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ANTIBACTERIAL AND FREE-RADICAL-SCAVENGING PROPERTIES OF *STACHYS SCHTSCHEGLEEVII* (LAMIACEAE)

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Abstract – *Stachys schtschegleevii* Sosn. (Lamiaceae alt. Labiatae) is one of 34 *Stachys* species found in Iran, and is morphologically similar to *Stachys inflata*. This plant has been used in the Iranian traditional medicine as a remedy for bacterial infections, rheumatic fever and other inflammatory conditions. The *n*-hexane, dichloromethane (DCM) and methanol (MeOH) extracts of the non-flowering aerial parts of this plant were assessed for their antibacterial and free-radical-scavenging activities using the micro-titer-based antimicrobial assay incorporating resazurin as an indicator of cell growth and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, respectively. While the *n*-hexane extract did not show any free-radical-scavenging activity, the MeOH extract showed the highest level of activity with a RC_{50} value of 2.94 x 10^{-2} mg/mL, which was about ten-fold less active than the positive control Trolox® (RC_{50} = 2.60 x 10^{-3} mg/mL). None of the extracts showed any antibacterial property against *Bacillus cereus* and *B. subtilis* at test concentrations. However, all extracts were active against ampicillin-resistant *Escherichia coli* and *Staphylococcus aureus*. The MeOH extract was the most potent (MIC range 1.56–6.25 mg/mL) among the extracts and was most active against ampicillin-resistant *E. coli* (MIC = 1.56 mg/mL).

Key words: Stachys schtschegleevii, Lamiaceae, antibacterial activity, free-radical-scavenger, activity, resazurin assay, DPPH assay

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INTRODUCTION

Stachys schtschegleevii Sosn. (Lamiaceae alt. Labiatae) is one of the 34 Iranian *Stachys* species (>300 species world-wide), and is morphologically similar to *Stachys inflata* (Rechinger, 1982; Mozaffarian, 1998; Rezazadeh et al., 2005) This plant, commonly known as 'Poulk', has been used in Iranian traditional medicine as a remedy for bacterial infections, rheumatic fever and respiratory inflammatory diseases (Rezazadeh et al., 2005). The analgesic and anti-inflammatory properties of the methanolic extract of the aerial parts of *S. schtschegleevii* have recently been reported (Rezazadeh et al., 2005). The antibacterial property of the essential oil obtained from the leaves of this plant is also known (Sonboli et al., 2005). Previous phytochemical studies on *S. schtschegleevii* revealed the presence of phenolic compounds (Nazemieyh et al., 2006), and mono- and sesquiterpenes (Norouzi-Arasi et al., 2004; Sonboli et al., 2005). In continuation of our bioactivity and phytochemical studies on the medicinal plants from the Iranian flora (Delazar et al., 2004; Delazar et al., 2007a,b; 2006a-d; 2010; Fathiazad et al., 2006; Nazemiyeh et al., 2006; 2008; Sarker et al., 2008; Asnaasahri et al., 2010), we now report on the antibacterial and free-

radical-scavenging activities of the *n*-hexane, dichloromethane (DCM) and methanol (MeOH) extracts of the non-flowering aerial parts of *S. schtschegleevii* using the micro-titer-based antimicrobial assay incorporating resazurin as an indicator of cell growth (Sarker et al., 2007) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Kumarasamy et al., 2002; 2007), respectively.

MATERIALS AND METHODS

Plant Materials

The non-flowering aerial parts of *Stachys schtschegleevii* Sosn. were collected from Hassan Abad Forest (Kaleibar, north-west of Iran) in July 2004. A voucher specimen (TUM-FPh 150) for this collection has been retained in the herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences, Iran.

Extraction of plant material

The ground non-flowering aerial parts of *S. schtschegleevii* (98.7 g) were Soxhlet-extracted sequentially with 500 mL of *n*-hexane, dichloromethane (DCM) and methanol (MeOH). Filtered extracts were dried using a rotary evaporator at 45° C. Extracts were re-suspended in MeOH to achieve the stock concentration 10 mg/mL for bioassays.

