

Antiinflammatory and antinociceptive activities of extracts and syringin isolated from *Carduus schimperi* Sch. Bip. ex A. Rich

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Abstract

Carduus schimperi Sch. Bip. ex A. Rich (Asteraceae) is a perennial herb and its roots are used in some localities in Ethiopia for orofacial inflammation in the form of warm aqueous macerate. In the present study, the *in vivo* antiinflammatory and antinociceptive effects of the aqueous root extracts of *C. schimperi* were investigated. The antiinflammatory effect was evaluated using carrageenan-induced mouse pedal (paw) oedema model, while the formalin test in mice was employed to study the antinociceptive activity. Administration of 400 mg/kg p.o. of the aqueous extract of the roots of *C. schimperi* produced significant ($P < 0.05-0.001$) antiinflammatory effects against carrageenan-induced acute inflammation and formalin-induced nociceptive pain stimulus in mice. Bioassay guided fractionation of the total extract indicated that the water fraction was by far the most potent in both models. Syringin, which was isolated for the first time from the active fraction of *C. schimperi* showed significant antiinflammatory and antinociceptive activities when tested at a dose of 100 mg/kg, p.o. The present findings indicated that *C. schimperi* possesses genuine antiinflammatory and antinociceptive properties, lending pharmacological support to folkloric or anecdotal use of the plant in the treatment and/or management of painful inflammatory conditions. Syringin appears to be one of the active ingredients of the plant.

Keywords: *Carduus schimperi*; syringin, antiinflammatory; antinociceptive

Introduction

Despite the immense technological advancement in modern medicine, many people in Africa (approximately 75% of the population) still rely on traditional healing practices and medicinal plants for their daily healthcare needs (Ojewole, 2004). Ethiopian traditional medicine is composed of a number of specific skills, namely, the use of plants, animal products and minerals as well as magic and superstition. The main body, however, is based on the use

of ethnobotany (Gedif, 2003). Despite the significant role of medicinal plants in supporting the Ethiopian national primary healthcare, little work has so far been done to properly document the associated knowledge and promote its practices. On the other hand, medicinal plants and the associated knowledge are being seriously depleted due to deforestation, environmental degradation and acculturation that have been taking place in the country for quite a long time. So, urgent ethnobotanical studies and subsequent conservation measures are needed to salvage medicinal plants and the associated knowledge from further loss (Giday 2009).

Carduus schimperi Sch. Bip. ex A. Rich (Asteraceae) is a perennial herb with a long white or brown taproot having leaves flat on ground or slightly raised from half-way up 3-20 cm long (Tadese, 2004). In Ethiopia *C. schimperi* is known by its vernacular names including “Kosheshila” in Amharic or “Chu’a” in Kembatigna. Its anecdotal use for orofacial inflammation in the form of warm aqueous root macerate is commonly claimed by local people though such ethnopharmacological report is lacking. However, the hydroalcoholic extracts of other members of the genus *Carduus* such as *C. pycnocephalus* have been reported to have potent antiinflammatory activity (Conforti *et al.*, 2009).

Jordon-Thaden and Louda (2003) and Barros *et al.* (2005) have reported the presence of flavonoids (linarin and pectolinarin), sterols and triterpenes (taraxasterol acetate, taraxasterol and erythrodiol-3-acetate), alkaloids (acanthoine, acanthoidine and ruscopine), polyacetylenes, acetylenes, hydrocarbons, coumarins, phenolic acids (hydroxybenzoic acid), lignans (pinoresinol), and a few other compounds in *Carduus* spp. However, no reports on the phytochemistry or pharmacological activity of *C. schimperi* could be found in the literature.

The present study was therefore undertaken to examine the possible antiinflammatory and antinociceptive activities of *C. schimperi* using carrageenan-induced paw oedema and the formalin test models, respectively.

