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Dr. Kiran R Giri
Department of Pharmacology, Indian Institute of Medical, Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India.

Correspondence
Dr. Kiran R Giri
Department of Pharmacology, Indian Institute of Medical, Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India.

Comparative study of anti-inflammatory activity of *Withania somnifera* (Ashwagandha) with hydrocortisone in experimental animals (Albino rats)

Dr. Kiran R Giri

Abstract
Title: Comparative Study of Anti-inflammatory activity of *Withania somnifera* (Ashwagandha) with Hydrocortisone in Experimental Animals (Albino Rats)
Objective: To compare the acute and chronic anti-inflammatory effect of *Withania somnifera* with Corticosteroid (Hydrocortisone).
Method: After approval from Institutional Animal Ethics Committee, albino rats of either sex (Wt. 150-250 gms) were divided into 4 groups of 6 animals in each. Acute inflammation was produced by sub-plantar injection of 0.1 ml of 1% freshly prepared Carrageenan in normal saline in right hind paw of rats. Adjuvant arthritis was induced by subcutaneous injection of complete Freund's adjuvant into the subplantar tissue of the right hind paw of each rats. Control groups were treated with normal saline, Test groups with Ethanolic root extract of WS (12mg/kg & 25 mg/kg p.o.), Standard group with hydrocortisone. Mean paw edema increase in mm (Mean±SE) and % inhibition of paw swelling was evaluated and analyzed statistically.
Result: Maximum percentage inhibition of edema exhibited with 12 mg/kg & 25 mg/kg of ethanolic extract of *Withania Somnifera* at 3 hrs were 36.36 % and 61.36 % respectively as compare to standard drug Hydrocortisone (40 mg/kg s.c.) 65.91%. In Freund,s adjuvant Arthritis model, on 3rd day Hydrocortisone percentage inhibition of paw edema is 31.58% as compare to *Withania somnifera* i.e. 21.83%.
Conclusion: Ethanolic extract of *Withania somnifera* elicited significant dose dependant acute and chronic anti-inflammatory activity in carrageenan comparable to hydrocortisone.

Keywords: *Withania somnifera*, Carrageenan, Freund's, Anti-inflammatory action.

Introduction
Ethnobotanicals are important for pharmacological research and drug development, not only when plant constituents are used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds [1].
It is not surprising that from conception to market most compounds face an uphill battle to become an approved drug. For approximately every 5,000 to 10,000 compounds that enter preclinical testing, only one is approved for marketing. Drug research and development is comprehensive, expensive, time-consuming and full of risk [2].
On the contrary many medicines of plant origin had been used since ages without any adverse effects. It is therefore essential that efforts should be made to introduce new medicinal plants to develop more effective and cheaper drugs. Plants represent a large natural source of useful compounds that might serve as lead for the development of novel drugs [2].
As we know that anti-inflammatory drugs are useful for many long standing diseases such as Rheumatoid arthritis, osteoarthritis, hence there is need of a drug with minimum side effects and can be useful for acute and chronic diseases.
Withania somnifera, also known as ashwagandha, Indian ginseng and winter cherry, has been an important herb in the Ayurvedic and indigenous medical systems for over 3000 years. Historically, the plant has been used as an aphrodisiac, liver tonic, anti-inflammatory agent, astringent and more recently to treat bronchitis, asthma, ulcers, emaciation, insomnia, and senile dementia [3].
Thirunavukkarasu *et al*. 2006 found the energy boosting properties of a formulation comprising of withania in I-R compromised heart and recommended its use as a dietary supplement for cardioprotection. The formulation favourably altered the myocardial energy

substrate, improved cardiac function and reduced infarct size [4]. In another study, Marutham, a polyherbal formulation containing W. somnifera has been found cardioprotective and antioxidant in isoproterenol-induced ischemic rats [5].

Withania has profound hypocholesteremic, hypolipidemic, Mary *et al*. 2003 however, the demonstrated the antiatherogenic activity of formulation containing WS [6].

Though claims have been made since long to our knowledge but very few scientific studies have been conducted so far to confer or refute long standing claims regarding anti-inflammatory property of *Withania somnifera*. The roots of *Withania somnifera* consist primarily of compounds known as withanolides, which are believed to account for its extraordinary medicinal properties. Laboratory analysis has revealed over 35 chemical constituents contained in the roots of *Withania somnifera* [7].

