SCREENING OF EXTRACTS AND FRACTIONS FROM AERIAL PARTS OF *STACHYS SCHTSCHEGLEEVII SOSN.* FOR ANTI-INFLAMMATORY ACTIVITIES

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ABSTRACT

Stachys schtschegleevii Sosn. is a native plant widely distributed in Iran and belongs to family of Lamiaceae and genus of Stachys. The plant is used in Iranian folk medicine in infective, rheumatic and other inflammatory disorders. In the present study the anti-inflammatory properties of different extracts and components isolated from aerial parts of *Stachys schtschegleevii Sosn.* were investigated. Intraperitoneal injection of hydroalcoholic extract 60 min before the induction of carrageenan-induced rat paw oedema significantly reduced the maximal oedema response attained during 4 hr and the total oedema response. A low dose of chloroform extract (100 mg/kg) caused significant inhibition of the carrageenan-induced inflammation, whereas a high dose of 400 mg/kg produced a pro-inflammatory response. One of the ethyl acetate extract caused a potent and dose-related inhibition of inflammation. This extract was fractionated into 11 major fractions according to increasing polarity of solvent mixtures. These results suggest that the hydroalcoholic extract of aerial parts of *Stachys schtschegleevii* attenuate the inflammatory response. The compounds in different fractions have also been identified to exhibit anti-inflammatory activity through thin layer chromagoraphy.

Keywords: *Stachys schtschegleevii Sosn.*, anti-inflammatory, phenylethanoid glycosides.

INTRODUCTION

The genus *Stachys* (Lamiaceae) is a woody annual or biennial herb, with 30 cm height and pink flowers. Whole plant densely covered with long white silky hairs, giving it a whitish appearance. *Stachys. Schtschegleevii* Sosn. is a native Iranian plant (Rechinger, 1982), and popularly known as "Sonbeleh Argavan", "Sonbeleh Badkonaki", and "poulk". Extracts obtained from the aerial parts of *S. schtschegleevii* have been used traditionally in North West of Iran in infective, asthma, rheumatic and other inflammatory disorders (Mozaffarian, 1982). Previous phytochemical studies have demonstrated the presence of several classes of compounds in the genus *Stachys*. These include phenylethanoid glycosides (Miyase *et al*., 1996; Nishimura *et al*., 1991; Takeda *et al*., 1997), diterpenes (Adinolfi *et al*., 1984), triterpenoids, steroids (Ross *et al*., 1975; Yamamoto *et al*., 1994) and flavonoids (Zinchenko, 1970; Lenherr *et al*., 1984; EL Ansari *et al*., 1991; EL Ansari *et al*., 1995; Skaltsa *et al*., 2000; Skaltsa *et al*., 2001). In the present study the anti-inflammatory properties of different extracts and components isolated from aerial parts of *Stachys schtschegleevi Sosn.* were investigated. Ethyl acetate, the most potent one, and chloroform extracts were subjected to chromatographic studies in order to sub-fractionation and identification of their active constituents.

METHODS AND MATERIALS

Extraction

Stachys schtschegleevii was collected in Iran (Kaleibar, Aynalu -1755 meter, Azerbaijan) and dried at room temperature. A herbarium specimen (TbzM-Fph 140) has been deposited at Tabriz University of Medical Sciences in Faculty of Pharmacy, Department of Pharmacognosy. Air-dried and finely powdered plant material (400 g), which consist of aerial parts from non-flowering stems of the plant, were extracted four times with 600 ml of 70% MeOH-H_2O while being macerated at room temperature for 48 hours each time (hydroalcoholic extract). The extracts were combined and methanol was evaporated, using a rotary evaporator under low pressure at 50°C. The resulting aqueous residue was extracted, successively, with chloroform ($CHCl_3$), ethyl acetate (EtOAc) and water saturated n-butanol (n-BuOH) (4×200 ml each). The solvents of the extracts were evaporated and residues were kept at 4°C until used. The dried extracts were dissolved in saline or DMSO in saline (1:4) and passed through a weighed paper filter. The filtered solutions were used for injection. Following the filtration, the filter was dried, weighed again and to obtain the real concentration of the extract, the unfiltered particles were calculated.

