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Treatment by hydroalcoholic and chloroformic extracts of *salvia candidissima* reduced hyperalgesia, edema and serum TNF–α level in arthritis model

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ABSTRACT

Medicinal plants are an important source of substances, which have been shown to induce non-specific immunomodulatory effects. Salvia species showed anti-inflammatory properties. This study was carried out to investigate the role of hydroalcoholic and chloroformic extracts of salvia candidissima on hyperalgesia, edema, and serum TNF-α level during adjuvant-induced arthritis. Inflammation was induced by a single subcutaneous injection of complete Freund's adjuvant into the rats' hind paw. Hydroalcoholic and chloroformic extracts were prepared from aerial parts of the plant, and different doses of the extracts were administered during 21 days of study. Hyperalgesia, edema, and serum TNF-α were assessed by radiant heat, plethysmometer, and ELISA technique, respectively. Administration of hydroalcoholic and chloroformic extracts of Salvia candidissima significantly reduced paw edema, hyperalgesia, and serum TNF-α level. Moreover, the hydroalcoholic extract was significantly more effective on the studied variables than the chloroformic extract. Then, it can be concluded that hydroalcoholic and chloroformic extracts of Salvia candidissima dose dependently reduce paw edema and hyperalgesia during CFA-induced arthritis. These effects can be mediated by decreased serum TNF-α levels.

Keywords: Inflammation, Pain, Medicinal plant, CFA, TNF–α

INTRODUCTION

Inflammation is a protective response of tissues to injury [1], which is divided into acute and chronic phases; acute phase refers to orchestrated reactions characterized by increased blood flow and vascular permeability along with the accumulation of fluid, leukocytes, and inflammatory mediators such as cytokines, and chronic phase is a dysregulated form of inflammation, which refers to specific humoral and cellular responses caused by the presence of pathogens in the site of injury [2, 3]. Hyperalgesia and edema are two important symptoms of inflammation [2]. Several cytokines, such as Interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6 and some chemokines, play key roles in acute inflammatory processes. TNF-α is a systemic marker of inflammation and a very potent pro-inflammatory cytokine, which is essential for the recruitment of neutrophils to the site of inflammation [3]. This cytokine is not only involved in acute inflammation but also in chronic inflammatory reactions [4]. TNF-α is a key cytokine involved in the pathogenesis of rheumatoid arthritis (RA), causing a chronic inflammatory state in which the synovial membrane is the primary site of attack. It is believed that TNF-α is a key mediator in the development of joint damage in RA [5]. Therapies directed against TNF-α are effective in treatment of RA and reduce pain scores

in this condition [6, 7]. There are several models for inducing arthritis. One of these models is complete Freund's adjuvant (CFA)-induced arthritis, which is widely used in etiopathogenic investigational drug and molecular studies, due to its similarity to human RA. It has been shown that local injection of CFA can mimic animal RA model and cause hyperalgesia and edema, which are associated with increased local and systemic secretion of cytokines, such as IL-1β, IL-6, and TNF-α [8].

Use of herbal products is growing worldwide, because they are available to consumers as over-the-counter (OTC) drugs in various forms of preparations and doses. Various factors are believed to contribute to the increasing trend for the use of herbal supplements, which are as follows: the desire for self-medication, easy availability, and the perceptions that herbs are safer, gentler, and less costly than conventional drugs [9, 10]. The genus *Salvia* (*Lamiaceae*) includes about 900 species spread all over the world, and 17 species of them are endemic in Iran [11]. Plants belonging to this genus are pharmacologically active and have been used in folk medicine all over the world. Many *Salvia* species and their isolated constituents have been shown to have significant antioxidant activity in enzyme-dependent and enzyme-independent systems [12, 13]. The phytochemical analysis of *Salvia* species showed the presence of several compounds, mostly belonging to the groups of phenolic acids, phenolic glycosides, coumarins, anthocyanins, flavonoids, polysaccharides, sterols, terpenoids, and essential oils [14, 15]. This species has several properties, such as antibacterial, antioxidant, antitumor, and cholinergic binding [13, 16-19]. Many Salvia species, such as *S. fruticosa*, *S. verticillata*, and *S. trichoclada* have been shown to have anti-inflammatory properties [20]. *S. candidissima* is herbaceous perennial native in western Greece and parts of Turkey, Iraq, and Iran [21]. There are not enough evidences about anti-inflammatory effects of this species, but some studies indicated that the chloroformic extract of *S. officinalis L.* leaves have strong anti-inflammatory properties after topical application. Ursolic acid, as an active component of sage, has been shown to have strong anti-inflammatory properties. It has been found that anti-inflammatory effect of ursolic acid was two-fold more potent than indomethacin [22]. Considering the increased inflammatory mediators in adjuvant-induced inflammation, the role of TNF-α in the induction inflammatory symptoms such as edema and hyperalgesia, unavailability and high cost of synthetic drugs, and the anti-inflammatory properties of *Salvia* species, we aimed to study the effects of hydroalcoholic and chloroformic extracts of *S. candidissima* on hyperalgesia, edema, and serum TNF–α level during adjuvant-induced arthritis in male Wistar rats.

