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Research Article

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Evaluation of Anti-Arthritic Activity of the Ethanolic Extract of *Catharanthus roseus* in Wistar Rats

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Abstract The ethanolic extracts of Catharanthus Roseus were investigated for its anti-arthritic activity in Wistar rats. The evaluation of anti-arthritic activity was carried out using cotton pellet granuloma method and Freund's adjuvant induced arthritis model. Prednisolone (5 mg/kg bw) was used as a standard drug. The ethanolic extract of *Catharanthus roseus* exhibited significant anti-arthritic activity as compared to other extracts. The doses of 200 mg/kg bw of the ethanolic extract of *Catharanthus roseus*, in chronic model of granuloma pouch in rats produced 48.0% and in arthritis model produced 44.0 % inhibition respectively with that of the standard drug Prednisolone (5 mg/kg) which produced 58.5% and 59% inhibition.

Keywords *Catharanthus roseus*, Anti-arthritic, cotton pellet granuloma, Freund's adjuvant

Introduction

Rheumatoid arthritis is a chronic, systemic inflammatory disorder or a long term auto immune multisystem illness in which the body‟s immune system attacks the body‟s tissues and joints mistakenly causing an inflammatory synovitis which often progresses the destruction of joint ankylosis and articlular cartilage [1]. An autoimmune disease is a condition which arises from an abnormal response to our normal immune system. The immune system is a host defence mechanism comprising complex organisation of cells and antibodies designed normally to "seek and destroy" invaders of the body. The synovium (inside of joints) is a thin delicate lining serves as an important source of nutrients for cartilage which thickens during RA resulting in inflammation and pain in and around the joints. Additionally, synovial cells synthesize joint lubricants and helps them move smoothly such as collagens, as well as fibronectin and hyaluronic acid that constitute the structural framework of the synovial interstitium [2].

Catharanthus roseus Linn (synonym: *Vinca rosea*; Madagascar periwinkle; Apocynaceae) a perennial plant is commonly seen in tropical counties and are native to Madagascar and Southern Asia [4,5]. The plant has spread all over tropical and subtropical parts of India and grows wild all over the plains and lower foothills in Northern and Southern hills of India. In Malaysia it is locally called as Kemunting Cina. The periwinkle logo as a symbol for hope for cancer patients is used by National Cancer Council of Malaysia [6]. The flowers produced by these plants are planted for decorative purposes are of colours such as pink, purple and white Madagas- car periwinkle is used traditionally for number of ailments such as high blood pressure, infection and diabetes mellitus. Stem produc- es a milky sap which is a source for more than 70 indole alkaloids. Vincristine and vinblastine were isolated from this

plant are well known anti-cancer drugs for Hodgkin's lymphoma and childhood leukemia respectively. The mechanism of action being binding to tubulin thus inhibit the metaphase of cellular mitosis. Hair loss, peripheral neuropathy, constipation and hyponatremia are the major side effects of this drugs.

Material and Method

Animals

The study was carried out in rats of Wister strains of either sex weighing 150-200 gm. 2-3 months old. They were procured from animal house of the biological signature analytical laboratory, Ghaziabad and were kept individually under standard laboratory condition. Food pellets and tap water biological signature analytical laboratory, Ghaziabad were provided and libitum. Ethical clearance for experimental studies was obtained from institutional animal Ethical Committee, Accuprec research lab Ahmedabad under reg. 1709/Rc/S/13/CPCSEA.

Plant Material

Catharanthus roseous was purchased from local market and its identity was confirmed. It was dried well to make powder. Coarse powder of the dried rind was prepared with the help of the grinder. Hydro alcoholic extract of the powered drug was prepared by Soxhlet's apparatus.

Preparation of Extract

The air-dried parts of the plants were powdered and extracted with 95% ethanol, chloroform per ether (40-60) and aqueous solvent systems by hot percolation method by using Soxhlet apparatus assembly at a controlled temperature. After complete extraction, marc was pressed to collect the micelle, mixed with the contents of RBF, filtered and concentrated to get the extract. The color and consistency of the extract was noted.

Extraction of Plant

The air-dried part of *Catharanthus roseous* were reducing to coarse powder. The dry powder of plants part (500 g) was subjected to successive solvent extraction procedure using various solvents petroleum ether, chloroform, acetone and methanol in the increasing order of polarity. The solvents were evaporated under reduced pressure to obtain a semisolid mass and then vacuum dried to yield solid residues. The dried extracts were stored in air tight container until the time of use.

