Anti-Inflammatory Activity of *Salvadora persica* L. against Carrageenan Induced Paw Oedema in Rat Relevant to Inflammatory Cytokines

Abeer Y. IBRAHIM*, Souad E. EL-GENGAIHI, Hemaia M. MOTAWEA, Amany A. SLEEM

National Research Center, 12622 Cairo, Egypt; abeeryousry@yahoo.com (*corresponding author)

**Abstract**

In this study, the anti-inflammatory effect of aqueous alcoholic crude extract and ethyl acetate extract of miswak sticks (*Salvadora persica* L.) was investigated in carrageenan induced rat paw oedema in respect to immunological parameters. Adult male sapargue dawely rats were classified into four groups, group I received the vehicle (0.25% gum acacia solution), group II received crude aqueous alcoholic extract orally at 100 mg/kg, group III received ethyl acetate extract (100 mg/kg) orally and group IV received indomethacin (20 mg/kg) orally, and served as standard reference. The oedema was quantified by measuring the hind paw thickness immediately before subplantar injection, and at 1, 2, 3 and 4 h. Blood samples were withdrawn after the 4th hour of carrageenan induction, centrifuged and sera were used for analysis of pro-inflammatory cytokines. Administration of aqueous alcoholic extract and ethyl acetate extract (100 mg/ml) significantly reduced the oedema thickness in a time dependent manner, the inhibition percentage of inflammation was 17% for crude extract and 27% for ethyl acetate extract. Also the two extracts reduced secretion of inflammatory mediators, interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) in serum. The ethyl acetate extract shows potent activity to be nearly the same of indomethacin activity on all determined parameters at the last hour of following up. These results may be due to the presence of flavonoids in ethyl acetate extract. Three major flavonoids were isolated from ethyl acetate extract and identified as apigenin rhamnoglucoside, luteolin glucoside and rutin. The experimental study revealed that *Salvadora persica* extracts display remarkable anti-inflammatory activity.

**Keywords:** anti-inflammatory, cytokines, flavonoids, *Salvadora*, interleukin

**Introduction**

A large number of plant species contain various bioactive compounds exhibiting health beneficial properties; anti-oxidative, anti-inflammatory and mainly antimicrobial effects. Numerous natural products have been tested on various animal models for the development of new anti-inflammatory therapeutics (Sannigrahi, 2010).

Acute inflammation is rapid, short-lived and characterized by accumulations of fluid, plasma proteins and leukocytes. At the site of inflammations, the injured vascular endothelial cells and the emigrated leukocytes release a large number of soluble mediators which modulate and maintain the inflammation (Rosenberg and Gallin, 1999). Inflammation exerts multiple actions on the growth, differentiation and functions of lymphoid and non-lymphoid cells and regulates various aspects of the immune response and its production during injury or infection (Sarlis et al., 1992). Chronic inflammation results in a progressive shift in type of cells present at site of inflammation and it is characterized by simultaneous destruction and healing of the injured tissue from incidence of inflammation. Carrageenan-induced mouse paw oedema has been used for assessment of the anti-inflammatory activity of many plant extracts and essential oils (Hajhashemi et al., 2003; Khalil et al.; 2006; Orhan et al., 2006). The toothbrush tree, *Salvadora persica* L., locally called miswak, is a member of the *Salvadoraceae* family, has been used by many Islamic communities as toothbrushes and has been scientifically proven to be very useful in the prevention of tooth decay, even when used without any other tooth cleaning methods (Salehi and Momenti, 2006). Chewing sticks that are made from the roots, twigs, or stems of *S. persica* are commonly used in the Middle East as a means of maintaining oral hygiene. Studies indicate that *S. persica* extract is comparable to other oral disinfectants and anti-plaque agents, such as triclosan and chlorhexidine gluconate, if used at a very high concentration (Almas, 2002; Almas et al., 2005). The present study aimed to investigate anti-inflammatory effect of *Salvadora persica* extract in oedema model focusing on four of anti-inflammatory mediators, namely; interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1).