Ethanolic root extract was used for comparing its anti-inflammatory property with hydrocortisone.

Antioxidant and antistress properties of *Withania somnifera* are well studied as compared to anti-inflammatory property [2]. This fact inspired us to evaluate about anti-inflammatory property of *Withania somnifera*.

2. Materials and Methods

Approval from Institutional Animal Ethical Committee

The research protocol was approved by the Institutional Animal Ethical Committee, Jawaharlal Nehru Medical College, Sawangi (Meghe), Wardha.

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Animals

The study was conducted using 60 Wistar Albino Rats, of either sex weighing 150-200g from institutional animal house, Sawangi (meghe), Wardha.

Materials

Collection of plant material– The plant material (Roots of *Withania Somnifera*) purchased from local herbarium "Shri Shail Herbarium, Nagpur".

Purchase of chemicals – Drugs Hydrocortisone was obtained from institutional medical store; Carrageenan from Himedia chemicals Mumbai; Complete Freund's adjuvant from MM supplier Pune.

Instruments

Digital Venire caliper
Feeding syringe & needle – For oral administration
Dispo van single use needle 26 no
Weighing machine
Soxelet apparatus

Methods

Preparation of plant material in powdered form-

Roots were already in dried form. Dried *Withania somnifera* was coarsely powered.

Preparation of extract–

Ethanolic extract of *Withania somnifera*: The powdered plant material of *Withania somnifera* roots was extracted with ethanol in a Soxhlet apparatus for 48 hrs. The extracts were filtered through Whatman filter paper (No.1) and concentrated by vacuum evaporation. The yield of extract as per solvent used was 3.25% w/w. The dried extracts were suspended in 2% gum acacia and used for experiments.

Phytochemical screening

Ashwagandha contains withanolides as its major active ingredients.

Extracts were tested for preliminary phytochemical studies using standard procedure.

Determination of doses [24]

Ethanolic root extract of *Withania somnifera*: 12 mg/kg p.o.
Ethanolic root extract of *Withania somnifera*: 25 mg/kg p.o
Hydrocortisone: 40mg/kg s.c.

Preparation of working solution

The required amount of powered extract of *Withania somnifera* measured as per the dose/bodyweight (12mg/kg & 25 mg/kg) of rats daily & fresh solution used to make with honey & water (2ml).

Grouping of animals - Animals were divided into 4 groups of six animals each.

Group 1: Treated with Normal Saline (Control).
Group 2: Treated with ethanolic roots extract of *Withania somnifera* (12mg/kg p.o)
Group 3: Treated with ethanolic roots extract of *Withania somnifera* (25 mg/kg p.o)
Group 4: Treated with Hydrocortisone (40mg/kg s.c.)

Anti-inflammatory activity

Carrageenan-induced paw edema

Acute inflammation was produced by sub-plantar injection of 0.1 ml of 1% freshly prepared Carrageenan in normal saline in right hind paw of rats. Control groups were treated with normal saline, Test groups with Ethanolic root extract of *Withania somnifera*,12mg/kg p.o. & 25 mg/kg p.o and Standard groups with Hydrocortisone (40mg/kg s.c) one hour before Carrageenan injection. The paw volume was measured at an interval of 1, 2, 3, 4, 6 hrs after Carrageenan injection by using vernier caliper [8].

The difference in paw thickness after and before induction of inflammation was calculated and presented as mean increase in paw thickness (mm). The ability of anti-inflammatory drug to suppress paw inflammation was expressed as a percentage of inhibition of paw edema [8] and this percentage can be calculated according to the following equation:

Percentage of inhibition (%) = $100 \times (1 - X / Y)$

Where X= mean increase in paw volume, thickness of treated rats

Y= mean increase in paw volume, thickness or weight of control rats. [8, 9]

Freund's Adjuvant Induced Arthritis [10, 11, 14]

Rats were divided into four groups of 6 animals each. Adjuvant arthritis was induced by subcutaneous injection of complete Freund's adjuvant into the subplantar tissue of the right hind paw of each rats.

