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IN VITRO ANTIBACTERIAL ACTIVITY OF METHANOL AND WATER EXTRACTS OF *ADIANTUM CAPILLUS VENERIS* AND *TAGETES PATULA* AGAINST MULTIDRUG RESISTANT BACTERIAL STRAINS

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Abstract

The aim of present study was to screen the antimicrobial activities of extracts of leaves and stems of *Adiantum capillus veneris* and *Tagetes patula* against multidrug-resistant (MDR) bacterial strains. Extracts from the leaves and stems of these plants were extracted with methanol and water and tested for their antibacterial activity by disc diffusion method against ten MDR bacterial strains i.e., *Citrobacter freundii, Escherichia coli, Providencia, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae, Proteus vulgaris, Salmonella typhi, Shigella* and *Vibrio cholerae.* Leaves methanol extract (LME) of Adiantum showed maximum Zone of Inhibition (ZI) against *Providencia, Klebsiella pneumoniae, Shigella, Vibrio cholerae, Staphylococcus aureus, Proteus vulgaris* and *Salmonella typhi*, whereas its stem methanol extract (SME) was very active against *Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhi.* Similarly LME of Tagetes showed highest ZI against *Escherichia coli* and *Vibrio cholerae* while SME showed highest ZI to *Escherichia coli, Vibrio cholerae, Providencia, Shigella* and *Klebsiella pneumoniae*. Leaves water extract (LWE) of Adiantum was very active against all ten bacterial strains while its stem water extract (SWE) showed maximum ZI against *Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhi, Shigella, Proteus vulgaris* and *Providencia*. LWE of Tagetes was only active against *Vibrio cholerae* whereas SWE was very active against *Salmonella typhi* and active against *P. vulgaris, Citrobacter freundii* and *Vibrio cholerae*. It was concluded from this study that extracts of both Adiantum and Tagetes have prominent activities against most of the MDR bacterial strains and needs further studies for utmost benefits.

Introduction

Among major health concerns infectious diseases accounts for 41% of the global disease burden along with non-infectious diseases (43%) and injuries (16%) measured in terms of Disability-Adjusted Life Years (DALYS) (Noumedem *et al*., 2013). The universal development of bacterial resistance to various antibiotics is one of the main reasons of these infectious diseases (Westh *et al*., 2004). The accumulation of various antibiotic resistance mechanisms inside the same strains leads to the development of MDR bacteria (Harbottle *et al*., 2006). Despite the fact that pharmacological companies have produced a number of new antibiotics in the previous decades, resistance to these drugs has increased (Nascimento *et al*., 2000; Rizvi *et al*., 2013). Due to this reason, the attention of researchers is turning towards herbal products, in search of development of improved drugs having antifungal, antibacterial and antiviral activities (Maiyo *et al*., 2010).

The research area in which medicine are derived from plants, animals or minerals and used for the cure of various disorders is known as ethnomedicine (Hazrat *et al*., 2013). Herbal medicines have been in use throughout the history of human beings against various infectious diseases either in the form of plant extracts or pure compounds (Parekh & Chanda, 2007). Different medicinal plants have been mentioned for the treatment of infectious diseases by many phytotherapy manuals because of their uncomplicated availability, reduced toxicity and few side effects (Khan *et al*., 2009). Numerous studies have been conducted around the world, describing the antimicrobial activities of various plant extracts (Islam *et al*., 2008; Izzo *et al*., 1995). Several plants have been investigated to treat gastrointestinal disorders, cutaneous and respiratory diseases, urinary tract infections (Somchit *et al*., 2003) as well as malarial infections (Khan *et al*., 2013).