Materials and Methods

Plant material

Fresh roots of *C. schimperi* were collected in and around Hossaena town about 230 km south of Addis Ababa, Ethiopia in November 2008. The authenticity of the plant material was confirmed by Mr. Melaku Wondaferash, the National Herbarium, College of Natural Sciences, Addis Ababa University, where voucher specimen (MW01) was deposited for future reference.

Animals

Swiss Albino mice of either sex with body weight of 20-35 g were used for the experiments. The animals were purchased from the animal breeding laboratories of the Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa, Ethiopia, housed under standard laboratory conditions and fed commercial rat feed and tap water *ad libitum*. All animal experiments were conducted in accordance with the internationally accepted laboratory animal use, care and guideline (ILAR, 1996) and approved by the Institutional Review Board of the School of Pharmacy, Addis Ababa University.

Chemicals and instruments

All the chemicals and reagents used for the experiments were analytical grade. Mass spectra were recorded on a Shimadzu LC-MS 2010 Advanced spectrometer attached to a Shimadzu LC-10ADvp pump with a Shimadzu SIL-HT auto injector; a Shimadzu CTO-10ACvp column oven and a Shimadzu Dgu-14a degasifier. The measurement was carried out at atmospheric pressure by electrospray ionization method with positive ion mode. The probe voltage and CDL temperature were fixed at 1.5 kV and 250 °C, respectively.

Proton and ^{13}C NMR spectra were recorded on JEOL JNM-L-400 spectrometer operating at 400 and 100 MHz, respectively, using MeOD as a solvent. Tetramethylsilane (TMS) was used as a standard.

Preparation of crude extract

Fresh roots of *C. schimperi* were washed with distilled water to remove dirt and soil, and the cleaned sliced roots (1 kg) were ground with mortar and pestle and extracted 3x. On each occasion the plant material was soaked in 1.5 liter of warm (60 °C) distilled water for 24 h with shaking. The extracts were filtered and the combined filtrates concentrated to dryness in a ventilated oven at a temperature not exceeding 40 °C. The dried extract was kept in a refrigerator before use. At the time of use, extract was reconstituted in distilled water at the required concentrations.

Preparation of solvent fractions and isolation of a compound

The crude aqueous extract (20 g) of *C. schimperi* suspended in 100 ml of distilled water was fractionated successively by partitioning first with chloroform (3 × 100 ml) and then with a 3:1 mixture of chloroform and methanol (3 × 100 ml). The remaining solution was labeled as water fraction. After being dried under reduced pressure at 40 °C, each fraction was used for bioassay and analysed by thin layer chromatography (TLC). The most active methanol fraction was subjected to silica gel preparative thin layer chromatography (PTLC) using butanol: acetic acid: water (4:1:5, upper phase) as a mobile phase. The purity of the whitish semi-crystalline substance obtained was checked by analytical TLC.

Identification of the isolated compound

The isolated compound was identified as syringin (Figure 1) by comparison of its spectral data (^1H and ^{13}C -NMR) with those reported in the literature (Agrawal 1992; Kiem *et al.*, 2003; Yan *et al.*, 2004).

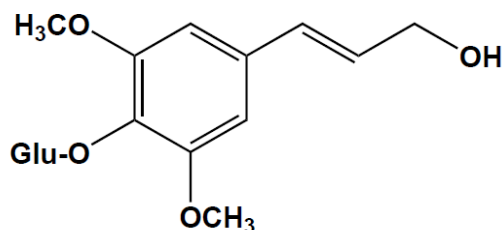


Figure 1. Chemical structure of syringin

***In vivo* antiinflammatory activity test**

In vivo antiinflammatory activity was evaluated on the basis of inhibition of carrageenan-induced mouse hind paw oedema as previously described by Boominathan *et al.* (2004) with some modifications. Mice were fasted for 12 h with free access to water until the experiment started. Extract, fraction and the isolated compound at doses of 400, 200 and 100 mg/kg, respectively, were administered orally to test groups using an oral gavage. Animals in the reference group received indomethacin (10 mg/kg p.o.), while control animals received distilled water (10 ml/kg p.o.). One h later, oedema was induced by injecting 0.1 ml of 1% carrageenan solution in normal saline into the right hind paw of each mouse. The volume of paw oedema was measured before, and 1, 2, 3, 4, and 5 h after induction of inflammation, using plethysmometer (Ugo Basile 7140, Italy).