MATERIALS AND METHODS

Plant collection and extract preparation and formulation

The aerial parts of *S. candidissima* were collected during its flowering stage from Urmia, West Azerbaijan Province, Iran. A voucher specimen (MPH-1755) of identified plant was deposited in Medicinal Plants and Drugs Research Institute Herbarium of Shahid Beheshti University, Tehran, Iran. The collected aerial parts were cleaned, air dried, chopped into small pieces, and powdered for extract preparation. In the next step, in order to provide hydroalcoholic extract, 250 g of the plant powder was exposed to 70% methanol for 72 h. For chloroformic extraction, 500 g of the plant powder was mixed with chloroform for 72 h. Extracts were filtered using filter paper. The clear extracts were separated from their solvents by rotary under vacuum at 55°C. Concentrated extracts were refrigerated at 4°C. Dried extracts were solved in dimethyl sulfoxide (DMSO).

Phytochemical and toxicity study

Standard phytochemical screening tests were used to screen the extracts. Qualitative analyses of the plant were performed to detect saponins, alkaloids, and terpenoids [23].

Median lethal dose (LD_{50}) was determined using the method described by Lorke [24]. Treatment was performed by intraperitoneal (i.p.) injection of different doses (25, 50, 100, 200, and 400 mg/kg) of hydroalcoholic or chloroformic extracts. Rats were monitored for 21 days after treatment. The final LD_{50} value was calculated as the square root of the product of the lowest lethal dose and highest nonlethal dose.

Laboratory animals

Seventy-two male Wistar rats, weighing 180 to 220 g were used in the present study. The animals were arranged in two sets of 6 groups (6 rats in each group). Rats were housed in standard environmental conditions (22 ± 2°C, humidity 60-70%), under a 12 h light/dark cycle). The animals were allowed a standard diet and water, except during the time of experiment. For habituation to the laboratory environment, animals were placed in the laboratory environment for at least half an hour before the start of the experiment. The study protocol was approved by the

local ethics committee for the use of animals in research and we followed the guidelines of ethical standards for investigations of experimental pain in animals [25].

Induction of inflammation

Inflammation was induced on day 0 by a single subcutaneous injection of 100 µL of CFA emulsion and 100 µL of double-distilled water into the right hind paw of the animals. Control rats only received injection of sterile mineral oil (100 µL). This animal model showed a rapid primary inflammation response to the adjuvant. Unilateral edema was observed during the first hour after CFA injection into hind paw (acute phase) and continued for three weeks (chronic phase) [26, 27].

Paw volume measurement

To confirm the inflammation induction, hind paw volume was measured before and after injections during different periods of study. The measurement was performed by displacement of an electrolyte solution containing NaCl 4-7% in a plethysmometer (model 7141; Ugo Basile; Comerio VA, Italy), as described previously by our lab. Briefly, rats were taken out of their cages and their hind paw was submerged to the tibiotarsal joint into an electrolyte-filled Perspex cell of the plethysmometer. The volume of displacement, which was equal to the paw volume, was indicated on a digital display system. Volume measurement was performed twice for each paw, and the averages were calculated. The edema was quantified by measuring the difference in foot volume between day 0 and the various time points [28, 29].

Thermal hyperalgesia assessment

Hyperalgesia was assessed according to our previous studies [28, 30]. In brief, thermal hyperalgesia were examined in both experimental and control groups using plantar test, in which an infrared heat source is directed through the floor of the chamber onto the plantar surface of animal's paw. An automatic timer was activated coincident with heat radiation and stopped with flicking of the paw. The time from onset of radiant heat to withdrawal of the hind paw was recorded by a digital timer and considered as paw withdrawal latency (PWL). If the rat failed to withdraw its paw from stimulus by 20 s, the test was terminated and the animal was assigned this cut-off value. PWL was measured two times for each paw at an interval of 5-10 min and the mean latency was calculated. The difference between right and left foot PWLs were calculated using 1-1 equation. Negative values indicated the presence of hyperalgesia in right paw (H, hyperalgesia; R, right; L, left; and t, time).

$$H = \frac{(Rt1 + Rt2 + Rt3)}{3} - \frac{(Lt1 + Lt2 + Lt3)}{3} \quad (1-1)$$

Blood sampling and serum TNF-α measurement

Rats were retro-orbitally bled under light anesthesia into heparinized tubes during the experimental procedure. The samples were centrifuged and then stored at -70°C. All blood samplings were done simultaneously for each group (8:00 to 8:15 am). Serum TNF-α was measured using standard rat enzyme-linked immunosorbent assay (ELISA) kit (Bender Med System, UK). The kit cross-reactivity with the rat serum TNF was 100%. Assessment procedure was performed according to the manufacturer's protocol [31].

Experimental procedure and experimental groups

Rats were randomly divided into different experimental groups: CFA (control); CFA + extract; CFA + DMSO (vehicle), and CFA + indomethacin (n = 6/group). CFA + extract group was divided into two subgroups of hydroalcoholic extract and chloroformic extract-treated. The subgroups separately received three different doses (25, 50, and 100 mg/kg) of the extracts. Injection of both polar (hydroalcoholic) and non-polar (chloroformic) extracts and indomethacin were performed from day 1 after CFA injection to day 21 at a certain time (8 to 9 am). Indomethacin, a standard non-steroidal anti-inflammatory agent, was injected (i.p.) at a dose of 5 mg/kg.

