

**Anti-Inflammatory Activity of Extracts of Leaves of *Hygrophila spinosa* T. Anders in Chronic Animal Models of Inflammation**

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**ABSTRACT**

*Hygrophila spinosa* T. Anders (Acanthaceae) is traditionally used in Indian medicine for the treatment of microbial infections, liver diseases, cancer, inflammation, rheumatism, diabetes, pain, fever etc. The aim of the present study is to evaluate the anti-inflammatory activity of chloroform and alcoholic extracts of the leaves of *H. spinosa* in chronic inflammation models in rats as our previous study revealed that these two extracts had anti-inflammatory activity in carrageenan induced paw oedema model. Anti-inflammatory activity was evaluated by cotton pellet-induced granuloma and Freund's adjuvant-induced arthritis in rats. Antioxidant activity of the extracts was revealed by their scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical and a flavonoid compound (apigenin) was also isolated and characterized from the alcoholic extract of the plant. Chloroform and alcoholic extracts showed anti-inflammatory activity both in cotton pellet-induced granuloma and Freund's adjuvant-induced arthritis in a dose dependent manner. The decrease in body weight due to injection of CFA was improved significantly by the above two extracts also. Both the extracts also exhibited antioxidant activity. The results demonstrated that *H. spinosa* has anti-inflammatory activity in chronic models of inflammation which support the traditional use of *H. spinosa* in the treatment of rheumatism.

**Keywords:** *Hygrophila spinosa*, Cotton pellet-induced granuloma, Freund's adjuvant-induced arthritis

## 1. Introduction

*Hygrophila spinosa* T. Anders (Acanthaceae) commonly known as ‘Talmakhana’ in Hindi is found in water-logged areas throughout India, contains a number of phytoconstituents viz. lupeol (Shailajan and Abhishek, 2008),  $\beta$ - sitosterol (Tiwari et al., 1967), stigmasterol (Khare, 2007), ascorbic acid,  $\beta$ - carotene (Sahoo and Acharya, 2005), nicotinic acid (Sharma et al., 2002), hentriacontane (Chopra et al., 1958), luteolin, luteolin-7-rutinoside (Balraj and Nagarajan, 1982), syringic acid, vanillic acid (Daniel, 2005) etc. In our earlier studies we have reported the other constituents, traditional uses, pharmacological activities of the plant (Patra et al., 2009a); various groups of phytoconstituents in petroleum ether, chloroform, alcoholic and aqueous extracts of the leaves of the plant (Patra et al., 2009b) and also few pharmacological studies of the above extracts (Patra et al., 2008, 2009c). The aim of this study was to determine the anti-inflammatory activity of chloroform and aqueous extracts of the leaves of *H. spinosa* in chronic inflammation models in rats.

## 2. Materials and methods

### 2.1 Plant material

*H. spinosa* plants were collected from Berhampur, Orissa, India and botanical identification was done through Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra, Ranchi (Voucher no. BITPcog. 463/07-08). Voucher specimen was preserved in the department for further verification.

### 2.2 Preparation of different extracts

The leaves were washed thoroughly, dried under shade and pulverized. The coarse powder was extracted successively with petroleum ether, chloroform and alcohol using soxhlet apparatus. The extracts were dried using a rotary vacuum evaporator and stored in a desiccator until further use.

### 2.3 Cotton pellet-induced granuloma in rats

Cotton pellet-induced granuloma in rats is used to screen drugs preventing transudative, exudative and proliferative components of chronic inflammation (Winter et al., 1957; Mizushima et al., 1972; Spector, 1969). Granuloma was induced by subcutaneous implant of a sterile cotton pellet (50 mg) in the dorsal area of anaesthetized rats. One day after the implant, the animals were divided into six groups (n=6) and were administered with vehicle/standard drug/test substances as mentioned in Table 1 for 10 days. On the 10<sup>th</sup> day the pellets were dissected out,

dried in an incubator at 60<sup>0</sup>C until a constant weight was obtained. The granuloma tissue formation (dry weight of granuloma) was calculated after deducting the weight of cotton pellet (50 mg) from the constant dry weight of pellet and taken as a measure of granuloma tissue formation. The anti inflammatory effect was assessed by degree of inhibition of the granuloma weight in the groups under study as compared with the control. The granuloma inhibition percentage for each group was calculated by comparison with the control group considered as 100% of inflammatory activity.

