

**EVALUATION OF ANTI ARTHRITIC ACTIVITY OF THE ROOT EXTRACT OF
ACALYPHA INDICA LINN. USING *IN VITRO* TECHNIQUES**

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Abstract

Acalypha indica L., belonging to family Euphorbiaceae, is an herb used in traditional medicine for the treatment of bronchitis, asthma, pneumonia, rheumatism, earache, syphilitic ulcers, bacterial and renal diseases. *Acalypha indica* crude root powder was extracted with solvents of increasing polarity viz. petroleum ether, chloroform and methanol successively by continuous hot percolation method. *Acalypha indica* methanol extract was evaluated using three different *in vitro* models to explore anti-arthritic potential such as inhibition of protein denaturation, proteinase inhibitory action and anti-hyaluronidase activity. The concentrations of 10 to 200 µg/ml of *Acalypha indica* methanol extract were prepared using DMSO. Diclofenac was used as the positive control. All *in vitro* determinations were done in triplicate. A dose dependent increase in percentage inhibition was observed for all the three models. The inhibitory concentration (IC₅₀) was found to be 52 µg/ml for protein denaturation assay, 37 µg/ml in proteinase inhibitory action and 18 µg/ml for anti-hyaluronidase activity. Diclofenac offered protective activity at even much lower concentrations compared to *Acalypha indica* methanol extract producing IC₅₀ values of 40 and 13 µg/ml for protein denaturation and proteinase inhibitory assays. *Acalypha indica* exhibited a very good anti-arthritic activity in all the methods checked confirming its traditional use. Further *in vivo* studies are to be carried out to confirm their activity and explore the mechanism by which this plant acts in protecting from autoimmune disease and rationalize their use.

Keywords: Autoimmune disease, hyaluronidase, bovine serum albumin, anti-rheumatic

1. Introduction:

Rheumatoid arthritis (RA) is an autoimmune disease that affects approximately 1% of the population. Prevalence of RA increases with age, approaching 5% in women over the age of 55. The incidence and prevalence of RA is 2-3 times greater in women than in men. Effective treatment of RA has been impeded by a paucity of accurate diagnostic and prognostic tests, owing in part to the heterogeneity of the disease¹. Pharmacological management of mild RA is geared towards symptomatic relief through use of conventional non-steroidal anti-inflammatory drugs (NSAIDs). Now the treatment is more focussed to use disease-modifying antirheumatic drugs (DMARDs) earlier in the course of therapy. Short-term glucocorticoids often are used to bring the level of inflammation quickly under control^{2,3}. The main aim of RA therapy is to decrease the patient's pain and joint inflammation, minimize the loss of function and reduce the progression of joint damage. Herbal drugs constitute a major part in all the traditional system of medicine. Traditional use of herbal medicines are gaining much importance now owing to its effectiveness, easy availability, low cost, less toxic compared to synthetic drugs and shortage of allopathic medical practitioners in rural areas. Significant attention is paid to the plant based drugs that are used in traditional medicine because these drugs exhibit few side effects and are inexpensive compared to allopathic medicines.

Acalypha indica Linn (Euphorbiaceae) is an annual herb found throughout the hotter parts of India, Ceylon, Africa and Philippines. *Acalypha indica* is commonly called in Tamil as Kuppaimeni and in Hindi as Khokali. The whole plant is traditionally used as expectorant and diuretic. It is a useful remedy for bronchitis, asthma, pneumonia and also for rheumatism. The juice of the leaves of *Acalypha indica* acts as an emetic for children. Decoction of the leaves is given in earache and

headache and applied as a local application in syphilitic ulcers. The leaf of this plant is used as an antiparasiticide and applied externally with common salt or quicklime or lime juice⁴. *Acalypha indica* is reported to possess anthelmintic⁵, antibacterial⁶ cardioprotective⁷, antidiabetic⁸ and antioxidant⁹ activities. The main active phytoconstituents present in the root extract of *Acalypha indica* were alkaloids acalyphus, acalyphine, quinine, amides such as acalyphamide and sterols, stigmasterol and a flavonol kaempherol and cyanogenic glycoside¹⁰. Therefore the present study was undertaken to evaluate the anti-arthritis activity of the root extract of *Acalypha indica* using three different *in vitro* models.

