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Full Length Research Paper

Effects of *Hibiscus asper* leaves extracts on carrageenan induced oedema and complete Freund's adjuvant-induced arthritis in rats

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The present study was undertaken to assess the anti-inflammatory potential of the aqueous and methanolic extracts of *Hibiscus asper* Hook. f. leaves (Malvaceae) against carrageenan induced acute inflammation and complete Freund's adjuvant (CFA)-induced arthritis in Wistar albino rats. In the carrageenan-induced acute inflammation, the methanolic extract of *H. asper* exhibited significant anti-inflammatory effect at the dose 200 and 400 mg/kg. Maximum inhibition (57.29%) was noted at the dose of 200 mg/kg after 2 h of drug treatment while with the aqueous extract, the maximum effect (46.36%) was obtained after 2 h at the same dose. Further, methanolic extract of *H. asper* leaves showed a significant dose-dependent protective effect against CFA-induced arthritis. At the dose of 400 mg/kg, the percentage of inflammation was 29.33 versus 45.56% for the control group on day 20 and 17.43 versus 60.80%, respectively to the treated (400 mg/kg) and control group at the end of the treatment period. These observations suggest possible therapeutic potential of the methanolic extract of *H. asper* leaves in inflammatory disorders like rheumatoid arthritis.

Key words: Anti-inflammatory, arthritis, *Hibiscus asper*; extracts, complete Freund's adjuvant, rats.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory auto-immune disease (Arend and Dayer, 1990). In spite of intensive research into arthritis and the development of numerous new antiarthritic agents, cures for the condition remain elusive (Kaithwas and Majumdar, 2010).

The use of inhibitors of phospholipid-derived mediators of inflammation, mainly the cyclo- oxygenase inhibitors, is limited by their often deleterious side effects (Cerella et al., 2010). Dietary management has provided only transient benefits (Kremer et al., 1985). Therefore, development of new and more powerful drugs with fewer side effects remains constant. In the undeveloped countries, a large number of herbal drugs are use by practitioners for the treatment of pain and rheumatism (Fotio et al., 2009). Among plant species, *Hibiscus asper* (Malvaceae) is an important medicinal plant widely distributed throughout tropical Africa and Madagascar. This specie belongs to the genus *Hibiscus* represented by 250 species (Sunil et al., 2009).

In the western region of Africa, this plant is widely used by traditional practitioners for the treatment of inflammation, anemia, jaundice, leucorrhoea, poison antidote, depression and dysmenorrhea (Schippers and Bosch, 2004; Foyet et al., 2011). In the western region of Cameroon, the leaves are highly recommended by tradipractitioners for the treatment of abscesses, urethritis and joint pain, but are also used as a potent sedative, tonic and restorative. It is also used to treat male infertility and skin infection (Burkill, 1985). In veterinary medicine, *H. asper* is used against the cutaneous infections of the domestic animals, as well as an antiparasitic drug (Schippers and Bosch, 2004). In our previous work, we demon strated

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that methanolic extract of *H. asper* leaves extract have antioxidant effects and improves spatial memory deficits in the 6-OHDA lesion rodent model of Parkinson's disease (Foyet et al., 2011). The present study was prompted by the claim of some Cameroonian traditional health practitioners that decoctions or infusions of *H. asper* leaves are effective remedies for the treatment or control of painful, arthritic and other inflammatory conditions. The aim of this study was, therefore, to investigate the anti-inflammatory potential of the extracts of *H. asper* leaves against carrageenan induced acute inflammation and complete Freund's adjuvant (CFA)-induced arthritis in Wistar albino rats.

MATERIALS AND METHODS

Plant material

H. asper Hook. f (Malvaceae) leaves were collected in Fotouni (West-region, Cameroon) in May 2010 and identified by Dr. Focho Derreck of the Department of Plant Biology (University of Dschang, Cameroon). A specimen was deposited at the National Herbarium, Yaounde, Cameroon under the number Lucha034.

