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ORIGINAL ARTICLE

ANTIBACTERIAL ACTIVITY OF THUJA ORIENTALIS AND GREEN TEA IN *PSEUDOMONAS AREOGENOSA* INFECTION

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Abstract: Seven isolates were identified as *Pseduomonas aeruginosa* from clinical samples. Antibiotic sensitivity test were done to determine their sensitivity to number of antibiotics, the results illustrated all that isolates were resistant to most used antibiotics. The ability of *Pseduomonas* isolates to produce haemolysin, protease and pyocyanin were detected in this study, all isolates had the ability to produce pyocyanin pigment, hemolysis and protease. The antimicrobial activity of the ethanolic extracts of Thuja orientalis and green tea against *P.aeruginosa* were investigated. The results showed that both these plant extracts have inhibitory effect against *Pseduomonas* isolates and it was shown that ethanolic extract of green tea was more efficient against *P. aeruginosa* isolated from eye infection while ethanolic extracts of Thuja orientalis was more effective against *P. aeruginosa* isolated from wound infection. Minimum inhibitory concentration (MIC) of ethanolic extract of Thuja orientalis be resoluted, it was 10 mg/ml. The effect of ethanolic extract of Thuja orientalis on the production of haemolysin, protease and pyocyanin was detected, the ethanolic extract of Thuja orientalis at MIC (10 mg/ml) completely inhibited *Pseudomonas* growth and haemolysis on blood agar, also inhibited protease and pyocyanin production. The ethanolic extracts of Thuja orientalis at sub MIC (5 mg/ml) had the ability to inhibit haemolysin production on blood agar. It also inhibited production of pyocyanin on nutrients agar and had effect on protease production. The minimum inhibitory concentration (MIC) of ethanolic extracts of the green tea was 100mg/ml and there was a significant induction of Protease IV expression in the groups treated with ethanolic extracts of the green tea in comparison with gentamicin and the highest induction in expression of Protease IV gene was at Sub MIC of gentamicin.

***Key words*:** Thuja orientalis, Green Tea, Antibacterial, *P.areogenosa*, Protease IV

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1. Introduction

Pseudomonas aeruginosa, Gram-negative bacterium is source of stern sickness in immuncomprmised individual and is a ordinary basis of nosocomial disease as well as infection of burn wounds, infections of urinary tract, bacteremia, in addition to pneumonia [Pier and Ramphal (2005)]. The ability of *P. aeruginosa* to resistance to antibiotics which acquired (plasmids, transposons) or natural might be due to the formation of biofilms that lead to increased colonization, alteration and resistance to antibiotics. Livermore (2002) and Ravichandran & Kareemulla (2018)]. The virulence factors of *p. aeruginosa* have extracellular enzymes, counting elastases, proteases and two hemolysins, pyocyanin and two siderophores (pyoverdine and pyochelin). In addition to exotoxin A. Protease IV is important virulence factor of *P. aeruginosa*. Protease IV plays important role in optical infections which credited to the devastation of proteins in host, counting fibrinogen and mechanism of the resistant organization [Engel *et al*. (1998)]. Thuja has a lot of names: Biota orientalis, Thuja orientalis, Platycladus orientalis, Oriental arborvita, all these are Thuja's scientific names [Jain and Garg (1997)]. Thuja

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is a useful herb remedy with a lots of health benefits, it can be used as diuretic, antibacterial, antifungal, sedative, antiasthmatic, antipyretic and parasiticide [Guang *et al.* (2009), Ridha (2019)]. Green tea acts as antibacterial activity against different types of pathogens [Reygaert (2014)]. Green tea contains a diversity of active compounds as polyphenols flavanoids, anthraquinone glycosides might assist to decrease the danger of cardiovascular diseases and some forms of cancers [Afzal *et al*. (2015)]. The present research was carried out to evaluate the antimicrobial activity of the alcoholic extracts of the dried fruit of Thuja orientalis and ethanolic extracts of the green tea and investigate their effect on several virulence determinants that are pyocyanin, protease production and hemolytic activity in *Pseudomonas* isolates.

