Assessment of anti-inflammatory properties of ethyl acetate extract of *Stachys schtschegleevii* Sosn.

[*1]Nazemiyeh, [2]Maleki N., [2]Mehmani F., [3]Kumarasamy Y., [3]Shoeb M., [2]Garjani A., [4]Sarker S.D.

[1]*Research Center for Pharmaceutical Nanotechnology, [2]Department of Pharmacology, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran. [3]School of Pharmacy, The Robert Gordon University, Schoolhill, Aberdeen, UK. [4]School of Biomedical Sciences, University of Ulster at Coleraine, Ireland, UK.*

ABSTRACT

Background: Stachys schtschegleevii Sosn. (commonly known as "Poulk" and "Sonbeleh Arasbarani") and *Stachys inflate* Benth. ("Sonbeleh Arghavani") are most widely used for medicinal purposes. *Stachys schtschegleevii* Sosn.(Lamiaceae) is widespread in North West of Iran and have been used traditionally to treat infections, asthma, rheumatic and other inflammatory disorders. In the present studies the anti-inflammatory activity of hydro-alcoholic extracts of both flowering and sterile tops of *S. schtschegleevii* were investigated.

Material and Methods: The methanolic extract of the sterile aerial parts was partitioned between chloroform, ethyl acetate and n-butanol. The column chromatography (CC) on silica gel was used to fractionate the ethyl acetate extract and the anti-inflammatory effects of each main fraction were evaluated by carrageenan-induced rat paw oedema assay. The reversed-phase preparative HPLC was used to isolate compounds from the active fractions and their structure (1-3) were elucidated by spectroscopic means. The ethyl acetate portion was fractionated into 11 major fractions.

Results: the most prominent anti-inflammatory effect was observed with fractions 8 to 10 of the ethyl acetate portion. Fraction 8 abolished considerably the mean maximal responses of inflammation from $87.00\pm3.5\%$ in control to $61.10\pm7.2\%$ *(p<0.001)* and $62.10\pm3.6\%$ *(p<0.001)* in 15 and 30 mg/kg fraction-treated groups, respectively. Preparative-HPLC analyses of fractions 5 and 8-10 led to the isolation and identification of three major compounds, chrysoeriol 7-*O*-β-[6"-(*p*- coumaroyl)]-glucoside (**1**), apigenin 7-*O*-β-[6"-(*p*-coumaroyl)]-glucoside (**2**), and acteoside (**3**).

Conclusion: It seems that caffeic acid derivatives such as acteoside may be implicated in anti-inflammatory effect of *Stachys schetschegleevii* Sosn.

Keywords: *Stachys schtschegleevii*; Carrageenan-induced rat paw oedema assay; Anti-inflammatory; Acteoside; Flavonoids; Caffeic acid derivatives; Phenylethanoids

INTRODUCTION

The genus *Stachys* (Lamiaceae) is known to contain a variety of plant secondary metabolites including phenylethanoid glycosides (1-3), di- and triterpenoids (4-6), steroids (5,6) and flavonoids (7-11). *Stachys schtschegleevii* Sosn. (commonly known as "Poulk" and "Sonbeleh Arasbarani") and *Stachys inflate* Benth. ("Sonbeleh Arghavani") are most widely used for medicinal purposes. These species are widespread in Arasbaran region, North West of Iran (12,13). Because of high similarity of *Stachys inflata* and *Stachys schtschegleevii*, sometimes the first one is used by un-professional practitioners or sold in local market instead of *S. schtschegleevii*. Our previous studies showed that hydro-alcoholic extract of sterile shoots of *S. inflate* has noticeable anti-inflammatory (14) and infarct size reducing effects (15). Screening of the anti-inflammatory effects of different fractions obtained from hydro-alcoholic extract showed that the ethyl acetate fraction has most prominent effect (14). The aqueous extracts obtained from the non flowering aerial parts of *S. schtschegleevii* have been used traditionally in the North West of Iran to treat infections, asthma, rheumatic and other inflammatory disorders. It has been shown that the methanolic extract of the flowering aerial parts