#### 2.4 Freund's adjuvant-induced arthritis in rats

Rats with an initial body weight of 150-200 gm are used in the study. On day 1, they are injected into the sub plantar region of the left hind paw with 0.1 ml of complete Freund's adjuvant (CFA, Sigma) (Wei et al., 1986). Dosing with the test compounds or the standard is started on the same day and continued for 12 days. Paw volumes of both sides and body weight are recorded on the day of injection, whereby paw volume is measured plethysmographically. On day 5, the volume of the injected paw is measured again, indicating the primary lesion and the influence of therapeutic agents on this phase (Newbould, 1963). From day 13 to 21, the animals are not dosed with the test compound or the standard. The severity of the induced adjuvant disease is followed by measurement of the non injected paw (secondary lesions) with a plethysmometer (Walz et al., 1971). The body weight was recorded at a weekly interval upto 21 days. On day 21, the severity of the secondary lesions is evaluated visually by observing redness/inflammation/nodules in hind paws, fore paws, ears, nose and tail (Vogel, 1997).

#### 2.5 Antioxidant activity

Antioxidant activity of different extracts was measured on the basis of the scavenging activity of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical (Burits and Bucar, 2000; Dasgupta and De, 2007 ). Various concentrations of the extracts were added to 0.004% methanolic solution of DPPH. After 30 min the absorbance at 517 nm was determined, and the percentage inhibition activity was calculated using the following formula.

$$\% \text{ inhibition} = [(Ac - At) / Ac] \times 100$$

Where, Ac = absorbance of control sample and At = the absorbance of test sample.

#### 2.6 Isolation of compound **1** from alcoholic extract

50 gm of alcoholic extract was column chromatographed on a silica gel column (mesh 60-120) and eluted with solvent mixtures of increasing polarity: chloroform (300 ml),

chloroform-acetone [(80:20, 300 ml), (60:40, 300 ml), (40:60, 300 ml), (20:80, 300 ml)], acetone (300 ml), acetone-methanol [(80:20, 300 ml), (60:40, 300 ml), (40:60, 300 ml), (20:80, 300 ml)] and methanol (300 ml). Fractions (20ml) were collected and monitored on TLC. Fractions (151 to 165) collected were pulled together as these fractions showed a single spot of same  $R_f$  value in TLC. It was evaporated in a water bath (70-80<sup>0</sup>C) to afford a solid residue. The residue was dissolved in EtOH with little warming on a water bath. It was left undisturbed in refrigerator when crystals of compound **1** was obtained (yield: 0.0015% w/w).

### 2.7 Characterization of compound **1**

The various instruments used for recording the data for compound **1** are: FTIR spectroscopy (Shimadzu, IRPrestige-21), elemental analysis (Elementar, Vario EL III), <sup>1</sup>H and <sup>13</sup>C NMR spectra (PABBO BB NMR spectrophotometer), mass spectroscopy etc. Other physicochemical characters as melting point, solubility and physical appearance were also recorded.

### 2.8 Statistical analysis

The results were expressed as mean  $\pm$  standard error mean (SEM). Statistical analysis of the data was carried out using one way analysis of variance (ANOVA) followed by Student's t-test to determine the significant difference between the control and the treated groups.  $P < 0.05$  was considered significant.

## 3. Results

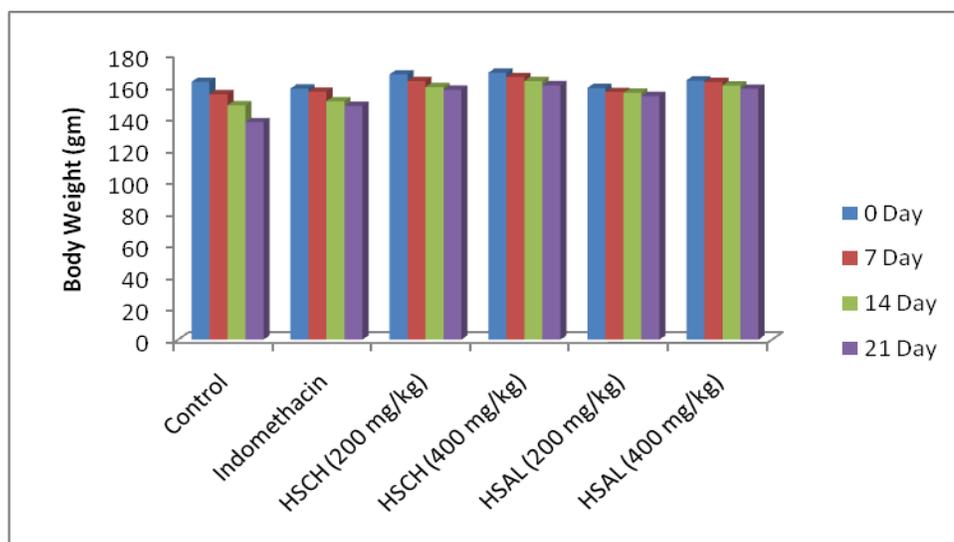
### 3.1 Cotton pellet-induced granuloma

The percentage inhibition of granuloma formation by chloroform and alcoholic extract (400 mg/kg) were 24.09 and 35.69% respectively ( $p < 0.05$ ) (Table 1). Hence the decrease in granuloma weight indicated the anti-inflammatory activity of alcoholic and chloroform extracts of *H. spinosa* in the treatment related to various types of inflammatory disorders.