2. Material and Methods

2.1 Drugs and chemicals: Bovine serum albumin, bovine hyaluronidase, casein and diclofenac were procured from Sigma Aldrich, USA, phosphate buffer, tris-hydrochloride buffer, acetate buffer, dimethyl sulfoxide and dimethyl amino benzaldehyde were purchased from SD Fine Chem. Limited, Mumbai and sodium sodium hyaluronate (Hyalgan) was obtained from Fidia Farmaceutici S.P.A Abano Terme (PD), Italy and imported by Lupin Ltd, India. All other reagents used were of analytical grade and were purchased from local drug suppliers.

2.2 Plant collection and authentication: The root of *Acalypha indica* have been collected from Coimbatore district, Tamil Nadu, and dried under shade. The root was identified and certified by Dr. C. Kunhikannan, Scientist-D, Biodiversity division, Institute of forest genetics & tree breeding, Coimbatore. The voucher specimen is preserved in the herbarium file of our department.

2.3 Preparation of root powder for extraction: The roots of *Acalypha indica* plant were separated, cleaned and well dried at room temperature to avoid the degradation of phytoconstituents. The dried root part of the crude drug was ground well for getting semi coarse powder.

2.3.1 Process of extraction: About 1000 gram of *Acalypha indica* crude drug root powder was extracted separately with 1000 ml of solvents of increasing polarity viz. petroleum ether, chloroform and methanol successively by continuous hot percolation method using Soxhlet extraction apparatus at a constant temperature of 30-45°C. The crude powder was extracted with each solvent for three consecutive days. After extraction, the extracts were collected and dried under air at room temperature to get a well dried extracts. Then the dried extracts were weighed and the percentage yield of each solvent extract was calculated from the weighed powder of each plant. The extract was reconstituted with the respective solvents for further analysis.

2.4 In vitro anti-arthritis activity

2.4.1 Preparation of reagents

5% Bovine serum albumin (BSA): Dissolved 5 g of BSA in 100 ml of water.

Phosphate buffer saline pH 6.3: Dissolved 8 g of sodium chloride (NaCl), 0.2 g of potassium chloride (KCl), 1.44 g of disodium hydrogen phosphate (Na_2HPO_4), 0.24 g of potassium dihydrogen phosphate (KH_2PO_4) in 800 ml distilled water. The pH was adjusted to 6.3 using 1N HCl and make up the volume to 1000 ml with distilled water.

25mM of tris-HCl buffer pH 7.4: Dissolved 3.94 g in 800 ml of deionized water and the pH was adjusted to 7.4 using 1M hydrochloric acid (HCl) and made up to 1000 ml with deionized water.

0.8% (w/v) casein: Dissolved 0.8 g in 100 ml of distilled water.

Dimethyl amino benzaldehyde solution: Dissolved 4.0 g of para-dimethyl amino benzaldehyde in 350 ml of 100% acetic acid and 50 ml of 10 N hydrochloric acid)

2.5 Inhibition of protein denaturation: The reaction mixture consisted of 0.5 ml of 5% aqueous solution of bovine serum albumin and 0.05 ml of various concentrations (25-100 $\mu\text{g/ml}$) of the methanol extract of *Acalypha indica*. The pH of the reaction mixture was adjusted to 6.3 using 1 N hydrochloric acid (HCl) and it was then incubated at 37°C for 20 minutes and then heated at 57°C for 3 minutes. The reaction mixture was allowed to cool and added 2.5 ml of phosphate buffer saline. Turbidity was measured at 340 nm¹¹. In control 0.05 ml distilled water was used instead of test extract while product control lacked bovine serum albumin and diclofenac sodium was used as the standard. The percentage inhibition of protein denaturation was calculated. The control represents 100% protein denaturation. All determinations were done in triplicate.