Antinociceptive activity tests

The antinociceptive activity of the extract was determined using the formalin test as described by Hunskaar *et al.* (1986). Animals were categorized into control treated (vehicle-distilled water), reference treated (indomethacin or morphine) and test group treated (extract, fraction or the isolated compound). Each group consisted of 6 mice (3 males and 3 females).

Formalin test

The formalin test was carried out according to the modifications of the test for mice by Hunskaar *et al.* (1986), and has two phases.

A. Early phase

This experiment was done on mice that had been individually exposed to observation chambers for an adaptation period of 2 h; the animals were deprived of food and water once they were in the observation chambers. The extract, fraction and isolated compound at 400, 200 and 100 mg/kg doses, respectively, indomethacin (10 mg/kg, p.o.) or morphine (5 mg/kg, s.c.) and the vehicle (10 ml/kg) were given orally for the test, reference and control groups, respectively. One h after administration of the extracts, indomethacin or vehicle, or 30 min after morphine injection, each mouse was taken out of the cage and with a minimum restraint, 20 μ l of 2.5% formalin was injected just under the skin of the dorsal surface of the right hind paw using a microsyringe. Then, the mouse was put back into the chamber and the time in sec the animal spent licking the injected paw or leg was recorded for 0-5 min after formalin injection.

B. Late phase

Experiments were carried out in the same manner as the early phase test. But, recording of licking time was done 20-30 min after formalin injection.

Data analysis

For the antiinflammatory test, the increase in paw volume i.e. percent inflammation (% I) was calculated according to the equation described by Delporte *et al.* (2005):

$$\% I = (V_f - V_i) / V_i \times 100$$

where, V_f and V_i are the final and initial paw volumes of each animal, respectively. The mean percent inflammation (% I) was then calculated and a curve of mean % I versus time was plotted. In addition, the antiinflammatory effect (% A) was calculated according to the formula given below and data were presented as mean \pm SEM:

$$\% A = \% I_c - \% I_e / \% I_c \times 100$$

Where, %Ic and %Ie are the mean inflammation values reached in control and experimental groups, respectively. The significance of drug-induced changes was estimated using one-way analysis of variance (ANOVA) followed by the Tukey's test to analyze the data and $P < 0.05$ was taken as statistically significant (Sayyah *et al.*, 2004) by using the software package INSTAT.

For the formalin test, the mean number of sec the animals spent licking the injected paw and the SEM in each treatment group were calculated for both early and late phases. Data were presented using bar graph plotted as treatment group versus mean number of sec spent in paw licking.

Results and Discussion

Extraction

The percentage yields of the aqueous extract and solvent fractions from the roots of *C. schimperi* are shown in Table 1.

Antiinflammatory activity

As shown in Table 2, the root aqueous extract of *C. schimperi* showed a significant ($P < 0.05$) effect on percent reduction of carrageenan-induced inflammation in mice with percent inhibition of 68.43 on the second h. The activity of the extract was also highly significant ($P < 0.001$) on the delayed stages of the oedematogenic response (third and fourth h) and continued to the fifth hour (59.67-52.39%) when compared to the vehicle. Indomethacin, the standard drug used in the study also exhibited very significant ($P < 0.01$) activity against carrageenan-induced oedema at the third and fourth h with percent reduction of 58.76 and 48.24, respectively. Indomethacin is a known cyclooxygenase inhibitor which is experimentally sh-

Table 1: Percentage yields of extracts and fractions obtained from the root extracts of *Carduus schimperi*

Plant material	Solvent used	Method of extraction	Percentage yield (w/w)
<i>C. schimperi</i>	Distilled water	Warm maceration	3.87 ^a
	Chloroform	Solvent-solvent fractionation	19.25 ^b
	Chloroform Methanol (3:1)	Solvent-solvent fractionation	27.50 ^b
	Distilled water	Solvent-solvent fractionation	53.25 ^b

^a Yields in g of dried extract per 100 g of fresh plant material.

