

Full Length Research Paper

Anti-inflammatory and anti-hyperalgesic activities of *Stachys athorecalyx* extracts on CFA-induced inflammation

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This study was aimed at investigating the possible anti-hyperalgesic and anti-inflammatory activities of pre-treatment and short term (6 days) administration of *Stachys athorecalyx* extracts during CFA-induced inflammation in male Wistar rats. Both methanolic and defatted extracts prepared from aerial parts of the plant were examined. The results showed dose related response in decreasing hyperalgesia and inflammation. The effective doses of both extracts were 100 mg/kg. The plant extracts were more potent in decreasing hyperplasia and inflammation compared with the standard drug (Indomethacin). By increasing the dose up to 200 mg/kg, there were no significant differences in pharmacological activities. Our results suggested that, aerial part of *S. athorecalyx* extracts suppress hyperalgesia and edema associated with acute and chronic CFA-induced inflammation.

Key words: *Stachys athorecalyx*, hyperalgesia, anti-inflammatory, CFA-induced inflammation.

INTRODUCTION

The usage of natural products is growing in many countries especially China, India and Iran. Also, there has been resurgence of interest in herbal medicine in western countries as alternative sources of drugs for often intractable diseases (Phillipson and Anderson, 1989). Since centuries some *Stachys* spp. are used traditionally for their health benefits. More than three hundred *Stachys* spp. is reported (Datta, 1991). In Iran this genus is represented by 34 species including *Stachys athorecalyx* (Mozaffarian, 1996; Rechinger, 1982). *S. athorecalyx* is a plant widely distributed in different areas of Iran (Amin, 1991) being popularly named "Sonboleh". In traditional medicine the infusions obtained from the aerial parts of *S. athorecalyx* have been used in treating infections, arthri-

tis and respiratory inflammatory disorders (Zargari, 1990). Previous pharmacological studies indicated that extracts or components of plants belonging to the genus *Stachys* exert significant antibacterial (Skaltsa et al., 1999), antitoxic (Zinchenko et al., 1981), antinephritic (Hayashi et al., 1994a), antihepatitis (Savchenko and Khvorostinka, 1978), anti-anoxia (Yamahara et al., 1990) and anti-anxiety effects (Rabbani et al., 2003; Rabbani et al., 2005). Other studies have shown that extracts of some *Stachys* spp. have anti-inflammatory and analgesic activities (Maleki et al., 2001; Khanavi et al., 2005). Phytochemical investigations on *Stachys* spp. demonstrated presence of several classes of compounds such as phenylethanoid glycosides (Nishimura et al., 1991; Miyas et al., 1996), terpenoids and steroids (Ross and Zinchenko 1975; Yamamoto et al., 1994), diterpenes (Piozzi et al., 1980) and flavonoids (Zinchenko, 1970; El-Ansari et al., 1991). Study on composition of the essential oil of this plant was done and reported previously (Rezazadeh et al., 2006). Although the anti-inflammatory and analgesic studies of some spe-

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cies of *Stachys* have been conducted, but *S. athorecalyx* has not been pharmacologically elucidated. Complete Freund Adjuvant (CFA)-induced inflammation is commonly recommended for acute and chronic animal inflammation model. It has been found out that CFA injected rats' demonstrate severe hyperalgesia and edema during first week after intervention (Zaringhalam et al., 2008). The current study was aimed at investigating the possible anti-hyperalgesic and anti-inflammatory activities of pre-treatment and short term (6 days) administration of *S. athorecalyx* extracts during CFA-induced inflammation in male Wistar rats.

MATERIALS AND METHODS

Plant material

The flowering aerial parts of *S. athorecalyx* including stems and leaves have been collected during flowering period (June and July 2001), identified in Central Herbarium of Iran (Research Institute of Forest and Rangelands, Tehran), and a voucher specimen was deposited there (83775 TARI). The aerial parts were cleaned, air dried and chopped into small pieces, powdered and stored.

Extract preparation

200 g of crushed aerial parts of plant was subjected to maceration with methanol (99.9%) for 72 h. Methanolic extract was filtered. In order to prepare defatted extract, methanolic extract was divided into two equal volumes and one of them extracted three times with Petroleum ether (40 - 60). Evaporation of extracts was done *in vacuo* using a rotary evaporator at temperature about 35 - 40°C to powder. The yields for methanolic and defatted extracts were determined. The dried extracts were kept at 4°C until used.

Phytochemical analyses

Standard phytochemical screening tests (Trease and Evans, 1983) were employed in screening of extracts. Qualitative analyses of plant for detecting saponins, alkaloids and terpenoids were utilized.