Preparation of extracts

The aqueous extract was prepared by maceration of 100 g of sun dried pulverized leaves of the plant in 1 L of boiled distilled water for 24 h. The extract was later filtered and the solvent eliminated by concentration in a rotor evaporator and dried in an oven at 45°C to give 9.5 g (9.5%) of aqueous extract (Foyet et al., 2008). The preparation of the methanolic extract was the same as previously described (Foyet et al., 2011). Basically, the leaves of *H. asper* were dried under shade and pulverized. 100 g of the pulverized leaves was macerated in 1 L of 90% methanol for 5 days at room temperature (25°C). It was later filtered, and the solvent was separated from the residues by gravity filtration and then evaporated in vacuum. The yield of 12.5 g of crude organic extract was 12.5%.

Chemicals

All reagents used in the study were of high purity. Carrageenan, indomethacin, chloroform, acetic anhydride, sulphuric acid, sodium pentobarbital, $NaNO_2$, $AlCl_3$, and NaOH were purchased from SIGMA Chemicals Co. (Dorset, UK). Complete Freund's adjuvant (CFA, complete fraction of Mycobacterium tuberculosis suspended in mineral oil) was procured from Difco, USA.

Phytochemical studies

The total phenolic content of the aqueous and methanol extracts of *H. asper* leaves was measured using the Folin-Ciocalteu method as described by Lee et al. (2003). The plant extracts (20 mg) were dissolved into $CHCl_3$ and added to the mixture of acetic anhydride (2 ml) and sulphuric acid (0.1 ml). The obtained colouration (greenish or violet red) determined if the plant extract contained sterols or triterpenes. For the determination of saponin content, the plant extracts were solubilised in water, shaken vigorously and the presence of the lather for more than 5 min indicated the presence of saponins in the medium. The flavonoid content of the plant extracts was determined by the method described by Lee et al. (2003). Briefly, 100 µl of appropriately diluted extract (1 mg/ml) was added to 400 µl of distilled water and 300 µl of 5% $NaNO_2$ was added to each volumetric flask. After 5 min, 300 µl of 10% $AlCl_3$ was added to the medium, followed 1 min later by 200 µl of 1 M NaOH and distilled water (240 µl) and the mixture was vortexed. Then, absorbance was measured at 510 nm, and the flavonoid contents of the samples were expressed in milligrams per serving of epicatechin equivalents.

Experimental animals

Studies were carried out using Wistar albino rats of either sex weighing 160 ± 20 g. They were raised in the animal house at the Faculty of Science of the University of Yaoundé I. The animals were grouped in polyacrylic cages (38 × 23 × 10 cm) with not more than six animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2°C) with dark and light circle (12/12 h). The rats were acclimatized to laboratory condition for 10 days before commencement of experiment. Prior to and after treatment, the animals were fasted for 12 and 7 h, respectively. However, water was made available *ad libitum*. Prior authorization for the use of laboratory animals in this study was obtained from the Cameroon National Ethical Committee (Reg. no. FWA-IRB00001954).

Carrageenan-induced rat paw oedema

Six groups of six animals were used. Acute inflammation was produced by the subplantar administration of 0.1 ml of 1% carrageenan in normal saline in the right paw of the rats. The different groups were treated with aqueous extract (200, 400 mg/kg, p.o.), methanolic extract (200, 400 mg/kg, p.o.) of *H. asper* leaves, indomethacin (10 mg/kg, p.o.) and control vehicle (normal saline, 10 ml/kg, p.o.). The paw volume was measured using plethysmometer (model 7150, Ugo Basil, Italy). Before injecting carrageenan, the average volume of the right hind paw of each rat was calculated from three readings which did not deviate more than 4% (Vo). At 0.5, 1, 2, 3, 4, 5 and 6 h after carrageenan injection, the paw volume of each rat was measured one time (Vt). The animals were pretreated with the extracts and indomethacin 30 min before the administration of carrageenan (Asongalem et al., 2004). The percentage of inhibition (PI) for each rat and each group was calculated using the following equation:

