

Antibacterial activity of different extracts from *Stachys turcomanica* aerial parts on typical food-borne pathogens

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Abstract

The present study was designated to evaluate the antibacterial activities of aqueous, methanolic and hydro alcoholic extracts from aerial parts of *Stachys turcomanica* against different pathogenic bacteria using broth micro-dilution and Agar Well Diffusion (AWD) assay. The extracts of *S. turcomanica* were active against the gram-positive bacteria, *Staphylococcus aureus* (inhibition zone = 16.5 mm) and virtually inactive against the other gram-positive and gram-negative bacteria (*Listeria monocytogenes*, *E. coli* and *S. typhimurium*). The hydro-alcoholic extract of this plant in broth micro-dilution assay, showed higher antimicrobial activity compared to aqueous and methanolic extracts (MIC= 12.5 mg ml⁻¹ for *E. coli* and *S. typhimurium*). Results showed good antibacterial activity for this extract on *S. aureus*, which suggest its capacity as a natural food preservative.

Keywords: *Listeria monocytogenes*, *E. coli* and *S. typhimurium* extracts, antibacterial activity, methanolic extracts and AWD

Introduction

Foodborne infections have been one of the major public health concerns worldwide and account for considerably high cases of illnesses. Recent studies report that *Bacillus cereus*, *Campylobacter jejuni*, *Escherichia coli*, *Salmonella*, *Shigella* and *Staphylococcus aureus* are considered to be the most frequent pathogens (Voravuthikunchai *et al.*, 2006). Also the increasing antibiotic resistance of some pathogens that are associated with diseases has increased the interest in the development of new types of effective and nontoxic antimicrobial compounds (Sobhy and El-Feky, 2007). The addition of chemical preservatives has long been an effective method to

control microbial contamination and the development of oxidative reactions, although in recent years, popular demand has shown a marked aversion to such synthetic chemical preservatives. This has resulted in a growing demand for natural products, principally, plant extracts, which are, in the consumer's mind, safer, functional and provide nutritional and health benefits. This demand has increased the importance of searching for alternative sources of natural preservatives rich in phenolic compounds (Viuda-Martos *et al.*, 2012).

A variety of plant species have been known to synthesize many bioactive secondary metabolites with antimicrobial and biological properties, like alkaloids, terpenoids (triterpenes and steroid saponins), phenolic

compounds, glycosides, flavonoids, tannins, and polysaccharides. In addition, phytomedicines are eco-friendly, inexpensive, easily prepared, and mitigate many of the side-effects that are often associated with synthetic antibiotics. Regarding the antibacterial activity of plant extracts/essential oils, the researchers have described several mechanisms of action, including cell membrane damage resulting in increased permeability, changes in intracellular pH and membrane potential, dissipation of cellular components, decrease in the cytoplasmic ATP concentration, which together induce bacterial death. Secondary effects that may be involved seem to be the inhibition of enzymes, loss of turgor pressure, alterations in macromolecules synthesis, and other cellular processes (Bulfon *et al.*, 2014).

The genus *Stachys* (Lamiaceae) includes about 200 – 300 species in the world (Khanavi *et al.*, 2009). This sub-cosmopolitan genus has two main centers of diversity in the Old World area. One is confined to *S. and E. anatolia*, Caucasia, N.W. Iran and N. Iraq, and the other to the Balkan Peninsula. Serbia is an area that belongs moderately rich in taxa belonging to the genus (seventeen species are acknowledged), however, eight species are recognized as endemic to the Balkans, or even narrower regions (Lazarevic *et al.*, 2010). In Iran, this genus is represented by 34 species (Mozaffarian *et al.*, 1996). Phytochemical studies on *Stachys* species have shown the presence of polyphenols including flavonoids, tannins, phenolic acids, and phenyl ethanoid glycosides. Many studies have shown various activities in this genus such as anti-inflammatory, anti-anxiety, antibacterial, anti-nephritic, anticancer, anti-helicobacter pylori, and anti-oxidant effects. Some species of this genus are used in folk medicine, especially *S. paalustris*, and *S. sylvatica* which are approved for healing wounds,

treating abdominal pains and as disinfectant, anti-spasmodic and anti-fever (Khanavi *et al.*, 2009). There was an investigation about the essential oil of *S. turcomanica*; the major constituents were identified as germacrene D (17.4 %), 7-epi-a-selinene (10.5%), -elemene (9.2%) and -pinene (8.6 %) (Firouznia *et al.*, 2009; Khanavi *et al.*, 2012).