Formulations

The dried extracts were dissolved in non pyrogene sterile water containing 0.9% (w/v) of sodium chloride using Tween 80 as solubilizing agent and passed through a weighed paper filter. The filtered solutions were used for intraperitoneal (i.p) injection. Following the filtration the filters were dried and weighed again, and to obtain the real concentration of the extracts, the unfiltered particles were calculated. Plant extracts (50, 100 and 200 mg/kg) were administered intraperitoneally in a volume lower than 1 ml. Indomethacin (Calbiochem-Novabiochem Corp., San Diego, CA), a standard non-steroidal anti-inflammatory agent, also was prepared with above mentioned method and injected in 5 mg/kg dosage (Tall et al., 2004). The paw sizes did not change after drugs administration. Control groups received only drugless vehicle.

Laboratory animals

Adult male Wistar rats, weighing 200 - 220 g were selected. Rats were housed in individual cages with free access to standard diet

And water and were kept in a temperature-controlled room (22.0 ± 0.5°C) under a 12 h light-dark cycle (lights on 06:00 - 18:00 h). The humidity of the room was controlled at 60 - 62%. 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 investigation of experimental pain in animals were followed (Zimmermann, 1983).

CFA-induced inflammation

CFA-induced inflammation was evoked on day 0 by a single subcutaneous injection (100 µL) of heat-killed *Mycobacterium tuberculosis* suspended in sterile mineral oil (10 mg/ml; CFA; Sigma, St Louis, MO, USA), into the right hindpaw. Control rats, only received sterile mineral oil (100 µL) injection. This animal model was chosen because it exhibits a rapid primary inflammation response to the adjuvant (Santora et al., 2007). First hours after CFA injection in the hindpaw unilateral edema was established (acute phase), and it was estimated during first week (chronic phase) (Philippe et al., 1997; Taniguchi et al., 2004).

Paw edema measurement

To confirm proper injection of CFA, paw volume was measured pre- and post-injection in both injected and contralateral paws during different periods of study. This measurement (paw volume) was conducted by displacement of an electrolyte solution in a plethysmometer (model 7141; Ugo Basile; Comerio VA, Italy). In brief, rats were taken out of their cages and held under soft paper wadding. Each hindpaw was submerged to the tibiotarsal joint into an electrolyte-filled Perspex cell of the plethysmometer. The volume of displacement, which is equal to the paw volume, was indicated on a digital display. For each paw, measurement was done twice and the average calculated. The edema was quantified by measuring the difference in foot volume between day 0 and the various time points. The newly measured volume was shown as the percentage of the day 0 volume (Woode et al., 2008).

Thermal hyperalgesia assessment

Withdrawal latencies from noxious heat using the plantar test were assessed in both experimental and control groups (Fraser et al., 2000). Rats were placed in Plexiglas boxes for 10 - 15 min before testing in order to habituate to test environment. Paw withdrawal latency (PWL) in response to radiant heat was measured using the plantar test apparatus (Ugo Basilar, Verse, Italy). The heat source was positioned under the plantar surface of the affected hind paw and activated. The digital timer connected to the heat source automatically recorded the PWL. If the rat did not withdraw its paw from stimulus by 20 s, the test was terminated and the rat was assigned this cut-off value. Each rat received three trials per hind-paw at an interval of 5 - 10 min. The mean latency of the withdrawal responses for each foot was calculated. Then, the value for the affected paw (CFA-injected paw) was subtracted from that for the other paw and the result considered as the hyperalgesia sign in the injured paw (Zaringhalam et al., 2008).

Experimental procedure

In order to determine the effect of *S. athorecalyx* extracts on inflammatory pain model and if a dose - response relationship exists, a series of experiments were performed. Inflammation was induced by CFA. Pre-treatment with *S. athorecalyx* extracts were assessed during acute phase of CFA-induced inflammation (first 24 h). In this design, rats were randomly divided into different experimental

groups ($n = 6$). Drugs injected 30 - 45 min before induction of inflammation by CFA. Hyperalgesia and edema measurement were performed before, 1, 4, and 24 h after the CFA injection. On the other hand, both CFA-induced inflammation and hyperalgesia persist till 6 days after intervention (chronic phase) (Zaringhalam et al., 2008). In the next part of this study since 1st to 6th days after CFA injection, the rats received either methanolic or defatted extracts once a day for 6 consecutive days. Hyperalgesia and edema were assessed on 0 (immediately before CFA injection), 3rd and 6th days (30 - 45 min after drugs administration). Indomethacin as a standard anti-inflammatory agent was used in positive control group (5 mg/kg, ip).

