of NDM-1 has been considered as a global threat because bacteria which possess this enzyme are resistant to almost all β-lactam antibiotics, aminoglycosides, fluoroquinolones and other classes of antimicrobial agents (Khosravi *et al*., 2011). Green tea (*Camellia Sinensis L.*) is prepared from *Camellia Sinensis* leaves and is rich in polyphenols, caffeine, antioxidant, anti-inflammatory and anti-cancer agents. In addition, this plant is beneficial to treat asthma, heart diseases, ulcers and various skin diseases (Kulandhaivel and Palaniswamy, 2012). There are many studies about the effects of herbal extracts on *P. aeruginosa* (Rao *et al*., 2010), but there are no reports based upon effect of herbal extracts on *P. aeruginosa* with

**Corresponding author:* e-mail: hashemi1388@yahoo.com

ESBL enzymes. Therefore, this study performed to diagnosis *P. aeruginosa* with ESBLs, determining the minimum inhibitory concentration (MIC) of methanolic extraction of green and black tea on clinical and standard *P. aeruginosa* strains.

MATERIALS AND METHOD

Bacterial identification

From January to September 2011, 120 strains of *P. aeruginosa* were isolated from 245 burn patients admitted to Shafa Hospital in Kerman after getting a clearance from the ethical committee, then Samples were obtained from pyogenic ulcers.

Isolation and clinical identification

Ulcers were washed by Physiological Serum and then specimens were collected with sterile swabs. Swab specimens were delivered to the laboratory in Stuart transport media and then they were cultured on Cetrimide and MacConkey medium. They were incubated at 37ºC overnight. After incubation period suspicious colonies were examined for pigment and smell production; smears were prepared too. Furthermore, biochemical tests such as oxidase test, sugar fermentation and growth in 42ºC were performed.

Minimum inhibitory concentration for antibiotics

MICs for 120 *P. aeruginosa* strains were determined by agar dilution method. The 0.5 MacFarland tube was prepared as a standard for preparing standard dilution of strains. Muller-Hinton agar media (Merck, Germany) with distinct dose of imipenem, meropenem, aztronam, cefotaxime and ceftazidime (GLAXO England Co.) were prepared. By Hand Inoculator Instrument (Mast Group, Merseyside, UK), 10µl from each bacterial suspension was inoculated on Muller-Hinton agar plus antibiotic; plates were incubated at 37ºC overnight (*P.aeruginosa ATCC27853* was used as the control strain).

Phenotypic detection of ESBL

ESBLs detection was done by Combination Disk Diffusion Test (CDDT) based on CLSI guideline. On Muller-Hinton agar the ceftazidime (30µg) and ceftazidime + clavulanic acid (30µg/10µg) disks, cefotaxime (30µg) and cefotaxime + clavulanic acid (30µg/ 10µg) disks were placed 30 mm apart; all disks were purchased from (Mast Group, Merseyside, UK). After overnight incubation at 37ºC, zone diameters were measured; if zone diameters were ≥5 mm for cephalosporin disks to cephalosporin-clavulanic acid disks, it would be ESBLs producers *P. aeruginosa*. *K. pneumoniae* ATCC700603 with ESBLs was used as a positive control.

Phenotypic detection of MBL

P. aeruginosa strains with MICs ≥8µg for imipenem and meropenem and ≥32 µg for ceftazidime were examined for MBL. E-test strips for MBL were used based on AB Biodisk (Swedish Co.) protocol. If IP: IPI was ≥8, *P. aeruginosa* produced MBL.

Preparation of plant extracts

The *Camellia Sinensis* leaves (L48I-8732 Number) were collected from Lahejan, Iran, during 2009. Leaves of the plant (500gr) were dried at 25^{o}C and then powdered using a mechanical grinder. One gram of dried plant was dissolved in 10ml Methanol (96%, v/v) for a period of 48 hours without any heating procedure. Extracts were filtered with Whatman No. 1 filter paper and then with a 0.45 µm membrane filter and evaporated under pressure in vacuum evaporator and then were preserved at -20ºC.