Testing for Animals

Testing of CBC and Total Serum protein estimation and histopathological study (Skin biopsy) were done from Precision Path Lab, Jaipur.

- Skin Biopsy

Chemicals

Sulphuric acid, sodium hydroxide, fehling's solution (A and B), hydrochloric acid (HCl), Mayer's reagent, Dragendorff's reagent, ferric chloride, Ammonia solution, chloroform and dichloromethane were purchased & distilled water was prepared in the department of pharmacology, Maharishi Arvind Institute of Pharmacy Jaipur.

Determination of acute toxicity (LD_{50})

The acute toxicity of petrolium ether, mathanolic and aqueous extract of plant *Catharanthus roseous* were determined in wister rat. The animal were fasted overnight prior to the experiment, fixed dose method of OECD guideline no. 420; (Annexure-2d) of CPCSEA was adopted for this purpose. Toxicity studies of petroleum ether and ethanolic extracts of *Catharanthus roseous* were carried out on rats, when topically applied in a concentration of up

to 5% did not show any toxic side effects or erythma on skin surface. Thus the prepared extracts were considered safe for topical administration.

Collection and Authentication of Plant: Identification of the root of Catharanthus Roseous Deputy conservator of Forest sikar

Reference no.: DCF/2022/18

Plant is authenticated by Bhima ram choudhary. Material was shade dried at room temperature and powdered mechanically and passed through a sieve #40.

Experimental Design

Acute oral toxicity study

Acute toxicity studies were conducted in female albino rats (150-200 g) body weight by *Staircase Method* of Ghosh [7]. The Experimental animals were subjected to acute oral toxicity studies as per revised OECD Organization of Economic Co-operation and Development guidelines (OECD No. 423) and acute class method.

***In Vivo* Anti-arthritic study Chemicals**

Freund‟s complete adjuvant (Sigma Aldrich) and all other chemicals and reagents used for the study were of analytical grade procured from approved organization.

Animals

Wistar rats of body weight 150–200 g were used for the study. The animals were maintained under standard environmental conditions and were fed with standard pellet diet and water ad libitum. All the experimental procedures were carried out in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA) guidelines and all the experimental procedures were approved.

Induction of Arthritis

To induce arthritis, animals were first anesthetized with a small amount of ether vapor, then a single injection of 0.2 ml Complete Freund‟s adjuvant dissolved in mineral oil (sterile) was injected delicately into the sub-plantar region of hind paw.

Treatment Regimen

The anti–arthritic activity was performed according to Jubie *et al* [8] method. After classifying and grouping animals according to their weight, each animal was marked and placed in a cage with a letter identifying the cage.

The animals were divided into five groups of six animals each. Each group was given a dose schedule as follows

- Group I -Vehicle control, 1% w/v DMSO, p.o; (nonarthritic);
- Group II - Negative control (0.1ml Complete Freund‟s adjuvant);
- Group III -Arthritic animals treated with standard, 0.75 mg/kg Methotrexate, p.o;
- Group IV - Arthritic animals treated with 200 mg/kg of Catharanthus Roseus, p.o;
- Group V -Arthritic animals treated with 400 mg/kg of Catharanthus Roseus, p.o;

Vehicle control animals were given 1% w/v of DMSO solution daily.

Negative control group was given a single injection of 0.2 ml Complete Freund‟s adjuvant inmineral oil into the sub-plantar region of hind paw on day 1 under light ether anesthesia.

The stock solution was prepared on a daily basis for the treatment of low dose, high dose and standard dose animals depending upon the body weight of animals. The plant extract was diluted in DMSO because of its solubilizing effect and no solubility in water. Freshly prepared drug was introduced into the group of animals through oral administration using oral cannula.

The drug treatment was continued with the respective groups for 40 days.
Every day animals were carefully and thoroughly inspected by examining the affected paw and animal's general status. The health status parameter included paw volume, animal body weight, arthritic score and behavioural observations such as locomotor activity. The body weights of all the animals were recorded in grams on weekly basis by using single pan weighing balance. Body movement was measured by observing the time taken by individual animal to move two meter distance and statistical analysis is performed. The animals were sacrificed on day 41 to study the histology of the joint.

Parameters

- **Paw edema volume**
    - Requirement: Plethysmometer, Mercury, Marker
- **Arthritic score**
- **Locomotor activity**
- **Body weight**

Results

Acute toxicity results

No toxic symptoms were observed after administration of different dose levels of extract up to maximum of 2000mg/kg p.o. according to OECD guideline 423; and in addition, the higher dose of 2000mg/kg dose was administered to a group of animals. No symptoms or adverse events were identified. Hence, safe tolerable dose was used as therapeutic dose for further pharmacological study. From this experiment, the minimum and maximum therapeutic dose level of *ECR* extracts were studied as 200mg/kg and 400 mg/kg.