Correspondence: nazemiyehh@yahoo.com

of S. *schtschegleevii* has also anti-inflammatory and antinociceptive properties (16). However, according to our results only sterile tops of the plant possessed anti-inflammatory effects.This finding is in agreement with Azerbaijan folk believes which only sterile tops of this plant are used as medicine. In the present work, hydro-alcoholic extracts of both flowering and sterile tops of *S. schtschegleevii* were subjected to anti-inflammatory assays, separately. Since, the extract of sterile tops was active, chloroform (CHCl3), ethyl acetate (EtOAc) and n-buthanol (BuOH) soluble fractions were prepared from hydro-alcoholic extract and their anti-inflammatory effects were investigated. Then the EtOAc fraction, the most potent one- was subjected to chromatographic study in order to sub-fractionate and to identify their active constituents. Further isolation of individual compounds was done using prep-HPLC and their structures were elucidated by means of MS and NMR (^{1}H and ^{13}C-NMR, 2D-NMR) as it has been previously described (17).

MATERIALS AND METHODS

Plant material
The sterile (non flowering) and flowering aerial parts of *Stachys schtschegleevii* Sosn. were collected from Arasbaran forest (Aynalou 1850 m ; E. Azerbaijan) in June 2000. A voucher specimen (TbzM-FPh 140) was deposited at the herbarium of Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.

Extraction
Air-dried and finely powdered aerial parts of *S. schtschegleevii* (400 g) were extracted four times with 600 ml of 70% MeOH in water using maceration for 48 h each time. The combined extracts were filtered and MeOH was evaporated, using a rotary evaporator at a temperature of ≤40°C. The resulting aqueous residue was extracted, successively, with chloroform (CHCl3; 4.08g), ethyl acetate (EtOAc; 7.69g) and n-butanol saturated with water (n-BuOH; 21.2g) (4×200 ml each). The solvents of the extracts were evaporated and residues were kept at 4 °C until used.

Standardization of hydro alcoholic and EtOAc extracts
To standardize the prepared extract, the amount of caffeic acid derivatives was measured by modified method of Nichiforesco (18). The spectra obtained (505 nm) in the presence of Arnow reagent, which produces a red colour when add to solution containing caffeic acid.

Fractionation of ethyl acetate extract
The dried EtOAc extract (6 g) was adsorbed on 24 g silica gel, and subjected to column chromatography (Kieselgel G 60, 230-400 mesh, 240g), eluting with EtOAc-CHCl3-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 column was washed with MeOH-water (70:30) to yield fractions f34-f38. All collected fractions (~100 ml portions) were monitored on TLC plates (Merck Silica gel 60 F$_{254}$, 0.25 mm) using mobile phases, EtOAc: MeOH: water (85:13:2), and EtOAc: formic acid: water (80:10:10). AlCl3 and FeCl3 spray reagents were used to ascertain fractions containing same chemical profiles, and were mixed to afford 11 main fractions, designated as F1-F11. The 5% AlCl3 solution, revealed all 5-hydroxy-flavonoids as fluorescent yellow spots under U.V. light (366 nm) (19). The 2% FeCl3 solution also detects caffeic acid derivatives as gray to black spots on the chromatogram (20). Solvents of all fractions were evaporated under reduced pressure at ambient temperature.

Analytical techniques
Separation of individual compounds in the fractions of EtOAc extract was accomplished by preparative HPLC (Shimadzu LC-8A HPLC with C$_{18}$ column: CLC Shim-pack prep-ODS, 20 x 250mm, 15μ and SPD-M10A-vp detector: detection at 220 and 280 nm), utilizing the solvent gradient system published previously (17). The structures of isolated compounds were determined by comparison of their physical properties (UV, MS, ^{1}H and ^{13}C-NMR, HMQC, HMBC) with those of published data.

