The aerial parts of *Stachys schtschegleevii* Sosn. as hydroalcoholic extract has antibacterial Activity on Multi-Drug Resistant Bacterial Isolates in Comparison to Ciprofloxacin

Peyman Mikaili [1], Nima H. Jazani [2] *, Jalal Shayegh [3], Negar Haghighi [4], Negar Aghamohammadi [4], Minoo Zartoshti [5]

[1.] Department of Pharmacology, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran
[2.] Center for food sciences and nutrition, Urmia University of Medical Sciences, Urmia, Iran
[3.] Department of Veterinary Medicine, Faculty of Agriculture and Veterinary, Shabestar branch, Islamic Azad University, Shabestar, Iran
[4.] Students Research Committee, Urmia University of Medical Sciences, Urmia, Iran
[5.] Dept. of Microbiology, Immunology and Genetics, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran
n_jazani@umsu.ac.ir, n_jazani@yahoo.com

Abstract: Several *Stachys* species have been used traditionally for their health benefits in various countries. About 34 species of *Stachys* are found in Iran. This plant possesses various medicinal properties, but there have been performed a few studies on the antibacterial effects of *Stachys schtschegleevii*. The aim of the present study was evaluation of the antibacterial activity of *Stachys schtschegleevii* on 100 Multi Drug Resistant isolates of *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella spp.* and *Escherichia coli*. *Stachys schtschegleevii* samples were collected from the fields of northwestern Iran. The flowering aerial parts of the plant were cut, and powdered, and then extracted by maceration at room temperature for 72 hours. Bacterial isolates were collected from clinical specimens from several wards of educational hospitals in Urmia, Iran during a 12 months period. The susceptibility of isolates to *Stachys schtschegleevii* extracts was determined using a broth microdilution method. Considering the wide application of ciprofloxacin in treatment of bacterial nosocomial infections, the antibacterial effect of ciprofloxacin on isolates was also determined. All the multi-drug resistant bacterial isolates were sensitive to different concentrations of *Stachys schtschegleevii* hydro-alcoholic extract. The most sensitive bacterial isolates to *Stachys schtschegleevii* extracts were *P.aeruginosa*, however, 69% of isolates were resistant to ciprofloxacin. The results demonstrated 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. Additionally, antifungal activity of this plant must be studied along with its antibacterial effect.
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1. Introduction

Resistant bacteria to the antimicrobials turned to be one of the most challenging problems of the recent century. The slogan of WHO in this year, is an evident support for this allegation. In this case, herbal medicines seem to be a new step toward fighting them. Iran is a country rich of medicinal plants and most of these plants are currently used by people as home remedies. Studying on antimicrobial activity of these herbs considered as noteworthy.

Stachys is a genus of shrubs and annual or perennial herbs. It belongs to family *Labiatae* that is considered as medicinal plant as an antipyretic, antiseptic, antispasmodic, astringent, carminative, diuretic, febrifuge, hypotensive, stomachic, styptic, tonic, vermifuge and antibacterial. It is taken orally as a medicinal tea in the treatment of fevers, diarrhea, sore mouth and throat, internal bleeding, and a tonic of liver and heart (Raymond M. Harley, 2004).

Stachys was named by Linnaeus in *Species Plantarum* in 1753 (Carolos, 1753).

The name is derived from the Greek word σταχυς (*stachys*), meaning "an ear of grain" (Umberto Quattrocchi, 2000) and refers to the fact that the inflorescence is often as a spike.

The plant's most useful constituents are betulinic acid, D-camphor, delphinidin, hyperoside, manganese, oleanolic acid, rosmarinic acid, rutin, ursolic acid, as well as various saponins and tannins. Also Preliminary phytochemical studies showed that the aerial parts of the genus *Stachys* contain flavonoids, which may be responsible for their antibacterial activity (M Saeedi, 2008).

Stachys are widely distributed in tropical and subtropical countries. Estimates of the number of

species in the genus vary from about 300 (Raymond M, 2004), to about 450 (David.J, 2008). 34 of them are found in Iran, of which 13 are endemic (Mozaffarian VA., 1996 and Rechinger KH. 1982). *Stachys schtschegleevii* is a native plants in Iran (Rechinger KH. 1982).

In the Iranian traditional medicine the extracts of the aerial parts of *Stachys schtschegleevii* (traditionally named *Poulk*) have been used in infectious, rheumatic and inflamamatory respiratory diseases (Shamsali Rezazadeh, 2005).

Since there are limited studies on the antibacterial effects of *Stachys schtschegleevii* extract on multi-drug resistant (MDR) bacteria, we evaluated its hydro-alcoholic extract on clinically resistant bacteria.

Resistant Gram-positive pathogens, such as *Staphylococcus aureus* have become a serious problem in clinical medicine. *S. 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 and long-lasting 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 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* are often spread and 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).

Pseudomonas aeruginosa is an opportunistic pathogen found as a 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 caused by *P. aeruginosa*. The problems of treating these infections is frequently complicated by antibiotic resistance, a problem that is increasing in the recent years.