Statistical analysis

The data were presented as the mean \pm standard error of mean (SEM). One way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison test (Statistica, 6.0), and unpaired student *t*-test were used to determine significant differences in inflammation and hyperalgesia where appropriate. Statistical significance was accepted at $P < 0.05$.

RESULTS

Phytochemical tests

Phytochemical screening of the extract revealed the presence of saponin glycoside, terpenoids and flavonoids. Alkaloid test was negative. The methanolic dried extract had 0.68% (w/w) of flavonoid on hyperoside basis.

Dose-response of *S. athorecalyx* extracts on CFA-induced acute hyperalgesia

CFA injection led to a dramatic increase in the hyperalgesia that was obvious at the 1st h and lasted for more than 24 h. At 1 h after CFA injection, extract-treated groups indicated dose-dependent decrease in the right paw hyperalgesia compared to control group. In both methanolic and defatted extracts treated groups, there were no significant differences in hyperalgesia variations between control and experimental groups at 50mg/kg doses during acute phase after CFA injection. Rats which received 100 and 200 mg/kg doses of extracts indicated a significant decrease in hyperalgesia compared to control group (CFA- injected rats). There were no significant differences between 100 and 200 mg/kg doses of either methanolic or defatted extracts in hyperalgesia variation during acute phase of study. Hyperalgesia indicated a significant decrease at 4th h compared to 1st h after intervention due to effective dose of extracts (100 mg/kg) ($P < 0.01$).

The significant decrease of hyperalgesia in 100 mg/kg dose of both extracts was significantly comparable to the hyperalgesia reduction in indomethacin-treated rats. Hyperalgesia variation was not significantly different among the experimental and control groups at 24 h after CFA injection (Figure 1A and B).

Dose-response of *S. athorecalyx* extracts on CFA-induced acute inflammation

The results of induction of acute inflammation in control rats showed a significant increase in paw volume 1 h after intraplantar injection of CFA ($P < 0.01$) which reached the peak of inflammation after 4 h ($P < 0.001$), and remained till 24 h. Pre-treatment with both methanolic and defatted extracts of *S. athorecalyx* inhibited CFA-induced inflammation dose dependently. Both methanolic and defatted extracts with 50 mg/kg indicated no significant effect on paw volume variation during 24 h after CFA injection. Peak inhibitory effect of extracts on edema was observed for 100 mg/kg at 1 and 4 h after CFA injection ($P < 0.01$ and $P < 0.001$ respectively). Effective dose of extracts (100 mg/kg) significantly abolished the maximal edema response attained on 4 h compared to 1 h after CFA injection ($P < 0.05$). There was no significant difference in paw volume between experimental and control group at 24th h of CFA injection. Paw volume variation due to 100 and 200 mg/kg of methanolic or defatted extracts did not show significant differences during the acute phase of this study. Indometacin as standard NSAID (5 mg/kg, i.p) demonstrated less inhibition than the effective dose of extracts (100 mg/kg) in all time point of this experiment ($P < 0.05$) (Figure 2A and B).

Dose-response of *S. athorecalyx* extracts short term (6 days) treatment on CFA-induced hyperalgesia

Upon 3rd and 6th days after CFA injection, hyperalgesia significantly increased compared to the control group ($P < 0.001$). There was no significant hyperalgesic difference between 3rd and 6th days in CFA injected rats ($P < 0.05$). Short term (6 days) injection of animals with all three doses of the extracts of *S. athorecalyx* (50, 100 and 200 mg/kg) in CFA-injected rats', caused a potent and dose-related reduction in thermal hyperalgesia compared to control group. Both methanolic and defatted extracts with 100 mg/kg dose induced significant reduction in hyperalgesia on 3rd and 6th days after CFA injection compared to 50 mg/kg dose ($P < 0.01$, $P < 0.001$ respectively). Dose of 100 mg/kg of both methanolic and defatted extracts significantly decreased the hyperalgesia on 6th day compared to 3rd day in CFA-injected group ($P < 0.05$). Indometacin (5 mg/kg, i.p.) showed less anti-hyperalgesic effects than both of extracts effective dose (100 mg/kg). There were no significant differences between 100 and 200 mg/kg doses of extracts in hyperalgesia reduction on 3rd and 6th days after CFA injection (Figure 3A and B) ($P > 0.05$).