Carrageenan-induced paw oedema
Male wistar rats (180-220 g) fasted over night with free access to water (18h) received a subplantar injection of 100μl of 1% carrageenan (Sigma, Germany) in saline in the right hind-paw. Paw thickness was measured from ventral to dorsal surfaces, using a dial calliper (21) immediately prior to carrageenan injection, and then at hourly intervals from 1-4 hours afterwards. Data are expressed as percent increase in paw thickness compared to pre-injection values. Fine suspensions of the hydro-alcoholic, CHCl3, n-BuOH and EtOAc extraxts (100-400 mg/kg) and its subfractions in saline were administered intraperitoneally (i.p). Fractions were used at doses of 15, and 30 mg/kg (nearly equivalent to 1/10 - 1/20 of total EtOAc extract dose; n=6).

Because of limitation of amounts which were 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 (2.5 mg/kg) treated rats were used as positive control. Swelling of both paw attained during 4hrs were a measure of the the inflammatory response in the drug-treated and control (vehicle-treated) groups and were determined by calculation of 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 AND DISCUSSION
While intraperitoneal injection of rats with the hydro-alcoholic extract of the sterile tops of *St. schtschegleevii* caused a potent and dose-related inhibition of the carrageenan induced inflammation, the hydro-alcoholic extract prepared from flowering tops of *St. schtschegleevii*, showed no anti-inflammatory effects at the examined doses, (Figure 1) .
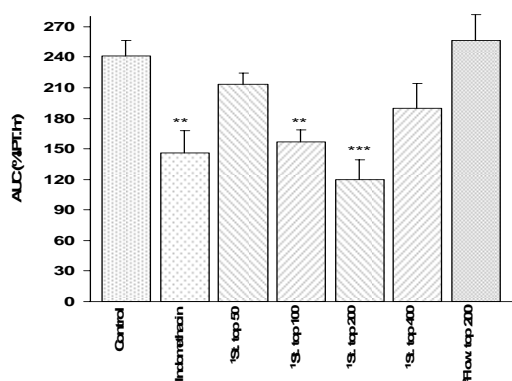
Figure 1. Effects of different doses of hydro-alcoholic extract (50, 100, 200 and 400 mg/kg; i.p.) prepared from aerial parts of *Stachys schtschegleevii*; and indomethacin as positive control on carrageenan-induced paw edema in the rats. *1*: sterile tops in different doses, *2*: flowering tops (because of no influence of different doses, i.e 50, 100, 200 and 400 mg/kg on inflammation only single dose of 200mg/kg has been shown) . Data represented as mean±s.e.m. ***p<0.01, ***p<0.00*1 compared to the control group using Mann-Whitney non parametric U-test.

Intraperitoneal injection of rats with ethyl acetate extract caused a potent and dose-related inhibition of the carrageenan-induced inflammation (Fig. 2) in comparison with others. The dose of 100 mg/kg of the extract induced a significant ($p < 0.05$) anti-inflammatory effect only at 2 hours after carrageenan injection.
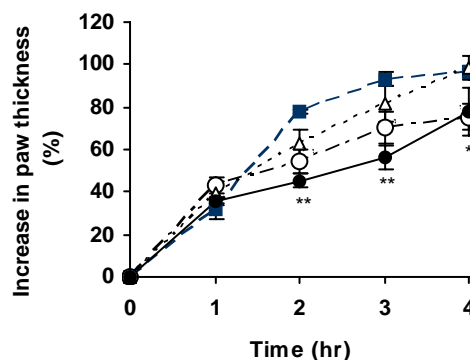
Figure 2. Effects of EtOAc extract [100 mg/kg (Δ), 200 mg/kg (O) and 400 mg/kg (●); i.p.] from aerial parts of non-flowering stems of *Stachys schtschegleevii* on carrageenan-induced paw edema in the rats. Data represented as mean±s.e.m. **p<0.05, ***p<0.00*1 compared to vehicle-treated group (■).