Antibacterial activity

The antibacterial activity of the extracts were assessed against six bacterial strains, *Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (NCTC 10400), *Escherichia coli* (ATCC 8739), ampicillin-resistant *Escherichia coli* (NCTC 10418), *Staphylococcus aureus* (NCTC 1803) and *Salmonella typhi* (NCTC 10203), obtained from the Biotechnology Laboratory, School of Biomedical Sciences, University of Ulster.

Disc diffusion assay

A conventional disc diffusion method (Bauer et al., 1966; Cruickshank, 1968) was employed for the initial assessment of antibacterial potential of the extracts. Sterile 6.0 mm diameter blank discs (BBL, Cocksville, USA) were impregnated with test substances at a dose of 500 µg/disc. These discs, along with the positive control disks (ciprofloxacin, 10 µg/disc) and the negative control disks, were placed on Petri dishes containing a suitable agar medium seeded with the test organisms using sterile transfer loop and kept at 4°C to facilitate maximum diffusion. The plates were kept in an incubator (37°C) to allow growth of the bacteria. The antibacterial activities of the test agents were determined by measuring the diameter of the zone of inhibition in terms of millimetre.

Resazurin micro-titre assay

The 96-well micro-titre assay using resazurin as the indicator of cell growth (Sarker et al., 2007; Genest et al., 2008) was employed for the determination of the minimum inhibitory concentration (MIC) of the active extracts.

Assessment of bacteriostatic/bactericidal property

Agar plates were seeded with the mixture from the well which was just before the well of the MIC, using sterile transfer loop and kept at 4°C to facilitate maximum diffusion. The plates were kept in an incubator (37°C) to allow growth of the bacteria. Any bacterial growth would indicate the bacteriostatic property of the extract, and no growth would be an indicator of bactericidal activity.

Free-radical-scavenging assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH), molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie AG, Bucks. Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Sigma-Aldrich, UK. The method used by Takao et al. (1994) was adopted with suitable modifications (Kumarasamy et al., 2002). DPPH (8 mg) was dissolved in MeOH (100 mL) to obtain a concentration of 80 µg/mL.

Qualitative assay

Test sample solutions were applied on a pre-coated silica gel TLC plate, sprayed with DPPH solution

using an atomizer, and left for 30 min for any color to develop. The color changes (purple on white) were recorded.

Quantitative assay

Serial dilutions were carried out with the stock solutions (10 mg/mL) of the plant extracts to obtain concentrations of $5x10^{-1}$, $5x10^{-2}$, $5x10^{-3}$, $5x10^{-4}$, $5x10^{-5}$, $5x10^{-6}$ $5x10^{-7}$, $5x10^{-8}$, $5x10^{-9}$ and $5x10^{-10}$ mg/mL. Diluted solutions (2 mL each) were mixed with DPPH (2 mL), and allowed to stand for 30 min for any reaction to take place. The UV absorbance was recorded at 517 nm. The experiment was carried out in triplicate and the average absorption was calculated for each concentration. The same procedure was followed for the positive control Trolox®. The RC_{50} value, which is the concentration of the test material that reduces 50% of the absorbance of the stable free-radical DPPH at 517 nm, was calculated as mg/mL.

RESULTS AND DISCUSSION

The conventional disc diffusion method was applied to assess any antibacterial property of *n*-hexane, DCM and MeOH extracts of the non-flowering aerial parts of *S. schtschegleevii* (Table 1). None of the extracts showed any antibacterial property against *Bacillus cereus* and *B. subtilis* at test concentrations. However, all extracts were active against ampicillin-resistant *Escherichia coli* and *Staphylococcus aureus*.