Dose-response of *S. athorecalyx* extracts short term (6 days) administration on CFA-induced inflammation

Plethysmometric estimation of edema demonstrated significant increment of right paw volume on 3rd and 6th

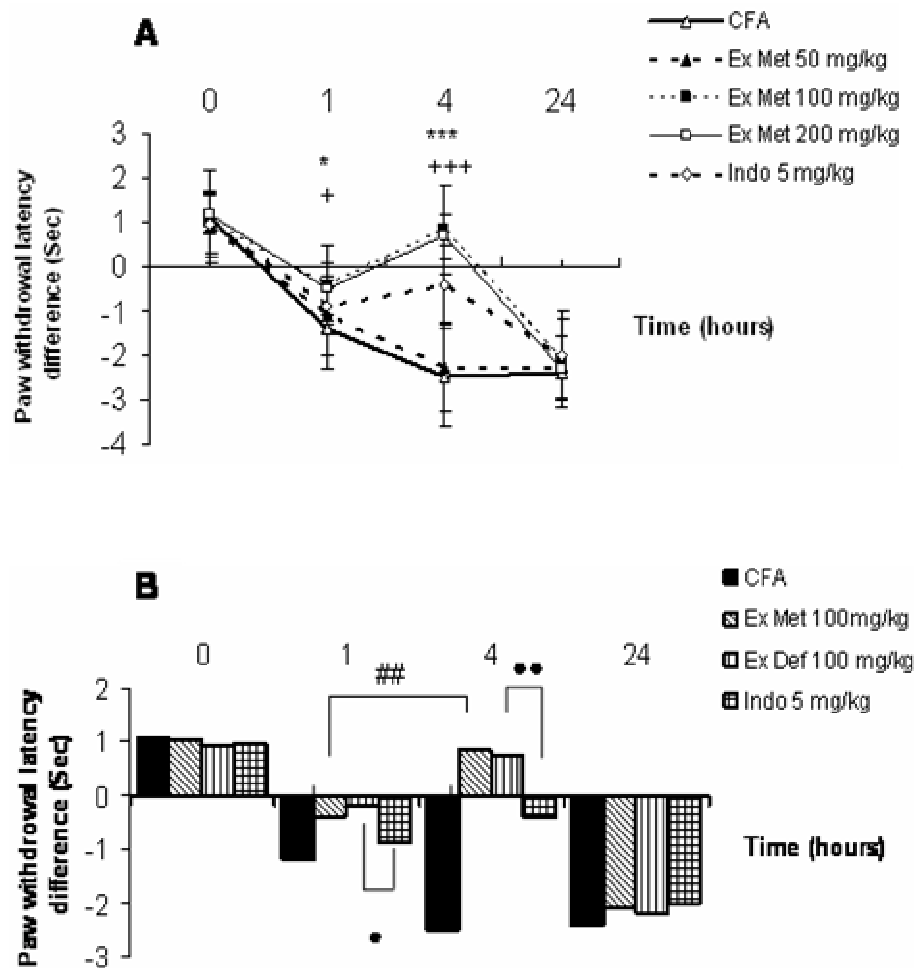


Figure 1A and B. Thermal hyperalgesia variation due to pre-treatment by different doses (50,100,200 mg/kg) of methanolic and defatted extracts of *S. athorecalyx* or indomethacin (5mg/kg) during acute phase of CFA-induced inflammation. The values are the mean \pm SE (n = 6). (A) Hyperalgesia significantly decreased due to methanolic extract (100, 200 mg/kg). (B) There was no significant hyperalgesic difference between methanolic and defatted extracts. * $P < 0.05$, *** $P < 0.001$ for comparing the hyperalgesia difference in CFA-injected control group. + $P < 0.05$, +++ $P < 0.001$ for comparing the hyperalgesia difference between control and methanolic extract treated groups (100 mg/kg). • $P < 0.05$, •• $P < 0.01$ for comparing the hyperalgesia difference between extract and indomethacin-treated groups. ## $P < 0.01$ for comparing the hyperalgesia difference between 1st and 4th hours in extract-treated groups (100 mg/kg).

days after CFA injection compared to day 0 and control group ($P < 0.001$). No increases in paw volumes were noted with saline injection. Paw volume significantly decreased due to extracts administration at all doses (50, 100 and 200 mg/kg) of both methanolic and defatted extracts for a short term (6 days) treatment in this study. Short period administration of extracts demonstrated that, they (methanolic and defatted extracts) were more effective at 100 and 200 mg/kg doses than 50 mg/kg during 6 days study (inflammatory phase). Results indicated that, extracts at 100 mg/kg doses significantly decreased paw edema on 6th day compared to 3rd day after CFA injection. Paw

volume attenuation due to effective dose of extracts (100 mg/kg) on 3rd and 6th days after CFA injection were significantly more than indomethacin (5 mg/kg) administration ($P < 0.05$). There were no significant differences in paw volume reduction between 100 and 200 mg/kg doses of extracts in CFA-treated rats during all time points of study (Figure 4A and B).