Standard strains

The standard strains *K. pneumoniae* ATCC700603 and *P. aeruginosa* 8821M, ATCC27853 and *PAO1* were received from Islamic Azad University of Tabriz and Microbiology Department of Kerman University of Medical Sciences.

Minimum inhibitory concentration for teas

Extracts of green and black teas were dissolved in DMSO and were prepared in several concentrations (0.078, 0.156, 0.312, 0.625, 1.25 and 2.5mg/ml) from them; then, each of them was poured in plates. The standard bacterial suspension (10µl) was inoculated to the plates with extracts and then incubated overnight at 37ºC; then results were recorded. Also, results of effect of extracts on isolated bacteria from patients were recorded.

STATISTICAL ANALYSIS

This study was descriptive-experimental-applicational. For analysis of results, MINITAB16 software was used. *P. value* and confidence intervals were <0.05 and 95%, respectively.

RESULTS

P. aeruginosa isolated from 120 pyo-ulcers of 245 patients were cultured; it means 48.97% of wounds were infected by *P. aeruginosa*. 77 (64%) of the isolates belonged to males and 23 (36%) to females. Most patients were from 11 to 20 years old. The resistant isolates were: Imipenem and Meropenem with MIC≥8µg/ml; Ceftazidime and Aztreonam with MIC≥32µg/ml and Cefotaxime MIC≥64µg/ml (based on CLSI guideline). Resistance to Cefotaxime, Ceftazidime, Aztreonam, Imipenem and Meropenem were 72(60%), 79(65.83%), 50 (41.66%), 24(20%) and 33(27.5%), respectively (table 1). ESBLs were found among 41 (34%) of the isolates. The isolates with ≥5 mm zone diameter for Cephalosporin+ Clavulanic acid than Cephalosporin without Clavulanic acid were considered as ESBL producers. There were no MBL among samples; E-test MBL strips were used for detection. 1.25mg/ml of the green tea extract was the highest concentration that inhibited the growth of all standard and clinical strains (table 2).

Table 1: Minimum inhibitory concentrations of different antibiotics against *P. aeruginosa* clinical isolates

Antibiotics	Minimum Inhibitory Concentration (µg/ml)								
	1	2	4	8	16	32	64	128	256
Imipenem	73 (60.83%)	8 (6.6%)	15(12.5%)	21 (17.5%)	1 (0.83%)	1 (0.83%)	----	----	1 (0.83%)
Meropenem	69 (57.5%)	4 (3.3%)	14 (1.6%)	27 (22.5%)	2 (1.6%)	2 (1.6%)	1 (0.83)	----	1 (0.83%)
Aztreonam	38 (31.6%)	1 (1.6%)	----	15 (12.5%)	16 (13.3%)	23 (19%)	22 (18.3%)	3 (2.5%)	2 (1.6%)
Ceftazidime	5 (4.1%)	18 (15%)	4 (3.3%)	7 (5.8%)	7 (5.8%)	7 (5.8%)	13 (10.8%)	41 (34.1%)	18 (15%)
Cefotaxime	3 (2.5%)	11 (9.1%)	2 (1.6%)	4 (3.3%)	10 (8.3%)	18 (15%)	19 (15.8%)	46 (38.3%)	7 (5.8%)

Table 2: Minimum inhibitory concentrations of black and green teas extraction for ESBL- producing *P.aeruginosa* clinical isolates

Methanolic Extract	Minimum Inhibitory Concentration (mg/ml) (no%)					
	0.078	0.156	0.312	0.625	1.25	2.5
Black Tea	4(9.7%)	5(12.19%)	3(7.31%)	5(12.19%)	17(41.46%)	7(17.08%)
Green Tea	3(7.31%)	5(12.19%)	3(7.31%)	4(9.7%)	26(63.41%)	----