The aim of this study was to determine the antibacterial activity of different extracts of aerial parts of *S. typhimurium* including hydro-alcoholic, aqueous and methanolic extracts on food-borne pathogens.

Material and Methods

Chemicals and Plant materials

Gentamicin (Sina daroo, Iran), methanol and Dimethyl Sulfoxide (DMSO) (Merck, Germany), Muller Hinton Agar and Muller Hinton Broth (Merck, Germany) were purchased. The aerial part of plant was collected in 2014 from the mountains of North Khorasan Province in Iran. The plant was identified by the Research Center of Natural Products Health (NPH), North Khorasan University of Medical Sciences (Iran).

Preparation of the methanolic extract

Plant samples were dried at room temperature under shade (Umer *et al.*, 2013; Yaghooti and Mohamadi Sani, 2015) and blended into fine powder and stored in the dark at a dry place. 30 g of the powdered aerial parts of the plant were extracted in 500 ml of methanol, water and hydro-alcohol for 72 h under agitation at room temperature of 23°C (Bousselsela *et al.*, 2012). The extracts were concentrated by rotary evaporation at 40°C (Sanches *et al.*, 2005) and the yield of extraction was determined. All the dried extracts were preserved in the refrigerator (Bousselsela *et al.*, 2012) at 4°C (Yaghooti and Mohamadi Sani, 2015) until further use. Plant extracts were dissolved in the solvent before use in the antimicrobial assay (Bousselsela *et al.*, 2012) (Table -1).

Table - 1. Yield of extraction

Weight of plant	Solvent	Weight of extracts	Yield of extraction
30 g	Methanol (100%)	6.37 g	21.24 %
30 g	Water (100%)	3.11 g	10.37 %
30 g	Hydro-alcohol (80%)	8.19 g	27.33 %

Organisms and inoculation conditions

The methanolic, aqueous and hydro-alcoholic extracts of *S. turcomanica* were individually tested against 4 bacteria strains, including *Staphylococcus aureus* (PTCC 1431), *Listeria monocytogenes* (PTCC 1298), *Salmonella typhimurium* (PTCC 1709) and *Escherichia coli* (PTCC 1399) which were obtained from the Persian Type Culture Collection, Iranian Research Organization for Science and Technology (PTCC, Iran). Bacterial strains were cultured overnight at 37°C in Mueller Hinton agar Yesil (Celiktas et al., 2007).

Antimicrobial assay

The antibacterial activity of the extracts was studied using the Agar well diffusion (AWD) assay (Kasim et al., 2014) and micro-dilution methods (Costa et al., 2009; Pandey et al., 2011; Ramalivhana et al., 2014) against three laboratory standards, including *Staphylococcus aureus* (PTCC 1431), *Listeria monocytogenes* (PTCC 1298), *Salmonella typhimurium* (PTCC 1709) and *Escherichia coli* (PTCC 1399) (Habibi et al., 2010). All tests were performed in two replicates.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Test

The minimal inhibitory concentrations (MICs) of all the extracts and reference antibiotics (Gentamicin Sina daroo, Iran) were determined by micro dilution techniques in Mueller-Hinton broth (Merck) (Sanches et al., 2005). The 96-well plates were prepared by dispensing into each well (Laciar et al., 2009) where placed 100µL of the extracts solution in DMSO (Rodrigues et al., 2012; Salvat et al., 2004). After that,

was added 100 µL of Mueller-Hinton (MH) broth and 20 µL of the inoculum (Laciar et al., 2009) (standardized at 1.5×10^6 CFU/ml by adjusting the optical density to 0.1 at 600 nm by Shimadzu UV-120-01 Spectrophotometer). One hundred microliter aliquot from the stock solutions of the extract and their serial dilutions initially prepared was transferred into the wells. The final volume in each well was 220 µL. The plates were covered with sterile plate sealer (2009) and followed by shaking for 30 s (Bento et al., 2009) and then incubated at 37°C for 24 hr (Laciar et al., 2009). Micro titer plates were then incubated at 37°C for 24 hr. After incubation, the wells were examined for microbial growth (Rodrigues et al., 2012; Naveed et al., 2013). MIC was defined as the lowest concentration of the EO in the medium in which there was no visible growth after incubation (no red color signifying live growth) (Laciar et al., 2009; Liu et al., 2014).

