Evaluation of Antibacterial Activity of *Cynodon dactylon* on Multi-Drug Resistant Bacterial Isolates in Comparing with Ciprofloxacin

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Abstract: *Cynodon dactylon* regarded to possess various medicinal properties as an anticancer, antidiabetic, anti-inflammatory and antioxidative agent, but there are a few studies on its antibacterial effects. The aim of the present study was the evaluation of the antibacterial activity of *Cynodon dactylon* on 100 Multi Drug Resistant isolates of S. aureus, *A. baumannii*, *P. aeruginosa*, *Klebsiella* and *E. coli*. *Cynodon dactylon* samples were collected from the fields of North West of Iran. Plant roots were cut, and powder was prepared. Powdered roots were extracted by maceration at room temperature for 72 hours. Bacterial isolates were collected from clinical specimens from different wards of educational hospitals in Urmia, Iran during a 12 months period. The susceptibility of isolates to *Cynodon dactylon* root extracts was determined using a broth microdilution method. Considering to the wide application of ciprofloxacin in treatment of bacterial nosocomial infections, the antibacterial effects of ciprofloxacin on isolates also determined. All the multi- drug resistant bacterial isolates were sensitive to different concentrations of *Cynodon dactylon* root hydroalcoholic extract, the most sensitive bacterial isolates to *Cynodon dactylon* root extracts were P.aeruginosa isolates, however 69% of isolates were resistant to ciprofloxacin. Results demonstrate that this herbal drug could represent a new source of antimicrobial agents, for the control of hospital acquired infections. However, more adequate studies must be carried out to verify the possibility of using it for fighting these bacteria in human body infections.

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1. Introduction

Cynodon dactylon family Poaceae is considered as a sacred herb (Balasubramanian, et al, 2008). It has been used in the folk medicine of many countries. It has been regarded to possess various medicinal properties as an antidiabetic agent in traditional system of Medicine. The aqueous plant extract is used as anti-inflammatory, cardioprotective diuretic, antioxidative, anti-emetic and purifying agent (Singh et al., 2007). Anticancer potential of C. dactylon in experimentally induced colon carcinogenesis in rats has been demonstrated before (Albert-Baskar and Ignacimuthu, 2010). Also it has been demonstrated that the Fresh juice of *Cynodon dactylon* (Bermudagrass) has DNA protective activity and immunomodulatory properties (Mangathayaru et al, 2009). The plant posses antimicrobial, and antiviral activity (Singh et al., 2007) and has also been used to treat urinary tract infection, calculi and prostatitis. Balasubramanian et al also showed that the Oral administration of plant extract of C. dactylon to be highly effective in preventing of white spot syndrome virus (WSSV) infection (2008). It also has significant application in treating dysentery, dropsy and secondary syphilis (Singh et al., 2007). *Rhizoctonia* sp. (Cy064), an endophytic fungus in the leaf of *Cynodon dactylon* used locally for treating hepatitis and metabolites were extracted from this fungus has anti-*H. pylori* effects (Ma et al, 2004).

Resistant Gram-positive pathogens, such as *Staphylococcus aureus* have become a serious problem in the medical community. *Staphylococcus aureus* is an organism with several virulent factors and resistance mechanisms at its disposal. It is also a significant cause of a wide range of infectious diseases in humans. *S. aureus* often causes life-threatening deep seated infections like bacteremia, endocarditis and pneumonia (Kanafani and Fowler 2006).

Acinetobacter baumannii is a gram-negative opportunistic bacillus. It is found in many hospital environments and can be colonize in human body in the hospital environments. The combination of its environmental colonization and its very high resistance to antimicrobials renders it as a successful nosocomial pathogen. The MDR strains of A. *baumannii* often spread to cause outbreaks throughout hospital wards. *A. baumannii* cause a wide range of clinical complications, such as pneumonia, septicemia, urinary tract infection, wound infection, and meningitis, especially in immunocompromised patients (Nordmann, 2004).

P. aeruginosa is an opportunistic pathogen found as part of the normal flora of the human skin (Larson and Ramphal, 2002). In immunocompromised host, *P. aeruginosa* can colonize and infect the burn and wound sites, it can rapidly disseminate from the wounds into other organs via the bloodstream and can produce severe infections such as endotoxic shock (Dale et al., 2004). Antibiotics are generally ineffective against most serious infections especially burn wounds infections by *P. aeruginosa*, treatment of these infections is frequently complicated by antibiotic resistance, a problem that is increasing in the recent years. *Klebsiella* sp. is a group of gram negative rods and they can cause different kinds of infections especially in a hospital setting. They are resistant to numerous antibiotics. Their resistance to antibiotics restricts the choice of antibiotics for therapy (Keynan and Rubinstein, 2007). Hospital acquired urinary tract infections account for 35-45% of the nosocomial infections (Kamat et al, 2009). *E. coli* is the main agent of this disease. Antibiotic therapy is the gold standard of treatment of such infections; however, long-term therapy may result in many side-effects and cause selection of resistant bacteria. So, we need new treatments that could replace antibiotic therapy (Jazani et al 2007).