**Materials and methods**

**Preparation of extracts**

*Salvadora persica* plant was collected from Aswan Botanical Garden, Egypt, and was identified by the Botanist in Agriculture Dept. of the Botanical Garden. The fresh miswak sticks were cut into small pieces and allowed to...
dry at room temperature (30 ± 2°C) for ten days then it was ground to powder in a ball mill. The powder (300 g) was exhaustively extracted with alcohol (70%) and then concentrated under reduced pressure using rotary evaporator to a small volume (100 ml). The crude ethanolic extract was fractionated using gradient solvents. Each extract was concentrated under reduced pressure to be free from solvents. The ethyl acetate extract and crude extract were used in this anti-inflammatory study. Chemicals, carrageenan and indomethacin, were purchased from Sigma-Aldrich GmbH, Sternheim, Germany.

**Animals and experimental design of anti-inflammatory activity in Carrageenan induced paw oedema model**

Carrageenan-induced rat paw oedema has been used for assessment of the anti-inflammatory activity of many plant extracts. The method used was carried out according to Winter et al. (1962).

Adult male sapargue dawely rats weighing between 150-180 g were maintained under normal laboratory conditions and kept in standard polypropylene cages at room temperature of 25-30°C, 60 to 65% relative humidity and provided with standard diet and water *ad libitum*. The experimental protocols were approved by institutional Animal Ethical Committee in National Research Center (registration no. 90123). Four groups each of eight rats were used and treated as follow:

- **Group I:** received the vehicle (0.25% gum acacia solution) orally using orogastric tube and served as control group;
- **Group II:** received crude aqueous alcoholic extract orally at 100 mg/kg;
- **Group III:** received ethyl acetate extract (100 mg/kg) orally;
- **Group IV:** received indomethacin (20 mg/kg) orally, and served as standard reference.

One hour following the previously mentioned treatments, paw swelling was induced by subcutaneous injection of 100 µl of 1% sterile lambda carrageenan suspension in saline into the right hind paw of all groups. The oedema was quantified by measuring the hind paw thickness immediately before subplantar injection, and at 1, 2, 3 and 4 h with a micrometer caliber. The increase in paw volume was calculated as the difference between the right paw volume (carrageenan) and the left paw volume (saline) measured at each time point. The percentage of inhibitory activity at 5th hour was calculated according to the following formula (Hajhashemi et al., 2003):

\[
\text{Percent Inhibition} = \left(\frac{(C - T)}{C}\right) \times 100
\]

where, ‘T’ represents increase in oedema volume in animal’s paw administered tested materials and ‘C’ represents increase in oedema volume in control.

Blood samples were withdrawn after the 4th hour of carrageenan induction, centrifuged at 3500 rpm. Sera aliquots were frozen at -80°C for analysis of pro-inflammatory cytokines.

**Determination of Interleukins, tumor necrosis factor-α and transforming growth factor-β1**

IL-1β and IL-6 levels were assayed in sera using enzyme-linked immunosorbent assay kits in accordance with the manufacturer’s recommendations (Quantikine, USA), tumoral necrosis factor-α (Hycult biotech, Nethelands) while transforming growth factor-β1 (Abnova, Taiwan). The assessment was done by ELISA reader (NJ 2000; Nihom Inter Med. Co), the sensitivity of assay was 20 pg/ml.

**Statistical analysis**

Data are expressed as mean ± standard deviation or Standard error, and they were analyzed by the one-way analysis of variance (ANOVA), followed by post-hoc Tukey’s test for multiple comparisons. The differences were considered significant if the probability was associated with p<0.01.

**Isolation and purification of major flavonoids from S. persica ethyl acetate extract**

As crude alcoholic and ethyl acetate extracts showed anti-inflammatory activity, both extracts were subjected to phytochemical examination using magnesium turning test (Mabry et al., 1970) which proved the occurrence of flavonoids.

The purification of major flavonoids was carried out according to Mabry et al. (1970). Ethyl acetate fraction was used for isolation of *Salvadora persica* main flavonoids using preparative TLC (GF254) technique. Ethyl acetate: methanol (9:1), ethyl acetate: methanol: water (6:2:1) and acetic acid 15% were used for fractionation as solvent systems. The isolated compounds were further purified on sephadex (LH-20) column using butanol saturated with water as solvent system.