There were no significant differences in paw volume and hyperalgesia variations when methanolic and defatted extracts compared with each other during this study ($P > 0.05$). There were no significant differences between CFA and CFA+ saline treated groups (vehicle of drugs) in hyper-

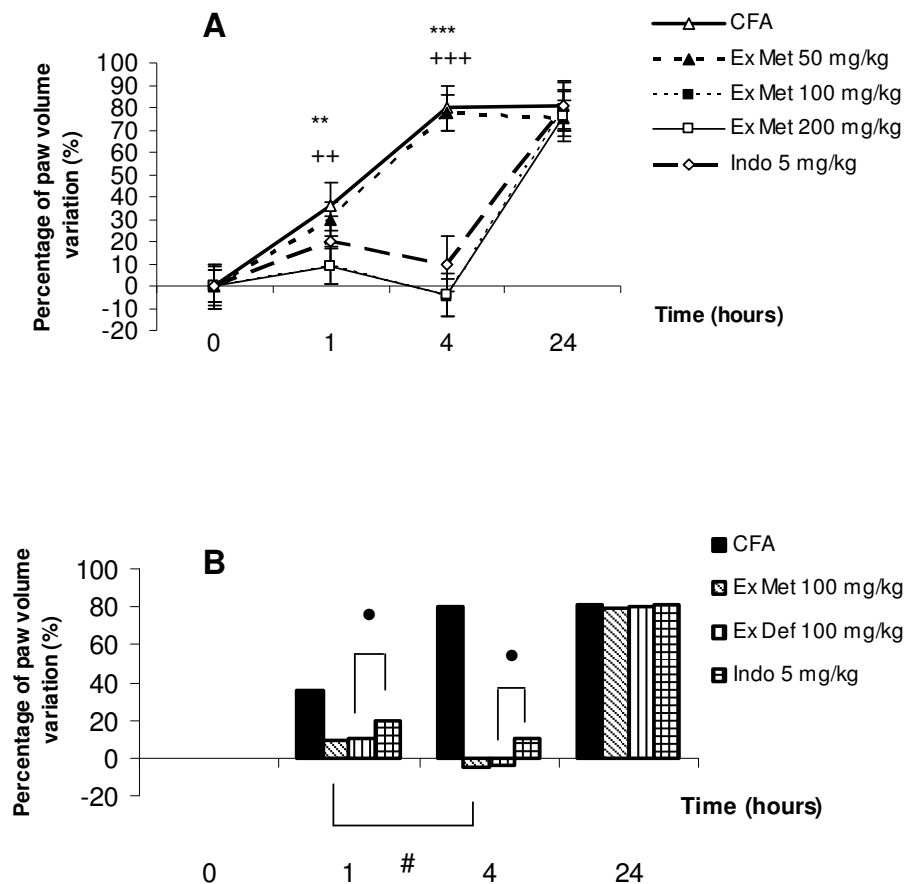


Figure 2 A and B. Time course effects of methanolic and defatted extracts of *S. athorecalyx* (50,100,200 mg/kg) or indomethacin (5mg/kg) on CFA-induced increase in ipsilateral paw volume. The values are the mean \pm SE (n = 6). (A) Methanolic extract administration (100,200 mg/kg) significantly decreased paw volume in CFA-injected groups. (B) There was no significant difference in paw volume variation between methanolic and defatted extracts-treated groups. ** P < 0.01, *** P < 0.001 for comparing the paw volume variation in CFA-injected control group. ++ P < 0.01, +++ P < 0.001 for comparing the paw volume variation between control and methanolic extract treated group (100 mg/kg). • P < 0.05 for comparing the paw volume variation between extract and indomethacin- treated groups. # P < 0.05 for comparing the paw volume variation between 1st and 4th hours in extract- treated groups (100 mg/kg).

