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IN VITRO ANTI-INFLAMMATORY AND ANTI-ARTHRITIC ACTIVITY IN METHANOLIC EXTRACT OF *COCCULUS HIRSUTUS* (L.) DIELS. *IN VIVO AND IN VITRO*

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ABSTRACT: The present study deals with the *in vitro* anti-inflammatory and anti-arthritic activity in methanolic extracts of *in vivo* (leaf and stem) and *in vitro* (callus) plant parts of *Cocculus hirsutus*. The previous phytochemical analysis of methanolic extract of *Cocculus hirsutus* has indicated the presence of several physiologically active phytochemicals such as phenols, flavonoids, triterpenoids, steroids, alkaloids etc. Since these compounds are of pharmacological interest, coupled with the use of this plant in traditional medicine, prompted us to check all *in vivo* and *in vitro* plant parts of *Cocculus hirsutus* for *in vitro* anti-inflammatory activity by HRBC (Human Red Blood Cell) membrane stabilization method and anti-arthritic activity by the inhibition of protein denaturation method. The methanolic extracts of all plant parts exhibited notable anti-inflammatory activity and remarkable anti-arthritic action. The membrane stabilization was found to be maximum in leaves (88.8% at a dose of 1000µg/ml) and that of protein denaturation was also found to be maximum in leaves (65.85% at a dose of 1000µg/ml) as compare to other *in vivo* (stem) and *in vitro* (callus) plant parts. Therefore, our studies support the isolation and the use of active constituents from *in vivo* and *in vitro* plant parts of *Cocculus hirsutus* in treating inflammations and rheumatism.

INTRODUCTION: India is one of the largest producers of medicinal herbs in the world. Plant derived drugs serve as a prototype to develop more effective and less toxic medicines. The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites such as alkaloids, flavonoids, phenols, saponins, sterols etc. There is a growing attention in correlating the phytochemicals of a medicinal plant with its pharmacological activity [1].

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The mechanism of inflammation injury is attributed, in part, to release of Reactive Oxygen species (ROS) from activated neutrophil and macrophages. Free radicals are important mediators that provoke or sustain inflammatory processes and consequently, their neutralization by antioxidants and radical scavengers can attenuate inflammation [2]. Rheumatoid arthritis is a chronic, systemic inflammatory disease predominantly affecting the joints and peri-articular tissues. Tumour necrosis factor alpha (TNF-alpha) is the product of macrophages has been demonstrated to play an important role in the pathogenesis of RA. The screening and development of drugs for their anti-inflammatory activity is still in progress and there is hope for finding anti-inflammatory drugs from indigenous medicinal plants [3, 4].

Cocculus hirsutus is an important medicinal plant. It is commonly known as Patalagarudi, Jaljamni and belongs to family Menispermaceae. It is distributed throughout India mainly in dry localities. The plant is a perennial climbing shrub with softly villous young parts. Earlier investigation on the plant resulted in the isolation of several bioactive alkaloids and triterpenoids [5, 6, 7, 8]. It is medicinally used by the Indian tribes for a wide range of ailments, including constipation, kidney problems, gonorrhea, spermatorrhoea, urinary troubles, diarrhea and hyperglycemia [9, 10, 11, 12]. The main aim of the present investigation was to study the *in vitro* anti-arthritic and anti-inflammatory activities in methanolic extracts of *in vivo* (leaf and stem) and *in vitro* (callus) plant parts of *Cocculus hirsutus*.

MATERIALS AND METHODS:

Plant material: The plant parts of *Cocculus hirsutus* were collected from the Kulish Smriti Van, Jaipur. The plant material was authenticated by herbarium of the Department of Botany, University of Rajasthan, Jaipur. A voucher specimen was deposited in the herbarium of the Department. The plant materials (leaves and stem) were air dried at room temperature under shade, and then powdered to a fine grade by using a laboratory scale mill. These shade dried parts of the plant were powdered and kept in air tight plastic bag until use.

