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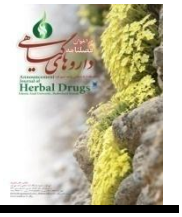

Investigation of antimicrobial effect of crude extract and three sub-fractions of *Platychaete aucheri (Boiss.) Boiss* against five standard microbial strains and clinical *Escherichia coli* isolates

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ABSTRACT

Background & Aim: *Platychaete aucheri (Boiss.) Boiss*is one of the Persian endemic plants and it belongs to *Asteraceae* family. The present study was aimed to evaluate the antimicrobial activity of crude methanolic extract and three sub-fractions of the flowering aerial parts of *Platychaete aucheri* against some gram-positive and gram-negative standard bacteria, *Candida albicans* and clinical *Escherichia coli* isolated from urinary tract infections (UTIs) and burn wounds.

Experimental: Total methanolic extract of *P.aucheri* was prepared by maceration method.Further chloroform, petroleum ether and aqueous fractions were obtained by using liquid-liquid extraction method. Antimicrobial effect examined by well diffusion and broth microdilution method based on the CLSI protocol. The standard tested microbial strains included *Escherichia coli* PTCC 1399, *Pseudomonas aeruginosa* PTCC 1430, *Staphylococcus aureus* PTCC1431, *Bacillus cereus* PTCC 1247 and *Candida albicans* PTCC 5027. Also, extract and fractions were tested against *Escherichia coli* isolates from urine samples and burn wounds of patients from Imam Khomeini and Shahid Motahari Hospitals of Tehran (Iran) during 2013 and 2014 respectively.

Results: Total extract and fractions had ability to prevent microbial growth. Total methanolic extract, chloroform and petroleum ether fractions demonstrated moderate antimicrobial activity against standard *P.aeruginosa* and *E.coli* with MIC values in the range of 35-42 mg/ml. MIC values against clinical isolates of *E.coli* were in the range of 60-72 mg/ml. The aqueous fraction showed lower antimicrobial activity in comparison to total extract and other fractions against standard and clinical isolates. Results confirmed that petroleum ether and chloroform fractions had relatively more anti bacterial activity than total methanolic extract and aqueous fraction.

Recommended applications/industries: Based on the non toxicity results in future studies, this plant can be used as a natural antibacterial source for therapeutic products to help UTIs and wounds treatment.

1. Introduction

Today due to increase of bacterial resistance to chemical drugs and also their potential adverse effects, searching on new medicinal plants is rising (Hakemi-vala *et al.*, 2014). Increasing in amounts of antibiotic usage during the past 60 years, caused emergence of new generations of bacteria that no longer response to them (Levy, 2002).

Platychaete aucher (Boiss.) Boiss,is one of the *Asteraceae* family and has limited dispersion in south of Iran (Mozaffarian, 2006). This family has more than 22750 species and 1620 genera (Zargari, 1989). The genus *platychaete* comprises 5 herbaceous perennial species (Zarrin *et al.*, 2010). This plant has beautiful yellow flowers and it can grow up to a height of about 60 cm. It is locally called "Kalajook" and exploited as a medicinal plant in Iranian traditional medicine.Oil exploited from aerial parts has specific aroma used in traditional Persian medicine as a sedative, carminative and also antibacterial and antifungal agent. Fumes obtained from burning used as an adjuvant therapy to treat measles and as an agent for repelling insects. Furthermore, this plant is used as a fragrance in handmade soaps (Asgarpanah *et al.*, 2016).

Literature survey revealed that there are three studies refer to phytochemical investigations of the aerial part (Rustaiyan *et al.*, 1990), seed oil components of this plant (Asgarpanah *et al.*, 2016) and essential oil of aerial parts that demonstrated the oil has large amounts of myrtenol and borneol (Javidnia *et al.*, 2008), both have antimicrobial activity (Tabanca *et al.*, 2001; Aleksic & Knazevic, 2014).

In addition, *E.coli*is a member of *Enterobacteriaceae* family that is the main cause of urinary tract infections (UTIs). Emerge of drug resistant isolates are seen worldwide including in Iran. In such situations, treatment by carbapenems such as imipenem is recommended (Abdi *et al.*, 2014)

Due to the wide spread use of *P.aucheri* in Iranian traditional medicine as an antimicrobial agent, we were prompted to evaluate the antimicrobial activity against two gram-positive, two gram-negative standard bacteria, standard *C. albicans* and clinical *E. coli* strains isolated from wounds of burnt patients and from urine of patients suffering from UTIs.

