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Research Article

EVALUATION OF ANTI-INFLAMMATORY ACTIVITY OF ETHANOLIC EXTRACT OF SEEDS OF *HOLARRHENA PUBESCENS* (BUCH.- HAM.) WALL

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ABSTRACT

Objective: To screen anti-inflammatory activity of ethanolic extract of seeds of *Holarrhena pubescens* using *in vivo* models for acute and chronic inflammation.

Methods: Dried seeds of *Holarrhena pubescens* were extracted with ethanol and the extract (HPE) were then screened to investigate the presence of phytconstituents by preliminary phytochemical tests. The median lethal dose (LD_{50}) of the test drug was assessed in acute toxicity study which was found to be 2000 mg/kg, and based on this study the oral doses of HPE were fixed to 100, 200 and 400 mg/kg body weight to carry out the anti-inflammatory activity. The anti-inflammatory activity of HPE was evaluated in this study using acute model, carrageenan-induced rat paw edema, as well as chronic models, like, cotton pellet induced granuloma formation and Freund's Complete adjuvant induced arthritis in rat models. Indomethacin (10 mg/kg body weight) was used as a standard drug.

Results: The preliminary phytochemical analysis showed the presence of alkaloids, carbohydrate, glycosides, triterpenoids and steroids in test material (HPE). HPE exhibited the anti-inflammatory efficacy in dose dependent manner. 400 mg/kg dose of HPE showed maximum 74.07% ($p<0.01$) inhibition of carrageenan-induced rat paw edema. The test drug at the same dose level also showed the significant ($p<0.01$) 62.63% inhibition of granuloma formation and 77.95% inhibition of adjuvant induced arthritic edema in rats.

Conclusion: The findings of the present study suggest the anti-inflammatory efficacy of ethanolic extract of seeds of *H. pubescens* and confirm the traditional use of the plant to treat inflammatory diseases.

Keywords: *Holarrhena pubescens*, Apocynaceae, Anti-inflammatory activity, Carrageenan-induced rat paw edema, Cotton pellet induced granuloma, Freund's Complete adjuvant induced arthritis, Seed

INTRODUCTION

Inflammation is a defense mechanism of body in response to the damage of its cells and tissues. The process is mediated by several kinins and cytokines, and involves series of events such as swelling, redness, heat and pain [1]. Nevertheless, the inflammatory process also leads to develop many diseases, like rheumatoid arthritis, psoriasis and inflammatory bowel disease [2]. The search for drugs to treat inflammatory diseases has been continued for a long time. Presently, many synthetic agents have been identified for their efficacy but also possess severe side effects [3]. Hence, the search continues for therapeutically active but less toxic anti-inflammatory agents.

Natural products have been an exemplary source for safer medicine since existence of human civilizations and this approach has been in practice over the years in various indigenous communities, which led to the development of diverse traditional health care system [4]. India has vast ethnobotanical knowledge based most ancient and a pluralistic traditional healthcare system Ayurveda [5]. This ancient system provides relatively organized database and exhaustive description about botanicals along with their applications in the treatment of many ailments including inflammation.

Holarrhena pubescens (Buch.- Ham.) Wall. (Apocynaceae), an Indian traditional medicinal plant, is commonly known as 'kurchi' in India. It is widely distributed in tropical countries like India, Burma, Sri Lanka, Pakistan, Nepal, and Africa. The deciduous tree is 0.6–18 m long [6,7]. Earlier studies have reported the isolation of several phytoconstituents like alkaloids, steroid alkaloids, triterpenoids and glycosides from different parts of the plant. Reports have been suggested the presence of steroid alkaloids majorly in this plant, namely, kurchine, kurchinine, pubescinine, holamide, conaine, conessine, norconessine, conessidine, are the few [8-11]. The isolation of glycosides as naringenin glycoside, naringin and naringenin 7-O-β-Dglucoside, together with triterpenoids lupeol, lupeol β-hydroxyhexadecanoate and ursolic acid from the leaves of this plant has been reported [12]. Traditionally, the plant has been used in the treatment of asthma, leprosy, eczema, colic dyspepsia, dysentery, and many other inflammatory diseases [13, 14]. The plant has been reported to possess antiplasmodial, immunomodulatory, antimalarial, febrifuge, antidysentric, antidiarrhoeal and anthelmintic properties [15-18]. However, till date, there have been no investigations reported the anti-inflammatory activity of *H. pubescens* seeds. Hence, the intention of this present study was to ascertain the anti-inflammatory activity of ethanolic extract of seeds of *H. pubescens* using *in vivo* models for acute and chronic inflammation.