Rats are divided into 4 groups

Group 1- Normal Saline. (Non Inflamed Control)
Group 2- Complete Freund's adjuvant (0.1ml injected in subplanter) region of Right hind paw. (Inflamed Control).
Group 3- Test drug ethanolic extract of *Withania Somnifera* (25mg/kg p.o.)
Group 4- Standard drug Hydrocortisone (40mg/kg s.c)

The groups consisted of complete Freund's adjuvant (CFA) - 0.1ml injected rats challenged with doses of the test drug and standard drug administered orally 2h before induction of arthritis. The drug administrations were continued daily at the same time of the day for 12 more days.

Development of adjuvant induced swelling in the paws of both the injected and non-injected paws of each rat were monitored daily as the percentage increase in paw edema [10]. The

percentage inhibition of paw edema compared with that of the inflamed control was taken as anti-arthritic activity. Paw edema of both hind limbs and body weights were recorded daily from the day of injection. [11, 12, 13]

Purposely, from day 13 to 21, the animals are not dosed with the test compound or the standard. As daily, on day 21st, the body weight is determined again and the severity of the secondary lesions is evaluated visually and graded according the following scheme [10, 11, 14].

Site	Degree of inflammation	Score
Ear	absence of nodules and redness	0
	presence of nodules and redness	1
Nose	no swelling of connective tissue	0
	intense swelling of connective tissue	1
Tail	absence of nodules	0
	presence of nodules	1
Fore paws	absence of inflammation	0
	inflammation of at least 1 joint	1
Hind Paws	absence of inflammation	0
	Slight inflammation	1
	Moderate inflammation	2
	Marked inflammation	3

Statistics

The data was subjected to statistical evaluation by applying the tests of significance as under Paired t-test one way ANOVA Dunnett's multiple comparison tests

3. Result

Acute study

Paw edema

Maximum percentage inhibition of edema exhibited with 12 mg/kg & 25 mg/kg of ethanolic extract of *Withania Somnifera* at 3 hrs were 36.36 % and 61.36 % respectively as compare to standard drug Hydrocotisone (40 mg/kg s.c.) 65.91%

Single dose of *Withania somnifera* has good duration of action as it could effectively suppress the inflammation after 6 hr of its administration.

As carrageenan produces biphasic response, test drug *Withania somnifera* has significant inhibition in first and second phase response.

Table 1: Effect of ethanolic root extract of *Withania somnifera*, Hydrocortisone on Carrageenan induced paw oedema in rats

Group	Treatment Dose(mg/kg, p.o)	*1hr*	*2hr*	*3hr*	*4hr*	*6hr*	*12hr*
1	Control	0.51±0.02	0.42±0.007	0.44±0.02	0.34±0.004	0.29±0.004	0.26±0.01
2	WS (25 mg/kg p.o.)	0.36±0.006* (29.41%)	0.18±0.007* (57.14%)	0.17±0.01* (61.36%)	0.14±0.01* (58.82%)	0.13±0.007* (55.17%)	0.15±0.06* (42.31%)
3	WS (12 mg/kg p.o.)	0.39±0.006* (23.53%)	0.28±0.004* (33.33%)	0.28±0.04* (36.36%)	0.22±0.004* (35.29%)	0.19±0.005* (34.48%)	0.17±0.005* (34.62%)
4	Hydrocortisone (40mg/kg s.c.)	0.27±0.005* (47.06%)	0.15±0.008* (64.29%)	0.15±0.01* (65.91%)	0.12±0.009* (64.71%)	0.12±0.004* (58.62%)	0.13±0.007* (50.00%)

n = 6. Results are expressed as mean ± SEM. Percentage inhibition is in brackets. The statistical analysis was carried out using one way ANOVA, Dunnet test. *P<0.05