2. Materials and Methods

2.1. Plant's collection

Fresh flowering aerial parts of *P. aucheri* were collected on May 2014 from Haji Abad country, Hormozgan province, Iran: (28ºC20′N 55ºC 50′E, 1800 m). Specimen was identified and voucher deposited in the Herbarium of Pharmaceutical science Branch, Islamic Azad University (IAU), Tehran, Iran under code number 5043-AUPF.

2.2. Extraction of plant material

The plant sample was cleaned from foreign materials and dried at room temperature (25-28ºC). Then it was milled into coarse powder by a lab mill machine. Air-dried plant was extracted by cold maceration using methanol and a ratio of 1 to 2 of sample to solvent (Merck, Germany) for 72h and after passing this time, total methanolic extract was collected. The extraction process was repeated for 3 times. The extract obtained was concentrated using rotary evaporator (Heidolphlaborata 4000) at 35ºC and the resulted product was a dark green gammy solid extract (80g). The adequate amount of total methanolic extract was kept in a sterile dark vial and the remains were used to prepare chloroform, petroleum ether and aqueous fractions by using liquid-liquid fractionation method (Handa *et al.*, 2008). The obtained crude extract and sub-fractions were dissolved in Tween20 (40%v/v) to prepare stock solutions. Extracts were kept in dark bottles at 4ºC.

2.3. Tested microorganisms

Gram-positive bacteria including *Staphylococcus aureus* (PTCC1431) and *Bacillus cereous*(PTCC1247) and gram-negative bacteria including *Escherichia coli* (PTCC1399) and *Pseudomonas aeruginosa* (PTCC1430) were tested. The yeast was *Candida albicans* (PTCC5027). Also we have tested 50 clinical isolates of *E. coli*from urine of patients who referred to Imam Khomeini hospital or burn wounds of patients who hospitalized in Shahid Motahari burn hospital

during 2013-2014. All samples identified based on standard bacteriologic protocols. Continuously, all were kept in TSB (Tryptic Soy Broth) and 15% glycerol at -20ºC until antimicrobial assay.

2.4. Antimicrobial survey

2.4.1. Agar well diffusion method. The antimicrobial activities of extract and fractions screened using agar-well diffusion method. Concentrations of 500, 250 and 125 mg/ml of each extract were prepared in 2 ml of sterile distilled water (for aqueous fraction) and in Tween20 40% (for crude extract, chloroform and petroleum ether fractions). Bacterial suspension of each microorganism was prepared equal to 0.5 MacFarland turbidity [1.5×10^8 colony forming unit (CFU/ml)]. Then, a microbial lawn was prepared by 100 µl of each suspension in Mueller Hinton Agar (Merck-Germany) plates, separately. As the next step, five equivalent wells (diameter of 5mm) were made in the seeded agar by a sterile pipet Pasteur. Continuously, all wells were filled with 100µl of different concentrations of the extracts and then plates were incubated at 37ºC for 24h. Then, zones of inhibitions produced by each dilution of crude extract and sub-fractions were measured in mm after 24h.The same procedure was accomplished for *C. albicans* using by Sabouraud Dextrose Agar (Merck-Germany) except Mueller Hinton Agar and incubated at 30ºC for 48h.

Amoxicillin, Ciprofloxacin and Amphotericin B (500 µg/ml) were tested simultaneously as a control for tested gram-positive, gram-negative bacteria and *C. albicans*, respectively. The activity of Tween 20 (40% v/v) was determined alone and it was found to exhibit no activity against all the tasted microorganisms. Also these steps were done for clinical *E. coli* samples isolated from urine and burn wounds.

2.4.2. Determination of Minimum Inhibitory Concentration (MIC). The MIC concentrations of the extract and fractions were determined by broth micro-dilution method based on CLSI 2012 (CLSI, 2012). In this survey, 96 wells microplates were used. Each wellwas filled with 100µl of sterile Mueller Hinton Broth (Merck-Germany) and the prepared extracts were diluted to a range of concentration of (1.95-250 mg/ml). Then 10 µl of 1.5×10^7 CFU/ml of microbial suspensions added and incubated at 37ºC for 24h. The MIC was detected as the lowest concentration of extract that inhibited the growth of each tested microorganism. Amoxicillin, Ciprofloxacin and Amphotericin B powders (Sigma Aldrich, Germany) were used as reference antibiotics for simultaneously test on gram-negative, gram-positive and *C. albicans*, respectively. The experiments were carried out triplicate.