In respect of high resistance of nosocomial isolates of mentioned bacteria to antimicrobials, introducing of the new antimicrobial agents against these kind of microorganisms is one of the most important goals in treatment of such infections (Perez et al., 2007). In this study we evaluated the antibacterial activity of *Cynodon dactylon* root on 100 Multi Drug Resistant isolates of *Staphylococcus aureus*, *Acinetobacter baumannii*, *P. aeruginosa*, *Klebsiella* and *E. coli*.

2. Material and Methods

Extract preparation: *Cynodon dactylon* samples were collected from the fields of Salmas road (Iran) and identities were confirmed by the Botanist. Plant roots were cut, chopped and dried and powder was prepared. Powdered roots were extracted by maceration at room temperature for 72 hours. The hydroalcoholic extracts were combined and concentrated to yield a dried powder. This hydroalcoholic extract was kept in refrigerator for all experiments (Garjani et al, 2009).

Bacterial strains and culture media: A total of 100 isolates *of Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella* and *E. coli*(20 isolates from each kind) were collected from clinical specimens of different wards of educational hospitals in Urmia, Iran during a 12 months period between April 2006-2007. The isolates were further processed by the standard methods to identify as the *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella* and *E. coli* isolates (Baron and Finegold, 1990). The susceptibilities of isolates to different antibiotics were tested using agar disk diffusion method and Multidrug resistant isolates was selected for further experiments. Isolated bacteria were maintained for long storage on skimmed milk medium (BBL) by adding 10% glycerol in -60°C, cultures were maintained for daily use on Nutrient agar (BBL) slants on 4°C. The Muller Hinton Agar (MHA) and Muller Hinton Broth (MHB) medium (Pronadisa) were used for detection of antibiotic resistance of isolates. *Acinetobacter calcuaceticus* PTCC 1318, *Enterococcus faecalis* ATCC29212, *Pseudomonas aeruginosa* ATCC27853, *Pseudomonas aeruginosa* PAO1, *E.coli* ATCC25922, *Klebsiella pneumoniae* ATCC10031, *Staphylococcus aureus* PTCC1112 and *Staphylococcus aureus* ATCC25923 have been used as reference strains.

Determination of antimicrobial activity of *Cynodon dactylon* root extracts: The susceptibility of isolates to *Cynodon dactylon* root extracts was determined using a broth microdilution method based on CLSI guidelines. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Cynodon dactylon* root extracts for isolates were determined in Muller-Hinton Broth (MHB; Oxoid) medium (Jazani et al, 2009) (Papadopoulos et al., 2006). 10 mg of *Cynodon dactylon* root powder was dissolved in 1000 µL of Dimetylsufoxide (DMSO, Sigma). The initial concentration of *Cynodon dactylon* root powder in the first tube contains MHB was 500 µg/mL. This was used to prepare serial doubling dilutions over the range 500-3.9 µg/mL. 1.5×10^6 inoculums of the isolates were added to each concentration in MHB. A tube containing growth medium without *Cynodon dactylon* root extracts and an un-inoculated tube were used as a positive and negative growth control respectively. Antibacterial activity was measured by

determining MICs and MBCs. The MIC was the lowest concentration of essential oil that resulted in a clear tube. Ten microlitres from each tube was spot-inoculated onto Nutrient Agar (NA) and incubated overnight at 37 °C to determine the MBC. The highest dilution that inhibits bacterial growth on nutrient agar after overnight incubation was taken as MBC (Baron and Finegold, 1990),(Papadopoulos et al., 2006). Experiments were performed at least three times and the modal value selected.

Determination of antimicrobial activity of ciprofloxacin: Considering to the wide application of ciprofloxacin in treatment of bacterial nosocomial infections, the antibacterial effects of ciprofloxacin on isolates also determined and the effectiveness was compared with *Cynodon dactylon* root extracts. Ciprofloxacin powder was kindly provided by Exir pharmaceutical company, Tehran, Iran. The pure content of active ciprofloxacin was 96% in the provided powder. For determining of the bacterial isolates sensitivity to ciprofloxacin, classic broth dilution susceptibility test were used (Sahm and Weissfeld, 2002). MIC and MBC of isolates to ciprofloxacin were determined. The initial concentration of antibiotic in the first tube was 500µg mL^{-1}, this solution was diluted serially in 8 steps. 1.5×10^{6} inoculums of the isolates were added to each concentration of ciprofloxacin in MHB. A tube containing growth medium without ciprofloxacin and an un-inoculated tube were used as a positive and negative growth control respectively. *In vitro* resistance was defined as MBC of 4 or more µg mL^{-1} for bacterial isolates (Chaudhry et al., 1999).