The purified compounds were analyzed using UV spectroscopy (Shimadzu UV-240IPC) with different reagents; sodium methoxide, sodium acetate, sodium acetate with boric acid, aluminum chloride and aluminum chloride with hydrochloric acid. The purified flavonoids were subjected to 1H-NMR (Joel ECA, 500MHz), 13C-NMR (Joel ECA, 125MHz) and mass-spectroscopy (Finningen, model 3200 at 70eV). The sugar moieties of the isolated flavonoid glycosides were investigated according to Gálvez et al. (2003).

**Results**

**Effect of S. persica extracts and indomethacin on oedema in Carrageenan model**

Carrageenan-induced paw edema as an *in vivo* model of inflammation is the most frequently used method to evaluate the anti-edematous effect of natural products. The results obtained are summarized in Tab. 1.

Carrageenan injection induced skin inflammation in rat hind paw which increased paw diameter to be 4.32
mm, however, administration of Salvadora extracts significantly reduced the inflammation in hind paw time dependently (Fig. 1). At the first hour, administration of Salvadora extracts didn’t show any significant reduction in oedema as compared to vehicle control group or reference drug. Through the following up period, the effect of ethyl acetate was increased in an ascending order to be similar to indomethacin at the last hour while crude extract remained the lowest effective one. The reduction in paw diameter was 27.08 and 27.31% for ethyl acetate extract and reference drug, respectively after four hours of carrageenan administration. It could be concluded that the ethyl acetate extract is more potent than the crude extract in treating oedema.

Effects of S. persica extracts and indomethacin on serum inflammatory cytokines concentration

In assay data, the IL-1β, IL-6, TNF-α and TGF-β1 secretion in serum were highly elevated by carrageenan induction but administration of S. persica extracts significantly reduced serum secretion of inflammatory mediators as compared to vehicle group (Tab. 2).

IL-1β was elevated in response to carrageenan induction (48.2 ng/l) inversely with extract pretreatment which caused a significant reduction in IL-1β secretion from 48.02 ng/l in vehicle control group sera to 33.53 and 29.77 ng/l for animals of crude and ethyl acetate extracts groups, respectively. The same effect was observed in IL-6 secretion as the two extracts produced depletion percentages of 10.5 and 19.5% for crude and ethyl acetate extracts, respectively and 19.8% for indomethacin administration. Data presented in Tab. 2 clearly indicate that ethyl acetate extract has similar effect to indomethacin on IL-1β and IL-6 with insignificant difference between them.

TNF-α secretion was magnified by carrageenan injection (371 pg/ml). TNF-α secretion was reduced, comparable to carrageenan group, as a respond to extract administration. Administration of ethyl acetate extract significantly reduced inflammatory mediator (TNF-α) secretion by 28.9% to be close to inhibition level of indomethacin administration (32.2%). Secretion of TGF-β1

| Tab. 1. Acute inflammatory activity of S. persica extracts and indomethacin (reference drug) on Carrageenan induced paw oedema in rats |
|---|---|---|---|---|
| Time in hours | Groups | Paw diameter (mm) | Oedema inhibition (%) |
| | | Zero time | 1 h | 2 h | 3 h | 4 h |
| Control vehicle | (Carrageenan+1 ml saline) | 2.66±0.09 | 3.66±0.07* | 3.75±0.13* | 3.9±0.13* | 4.32±0.10* |
| Crude extract | (100 mg/kg) | 3.13±0.11 | 3.85±0.18* | 3.77±0.14* | 3.61±0.13* (7.4%) | 3.56±0.14* (17.59%) |
| Ethyl acetate | (100 mg/kg) | 2.88±0.07 | 3.64±0.05* (0.55%) | 3.37±0.01* (10.13%) | 3.25±0.1* (16.66%) | 3.15±0.06* (27.08%) |
| Indomethacin | (20 mg/kg) | 3.00±0.09 | 3.37±0.04* (10.13%) | 3.28±0.06* (12.53%) | 3.16±0.01* (18.97%) | 3.14±0.01* (27.31%) |

Data are presented as mean ± standard error followed by oedema inhibition percentage, *P<0.01

Fig. 1. Effect of Salvadora persica extracts on rat skin oedema thickness in carrageenan model. Values are mean of n, n=8
reached a high concentration in serum by carrageenan induction however, administration of the two extracts decreased TGF-β1 concentration in serum from 100.69 pg/ml in vehicle control group to be 61.33 pg/ml and 74.15 pg/ml in case of ethyl acetate and crude extracts, respectively. On the other hand, indomethacin reached nearly the same value (59.51 pg/ml).