$$PI(\%) = \frac{(Vt - V0)control - (Vt - V0)treated}{(Vt - V0)} \times 100$$

CFA-induced arthritis in rats

Complete Freund's adjuvant (0.1 ml) was injected into the right hind foot pad of 24 rats divided into four groups (day 0). The treatment was initiated on day 14 after CFA injection, when the joints are supposed to be well inflamed (Young et al., 2007). Experimental groups of animals received an oral administration of 200 or 400 mg/kg body weight of the methanol extract of *H. asper* leaves during 13 days. Rats of the control group received normal saline (10 ml/kg, p.o.). Indomethacin (10 mg/kg) was used as reference drug and daily administered to rats during 13 days. The volume of inflamed paws was measured every day (Vt) and compared with the volume before the CFA injection (Vo), using a plethysmometer throughout the study. The percentage of inflammation (Pi) for each rat and each group was calculated using the following equation (Ashok et al., 2006):

Table 1. Phytochemical composition of the aqueous and methanolic extracts of *H. Asper.*

Compound	Concentration (mg/ml)	Aqueous extract of *H. asper*	Methanolic extract of *H. asper*
Total phenolic compounds (mg of GAE)	0.5	0.03±0.01	0.21±0.03
	1	0.15±0.07	0.45±0.3
Flavonoids (mg of ECE)	0.5	0.07±0.03	0.09±0.01
	1	0.13±0.02	0.25±0.11
Saponins		-	-

GAE, Gallic acid equivalents, ECE, epicatechin equivalents; n = 3; - indicate the absence of saponins.

$$Pi\ (\%) = \frac{Vt - V0}{V0} \times 100$$

The body weight was measured on days 5, 14 and 26. At the end of the experiment, all rats were killed with an overdose of sodium pentobarbital (100 mg/kg b.w., i.p., Sigma).

Statistical analysis

The animal's paw volumes were statistically analyzed with analysis of variance (one-way ANOVA). All results are expressed as mean ± standard error of the mean (S.E.M). Significant differences were determined by Tukey's post hoc test using MINITAB-15 software. F values for which $p<0.05$ were regarded as statistically significant.

RESULTS

Phytochemistry

Preliminary phytochemical screening of the aqueous and methanol extract of *H. asper* leaves revealed the presence of flavonoids and polyphenols (Table 1) and the absence of saponins.

Effect of the methanolic extract of *H. asper* leaves on carrageenan induce inflammation

The aqueous extract (200 and 400 mg/kg) and methanolic extract (200 and 400 mg/kg) exhibited significant ($P < 0.05$) reduction in paw oedema volume of rats. The percentage inhibition of both extracts was comparable to that of indomethacin (100 mg/kg), a standard anti-inflammatory drug. For the methanolic extract, the maximum inhibition (57.29 and 41.52%) was obtained after 2 h with the dose of 200 mg/kg and after 2 h with the dose of 400 mg/kg, respectively (Table 2). While the maximum activity of the aqueous extract (46.36%), was obtained at the dose of 200 mg/kg and after 4 h (Table 3).

Effect of the methanolic extract of *H. asper* leaves on adjuvant-induced arthritis

After inoculation with the CFA suspension, the vehicle-treated rats developed visible clinical signs of arthritis characterized by oedema and/or erythema in paws around day 7. The arthritis continued to grow until day 26 after CFA injection (Figure 1). In the animals treated with the methanolic extract of *H. asper* leaves, the percentage of the inflammatory response was clearly reduced. Administration of 400 mg/kg of the methanolic extract of *H. asper* leaves lead to a significant ($p<0.05$) decrease in the percentage of inflammation on days 16, 18, 20, 22 and 24, showing 35.48, 19.23, 33.33, 26.08 and 23.33%, respectively (Table 4). As shown in Figure 2, methanolic extract of *H. asper* leaves at the dose of 400 mg/kg could also significantly ($p<0.05$) reduce the hind paw volume of the controlateral paw at days 18, 20 and 22, and markedly improved the loss of body weight (Table 5) compared to the vehicle-treated arthritic.