2. Materials and Methods

2.1 Isolation of bacteria

Pseudomonas spp. were isolated from 7 clinical samples (wound, burn, eye swab) from hospitals in Baghdad. *Pseudomonas* isolates were cultured on Mac Conkey agar and recognized through microbial and biochemical test with the use of API profile index [Harley & Prescott (2002)].

The modified Kirby Bauer method [Vandepitte *et al.* (2003)] was used to conclude the vulnerability of bacterial isolates to antibiotics. Antibiotics are Imipenem, Levofloxacin, azithromycin, ciprofloxacin, cephalexin, piperacillin and gentamicin.

2.2 Detection of some virulence factors

Haemolysin production by *P. aeruginosa* isolates were detected. Human blood agar dishes were immunized through the examination organism and then keep warm at 37°C for 24 hours. The dishes were then looked at for the presence of haemolysis around the colony. For Protease production skimmed milk agar plates were inoculated with the test organism by streaking in straight lines and then plates were keep warm at 37°C for 24 hours. The plates were then observed for clear areas near the streak that was an indication of casein digestion and production of protease. The *P. aeruginosa* isolates is aptitude to produce pyocyanin pigment and been detected by using Nutrient agar. The plates were then observed for the produce of blue- green pigment pyocyanin. Congo-red agar medium was used to detect the capability of *P. aeruginosa* isolates to manufacture slime layer, according to Freeman *et al*. (1989). The black colonies with a dry crystalline consistency were considered as positive result.

2.3 Biofilm Formation

The bioflim formation by Tissue Culture Plate (TCP) technique of. *P. aeruginosa* isolates was demonstrated by [Stepanoviæ *et al.* (2007)].

2.4 Preparation of Plant Extracts

2.4.1 Alcohol extraction of the Thuja orientalis

The dried fruit of the Thuja orientalis was collected from the local markets and ground into fine powder and extracted with hot ethanol by adding 4 gm of thuja powder into 100ml of ethanol (95%). Then it was left in water bath for 18hour at 40°. The flask was then allowed to cool and filtered. Later it allows drying in the incubator at 37° for 72 hours and then four concentrations 40, 20, 10, 5, 2.5 mg/ml were set to use in this experiment [Sharad *et al.* (2008)]. The antibacterial activity of alcoholic extract of thuja was determined by using well diffusion assay. The antimicrobial inhibition activity was measured by seeding 0.1ml from bacterial suspensions at OD of 0.5nm and spread with sterile spreader. Later, wells of 0.5mm were made in the culture medium (nutrient agar) and to which the plant extracts with the all concentrations were added. Finally, the zone of inhibition was determined in plates were kept warm on 37°C for 18 hours.

2.4.2 Alcohol extraction of the green tea

Ahmad brand green tea leaves were obtained from a local retail market and then grinded to powder using a coffee blender. Three serial dilutions 100, 150, 200 mg/ml of green tea leaves were geared up by suspending 1, 1.5 and 2 gm respectively in 10 ml of 95% ethanol. Each concentration was mixed and preserved at 40°C until used [Kumar *et al.* (2012)].

2.4.3 Minimum Inhibitory Concentration (MIC) Determination

The MICs for alcoholic extract of thuja, alcoholic extract of green tea and Gentamicin against *P. aeruginosa* were determined. The lowest concentration that reduced the visible bacterial growth act as MIC [Morello *et al*. (2006)].

2.4.4 The effect of Thuja orientalis extract on some virulence factors

P. aeruginosa isolates were diluted in 1:100 in LB medium and cultured with alcoholic extract of Thuja at

sub MIC concentration for 16 hours. Bacterial cultures were taken and spread on blood agar, Skimmed milk agar (SMA), Nutrient agar (NA) and Congo-red agar medium. After incubation for 24 hours at 37ºC the plates were examined for the production of haemolysin, protease, pyocyanin and slime layer respectively.

2.5 Effects of Green Tea on Protease IV gene expression

This experiment was conducted to detect the effect of green tea in virulence factor of *P. aeruginosa* (Table 1).