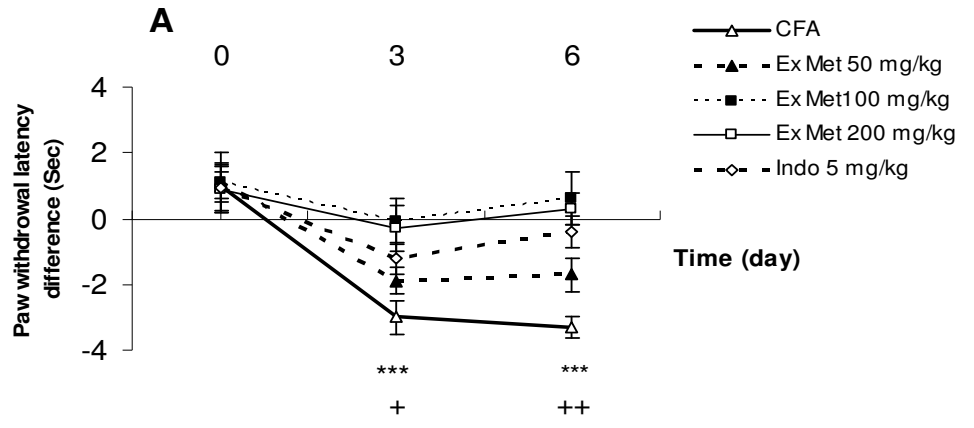
algnesia and paw volume variations during this study (data not shown).

DISCUSSION

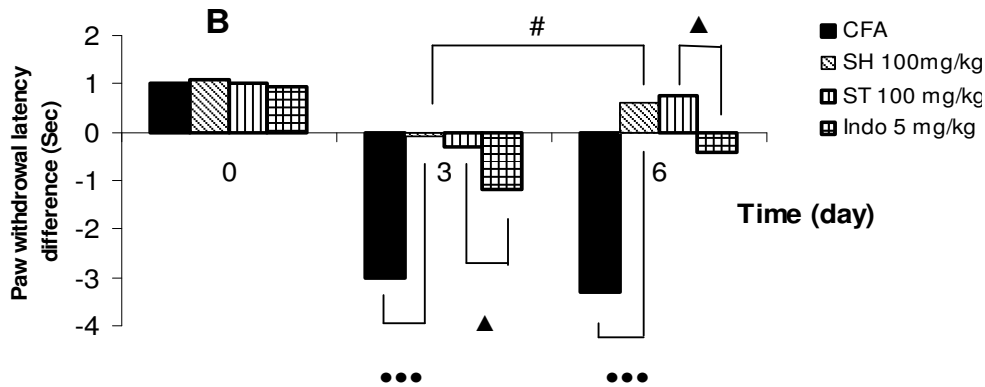
The main objective of this study was to examine the effect of pre-treatment and short term (6 days) administration of methanolic and defatted extracts of *S. athorecalyx* on CFA-induced inflammation. The results demonstrated that, both methanolic and defatted extracts of *S. athorecalyx* were capable to attenuate acute and chronic phases of CFA-induced inflammation in dose-related situation. The results also highlighted that, effective doses of extracts were more potent than anti-inflammatory and anti-hyperalgesic dose of indomethacin (5 mg/kg

i.p.).

The CFA-induced inflammation test is highly sensitive and most frequently used as an inflammatory model, which has long been accepted as a useful phlogistic tool for investigating new anti-inflammatory drugs (Woode et al., 2008; Hamada et al., 2000; Jones and Ward, 1966). This study demonstrated that, pre-treatment with the total and defatted extracts of *S. athorecalyx* (100 and 200 mg/kg) caused marked inhibition of CFA-induced hind paw hyperalgesia and edema and the 100 and 200 mg/kg extracts were similar in potency. Previous studies stated that, hydroalcoholic extract of *S. inflata* was capable of attenuating carrageenan-induced inflammation (Maleki et al., 2001; Hajhashemi et al., 2007). Anti-inflammatory and anti-hyperalgesic effects were observed during the acute



Hyperalgesia variation during short term drugs administration in CFA-induced inflammation