^b Yields in g of dried fractions per 100 g of the aqueous extract.

Table 2. Effect of the aqueous rot extract of *Carduus schimperi* (400 mg/kg, p.o.) and indomethacin (10 mg/kg, p.o.) on percent inflammation over a period of five hours after carrageenan mouse paw injection

Test substance	Percent inflammation				
	1 h	2 h	3 h	4 h	5 h
Vehicle	80.67 ± 1.31	76.33 ± 1.40	79.01 ± 1.23	71.20 ± 1.33	63.74 ± 1.94
Indomethacin	75.05 ± 2.39	70.28 ± 1.56	58.76 ± 1.74 ^{***}	48.24 ± 1.99 ^{***}	44.21 ± 1.15 ^{***}
<i>C. schimperi</i>	74.55 ± 1.82	68.43 ± 1.68 [*]	59.67 ± 1.72 ^{***}	52.39 ± 2.25 ^{***}	48.27 ± 1.92 ^{***}

Values are mean ± SEM, n = 6, ^{*}P < 0.05, ^{**}P < 0.01, ^{***}P < 0.001 compared to vehicle.

own to attenuate carrageenan response in the second phase of inflammation mainly mediated by prostaglandins (Deciga-Campos et al., 2007; Goulart et al., 2007; Küpeli et al., 2007).

Carrageenan-induced hind paw oedema model is a widely used screening protocol to test antiinflammatory activity of natural products (Tunalier et al., 2007). It is believed to be biphasic. The first phase, within 2 h, involves the release of serotonin and histamine and the second phase, after 2 h, is mediated by prostaglandins, cyclooxygenase products. Thus, as shown in Table 3, the mean percent antiinflammatory effect (24.48 and 26.42) displayed by the aqueous extract of *C. schimperi* was particularly high on the delayed stages (3 h and 4 h, respectively) of the oedematogenic response induced by carrageenan when compared to vehicle. Indomethacin, the positive control used in this study, exhibited a better antiinflammatory activity (25.29% and 30.03%) at 3 and 4 h, respectively. Since the antiinflammatory effect of the aqueous extract of *C. schimperi*, like indomethacin, is mainly in the second phase of inflammation, it suggests that the antiinflammatory activity might be due to inhibition of prostaglandin release. However, the early antiinflammatory effect of the aqueous extract of *C. schimperi* (10.36%) at the 2nd h against inflammation induced by carrageenan due to histamine, serotonin and kinin-like substances release (Küpeli et al., 2007) should not be ruled out.

Oral pretreatment of mice with the water fraction of *C. schimperi* (200 mg/kg) resulted in a very significant (P < 0.01) inhibition of carrageenan-induced hind mouse paw oedema (Table 3). The water fraction of *C. schimperi* showed 11.71% antiinflammatory activity at the second h. This activity was also highly significant (P < 0.001) ranging from 27.55-34.85 % in the subsequent h of oedema formation (3-5 h). Indomethacin also showed highly significant (P < 0.001) inhibitory effect, 25.29-34.61% at the late stages (3-5 h), when compared to vehicle.