### 3.2 Freund's adjuvant-induced arthritis

Injection of complete Freund's adjuvant into the rat paw induces inflammation as primary lesion with a maximum after 3 to 5 days. Secondary lesions occur after a delay of approximately 11 to 12 days which are characterized by inflammation of non-injected sites (hind leg, forepaws, ears, nose and tail), a decrease of weight and immune responses. The decrease in body weight due to injection of CFA was improved significantly by chloroform and alcoholic extracts of *H. spinosa* (Fig. 1). Further, there was significant inhibition of oedema both after 5 days (only by

alcoholic extract at 400 mg/kg dose) and 21 days (by both chloroform and alcoholic extract) of CFA administration (Table 2). The percentage inhibition of paw oedema by chloroform and alcoholic extract (400 mg/kg) after 21 days of CFA treatment was 22.72 and 29.54% respectively. Further, the extent of inhibition by both the extracts was more than the standard drug, indomethacin (20.45%). The severity of secondary lesions was also decreased by both the above extracts. These findings confirm that the *H. spinosa* can be used for the treatment of chronic inflammation and arthritis.



**Fig. 1.** Effect of different extracts of *H. spinosa* leaf on body weight of animals in CFA induced arthritic rats. HSCH, chloroform extract; HSAL, alcoholic extract

**Table 1.** Effect of different extracts of *H. spinosa* leaf on cotton pellet-induced granuloma

Treatment	Dose (mg/kg, p.o.)	Weight of dry granuloma (mg)	Percentage inhibition of granuloma
Vehicle (Control)	-	122.36 ± 2.32	-
Indomethacin	10	65.42 ± 1.52*	46.53*
Chloroform	200	109.65 ± 1.05*	10.38*
	400	92.88 ± 0.98*	24.09*
Alcoholic	200	102.28 ± 1.06*	16.41*
	400	78.68 ± 0.86*	35.69*

Values are expressed as mean ± S.E.M. (n = 6); \*p < 0.05 vs. control.

**Table 2.** Effect of different extracts of *H. spinosa* on adjuvant induced arthritis

Treatment	Dose (mg/kg)	Increase in paw volume (in ml)		Percentage inhibition	
		After 5 days	After 21 days	After 5 days	After 21 days
Control	-	0.56 ± 0.02	0.44 ± 0.04	-	-
Indomethacin	10	0.54 ± 0.02	0.35 ± 0.05*	3.57	20.45*
Chloroform	200	0.54 ± 0.02	0.40 ± 0.03	3.57	9.09
	400	0.52 ± 0.01	0.34 ± 0.02*	7.14	22.72*
Alcoholic	200	0.53 ± 0.02	0.38 ± 0.03*	5.35	13.63*
	400	0.50 ± 0.02*	0.31 ± 0.04*	10.71*	29.54*

Values are expressed as mean ± S.E.M. (n = 6); \* p < 0.05 vs. control

### 3.3 Antioxidant activity

Both the extracts exhibited antioxidant activity in DPPH method and the potency of different extracts are in the following order: alcoholic>chloroform (Table 3).

**Table 3.** Free radical scavenging activity of different extracts of *H. spinosa* in DPPH method

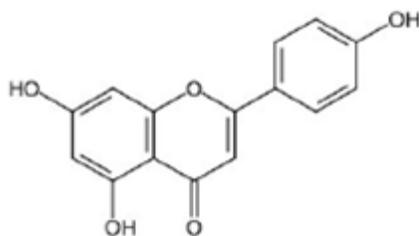
Concentration (µg/ml)	% free radical scavenging	
	Chloroform Extract	Alcoholic Extract
25	10.36±0.69	13.14±1.24
50	18.17±1.03	20.80±0.75
100	24.83±0.78	29.96±0.79
200	35.8±2.48	41.17±1.58
400	49.81±4.42	56.05±3.27

Values are mean±SEM (n=3)