To confirm MICs, a portion of liquid from each well with no visible growth inoculated in MHA plates and incubated at 37ºC. After 24 h, the concentration with lowest growth of microorganism will be taken as the MIC. These steps were repeated for clinical *E. coli* isolates.

2.5. Validation method

To validate the procedure, these three steps were performed:

1- Using negative and positive control in both cup plate and broth microdilution methods.
2- Using of Amoxicillin, Ciprofloxacin and Amphotericin B 500µg/ml as a positive control in all antimicrobial tests.
3- Triplicate performing of all the mentioned tests

2.6. Antimicrobial susceptibility test (AST)

Antimicrobial susceptibility test was done for 50 clinical *E. Coli* isolates based on Kirby-Bauer method protocol (Baure *et al*., 1966). All tested antibiotics were purchased from (Mast co, UK). *Escherichia coli* (PTCC1399) were tested, simultaneously.

3. Results and discussion

In the present study, aerial parts of *Platychaete aucheri* was assayed for its in vitro antimicrobial activity against 5 standard microorganisms and also 50 clinical isolates from infected burn wounds and urine samples.Of 50 clinical isolates 40 were isolated from urine samples and 10 from burn wounds.

The antimicrobial activities of total methanolic extract and different sub-fractions were evaluated on two gram-negative and two gram-positive bacteria and

a yeast as standard strains using cup plate method. In this part of the study the most antimicrobial effect of total methanolic extract, petroleum ether and chloroform fraction of *P. aucheri*was on *Pseudomonas aeruginosa* with inhibition zone diameters ranging from 15 to 16.67 mm while the aqueous fraction showed the best antimicrobial effect against *Staphylococcus aureus* with inhibition zone diameter of 17mm (Table 1).

Table 1. The mean inhibition zone diameters (mm) of total extract and sub-fractions of *P. aucheri* against standard microbial strains

Microorganism	Concentration (mg/ml)				Antibiotics	
	Extract or fraction	500	250	125		
Staphylococcus aureus PTCC1431	TME	12.00	8.33	6.33	30.32	Amoxicillin
	CLF	14.00	11.33	8.00		
	PTE	12.00	11.67	9.00		
	AQS	17.00	14.00	10.00		
Bacillus cereus PTCC 1247	TME	11.33	11.00	8.33	29.33	
	CLF	13.67	12.33	9.33		
	PTE	10.67	11.33	10.33		
	AQS	NA	NA	NA		
Escherichia coli PTCC 1399	TME	13.67	13.00	11.67	33.67	Ciprofloxacin
	CLF	13.67	12.67	10.67		
	PTE	12.33	11.33	9.00		
	AQS	NA	NA	NA		
Pseudomonas aeruginosa PTCC 1430	TME	15.00	16.00	14.33	35.67	
	CLF	16.67	15.67	14.00		
	PTE	15.00	13.67	12.33		
	AQS	7.33	6.33	NA		
Candida albicans PTCC 5027	TME	13.33	12.67	11.00	25.67	Amphotericin B
	CLF	13.67	12.67	11.00		
	PTE	12.67	11.67	11.00		
	AQS	7.33	6.67	6.33		

Abbreviations: TME = Total methanolic extract, CLF = Chloroform, PET = Petroleum ether, AQS = Aqueous

Also, the Minimum Inhibitory Concentration (MIC) of the total extract and sub-fractions were determined by broth microdilution method. Best results for total methanolic extract was on *E.coli* with the MIC of 41.67mg/ml and for petroleum ether fraction was on *E.coli* and *S.aureus* with the MIC value of 41.67 mg/ml. The best result for chloroform fraction was detected against *E.coli* with MIC value of 36.46 mg/ml.