Biochemical estimations

On day 41, after anesthesia (using ether vapor), cardiac puncture was done and a centrifuge tube was introduced to withdraw blood. Blood with and without anticoagulant was centrifuged for 15 min (3000 rpm) and the plasma and serum was collected. Total proteins such as albumin and globulin, Rheumatoid factor (R_f) and C-Reactive protein (CRP) levels were quantified.

Estimation of total protein

Spectrophotometric method using erythrosine B dye was carried out for the determination of total proteins in blood plasma from rats.

Materials and reagents

- Bovine serum albumin (BSA-Sigma) solutions
- 3',3",5',5"-tetrabromophenolphthalein ethyl ester (TBPEE)
- Ultraviolet and visible spectrophotometer

Other Methods

Histological analysis of ankle joints

X-ray radiography

Statistical Analysis

The data was analyzed in terms of Mean ± Standard error of Mean (SEM). For statistical analysis, multiple comparisons of data were made using one and two way analysis of variance (ANOVA) followed by Dunnet‟s test

was used for post hoc analysis. Significance was statistically acceptable at a level of $P < 0.05$. Software program GraphPad Prism was used for all data analysis [9].

Rheumatoid arthritis is influenced by the following factors such as gender, age, environmental factors and reproductive status, various studies demonstrate that genetic factors also play a major role on an individual's susceptibility to RA. It is characterized by periods of disease flares and remissions. Chronic inflammation of rheumatoid arthritis can cause permanent joint destruction and deformity. It leads to warm, swollen, painful and stiff joints which gets worsened following rest. Usually multiple joints of the fingers and hands, wrists, feet and knees typically gets affected in a symmetrical distribution (affecting both sides of the body). It may also affect other parts of the body and this may result in a low red blood cell count, inflammation around the lungs, and inflammation around the heart [3].

Results

Acute toxicity results

No toxic symptoms were observed after administration of different dose levels of extract up to maximum of 2000mg/kg p.o. according to OECD guideline 423; and in addition, the higher dose of 2000mg/kg dose was administered to a group of animals. No symptoms or adverse events were identified. Hence, safe tolerable dose was used as therapeutic dose for further pharmacological study. From this experiment, the minimum and maximum therapeutic dose level of *ECR* extracts were studied as 200mg/kg and 400 mg/kg.

Table 1: Ash values of whole plant of *Catharanthus roseus*

S. No.	Type of ash	Percentage (W/W)
1.	Total Ash	5.257% w/w
2.	Acid Insoluble Ash	1.611% w/w
3.	Sulphated Ash	7.993% w/w

Table 2: Extractive values of whole plant of *Catharanthus roseus*

S. No.	Type of extractive value	Percentage
1.	Ethanol	23.758% w/w
2.	Water	18.964% w/w

Table 3: Nature of phytoconstituents present in the whole plant of *Catharanthus roseus*

Phytoconstituents	Observation
Carbohydrates	+
Alkaloids	+
Proteins & Amino acids	+
Tannins & Phenolics	+
Flavonoids	+
Triterpenoids	-
Saponins	+
Fixed oils	+
Glycosides	-
Gums	-
Mucilage	+

(+) Indicates the presence of chemical constituents, (-) Indicates the absence of chemical constituents

Table 4: Fluorescence analysis of the raw material and whole plant extract of *Catharanthus roseus*

Sample		Reagent used	Visible	UV
Catharanthus roseus	1.	1(N) NaOH	Yellowish green	Green
(Linn.) raw	2.	1(N) NaOH in Alcohol	Yellowish green	Green
powder	3.	1(N) HCI	Light brown	Green
(Whole plant)	4.	50% HNO_3	Orange	Green
50% ethanolic	1.	1(N) NaOH	Yellowish brown	Green
extract of	2.	1(N) NaOH in Alcohol	Yellowish brown	Green
Catharanthus roseus	3.	1(N) HCI	Light brown	Green
(Linn.) (leaves)	4.	50% HNO_3	Orange	Green

Table 5: R_f values of the TLC studies on the ethanolic extract of *Catharanthus roseus*

S. No.	Extract (10 mg/ml)	Solvent System	TLC study for	R_f values
1	Mother extract	Acetonitrile: methanol (8:2)	Alkaloid	0.54
2	Alkaloidal fraction	Acetonitrile: methanol (8:2)	Alkaloid	0.51
3	Methanolic fraction	Acetonitrile: methanol (8:2)	Alkaloid	0.80
4	Ethyl acetate fraction	Acetonitrile: methanol (8:2)	Alkaloid	0.56