DISCUSSION

ESBL and MBL-producing bacteria are major causes of mortality and morbidity. Therefore, detection of ESBL and MBL-producing *P. aeruginosa* would be critical to control the spreading infectious diseases with antibiotic resistance bacteria. Over the past 20 years, *P. aeruginosa* has been found to be the most prevalent bacteria in burn wards in Iran (Estahbanati *et al*., 2002). In the present study clinical *P. aeruginosa* isolates were resistance to Cefotaxime, Ceftazidime, Aztreonam. The present study showed that 72 (60%), 79 (65.83%), 50 (41.66%), 24 (20%) and 33 (27.5%) of clinical isolates of *P. aeruginosa* were resistance to Cefotaxime, Ceftazidime, Aztreonam, Imipenem and Meropenem, respectively. Different studies in Iran and other countries had shown that prevalence of antibiotic resistance is high (Jean and Hsueh, 2011). In a study in Tehran (IRAN) hospitals could isolate *P.aeruginosa* strains from burn wounds with resistance to: Gentamicin (93.7%), Ceftazidime (96%), Amikacin (93.4%), Kanamycin (96%), Tetracyclin (91%) and Ciprofloxacin (86.7%) (Shahcheraghi *et al*., 2003). Prevalence of drug resistance in burn ward has been reported from other parts of the world (Yousefi *et al*., 2010; Kumar *et al*., 2012). Also, a research in Tehran showed that 3 strains from 70 isolates of *P.aeruginosa* isolated from burn ward had ESBLs whereas there were no MBL (Japoni *et al*., 2006).In a study in Saudi Arabia, 39 (19.5%) *P. aeruginosa* strains were ceftazidime resistant and 23 (59%) and 16 (41%) of these 39 appeared to be ESBL and MBL positive respectively (Al-Agamy *et al*., 2012). A research in Iran, revealed that 148 (87.05%) of *P. aeroginosa* isolates were MDR and 67 (39.41%) of the strains were ESBL positive (Mirsalehian *et al*., 2010). The prevalence of MBL 0 (0.0%) in the present study was lower than the prevalence of MBL in the USA (Aboufaycal *et al*., 2007), Korea (Chin *et al*., 2011), India (De *et al*., 2010), and Saudi Arabia. The prevalence of ESBLs in Kerman was higher than Shiraz but lower than Tehran; it could be due to hospitalization time, care conditions and advised antibiotics. Antibiotic resistance among burn patients in burn ward is mostly due to ESBL production. Resistance to many antibiotics, especially third generation cephalosporins is due to these enzymes. Polyphenolic components of green tea are the main active compounds, which act against bacteria. In this study we found that the MIC of green tea extract against *P. aeroginosa* was 1.25mg/ml. A study in America showed green tea extract had inhibitory effect on quorum sensing in *P. aeroginosa* (Mihalik *et al*., 2008). The results of our study is consistent with other studies that have previously been reported that green tea has antibacterial activity against resistant bacteria strains such as MRSA, *P. aeruginosa* and vancomycin-resistant enterococci (Radji *et al*., 2013).

CONCLUSION

The prevalence of ß-lactamase-producing isolates, and their isolation from life-threatening infections, is dramatically increasing worldwide. Intensity pressure for usage of antimicrobial drugs by patients resulted in eradication of normal flora and situation of MDR isolates substitution. The results of this study indicate that *Camellia Sinensis leaves* Methanolic extract can be used in treating diseases caused by ESBL-producing *P. aeruginosa*. As a suggestion, further research should be done to investigate the antimicrobial effects of other herbal essences and extracts. In addition, *in vivo* examine of this extract shows its effects on infections associated resistant bacteria.

REFERENCES

Aboufaycal H, Sader HS, Rolston K, Deshpande LM, Toleman M, Bodey G, Raad I and Jones RN (2007). blaVIM-2 and blaVIM-7 carbapenemase-producing Pseudomonas aeruginosa isolates detected in a tertiary care medical center in the United States: Report from the MYSTIC program. *J. Clin. Microbiol.,* **45**(2): 614-615.