Callus Induction and Establishment: For callus establishment the fresh and healthy plant twigs were collected and washed with detergent followed by running tap water. To overcome the problem of *in vitro* oxidative browning, the explants were given a pretreatment with an antioxidant solution comprising ascorbic acid (50 mg/L), polyvinyl pyrrolidone (100 mg/L) and citric acid (100 mg/L) for One hour. The explants were then surface sterilized with 0.1% mercuric chloride for One minute and rinsed with sterile distilled water. Sterilized leaf discs and nodal segments were used as explants for the induction of callus. The explants were inoculated on MS medium [13] consisting of basal salts and vitamins with 3% (w/v) sucrose and 0.8% agar with various concentrations of growth regulators like NAA (0.25-2.0 mg/l) and BAP (0.5-2.0 mg/l) alone or in the combination. The induced callus was then subcultured on MS media with various concentrations of BAP (0.25-2.0mg/l) with the aim to stimulate the rate of cell division for enhancement of callus growth. These cultures were allowed to grow upto their maximum growth age (6 weeks). The developed undifferentiated homogenous cell mass was repeatedly subcultured to maintain cell growth. The collected cell mass was allowed to dry at room temperature and then used for further investigation.

Preparation of Extract: All the powdered plant materials (leaf, stem and callus) were extracted separately with methanol using Soxhlet apparatus for 48 hours. The solvent was distilled at lower temperature under reduced pressure and concentrated on water bath to get the crude extract which is stored in desiccators for future use. All chemicals and reagents used including the solvents were of analytical grade.

***In vitro* anti-inflammatory activity by HRBC membrane stabilization method:** The principle involved here is stabilization of human red blood cell membrane by hypo tonicity induced membrane lysis. The assay mixture contains 1ml phosphate buffer [pH 7.4, 0.15 M], 2 ml hypo saline (0.36%), 0.5 ml HRBC suspension (10% v/v) with 0.5 ml of each plant extracts (leaf, stem and callus) of various concentrations (100, 250, 500, 1000 µg/ml), standard drug diclofenac sodium (100, 250, 500, 1000 µg/ml) and control distilled water instead of hypo saline to produce 100 % hemolysis were incubated at 37^{o}C for 30 min and centrifuged respectively [14, 15]. The hemoglobin content in the suspension was estimated using spectrophotometer at 560 nm. The percentage hemolysis produced in the presence of distilled water was taken as 100 %. Percentage of HRBC membrane stabilization or protection was calculated using the formula;

Percentage stabilization = 100 - [(optical density of test solution) ÷ (optical density of control) × 100].

***In vitro* anti-arthritic activity by inhibition of protein denaturation method:**

1. The Test solution (0.5ml) consists of 0.45ml of Bovine serum albumin (5% W/V aqueous solution) and 0.05ml of test solution of each

plant extracts (leaf, stem, callus) of various concentrations (100, 250, 500, 1000 µg/ml).

2. Test control solution (0.5ml) consists of 0.45ml of bovine serum albumin (5% W/V aqueous solution) and 0.05ml of distilled water.

3. Product control (0.5ml) consists of 0.45ml of distilled water and 0.05 ml of test solution.

4. Standard solution (0.5ml) consists of 0.45ml of Bovine serum albumin (5% W/V aqueous solution) and 0.05ml of diclofenac sodium (250µg/ml).

All the above solutions were adjusted to pH 6.3 using 1N HCl. The samples were incubated at 37°C for 20 min and the temperature was increased to keep the samples at 57°C for 3 min. After cooling, add 2.5 ml of phosphate buffer to the above solutions. The absorbance was measured using UV-Visible spectrophotometer at 416 nm [15, 16, 17]. The percentage inhibition of protein denaturation can be calculated as;

Percentage Inhibition = [100 - (optical density of test solution – optical density of product control) / (optical density of test control) × 100.