Chronic study result

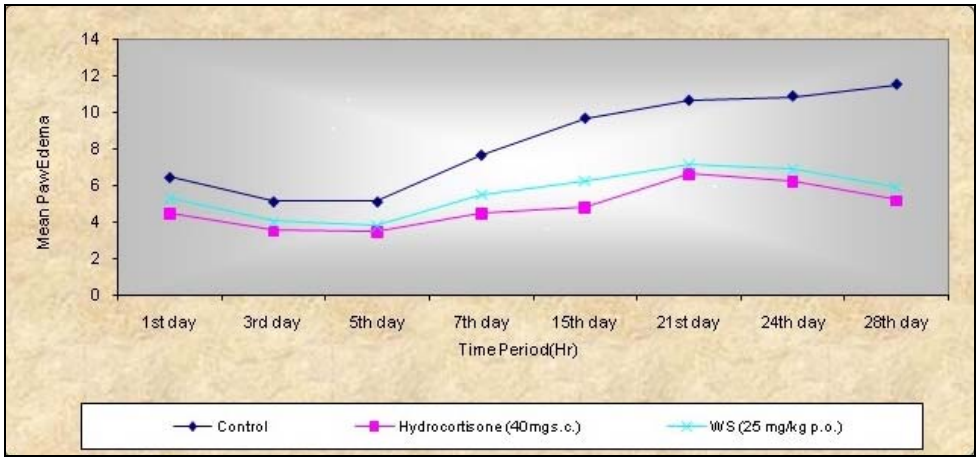
Paw edema

The maximum effect was observed on 28th day and the effect is very similar to standard drug Hydrocortisone.

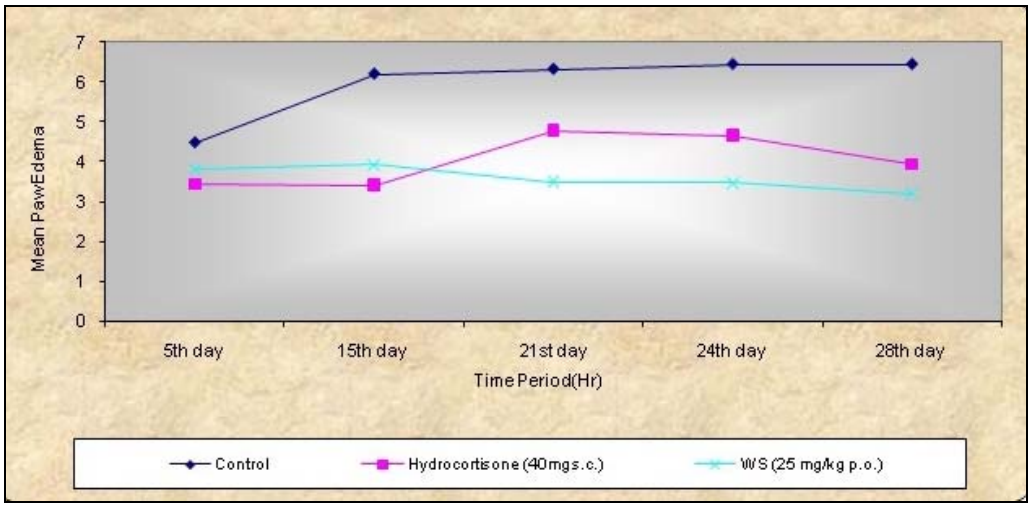
In all the groups the inhibition started decreasing after day 15th continue till day 21st, again reached the maximum on day 28th.

In Non-injected group paw edema and inflammation appeared on 5th day indicating development of secondary lesion, significant % inhibition is seen on day 15th i.e. 36.39% by *Withania somnifera* as compare to 44.77% by Hydrocortisone.

The cordial signs of the chronic inflammation like redness, swelling, arthralgia and immobility of affected joints were significantly less on the drug treated animal than that of the control.