Table 3. Antiinflammatory effects of the aqueous extract (400 mg/kg, p.o.), solvent fractions (200 mg/kg, p.o.), syringin isolated from the root extract of *Carduus schimperi* (100 mg/kg, p.o.), and indomethacin (10 mg/kg, p.o.) against carrageenan-induced mouse paw oedema

Test substance	Percent antiinflammatory activity (% A)				
	1 h	2 h	3 h	4 h	5 h
Aqueous extract	7.59	10.36	24.48	26.42	24.33
Chloroform fraction	4.31	2.90	8.18	4.86	4.86
Chloroform-methanol (3:1) fraction	3.92	5.58	11.49	11.49	10.94
Aqueous fraction	5.33	11.71 [*]	27.55 ^{**}	31.77 ^{**}	34.85 [*]
Indomethacin	5.89	8.05	25.29 ^{**}	30.03 ^{**}	34.61 [*]
Syringin	8.29	11.89 [*]	31.83 ^{**}	32.39 ^{**}	33.07 [*]

Values are mean % A calculated from % I, n = 6, ^{*}P < 0.01, ^{**}P < 0.001 compared to vehicle.

The potent inhibitory effect of the water fraction on carrageenan-induced paw oedema at the late stage suggests that its main mechanism of action may involve prostaglandin biosynthesis. This fraction may also possess some influence on other inflammatory mediators such as bradykinin released during the second h after carrageenan injection. However, there was no any significant antiinflammatory activity (% A) shown by either the chloroform or chloroform-methanol (3:1) fraction against carrageenan-induced hind paw oedema in mice.

Phytochemical investigation of the active aqueous fraction resulted in the isolation of the sinapyl alcohol glycoside syringin which was found to be even more active than the aqueous extract. Syringin at a dose of 100 mg/kg, p.o showed a very significant ($P < 0.01$) inhibition (11.89-33.07%) of carrageenan-induced mice paw oedema over a period of 2-5 h of inflammation (Table 3).

Considering the fact that subplantar injection of carrageenan in mice is biphasic, where the early phase involves the release of serotonin, histamine and kinins, and the late phase mediated by prostaglandins, the present results suggest that the antiinflammatory effects of both the extract and the isolated compound syringin are probably due to inhibition of synthesis and/or release of the mediators, especially the cyclooxygenase products.

Previous work carried out by Choi *et al.* (2004) indicated that syringin and its hydrolysis product sinapyl alcohol isolated by activity-guided fractionation of the ethyl acetate extracts of the stem bark of *Magnolia sieboldii* (Magnoliaceae), inhibited increased vascular permeability induced by acetic acid in mice. Moreover, sinapyl alcohol was shown to reduce acute paw oedema induced by carrageenan in rats better than syringin. In addition, both substances potently inhibited lipopolysaccharide (LPS)-induced nitric oxide (NO), prostaglandin E₂ (PGE₂), and tumor necrosis factor. Sinapyl alcohol also reduced the expression levels of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX)-2 in a concentration-dependent manner. Choi *et al.* (2004) also suggested that syringin might undergo *in vivo* transformation to sinapyl alcohol after oral administration since sinapyl alcohol was found to be more potent than syringin.

In another study, Yan *et al.* (2004) reported the presence of syringin in the *n*-butanol extract of the stems and barks of *Edgeworthia chrysantha* Lindl (Thymelaceae). Moreover, these authors showed syringin and its aglycone to inhibit TNF α -secretion, and also to possess anti-hypersensitivity as well as antiinflammatory effects on autoimmune diseases like uveitis.

Antinociceptive activity

As shown in Figure 2, the aqueous extract of *C. schimperi* (400 mg/kg) produced inhibition on formalin-induced pain responses in mice. This extract significantly ($P < 0.05$) reduced the licking time (82.60 sec) at the late phase of formalin-induced pain. The positive control drug, morphine (5 mg/kg), very significantly ($P < 0.001$) attenuated the pain responses of both the early and late phases by 30.17 and 21.67 sec, respectively, whereas indomethacin (10 mg/kg) was very efficient in the late phase (83.00 sec licking time) in comparison to