The control represents 100% protein denaturation. The results were compared with diclofenac sodium (250µg/ml).

RESULTS AND DISCUSSION: Callus induction and establishment was best reported on MS media supplemented with NAA (0.5mg/l) and BAP (1.0 mg/l) using nodal explants. These static cultures were allowed to grow upto their maximum growth age i.e. 6 weeks.

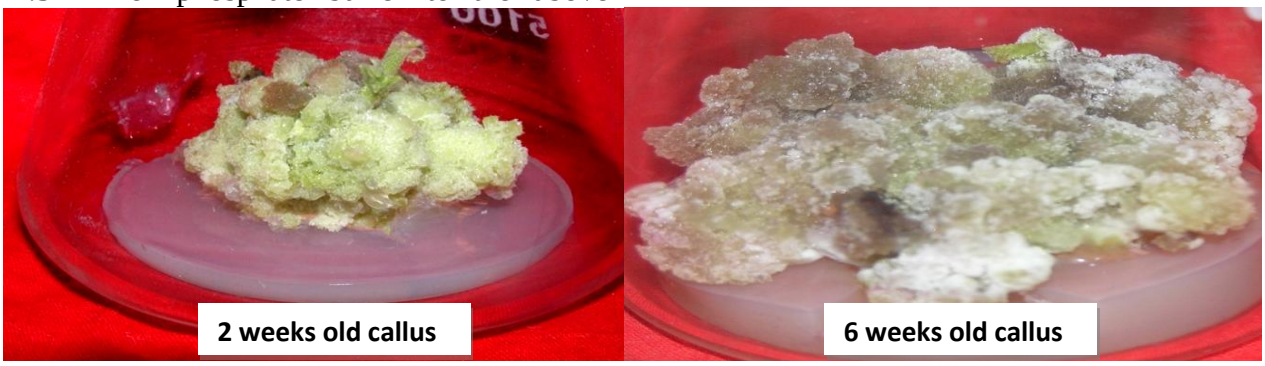

FIG. 1: STATIC CALLUS CULTURES OF *COCCULUS HIRSUTUS* GROWN ON MS MEDIUM SUPPLEMENTED WITH NAA (0.5 MG/L) AND BAP (1.0 MG/L) AT DIFFERENT TIME INTERVALS.

***In vitro* anti-inflammatory activity by HRBC membrane stabilization method:** The investigation is based on the need for newer anti-inflammatory agents from natural source with potent activity and lesser side effects as substitutes for chemical therapeutics. The effect of methanolic extracts of all plant parts (leaf, stem and callus) of *Cocculus hirsutus* on stabilization of HRBC membrane is shown in **Table 1 and Figure 2.**

The maximum percentage stabilization was observed in methanolic extract of leaves (88.8% at 1000µg/ml) as compared to other *in vivo* (stem) and *in vitro* (callus) plant parts. It possesses significant activity comparable with that of the standard diclofenac sodium [18, 19]. All the plant parts of *Cocculus hirsutus* have significant anti-inflammatory activity which may be due to presence of chemical profile such as flavonoids, tri-terpenoids and phenols.

TABLE 1: *IN VITRO* ANTI INFLAMMATORY ACTIVITY OF METHANOLIC EXTRACT OF *Cocculus hirsutus IN VIVO* AND *IN VITRO*

Concentrations (µg/ml)	% stabilization on HRBC membrane			
	Plant parts			Standard (Diclofenac sodium)
	Leaf	Stem	Callus	
100	66.5	48.25	61.11	70.14
250	75.25	52.78	66.38	82.74
500	82.5	57.35	74.2	88.39
1000	88.8	62.25	78.5	90.10