Klebsiella spp. are 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 treating such infections; however, long-term therapy may result in many side-effects and cause selection of resistant bacteria. Thus, 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 goal in treatment of such infections (Perez et al., 2007).

In this study we evaluated the antibacterial activity of hydro-alcoholic extract of aerial parts of *Stachys schtschegleevii* on 100 Multidrug resistant isolates of *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella* and *E. coli*.

2. Material and Methods

For data collection, the junior medical students were grouped into several groups. Then, the information gathered from ethno-pharmacologists, herbal-drug sellers and rural native-healers, from different regions of Iran, especially Northwest, Southwest, Central and Northern provinces. All data collected, were summarized for every species. For each repeated report of a certain indication we added “a point” to the specification of that plant. If the number of every reported indication was more than 7-15 times we reported that indication or pharmacological effect in our final report in this article. We thought that, the higher frequency of the reports of an activity or indication, may mean the most reliable applications of that plant in Iranian traditional medicine. We report here, the plants used in Iranian traditional herbal medicine with cold nature.

3. Results

Extract preparation: *Stachys* samples were collected from the fields of West Azerbayjan province, the northwestern Iran, and identities were confirmed by the Botanist. The flowering aerial parts of the plant were cut, chopped, dried and powdered. The powders were extracted by maceration at room temperature for 72 hours. The hydro-alcoholic extracts were combined and concentrated to yield a dried powder, and it 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 *Stachys* extracts: The susceptibility of isolates to *Stachys* extracts was determined using a broth microdilution method based on CLSI guidelines. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Stachys* extracts for isolates were determined in Muller-Hinton Broth (MHB; Oxoid) medium (Jazani et al, 2009) (Papadopoulos et al., 2006). 10 mg of *Stachys* powder was dissolved in 1000 µL of Dimetylsufoxide (DMSO, Sigma). The initial concentration of *Stachys* 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 *Stachys* 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 *Stachys* 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).

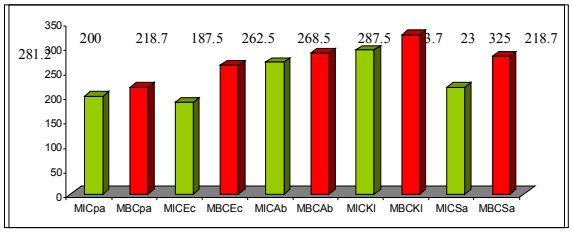

Fig 1: Antibacterial activity of *Stachys* hydroalcoholic extract against 100 nosocomial isolates of multi drug resistant gram negative and gram positive bacteria.

Pa: *Pseudomonas aeruginosa*, Ec: *E. coli*, Ab: *Acinetobacter baumannii*, Kl: *Klebsiella Sp*, Sa: *Staphylococcus aureus*. MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration.

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 *Stachys* hydroalcoholic extract has been shown in Figure 1**.**

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

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

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

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

4. Discussions and Conclusion

The antimicrobials have increasingly been 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 *Stachys schtschegleevii* extract on multi drug resistant bacteria.

Saaedi et al. performed a study to evaluate the antimicrobial activity of the methanol extracts of dried flowering aerial parts of *Stachys byzantina* , *S. inflata* , *S. lavandulifolia* and *S. laxa* (Labiatae) using the disc diffusion method and determination of (MIC) values against *Staphylococcus aureus*, *Streptococcus sanguis*, *Escherichia coli*, *Pseudomonas aeroginosa*, *Klebsiella pneumoniae*, *Aspergilus niger* and *Candida albicans*. The antimicrobial activities and MICs of *methanol extract of S. byzantine* (14.1%), *S. inflate* (14.3%), *S. lavandulifolia* (10.1%) and *S. laxa* (10.6%) exhibited concentration-dependent antibacterial activity against bacteria tested. The methanol extracts were more active against *Streptococcus sanguis* and *Staphylococcus aureus*. Their preliminary phytochemical studies also showed that the aerial parts of the genus *Stachys* contain flavonoids, which may be responsible for their antibacterial activity. (M Saeedi, 2008)

Chitsaz et al. studied aqueous and methanolic extracts of *St. schtschegleevi* on *Staphylococcus aureus*, *Streptococcus pyogen*, *E coli* and *Pseudomonas aeroginosa*, using well diffusion method and determining MIC and MBC by standard macro dilution NCCLS method.

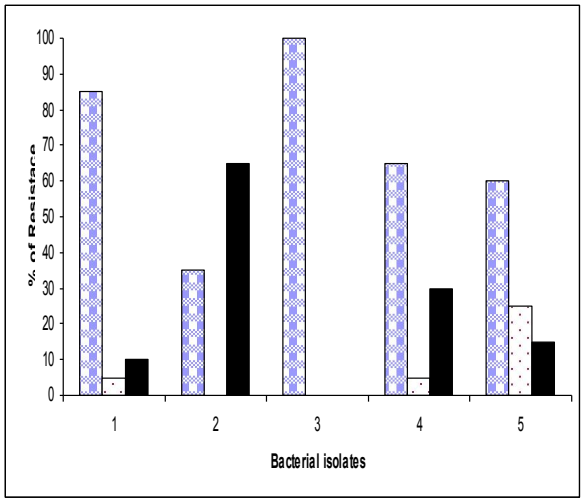

Figure 2: The rates of resistance to Ciprofloxacin for 100 clinical isolates of multi-drug resistant bacteria. Resistant (checked), Intermediate (spotted), Sensitive (black). 1: *Staphylococcus aureus,* 2: *E. coli,* 3: *Acinetobacter baumannii,* 4: *Klebsiella Sp.* And 5: *Pseudomonas aeruginosa.*