Adiantum and Tagetes have worldwide distribution. They are found in pak-indian subcontinent, western Himalaya, Mexico, warmer parts of America and other tropical and subtropical regions of the world (Nisar *et al*., 2012; Reddy, 2010). Adiantum is used as expectorant, astringent, demulcent, antitussive, emmenagogue, febrifuge, diuretic and catarrhal affections. The herb is boiled in mead or wine and drunk in case of hard tumors in the liver, spleen and other viscera (Ahmad *et al*., 2012). Different extracts of adiantum has previously shown good activities against various strains of bacteria (*Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae*) and fungi (*Candida albicans*) (Kumar & Nagarajan, 2012). Similarly Tagetes is also used as folk medicine for the treatment of vomiting, fever, diarrhea, hepatic disorders and skin infections. It is evident from the earlier studies that this plant has antibacterial activities against *Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus, Pseudomonas aeruginosa* and *Enterococcus faecalis* (Jain *et al*., 2012). The present work was therefore designed to investigate the antibacterial activities of methanol and water extracts of Adiantum and Tagetes against MDR bacterial strains isolated from community acquired and nosocomial infections.

Materials and Methods

Plant material and extraction: Adiantum and Tagetes were collected from different areas of Swat and Peshawar and were identified from the Department of Botany, University of Peshawar. In order to collect different extracts, the leaves and stems were separately shadow dried by the method of Shalini & Sampathkumar (2012). The leaves and roots were separately ground to homogenous powder. 100g of powder i.e., from leaves and stems were separately soaked in 1 L of each methanol and distilled water for 24 h at 25°C and then filtered using Whattman No1 filter paper. The filtrates were collected in separate flasks and the same process was repeated in triplicate by previously described methods (Gracelin *et al*., 2012). The filtrates (crude extracts) obtained were concentrated in rotary evaporator keeping the water bath at 60^{o}C. The isolated extracts were re-suspended in a minimum required volume of corresponding solvents and placed on the water bath at 60^{o}C to evaporate the extra solvents for the isolation of pure extracts. Then all the extracts were preserved in separate containers at 5^{o}C for further experimentations according to previous methods (Cannell, 1990).

Collection and identification of bacterial cultures: The samples were collected from the main laboratory of Lady Reading Hospital Peshawar and Pakistan council of scientific and industrial research (PCSIR) laboratory Peshawar. Bacterial species i.e., *Citrobacter freundii, Escherichia coli* and *Providencia* species were isolated from urine samples, *Pseudomonas aeruginosa* and *Staphylococcus aureus* from pus samples while *Klebsiella pneumoniae, Proteus vulgaris, Salmonella typhi, Shigella* and *Vibrio cholerae* were isolated from water sample. The isolated bacterial species were sub-cultured on differential and selective media e.g., MacConkey & CLED agar, and were identified through their specific morphological, staining and biochemical characteristics according to previously described methods (Collee & Marr, 1996).

Assessment of drug resistance pattern of the test-bacterial strains: Before the evaluation of antimicrobial activity of plant extracts, all the microorganisms were first tested against commonly used antibiotics. Disk diffusion method was used for measuring the antimicrobial activity and the media used was the Muller Hinton agar. The sensitivity of 14 antibiotics was tested against the previously mentioned 10 bacterial strains (Table 1) and the process was repeated thrice. All the plates were incubated at 37^{o}C for 24h (Ushimaru *et al*., 2007).

Evaluation of antimicrobial activity of extracts: Well diffusion method of Janovska *et al.,* (2003) was followed with some modifications for the assessment of antimicrobial activities of methanol and water extracts of Adiantum and Tagetes. Approximately 1 mg of plant extract was dissolved in 1 ml of dimethyl sulfoxide (DMSO). Pre-autoclaved Muller Hinton agar plates were inoculated with a 10^{-5} dilution of bacterial cultures, using sterile cotton swabs to achieve uniform growth. Sterile cork borer was used to bore wells in the agar, to test the activity of plant extracts. 60µl of each extract i.e., LME, LWE, SME and SWE was introduced through micropipette aseptically into specifically marked wells in the agar plates. All the plates were incubated at 37^{o}C for 24h and the process was repeated for three times.

Statistical analysis: The SPSS version 16.0 and Microsoft excel was used to evaluate the percentages of inhibitions and bactericidal activities.

Results

Drug resistance pattern of the test-bacterial strains: The MDR bacterial strains were tested for antibiotic sensitivity patterns against 14 commonly used antibiotics. Most of the tested strains were found resistant to the used antibiotics. *Citrobacter freundii* was the most resistant strain (92.8%) among all the tested organisms that showed relatively low sensitivity only to tetracycline (TET) (10mm). Other most resistant strains (85.7%) were *Klebsiella pneumoniae* which showed sensitivity only to cefoperazone-sulbactam,

Table 1. Drug resistance pattern of the test-bacterial strains.