The minimum inhibitory concentration (MIC) values of the extracts were determined by the resazurin micro-titer assay (Sarker et al., 2007). The MeOH extract was the most potent (MIC range 1.56–6.25 mg/mL) among the extracts, and was most active against ampicillin-resistant *E. coli* (MIC = 1.56 mg/mL) (Table 1). The active extracts were found to be bacteriostatic rather than bactericidal. This finding was similar to the bacteriostatic activity of *Stachys glutinosa* against *Vibrio cholerae* reported recently (Giorgio et al., 2006). However, unlike any mono- or sesquiterpenes, which are generally nonpolar in nature, the antibacterial activity of *S. schtschegleevii* was mainly due to medium polarity or polar compounds, e.g. phenolics, present in the polar MeOH extract. The antimicrobial activity was also reported with other species of the genus *Stachys* (Skaltsa et al., 2003; Benli et al., 2007; Salehi et al., 2007).

The DPPH antioxidant assay is based on the ability of 2,2-diphenyl-1-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of free-radical-scavengers. The odd electron in the DPPH radical is responsible for the absorbance at 517 nm, and also for visible deep purple color (Kumarasamy et al., 2007). When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. In the TLC-based qualitative free-radical-scavenging assay using the DPPH spray, the DCM and MeOH extracts of *S. schtschegleevii* exhibited prominent antioxidant properties indicated by the presence of a yellow/white spot on a purple background on the TLC plates. The *n*-hexane extract did not show any detectable activity. The DPPH scavenging capacity of the extracts was compared with known antioxidant, Trolox®. The DPPH radical scavenging activities of the positive control as well as the test extracts are presented in Table 1.

In the quantitative DPPH assay, while the MeOH extract displayed a strong free-radical-scavenging activity with a RC_{50} value of 2.94×10^{-2} mg/mL, the RC_{50} value of the DCM extract was 3.24×10^{-1} mg/mL. The RC_{50} value of the positive control Trolox® was 2.60×10^{-3} mg/mL. The free-radical-scavenging potential of the MeOH extract was about ten-fold less than that of the positive control on the basis of the RC_{50} values. The results obtained in this study (Table 1) indicate that the significant free-radical-scavenging activities were associated with the medium polarity and polar extracts, e.g. DCM and MeOH. This suggested that the compounds responsible for the free-radical-scavenging properties of these plant extracts were possibly due to the phenolic compounds, e.g. flavonoids and phenylethanoid glycosides, previously reported from this plant (Nazemiyeh et al., 2006). The free-radical-

Table 1. Antibacterial and free-radical-scavenging activities of the extracts of *Stachys schtschegleevii*.

Extracts	*Free-radical-scavenging activity*[a]		Antibacterial activity									
	Qualitative	*Quantitative (RC_{50} in mg/mL)*	Disc diffusion assay (Zone of inhibition in mm)						Resazurin assay (MIC in mg/mL)			
			BC	BS	EC	AEC	SA	ST	EC	AEC	SA	ST
n-Hexane	+	-	-	-	-	8	12	-	-	25.0	12.5	-
DCM	+	3.24×10^{-1}	-	-	7	8	10	7	25.0	25.0	6.25	12.5
MeOH	+	2.94×10^{-2}	-	-	10	10	10	10	3.12	1.56	3.12	6.25
Trolox	+	2.60×10^{-3}	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Ciprofloxacin	NA	NA	32	30	30	30	30	30	2.5×10^{-7}	2.5×10^{-6}	2.5×10^{-8}	2.5×10^{-7}

[a] Determined by the DPPH assay.
= No activity detected at test concentrations; + = Activity
NA = Not applicable
BC = *Bacillus cereus* (ATCC 11778), BS = *Bacillus subtilis* (NCTC 10400), EC = *Escherichia coli* (ATCC 8739), AEC = Ampicillin-resistant *Escherichia coli* (NCTC 10418), SA = *Staphylococcus aureus* (NCTC 1803) and ST = *Salmonella typhi* (NCTC 10203).

scavenging properties were also previously reported from a number of other species of the genus *Stachys*, and in most of these cases, the activity was due to the presence of polyphenolic compounds (Morteza-Semnani et al., 2006; Haznagy-Radnai et al., 2007; Vundac et al., 2007; Asnaashari et al., 2010).

The present findings support, at least to some extent, the traditional uses of this plant, particularly its use for the treatment of bacterial infections and inflammation.

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