Because of usage of this plant in traditional Iranian medicine as an antimicrobial effect in one hand and based on the acceptable antimicrobial effect of tested extract and fractions against standard *E.coli* (PTCC 1399) in this study (Table 2), we prompted to evaluate antibacterial activity against 50 pathogenic strains of *E.coli* that were isolated from different patients who suffered from urine infections and/or skin burn wounds. The MIC values on clinical isolates were ranged from 31.25 to 250 mg/ml. All of the extracts had inhibition effect on bacterial growth of mentioned *E.coli* strains, but petroleum ether and chloroform fractions had more significant effects by the mean MIC values of 61.87mg/ml and 63.75 mg/ml, respectively (Table 3). The other respect result of this study is related to effectiveness of these extracts on 2 *E.coli* isolates from burn wounds which were imipenem intermediate, meropenem and ertapenem intermediate resistant.

Table 2. Minimum inhibitory concentration (mg/ml) of total methanolic extract and sub-fractions of *P.aucheri* against standard microbial strains.

Microorganism	Extracts or fractions (mg/ml)				Antibiotics	
	TME	CLF	PET	AQS		
Staphylococcus aureus PTCC1431	104.17	41.67	41.67	104.17	0.0019	Amoxicillin
Bacillus cereus PTCC 1247	250.00	208.33	208.33	NA	0.01	
Escherichia coli PTCC 1399	41.67	36.46	41.67	250.00	0.0004	Ciprofloxacin
Pseudomonas aeruginosa PTCC 1430	104.17	46.87	104.17	208.33	0.0003	
Candida albicans PTCC 5027	104.17	104.17	52.08	208.33	0.0002	Amphotericin B

Abbreviations: TME = Total methanolic extract, CLF = Chloroform, PET = Petroleum ether, AQS = Aqueous

In this study, the antimicrobial susceptibility pattern of 50 clinical *E.coli* isolates was also evaluated against some common antibiotics (Figure 1).

Table 3. Minimum inhibitory concentration(mg/ml) of total methanolic extract and sub-fractions of *P.aucheri* against clinical isolates of *E.coli* (burn wounds and urine samples).

Microorganism	Extracts (mg/ml)				Antibiotic
	TME	CLF	PET	AQS	Ciprofloxacin
Clinical *E.coli* isolates	97.54	63.75	61.87	227.50	0.0006

Abbreviations: TME = Total methanolic extract, CLF = Chloroform, PET = Petroleum ether, AQS = Aqueous.

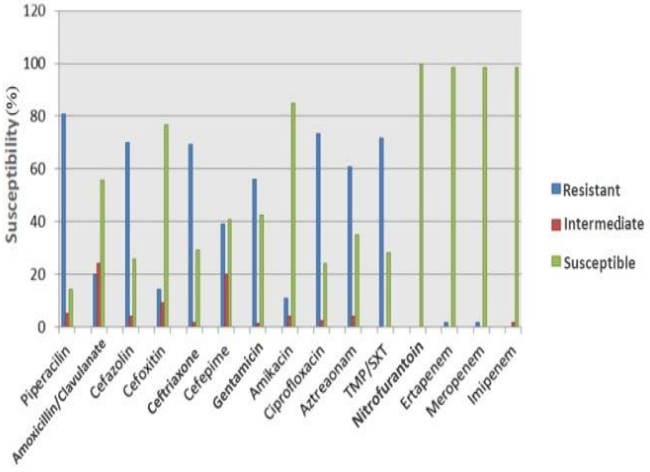

Fig 1. Antibiotic Susceptibility Test (AST) results of 50 clinical *E.coli* isolates

4. Conclusion

In summary, results of this pilot study presented that extract and sub-fractions of *P.aucheri* hold potential antimicrobial effects against wide spectrum of pathogenic microorganisms. Total methanolic extract, petroleum ether and chloroform fractions had more significant antibacterial effect on standard bacteria while chloroform and petroleum ether fractions showed more strong antibacterial effect on the clinical *E.coli* strains.

Based on the results and because of better antibacterial effect on gram-negative bacteria, it is potential for the plant to be used as an antibacterial agent against wound causing pathogens. However the results of antimicrobial effect of *P.aucheri* were satisfied on *E.coli* isolates from urine of patients but survey on more clinical isolates is recommended.

Also study of cytotoxicity effect of extracts on cell cultures and survey of the antimicrobial effect against other microbial stains is suggested. On the other hand, according to traditional usage of plant to repel insects, investigation of probable anticholinesterase effect of this plant is recommended.

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