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Fractionation of ethyl acetate extract (EtOAc)

Dried EtOAc extract was adsorbed on silica gel, and subjected to column chromatography, eluting with EtOAc-$CHCl_3$-MeOH mixtures to afford 24 fractions, (40:10:0; frac. f1-f6), (40:10:10; frac. f7-f14), (40:10:20, frac. f15-f21) and (40:10:40; frac. f22-f24). The column was then eluted with EtOAc-water-MeOH (40:10:40) to obtain fractions f25-f33. Finally it was washed with MeOH-water (70:30) to yield fractions f34-f38. All collected fractions (~100 ml portions) were monitored on TLC plates using EtOAc: MeOH: water (85:13:2) and EtOAc: formic acid: water (80:10:10). $AlCl_3$ and $FeCl_3$ spray reagents were used to ascertain fractions containing flavonoids and caffeic acid derivatives, respectively, and were mixed to afford 11 main fractions, designated as F1-F11.

Analytical techniques

Separation of individual compounds in EtOAc and chloroform extracts was done by prep- HPLC. The structures of isolated compounds were determined by comparison of their physical properties (UV, IR, MS, ^1H and ^{13}C-NMR, HMQC, HMBC) with those of published data. The 5% $AlCl_3$ solution, when sprayed over a dried chromatogram, reveals all 5-hydroxy-flavonoids (Markham, 1982) as fluorescent yellow spots when viewed under U.V. light (366 nm). The 2% $FeCl_3$ solution also reveals caffeic acid derivatives as gray to black spots on the chromatogram. The main flavonoides and caffeic acid derivatives were further purified using prep-HPLC. A Shimadzu LC-8A HPLC with C_{18} column (CLC Shim-pack prep-ODS, 20x250mm, 15µ) and SPD-M10A-vp detector (detection at 220 and 280 nm) were used. The mobile phase, running at 20ml/min consisted of a linear gradient of 30-70% methanol for 50 min followed by 70-90% methanol for 2min and 90% methanol for 10 min. The isolated compounds were identified using UV/Visible and NMR spectroscopy. Ultraviolet analysis was performed using a Shimadzu 2100 spectrophotometer. NMR spectra were recorded in DMSO-6d on 300 MHz Brucker spectrometer. Fraction 11 was the result of washing the column by MeOH 70%, so it was full of polar components and also trace of different salts.

Carrageenan-induced paw oedema

Carrageenan-induced rat paw oedema was used as an acute inflammation model. Male wistar rats received a subplantar injection in the right hind-paw of 100µl of 1% carrageenan in saline. Footpad volume was measured by plethysmometer (UGO BASILE 7140, Italy) prior to carrageenan injection and then at hourly intervals from 1-4h afterwards (Maleki *et al*., 2001). Data are expressed as percent increase in paw volume compared to the pre-injection values. The inflammatory response in the drug-treated and control groups were also measured as the area under the time-course curves (AUC). Fine suspensions of the hydro-alcoholic, $CHCl_3$, n-BuOH and EtOAc extracts (100-400 mg/kg) and its subfractions in saline were administered intraperitoneally (i.p). Fractions were used in doses of 15, and 30 mg/kg (nearly equivalent to 1/10 to 1/20 of total EtOAc extract dose; n=6). Because of the limited amounts obtained from column chromatography, only one dose of some fractions was studied. Each dose was administered in a total volume of 0.5 ml, one hour prior to the induction of oedema. The control animals received drug vehicle. Indomethacin treated rats (2.5 mg/kg) were used as positive control. The inflammatory response in the drug-treated and control (vehicle-treated) groups were measured as both the paw swelling attained during 4h of the oedema response and the area under the time-course curves (AUC).

STATISTICS

All results are expressed as mean ± standard error of the mean (S.E.M.). Carrageenan-induced inflammation was assessed by one-way analysis of variance (ANOVA), and the significant differences were examined by the Newman-Keuls range test. To compare the area under the curve between groups, the Mann-Whitney non-parametric U-test was employed. Differences between groups were considered significant at a level of $p<0.05$.