S.#	Microorganisms	Antibiotic discs with ZI (mm) representing sensitivity, while (-) represents resistance													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
		AMP	AMX	FC	CPH	CIP	CTX	CRO	CZS	GEN	MXF	NA	NOR	TET	TS
1.	*Escherichia coli*	-	-	-	-	-	-	-	-	18	10	-	-	20	-
2.	*C. freundii*	-	-	-	-	-	-	-	-	-	-	-	-	10	-
3.	*K. pneumoniae*	-	-	-	-	-	-	-	15	15	-	-	-	-	-
4.	*S. typhi*	-	-	-	-	-	15	-	-	-	10	9	-	11	-
5.	*Shigella*	20	-	-	-	28	30	-	20	12	19	19	29	11	-
6.	*P. vulgaris*	-	-	-	-	-	-	-	20	13	-	-	-	10	-
7.	*Providencia*	-	-	-	-	-	22	18	-	-	-	-	-	-	-
8.	*P. aeruginosa*	-	-	-	-	30	16	-	30	14	28	-	30	-	12
9.	*S. aureus*	-	-	-	-	30	25	-	25	18	25	-	30	12	-
10.	*Vibrio cholerae*	-	-	-	-	-	21	21	-	12	-	21	-	-	-

AMP = ampicillin, AMX= amoxicillin, CF= cefaclor, CPH= cephradine, CIP= ciprofloxacin, CTX= cefotaxime, CRO= ceftriaxone, CZS= cefoperazone-sulbactam, GEN- gentamicin, MXF= moxifloxacin, NA= naladixic acid, NOR= norfloxacin, TET= tetracycline, TS= trimethoprim-sulfamethoxazole

(CZS) (15mm) and gentamicin (GEN) (15mm) followed by *Providencia* (85.7%), that showed sensitivity to cefotaxime (CTX) (22mm) and ceftriaxone (CRO) (18mm). *Escherichia coli* and *Proteus vulgaris* were 78.6% resistant while *Salmonella typhi* and V. cholera were 71.4% resistant to all tested antibiotics. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were found 50% resistant meanwhile Shigella was 35.8% resistant against all 14 antibiotics (Table1). The results were recorded after 24h incubation, according to the ZI of each antibiotic for all tested bacterial strains.

Evaluation of antimicrobial activity of plant extracts: The methanol and water extracts of leaves and stems of Adiantum and Tagetes were tested against ten MDR bacterial strains. A volume of 60µl (1mg/1ml) of each extract was applied for antimicrobial activity evaluation through well diffusion method.

Both LME and SME of Adiantum showed antibacterial activity against all the test bacterial strains. LME of Adiantum showed maximum Zone of inhibition (ZI) against *Providencia* (29mm), *Klebsiella pneumoniae* (28mm ±1.00), *Shigella* (29mm ±1.00), *Vibrio cholerae* (28mm ± 1.00), *Staphylococcus aureus* (27 ± 1.00mm), *Proteus vulgaris* (24mm ± 1.00) and *Salmonella typhi* (24mm ±1.00),while its SME showed maximum ZI against *Escherichia coli* (29mm ± 1.00), *Klebsiella pneumoniae* (24mm ±1.00), and *Salmonella typhi* (24mm ±1.00). Similarly LME of Tagetes showed highest ZI against *Escherichia coli* (23mm ±1.00) and *Vibrio cholerae* (15mm ±0.58) while SME showed highest ZI against *Escherichia coli* (26mm ±1.00), *Vibrio cholerae* (20mm ±1.00), *Providencia* (18mm ±0.58), *Shigella* (18mm ±0.58) and *Klebsiella pneumoniae* (17mm ±0.58). LWE of Adiantum very active against all ten bacterial strains while it's SWE showed maximum ZI against *Escherichia coli* (20mm ±1.00), *Klebsiella pneumoniae* (20mm ±1.00) and *Salmonella typhi* (20mm ±1.00), *Shigella* (15mm ±0.58), *Proteus vulgaris* (15mm ±0.58) and *Providencia* (15mm ±0.58). LWE of Tagetes was only active against *Vibrio cholerae* (14mm ±0.58) while its SWE was very active against *Salmonella typhi* (19mm ±0.58) and active against *Proteus vulgaris* (18mm ±0.58), *Citrobacter freundii* (16mm ±0.58) and *Vibrio cholerae* (16mm ±0.58).