Identification of S. persica isolated flavonoids

Purification of the ethyl acetate extract gave three main flavonoids, each of them showed a single spot with different solvent systems. Using acetic acid (15%) as solvent system, the Rf’s of the purified three compounds were 0.51, 0.57 and 0.19 corresponding to apigenin rhamnogluco side, luteolin glucoside and rutin, respectively.

<table>
<thead>
<tr>
<th>Compound 1 (Apigenin rhamnogluco side)</th>
</tr>
</thead>
</table>
| The UV spectral data of compound 1 indicated that this compound is flavone glycoside as it gave two absorption bands at 268 nm and 333 nm and it was glycosylated at C-7 position on A-ring. A bathochromic shift of band at 268 nm to 295 nm with addition of sodium methoxide and shift with aluminum chloride to 308 nm. Also a shoulder at 340 nm indicates the presence of free hydroxyl groups at C-5, C-7 and C-9 in the upfield regions at δ 146.7, 152.1 and 157, respectively, which agreed with 5, 7-hydroxylated C-ring of flavone (Horie et al., 1998). The linkage between aromatic ring and the sugar H-1" (δ 5) and the C-7 position of the flavone was supported by the observation of carbon signals at 103 ppm associated with the C-3 in the 13C-NMR spectra. 13C-NMR spectrum displayed the expected flavonoid structure is flavone with two free hydroxyl groups at C-5 and C-4` position and was glycosylated at C-7 position. Partial hydrolysis of this compound showed that it has two sugars; rhamnose and glucose. It is obvious from the above data that this compound is apigenin glycoside glycosylated with rhamnose and glucose, this expectation is coincided with data reported by Mabry et al. (1970) and Dordevic et al. (2000). This expectation was also supported by 1H-NMR, IR and Ms spectral data which showed four characteristic signals of flavone aromatic ring at 6.2 ppm, 7.5 ppm, 7.685 ppm and 8.52 ppm for H-6, H-5`, H-3` and H-6`, respectively. The carbonyl group of C-ring gave IR band at 1700 cm⁻¹ and characteristic signal of rhamnose methyl group at 1.085 ppm to 1.233 ppm which gave a sharp band at 1416.5 cm⁻¹ in IR chromatogram. The recorded protons signals of rhamnose were as follow; signal at 3.282 for H-5`, 3.556 ppm for H-2`, 3.826 ppm for H-6` and 4.6 ppm for H-1`. Signals at 3.11 ppm are for H-2 of glucose while band at 3424.2 cm⁻¹ on IR is for glucose hydroxyl groups. The UV spectral data and 1H-NMR spectra are in accordance with Mabry et al. (1970) and El Alfy et al. (2010). It could be suggested that this compound is apigenin rhamno-glucoside.