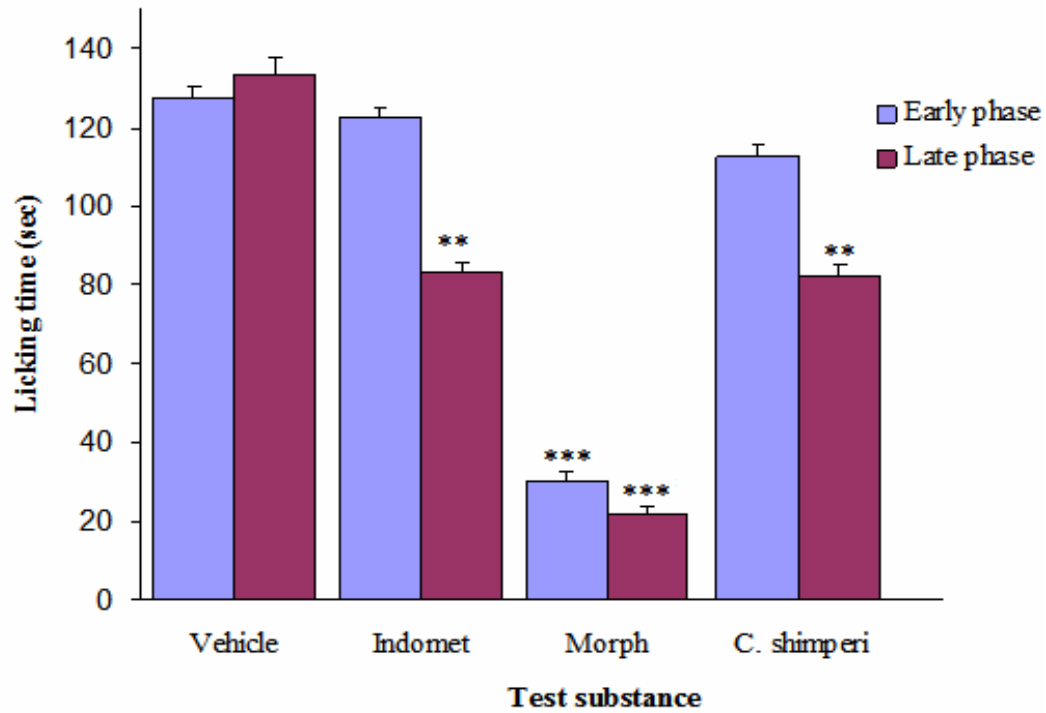


Figure 2. Antinociceptive activities of the aqueous extract of *Carduus shimperi* (400 mg/kg, p.o), morphine (morph, 5 mg/kg, sc.) and indomethacin (indomet, 10 mg/kg, p.o) compared with vehicle on the early and late phases of formalin induced nociception in mice. The values are means \pm SEM, n = 6, **P < 0.01, ***P < 0.001 compared to vehicle