3. Results and Discussion

Pseudomonas spp. were isolated from 7 clinical samples {wound, burn, eye swab} from hospitals in Baghdad. The isolates were recognized as *P. aeruginosa* dependent to the morphological and biochemical examination with the using of API profile index [Harley and Prescott (2002)]. Antibiotic sensitivity test were done to determine the sensitivity of 7 isolates to number of antibiotics The results indicated that isolates were resistant to the most used antibiotics, *P. aeruginosa* isolate no. 20, 5 was senstitive to Aztreonam. In the present *P. aeruginosa* were resistant to a wide variety of antibiotics might be to bacterial external membranes which limited diffusion of antibiotics and the competent taking away of antibiotics by efflux pumps that gaide *P. aeruginosa* has the hereditary capability to resistant and alter of antibiotics [Lambert (2002)]. The ability of the isolates (7) to produce hemolysin, protease and pyocyanin were detected in this study. All isolates produced zone of beta-haemolysis around the colonies on blood agar plate, The hydrolysed obvious zones around the colonies on SMA considered positive result. This is evidence of casein hydrolysis (casein is the most predominant protein in milk) and it indicated the production of protease enzyme. All *P. aeruginosa* isolate had the ability to produce protease, as shown in Fig. 1a and Pyocyanin on NA, the presence of blue-green pigment were considered positive result, as shown in Fig. 1b. The results by Congo-red agar method showed that all *P. aeruginosa* isolates were slime (Fig. 1c).

Table 1: The primer of housekeeping gene and its sequence used in q-PCR amplification table.

Target gene	Primer name	Primer sequence 5 3
Housekeeping gene	actin gene primer	F GGGTGGAGCCAAACGGGTC 530bp R GGAGTTGCTGTTGAAGTCGCA
target genes	Proteas IV	F TCCGCAGG TAG CACTCAGTTC 353bp R AAGCCGGATTCATAGGTGGTG

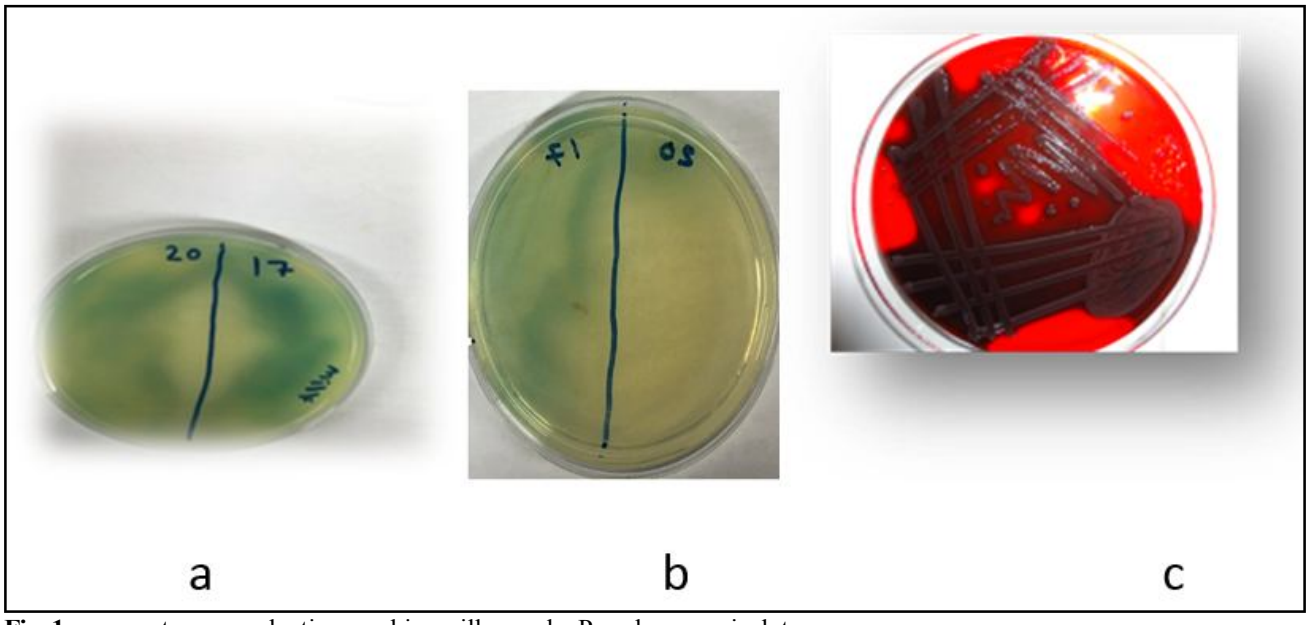

Fig. 1: a: protease production on skim milk agar by Pseudomonas isolate
b: pyocyanin production on nutrient agar by Pseudomonas isolate
c: Strong Slime Layer Production by Pseudomonas on Congo- red Agar medium

