

ANTI-ARTHRITIC ACTIVITY OF HYDROALCOHOLIC EXTRACT OF *DACTYLORHIZA HATAGIREA***Mrinal Aryan^{*1}, C.K. Tyagi¹, Hemant Kumar Sharma¹, Prabhakar Budholiya¹, Geeta Parkhe²**¹College of Pharmacy, Sri Satya Sai University of Technology and Medical Sciences, Sehore (M. P.)²Scan Research Laboratories, Bhopal (M.P.)*Corresponding Author's E mail: mrinalaryan6@gmail.com

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ABSTRACT

Dactylorhiza hatagirea (D. Don) Soo (*D. hatagirea*) is a perennial orchid, native to the Himalayan region. This species is distributed in India, Pakistan, Afghanistan, Nepal, Tibet and Bhutan. In India, it is distributed in Jammu and Kashmir, Uttarakhand and Himachal Pradesh. Tubers of this species are supposed as a potent medicine in Leucorrhea in Traditional System of Medicine (TSM) rich source of mucilage and are used to make Salep as food and medicine, useful as expectorant and astringent. The present study was designed to investigate anti-arthritis activity of hydroalcoholic roots extract of *D. hatagirea* in Freund's complete adjuvant (FCA)-induced arthritis in rats. Qualitative analysis of various phytochemical constituents and quantitative analysis of total flavonoids were determined by the well-known test protocol available in the literature. Quantitative analysis of flavonoids was carried out by aluminium chloride method. *D. hatagirea* was evaluated for anti-arthritis action by Freund's adjuvant induced arthritis test in adult Albino rats (180-250gm). Rats were injected 0.1 ml of complete Freund's adjuvant into the planter region of the left hind paw. Diclofenac sodium used as standard drug. Statistical analysis was performed using One way analysis of variance (ANOVA) followed by Dunnet's test. $P < 0.05$ was considered statistically significant. The hydroalcoholic extract of *D. hatagirea* in doses of 400 and 800 mg/kg showed 47.53% and 69.64% protection against increase in paw volume, respectively. *D. hatagirea* showed dose-dependent action in all the experimental models. The present study indicates that *D. hatagirea* has significant anti-arthritis properties.

Keywords: Arthritis, *Dactylorhiza hatagirea*, Diclofenac sodium, Freund's complete adjuvant, Paw volume

INTRODUCTION:

Rheumatoid arthritis (RA) is a systemic autoimmune disease of unknown etiology, affecting 1-1.5% of the population worldwide ¹. The disease is characterized by articular inflammation and by the formation of an inflammatory and invasive tissue, rheumatoid pannus that eventually leads to the destruction of joints. Anti-inflammatory drugs and analgesics including steroids are used to suppress the symptoms, while disease-modifying antirheumatic drugs (DMARDs), newer therapies such as anti-CD20 therapy (rituximab) and abatacept, anti-tumor necrosis factor (TNF)- α therapy (etanercept, infliximab and adalimumab) are often required to inhibit or halt the underlying immune process.

However, all of these agents are associated with numerous side effects. In recent days, researchers are directed toward traditional system of medicine for the discovery of drugs that are long acting anti-inflammatory with minimum side effects. Although there is no ideal animal model for RA at this time, rat adjuvant arthritis shares many features of