Before the onset of experiments, the rats were weighed. Inflammation was induced by injection of CFA. The effects of the treatment with different doses of hydroalcoholic and chloroformic extracts of *S. candidissima*, were assessed during acute and chronic phases of CFA-induced inflammation. To determine if dose-response relationship existed, different experiments were performed with different doses (25, 50, and 100 mg/kg) of the extracts. All doses of the extracts used during different stages of treatment were obtained from the same batch. Administration of extracts was performed daily at the same times, after injection of CFA during the 21 days of study. Serum TNF-α level,

hyperalgesia, and edema were measured on days 0, 3, 7, 14, and 21 of the study. Indomethacin as a standard anti-inflammatory agent was injected at the same times. Total volume of each injection was about 1 ml/rat.

Statistical analysis

Data analysis was performed using the software SPSS (version 19). One-way ANOVA and post-hoc Tukey's tests were used for intra-group comparison of hyperalgesia, edema, and serum TNF-α level, and unpaired student t-test was used for a more detailed comparison of changes between groups. All results were presented as mean ± standard error (SEM) and statistical significance was accepted at $p<0.05$. All graphs were plotted using Excel software.

RESULTS

Phytochemical and toxicity studies

Phytochemical study of the extracts showed the presence of flavonoids, saponin glycosides, and terpenoids. Our alkaloid test result was negative. The dried hydroalcoholic extract had 0.83% (w/w) of flavonoid on the basis of hyperoside.

Our results indicated that LD_{50} of both extracts were near 315 mg/kg. According to our findings, when rats received doses above LD50 of the extracts, they showed decreased mobility, respiratory distress, cyanosis, immobility, and death.

Hyperalgesia variations during different stages of study

Plantar injection of CFA caused significant increase in hyperalgesia. In the CFA group, hyperalgesia significantly increased on the 3rd, 7th, 14th, and 21st days of the study compared to day 0. In this case, on day 7 after CFA injection, hyperalgesia was significantly greater than days 3, 14, and 21 ($p<0.001$ for all).

Administration of hydroalcoholic or chloroformic extracts of *S. candidissima* at doses of 25, 50 and, 100 mg/kg significantly decreased hyperalgesia in a dose-dependent manner on days 3, 7, 14, and 21 compared to the CFA control group. Reduction of hyperalgesia in the CFA-injected rats were significantly greater during treatment with 50 and100 mg/kg doses of the extracts, compared to the dose of 25 mg/kg on days 7, 14, and 21 ($p<0.001$ for days 3 and 7, $p<0.01$ for day 14, and $p<0.05$ for day 21). Also, there were no significant differences between the anti-hyperalgesic effects of 50 and 100 mg/kg doses of the extracts during different stages of study, and thus the dose of 50 mg/kg of the extracts was considered as effective dose (Table 1). Administration of the effective dose of extracts (hydroalcoholic or chloroformic) in CFA-treated rats significantly reduced hyperalgesia compared to the indomethacin-treated group (5 mg/kg) on 7th and 14th days of study ($p<0.001$ for both). Moreover, our results indicated that administration of the effective dose of hydroalcoholic extract of *S. candidissima* was significantly more effective than chloroformic extract in the reduction of CFA-induced hyperalgesia on days 7 and 21 of the study ($p<0.01$) (Figure 1).

Paw volume variations during different stages of study

Paw volume significantly increased in CFA-injected rats on days 3, 7, 14, and 21 compared to day 0 ($p<0.01$ for day 3 and $p<0.001$ for all other days). In this study, we found that administration of hydroalcoholic or chloroformic extracts of *S. candidissima* at doses of 25, 50, and 100 mg/kg in CFA-inflamed rats caused a significant dose-dependent reduction in paw edema. Both hydroalcoholic and chloroformic extracts at doses of 50 and 100 mg/kg significantly reduced paw edema on days 3, 7, 14, and 21 compared to the CFA-treated control rats ($p<0.001$). Also, the results showed that 50 and 100 mg/kg doses of the extracts were more effective than dose of 25 mg/kg in the reduction of edema on days 7, 14, and 21 ($p<0.05$). There were no a significant differences between doses of 50 and 100 mg/kg in terms of paw volume reduction. Consequently, the dose of 50 mg/kg was considered as effective dose (for both extracts) (Table 2).

Administration of the effective dose of hydroalcoholic or chloroformic extracts of *S. candidissima* caused significantly less reduction in paw edema than indomethacin-treated (5 mg/kg) rats on day 3 ($p<0.05$), but in CFA + extract treated rats paw edema reduction on days 7, 14, and 21 was significantly more than indomethacin-treated group ($p<0.01$ for day 7 and $p<0.001$ for other days). Finally, our results showed that administration of the effective dose of hydroalcoholic extract was significantly more potent than chloroformic extract in reduction of paw edema in CFA-treated rats ($p<0.001$) (Figure 2).