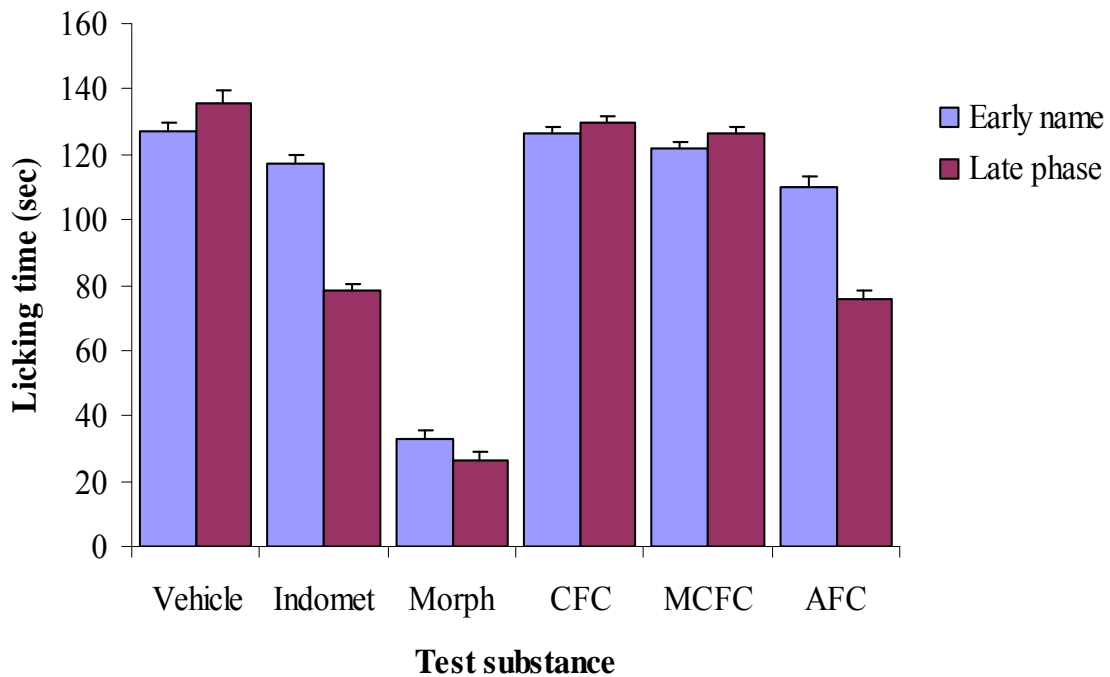


Figure 3. Antinociceptive activities of fractions of *Carduus shimperi* (200 mg/kg, p.o), morphine (morph, 5 mg/kg, sc.) and indomethacin (indomet, 10 mg/kg, p.o) compared with vehicle on the early and late phases of formalin induced nociception in mice. The values represent means \pm SEM, n = 6, AFC = aqueous fraction; CFC = chloroform fraction; MCFC = 3:1 chloroform-methanol fraction.