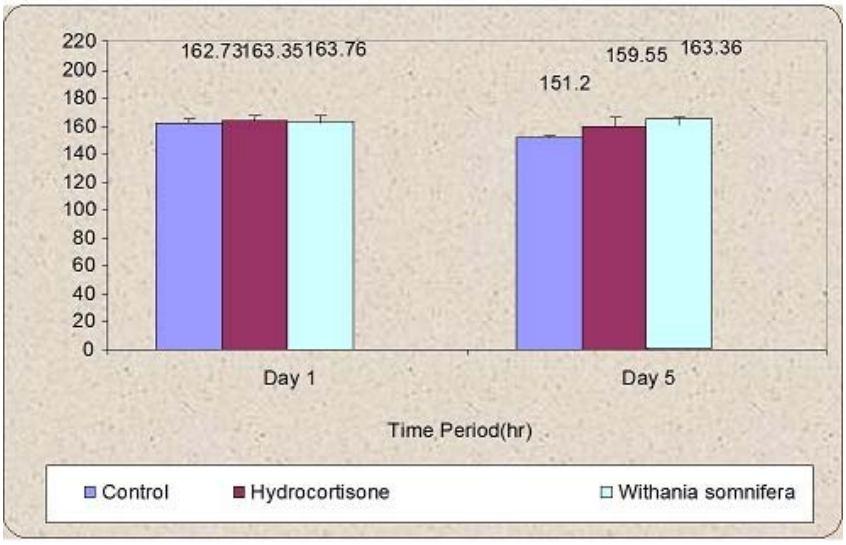
Graph 2: Effect of ethanolic root extract *of Withania somnifera*, Hydrocortisone against Complete Freund's Adjuvant Induced arthritis in rats. (Right injected paw)



Graph 3: Effect of ethanolic roots extract of *Withania somnifera*, Hydrocortisone against Complete Freund's Adjuvant Induced arthritis in rats. (Left Non Injected Paw)

Body weight changes

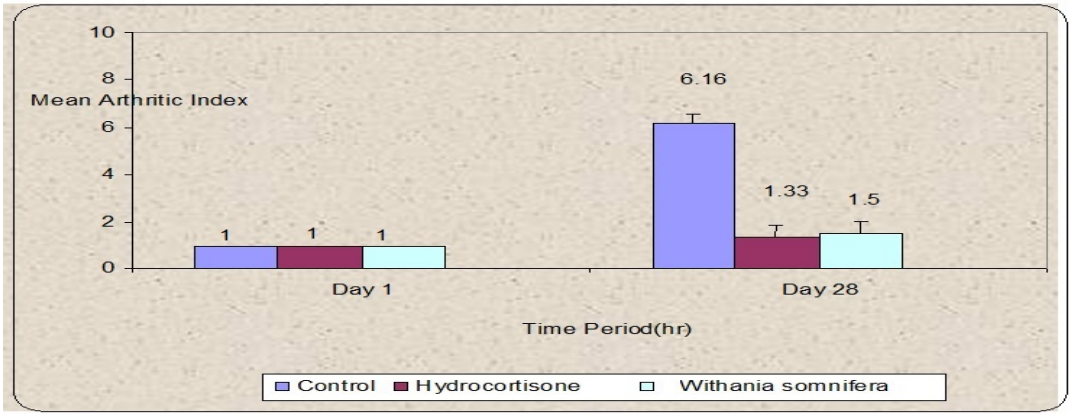
Withania somnifera inhibit the loss of body weight on adjuvant induced arthritis animals as compare to control group.



Graph 4: Changes in body weight in adjuvant induced arthritis in rats

Arthritic Index

Comparing Day 1 and Day 28, significant increase in arthritic index in C.F.A group while non-significant increase in test and standard drug group indicating that *Withania somnifera* and hydrocortisone effectively decrease redness, swelling in both injected and non-injected paws.



Graph 6: Changes in Arthritic Index after treatment with ethanolic root extract of *Withania somnifera*, Hydrocortisone in rats

4. Discussion

A study by al-Hindawi used 10mg/kg methanolic root extract to study the anti-inflammatory property [15]. A study on anti-inflammatory and antiarthritic activity of Withaferin A by P.D. Sethi used 25 mg/kg and 12mg/kg root extract [16].

This indicates that active principle must be fat soluble, hence in present study we used ethanolic roots extract of *Withania somnifera* (25mg/kg p.o).

Carrageenan-induced hind paw edema is the standard experimental model of acute inflammation [8, 9].

The time course of edema development in carrageenan-induced paw edema model in rats is generally represented by a biphasic curve. The first phase of inflammation occurs within an hour of carrageenan injection and is partly attributed to trauma of injection and also to histamine, and serotonin components. The second phase is associated with the production of bradykinin, protease, prostaglandin, and lysosome. Prostaglandins (PGs) play a major role in the development of the second phase of inflammatory reaction which is measured at 3h. [17] Hence we had also used carrageenan induced paw edema model for evaluation of acute anti-inflammatory property.