RESULTS

Effects of extracts from aerial parts of Stachys schtschegleevii on carrageenan-induced paw oedema

Induction of acute inflammation in control rats resulted in a prominent increase in paw thickness, began 1 h after intraplantar injection of carrageenan and reached a peak of inflammation after 4 hours (fig. 1). Intraperitoneal injection of rats with total hydroalcoholic extract of aerial parts of *S. schtschegleevii*, obtained from non-flowering stems, caused a potent and dose-related inhibition of the carrageenan-induced inflammation. The extract with the dose of 50 mg/kg induced a significant ($p<0.05$) anti-inflammatory effect only at the second hour after carrageenan injection. The dose of 200 mg/kg abolished the inflammation more significantly ($p<0.01; 0.001$) at all time points, whereas in the presence of the higher dose of 400 mg/kg the anti-inflammatory action was seen only at the point of 2 h. A standard drug indomethacin (2.5 mg/kg, i.p) showed less potent inhibition than 200 mg/kg of the total extract (fig. 1).

As shown in fig. 2 intraperitoneal injection of 100 mg/kg of chloroform extract of aerial parts of *Stachys schtschegleevii* inhibited inflammatory response markedly in carrageenan-induced rat paw oedema at hours 3 and 4 ($p<0.01$); the dose of 200 mg/kg had no inflammatory/anti-inflammatory effects, whereas the dose of 400 mg/kg induced a significant ($p<0.01$) pro-inflammatory effect 4 hours after carrageenan injection.

Table 1: The main components of different fractions of ethyl acetate extract of *Stachys schtschegleevii*

Primary fractions	Main fractions	Components of fractions
f1	F1	————
f2-f4	F2	Caffeic acid derivatives (Trace of flavonoids)
f5, f6	F3	————
f7	F4	Flavonoids
f8	F5	Flavonoids: 1) Chrysoeriol-7-O-β-[6"(p- coumaryl)]-glucoside , 2) Apigenin-7-O-β-[6"(p- coumaryl)]-glucoside)
f9, f10	F6	Flavonoids and Caffeic acid derivatives
f11	F7	Flavonoids and Caffeic acid derivatives
f12-f16	F8	Caffeic acid derivatives and Flavonoids (*acteoside*)
f17-f24	F9	Caffeic acid derivatives (*acteoside*)
f25, f26	F10	Caffeic acid derivatives
f27-f38	F11	Full of colored and degraded components (trace of different salts)

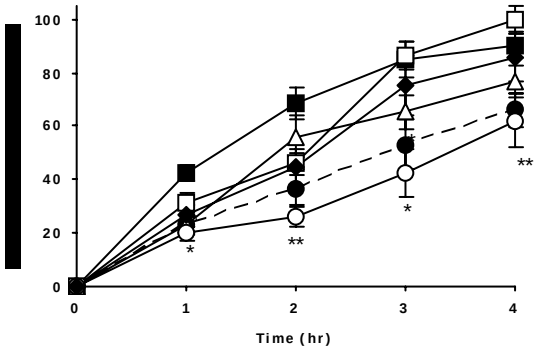


Fig. 1. Effects of different doses of total hydroalcoholic extract [50 mg/kg (□); 100 mg/kg (△); 200 mg/kg (○); 400 mg/kg (◆), i.p.] from aerial parts of non-flowering stems of *Stachys schtschegleevii* and indomethacin (2.5 mg/kg, ●), on carrageenan-induced paw oedema in the rat. Data represented as mean±s.e.m. **p<0.05, **p<0.01 and ***p<0.001* compared to the same points in the control group (saline, ■).