human RA and the sensitivity of this model to anti-arthritic agents support the view that the adjuvant arthritis is the best available model of RA². Herbal medicines are being accepted and used increasingly by general populations in both eastern and western countries because of the ethnic acceptability and compatibility having fewer side effects³. *Dactylorhizahatagirea* (*D. hatagirea*, Family: Orchidaceae) is a terrestrial ground dwelling perennial herb orchid, the stem is erect, hollow and obtuse and bears palmately lobed and lanceolate leaves with sheathing leaf base. The cylindrical and terminal spike bears rosy purple flowers with green bracts. Flowers are 1.7-1.9 cm long with curved spur. The inflorescence consists of a compact raceme with 25-50 flowers developed from axillary buds. The dark purple spotted lip of the flower is rounded and lobed (1-5)⁴. The plant is native and near endemic to Indian Himalayan region. Its distribution extends to Pakistan, China, Afghanistan, Nepal, Tibet and Bhutan. In India, it is reported from Jammu and Kashmir, Sikkim, Arunachal Pradesh, Uttarakhand and Himachal Pradesh. Generally, it is widely and narrowly distributed at an altitudinal ranges between 2500 m and 5000 m amsl in open grassy slopes and alpine meadows⁵. It is commonly known as HathpanjaorHatajari in Uttarakhand; Salam panja in Kashmir and WanglakorAngmo-lakpa in various parts of Ladakh. It is widely used to cure various diseases like dysentery, diarrhea, chronic fever, cough, stomach ache, wounds, cuts, burns, fractures and general weakness. The tuber of this species yield a high quality 'Salep' which is used as a tonic to increase immunity (immunomodulator), vajikaran drug (aphrodisiac), increases the sexual performance as well as nervine tonic for its astringent⁶. Due to over exploitation of rhizomes, harvest for medicine and habitat fragmentation or reduction, It has been identified as a critically endangered by Conservation Assessment and Management Plan(C.A.M.P.)⁷ and is listed under Appendix II of Convention of International Trade in Endangered Species (CITES)⁸. The pharmacological interest of this species is mainly due to the dactylorhin (glucoside) content in the tuber which acts as a neuroprotective agents against dementia, Alzheimer's disease, depression, anxiety and irritable bowel syndrome and significantly it improves the memory in case of treating with inhibitor such as scopolamine, cycloheximide or alcohol⁶. The economic potential of the species could be assessed on the basis of its high demands at national and international market value i.e. Rs. 2700–3200 kg–1(dry tubers) and annual consumption of 'Salep' obtained from the species in India is about 7.38 tons (valued at about Rs. 50 lakhs)⁸. Despite the economic importance and the level of endangerment of *D. hatagirea*, we are not aware of any studies about population structure and

conservation managements are still unknown. Hence, the aim of this study is to prove the therapeutic potential of the plant *D. hatagirea* as an anti-arthritic agent against Freund's complete adjuvant (FCA) induced arthritis.

Materials and Methods

Collection of plant material

Fresh root of plant *D. hatagirea* were collected from local market of Bhopal (M.P.). The plant material were washed thoroughly with normal tap water followed by sterile distilled water. Then plant material was dried under shaded condition at room temperature. Fresh plant materials of *D. hatagirea* were crushed to powder using grinding machine. Powder was stored at 4°C in tight air container bottle.

Drugs and Chemicals

Complete Freund's adjuvant (CFA) was procured from Sigma Aldrich chemicals Pvt. Ltd, Hyderabad, India. Diclofenac Na was obtained from Akums Drugs and Pharmaceuticals, India. Petroleum ether, Chloroform obtained from SD fine chemicals Pvt. Ltd, Mumbai, India and all other chemicals used in this study were obtained commercially and were of analytical grade.

Preparation of extracts

42 gm powdered sample was weighed and taken separately. The powder was moistening with ammonia and evaporated to dryness^{9, 10}. The maceration method was followed for the extraction. 42 gm powder of dried plant material was added into 100 ml of 70 % methanol in an Erlenmeyer flask (250 ml capacity) and resulting mixture was vortexed well. The maceration process was carried out in shaker incubator at with 50 rpm for 48-72 hrs. After this process, the extracts were filtered dried extract was stored in refrigerator for their future use in phytochemical analysis.

Phytochemical screening of the extract

The extract of *D. hatagirea* was subjected to qualitative analysis for the various phytoconstituents like alkaloids, carbohydrates, glycosides, phytosterols, saponins, tannins, proteins, amino acids and flavonoids^{11, 12}.

Total flavonoids determination

The total flavonoid content was determined using the method of Olufunmisoet al¹³. 1 ml of 2% AlCl₃ methanolic solution was added to 3 ml of extract or standard and allowed to stand for 15 min at room temperature; the absorbance of the reaction mixture was measured at 420 nm using UV/visible spectrophotometer. The content of flavonoids was calculated using standard graph of quercetin and the results were expressed as quercetin equivalent (mg/100mg).