MATERIALS AND METHODS

Chemicals and drugs

All the chemicals and reagents used in this study were of analytical grade. The chemicals were obtained in high purity either from S.D. fine chemicals Pvt. Ltd; Mumbai, India or E. Merck (India) Ltd., Mumbai. Carrageenan (Hi–Media Research Laboratories Pvt. Ltd., Mumbai), Tween80 (S.D. fine Chemicals Pvt. Ltd., Mumbai), Freund's Complete adjuvant (FCA) and Indomethacin (Indo) (Sigma Aldrich St. Louis, USA) was procured.

Plant material

The seeds of *Holarrhena pubescens* were collected from Mangalore, Karnataka during May 2010. The plant was authenticated by Prof. Dr. Krishna Kumar, Dept. of Applied Botany, Mangalore University, Mangalore. A voucher specimen (voucher no. D-82) was deposited in the herbarium of NGSM Institute of Pharmaceutical Sciences, Paneer, Derelakatte, Mangalore, India.

Extraction of plant material

The seeds were shade dried at room temperature and pulverized to coarse powder. The powdered plant material was extracted with 95% v/v ethanol by cold-maceration method for four days. The extract was filtered through muslin cloth and concentrated to one third of its initial volume by using rotary evaporator (Superfit, India) at 40°C. The remaining solvent was evaporated completely by using

water bath under 40°C to dryness. The ethanolic extract of seeds of *H. pubescens* (HPE) was used for the experimental studies.

Preliminary phytochemical screening

The preliminary phytochemical screening of ethanolic extract of seeds of *H. pubescens* (HPE) was carried out for investigating the presence of alkaloids, carbohydrate, protein, flavonoids, glycosides, triterpenoids, resins, saponins, steroids, tannins and starch using the standard methods and procedures [19].

Preparation of drug materials

The ethanolic extract of seeds of *H. pubescens* (HPE) and standard drug (Indomethacin) were used as a suspension in 1% solution of tween 80 in water to screen biological activity. The solution of tween 80 (1%) in water was served as vehicle alone in study.

Animals

Studies were carried out by using Albino Wistar rats (180–200 g) of either sex. All animals were obtained from K.S. Hegde Medical Academy (KSHEMA), Deralakatte, Mangalore. Animals were grouped and housed in polyacrylic cages and kept at ambient temperature (25 ± 2) ºC, relative humidity (60 ± 5) % and 12 h light and dark cycle. They had been given standard pellet diet (Hindustan Lever Limited, Mumbai, India) and water *ad libitum* throughout the course of the study. The study protocols were approved by Institutional Animal Ethical Committee (KSHEMA /AEC/077/2008).

Acute toxicity study

Acute toxicity study was conducted to determine the median lethal dose (LD_{50}) of test material, HPE (ethanolic extract of seeds of *Holarrhena pubescens*) in adult female albino wistar rats (nulliparous and non-pregnant) by following up and down procedure of OECD guideline no. 425 [20]. Animals were administered the extract preparations orally, and observed at half hour intervals for 4 h, then after 24 h. The test material (HPE) was found to be safe up to an oral dose of 2000 mg/kg. Based on the study, three dose levels of extract were selected of which middle dose was approximately one tenth of the LD_{50}, low dose was half of that one tenth dose, and a high dose was twice of that one tenth dose, so, that was 200mg/kg, 100mg/kg and 400mg/kg respectively, to carry out the *in vivo* biological activity studies.