In present study, we used Hydrocortisone as a standard drug for comparison, because the roots of *Withania somnifera* consist primarily of compounds known as withanolides, which are believed to account for its extraordinary medicinal properties. Withanolides are steroidal and bear a resemblance, both in their acappearance with steroids [18, 19].

Research by Anabalangan K *et al*. has explored the capacity of Ashwagandha to ease the symptoms of arthritis and other inflammatory conditions. These studies have proven that the herb acts as an effective anti-inflammatory agent and its naturally occurring steroidal content is much higher than that of hydrocortisone, a commonly-prescribed anti-inflammatory drug [20].

In present study, we found that peak % inhibition of paw swelling occur at 3hrs in carrageenan induced paw edema. % inhibition of paw edema is 61.36% at dose 25mg/kg *Withania somnifera*, 36.36% at 12mg/kg *Withania somnifera* as compare to Hydrocortisone i.e. 65.9% at the end of 3hrs.

For accuracy in measurement in present study digital vernier caliper was used Duffy JC & Kasim Mahmood Juma'a1 used Vernier Caliper for the measurement of paw edema [8, 9].

Complete Freund's adjuvant (CFA) administration is known to produce both primary and secondary lesions. With majority of consequent pathological changes similar to that observed in rheumatoid arthritis.

In present chronic study, we found that on 3rd day Hydrocortisone percentage inhibition of paw edema is 31.58% as compare to *Withania somnifera* i.e. 21.83%.

A study by Hindawi *et al.* found *Withania somnifera* inhibited the granuloma formation in cotton-pellet implantation in rats and the effect was comparable to hydrocortisone sodium succinate (5 mg/kg) treatment [15]. In a study by P.D. sethi, dose of hydrocortisone used for comparison is 25mg/kg p.o and 5mg/kg i.p [16].

In Fundamentals of Experimental Pharmacology by M.N. Ghosh Anti-inflammatory dose of hydrocortisone mentioned is 10 to 40 mg /kg s.c. [21] Hence in present study we had given 40 mg/kg s.c. injection of Hydrocortisone as a standard drug.

From day 13 to 21 there is decline in % inhibition, indicating that decrease in drug concentration lead to decrease in anti-inflammatory activity, on day 21st % inhibition is 32.93% with *Withania somnifera* as compare to 37.82% with Hydrocortisone.

In a study by Begum VH, it was mentioned that the effectiveness of Ashwagandha in a variety of rheumatologic conditions may be due in part to its anti-inflammatory properties. Rats given powdered root of *Withania somnifera* orally one hour before being given injections of an inflammatory agent over a 3day period showed that Ashwagandha produced anti-inflammatory responses comparable to that of hydrocortisone sodium succinate [22].

In Non-injected group paw edema and inflammation appeared on 5th day indicating development of secondary lesion, significant % inhibition is seen on day 15th i.e. 36.39% by *Withania somnifera* as compare to 44.77% by Hydrocortisone

The increased body weight during treatment of root extract of *Withania somnifera* may be due to the restoration of absorption capacity of intestine. Rise in corticosteroids which can cause the increase in the body weight, was observed in standard Hydrocortisone group [23].

In present study significant increase in arthritic index is seen in inflammed control group on day 28th i.e. 6.16±0.40. While non-significant rise seen in all the 2 groups, standard drug Hydrocortisone arthritic index is 1.33+0.51 as compare to 1.50+0.50 of *Withania somnifera*. *Withania somnifera* shows significant inhibition of secondary lesion, decrease the severity of spread of lesion; the results are comparable with Hydrocortisone and indicate that *Withania somnifera* is also useful in chronic inflammatory conditions.

5. Conclusion

1. Ethanolic extract of *Withania somnifera* elicited significant dose dependant acute anti-inflammatory activity in carrageenan induced paw edema comparable to hydrocortisone
2. Ethanolic extract of *Withania somnifera* elicited significant chronic anti-inflammatory activity in Freund's adjuvant induced Arthritis comparable to hydrocortisone
3. A single dose of *Withania somnifera* has a good duration of action as it could effectively suppress the inflammation after 6 hr of its administration.
4. *Withania somnifera* posses significant anti-inflammatory activity

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