Discussion

The escalating emergence of antibiotic resistance have diverted the attention of researchers towards the medicinal plants in search of new, less toxic and effective drugs. In this regard the present study has been carried out to evaluate the antimicrobial activities of methanol and water extracts of Adiantum and Tagetes.

In the present study 10 bacterial strains were used which were MDR to most of the given antibiotics (Table 1). Our results showed that *Citrobacter freundii* was the most resistant strain (92.8%) among all the tested bacterial strains. This finding goes in line with the studies in other parts of Pakistan where 100% MDR *Citrobacter* was reported (Shaikh *et al*., 2003). Moreover, 92.8% MDR *Citrobacter* seen in this study concise with 100% MDR rate in Ethiopia (Biadglegne *et al*., 2009) and 86.95% in Nepal (Thapa *et al*., 2010). Similarly 85.7% MDR *Klebsiella pneumoniae* found in our study is almost in accordance with 81.8% MDR investigated locally (Hannan *et al*., 2013) in early 2013. 85.7% MDR *Providencia* evaluated in our study is almost in line with the study of Mario *et al*., (Tumbarello *et al*., 2004) (75%). We have also investigated that *Escherichia coli*, *P. vulgaris*, *Salmonella typhi*, *V. cholera*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Shigella* were somewhat more MDR (Fig. 1) than found in other parts of the world, as evident from multivariate studies (Amaya *et al*., 2011; Zavascki *et al*., 2010) on these bacterial strains.

Several studies on Adiantum and Tagetes reveal its potency against MDR bacterial strains. For example *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* were sensitive to LME, LWE, SME and SWE of Adiantum in our study proved to be almost in accordance with the findings of Kumar & Nagarajan (2012) and Mahboubi *et al*., (2012) from India and Iran respectively. Similarly our extracts of Tagetes were active against *Escherichia coli*, *V. cholera*, *Providencia*, *Shigella*, *P. vulgaris*, *Salmonella typhi*, *Klebsiella pneumoniae* and *Citrobacter freundii* (Table 2) which are almost in agreement with the studies from India by Jain *et al*., (2012) and from Venezuela Andes by Rondon *et al*., (2006). We have find out in this study that most of the extracts of Adiantum and Tagetes were very effective against the MDR bacterial strains as compared to other studies (Bindu *et al*., 2012; Bukhari *et al*., 2012; Ramya *et al*., 2012) which might be due to the variation in geographical conditions, procedures etc.

In comparison to the antibiotics used in this study, the plants extracts were far more active against the test bacterial strains (Fig. 2). *Citrobacter freundii* was found to be resistant to 13 (92.8%) out of 14 test-antibiotics, while it was sensitive to all the extracts of Adiantum and SME and SWE of Tagetes. Similarly *Klebsiella pneumoniae* and *Providencia* were resistant to 12/14 (85.7% MDR) antibiotics but at the same time they were sensitive to all extracts of Adiantum and SME of Tagetes. 78.6% MDR *Escherichia coli* and *Proteus vulgaris* were sensitive to all extracts of both plants (except *Proteus vulgaris* was resistant to SME of Tagetes). *Salmonella typhi* and *Vibrio cholera* were 71.4% MDR and were sensitive to all extracts of both plants. 50% MDR *Staphylococcus aureus* and *Pseudomonas aeruginosa* were sensitive to all extracts of Adiantum and SWE of Tagetes. Shigella was 35.8% resistant against all 14 antibiotics while sensitive to all extracts of both plants except LME of Tagetes.