***In vivo* anti-inflammatory activity studies**

The anti-inflammatory activity of test drug was studied by carrageenan induced rat paw edema, cotton pellets induced granuloma formation in rat and Freund's Complete Adjuvant (FCA) induced arthritis in rat models.

Carrageenan induced rat paw edema

Acute anti-inflammatory activity of HPE was evaluated by carrageenan induced rat paw edema according to the method of Winter et al. [21]. Paw edema was induced by injecting 0.1 ml of 1% (w/v) carrageenan suspension in 0.9% (w/v) sterile saline into the plantar tissue of the left hind paw of all animals. Different groups of animals (n=6) were respectively treated orally with vehicle (tween 80, 3 ml of 1% solution), indomethacin (Indo 10 mg/kg body weight.) and HPE (100, 200 and 400 mg/kg body weight) at 1 h prior to the injection of carrageenan. The right paw served as reference to measure the degree of inflammation in the left one. Increase in the paw volume was measured by plethysmograph at hourly intervals for up to 4 h after carrageenan injection. The percentage inhibition of edema volume was calculated by using following formula [22]

$$\text{Percentage Inhibition} = (1 - V_t / V_c) \text{X} 100$$

Where V_t is the average paw edema volume of extracts and indomethacin treated groups; and V_c is the average paw edema volume of the control group that only received the vehicle.

Cotton pellets induced granuloma in rats

The granuloma in albino wistar rats was induced by implanting cotton pellets [23]. All animals were anaesthetized with ether after shaving the fur and 10 mg of sterile cotton pellets were inserted, one in each axilla. Test drug (HPE 100, 200 and 400 mg/kg), standard drug (Indo 10mg/kg) and vehicle were administered orally to animals of respective groups (n=6) for seven consecutive days. On eighth day animals were anaesthetized again to remove cotton pellets surgically and made free from extraneous tissues. The moist pellets were weighed, dried at 60° C for 24 h and then re-weighed. Increment in dry weight of pellets was taken as measure of granuloma formation. The percentage inhibition of cotton pellet weight was evaluated.

Adjuvant induced arthritis in rats

Arthritis was induced by the injection of 0.1mL of Freund's Complete Adjuvant (FCA) containing 1mg/ml of heat killed *Mycobacterium tuberculosis* in paraffin oil and mannide monooleate (Sigma Aldrich St. Louis, USA) into the subplantar region of right hind paw of rat on day 0 of the experiment [24]. Two hours prior to the injection of FCA each group of animals (n=6) were treated orally with indomethacin (Indo10 mg/kg), HPE (100, 200 and 400 mg/kg) and vehicle. Treatments were continued till day 14. Paw volume was measured on day 0, 3, 7 and 14 and the percentage inhibition was determined.

Statistical analysis

Values were expressed as mean ± S.E.M. Statistical significance of weight or volume change was determined by ANOVA, followed by Dunnet's *t*-test; values with $P<0.05$ and $p<0.01$ were considered as statistically significant. GraphPad Prism version 4.0, GraphPad Software Inc., was used for statistical analysis.

RESULTS

Preliminary phytochemical screening

The ethanolic extract of seeds of *Holarrhena pubescens* (HPE) showed the presence of alkaloids, carbohydrate, glycosides, triterpenoids and steroids in preliminary phytochemical tests (Table 1).

Acute toxicity study

The acute toxicity study of ethanolic extract of seeds of *Holarrhena pubescens* (HPE) showed no mortality and significant behaviour changes in rats up to the dose level of 2000 mg/kg. Hence, the drug was found to be safe up to 2000 mg/kg oral dose and for the further studies the doses of the test drug were selected to be 100, 200 and 400 mg/kg body weight.