Table 1. Effects of long term treatment with different doses of hydroalcholic and chloroformic extracts of *S. candidissima* on hyperalgesia variations

	Hydroalcholic extracts			Chloroformic extracts	
Group	N	Mean value of withdrawal latencies between right and left hindpaws	Group	N	Mean value of withdrawal latencies between right and left hindpaws
CFA			CFA		
0 d of treatment	6	0.11±0.05	0 d of treatment	6	0.11±0.05
3 d after treatment	6	-2.45±0.18	3 d after treatment	6	-2.45±0.18
7 d after treatment	6	-5.88±0.14	7 d after treatment	6	-5.88±0.14
14 d of treatment	6	-3.46±0.13	14 d of treatment	6	-3.46±0.13
21 d of treatment	6	-2.43±0.08	21 d of treatment	6	-2.43±0.08
CFA+ Extract (25 mg/kg)			CFA+ Extract (25 mg/kg)		
0 d of treatment	6	.1±0.05	0 d of treatment	6	.183±0.07
3 d after treatment	6	-2.21±0.13	3 d after treatment	6	-2.13±0.1
7 d after treatment	6	-3.01±0.21	7 d after treatment	6	-3.2±0.1
14 d of treatment	6	-1.91±0.7	14 d of treatment	6	-1.93±0.11
21 d of treatment	6	-1.63±0.01	21 d of treatment	6	-1.93.±0.12
CFA+ Extract (50 mg/kg)			CFA+ Extract (50 mg/kg)		
0 d of treatment	6	.11±0.11	0 d of treatment	6	.11±0.07
3 d after treatment	6	-1.41±0.75	3 d after treatment	6	-1.39±0.08
7 d after treatment	6 ***	-1.32±0.45	7 d after treatment	6 ###	-1.85±0.18
14 d of treatment	6	-1.34±0.76	14 d of treatment	6	-1.43±0.03
21 d of treatment	6 ± ±	-0.8±.0.81	21 d of treatment	6	-1.22±0.07
CFA+ Extract (100 mg/kg)			CFA+ Extract (100 mg/kg)		
0 d of treatment	6	.13±0.13	0 d of treatment	6	.085±0.07
3 d after treatment	6	-1.31±0.75	3 d after treatment	6	-1.51±0.11
7 d after treatment	6	-1.35±0.11	7 d after treatment	6	-1.65±0.1
14 d of treatment	6	-1.38±0.11	14 d of treatment	6	-1.41±0.11
21 d of treatment	6	-1.25±0.04	21 d of treatment	6	-1.36±0.04
Indomethacin (5mg/kg)			Indomethacin (5mg/kg)		
0 d of treatment	6	-.133±0.1	0 d of treatment	6	-.133±0.1
3 d after treatment	6	-1.13±0.08	3 d after treatment	6	-1.13±0.08
7 d after treatment	6	-3.78±0.34	7 d after treatment	6	-3.78±0.34
14 d of treatment	6	-2.15±0.1	14 d of treatment	6	-2.15±0.1
21 d of treatment	6	-1.26±0.12	21 d of treatment	6	-1.26±0.12

**** P<0.001, vs day 0 of hydroalcholic (50mg/kg) group.*

P<0.001, vs day 0 of chloroformic (50mg/kg) group.; ±± <0.01vs day 21 in CFA group; ¶ <0.05vs day 21 in CFA group

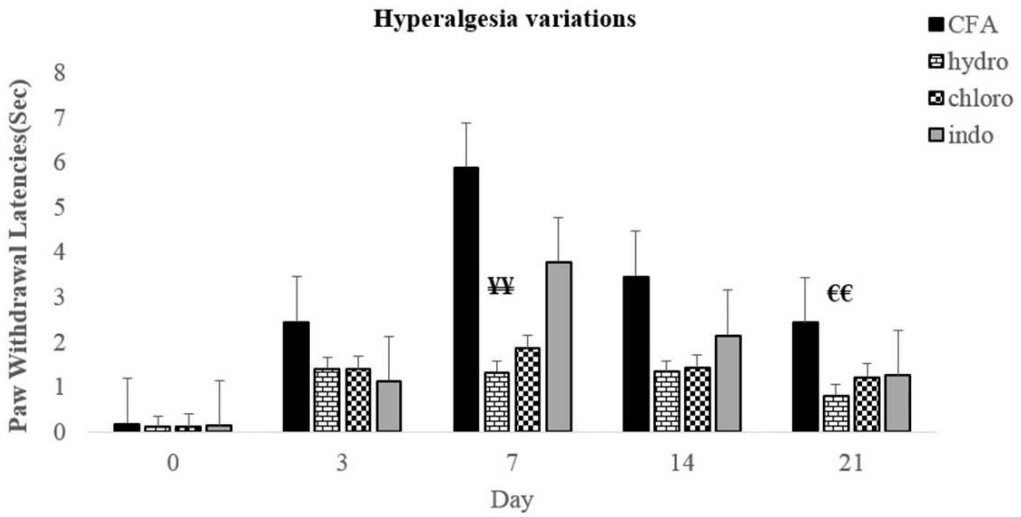

Figure 1. Comparison of paw withdrawal latencies between hydroalcholic and chloroformic extract treated rats.
Administration of effective dose of hydroalcholic extract was more potent than chloroformic extract in hyperalgesia reduction in CFA-inflamed rats. Data are presented as mean±SEM (n=6/group).; ¥¥ P<0.01: for comparing hydroalcholic with chloroformic extract- treated (50mg/kg) groups on day 7.; €€ P<0.01: for comparing hydroalcholic with chloroformic extract- treated (50mg/kg) groups on day 21.