3. Results

A total of 100 multi-drug resistant isolates with nosocomial origin of gram negative and gram positive bacteria were collected from clinical specimens submitted to the educational hospital clinical microbiology laboratories of selected hospitals in Urmia, Iran. The Sensitivity of bacterial isolates to *Cynodon dactylon* root hydroalcoholic extract has been shown in Figure 1**.**

Also the MIC and MBC of *Cynodon dactylon* root hydroalcoholic extract against standard bacterial strains has been shown in Table 1**.**

The Sensitivity of bacterial isolates to ciprofloxacin has been shown in Figure 2. 69 isolates (69% of all isolates) were resistant (MBC ≥ 4 or µg mL^{-1}) and the other isolates were sensitive to ciprofloxacin (MBC ≤ 4 µg mL^{-1}) (Figure 2).

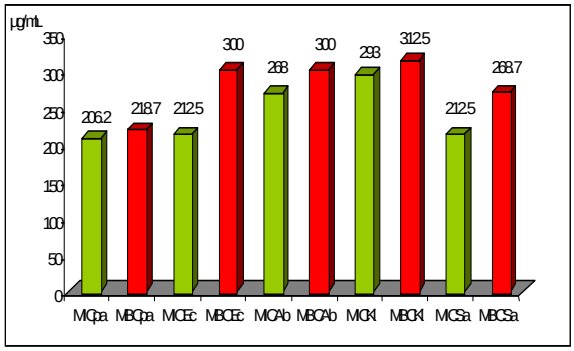

Figure 1: Antibacterial activity of *Cynodon dactylon* root hydroalcoholic extract against 100 nosocomial isolates of multi drug resistant gram negative and gram positive bacteria. Pa: *Pseudomonas aeruginosa*, Ec: *E. coli*, Ab: *Acinetobacter baumannii*, Kl: *Klebsiella Sp*, Sa: *Staphylococcus aureus*. MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration.

Table 1: The MIC and MBC of *Cynodon dactylon* root hydroalcoholic extract against standard bacterial strains.

Standard Bacterial isolates	*Cynodon dactylon* root hydroalcoholic extract(µg/mL)
Acinetobacter calcuaceticus PTCC 1318	MIC= MBC=125
Enterococcus faecalis ATCC29212	MIC= MBC=250
Pseudomonas aeruginosa ATCC27853	MIC= MBC=250
E.coli ATCC25922	MIC= MBC=125
Klebsiella pneumoniae ATCC10031	MIC=125, MBC=250
Staphylococcus aureus PTCC1112	MIC= MBC=250
Staphylococcus aureus ATCC25923	MIC= MBC=125
Pseudomonas aeruginosa PAO1	MIC= MBC=250

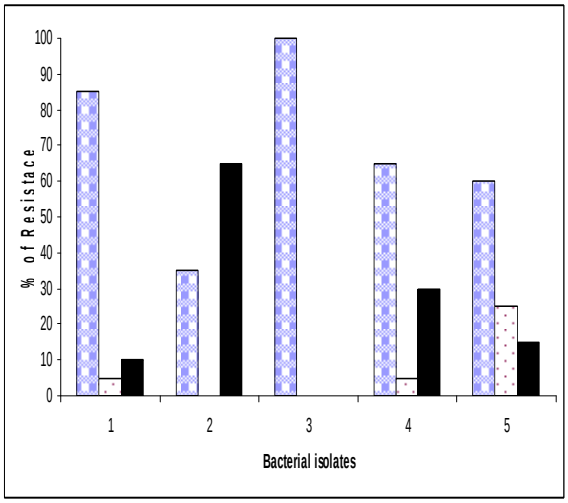

Figure 2: The rates of resistance to Ciprofloxacin for 100 clinical isolates of multi-drug resistant bacteria. Resistant (checked), Intermediate (spotted), Sensitive (black). 1: *Staphylococcus aureus*, 2: *E. coli*, 3: *Acinetobacter baumannii*, 4: *Klebsiella Sp.* And 5: *Pseudomonas aeruginosa*

4. Discussions

Antibiotics are generally ineffective against most serious infections by multi drug resistant bacteria, treatment of these infections is frequently complicated by antibiotic resistance, a problem that is increasing in recent years, so introducing of the new antimicrobial agents against these kinds of bacteria is one of the most important goals in treatment of such infections. However there are limited studies on investigation of the antibacterial effects of *Cynodon dactylon* root extract on multi drug resistant bacteria.

C. dactylon is used in traditional medicine as an anti-inflammatory, diuretic, anti-emetic and purifying agent, and to treat dysentery (Artizzu et al, 1996).