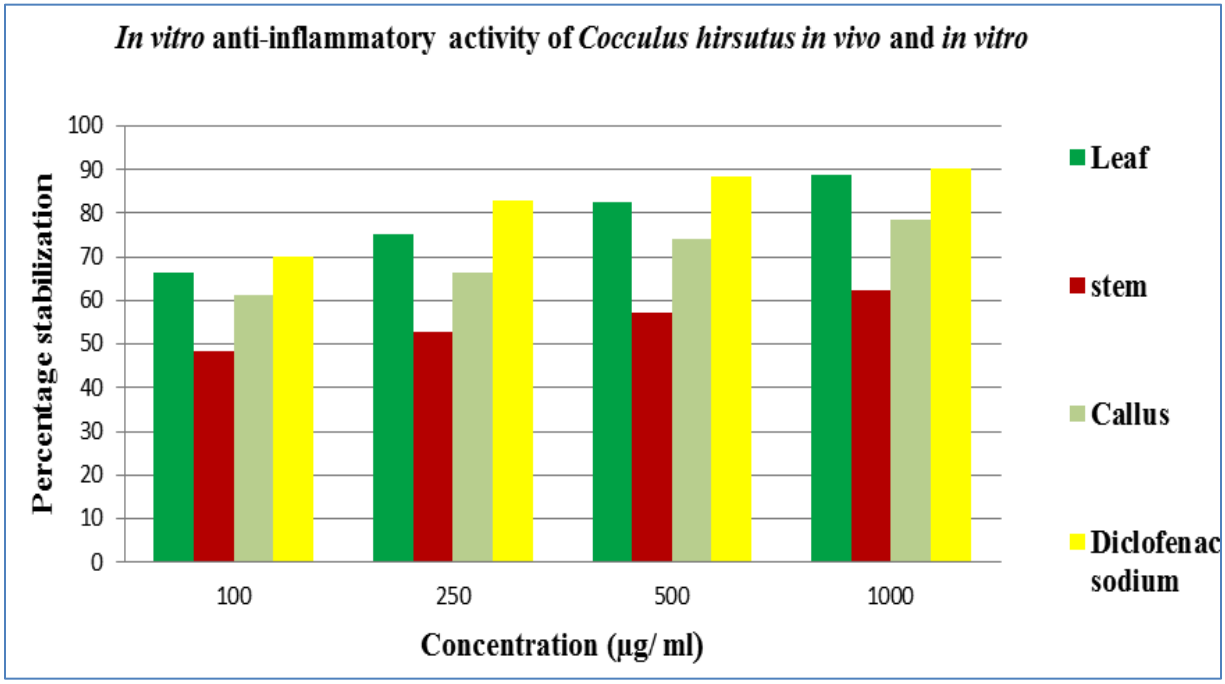

FIG. 2: EFFECT OF VARIOUS EXTRACTS OF *IN VIVO* AND *IN VITRO* PLANT PARTS OF *COCCULUS HIRSUTUS* ON ANTI-INFLAMMATORY ACTIVITY BY HRBC MEMBRANE STABILIZATION

***In vitro* anti-arthritic activity by inhibition of protein denaturation method:** The effects of methanolic extract of all plant parts (leaf, stem and callus) of *Cocculus hirsutus* on inhibition of protein denaturation are shown in **Table 2 and Figure 3.** Extracts of all plant samples at different concentrations (dose levels) provided significant protection against denaturation of proteins. The maximum percentage inhibition was observed in methanolic extract of leaves (65.85% at 1000µg/ml) as compared to other *in vivo* (stem) and *in vitro* (callus) plant parts.

It possesses significant activity comparable with that of the standard diclofenac sodium. Most of the investigators have reported that denaturation of protein is one of the cause of rheumatoid arthritis. Production of auto-antigens in certain rheumatic diseases may be due to *in vivo* denaturation of proteins [19, 20].

Mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding. From the results of present study it can be stated that methanolic extracts of all the plant parts of *Cocculus hirsutus* is capable of controlling the production of auto antigen and inhibits denaturation of protein in rheumatic disease.