The present investigation confirms that fractions of Adiantum and Tagetes have significant antibacterial activity. Different fractions have different antibacterial activities against MDR bacterial strains. The methanol and water extracts of *Adiantum capillus veneris* proved to be more active than *Tagetes patula*. It is recommended that research should be conducted for compound characterization of extracts for more effective antibacterial activities.

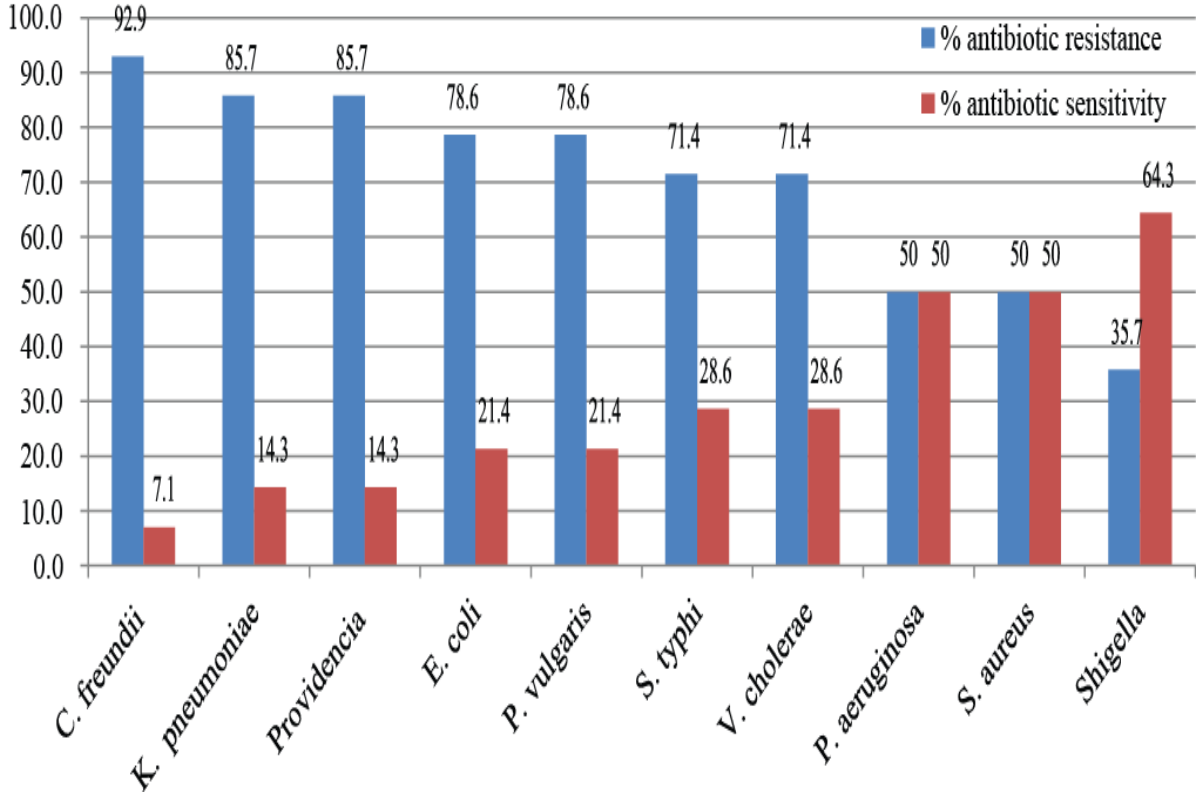

Fig.1. % antibiotic resistance and sensitivity of MDR bacterial strains.