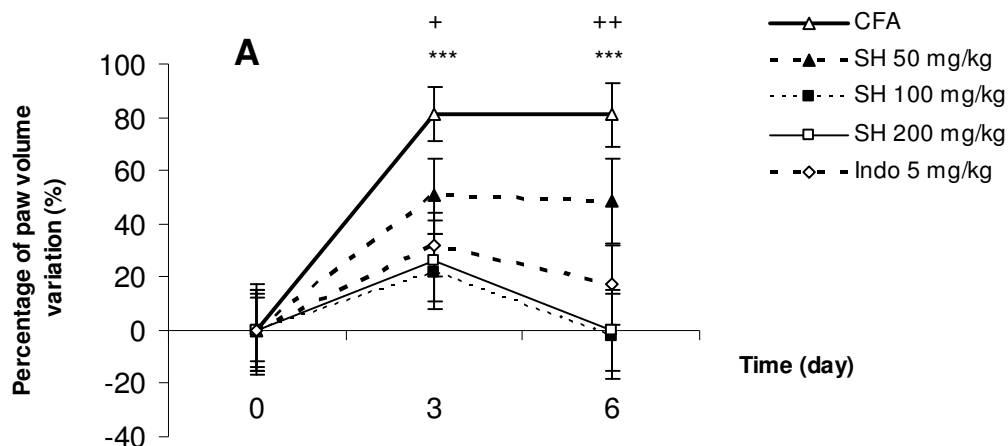


hyperalgesia variation during short term drugs administration in CFA-induced inflammation

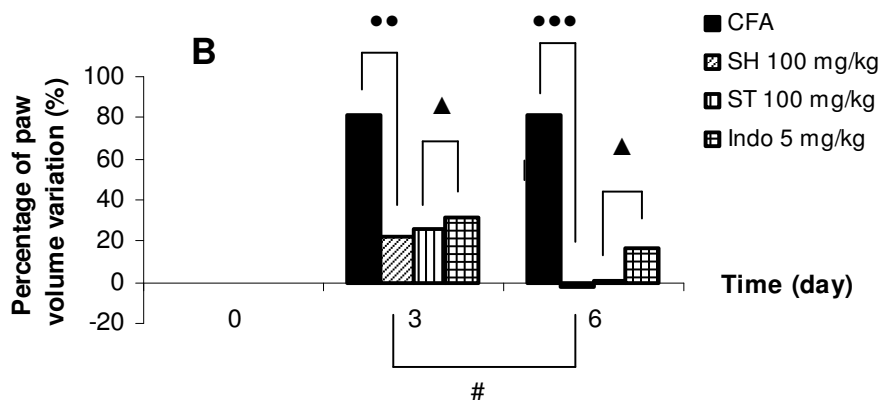
Figure 3 A and B. Thermal hyperalgesia varies due to short term (6 days) treatment by different doses (50,100,200 mg/kg) of methanolic and defatted extracts of *S. athorecalyx* or indomethacin (5mg/kg) during CFA-induced inflammation. The values are the mean \pm SE (n = 6). (A) Hyperalgesia significantly decreased due to methanolic extract (50,100, 200 mg/kg). (B) Results indicated no significant hyperalgesic difference between methanolic and defatted extracts. *** P < 0.001 for comparing the hyperalgesia difference in CFA-injected control group. + P < 0.05, ++ P < 0.001 for comparing the hyperalgesia difference between control and methanolic extract-treated groups (50 mg/kg). ●●● P < 0.001 for comparing the hyperalgesia difference between control and methanolic extract treated groups (100 mg/kg). ▲ P < 0.05 for comparing the hyperalgesia difference between extract and indomethacin- treated groups. # P < 0.01 for comparing the hyperalgesia difference between 3rd and 6th days in extract- treated groups (100 mg/kg).

phase of inflammation in this study. This finding is consistent with that reported by Khanavi et al. (2005), and Rezazadeh et al. (2005). The inhibition of CFA- induced paw edema by extracts during this study suggested that they possess a significant effect against acute inflammation. The phytochemical screening on the leaves of *S. athorecalyx* indicated presence of various classes of chemicals such as terpenoids, saponin glycosids and flavor-

noids. Flavonoids such as quercetin are known to be effective in acute inflammation symptoms reduction (Rajnarayana et al., 2001). This kind of flavonoids possess potent inhibitory effects against different enzymes such as protein kinase C, phospholipase A2, phosphor-diesterases and others (Middleton, 1998). Also, there are different reports on the analgesic effects of terpenoids, essential oils and saponins (Reanmongkol et al., 2005; De



Paw volume variation during short term drugs administration in CFA-induced inflammation



Paw volume variation during short term drugs administration in CFA-induced inflammation

Figure 4 A and B. Time course effects of methanolic and defatted extracts of *S. athorecalyx* (50, 100, 200 mg/kg) or indomethacin (5mg/kg) on CFA-induced increase in the ipsilateral paw volume. The values are the mean \pm SE (n = 6). (A) Methanolic extract administration (50,100,200 mg/kg) significantly decreased paw volume in CFA-injected groups. (B) There was no significant difference in paw volume variation between methanolic and defatted extracts- treated groups. *** P < 0.001 for comparing the paw volume variation in CFA-injected control group. + P < 0.01, ++ P < 0.001 for comparing the paw volume variation between control and methanolic extract treated groups (50 mg/kg). ** P < 0.01, *** P < 0.001 for comparing the paw volume variation between control and methanolic extract treated groups (100 mg/kg). \blacktriangle P < 0.05 for comparing the paw volume variation between extract and indomethacin- treated groups. # P < 0.05 for comparing the paw volume variation between 3rd and 6th days in extract- treated groups (100 mg/kg).