Table 2. Effects of treatment with different doses of hydroalcholic and chloroformic extracts of *S. candidissima* on paw volume variation

Hydroalcholic extracts					Chloroformic extracts
Group	N	Paw edema variation	Group	N	Paw edema variation
CFA			CFA		
0 d of treatment	6	1.1±0.086	0 d of treatment	6	1.1±0.086
3 d after treatment	6	1.53±.008	3 d after treatment	6	1.53±0.08
7 d after treatment	6	1.96±0.012	7 d after treatment	6	1.96±0.012
14 d of treatment	6	2.3±0.086	14 d of treatment	6	2.3±0.086
21 d of treatment	6	2.8±0.076	21 d of treatment	6	2.8±0.086
CFA+ Extract (25 mg/kg)			CFA+ Extract (25 mg/kg)		
0 d of treatment	6	1.07±.0.013	0 d of treatment	6	1.09±0.049
3 d after treatment	6	1.63±0.078	3 d after treatment	6	1.73±0.061
7 d after treatment	6	1.43±.0.066	7 d after treatment	6	1.54±0.085
14 d of treatment	6	1.49±0.053	14 d of treatment	6	1.59±0.086
21 d of treatment	6	1.63±.0.035	21 d of treatment	6	1.65±0.049
CFA+ Extract (50 mg/kg)			CFA+ Extract (50 mg/kg)		
0 d of treatment	6	1.11±0.075	0 d of treatment	6	1.08±.0.073
3 d after treatment	6	1.44±.0.066	3 d after treatment	6	1.52±0/075
7 d after treatment	6 ***	1.19±0.069	7 d after treatment	6 ###	1.39±0.067
14 d of treatment	6	1.25±0/047	14 d of treatment	6	1.61±0.023
21 d of treatment	6 6+++	1.35±0.047	21 d of treatment	6 ¶¶¶	1.62±0.024
CFA+ Extract (100 mg/kg)			CFA+ Extract (100 mg/kg)		
0 d of treatment	6	1.05±0.054	0 d of treatment	6	1.09±0/062
3 d after treatment	6	1.56±0.052	3 d after treatment	6	1.64±0.032
7 d after treatment	6	1.21±0.033	7 d after treatment	6	1.45±0.054
14 d of treatment	6	1.37±0.049	14 d of treatment	6	1.53±0.086
21 d of treatment	6	1.45±0.073	21 d of treatment	6	1.61±0.048
Indomethacin (5mg/kg)			Indomethacin (5mg/kg)		
0 d of treatment	6	1.13±0.05	0 d of treatment	6	1.13±0.05
3 d after treatment	6	1.21±0.07	3 d after treatment	6	1.21±0.07
7 d after treatment	6	1.61±0.07	7 d after treatment	6	1.61±0.07
14 d of treatment	6	1.91±0.13	14 d of treatment	6	1.91±0.13
21 d of treatment	6	2.7±0.12	21 d of treatment	6	2.7±0.12

**** P<0.001, vs day 0 in hydroalcholic (50mg/kg) group.; ### P<0.001, vs day 0 in chloroformic (50mg/kg) group.*
+++ <0.001vs day 21 in CFA group; ¶¶¶ <0.001vs day 21 in CFA group