2.6 Proteinase inhibitory action: The reaction mixtures consisted of 2.0 ml of 0.06 mg/ml trypsin, 1.0 ml of 25 mM tris-HCl buffer pH adjusted to 7.4 and 1.0 ml of methanol extract of *Acalypha indica* solution. The mixtures were incubated at 37°C for 5 minutes. It was then added with 1.0 ml of 0.8% (w/v) casein. The mixtures were incubated for additional 20 minutes. Then 2.0 ml of 70% (v/v)

perchloric acid was added and the cloudy solution was centrifuged at 2500 rpm for 5 minutes. Diclofenac was used as the standard¹². Absorbance of the supernatant was measured at 217 nm keeping buffer as blank. The percentage inhibition of proteinase was calculated. All determinations were done in triplicate.

2.7 Anti-hyaluronidase activity: Hyaluronidase activity was determined spectrophotometrically by measuring the amount of N- acetyl glucosamine formed from sodium hyaluronate. 50 µL of bovine hyaluronidase (7900 units/ml) dissolved in 0.1M acetate buffer (pH 3.5) was mixed with 100 µL of a designated concentration of sample (total MeOH extract) dissolved in 5% dimethyl sulfoxide and then incubated in a water bath at 37°C for 20 minutes. The control group was treated with 100 µL of 5% DMSO instead of the sample. To this 100 µL of 12.5mM CaCl₂ was added to the reaction mixture and then the mixture was incubated in a water bath at 37°C for 20 minutes. The Ca²⁺ activated hyaluronidase was treated with 250 µL of sodium hyaluronate (1.2 mg/ml) dissolved in 0.1M acetate buffer (pH 3.5) and then incubated in a water bath at 37°C for 40 minutes. About 100 µL of 0.4N NaOH and 100 µL of 0.4N potassium bromate (K₃BO₃) were added to the reaction mixture and then incubated in a boiling water bath for 3 minutes. After cooling to room temperature, 3 ml of dimethyl amino benzaldehyde solution was added to the reaction mixture which was again incubated in a water bath at 37°C for 20 minutes. Optical density was measured at 404 nm for the reaction mixture using a spectrophotometer. All determinations of the assay were done in triplicate for reproducibility¹³.

2.8 Calculation of percentage inhibition

$$\% \text{ inhibition} = \left\{ \frac{\text{Abs of control} - \text{Abs of test}}{\text{Abs of control}} \right\} \times 100$$

3. Results and Discussion

Inflammatory arthritis is a synovial disease characterized by chronic inflammation of the joints and can result in disability owing to joint destruction. *In vitro* anti-arthritic activity was performed using most popular methods such as inhibition of protein denaturation, proteinase inhibitory action and anti-hyaluronidase activity. Concentrations ranging from 10- 200 µg/mL were tested to find out the percentage inhibition and 50% inhibitory concentrations. In inhibition of protein denaturation assay it was found that the methanol extract of *Acalypha indica* at concentration of 25 µg/ml it offered an percentage inhibition of 31.18 and in 50µg/ml it has increased further to 48% and likewise at 75µg/ml it was 70% and in 100µg/ml it could offer 80.36% activity. A dose dependent increase in the percentage inhibition was observed for all the concentrations tested with a dose dependent increase in anti-arthritic activity (Table 1). The IC₅₀ values of the *Acalypha indica* methanol extract was found to be 52 µg/ml. In this method diclofenac was used as standard for comparing its anti-arthritic activity and when compared to our extract it was found that diclofenac could offer anti-arthritic potential at much lower concentrations with an IC₅₀ value of 40 µg/ml. In case of arthritis autoantigens were produced due to protein denaturation. A decrease in the absorbance reflects the inhibition of the protein denaturation, which further reveals that the compound has the capability to control the production of autoantigens by inhibiting denaturation of proteins in rheumatic disease¹⁴.