The dose of 200 mg/kg abolished inflammation significantly ($p < 0.05$) at 2, 3 and 4 hrs after injection of carrageenan. In the presence of the higher dose of 400 mg/kg the, anti-inflammatory action was more significant at 2, 3 and 4 hours after injection ($p < 0.01$). The ethyl acetate extract significantly reduced the maximal edema response from 96.50±4.7% increase in controls to 75.30±6.2% (200 mg/kg) and 78.00±11.2% (400 mg/kg) increase in the extract treated rats ($p < 0.01$) (Fig. 2). The standardization result indicated that the EtOAc extract contained 3.8% of caffeic acid derivatives (according to caffeic acid).

EtOAc extract was submitted to column chromatography in order to isolate the active fractions and ingredients. Table 1 summarises the main components of 11 fractions obtained from the ethyl acetate extract of *S. schtschegleevii*. In fractions 1 and 3, there were no detectable amounts 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.

Fraction 11 was obtained by washing of the column with MeOH 70%, and it contained a complex mixture of highly polar components, and

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 and a trace amount of flavonoids
f5, f6	F3	-------------
f7	F4	Flavonoids
f8	F5	Flavonoids: Chrysoeriol 7-*O*-β-[6"-(*p*- coumaroyl)]-glucoside (**1**) Apigenin 7-*O*-β-[6"-(*p*- coumaroyl)]-glucoside (**2**)
f9, f10	F6	Flavonoids and caffeic acid derivatives
f11	F7	Flavonoids and Caffeic acid derivatives
f12-f16	F8	Caffeic acid derivatives (acteoside, **3**) and flavonoids
f17-f24	F9	Caffeic acid derivatives: acteoside (**3**)
f25, f26	F10	Caffeic acid derivatives: acteoside (**3**)
f27-f38	F11	Full of coloured and degraded components (trace amounts of different salts)
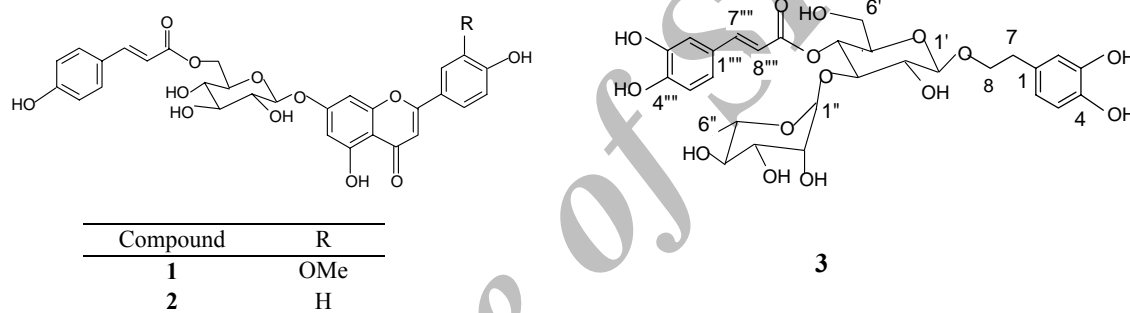
Compound	R
1	OMe
2	H

Figure 3. Major compounds which were identified in various fractions of the ethyl acetate extract of *Stachys schtschegleevii* which were identified.

also a trace amount of different salts. The lipophilic compounds which were present in fraction 1, could inhibit the inflammatory response at a dose of 30 mg/kg, slightly.