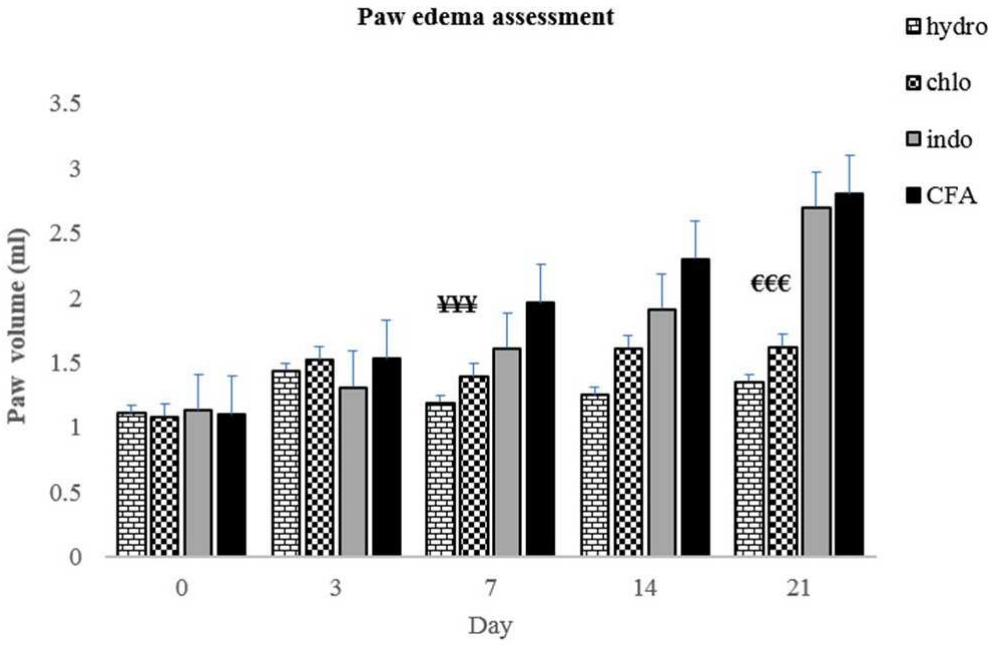

Figure 2. Comparison of paw edema between hydroalcholic and chloroformic extracts in CFA-treated rats.
Effective dose of hydroalcholic extract was more potent than chloroformic extract in paw volume reduction during CFA-induced inflammation. Data are presented as mean±SEM (n=6/group).
¥¥¥ P<0.001: for comparing hydroalcholic with chloroformic extract- treated (50mg/kg) groups on day 7.
€€€ <0.001: for comparing hydroalcholic with chloroformic extract- treated (50mg/kg) groups on day 21.

Table 3. Effects of treatment with different doses of hydroalcholic and chloroformic extracts of *S. candidissima* on serum *TNF-α* variation

		Hydroalcholic extracts		Chloroformic extracts	
Group	n	Serum *TNF-α* variation	Group	N	Serum *TNF-α* variation
CFA			CFA		
0 d of treatment	6	0±1.12	0 d of treatment	6	0±1.12
3 d after treatment	6	850.14±19.11	3 d after treatment	6	850.14±19.11
7 d after treatment	6	740±23.75	7 d after treatment	6	740±23.75
14 d of treatment	6	639±13.4	14 d of treatment	6	639±13.4
21 d of treatment	6	600±23.2	21 d of treatment	6	600±23.2
CFA+ Extract (50 mg/kg)			CFA+ Extract (50 mg/kg)		
0 d of treatment	6	0±1.2	0 d of treatment	6	0±1.12
3 d after treatment	6	475±24.6	3 d after treatment	6	522.41±22.7
7 d after treatment	6 *** ¥	20.5±1.87	7 d after treatment	6 ###	28.64±4.4
14 d of treatment	6	50.5±2.42	14 d of treatment	6	125.45±4.33
21 d of treatment	6 €€€ ±±±	52.6±1.82	21 d of treatment	6 ¶¶¶	154.51±4.07
Indomethacin (5 mg/kg)			Indomethacin (5mg/kg)		
0 d of treatment	6	0±1.2	0 d of treatment	6	0±1.2
3 d after treatment	6	321.21±17.05	3 d after treatment	6	321.21±17.05
7 d after treatment	6	347.15±4.3	7 d after treatment	6	347.15±4.3
14 d of treatment	6	443.65±19.5	14 d of treatment	6	443.65±19.5
21 d of treatment	6	403.87±10.2	21 d of treatment	6	403.87±10.2

**** P<0.001, vs day 0 in hydroalcholic (50mg/kg) group; ### P<0.001, vs day 0 in chloroformic (50mg/kg) group*
¥ P<0.05, vs day 7 in chloroformic (50mg/kg) group; ±±± <0.001vs day 21 in CFA group
¶¶¶ <0.001vs day 21 in CFA group; €€€ <0.001vs day21 of chloroformic (50mg/kg) group.

Serum TNF-α level variation during different stages of study

Plantar injection of CFA caused a significant increase in serum TNF-α level on days 3, 7, 14, and 21 compared to the day 0 ($p<0.001$ for all). In this case, increased serum TNF-α level in CFA-injected group was significantly higher on days 7, 14, and 21 compared to day 3 ($p<0.001$ for all). Administration of hydroalcoholic or chloroformic extract of *S. candidissima* in CFA-treated rats (at doses of 25, 50, and 100 mg/kg) caused a significant decrease in serum TNF-α level on days 3, 7, 14, and 21, as compared to the CFA group ($p<0.001$). Administration of extracts at doses of 50 and 100 mg/kg were more effective in the reduction of serum TNF-α level in CFA-inflamed rats compared to dose of 25 mg/kg during different stages of study ($p<0.001$). Also, there were no significant differences in serum TNF-α level, between 50 and 100 mg/kg doses in CFA + extracts treated rats; therefore, 50 mg/kg was considered as the effective dose. Administration of 50 mg/kg (effective dose) of hydroalcoholic or chloroformic extract in CFA-treated rats caused a significant reduction in serum TNF-α level compared to the group received indomethacin (5 mg/kg) on days 7,14, and 21 of study ($p<0.001$).

Furthermore, our results indicated that hydroalcoholic extract of *S. candidissima* was significantly more effective than chloroformic extract in the reduction of CFA-induced increase in serum TNF-α level on days 7 and 21 of the study ($p<0.001$ for all) (Table 3).

DISCUSSION

This study confirmed that administration of hydroalcoholic and chloroformic extracts of *S. candidissima* in CFA-injected rats, significantly and dose-dependently decreased edema, hyperalgesia, and serum TNF-α level. Also, our results indicated that the effective dose of extracts showed significantly more potent anti-inflammatory effect than indomethacin.