Determination of minimum bactericidal concentration (MBC)

MBC is defined as the lowest concentration which no growth was noted on Muller-Hinton agar (Bento et al., 2009). For the determination of the MBC, 50 µL of the four last concentrations of the extract were removed, in the micro-dilution plate with growth absence, and were inoculated in a plate contend (Costa et al., 2009). The same ones were incubated at 37°C during 24 hours and, after this, the reading was made. The minimum bactericidal concentration (MBC) was considered as the starting point that did not allow to the reactivation of the microorganism in the medium without antimicrobials (Costa et al., 2009).

Table – 2. MIC (mg ml⁻¹) for different extracts of *S. turcomanica*

Type of extract	<i>S. aureus</i>	<i>L. monocytogenes</i>	<i>S. typhimurium</i>	<i>E. coli</i>
Methanolic	50	50	25	25
Aqueous	50	50	25	25
Hydro-alcoholic	25	25	12.5	12.5

Values are the mean of two replicate

Table - 3. MBC (mg ml⁻¹) for different extracts of *S. turcomanica*

Type of extract	<i>S. aureus</i>	<i>L. monocytogenes</i>	<i>s. typhimurium</i>	<i>E. coli</i>
Methanolic	100	>100	>100	>100
Aqueous	100	>100	>100	>100
Hydro-alcoholic	100	>100	>100	>100

Values are the mean of two replicate

Agar-Well Diffusion Assay

Agar-well diffusion method by Durodola (1977) and Cheruiyot *et al.* (2009) was used in this microbiological assay.

Petri plates containing 20 ml of Mueller Hinton Agar were seeded with samples of 24 hr broth cultures of the bacterial isolate. Cultures of *Staphylococcus aureus* (PTCC 1431), *Listeria monocytogenes* (PTCC 1298), *Salmonella typhimurium* (PTCC 1709) and *Escherichia coli* (PTCC 1399) were inoculated separately on the surface of Mueller Hinton agar plates by surface spreading using a sterile cotton swab and each bacterium evenly spread over the entire surface of an agar plate to obtain a uniform inoculum. The sensitivity testing of the extracts was done using the agar well diffusion method whereby, wells of 6 mm diameter and 5 mm depth were made on the solid agar using a sterile glass borer and Gentamicin (Sigma, UK) (10 µg/ml). 50 µl of the plant extracts were tested after (Elkhalfi *et al.*, 2013). All the tests were run in triplicates for quality results. The set up was incubated for 24 hr at 37°C for anti-microbial test and the zones of inhibition were measured using a ruler (AIM®) and a pair of divider, and then results reported in millimeters (mm). The inhibition was measured as a basis for activity (Kasim *et al.*, 2014).

Results

Results of MIC and MBC

The minimum inhibitory (MIC) and bactericidal (MBC) concentrations, two parameters that respectively quantify the bacteriostatic and bactericidal potential of bioactive compounds, were determined using dry extracts and the serial dilution method for the 3 plant extracts with a substantial antibacterial activity against the *S. aureus*, *L. monocytogenes*, *S. typhimurium* and *E. coli*. As shown in Table - 2 and 3, the obtained values were in the ranges of 12.5 - 50 mgml⁻¹ for MIC and 100 and more than 100 mgml⁻¹ for MBC, and on the whole are in agreement with the inhibitory activities shown above. The hydro-alcoholic extract was the strongest bacteriostatic effects on the bacteria test and gram-negative bacteria were the most sensitive to this extract.

Results of Agar Well Diffusion (AWD) assay

The results of the antimicrobial activities of the extracts by using Agar Well Diffusion (AWD) assay are summarized in Table - 4. All extracts were virtually inactive against the *L. monocytogenes*, *S. typhimurium* and *E. coli* except *S. aureus*. The hydro-alcoholic was the strongest extract which showed the highest inhibition zone on *S. aureus* (11.5 mm).