Fraction 2, which contained non-polar and free caffeic acid derivatives (such as *p*- coumaric acid and caffeic acid (Table 1), had no anti-inflammatory effect against the carrageenan-induced paw oedema at two doses of 15 and 30 mg/kg

Fractions 3, which did not contain any detectable amounts of flavonoids or caffeic acid derivatives, and fraction 4, which contained flavonoids as the major compounds (Table 1), at a dose of 15 mg/kg, had no effect on inflammatory response. Similarly, no significant anti-inflammatory effect was observed by a dose of 15 mg/kg of fraction 7. Because of the small amounts of these fractions, the anti-inflammatory effects at a higher dose (30 mg/kg) could not be studied.

Fraction 5 that contained acylated glycosyl-flavonoids (Table 1) showed a significant anti-inflammatory effect. It reduced the mean

maximal oedema response from 85.00±6.0% in control to 74.10±5.6% and 48.80±4.8% *(p<0.001)* with doses of 15 and 30 mg/kg, respectively Table 2).

Fractions 6 and 7 could reduce inflammation, but did not show considerable effects.

Fractions 8 that contained caffeic acid derivatives (acteoside, **3**) and glycosylflavonoids, (Table 1), abolished considerably the mean maximal responses from 87.00±3.5% in control to 61.10±7.2% *(p<0.001)* and 62.10±3.6% *(p<0.001)* in 15 and 30 mg/kg fraction-treated groups, respectively (Table 2). The fraction also produced a 31.8% reduction in the mean total oedema responses with both doses. However, the anti-inflammatory effect was not dose-dependent and low dose of 15 mg/kg reduced the percentage of oedema response at all times.

Fractions 9 and 10 had the greatest anti-inflammatory effect with both doses of 15 and 30 mg/kg. The mean maximal effects elicited during 4 h decreased from 90.00±3.0% to 51.20± 2.2% (15 mg/kg, *p<0.001*) and 54.60±2.9% (30

Table 2. Increase in paw thickness (%) at 1, 2, 3, and 4 hrs after injection of carrageenan into the paws and mean total oedema response measured as Area Under Curve (AUC) of paw oedema in control and rats which received fractions of ethyl acetate extract from aerial parts of non-flowering stems of *Stachys schtschegleevii*. Data are represented as mean±s.e.m.

Group	Increase in paw thickness (%)				AUC
	1h	2h	3h	4h	
Fraction 1					
control	47±1.7	63±2.3	83±1.9	92±2.8	100
15 mg/kg	40±2.2	60±2.3	80±1.9	95±4.5	92.0
30 mg/kg	39±6.3	48±6.2*	60±6.9***	77±6.9*	78.0*
Fraction 2					
control	45±3.2	64±2.7	83±2.5	90±3.5	100
15 mg/kg	39±2.6	69±9.7	89±5.7	96±5.7	90.5
30 mg/kg	29±0.9	59±5.0	83±4.9	94±4.3	91.4
Fraction 3					
control	45±3.3	65±2.7	84±2.4	89±3.5	100
15 mg/kg	52±9.2	73±10.2	85±8.0	94±9.2	107.7
30 mg/kg	-	-	-	-	-
Fraction 4					
control	40±3.9	52±3.8	66±4.2	90±2.6	100
15 mg/kg	41±5.7	44±3.5	62±6.5	83±4.0	93.0
30 mg/kg	-	-	-	-	-
Fraction 5					
control	29±2.2	50±3.0	74±4.5	85±6.0	100
15 mg/kg	36±2.3	42±1.5	60±4.4*	74±5.6	80.9*
30 mg/kg	23±2.2	40±6.8	51±6.7***	49±4.8***	77.0*
Fraction 6					
control	30±2.2	51±6.0	78±8.0	90±6.5	100
15 mg/kg	23±1.7	36±3.3*	68±6.0	88±4.0	85.5
30 mg/kg	26±2.4	39±1.5*	63±5.4	76±4.8	78.2*
Fraction 7					
control	39±3.0	53±3.4	69±3.1	77±2.8	100
15 mg/kg	25±4.3	60±4.4	63±4.7	79±9.9	94.2
30 mg/kg	-	-	-	-	-
Fraction 8					
control	39±1.2	55±2.5	73±3.0	87±3.5	100
15 mg/kg	22±2.1***	42±4.6	55±6.8**	61±7.2***	68.2**
30 mg/kg	43±0.9	44±1.4	51±2.2***	62±3.5***	68.2**
Fraction 9					
control	35±1.0	50±2.8	73±3.0	90±3.0	100
15 mg/kg	28±0.6	33±3.7***	40±1.8***	51±2.1***	64.9***
30 mg/kg	40±5.3	33±4.1***	39±2.3***	54±2.8***	67.2***
Fraction 10					
control	36±4.2	49±4.5	66±6.3	83±3.9	100
15 mg/kg	30±2.5	37±5.0*	28±3.5***	40±7.0***	58.0***
30 mg/kg	30±3.3	31±6.0***	28±3.7***	36±6.9***	56.6***
Fraction 11					
control	46±3.2	68±2.3	92±1.9	98±1.9	100
15 mg/kg	49±1.1	63±3.7	82±3.6	92±3.7	91.0
30 mg/kg	-	-	-	-	