3.1 Biofilm assay

The TCP method was used to detect biofilm production, the results showed that 4 isolates (57.14%) were biofilm producer, from that 2 isolates from wound and 1 isolate from eye infection were moderate biofilm production and only 3 isolates (42.85%) were weak or non-biofilm producers from the total isolates (Table 2). Pournajaf *et al.* (2018) indicated that 83.75% of clinical *Pseudomonas aeruginosa* isolates formed biofilm. Biofilm production is thought to be a major factor which protect the bacteria and associated with their resistant to antimicrobial agents [Baker *et al*. (2016)].

3.2 Minimum inhibitory concentration of alcoholic plant extracts

The effect of alcoholic extracts of Thuja and green tea on two Pseudomonas aeruginosa isolates, one from a wound and the other from an eye infection, was examined. Both isolates produced significant levels of pyocyanin and were highly resistant to antibiotics when compared to other isolates. The inhibition zones of alcoholic extract of thuja against *P.aeruginosa* isolated from wound were (19, 16, 14, 11, 0, 0) mm, for the alcoholic extract at 40, 20, 10, 5 and 2.5 mg/ml respectively. The minimum inhibitory concentration (MIC) was determined [Morello *et al*. (2006)], the first plate showing no visible growth considered as MIC, the result showed that the lowest concentration (MIC) of the alcoholic extract of thuja that inhibits the growth of *P.aeruginosa* was 10 mg/ml. Similar results were also indicated by Oh (2000) and Al-Mariri & Safi (2014) in which they reported the bacteriacidal property of Thuja orientalis aqueous extract against some bacterial and fungal infections. It was shown that the higher antimicrobial activity was seen against *Bacillus cereus* which is reached to 35 mm. However, the antimicrobial activities of alcohol extract of Thuja orientalis have also been demonstrated by additional authors including [Sharad *et al.* (2008) and Akhtar *et al*. (2014)]. The results demonstrated that the inhibition zones of green tea extract against *P. aeruginosa* isolated from eye were (18, 20, 23) mm, due to the three concentrations of alcoholic extract 100, 150, 200 mg/ml respectively. The MIC of green tea against *P. aeruginosa* was 100mg/ml.

The study demonstrated resulting that the green tea extract show the attendance of effective antibacterial activity. These comments may be accredited to active complex of green tea as catechin and polyphenols. which have antibacterial property [Saikia *et al.* (2006)]. From the results of the antimicrobial screening of the two extracts of Thuja and green tea used in this study, it was found that the two plants haves promising antimicrobial activity and this is due to the chemical composition of these plants which is found to be rich in glycosides, flavonoids and triterpenoids.

3.3 The effect of Thuja extracts on some virulence factors

The effect of Thuja extract on the production of haemolysin, protease and pyocyanin was detected phenotypically using blood agar plate, SMA plate and NA plates respectively, the results showed that alcoholic extract of Thuja inhibit *P.aeruginosa* growth completely on blood agar (no haemolysis) and slime layer formation in the corresponding MIC. It also inhibited skimmed milk agar digestion and production of protease enzyme and inhibited pyocyanin production on nutrient agar as shown in Figs. 2a, 2b, 2 c. Alcoholic extract of Thuja at sub MIC (5 mg/ml) inhibit heamolysin and slim layer production as well as affect the protease and pyocyanin production as exposed in Figs. 3a, 3b, 3c.

The results showed that alcoholic extract of Thuja reduced virulence factor production of *P.aeruginosa* isolates.

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

The effect of green tea in comparison with gentamicin on Protease IV gene expression by *P. aeruginosa* isolated from eye infection was determined using RT- qPCR, because there was no phenotypic test

Table 2: Biofilm Production by Pseudomonas isolates.