Araujo et al., 2005). It is known that the acute phase of CFA-induced inflammation is related to the producing substances such as histamine, arachidonic metabolites via cyclooxygenase and bradykinins (Dawson et al., 1991 1991; Salvemini et al., 1996; Boughton-Smith et al.,

1999). Then, it seems that the anti-inflammatory and hyperalgesic activity of pre-treatment with *S. athorecalyx* extracts may be related to inhibition of release or synthesis of inflammatory enzymes and their products by its chemical constituents. However, further studies are

needed to isolate the active constituents responsible for the observed effect and to reveal the possible mechanisms of action responsible for the analgesic and anti-inflammatory activities of *S. athorecalyx*.

Moreover, short term administration of all three doses of the extracts (50, 100 and 200 mg/kg) significantly inhibited the hyperalgesia and edema associated with the chronic phase of the CFA-induced inflammation, but the effect of the 100 and 200 mg/kg dose were predominant. In the short term (6 days) extracts administration, the extracts at 50 mg/kg dose appeared to be effective in edema and hyperalgesia reduction than used in pre-treatment situation. This may be as a result of more than one time extract administration and possible interactions between constituents of the extract sample (Vongtau et al., 2004). There was no significant difference between short term treatment with 100 and 200 mg/kg doses. The apparent lack of significant difference in the effects of the two doses in the test may be an indication that a ceiling effect may have been attained at 100 mg/kg after which increase in dose may just bring about minimal increase in observed effects (Vongtau et al., 2004). Furthermore, the results have shown that *S. athorecalyx* extracts significantly inhibited the development of chronic swelling and hyperalgesia induced by CFA. Previous studies assessed only pre-treatment effects of other species of *Stachys* on carrageenan-induced acute inflammation, and there was no evidence about the effects of *S. athorecalyx* extracts on CFA-induced inflammation. The composition of CFA adjuvant is complex and the immune response during chronic inflammation is a multi-stage process of intercellular cooperation (Walz et al., 1972). There was evidence that CFA-induced chronic inflammation was accompanied by increase in cytokines and free radicals activity (Zaringhalam et al., 2008). Free radicals are implicated in a number of pathological processes such as inflammation, aging and atherosclerosis. Antioxidants protect living systems from damages which related to the production of reactive oxygen species (Halliwell and Gutteridge, 1990). It was indicated that, species of *stachys* have high levels of antioxidant activity (Hajhashemi et al., 2007). Several flavonoids isolated from medicinal plants have been discovered to possess significant antioxidant effects (Duke, 1992).

Therefore, it is possible that at least part of anti-hyperalgesic and anti-inflammatory effects observed with *S. athorecalyx* extracts may be attributable to their flavonoid component. It was also mentioned that, chronic hyperalgesia and inflammation are related to leukocytes accumulation. It was found out that acteoside; a phenylethanoid glycoside of some species of *Stachys* has a suppressive effect on the accumulation of leukocytes during the chronic inflammation (Hayashi et al., 1994b). The anti-inflammatory effects exhibited by *S. arthorecalyx* extracts in this study may have been at least partly due to the inhibition of leukocytes activity (Maleki et al., 2001) however, more experiments are needed to confirm that. The co-existence of both anti-hyperalgesic and anti-inflamma-

tory effects seen in treatment with these extracts is well-defined for various non-steroidal anti-inflammatory drugs (NSAIDs) particularly the indomethacin (Vongtau et al., 2004). It is therefore interesting that the extracts behaved more potent than indomethacin during this study which correlates well with the traditional application of the plant. As mentioned earlier, there was no significant difference between methanolic and defatted extracts in their anti-hyperalgesic and anti-inflammatory effects in similar doses, suggesting that, there was no clear correlation between polar and non-polar extracts of plant and those pharmacological properties. In conclusion, our results suggested that, aerial part of *S. athorecalyx* extracts suppressed hyperalgesia and edema associated with acute and chronic CFA-induced inflammation and may have a beneficial role in the treatment of inflammatory pain. However, further studies are needed to determine the precise mechanism of action of the anti-hyperalgesic and anti-inflammatory effects of *S. athorecalyx* extracts.

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