*p<0.05,*p<0.01 and ***p<0.001 compared to the same points in the control group using one-way ANOVA and Newman-Keuls post-test.

Tabl 3. ^{13}C-NMR(75 MHz) and ^{1}H-NMR(300 MHz) spectroscopic data for *1-4* (DMSO-d_6).

Position		*1*			*2*	
Aglycone	$\delta_{C\,(ppm)}$	$\delta_{H\,(ppm)}$	**HMBC**	$\delta_{C\,(ppm)}$	$\delta_{H(ppm)}$	**HMBC**
2	164.21		H3 (^{2}J) H2',6'(^{3}J)	162.64		H3 (^{2}J) H2',6'(^{3}J)
3	103.30	6.89, s	-	102.95	6.78,s	-
4	181.90		H3(^{2}J)	181.85		H3(^{2}J)
5	161.07	12.98 (OH)	H6(^{2}J)	161.26	12.98 (OH)	H6(^{2}J)
6	99.54	6.46, d(J=2.2)	H8(^{3}J)	99.51	6.47, d(J=2.19)	H8(^{3}J)
7	162.61		H6,8(^{2}J) H1''(^{3}J)	164.08		H6,8(^{2}J) H1''(^{3}J)
8	94.82	6.81, d(J=2.2)	H6(^{3}J)	94.82	6.79, d(J=2.19)	H6(^{3}J)
9	156.82		H8 (^{2}J)	156.82		H8 (^{2}J)
10	105.32		H3,6,8(^{3}J)	105.32		H3,6,8 (^{3}J)
1'	121.27		H5'(^{3}J) H2',6'(^{2}J)	120.94		H3',5'(^{3}J)
2'	113.7	7.54, d(J=1.98)	H6'(^{3}J)	128.42	7.91, d(J=8.79)	H6'(^{3}J) H3'(^{2}J)
3'	147.97		H5'(^{3}J) H$_3$CO-(^{3}J)	115.58	6.91, d(J=8.79)	H5'(^{3}J)
4'	150.90		H2',6'(^{3}J)	161.07		H2',6'(^{3}J)
5'	115.91	6.90, d(J=8.67)	H6'(^{2}J)	115.58	6.91, d(J=8.79)	H3'(^{3}J) H6'(^{2}J)
6'	120.4	7.55, dd(J=8.67, 1.98)	H2' (^{3}J) H5'(^{2}J)	128.42	7.91, d(J=8.79)	H2' (^{3}J) H5'(^{2}J)
OCH$_3$	55.96	3.88,s				
Glucose						
1''	99.42	5.14, d(J=7.26)		99.39	5.13, d(J=7.26)	
2''	72.91	n		72.91	n	
3''	76.19	n		76.19	n	
4''	69.96	n		70.04	n	
5''	73.77	3.18,m		73.77	3.18,m	
6''	63.35	4.46, dd(J=12.27, 2.48) 4.13, dd dd(J=12.27, 2.48)		63.35	4.48, dd(J=12.27, 2.48) 4.15, dd dd(J=12.27, 2.48)	
Acyle(s)						
α	113.70	6.29, d(J=15.78)		113.76	6.30, d(J=16.21)	
β	144.79	7.47, d(J=15.78)		144.79	7.48, d(J=16.21)	
1'''	124.83		Hα (^{3}J) H3''' 5'''(^{3}J)	124.83		Hα (^{3}J) H3''' 5'''(^{3}J)
2'''	129.91	7.34, d(J=8.58)	Hβ(^{3}J)	129.91	7.33, d(J=8.55)	Hβ(^{3}J)
3'''	115.75	6.69, d(J=8.58)	H5'''(^{3}J)	115.75	6.69, d(J=8.55)	H5'''(^{3}J)
4'''	159.66		H2'''(^{3}J) H6'''(^{3}J)	159.64		H2'''(^{3}J) H6'''(^{3}J)
5'''	115.75	6.69, d(J=8.58)	H3'''(^{3}J)	115.75	6.69, d(J=8.55)	H3'''(^{3}J)
6'''	129.91	7.34, d(J=8.58)	H$_\beta$(^{3}J) H2'''(^{3}J)	129.91	7.34, d(J=8.55)	H$_\beta$(^{3}J) H2'''(^{3}J)
CO	166.43		Hβ(^{3}J) Hα (^{2}J) H6''(3J)	166.30		Hβ(^{3}J) Hα (^{2}J) H6''(3J)