White spot disease is one of the major causes of severe mortality in farmed black tiger shrimp all over the world. The antiviral activity of extract of *C.dactylon* on white spot syndrome virus (WSSV) in black tiger shrimp has been shown by in vivo testing after oral administration previously (Balasubramanian et al, 2008).

Artizzu et al reported that the essential oil of the aerial parts of *C.dactylon* did not exhibit antimicrobial properties, but agropyrene one of the compounds of this essential oil exhibited weak activity against *Candida albicans*, *Saccharomyces cerevisiae*, *Staphylococcus aureus* and *Bacillus subtilis*(1996), however in the present study we examine the root of the plant for antimicrobial effects.

Parekh et al(2005) reported that the aqueous extracts of *C.dactylon* was inactive against some of defined bacterial strains, while methanol extracts could inhibit only *S. epidermidis* and *B. subtilis*, however they mentioned that successful prediction of plant compounds from is largely dependent on the type of solvent used in the extraction procedure. water as the solvent is used with more frequency in compare with organic solvent (for example methanol), but organic solvents provide more consistent antimicrobial activity compared to those botanical compounds extracted in water (Parekh et al, 2005), in this research we also use organic solvent(hydroalcoholic extraction method) for evaluation of the antibacterial activity of the *C.dactylon* root.

Srinivasan et al (2001) prepared an extract from leaves, flowers and bulb of the *C.dactylon* and examined its antimicrobial activities against 10 different strains of fungi and gram positive and gram negative bacteria, however they didn't find any antimicrobial effects for this extract, however they didn't use the root of the plant for preparing this extract. In this research we used only the root extract of the *C.dactylon* for determining the antibacterial effects (Srinivasan et al, 2001).

Punitha et al (2008) isolated *Vibrio harveyi* from the wounds of the infected fish, and determined the *in vivo* and *in vitro* antibacterial activity of *C.dactylon* on this bacterium. In the challenge experiments of the fish with this isolate, the control group had the highest and fastest mortality, however survival rate was significantly increased in the group fed with this herb, Also different kinds of organic solvent extracts of the *C.dactylon* showed antibacterial effects on this bacterial isolate (Punitha et al, 2008), this finding is in agreement with our findings in showing the antibacterial effects of *C.dactylon*.

It has been previously demonstrated that *A. fumigatus* residing in *C. dactylon* is a versatile producer of new and bioactive metabolites, also it has been shown that these metabolites have antifungal effects on *C. albicans*, *T. rubrum* and *A. niger* **(**Liu, et al., 2004).

The antibacterial effects of the *Rhizoctonia* sp., an endophytic fungus in the leaf of *C. dactylon* has been shown previously (Ma et al 2004). This finding is also in agreement with our data, because we didn't any efforts for isolating the entophytic fungus from the surface of the roots of this plant, so the antibacterial effects of the *C. dactylon* has been observed in this research may be at least in part due to entophytic fungi living as Symbiotic microorganisms on the surface of this herbal plant.

Also two new metabolites from the endophytic fungus *Chaetomium globosum* residing inside the root of *C.dactylon* have been isolated recently. These Compounds showed antimicrobial activity against the gram-positive bacteria. This finding is also in agreement with the hypothesis that the antibacterial effects has been showed in the present study can be attributed to the Symbiotic fungi living on the root of *C. dactylon* (Ge et al, 2010).

In contrast with the results obtained by Parekh et al (2005), the findings of this study indicate that *C.dactylon* root hydroalcoholic extract had a significant antibacterial effect on all multi-drug resistant isolates of gram negative and gram positive bacteria. These difference may due in part to the different extractions methods used in these studies, moreover Parekh et al(2005) sterilized the extracts by autoclave at 121 ºC and 15 lbs pressure, this procedure may destroy bioactive compounds in the extract, however in our study we use 0.45 µ filters for sterilizing of the *C.dactylon* root hydroalcoholic extract.

In the present study all the multi- drug bacterial isolates were sensitive to different concentrations of *C.dactylon* root hydroalcoholic extract, the most sensitive bacterial isolates to Bermudagrass root extracts were *P. aeruginosa* isolates(Fig 1), Also *A.calcuaceticus* PTCC 1318, *E.coli* ATCC25922 and *S.aureus* ATCC25923 were the most sensitive strains among the standard isolates (MIC= MBC=125) (Table 1), however clinical isolates showed high resistance to ciprofloxacin (Fig 3). In the present study results showed that the *C.dactylon* root hydroalcoholic extract possessed antibacterial effect against all multi-drug resistant bacterial isolates, furthermore, beside the confirmation of the popular use, the obtained results demonstrate that this herbal drug could represent a new source of antimicrobial agents, for the control of hospital acquired infections. However, more adequate studies must be carried out to verify the possibility of using it for fighting these bacteria in human body infections.

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