***In vivo* anti-inflammatory activity studies**

Carrageenan induced rat paw edema

The effect of test drug on carrageenan induced rat paw edema is showed in Table 2. Carrageenan showed most inflammation in animals of control group at third and fourth hour after injection. The test drug (HPE) exhibited the inhibition of rat paw edema in dose dependent manner. HPE 400 mg/kg b.w., p.o. dose showed significant ($p<0.01$) 53.16 and 74.07% inhibition of rat paw edema during 3h and 4h, respectively. HPE 200 mg/kg dose showed significant ($p<0.05$) 43.51% inhibition only at 4h, whereas, 100 mg/kg dose of HPE showed mild effect throughout the study and which was also found to be non-significant. The standard drug indomethacin showed the significant ($p<0.01$) inhibition of rat paw edema by 64.55 and 80.55% at 3h and 4h, respectively.

Cotton pellets induced granuloma in rats

HPE inhibited the cotton pellets induced granuloma tissue formation in rats in dose dependent manner (Table 3). HPE 400 mg/kg dose showed significant ($p<0.01$) 58.06 and 62.63% inhibition of moist and dry cotton pellets weight, respectively. Whereas, HPE 100 and 200 mg/kg dose showed significant ($p<0.05$) 33.11 and 44.19% inhibition of dry cotton pellets weight, respectively. The standard drug indomethacin (Indo) 10 mg/kg dose showed 62.44and 76.19% inhibition of moist and dry cotton pellets weight, respectively.

Table 1: Results of preliminary phytochemical tests of ethanolic extract of seeds of *Holarrhena pubescens* (HPE)

S. No.	Tests	Inference
1.	Alkaloids	
	a) Dragendorff's test	*+ve*
	b) Hager's test	*+ve*
	c) Wagner's test	*+ve*
	d) Mayer's test	*+ve*
2.	Carbohydrates	
	a) Anthrone test	*+ve*
	b) Benedict's test	*+ve*
	c) Fehling's test	*+ve*
	d) Molisch's test	*+ve*
3.	Proteins	
	(a) Biuret test	*-ve*
	(b) Million's test	*-ve*
4.	Flavanoids	
	a) Shinoda's test	*-ve*
5.	Glycosides	
	a) Molisch's test	*+ve*
6.	Triterpenoids	
	a) Liebermann – Burchard's test	*+ve*
7.	Resins	*-ve*
8.	Saponins	*-ve*
9.	Steroids	
	a) Liebermann -Burchard's test	*+ve*
	b) Salkowski reaction	*+ve*
10.	Tannins	*-ve*
11.	Starch	-ve

+ve represent presence and –ve represent absence

Table 2: Anti-inflammatory activity of ethanolic extract of seeds of *Holarrhena pubescens* (HPE) in carrageenan induced rat paw edema model.

Groups	Dose (mg/kg b.w., p.o.)	Increase in paw volume (ml)			
		1 h	2h	3 h	4 h
Control	-	0.40±0.09	0.51±0.13	0.79±0.17	1.08±0.11
Indo	10	0.32±0.14 (20)	0.39±0.27 (23.52)	0.28±0.12 ** (64.55)	0.21±0.17** (80.55)
HPE	100	0.38±0.23 (5)	0.47±0.2 (7.84)	0.64±0.24 (18.98)	0.82±0.20 (24.07)
HPE	200	0.36±0.17 (10)	0.45±0.18 (11.76)	0.54±0.18 (31.64)	0.61±0.22* (43.51)
HPE	400	0.35±0.15 (12.5)	0.42±0.06 (17.64)	0.37±0.23** (53.16)	0.28±0.08** (74.07)

All the result are expressed in term of Mean ± S.E.M., n=6 animals in each group; number in parenthesis indicates percentage inhibition in increase in paw volume. Statistical significance was determined by ANOVA, followed by Dunnet's t-test. * p<0.05, ** p<0.01, statistically significant.

Table 3: Effects of ethanolic extract of seeds of *Holarrhena pubescens* (HPE) on cotton pellets induced granuloma formation in rats.