^{n} Signal pattern are unclear due to overlaping .

Table 4. ^{1}H-NMR(300 MHz) and ^{13}C-NMR(75 MHz) spectroscopic data for *3* (CD$_3$OD).

Position	3	
Phenylethanoid	$\delta_{H\ (ppm)}$	$\delta_{C\ (ppm)}$
1	-	131.61
2	6.701, d, J=1.8	117.28
3	-	146.96
4	-	144.80
5	6.68 ,d, J= 7.93	116.67
6	6.56,dd, J=7.93 , 1.8	121.47
7 (β)	2.79 , m	36.71
8(α)	3.81 *	72.17
	4.05 *	
Caffeoyl		
1''''	-	127.79
2''''	7.063, d, J=1.8	115.38
3''''	-	146.25
4''''	-	149.94
5''''	6.78 ,d, J= 8.25	116.50
6''''	6.954 ,dd, J=8.25 ,1.8	123.46
7''''(β)	7.59,d,J= 15.87	148.22
8''''(α)	6.28 ,d,J=15.87	114.82
C=O	-	168.50
Glucose		
1'	4.38 , d, J= 7.5	104.31
2'	3.40 *	76.33
3'	3.8 , t ,J=9.5	81.86
4'	-	70.59
5'	3.59 *	76.12
6'	3.52 * 3.60 *	62.48
Rhamnose		
1''	5.19, brs	103.21
2''	3.92 *	72.47
3''	3.78 *	72.40
4''	3.30 *	73.93
5''	3.54 *	70.68
6''	1.09 , d, J=6.1	18.65

* Overlapping with other peaks

mg/kg, *p<0.001*) by fraction 9, and from 82.70±3.9% to 40.00±7.0% (15 mg/kg, *p<0.001*) and 36.40±6.9% (30 mg/kg, *p<0.001*) by fraction 10 (Table 2). However, like fraction 8, the anti-inflammatory effects were not dose-dependent.

The last fraction (fr 11), had no inflammatory effect.