Animals

All the experiments were carried out using Swiss albino male rats weighing between 180-250 gm. All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Ethical Committee No.COPSSUTMS/ANIL/19-07. All animals were housed in polypropylene cages and maintained under standard laboratory conditions. Animals were housed at a temperature of $24\pm 2^{\circ}\text{C}$ and relative humidity of 65-75% RH. They were fed with a standard diet and water was given *ad libitum* and they were left for a week for acclimatization to animal house conditions. All experiments were conducted after overnight fasting but there was free access to water.

Acute oral toxicity

Acute oral toxicity was performed according to Organization for Economic Co-operation and Development (OECD) guideline No. 420¹⁴. Swiss albino rat's fasted overnight, accessing water *ad libitum* were used in this study. The extract was administered orally at a dose of 2000 mg/kg body weight and the animals were observed for mortality or any abnormal behavior for first 24 h, then for next 14 days. Further behavioral responses, neurological responses as well as autonomic responses were observed.

Freund's adjuvant induced arthritis

The male albino rats were divided into five groups, *i.e.* control, standard, drug treated (two groups of hydro-alcoholic extract of *D. hatagirea* roots (low and high dose).

Experimental protocol¹⁵

- **Group I (Normal control group):** Rats received 1% CMC (1 ml/kg body weight) only daily for 14 days
- **Group II (Negative control group):** Received 1% CMC (1 ml/kg body weight) +FCA (0.1 ml) daily for 14 days
- **Group III (Standard group):** Diclofenac sodium 15 mg/kg suspended in CMC+FCA (0.1 ml) daily for 14 days
- **Group IV (Treatment control group):** hydro-alcoholic extract of *D. hatagirea* roots at dose of 400 mg/kg orally suspended in CMC +FCA (0.1 ml) daily for 14 days
- **Group V (Treatment control group):** hydro-alcoholic extract of *D. hatagirea* roots at dose of 800 mg/kg orally suspended in CMC +FCA (0.1 ml) daily for 14 days

Arthritis was induced by injecting 0.1 ml of Freund's complete adjuvant (FCA) into the planter region of the left hind paw. The paw volumes of both the hind paws were measured using a plethysmometer

and body weight was recorded on the day of adjuvant injection. The hydro-alcoholic extract of *D. hatagirea* roots (400 and 800 mg/kg) and diclofenac sodium (15 mg/kg) doses were administered orally for 14 days from the day of Freund's adjuvant injection. The changes in the paw volume were measured on various days up to 21 days following FCA injection. The change in the inflammatory reaction was measured by using mercury plethysmometer on 1st, 7th, 14th and 21st day from the day of adjuvant injection. The animals were weighed, using digital weighing balance on 1st, 7th, 14th and 21st day from the day of adjuvant injection.

Percentage inhibition of edema was calculated

$$\text{Percentage inhibition} = \frac{V_c - V_t}{V_c} \times 100$$

Where, V_c = Joint edema of negative control group; V_t = Joint edema of treatment group;

Data Analysis

The data is expressed as mean ± Standard Deviation (SD). Results were analyzed using one-way ANOVA followed by Dunnet's test. Differences were considered as statistically significant at P < 0.05, when compared with control.

Result

The percentage yields of hydroalcoholic extract obtained from *D. hatagirea* are depicted in the table 1. Preliminary phytochemical studies of the extract were done according to the published standard methods. These tests were broad in scope and used to determine the presence of flavonoids and saponins but all constitute was absents in the extract Table 2.