Inflammation is a beneficial response, which is normally resolved by restoration of normal tissue structure and function; however when inflammation persists and becomes chronic, it can lead to tissue damage and loss of function. Chronic inflammation can be associated with variations in cytokine secretion [32]. CFA model has been used extensively, not only to analyze the cellular and molecular aspects of inflammation, but also toevaluate the anti-inflammatory and anti-nociceptive effects of newly developed drugs on chronic arthritis [33]. Our previous studies revealed that CFA-induced monoarthritis consists of two phases: first, inflammatory and second, arthritic phases. The first phase results in rapid elevation of the secretion of inflammatory mediators, chemokines, cytokines (such as IL-1, IL-6, and TNF-α), and free radicals [23, 30]. TNF-α is involved in various pathological processes, such as inflammation and pain induction. This cytokine plays a critical role in the development of hyperalgesia. In the peripheral nervous system, TNF-α can promote nociceptor sensitization through modulation of the activity of several ion channel, such as capsaicin receptor (TRPV1), Na^+, Ca^{2+}, and K^+ channels [34-36]. Also, this cytokine induces sensitization in the central part of pain-conducting system, particularly lamina II of spinal cord [37, 38]. TNF-α is involved in the induction of edema. In this way, expression of vascular endothelial intercellular adhesion molecule-1 (ICAM-1) and CD-18 integrin are increased on the surface of neutrophils by this cytokine. This condition could induce endothelial injury, increased vessel permeability, and edema [39]. Flavonoids can inhibit secretion of pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6 [40]. Studies indicated the presence of flavonoids, terpenoids, and antioxidant factors in the Salvia species [31, 41]. Our results also confirmed the presence of flavonoids and terpenoids in *S. candidissima* extracts, which is in agreement with the findings of other studies [42-44]. Studies also have shown that flavonoids prevent the induction of inflammation by inhibiting nuclear factor kappa B (NF-κB) as an important inflammatory factor. Some studies revealed that flavonoid may increase the release of anti-inflammatory cytokines, such as IL-10 [45]. In the case of terpenoids, also there is an evidence of anti-inflammatory effects through inhibition of nitric oxide production by macrophages infiltrating into the site of inflammation [46]. In addition, it has been shown that antioxidants have protective effects against damages related to the inflammation and production of reactive oxygen species [26]. Asadi et al. showed antioxidant property of 6 species of *Salvia (S. hydrangea, S. lachnocalyx, S. macilenta, S. multicalis, S. sclarea and S. xanthocheila*) [47]) Hosseinzadeh et al. also found that *S. leriifolia* has potent anti-inflammatory and anti-analgesic effects on inflamed rats, which could be related to its components, such as flavonoids [48].

Furthermore, the survey conducted in this study showed that daily administration of 25, 50, and 100 mg/kg doses of hydroalcoholic and chloroformic extracts of *S. candidissima* significantly reduced the edema and hyperalgesia induced by CFA injection, but the hydroalcoholic extract showed stronger effects than chloroformic extract. It seems that the anti-inflammatory properties of polar components of *S. candidissima* extract are more than non-polar

components, a matter which needs more investigation. On the other hand, our results indicated that although, long term administration of effective dose of hydroalcoholic and chloroformic extracts of *S. candidissima* showed stronger anti-hyperalgesic and anti-inflammatory effects than indomethacin, but in the first days, indomethacin had stronger anti-inflammatory effects than the extracts. These effects can be related to chemical properties of indomethacin components compared to the cumulative anti-inflammatory effects of the extracts' components during long-term administration, a point which needs more investigation. According to our results, it can be concluded that long-term administration of hydroalcoholic and chloroformic extracts of *S. candidissima* can effectively reduce hyperalgesia and edema during CFA-induced arthritis. It seems that at least a part of these anti-inflammatory effects is mediated by reduction of serum TNF-α level due to the existence of some anti-inflammatory components, such as flavonoids and terpenoids in the *S. candidissima* extracts. More investigations are recommended to assess the role of the effective components of extracts.

Conflict of interests

The authors declare that they have no conflict of interests.