<table>
<thead>
<tr>
<th>Compound 2 (Luteolin glucoside)</th>
</tr>
</thead>
</table>
| The UV spectra of this compound suggested the presence of luteolin glucoside with a single sugar position. Compound 2 has two characteristic bands at 349 and 253 nm. The bathochromic shift with AlCl₃ produced two peaks at 426 and 328 nm indicating the presence of free OH in position 5, also the band shift with NaOAc+H₃BO₃ at 370 nm indicating the presence of free OH groups at 3 and 4 positions (Mabry et al., 1970; Meririnhos et al., 2005). Comparison of NMR spectra of compound 2 with those published by Wang et al. (1998) and Lu and Foo (2000), compound 2 could be identified as luteolin-7-O-β-D-glucoside. The doublet at δ 5.4 (J 1.9Hz) for anomeric proton in 1H-NMR data indicating a β-configuration for glucose moiety. Compound 2 showed a singlet at 6.7 ppm in 1H-NMR spectra consistent with the H-3 of flavone and this was supported by the observation of carbon signals at 103 ppm associated with the C-3 in the 13C-NMR spectra. 13C-NMR spectrum displayed C-5, C-7 and C-9 in the upfield regions at δ 146.7, 152.1 and 157, respectively, which agreed with 5, 7-hydroxylated A-ring of flavone (Horie et al., 1998). The linkage between the sugar H-1" (δ 5) and the C-7 position of the flavone is occurred in the upfield region at δ 152.8, (Tab. 3). Samples were analyzed in DMSO, 500 MHz exhibited molecular ion at m/z 446.5 whose fragmentation provided a characteristic m/z at 285, a typical mass for luteolin aglycone.

---

### Tab. 2. Effect of S. persica on serum inflammatory cytokines secretion in carrageenan induced paw oedema in rat

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Interleukin-1β (pg/ml)</th>
<th>Interleukin-6 (pg/ml)</th>
<th>Tumor necrosis factor-α (pg/ml)</th>
<th>Transforming growth factor-β1 (pg/ml)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (v-)</td>
<td>17.83±0.26a</td>
<td>211.52±0.99a</td>
<td>224.92±1.11</td>
<td>30.82±0.66a</td>
<td></td>
</tr>
<tr>
<td>Control (Carrageenan)</td>
<td>48.02±0.58</td>
<td>405.70±4.37</td>
<td>371±0.77</td>
<td>100.69±4.49</td>
<td></td>
</tr>
<tr>
<td>Crude extract (100 mg/kg)</td>
<td>33.53±0.72a</td>
<td>363.13±9.43a</td>
<td>290.71±2.48</td>
<td>74.15±1.35a</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate extract (100 mg/kg)</td>
<td>29.77±0.51a</td>
<td>326.62±1.11a</td>
<td>263.61±0.57</td>
<td>61.33±0.85a</td>
<td></td>
</tr>
<tr>
<td>Indomethacin (20 mg/kg)</td>
<td>26.80±0.34a</td>
<td>325.15±0.54a</td>
<td>251.36±4.92</td>
<td>59.52±6.51a</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as the means± S.D. a P<0.01, compared to vehicle control group. Groups have the same letter means do not differ significantly.
Compound 3 (Rutin)

The UV spectrum of compound 3 showed two major absorption bands at 359 and 257 nm, which indicated the presence of flavonol structure. The bathochromic shift with sodium methoxide supported the presence of 4'-hydroxyl group and with sodium acetate indicated the 7-OH function, while AlCl₃ and AlCl₃/HCl spectrum showed 5-OH and ortho di-OH in B-ring. The above data mean that 3-OH is absent or substituted. These results were supported by ¹H-NMR and ¹³C-NMR. The chemical shifts of protons and carbons essentially identical with those reported for rutin by Fathiazad et al. (2006) and Brietmaier and Voelter (1989). Also chemical shift at 6.21 ppm (J=2) was for proton of C-6 while shift at 6.4 ppm (J=2) for C-8 proton. The chemical shifts at 7.6, 6.81, 7.56 ppm for protons of C-2, C-5 and C-6, respectively. The OH group on C-3, C-4, C-5 and C-7 showed chemical shifts at 9.1, 9.74, 12.6 and 10.9 ppm. The characteristic signal of β-D-glucose was at 5.21 ppm while the characteristic signal of H-1 rhamnose and methyl group were at 5.12 and 1.24 ppm respectively. ¹⁳C-NMR readings (Tab. 3) were identical with reported data by Fathiazad et al. (2006) also Brietmaier and Voelter (1989).

Discussion

The present study was conducted to investigate the possible anti-inflammatory effects of S. persica extracts in carrageenan model relevant to their ability to block leukotriene secretion in animal serum. Effort was also directed towards the isolation and identification of the chemical constituents of the biologically active extract.