Table 1 % Yield of hydroalcoholic extract

S. No.	Plant	% Yield (w/w)
1	<i>Dactylorhizahatagirea</i>	7.75

Table 2 Results of Phytochemical screening of *Dactylorhizahatagirea*

S. No.	Phytochemicals	Tests	Observation	Inference
1.	Alkaloids	Iodine Test	No Blue colour	-
		Wagner's Test	No reddish brown precipitate	-
		Dragendorff's Tests	No orange brown precipitate	-
2.	Flavonoids	NaOH Tests	Colourless	+
		Shinoda Tests	Colourless	-
3.	Glycosides	Keller-Kiliani Test	No Bluish green colour	-
4.	Phenols	Phenol Tests	No Blue colour	-
5.	Saponins	Foam Test	Layer of foam	+
6.	Tannins	Gelatin Test	No white precipitate	-
7.	Carbohydrates	Molisch's test	No violet colour	-
		Fehling's test	Reddish orange precipitate	-
8.	Proteins	Millon's test	No white precipitate	-

The content of total flavonoid compounds (TFC) was expressed as mg/100mg of quercetin equivalent of dry extract sample using the equation obtained from the calibration curve: $Y = 0.06X + 0.019$, $R^2 = 0.999$, where X is the quercetin equivalent (QE) and Y is the absorbance. The results are given in table 3 and fig 1.

Table 3 Estimation of Total flavonoid content

S. No.	Solvents → Bioactive compound ↓	Hydroalcoholic extract <i>Dactylorhizahatagirea</i>
1.	Total flavonoid (Quercetin equivalent (QE) mg/100mg)	1.256

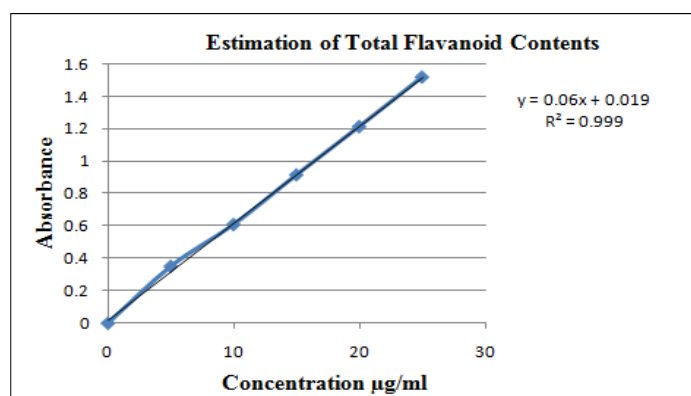


Figure 1 Graph of estimation of total flavonoid content

No adverse changes and mortality were observed in animals, which orally received hydroalcoholic extract (2000 mg/kg) of *D. hatagirea* roots. This indicates that 2000 mg/kg is maximum safe dose. So 400 and 800 mg/kg of body weight, of the maximum safe dose were selected for studying *in vivo* anti-arthritic activity. There is a significant increase in rat paw volume in FCA injected control rats when compared to the standard and drug treated rats. Hydro-alcoholic extract treatment at the dose of 400 mg/kg and 800 mg/kg showed significant reduction in rat paw edema volume when compared with the control group. Table 4 shows the effect of extract on Freund's adjuvant model induced arthritis. After 21 days it was found that hydro-alcoholic extract of *D. hatagirea* roots significantly shows dose dependant inhibition in paw thickness i.e. the chronic inflammation induced by adjuvant shows decrease in paw thickness. Standard diclofenac sodium significantly decreased the paw thickness 6.75 ± 0.04 mm i.e. 70.64% inhibition after induction of Freund's adjuvant; whereas the extract at high dose (800 mg/kg) significantly decreased the paw thickness. It was found that in case of the high dose of the hydro-alcoholic extract, the percent protection against increase in paw volume was found to be 69.64% as compared to that of the low dose which was found to be 47.53%.