Intraperitoneal injection of rats with ethyl acetate extract of *Stachys schtschegleevii* caused a potent and dose-related inhibition of the carrageenan-induced inflammation (fig. 3). The extract with the dose of 100 mg/kg induced a significant (*p<0.05*) anti-inflammatory effect only at 2 hours after induction of inflammation. The dose of 200 mg/kg abolished inflammation significantly (*p<0.05*) at 2, 3 and 4 hours after injection of carrageenan. In the presence of the higher dose of the extract (400 mg/kg) the anti-inflammatory action was seen more significantly at 2, 3 and 4 h (*p<0.01).*

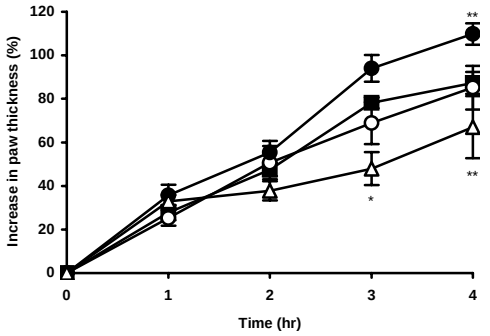


Fig. 2: Effects of chloroform extract [100 mg/kg (Δ), 200 mg/kg (○) and 400 mg/kg (●); i.p.] from aerial parts of non-flowering stems of *Stachys schtschegleevii* on carrageenan-induced paw oedema in the rat. Data represented as mean±s.e.m. ***p<0.01* compared to vehicle-treated group (■).

Phytochemical analysis

Active EtOAc extract was submitted to chromatographic procedure in order to isolate the active fractions and metabolites (table 1). In Fractions 1 and 3, there was no detectable amount of flavonoid or caffeic acid derivative. Fraction 2 contained significant amounts of caffeic acid derivatives. Flavonoids were the major components of Fractions 4 and 5, whereas Fractions 6 to 10 contained significant amounts of caffeic acid derivatives. The main flavonoids and caffeic acid derivatives, chrysoeriol 7-*O*-β-[6"-(*p*- coumaroyl)]-glucoside (**1**), apigenin 7-*O*-β-[6"-(*p*- coumaroyl)]-glucoside (**2**), and acteoside (**3**) (fig. 4), present in the fractions 4 to 10, were further purified using prep-HPLC and identified by spectroscopic analyses. Phytochemical screening of the chloroform extract revealed the presence of four compounds, I, II, III,

and IV. Compound I, II and III were identified as penduletin, cirsimaritin (scutellarein 6,7-dimethyl ether) and xanthomicrol (6,7,8-trimethoxy 5,4′-dihydroxy-flavone), respectively (fig. 5).

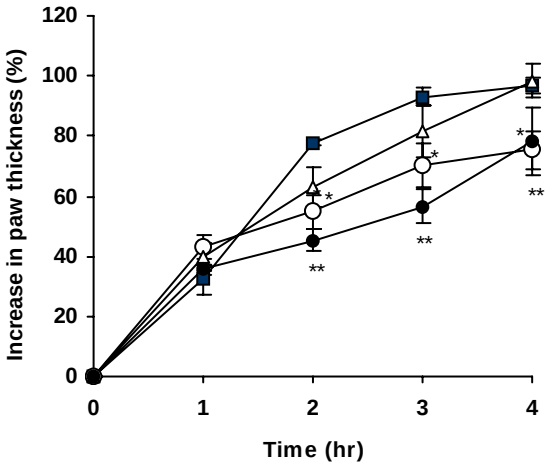
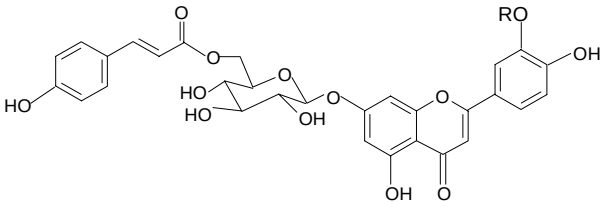
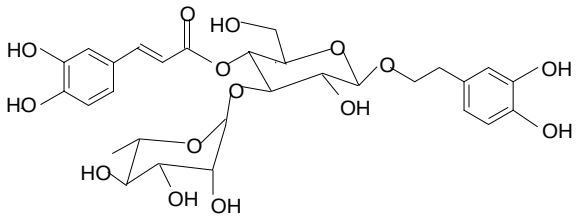


Fig. 3: Effects of EtOAc extract [100 mg/kg (Δ), 200 mg/kg (○) and 400 mg/kg (●); i.p.] from aerial parts of non-flowering stems of *Stachys schtschegleevii* on carrageenan-induced paw oedema in the rats. Data represented as mean±s.e.m. *$p<0.05$, ***$p<0.001$ compared to vehicle-treated group (■).