Groups	Dose (mg/kg b.w., p.o.)	Moist cotton pellet		Dried cotton pellet	
		Weight (mg)	% inhibition	Weight (mg)	% inhibition
Control	-	279.33±21.3	-------	76.54±1.3	--------
Indo	10	104.9±13.5**	62.44	18.22±2.1**	76.19
HPE	100	216.42±23.6*	22.52	51.19±1.8*	33.11
HPE	200	186.67±17.1*	33.17	42.71±2.0*	44.19
HPE	400	117.13±9.4**	58.06	28.6±1.0**	62.63

All the result are expressed in term of Mean ± S.E.M. n=6 animals in each group; Statistical significance was determined by ANOVA, followed by Dunnet's t-test. * p<0.05, ** p<0.01, statistically significant.

Adjuvant induced arthritis in rats

In adjuvant induced animals, a dose dependent reduction in foot thickness was observed by ethanolic extract of seeds of *H. pubescens*. As shown in Table 4, HPE at 400 mg/kg dose inhibited the arthritic edema in rats by 34.06% (p<0.05) and 77.95% (p<0.01) on 7th and 14th day of study, respectively. HPE 100 and 200 mg/kg dose respectively showed significant (p<0.05) 56.45 and 61.29% inhibition of adjuvant induced rat paw edema compared to the control group on day 14 of the study. Indomethacin (10 mg/kg dose) inhibited the arthritic edema in animals by 57.14 and 86.55% (p<0.01) on 7th and 14th day of study, respectively.

Table 4: Anti-inflammatory activity of ethanolic extract of seeds of *Holarrhena pubescens* (HPE) in adjuvant induced arthritis in rats.

Groups	Dose (mg/kg b.w., p.o.)	Increase in paw volume (ml)			
		0 day	3 day	7 day	14 day
Control	-	0.54±0.24	0.73±0.1	0.91±0.26	1.86±0.19
Indo	10	0.42±0.16 (22.22)	0.48± 0.22 (34.24)	0.39±0.15** (57.14)	0.25±0.13** (86.55)
HPE	100	0.47±0.21 (12.96)	0.62± 0.13 (15.06)	0.73±0.19 (19.78)	0.81±0.24* (56.45)
HPE	200	0.46±0. 09 (14.81)	0.58±0.2 (20.54)	0.66±0.14 (27.47)	0.72±0.16* (61.29)
HPE	400	0.44±0.12 (18.51)	0.56±0.19 (23.28)	0.60±0.13* (34.06)	0.41±0.18** (77.95)

All the result are expressed in term of Mean ± S.E.M., n=6 animals in each group; number in parenthesis indicates percentage inhibition in increase in paw volume. Statistical significance was determined by ANOVA, followed by Dunnet's t-test. * p<0.05, ** p<0.01, statistically significant.

DISCUSSION

In the present study, the anti-inflammatory activity of HPE was evaluated by carrageenan-induced rat paw edema, cotton pellet induced granuloma formation and adjuvant induced arthritis in rat models. The doses of HPE were fixed to 100, 200 and 400 mg/kg b.w., p.o. to carry out the biological activity as the median lethal dose (LD_{50}) of the test drug was found to be 2000 mg/kg in acute toxicity study. The carrageenan induced rat paw edema is an experimental model for acute inflammation study and which is widely used to screen most of the anti-inflammatory agents. The carrageenan induced rat paw edema development is a biphasic response, where the initial stage is dependent on the release of signature mediators like histamine, kinin and bradykinin, and the last phase is attributed to the synthesis of prostaglandins [25, 26]. Mostly, the anti-inflammatory drugs are found to be effective during late phase, as in this study indomethacin showed the inhibition of rat paw edema in third and fourth hour. Like indomethacin, HPE 400mg/kg b.w. p.o. dose also showed a significant ($p<0.01$) inhibition of rat paw edema during third and fourth hour. As shown in Table 2, the ethanolic extract of seeds of *H. pubescens* (HPE) exhibited anti-inflammatory potential in dose dependent manner in this present study. Though, HPE at 100 and 200 mg/kg oral doses showed inhibition of rat paw edema at 4h, HPE 400 mg/kg oral dose showed most potent anti-inflammatory effect (74.07%) at the same time (4h). This response may be due to the inhibition of biosynthesis of prostaglandins [27].