Table 4 Effect of hydroalcoholic extract of *Dactylorhizahatagirea* roots on FCA induced arthritis in rats

Group	Dose of extract (mg/kg, p.o.)	Change in paw thickness (mm)±SD (% inhibition)			
		1 Day	7 Day	14 Day	21 Day
Group I (Normal control)	1% CMC (1 ml/kg body weight)	$4.07 \pm 0.16^{**}$ *	$4.07 \pm 0.16^{***}$	$4.07 \pm 0.09^{***}$	$4.07 \pm 0.08^{***}$
Group II (Negative control)	1% CMC (1 ml/kg body weight) +FCA (0.1 ml)	5.71 ± 0.04	13.64 ± 0.03	18.20 ± 0.02	23.01 ± 0.04
Group III (Standard)	Diclofenac sodium (15	$4.49 \pm 0.03^{**}$ (21.23)	$7.96 \pm 0.03^{***}$ (41.64)	$7.50 \pm 0.03^{***}$ (58.78)	$6.75 \pm 0.04^{***}$ (70.64)

control)	mg/kg) +FCA (0.1 ml)				
Group IV (Treatment Control)	Hydro-alcoholic (400 mg/kg) +FCA (0.1 ml)	4.93 ± 0.09* (13.62)	9.71 ± 0.04*** (28.75)	11.60 ± 0.07** * (36.24)	12.07 ± 0.04*** (47.53)
Group V (Treatment Control)	hydro-alcoholic (800 mg/kg) +FCA (0.1 ml)	4.55 ± 0.04** (20.23)	8.08 ± 0.03*** (40.74)	7.44 ± 0.09*** (59.21)	6.98 ± 0.05*** (69.64)

Each values represents the mean±SEM; (n=6), *p<0.05, **p<0.01, ***p< 0.001 respectively when compared with toxicant control group (one-way ANOVA followed by Dunnett's test). Values in parentheses indicate percent inhibition activity (H), calculated as 100 x (value of negative control – value of treatment) / value of negative control

DISCUSSION

Rheumatoid arthritis is an autoimmune disorder, the immunologically mediated complete Freund's adjuvant induced arthritic model of chronic inflammation is considered as the best available experimental model of rheumatoid arthritis. Complete Freund's adjuvant-induced arthritis is a model of chronic polyarthritis with features that resemble rheumatoid arthritis¹⁶.

The determination of paw swelling is apparently simple, sensitive and quick procedure for evaluating the degree of inflammation and assessing of therapeutic effects of drugs¹⁷. In adjuvant-induced arthritis model rats developed a chronic swelling in multiple joints with influence of inflammatory cells, erosion of joint cartilage and bone destruction and remodeling which have close similarities to human rheumatoid disease. These inflammatory changes ultimately result in the complete destruction of joint integrity and functions in the affected animal. Also, the CFA administered rats showed soft tissue swelling around the ankle joints during the development of arthritis, which was considered as edema of the particular tissues¹⁸.

In our study the hydroalcoholic extract of *D.hatagirea* exhibited a significant anti-arthritic activity in a dose dependent manner. In the present study, we showed that hydroalcoholic extract of *D.hatagirea* could significantly inhibit the progression of the rheumatoid arthritis in treated animals. However, standard drug and hydroalcoholic extract significantly suppressed the swelling of the paws in both acute and chronic phase which may be due to the suppression of inflammatory mediator released due to induction of Freund's adjuvant. Though the actual mechanism of suppressing inflammation is not known but it can be correlated with the presence of flavonoids in suppressing the inflammation and anti-oxidant activity. Numerous studies have suggested a role of oxidative stress in the pathogenesis of rheumatoid arthritis¹⁹. Therefore; it was assumed that the reported and well established antioxidant properties of *D.hatagirea* and its ability to block the COX-2 pathway during the progression of inflammation justify the usage of the plant extract in the treatment of rheumatoid arthritis. There was a

significant reduction in the paw volume in Freund's complete adjuvant induced arthritic rats. The cardinal signs of the chronic inflammatory reactions like redness, swelling, arthralgia and immobility of affected joints were significantly less in the drug treated animal than those of the control. The pathogenesis or reasons for development of arthritis following injection of FCA are not fully understood.

CONCLUSIONS

From the results obtained in the present study, it may be concluded that *D.hatagirea* possesses significant anti-arthritic activity. Further, research may be planned as an extension of this work which could prove *D.hatagirea* as a potent antiarthritic agent.

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