Proteinase inhibitory action was also done for the *Acalypha indica* methanol extract and the extract could produce 21% inhibitory action at 10 µg/ml and a dose dependant activity was produced with a greater inhibitory percentage of 79 at 100 µg/ml and with an effective IC₅₀ value of 37 µg/ml. However studies done with standard drug diclofenac exhibited remarkable activity in all the concentrations tested with a maximum percentage inhibition of 92.68 at 100 µg/ml dose with an IC₅₀ of 13 µg/ml.

In assay conducted with the enzyme hyaluronidase present mainly in extracellular matrix a greater activity could be observed in all the tested concentrations. Methanol extract of *Acalypha indica* produced 29.3% inhibition at 12.5 µg/ml and this gradually increased to 83.81% at 100 µg/ml and to 83.93 at 200 µg/ml. However the % inhibition decreased when concentration was increased to above 200 µg/ml and dose dependent effect could be noted up to 200 µg/ml. The IC₅₀ score was found to be 18 µg/ml. Hyaluronidases are the family of enzymes that degrade hyaluronic acid. By catalyzing the hydrolysis of hyaluronan, a constituent of the extracellular matrix, hyaluronidase lowers the viscosity of hyaluronan, thereby increasing tissue permeability. In our experiment we have observed that the root extract of *Acalypha indica* have the potential activity to inhibit a key enzyme involved in RA, hyaluronidase, an enzyme that destroys the hyaluronic acid backbone of cartilage matrix. It is indeed

found that the elevated serum level of hyaluronic acid is a reliable biomarker of arthritis progression¹⁵. Hence, it can be confirmed that our plant *Acalypha indica* root extract has the potential to inhibit the enzymes involved in disease progression of RA.

Conclusion

The plant extract obtained from *Acalypha indica* used commonly in traditional Indian system of medicine produced an excellent *in vitro* anti-arthritic activity when tested using standard methods. However, treatment with plant extracts although may have some unpredictability in the effectiveness; being non-toxic, side effect less alternative, purified plant extracts and their isolated phytoconstituents can be very useful against rheumatoid arthritis. Further to corroborate the *in vitro* anti-arthritic activity, studies are to be carried out *in vivo* to confirm their activity and also the active principles has to be identified and isolated to explore the possible mechanism by which this plant acts in protecting from autoimmune disease, rheumatoid arthritis.

Acknowledgement

The authors thank Thiru. C. Soundararaj, Managing Trustee, S.N.R. Sons Charitable Trust, Coimbatore for giving us full strength and financial support for carrying out this work at Department of Pharmaceutical Analysis, College of Pharmacy, Sri Ramakrishna Institute of Paramedical Sciences, Coimbatore, Tamil Nadu, India.

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Table 1: Percentage inhibition on Protein Denaturation assay

DRUGS	Concentration in $\mu\text{g/ml}$				
	25	50	75	100	IC ₅₀
<i>Acalypha indica</i>	31.18 \pm 0.62	48.00 \pm 0.84	70.00 \pm 0.98	80.36 \pm 0.72	52.00 \pm 0.50
Diclofenac	42.92 \pm 0.03	56.62 \pm 0.28	80.24 \pm 0.96	94.08 \pm 0.04	40.00 \pm 0.35

Table 2: Percentage Inhibition in Proteinase Assay

DRUGS	Concentration in $\mu\text{g/ml}$					
	10	20	40	80	100	IC ₅₀
<i>Acalypha indica</i>	21.00 \pm 0.12	39.34 \pm 0.38	53.16 \pm 0.60	65.12 \pm 0.45	79.00 \pm 0.96	37.00 \pm 0.45
Diclofenac	46.28 \pm 0.08	58.58 \pm 0.11	77.10 \pm 0.20	85.20 \pm 0.51	92.68 \pm 0.30	13.00 \pm 0.09

Table 3: Percentage Inhibition in Hyaluronidase Enzyme

DRUGS	Concentration in $\mu\text{g/ml}$					
	12.5	25	50	100	200	IC ₅₀
<i>Acalypha indica</i>	29.3 \pm 0.86	82.3 \pm 0.20	83.43 \pm 0.38	83.81 \pm 0.02	83.93 \pm 0.09	18.00 \pm 0.96