DISCUSSION

Carrageenan-induced oedema has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic. The early phase (1 to 2 h) of the carrageenan model is mainly mediated by histamine,serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings. The late phase (3 to 4 h) is sustained by prostaglandin release and mediated by bradykinin, leukotrienes, polymerphonuclear cells and prostaglandins produced by tissue macrophages (Gupta et al., 2006; Olivera de Melo et al., 2006). The significant ($p<0.05$) suppressive activity of the aqueous and methanolic extract of *H. asper* leaves in both phases shows its potent anti-inflammatory effect. This result is quite similar to the one observed for indomethacin at 5 mg/kg, which inhibited the oedema by 57.82%. Both results were statistically significant ($p<0.05$). Ueno et al. (2000) found that the injection of

Table 2. Effect of methanolic extract of *H. asper* leaves and indomethacin on carrageenan induced paw oedema in rats.

Treatment	Dose (mg/kg)	Variation of volume (ml)						
		0. 5 h	1 h	2 h	3 h	4 h	5 h	6 h
Control	-	0.21±0.04	0.34±0.11	0.50±0.08	0.69±0.09	0.53±0.08	0.55±0.06	0.43±0.05
Indomethacin	10	0.14±0.06 (32.94)	0.22±0.03 (34.56)	0.23±0.02* (54.27)	0.31±0.06** (55.60)	0.22±0.03** (57.82)	0.31±0.04**(44.09)	0.29±0.04 (33.53)
H. asper (meth.)	200	0.17±0.04 (20.00)	0.23±0.02 (33.09)	0.21±0.03** (57.29)	0.35±0.02** (50.18)	0.25±0.01** (53.08)	0.28±0.01**(49.55)	0.27±0.01 (36.99)
H. asper (meth.)	400	0.18±0.03 (15.29)	0.28±0.05 (16.91)	0.32±0.07 (35.18)	0.41±0.06** (41.52)	0.33±0.07* (37.91)	0.37±0.07(33.64)	0.29±0.07 (32.37)

n = 6. Results are expressed as mean ± SEM. Percentage inhibition is in brackets. The statistical analysis was performed on absolute data. *P<0.05, **P<0.01.

Table 3. Effect of the aqueous extract of *H. asper* leaves and indomethacin on carrageenan induced paw oedema in rats.

Treatment	Dose (mg/kg)	Variation of volume (ml)						
		0.5 h	1 h	2 h	3 h	4 h	5 h	6 h
Control	-	0.21±0.04	0.34±0.11	0.50±0.08	0.69±0.09	0.53±0.08	0.55±0.06	0.43±0.05
Indomethacin	10	0.14±0.06 (32.94)	0.22±0.03 (34.56)	0.23±0.02* (54.27)	0.31±0.06** (55.60)	0.22±0.03** (57.82)	0.31±0.04** (44.09)	0.29±0.04 (33.53)
H. asper (aq)	200	0.14±0.04 (34.12)	0.26±0.06 (23.53)	0.35±0.06 (29.65)	0.41±0.04** (40.79)	0.31±0.03* (41.23)	0.30±0.02** (46.36)	0.23±0.04* (46.24)
H. asper (aq)	400	0.19±0.04 (9.41)	0.20±0.01 (40.44)	0.35±0.04 (30.65)	0.40±0.04** (42.60)	0.34±0.04 (35.55)	0.30±0.06** (45.00)	0.26±0.04 (39.88)

n= 6. Results are expressed as mean ± SEM. Percentage inhibition is in brackets. The statistical analysis was performed on absolute data. *P<0.05, **P<0.01.

carrageenan into the rat paw induces the liberation of bradykinin, which later induces the biosynthesis of prostaglandin and other autacoids, which are responsible for the formation of the inflammatory exudate. Besides, in the carrageenan-induced rat paw oedema model, the production of prostanoids has been through the serum expression of COX-2 by a positive feedback mechanism (Nantel et al., 1999).