Paw swelling is one of the major factors in assessing the degree of inflammation and efficacy of the tested drugs (Begum and Sadique, 1988; Mizushima et al., 1972). The crude and the ethyl acetate extracts of S. persica were investigated for their acute anti-inflammatory activity using oedema model. The two extracts significantly inhibited oedema in a time dependent manner and the maximum inhibition percentages were recorded with ethyl acetate extract. In addition, ethyl acetate extract reached the same inhibition level of the reference drug indomethacin at end of the follow up period.

Plasma levels of IL-6 appear to reflect the changes in inflammation (Gadient and Otten, 1995) and it is an important immune mediator co-ordinating the activity of different immune cells with an important role in acute phase response (Harbus et al., 1993). Administration of S. persica extracts reduced the elevation of IL-6 by carrageenan induction. However, the ethyl acetate extract showed similar effect to the reference drug indomethacin, meaning that it is a potent agent in reducing inflammation.

Tumor necrosis factor α (TNF-α) is a pleotropic inflammatory cytokine produced by the immune system that suppresses tumor cell proliferation. Subsequent studies established that TNF-α is a key mediator of inflammation (Vasanthi et al., 2007; Warren et al., 1988,) so, it is an important parameter that should be included in determining the anti-inflammatory activity of plant extracts. Also, interleukin-1, an important cytokine produced mainly by blood monocytes, mediates the panoply of host reactions collectively known as acute phase response also known as Endogenous Pyrogen (EP), mononuclear cell factor and lymphocyte activating factor. TNF-α and IL-1β are potent proinflammatory cytokines capable of inducing multiple signaling cascades that can serve in host defense and paradoxically contribute to inflammatory tissue injury (McCulloch et al., 2006). Both IL-1α and IL-1β can trigger fever by enhancing prostaglandin E2 (PGE2) synthesis by the vascular endothelium of the hypothalamus and can stimulate T cell proliferation (Limeli et al., 2008). Results of the present study revealed that the effects of S persica extracts on IL-1β and TNF-α were more pronounced than their effects on IL-6. Both of the two extracts significantly reduced the secretion of IL-1β and TNF-α. The represented effect of ethyl acetate extract was similar to the standard drug indomethacin. TGF-β distributed in tissues and synthesized by many different cells (Kropf et al., 1997). It is involved in wound repair processes and in starting inflammatory reaction and then in the resolution through chemotactic attraction of inflammatory cells and fibroblast (Lawrance, 1996). Administration of extracts before carrageenan induction appeared to be preferable; they decreased TGF-β concentration in serum. The ethyl acetate extract was superior to crude one and gave the same results of indomethacin with insignificant difference between them.

These findings show that the ethyl acetate extract was able to inhibit acute inflammatory mediator release from mast cells and leukocytes, also to reduce inflammation by inhibition of TGF-1 which might participate in the early inflammatory responses (Giri et al., 1993; Pittet et al., 2001). The obtained results suggest that Salvadora persica ethyl acetate extract may decrease the vascular permeability, promote the resolution of oedema and exhibit inhibitory effect on proinflammatory cytokines secretion. Our results are in coincided with those of Saahil et al. (2007), Guardia et al. (2001) and Rajesh et al. (2011) who reported that Salvadara persica roots possess anti-oxidant activity and anti-inflammatory activity. Also S. persica, toothbrush stick, is used for oral hygiene, and to treat gum inflammation (Sher et al., 2010).

Biochemical investigation carried out through this investigation revealed the presence of apigenin rhamnoglucoside, luteolin glucoside and rutin as the major flavonoids in the ethyl acetate extract of S. persica. Many plant derived compounds have been tested for their anti-inflammatory activity as ability to block leukotriene synthesis in cells from rat, mouse or human sources. Some plant derived chemical constituents like flavanoids, coumarins, quinones, pentacyclic triterpenes, sesquiterpenes, alkaloids and polyacetylates have been reported to be 5-Lipoxygenase
Conclusions

In conclusion, *Salvadora persica* extracts have anti-inflammatory properties. The potential efficacy of *S. persica* extracts to treat inflammation is based in a part on the hypothesis that it will suppress the proinflammatory cytokines resulting in less oedema.

References