Al-Agamy MH, Shibl AM, Tawfik AF, Elkhizzi NA and Livermore DM (2012). Extended-spectrum and metallo-beta-lactamases among ceftazidime-resistant Pseudomonas aeruginosa in Riyadh, Saudi Arabia. *J. Chemother.,* **24**(2): 97-100.

Altoparlak U, Aktas F, Celebi D, Ozkurt Z and Akcay MN (2005). Prevalence of metallo-beta-lactamase among Pseudomonas aeruginosa and Acinetobacter baumannii isolated from burn wounds and *in vitro* activities of antibiotic combinations against these isolates. *Burns.,* **31**(6): 707-710.

Azizkhani M, Misaghi A, Basti AA, Gandomi H and Hosseini H (2013). Effects of Zataria multiflora Boiss. essential oil on growth and gene expression of enterotoxins A, C and E in Staphylococcus aureus ATCC 29213. *Int. J. Food Microbiol.,* **163**(2-3): 159-165.

Bag A, Bhattacharyya SK, Pal NK and Chattopadhyay RR (2012). *In vitro* antibacterial potential of Eugenia jambolana seed extracts against multidrug-resistant human bacterial pathogens. *Microbiol. Res.,* **167**(6): 352-357.

Chin BS, Han SH, Choi SH, Lee HS, Jeong SJ, Choi HK, Choi JY, Song YG, Kim CK, Yong D, Lee K and Kim JM (2011). The characteristics of metallo-beta-lactamase-producing gram-negative bacilli isolated from sputum and urine: A single center experience in Korea. *Yonsei. Med. J.,* **52**(2): 351-357.

De AS, Kumar SH and Baveja SM (2010). Prevalence of metallo-beta-lactamase producing pseudomonas aeruginosa and acinetobacter species in intensive care areas in a tertiary care hospital. *Indian J. Crit. Care Med.,* **14**(4): 217-219.

Dhillon RH and Clark J (2012). ESBLs: A Clear and Present Danger? *Crit. Care Res. Pract.,* 625170.

Drawz SM and Bonomo RA (2010). Three decades of beta-lactamase inhibitors. *Clin. Microbiol. Rev.,* **23**(1): 160-201.

Estahbanati HK, Kashani PP and Ghanaatpisheh F (2002). Frequency of pseudomonas aeruginosa serotypes in burn wound infections and their resistance to antibiotics. *Burns,* **28**(4): 340-348.

Japoni A, Alborzi A, Kalani M, Nasiri J, Hayati M and Farshad S (2006). Susceptibility patterns and cross-resistance of antibiotics against pseudomonas aeruginosa isolated from burn patients in the South of Iran. *Burns,* **32**(3): 343-347.

Jean SS and Hsueh PR (2011). High burden of antimicrobial resistance in Asia. *Int. J. Antimicrob. Agents,* **37**(4): 291-295.

Jiang X, Zhang Z, Li M, Zhou D, Ruan F and Lu Y (2006). Detection of extended-spectrum beta-lactamases in clinical isolates of pseudomonas aeruginosa. *Antimicrob. Agents Chemother.,* **50**(9): 2990-2995.

Khosravi Y, Tay ST and Vadivelu J (2011). Analysis of integrons and associated gene cassettes of metallo-beta-lactamase-positive pseudomonas aeruginosa in Malaysia. *J. Med. Microbiol.,* **60**(Pt 7): 988-994.

Kulandhaivel M (2012). *In vitro* antimicrobial activity of camellia sinensis and myristica fragrans against staphylococcus aureus pseudomonas aeruginosa Candida albicans. *Int. J. Pharm. Bio. Arch.,* **3**(3): 604-609

Kumar SH, De AS, Baveja SM and Gore MA (2012). Prevalence and risk factors of metallo beta-lactamase producing pseudomonas aeruginosa and acinetobacter species in burns and surgical wards in a tertiary care hospital. *J. Lab Physicians,* **4**(1): 39-42.