Compound	R
chrysoeriol 7-*O*-β-[6”-(*p*- coumaroyl)]-glucoside (**1**)	Me
apigenin 7-*O*-β-[6”-(*p*- coumaroyl)]-glucoside (**2**)	H



Acteoside (**3**)

Fig. 4. Identified major compounds in various fractions of the ethyl acetate extract of *Stachys schtschegleevii*

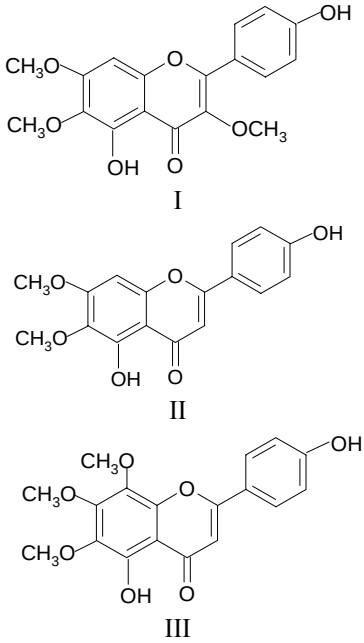


Fig. 5: Structures of penduletine (I), xanthomicrol (II) and cirsimaritin (III) obtained from chloroform extract of *Stachys schtschegleevii*

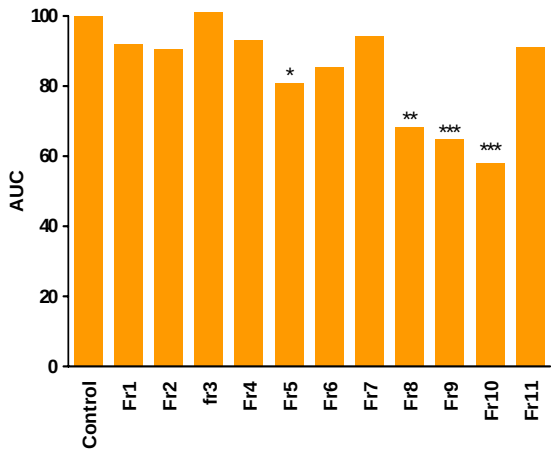


Fig. 6: Effects of different Fractions of ethyl acetate extract (15 mg/kg; i.p.) from aerial parts of non-flowering stems of *Stachys schtschegleevii* on total oedema response measured as Area Under Curve (AUC) of carrageenan-induced paw oedema response in the rat during 4 hours. Data represented as percentage of the mean of control response. *p<0.05, **p<0.01, and ***p<0.001 compared to control group.

Effects of fractions obtained from ethyl acetate extract of Stachys schtschegleevii Sosn. on carrageenan-induced paw oedema

Fig. 6 shows the effects of different Fractions of ethyl acetate extract (15 mg/kg; i.p.) from aerial parts of non-flowering stems of *Stachys schtschegleevii* on total oedema response measured as Area Under Curve (AUC)

of carrageenan-induced paw oedema response in the rat during 4 h. Data represented as percentage of the mean of control response. Fractions 5, 8, 9 and 10 that contained acylated glycosylflavonoids (table 1) and caffeic acid derivatives (acteoside, **3**) showed significant anti-inflammatory effects. The fraction 10 had the greatest anti-inflammatory effect ($p<0.001$).