Prostaglandin-E2 (PGE2), a powerful vasodilator, synergizes with other inflammatory vasodilators such as histamine and bradykinin and contributes to the redness and increased blood flow in areas of acute inflammation. Therefore, it is suggested that the mechanism of action of the methanolic extract of *H. asper* extracts may be related to histamine and prostaglandin synthesis inhibition, as described for the anti-inflammatory mechanism of indomethacin in the inhibition of the inflammatory process induced by carrageenan (Neto et al., 2005; Cerella et al., 2010).

The results of the present study also indicate that the Methanolic extract of *H. asper* leaves exhibits anti-arthritic effects in rats with Freund's adjuvant-induced arthritis. The model of adjuvant-induced arthritis in rats has been extensively used in the study of inflammatory processes (Barsante et al., Mayer et al., 2005). Freund Adjuvant is an antigen solution emulsified in mineral oil that is used as an immune-potentiator. The complete form (CFA) is composed of inactivated and dried mycobacteria and is effective in stimulating cell-mediated immunity and may lead to the potentiation of the production of certain immunoglobulins. Shortly after the administration of CFA into hind paw; pronounced swelling appears in the hind paw which persists for weeks (primary reaction). After few days, the contralateral paw as well as front paw also becomes swollen and arthritic nodules appear in ear and tail (delayed systemic response) (Lee et al., 2009; Kaithwas and Majumdar, 2010). Rheumatoid arthritis (RA), which is associated with systemic inflammatory disorders, is a chronic inflammatory disease involving multiple joints. It is an autoimmune disorder of unknown etiology that is characterized by progressive joint destruction, deformity, disability and premature death in most patients. Recent studies have revealed the key roles of pro-inflammatory cytokines, such as tumor necrosis factor-a (TNF-a), interleukin-1b (IL-1b), IL-6 and IL-8 in the pathogenesis of RA (Feldmann and Maini, 1999; Cai et al., 2005).