Table - 4. AWD assay and inhibition zones (mm) for different extracts of *S. turcomanica*

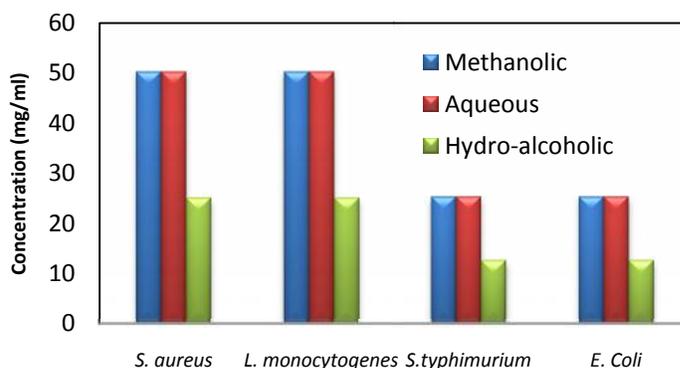
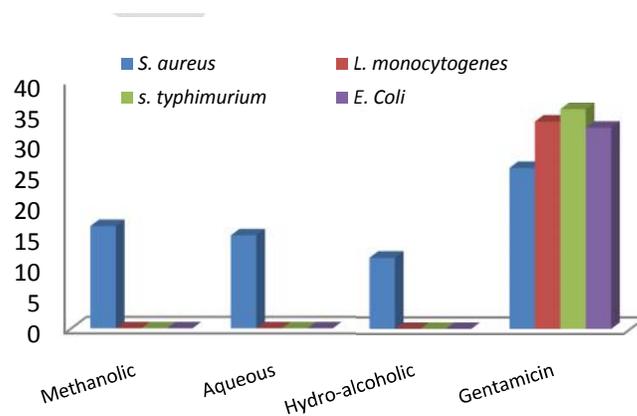
Type of extract	<i>S. aureus</i>	<i>L. monocytogenes</i>	<i>s. typhimurium</i>	<i>E. coli</i>
Methanolic	16.5	-	-	-
Aqueous	15	-	-	-
Hydro-alcoholic	11.5	-	-	-
Gentamicin	26	33.5	35.5	32.5

Values are the mean of two replicate

Discussion

Natural products, such as a plant extract, either as pure compounds or as standardized extracts, provide unlimited opportunities for control of microbial growth owing to their chemical diversity. Besides antimicrobial, several plants are being used in different areas of human health such as traditional medicine, functional foods, dietary supplements and recombinant protein manufacturing. Phytochemicals, especially flavonoids, polyphenols, anthocyanins and carotenoids, share the major market (Negi, 2012). Flavonoids (derivatives of phenylchromone ring) are a large group of compounds naturally occurring in higher and lower plants. Flavonoids have been shown to be able to affect various biological functions: capillary permeability, cellular secretory processes involved in the inflammatory response and inhibition of enzymes, receptors and carriers (Sanches *et al.*, 2005).

Some studies show that gram-negative bacteria are more resistant to essential oil others claim the same for gram-positive bacteria. In our study the gram-negative bacteria were in both categories (Klein *et al.*, 2013). The internal stability of the bacterial cells depends on the interaction between a series of physiological factors, and the disturbance of this stability, may determine the bacteria's death or the inhibition of its growth. To provide products, which reduce the toxicity risk and at the same time are obtained from a new natural and renewable source and become a growing and economically viable option.

**Fig.-1. MIC (mg ml⁻¹) of extracts from *S. turcomanica*****Fig. - 2. Parameters of Agar Well diffusion (AWD) assay (mm) in *S. turcomanica* extracts**

The use of vegetal extracts for antibacterial activity is a consummated fact (Nogueira *et al.*, 2014).

Conclusion

The results of this study indicated that extracts obtained from aerial parts of *S. turcomanica* possess antibacterial properties. On the basis of the experimental results, it can be postulated that the extracts of *S. turcomanica* have the potent anti-bacterial

properties against some representative food-borne pathogens. Specifically, the hydro-alcoholic extract was more active against gram-negative bacteria which indicate the presence of active compounds. Therefore, it could be used as possible food anti-microbial preservative in food industry, but the *in vivo* studies should be done to evaluate the probable adverse effect on food sensory properties.

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