Amount of Adherent Layer	Number and Percentage % of Isolates	Percentage ofTotal Isolates That Produce Biofilm %
Strong	1 isolate (14.28%)	(57.14%)
Moderate	3 isolate (42.86%)	
Weak	3 isolate (42.86%)	-

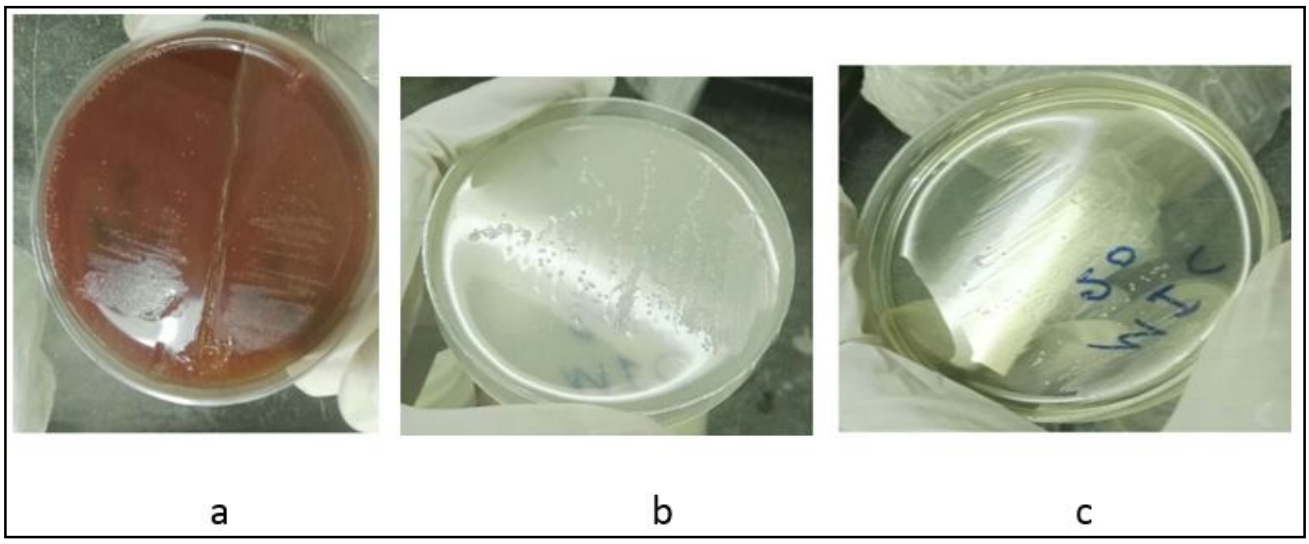

Fig. 2: a: Thuja alcoholic extract effect (10 mg/ml) on the manufacture of heamolysin on blood agar
b:the Thuja alcoholic extract effect (10 mg/ml)on the manufacture of protease on skim milk agar
c: the Thuja alcoholic extract effect (10 mg/ml) on the manufacture of pyocyanin on nutrient agar by *P.aeruginosa* isolate