### Characterization of compound 1

5,7-Dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (**1**) i.e., apigenin (Fig. 2) was isolated as whitish yellow crystals and its elemental composition was determined to be C<sub>15</sub>H<sub>10</sub>O<sub>5</sub> (Anal. Calcd for C<sub>15</sub>H<sub>10</sub>O<sub>5</sub>: C, 66.67; H, 3.73 and O, 29.60. Found: C, 66.55; H, 3.73 and O, 29.57). Melting point: 337-339<sup>0</sup>C and soluble in warm EtOH. It also contains hydroxyl (Lucas

reagent), phenol (FeCl<sub>3</sub> test) and ketone (2,4-dinitrophenyl hydrazene) functional groups which was found from functional group analysis (Siddiqui and Ali, 1997; Nadendl, 2005) and supported by IR spectroscopy. Phytochemical analysis (Shinoda test) of the compound confirmed its flavonoid nature (Khandelwal, 2005). IR (KBr) cm<sup>-1</sup>: 3312.54 (OH), 3064.46 (CH), 2952.80 (CH), 1664.64 (C=O), 1608.15 (C=C), 1144.22 (C-O-C) etc. UV λ<sub>max</sub>(EtOH): 336 nm. MS m/z: 270 (M<sup>+</sup>) (Calcd for C<sub>15</sub>H<sub>10</sub>O<sub>5</sub>: 270.24), 242, 220, 152 and 120. NMR spectrum of this compound superimposed with the data published in previous studies (Ersoz et al., 2002; Fathiazad et al., 2006; Breitmaier and Voelter, 1989; Harborne and Mabry, 1982). The structure was simulated using ACD/NMR program (ACD/ChemSketch-Product Version: 10.00) to obtain the chemical shifts of both proton and carbon where the experimental data matched with the simulated data.



**Fig. 2. Structure of compound 1**

#### **4. Discussion**

The cotton pellet granuloma method has been widely employed to assess the transudative, exudative and proliferative components of chronic inflammation (Spector, 1969). The fluid absorbed by the pellet greatly influences the wet weight of the granuloma and the dry weight correlates well with the amount of granulomatous tissue formed (Swingle and Shideman, 1972). Monocyte infiltration and fibroblast proliferation rather than neutrophil infiltration and exudation take place in chronic inflammation (Dunne, 1990). Efficacy of anti-inflammatory agents in chronic inflammatory states is indicated by their ability to inhibit the increase in the number of fibroblasts during granular tissue formation (Gupta et al., 2003). We found a dose-dependent inhibition of granuloma formation by chloroform and alcoholic extracts of *H. spinosa* leaf which reflected its efficacy to inhibit the proliferative phase of the inflammation process, i.e. increase in the number of fibroblasts and synthesis of collagen and mucopolysaccharides during granuloma tissue formation (Arrigoni-Martellie, 1977).

Determination of paw volume and changes in body weight of the animals has been used in evaluating anti-inflammatory activity and therapeutic effects of the treatment. In the present study, it was found that the alcoholic and chloroform extracts showed significant antiarthritic effect evidenced by increase in body weight and decreased oedema. Prostaglandins greatly potentiate exudates by inducing relaxation of arteriolar smooth muscle and increasing the blood supply to the tissue (Williams, 1979). Further, Rheumatoid arthritis (RA) is a chronic, cytokine-mediated destructive inflammatory polyarticular joint disease, characterized by massive synovial proliferation, systemic and local inflammation resulting in cartilage and bone destruction (Koch, 1998; McCoy et al., 2002). Prostaglandins are involved in a number of biologic activities relevant to the pathogenesis of RA, the pro-inflammatory aspects of this disease are also mediated by PGE<sub>2</sub> (Dayer and Fenner, 1992; Lippiello et al., 1979; Davies et al., 1984). The ability of the extracts to reduce CFA induced arthritis may thus be related to its inhibitory action of prostaglandin synthesis.

Several anti-inflammatory, digestive, antinecrotic, neuroprotective and hepatoprotective drugs have recently been shown to have an antioxidant and/or radical-scavenging mechanism as part of their activity (Repetto and Llesuy, 2002). Free radicals are important mediators that provoke or sustain inflammatory processes and, consequently, their neutralisation by antioxidants and radical scavengers can attenuate inflammation (Geronikaki and Gavalas, 2006; Pedrera et al., 2006).

Several flavonoids isolated from medicinal plants have been discovered to possess significant anti-inflammatory effects (Duke, 1992). According to Oktay et al. (2003) a highly positive relationship between total phenolics and antioxidant activity appears to be the trend in many plants. The anti-inflammatory activity is a common property of many flavonoids, tannins and sterols (Goncalves et al., 2008; Singh et al., 1997; Vimala et al., 1997) and these phytoconstituents also possess antioxidant property (Oktay et al., 2003). The presence of the said constituents in the extracts of *H. spinosa* may be responsible for the observed activities.

In summary, our results suggest that *H. spinosa* has considerable potency in anti-inflammatory action and has prominent effects on adjuvant-induced arthritis by alleviating paw edema. Hence, the results of the present study support the traditional use of *H. spinosa* in the treatment of rheumatism.

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