TABLE 2: *IN VITRO* ANTI ARTHRITIC ACTIVITY OF METHANOLIC EXTRACT OF *COCCULUS HIRSUTUS IN VIVO* AND *IN VITRO*

Concentrations (µg/ml)	% inhibition of protein denaturation			
	Plant parts			Standard (Diclofenac sodium)
	Leaf	Stem	Callus	
100	52.15	32.14	49.19	-
250	55.75	37.58	53.31	75.20
500	61.11	42.24	57.11	-
1000	65.85	46.15	60.21	-

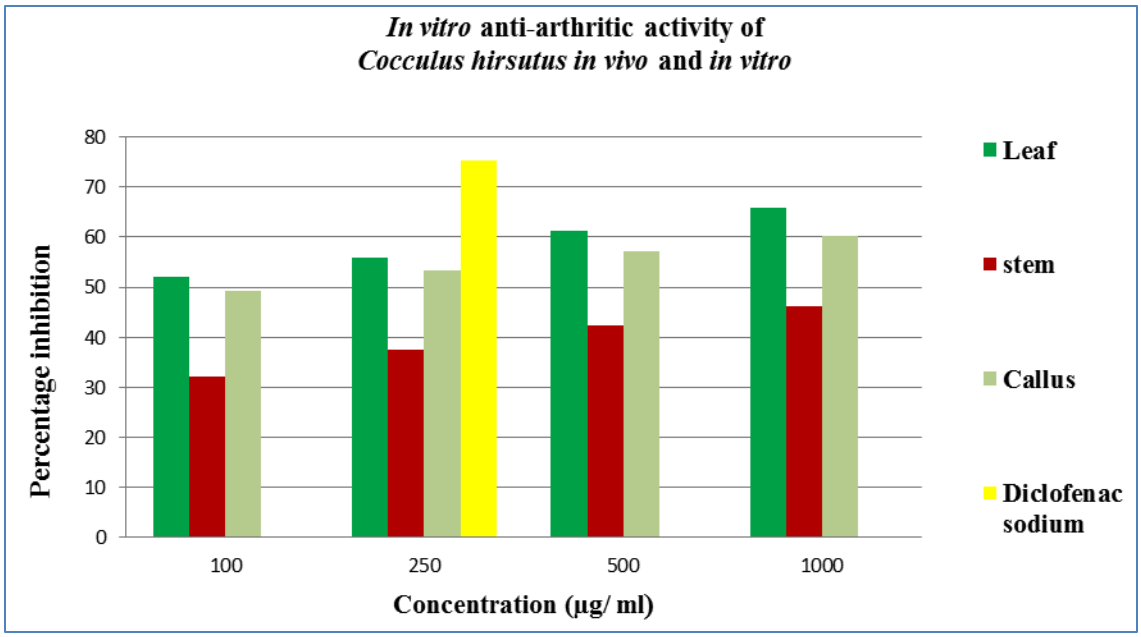

FIG. 3: EFFECT OF VARIOUS EXTRACTS OF *IN VIVO* AND *IN VITRO* PLANT PARTS OF *COCCULUS HIRSUTUS* ON ANTI-ARTHRITIC ACTIVITY BY PERCENTAGE INHIBITION OF PROTEIN DENATURATION

CONCLUSION: This is the first comparative *in vitro* study on anti-inflammatory and anti-arthritic activities of *in vivo* and *in vitro* plant parts of *Cocculus hirsutus*. The methanolic extract of the leaves of *Cocculus hirsutus* showed maximum anti-inflammatory and anti-arthritic activities as compared to other *in vivo* and *in vitro* plant parts. The plant contains many secondary metabolites e.g. flavonoids, sitosteroids, alkaloids, tri-terpenoids and phenolics. Hence proper isolation of the active principles might help in the findings of new lead compounds in the fields of anti-arthritic and anti-inflammatory drug research. This established a significant scope to develop a broad spectrum use of *Cocculus hirsutus* in herbal medicine and as a base for the development of novel potent drugs against inflammations and arthritis.

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