By well diffusion method, inhibition zone for *Staphylococcus aureus* and *Streptococcus pyogen* was 23.67 mm and 17.30mm, respectively. The corresponding values of MIC and MBC were 6.25, 12.5 for *Staphylococcus aureus* and 12.5, 12.5 for *Streptococcus pyogen*, respectively.

According to the results of their study, the aqueous extract had no anti-bacterial activity against tested bacteria and methanolic extract was not active against tested gram-negative bacteria (Chitsaz, 2006). However, in our study the most sensitive bacterium to *St. schtschegleevii* was *Pseudomonas aeroginosa*.

In another study, Helen D. Skaltsa *et al.* studied the antimicrobial activity of essential oil of eight *Stachys* species, including *St. alopecuros* (L.) Bentham., *St. scardica* (Griseb.) Hayek, *St. cretica* L. subsp. *cretica*, *St. germanica* L. subsp. *heldreichii*

(Boiss.) Hayek, *St. recta* L., *St. spinulosa* L., *St. euboica* Rech. and *St. menthifolia* Vis. The studied species were of bacteria including: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 35210), *Bacillus subtilis* (ATCC 10907), *Bacillus cereus* (clinical isolates), *Micrococcus flavus* (ATCC 10240), *Staphylococcus epidermidis* (ATCC 2228), as well as five fungi, including: *Aspergillus niger* (ATCC 6275), *Penicillium ochrochloron* (ATCC 9112), *Epidermophyton floccosum* (clinical isolates), *Candida albicans* (clinical isolates) and *Trichophyton mentagrophytes* (clinical isolates). The results of the study showed the tested essential oils had better activity against bacterial species than fungi, but *St. scardica* was the most active on both bacteria and fungi. *Pseudomonas aeruginosa* (Pa) was the most resistant strain. In addition, *Pseudomonas aeruginosa* (Pa) was the most resistant strain to all essential oils (Helen D. Skaltsa**,** 2003).

This is in contrast with our study's result, in which the most sensitive bacteria to *St. schtschegleevi* Sosn. was *Pseudomonas aeruginosa* (Pa).

In another similar study in Turkey, G Dugler and C Aki investigated antimicrobial activities of the ethanol extract of the leaves of *Stachys pseudopinardii*, *R. Bhattacharjee* (Lamiaceae), against *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 7064, *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 10538, *Proteus vulgaris* ATCC 6899, *Salmonella typhimurium* CCM 5445 and *Pseudomonas aeruginosa* ATCC 27853, as well as *Candida albicans ATCC 10239*, *Debaryomyces hansenii* DSM 70238, *Kluyveromyces fragilis* ATCC 8608 and *Rhodotorula rubra* DSM 70403, by disc diffusion and microdilution methods. In their study, all extracts showed strong antibacterial activity against *Bacillus cereus* ATCC 7064, with an inhibition zone of 25.0 mm (MIC=16 µg/mL) and (MBC=32 µg/mL). *Debaryomyces hansenii* DSM 70238 was among the most susceptible of the yeast cultures, with an inhibition zone of 17.0 mm and MIC=32 µg/mL and minimum fungicidal concentration (MFC) of 32 µg/mL. The extract exhibited moderate activity against the other test microorganisms (G Dugler and C Aki,2009).

As the study showed, the ethanol extract of the leaves of *Stachys pseudopinardii*, had significant antimicrobial activity and it may be useful in the treatment of mentioned infections. Therefore, we can test our extracts against fungi, too.

In the present study all the multi-drug bacterial isolates were sensitive to different concentrations of *Stachys* hydroalcoholic extract, the most sensitive bacterial isolates to *Stachys* extracts were *P. aeruginosa* isolates (Fig 1). Also *A.calcuaceticus* PTCC 1318, *E.coli* ATCC25922 and *S.aureus* ATCC 25923 and *Klebsiella pneumoniae* ATCC10031 were the most sensitive strains among the standard isolates (MIC= MBC=125) (Table 1), however clinical isolates showed high resistance to ciprofloxacin (Figure 3).

In the present study results showed that the *Stachys* hydroalcoholic extract possessed antibacterial effect against all multi-drug resistant bacteria 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, studies that are more adequate must be carried out to verify the possibility of using it for fighting these bacteria in human body infections.

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Corresponding Author

Dr. Nima H. Jazani

Dept. of Microbiology, Immunology and Genetics, Faculty of Medicine,

Urmia University of Medical Sciences,

Nazloo Road, Urmia, Iran

E-mails: n_jazani@umsu.ac.ir, n_jazani@yahoo.com

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