DISCUSSION

Initial screening of various extracts of *Stachys schtschegleevii Sosn.* revealed that the EtOAc extract of the non-flowering stems had the greatest anti-inflammatory effects against carrageenan-induced paw oedema. Fractionation of the EtOAc extract and determination of major components present in each fraction (e.g. flavonoids or caffeic acid derivatives) could provide information on the compounds responsible for anti-inflammatory activity of *Stachys schtschegleevii*. The most prominent anti-inflammatory effect was observed with fractions 8-10, which contained phenylethanoid glycosides (caffeic acid derivatives). Phenylethanoid glycosides (caffeic acid derivatives) are of common occurrence within the species of the genus *Stachys*. It has been shown that phenylethanoid glycosides (such as acteoside) could suppress the accumulation of leukocytes in the glomeruli (Hayashi *et al*., 1994) and this effect possibly is mediated via the inhibition of up-regulation of ICAM-1 in nephritic cells (Hattori *et al*., 1996; Hayashi *et al*., 1996). It has been demonstrated that acteoside inhibits endothelial NO production/release in rat mesenteric arteries (Tam *et al*., 2002). Phenethyl glycosides also have antioxidant activity (Li *et al*., 2000). The discrepancy between the inhibitory effect upon low doses and the ineffectiveness of the higher dose (400 mg/kg) of the extract might be explained by this hypothesis that some of the active constituent(s) of *Stachys schtschegleevii* at high concentrations may exhibit pro-inflammatory properties. A considerable anti-inflammatory effect was seen by the lower dose of chloroform extract (100 mg/kg), whereas increasing the dose of extract increased the inflammatory response. At least three flavonoids were identified in chloroform extract of *Stachys schtschegleevii* as penduletin, cirsimaritin and xanthomicrol. Xanthomicrol is a weak COX inhibitor, while it can inhibit lipoxygenase enzyme slightly but significantly (Ferrándiz *et al*., 1990; Skaltsa *et al*., 2000). Leukotriens are the products of lipoxygenase pathway that can exacerbate the inflammatory response via different pathways. The inhibitory effect of the chloroform extract at lower doses may be explained both by the inhibitory effect of xanthomicrol on lipoxygenase enzyme and COX. Pro-inflammatory effect with high dose (400 mg/kg) of chloroform extract may be explained by the greater inhibitory effect of xanthomicrol on lipoxygenase rather than COX. Inhibition of lipoxygenase pathway of arachidonic acid metabolites makes them available for COX pathway metabolism (xanthomicrol is a weak COX-inhibitor) and more synthesize of inflammatory prostaglandins. It has been shown that cirsimaritin, concentration-dependently, inhibited the superoxide anion generation and O_2 consumption of neutrophils, but slightly enhanced the superoxide anion generation in PMA-activated or arachidonic acid-stimulated NADPH oxidase (Wang *et al*., 2002). Also, it was demonstrated that cirsimaritin displayed 50% COXI and COXII inhibitory activity and it had a weak antioxidant activity (Kelm *et al*., 2000). The anti-inflammatory effect of the chloroform extract of *Stachys schtschegleevii* can be also explained by inhibitory effect of cirsimaritin on COXI and II. The increase in inflammatory response by dose of 400 mg/kg of chloroform extract was significant at hours 3 and 4; the second phase of inflammation in carrageenan-induced inflammation which is largely depended on arachidonate metabolites (Holsapple *et al*., 1980; Salvemini *et al*., 1996). The increase in oedema response in second phase of inflammation indicates that high doses of chloroform extract of *Stachys schtschegleevii* could interfere with production of arachidonate metabolites with an unknown mechanism. The increase in inflammatory response with high concentrations of extract has been reported for other extracts too. It was reported that COX was stimulated by some flavonoids at high substrate arachidonic acid concentrations (Middleton *et al*., 2000). Also, it has been shown that some components of tart cherries had little or no activity at low concentration on the activity of COXI and COXII, whereas at higher concentrations actually increased the activity of these enzymes (Wang *et al*., 1999). Also, it has been shown that some phenolic compounds have pro-oxidant activity with high doses (Cao *et al*., 1997; Yamanaka *et al*., 1997; Galati *et al*., 2002). Increasing the superoxide anion generation, in arachidonic acid-stimulated NADPH oxidase model, by cirsimaritin (Wang *et al*., 2002) at high dose may explain the divers effect of the chloroform extract of *Stachys schtschegleevii*. The inflammagen effects of higher dose of the chloroform extract of *Stachys schtschegleevii* might result from other unknown components in the extract that in high concentration they could overcome the anti-inflammatory effects of xanthomicrol or cirsimaritin.

ACKNOWLEDGEMENTS

This study was supported by the Research Vice Chancellors of Tabriz University of Medical Sciences.

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