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REFERENCES

[1] Khan K.M., Ambreen N., Mughal U.R., et al. *Eur J Med Chem*, **2010**, 45(9):4058-64.
[2] Murakami M. The molecular mechanisms of chronic inflammation development. Frontiers E-books, **2012**.
[3] Shaikh P.Z., Sharma M., Shah A., et al. *Int J of Pharm & Life Sci(IJPLS)*, **2011**, 2(10):1154-73.
[4] Feghali C.A., Wright T.M. *Front Biosci*, **1997**, 2(1):d12-d26.
[5] Inglis J.J., Nissim A., Lees D.M., et al. *Arthritis Res Ther*, **2005**, 7(4):R807-16.
[6] Hultgren O., Kopf M., Tarkowski A. *J Immunol*, **1998**, 160(10):5082-7.
[7] Vasanthi P., Nalini G., Rajasekhar G. *APLAR J Rheumatol*, **2007**, 10(4):270-4.
[8] Zhang Q., Schäfer M., Elde R., et al. *Neuroscience*, **1998**, 85(1):281-91.
[9] Miller L.G., Hume A., Harris I.M., et al. *The Journal of Human Pharmacology and Drug Therapy*, **2000**, 20(7):877-87.
[10] Abebe W. *Journal of dental hygiene*: JDH/*American Dental Hygienists' Association*, **2002**, 77(1):37-46.
[11] Mozaffarian V. A dictionary of Iranian plant names: Latin, English, Persian: Farhang Mo'aser, **1996**.
[12] Hohmann J., Zupkó I., Rédei D., et al. *Planta Med: natural products and medicinal plant research*, **1999**, 65(6):576-8.
[13] Zupkó I., Hohmann J., Redei D., et al. *Planta Med*, **2001**, 67(4):366-8.
[14] Lu Y., Foo L.Y. *Phytochemistry*, **2002**, 59(2):117-40.
[15] Ghannadi A., Moattar S.s.-S.f. *DARU Journal of Pharmaceutical Sciences*, **1999**, 7(3):23-5.
[16] Ulubelen A., Öksüz S., Topcu G., et al. *J Nat Prod*, **2001**, 64(4):549-51.
[17] Tepe B., Daferera D., Sokmen A., et al. *Food Chem*, **2005**, 90(3):333-40.
[18] Ren Y., Houghton P.J., Hider R.C., et al. *Planta med*, **2004**, 70(3):201-4.
[19] Li H.-Y., Li Y., Yan C.-H., et al. *J Asian Nat Prod Res*, **2002**, 4(4):271-80.
[20] Çadirci E., Süleyman H., Gürbüz P., et al. *Turk. J. Biol*, **2012**, 36(1):59-64.
[21] Clebsch B. The new book of salvias: sages for every garden: Portland, Or.: Timber Press 344p.-illus, col. illus. ISBN, **2003**.
[22] Baricevic D., Sosa S., Della Loggia R., et al. *J Ethnopharmacol*, **2001**, 75(2):125-32.
[23] Zaringhalam J., Akbari A., Tekieh E., et al. *J Chin Integr Med*, **2010**, 12:1180-9.
[24] Lorke D. *Arch Toxicol*, **1983**, 54(4):275-87.
[25] Zimmermann M. *Pain*, **1983**, 16(2):109-10.
[26] Taniguchi N., Kanai S., Kawamoto M., et al. *Evid Based Complement Alternat Med*, **2004**, 1(2):187-91.
[27] Philippe L., Gegout-Pottie P., Guingamp C., et al. *Am J Physiol Regul Integr Comp Physiol*, **1997**, 273(4):R1550-R6.
[28] Rezazadeh S., Zaringhalam J., Manaheji H., et al. *J Med Plant Res*, **2009**, 3(5):368-76.
[29] Woode E., Ainooson G.K., Boakye-Gyasi E., et al. *J Med Plant Res*, **2008**, 2(8):180-8.
[30] Zaringhalam J., Tekieh E., Manaheji H., et al. *Rheumatol Int*, **2013**, 33(9):2291-9.
[31] Fraser G.L., Gaudreau G.A., Clarke P., et al. *Br J Pharmacol*, **2000**, 129(8):1668-72.

[32] Rodriguez-Vita J., Lawrence T. *Cytokine Growth Factor Rev*, **2010**, 21(1):61-5.
[33] Lee J., Kim K.A., Jeong S., et al. *J Ethnopharmacol*, **2009**, 126(2):258-64.
[34] Liu B.-G., Dobretsov M., Stimers J.R., et al. *Open Pain J*, **2008**, 1:1.
[35] Jin X., Gereau R.W. *J Neurosci*, **2006**, 26(1):246-55.
[36] Czeschik J.C., Hagenacker T., Schäfers M., et al. *Neurosci Lett*, **2008**, 434(3):293-8.
[37] Zhang L., Berta T., Xu Z.-Z., et al. *PAIN*®, **2011**, 152(2):419-27.
[38] Kawasaki Y., Zhang L., Cheng J.-K., et al. *J Neurosci*, **2008**, 28(20):5189-94.
[39] Lo S., Everitt J., Gu J., et al. *J Clin Investig*, **1992**, 89(3):981.
[40] Hougee S., Sanders A., Faber J., et al. *Biochem Pharmacol*, **2005**, 69(2):241-8.
[41] Ahmed B., Al-Howiriny T.A., Al-Rehaily A.J., et al. *Z Naturforsch* C, **2004**, 59(1-2):9-14.
[42] El-Sayed N., El-Eraky W., Ibrahim M., et al. *Fitoterapia*, **2006**, 77(4):333-5.
[43] Kamatou G.P., Viljoen A.M., Steenkamp P. *Food Chem*, **2010**, 119(2):684-8.
[44] Topcu G., Tan N., Ulubelen A., et al. *Phytochemistry*, **1995**, 40(2):501-4.
[45] Comalada M., Ballester I., Bailon E., et al. *Biochem Pharmacol*, **2006**, 72(8):1010-21.
[46] Han Y. *Int Immunopharmacol*, **2005**, 5(6):1049-56.
[47] Asadi S., Ahmadiani A., Esmaeili M.A., et al. *Food Chem Toxicol*, **2010**, 48(5):1341-9.
[48] Hosseinzadeh H., Haddadkhodaparast M.H., Arash A.R. *Phytother Res*, **2003**, 17(4):422-5.