Mihalik K, Chung D, Crixell S, McLean R and Vattem D (2008). Quorum sensing modulators of pseudomonas aeruginosa characterized in *Camellia sinensis. Asian J. Trad. Med.,* **3**: 12-23.

Mirsalehian A, Feizabadi M, Nakhjavani FA, Jabalameli F, Goli H and Kalantari N (2010). Detection of VEB-1, OXA-10 and PER-1 genotypes in extended-spectrum beta-lactamase-producing pseudomonas aeruginosa strains isolated from burn patients. *Burns,* **36**(1): 70-74.

Pasteran F, Faccone D, Petroni A, Rapoport M, Galas M, Vazquez M and Procopio A (2005). Novel variant (bla(VIM-11)) of the metallo-{beta}-lactamase bla (VIM) family in a GES-1 extended-spectrum-{beta}-lactamase-producing pseudomonas aeruginosa clinical isolate in Argentina. *Antimicrob. Agents Chemother.,* **49**(1): 474-475.

Poole K (2011). Pseudomonas aeruginosa: Resistance to the max. *Frontiers in microbiology,* **2**: 1-11.

Radji M, Agustama RA, Elya B and Tjampakasari CR (2013). Antimicrobial activity of green tea extract against isolates of methicillin-resistant Staphylococcus aureus and multi-drug resistant pseudomonas aeruginosa. *Asian Pac. J. Trop. Biomed.,* **3**(8): 663-667.

Rao PV, Goudu AS, Sasikala S and Naidu MD (2010). Efficacy of antimicrobial activity of rhinacanthus nasutus (linn) leaves in different extractions. *Int J Pharm. Bio. Sci.,* **1(2):** 1-4

Shahcheraghi F, Feizabadi MM, Yamin V, Abiri R and Abedian Z (2003). Serovar determination, drug resistance patterns and plasmid profiles of Pseudomonas aeruginosa isolated from burn patients at two hospitals of Tehran (IRAN). *Burns,* **29**(6): 547-551.

Tawfik AF, Shibl AM, Aljohi MA, Altammami MA and Al-Agamy MH (2012). Distribution of ambler class A, B and D beta-lactamases among Pseudomonas aeruginosa isolates. *Burns,* **38**(6): 855-860.

Tredget EE, Shankowsky HA, Rennie R, Burrell RE and Logsetty S (2004). Pseudomonas infections in the thermally injured patient. *Burns,* **30**(1): 3-26.

Viedma E, Juan C, Villa J, Barrado L, Orellana MA, Sanz F, Otero JR, Oliver A and Chaves F (2012). VIM-2-producing multidrug-resistant Pseudomonas aeruginosa

ST175 clone, Spain. *Emerg. Infect Dis.,* **18**(8): 1235-1241.

Villegas MV, Correa A, Perez F, Miranda MC, Zuluaga T and Quinn JP. Colombian Nosocomial Resistance Study G (2004). Prevalence and characterization of extended-spectrum beta-lactamases in Klebsiella pneumoniae and Escherichia coli isolates from Colombian hospitals. *Diagn. Microbiol. Infect Dis.,* **49**(3): 217-222.

Villegas MV, Lolans K, del Rosario Olivera M, Suarez CJ, Correa A, Queenan AM and Quinn JP (2006). Colombian Nosocomial Resistance Study G. First detection of metallo-beta-lactamase VIM-2 in Pseudomonas aeruginosa isolates from Colombia. *Antimicrob Agents Chemother.,* **50**(1): 226-229.

Yousefi S, Farajnia S, Nahaei MR, Akhi MT, Ghotaslou R, Soroush MH, Naghili B and Jazani NH (2010). Detection of metallo-beta-lactamase-encoding genes among clinical isolates of pseudomonas aeruginosa in northwest of Iran. *Diagn. Microbiol. Infect Dis.,* **68**(3): 322-325.