The cotton pellet granuloma method is a widely used test model for assessing transudative, exudative and proliferative components of chronic inflammation and to evaluate anti-inflammatory substances [28, 29]. The cotton pellet induction in rat develops granulaoma as a sign of chronic inflammation by accumulation of macrophages and lymphocytes around the foreign particles together with epitheloid and giant cells which are derived from macrophages [30]. The inhibition of granulomatous tissue formation in rat indicates the anti-proliferative effect of drugs, and which is measured by percentage inhibition of cotton pellet weight. The wet weight of the pellet is influenced by absorption of fluid while the dry weight correlates with the amount of granulomatous tissue formed [31]. In the present study, HPE showed the inhibition of cotton pellet weight in dose dependent manner. The anti-inflammatory effect showed by test drug was comparable to the effect of standard drug, indomethacin. The ethanolic extract of seeds of *H. pubescens* at 400mg/kg b.w., p.o. dose exhibited significant ($p<0.01$) 58.06% and 62.63% inhibition of moist and dry cotton pellet weight respectively, and the effect was found to be most potent among all the doses of test drug. Hence, the findings suggested that the ethanolic extract of seeds of *H. pubescens* significantly ($p<0.05$ and $p<0.01$) inhibited the proliferative phase of inflammation.

Freund's Complete adjuvant-induced arthritis have been used as a model of sub-chronic or chronic inflammation in rats, as well as for the evaluation of anti-inflammatory effects of drugs [32]. The wide utilization of this model may be due to the reason that anti-inflammatory activity of therapeutic agents which has been detected in this model can be useful in the treatment of rheumatoid arthritis in human [33]. The arthritis was induced in this study by a sub-cutaneous injection of Freund's Complete adjuvant (FCA), i.e. heat killed *Mycobacterium tuberculosis* in paraffin oil and mannide monooleate, in the rat paw's plantar surface. The arthritis was developed in the joints of hind limbs of rats by reduction of motor activity and increased paw diameter was observed due to inflammation and edema [34, 35]. The initial inflammatory response was observed within hours, but more critical signs of inflammation were developed with days and observed till the 14th day of the study. Indomethacin, the standard drug showed anti-arthritic activity by reducing FCA induced rat paw edema from the beginning of the treatment but the significant ($p<0.01$) response was observed during 7th and 14th day (57.14 and 86.55%, respectively) of the study. HPE showed the efficacy during the study in dose dependent manner and HPE 400mg/kg dose showed the maximum reduction of rat paw edema among the other doses which was also comparable to the effect of standard drug. The inhibition of rat paw edema was indicated the therapeutic efficacy of the test drug in chronic inflammatory state and the effect was observed may be due to the inhibition of prostaglandin biosynthesis [36].

Furthermore, the preliminary phytochemical test of ethanolic extract of seeds of *H. pubescens* revealed the presence of many active constituents such as, alkaloids, carbohydrate, glycosides, triterpenoids and steroids (Table 1). As per the previous reports, the presence of said constituents may be responsible for the anti-inflammatory efficacy of the test drug [37-41].

CONCLUSION

In conclusion, the results of this study suggest that the ethanolic extract of seeds of *H. pubescens* possess potent anti-inflammatory efficacy in both acute and chronic inflammatory models. Hence, the study confirms the traditional use of the plant in inflammatory conditions. Further detail phytochemical analysis and mechanism study of anti-inflammatory activity of seeds of *Holarrhena pubescens* may help to develop new class of potent anti-inflammatory agent with low toxicity and better therapeutic index.

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