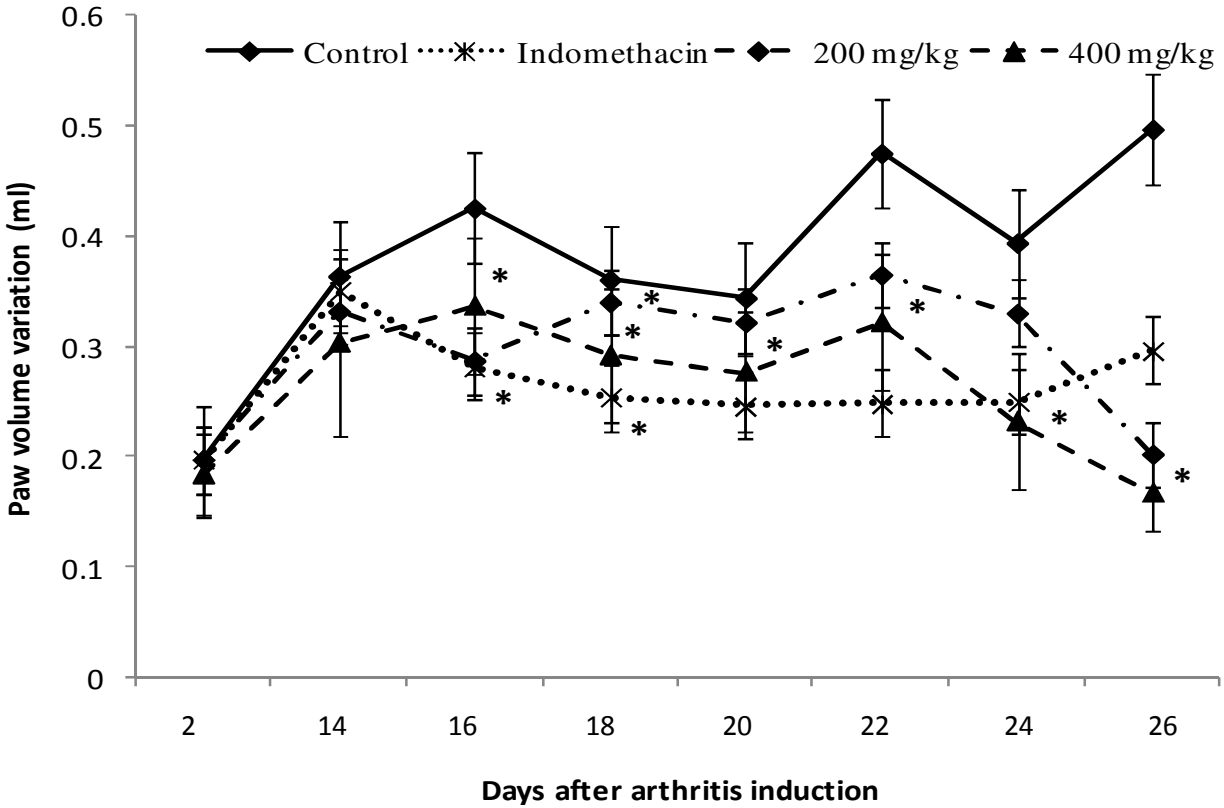

Figure 1. Effects of the methanolic extract of *H. asper* leaves and indomethacin on the chronic inflammation induced by Freund Complete Adjuvant (Injected paw). Data are given as means ± S.E.M. *p<0.05 is considered significant, compared to control.

Table 4. Effects of methanolic extract of *H. asper* and indomethacin on the inflammation induced par Freund complete adjuvant.

Treatment	Inflammation (%)							
	Day 2	Day 14	Day 16	Day 18	Day 20	Day 22	Day 24	Day 26
Control	30.08± 3.70	40.5±4.35	51.62±5.50	49.82±6.12	45.56±4.21	59.71±4.78	49.31±4.57	60.80±5.12
H. asper (200 mg/kg)	30.22±5.02	39.54±5.91	34.80±4.18	45.76±3.86	41.16±5.81	48.16±5.82	38.00±3.52	20.04±7.65*
H. asper (400 mg/kg)	30.87±5.31	37.72±6.23	39.48±4.67	35.23±4.57*	29.33±4.61*	42.08±3.97	21.33±3.88*	17.43±6.48*
Indomethacin (10 mg/kg)	27.25±5.48	39.62±3.42	34.03±4.36	26.92±2.23*	23.83±7.24*	24.39±5.27*	25.00±9.25*	36.08±9.89

Data are given as means ± S.E.M. Significant parameters were obtained statistically from one way ANOVA and Tukey's post hoc test *p<0.05 is considered significant, compared to control.