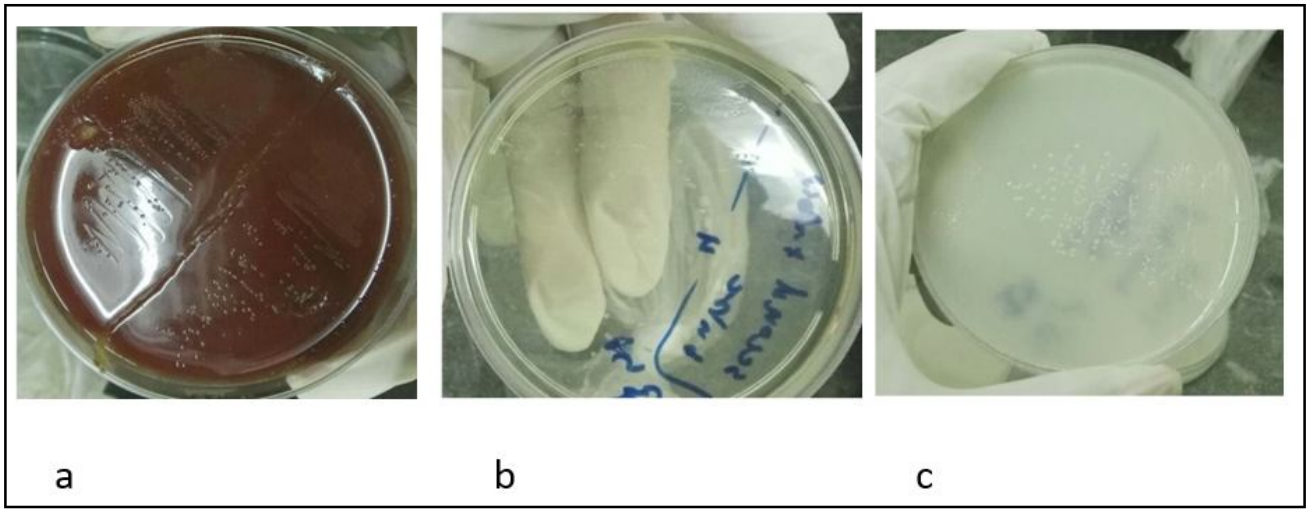

Fig. 3: a: the Thuja alcoholic extract effect (5 mg/ml) on the manufacture of haemolysin on blood
b: the Thuja extract effect (5 mg /ml)on the manufacture of pyocyanin by *P.aeruginosa* isolate
c: the Thuja alcoholic extract effect (5 mg /ml) had effect on protease production on skim milk agar by *P.aeruginosa* isolate

Table 3: *In vitro* inhibitory and minimum inhibitory effects of Thuja orientalis and green on *P. aeruginosa* isolation.

Plant extract	Concentrations mg/ml extract	inhibition zone-mm	MIC mg/ml
Thuja orientalis	2.5 gm/ml	0.0 mm	10mm
	5 gm/ml	11mm	
	10 gm/ml	14mm	
	20 gm/ml	16mm	
green tea	100 gm/ml	18mm	100mm
	150 gm/ml	20mm	
	200 gm/ml	23 mm	

Table 4: Gene expression levels of on Protease IV gene of *P. aeruginosa*.

*Sampling	H.K.	Protease IV.	Δct	ΔΔdct	Folding
1	17.2	24.1	6.9	6.2	1.00
2	25.9	31.4	5.5	5.9	1.22
3	22.8	27.0	4.4	4.9	2.55
4	21.4	22.3	4.0	4.2	3.89
5	20.8	25.4	3.5	3.6	6.27

* Sampling (1-control negative, 2-bacterial suspension with green tea 100 mg/ml, 3-bacterial suspension with green tea 50 mg/ml, 4-bacterial suspension with Gentamicin 10 mg/ml, 5-bacterial suspension with Gentamicin 5 mg/ml).

available to detect the green tea extract effect on protease IV production. The results in Table 3 showed a significant induction ($P < 0.05$) of the expression in the groups treated with gentamicin as compared 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) (Table 4).

A study results of the expression of Protease IV, the induction of Protease IV synthesis and down regulation of degradation in response to the ecological feature in the bacterial QS systems modification [Lee *et al.* (2013)]. The gene expression results of green tea showed that there were lower fold protease IV than Gentamicin, these indicate the superiority effect of green tea than gentamicine, these results are due to that green tea act as antimicrobial by interaction with the microbial cell surface, eventually resulting in impairment of bacterial activities by decreasing virulence production of the protease TV. The results were in conformity with Subhashini *et al.* (2010) who confirmed that antimicrobial effect of green tea is due to active compound as polyphenol, flavonoids, tannins, alkaloids, saponins. Tabas and Glass ((2013) showed 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 guide to the activation of NF-κB and inspiration of immune response and inhibit NF-κB activation through the modulation of inflammatory pathways. The results of the green tea-treated group showed that green tea is a more effective antibacterial against P. aeruginosa than gentamicine. Many studies have shown that the active element in tea is disclosed by passing through the bacterial cell wall and cellular membrane, causing phospholipid bilayer agreement to be disrupted, as well as polysaccharides molecules. [Longbottom *et al.* (2004) and Raut and S.M. Karuppayil (2014)].

4. Conclusion

The study demonstrated that the two extracts of Thuja and green tea have promising antimicrobial activity inhibit several virulence determinants in *Pseudomonas* isolate, the results support that two extracts of Thuja and green tea should be considered potential antivirulence agents and treatments for *Pseudomonas* infections.

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