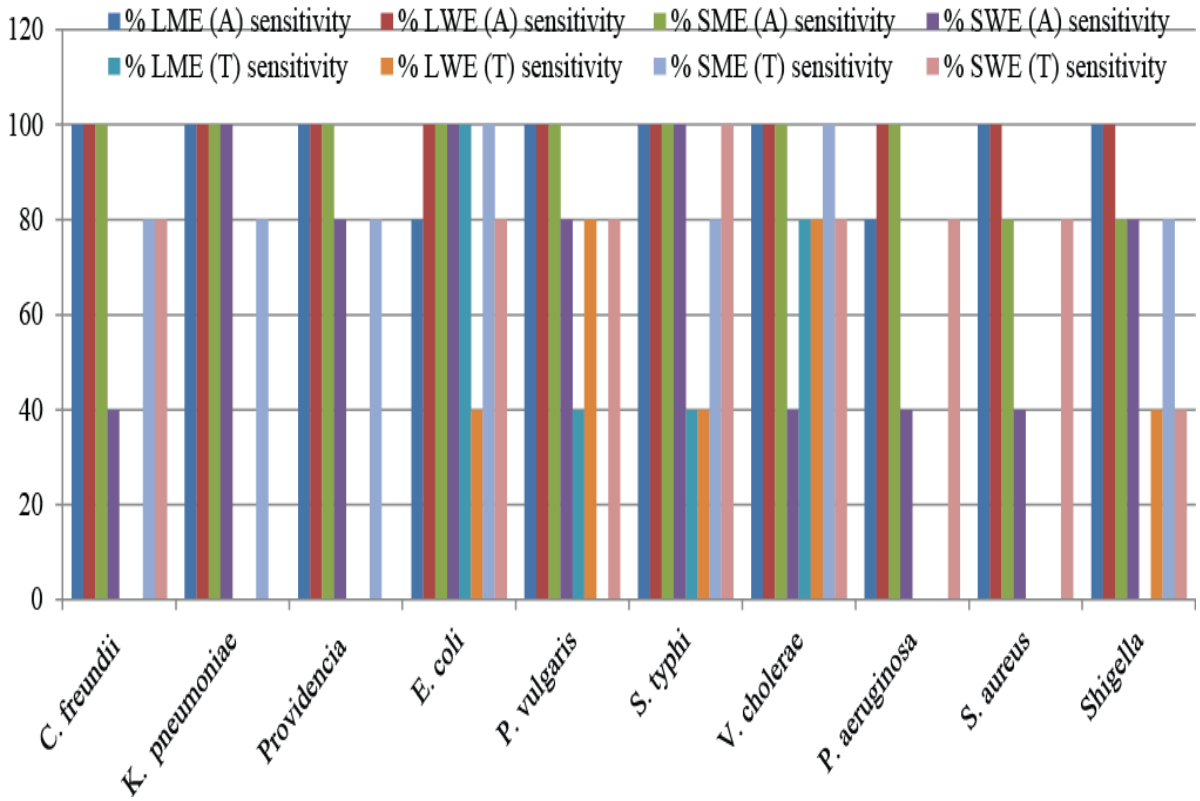

Fig. 2.Percentage sensitivity of all methanol and water extracts against MDR bacterial strains
A= *Adiantum capillus veneris*, T= *Tagetes patula*, ZI (mm): 9mm= inactive (0% sensitivity), 9-12mm= partially active (40% sensitivity), 13-18mm= active (80% sensitivity) and 18mm= very active (100% sensitivity)

Table 2. Antimicrobial activity of plant extracts.

S. #	Microorganisms	LME		LWE		SME		SWE	
		A.C. Veneris	***T. patula***	***A.C. Veneris***	***T. patula***	***A.C. Veneris***	***T. patula***	***A.C. Veneris***	***T. patula***
1.	*Escherichia coli*	18	23	20	12	29	26	20	14
2.	*C. freundii*	21	-	20	-	20	16	10	16
3.	*K. pneumoniae*	28	-	24	-	24	17	20	-
4.	*S. typhi*	24	10	21	11	24	15	20	19
5.	*Shigella*	29	-	20	11	18	18	15	11
6.	*P. vulgaris*	24	10	24	13	20	-	15	18
7.	*Providencia*	29	-	20	-	20	18	15	-
8.	*P. aeruginosa*	15	-	24	-	20	-	10	15
9.	*S. aureus*	27	-	20	-	18	-	12	14
10.	*Vibrio cholerae*	28	15	24	14	20	20	10	16

Extracts with ZI representing sensitivity in millimeter (mm), *A.C. Veneris: Adiantum capillus veneris, T. patula: Tagetes patula.* ZI (mm): 9mm= inactive, 9-12mm= partially active, 13-18mm= active and 18mm= very active (Junior & Zanil, 2000)

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