The most prominent anti-inflammatory effect was observed with fractions 8-10, which contained phenylethanoid glycosides (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**) (Figure 3), which were present in the fractions 4 to 10, were further purified using prep-HPLC and identified by spectroscopic analyses (Tables 3 and 4). Phenylethanoid glycosides (caffeic acid derivatives) are of common occurrence within the species of the genus *Stachys*. For example, acteoside (**3**), campneoside II, forsythoside B, leucosceptoside B and betonyosdes were the major caffeic acid derivatives extracted from *Stachys officinalis* (3), and stachysoside, acteoside, decaffeoylacteoside, isoacteoside, leucosceptoside A, martynoside were isolated from *Stachys sieboldii* (4).

It has previously been demonstrated that these compounds could affect the inflammatory processes. It has also been shown that acteoside (**3**) could suppress the accumulation of leukocytes in the glomeruli (22,23) and this effect was possibly mediated via the inhibition of up-regulation of ICAM-1 in nephritic cells (24). This was consistent with our previous results which indicated the inhibition of myeloperoxidase activity and neutrophil accumulation in extract treated animals (15, 16).

An inflammatory response implicated macrophages and neutrophils, which secret a number of mediators responsible for initiation, progression and persistence of acute inflammation (25). Nitric oxide (NO) is one of the most important mediators, and is produced in

macrophages by inducible nitric oxide synthase, iNOS, (26). Nitric oxide is responsible for vasodilatation, increase in vascular permeability and oedema formation at the site of inflammation (26). It has been observed that acteoside (**3**), which is one of the major compounds found in the fractions of the the EtOAc extract, could inhibit endothelial NO production/release in rat mesenteric arteries (28). Therefore, it can be assumed that various fractions of the EtOAc extract affected the inflammatory response by inhibition of neutrophil action.

Acteoside (**3**) and several other phenolic glycosides were also found to decrease some leukocyte functions, including rosette formation, mitogen-induced blast transformation and phagocytic activity *in vitro* (29).

The doses of 15 and 30 mg/kg of fractions 8-10 of the EtOAc extract had similar anti-inflammatory properties. Probably, because of the achievement of 100% response by the smaller dose, increasing the dose could not increase the response. Fractions 5 and 6 produced significant *(p<0.05)* anti-inflammatory effect at a dose level of 30 mg/kg. However, these effects were less than thoes effects observed by fractions 8-10 *(p<0.001)*. It seems that the anti-inflammatory effects of fractions 5 and 6 are related to flavonoid components rather than caffeic acid derivatives. Glycosylflavonoids including chrysoeriol 7-*O*-β-[6''-(E) *p*- coumaroyl]-glucoside (**1**) and apigenin 7-*O*-β-[6''-(E) *p*-coumaroyl]-glucoside), were the major components of fractions 5 and 6. The anti-inflammatory effects of these components have been previously established (30-32). By

increasing the polarity of the fractions, the type and the number of bounded sugars, the position of glycosylation, and briefly the structure of extracted flavonoids could be changed. The flavonoids may contain more glycosyl substituents or long chain of sugars in their structure, and as result the steric hindrance of glycosides could influence the interaction between the effective moiety of flavonoids and the enzymes that are involved in inflammatory response. Also it appears that a certain degree of lipophilicity is required for the anti-inflammatory effects (33). Lipophilic substituents enhance the inhibitory properties of the extract, whereas additional polar groups diminish them as is apparent when the activities of fractions 5 - 7 were compared. On the other hand, crystallisation of flavonoids with long chain of sugars might lead to decrease in solubility and reduce anti-inflammatory response.

CONCLUSION

In conclusion, this study has shown that ethyl acetate extract of sterile tops of *S. schtschegleevii* possess significant anti-inflammatory effects while the extracts of flowering tops were inactive. Phytochemical screening of this extract indicated the presence of flavonoids and caffeic acid derivatives.

It may be assumed that the anti-inflammatory effect of the EtOAc extract of *Stachys schtschegleevii* could be due to the presence of various phenylethanoids and flavonoids, especially acteoside (**3**).

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