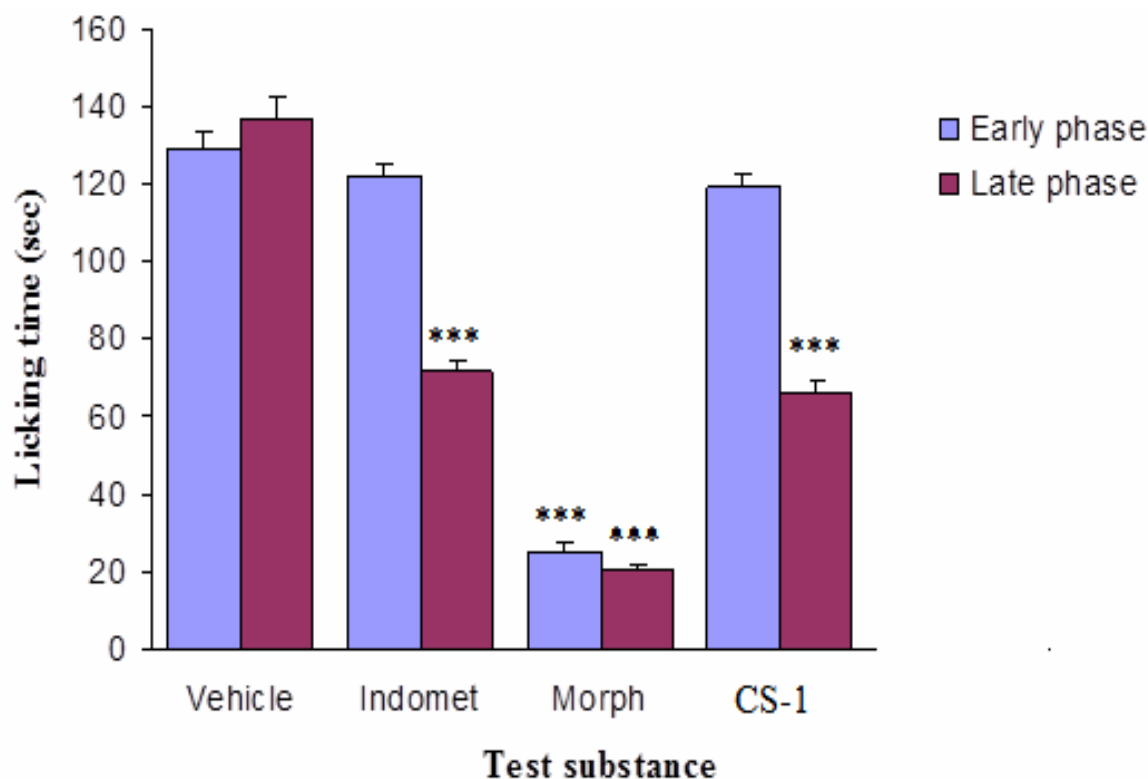


Figure 4. Antinociceptive activities of syringin isolated from the aqueous fraction of root extract of *Carduus schimperi* (CS-1, syringin 100 mg/kg, p.o), morphine (morph, 5 mg/kg, sc.), and indomethacin (indomet, 10 mg/kg, p.o) compared with vehicle on the early and late phases of formalin induced nociception in mice. The values represent means \pm SEM, n = 6, ***P < 0.001 compared to vehicle.

the vehicle with licking time of 127.33 and 133.33 sec in the early and late phase, respectively.

As reported by Umukoro and Ashorobi (2007) formalin-induced paw licking is often used to distinguish between central and peripheral analgesic actions. In the present study, the aqueous extract of *C. schimperi* was found to be highly potent against nociceptive response in the late phase of the formalin test. Thus, this result tends to suggest that the antinociception effect of the extract might be due to its peripheral antiinflammatory effect with the possible presence of constituents with prostaglandin synthesis inhibitory activity. Similarly, indomethacin showed significant inhibition of pain induced by formalin in the second phase.

It is worth mentioning that the aqueous fraction of *C. schimperi*, administered at a dose of 200 mg/kg, p.o produced significant ($P < 0.05$) antinociception (110 and 75.5 sec) on both the first and second phases of formalin-induced pain, respectively (Figure 3), although the effect was more pronounced on the second phase. On the other hand, the 3:1 chloroform-methanol and chloroform fractions of *C. schimperi*, given at a dose of 200 mg/kg, p.o, failed to show any significant effect on both the first and second phase. Considering the potent inhibitory property of *C. schimperi* extracts on the second phase of formalin induced pain, it might be possible to suggest that the total extract of this plant exert its analgesic action because of its antiinflammatory properties. However, the aqueous fraction of *C. schi-*

mperi, unlike the aqueous crude extract, showed significant inhibition of the neurogenic pain implying its central involvement.

As depicted in Figure 4, at an oral dose of 100 mg/kg, the isolated compound syringin displayed potent antinociceptive activity decreasing very significantly ($P < 0.001$) the licking and biting time (65.5 sec) in the second phase of formalin-induced pain. In the control group, the licking time induced by 2.5% formalin was 129.17 and 136.83 sec during the first and second phases, respectively.

The antinociception mechanism of the isolated compound syringin is also correlated with that of the aqueous fraction of *C. schimperi* in that syringin was also very active in the second phase of formalin-induced pain. Thus, the result of the present study suggests that the peripheral antinociceptive effects of syringin against the nocifensive responses of mice induced by formalin may be mediated via inhibition of inflammatory mediators such as cyclooxygenases and/or lipoxygenases, which corroborate the antiinflammatory activity of syringin.

From the present study, it can be concluded that extracts of *C. schimperi* possess significant antiinflammatory and antinociceptive activities. It can also be proposed that the antinociceptive activity of the plant is mainly due to its antiinflammatory effect. Syringin, the 4-*O*-glucoside of sinapyl alcohol isolated for the first time from the aqueous extract of the roots of *C. schimperi* appears to be one of the active ingredients of the plant. The results of this study support the traditional use of the roots of *C. schimperi* for the treatment of orofacial inflammation.

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Conflict of interest

There is no conflict of interest declared by the authors.

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