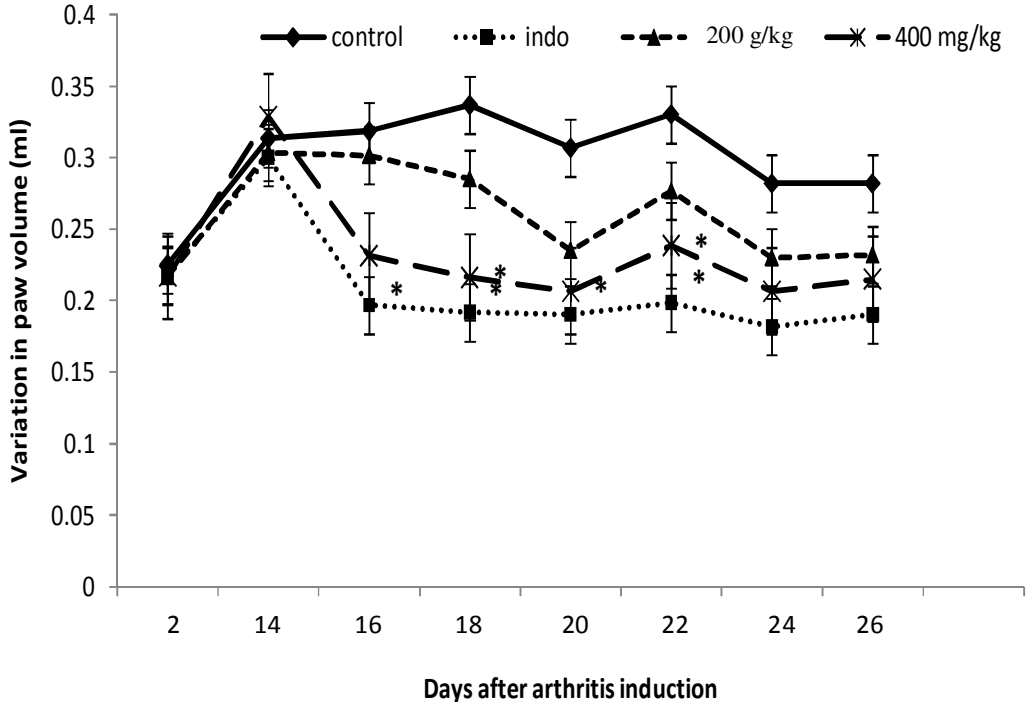

Figure 2. Effects of methanolic extract of *H. asper* leaves and indomethacin on the chronic inflammation induced by Freund complete adjuvant on controlateral (Non-Injected) paw. Data are given as means ± S.E.M. *p<0.05 is considered significant, compared to control.

Table 5. Effects of methanolic extract of *H. asper* leaves treatment on the body weight of arthritic animals.

Treatment	Difference in body weight (g)		
	Day 5	Day 14	Day 26
Control (-)	3.54±1.77	2.16±2.04	1.74±0.92
H. asper (200 mg/kg)	-1.52±2.18	5.00±2.34	4.71±2.01
H. asper (400 mg/kg)	2.92±1.08	4.52±1.08	13.53±5.21*
Indomethacin (10 mg/kg)	4.97±1.74	13.22±3.72*	17.47±3.45*

-: Indicates decrease in body weight. Values are mean ± S.E.M of 6 animals. One-way ANOVA followed by Tukey's post hoc test. * p<0.05, as compared to arthritic control.

In the present study, we showed that methanolic extract of *H. asper* leaves could significantly inhibit the progression of the rheumatoid arthritis in treated animals.

This extract was used because it was more active in the carrageenan-induced oedema. The effect of the methanolic extract of *H. asper* leaves was dose-dependent and for a long period compared to the standard. In particular, the chronic anti-arthritic effect of this extract was also significantly ($p<0.05$) effective on the second signals (after day 16) of left hind paw from an immunological reaction after treatment of CFA in rats. These results indicated that the extract may probably reduced vascular permeability and suppress the abundant production of pro-inflammatory cytokines in the blood serum.

Earlier observations by Rekha et al. (2010) supported the alterations in the metabolic activities of diseased rats. Earlier findings suggest that absorption of 14C-glucose and 14C-leucine in rat's intestine was reduced in inflamed rats (Sigthorsson et al., 1998), and it shows that the anti-inflammatory drugs have corrected the decreased absorption capacity of intestine during inflammation (Gareth et al., 1993; Brunet-Guedj et al., 2006). The increased body weight during the treatment with indomethacin and the methanolic extract of *H. asper* leaves as observed in this work may be due to the restoration of the absorption capacity of the intestine.

The presence of phytoconstituents like flavonoids and polyphenols has been previously found to be responsible for anti-inflammatory activities in plants (Ilavarasan et al.,

2005). Theses constituents may be responsible for the anti-inflammatory activities observed in this study since they are present in the methanolic extract of *H. asper* leaves.

Conclusion

Based on these results, we can conclude that oral administration of the aqueous and methanolic extract of *H. asper* leaves results in anti-inflammatory and anti-arthritic activity, respectively. However, more clinical investigations are required to substantiate this report.

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