Table 6: Effect of *Catharanthus roseus* Extracts on Changes in Paw Volume in Cfa Induced Arthritis in Rats

Group	0^{th} day	9th day	18th day	25th day	40th day
Control	0.65 ± 0.01	0.66 ± 0.01	0.65 ± 0.02	0.67 ± 0.01	0.67 ± 0.02
Negativecontrol	0.66 ± 0.02	0.75 ± 0.01	0.80 ± 0.01	0.86 ± 0.02	0.91 ± 0.02
Standard	0.59 ± 0.01	0.48 ± 0.01	0.43 ± 0.02	0.40 ± 0.01	0.35 ± 0.02
200 mg/kgCR	0.62 ± 0.02	0.51 ± 0.01	0.42 ± 0.02	0.40 ± 0.01	0.38 ± 0.01*
400 mg/kgCR	0.58 ± 0.02	0.42 ± 0.01	0.36 ±0.01*	0.32 ±0.01**	0.27 ± 0.01*

Values are expressed in mean ± SEM, n = 6, *p<0.05 are significant compared to standard, LDMP (200 mg/kg); HDMP (400 mg/kg)

Table 7: Effect of ethanolic extracts of LDCR and HDCR on CFA induced arthritic rats showing changes in body weight and locomotor activity

	Physical and behavioural changes									
	0^{th} day		9^{th} day		18^{th} day		25^{th} day		40^{th} day	
	BW	M	BW	M	BW	M	BW	M	BW	M
Group	(gms)	(sec)	(gms)	(sec)	(gms)	(sec)	(gms)	(sec)	(gms)	(sec)
Control	180±0.02	20	175±0.01	22	170±0.01	24	173±0.02	22	174±0.01	23
Negative control	170±0.01	20	160±0.01	30	150±0.02	35	100±0.02	50	92±0.02	55
Standard	170±0.01	20	160±0.02	30	140±0.01	32	130±0.01	32	175±0.01	25
LDCR	160±0.02	20	150±0.02	30	140±0.02	30	120±0.01	40	100±0.01	40
HDCR	200±0.02	20	180±0.02	25	160±0.02	25	165±0.01	30	195±0.01*	25

Values are expressed in mean ± SEM, n =6, *p < 0.05 are considered significant compared to standard. LDCR (200 mg/kg); HDCR (400 mg/kg); BW – Body weight; M – Movement

Table 8: Effect of ethanolic extracts of LDMP and HDMP on serum parameters in CFA induced arthritic rats

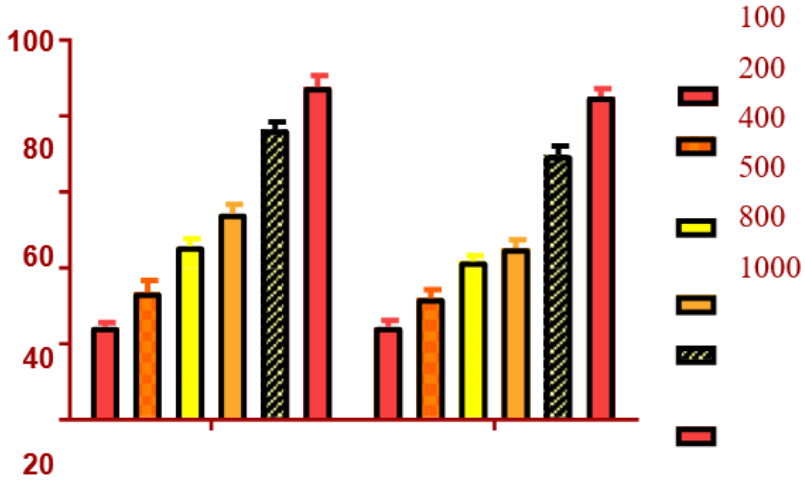
Group	CRP (mg/l)	R_f (IU/ml)	Total Protein (g/dl)
Control	1.65 ± 0.01	-	8.0 ± 0.54
Negative control	6.92 ± 0.22**	58.01 ± 1.50	5.4 ± 0.15*
Standard	3.68 ± 0.30$^{**\#}$	39.15 ± 0.23$^{\#}$	7.6 ± 0.42*
LDCR	4.12 ± 0.18$^{**\#}$	40.03 ± 0.02$^{\#}$	7.1 ± 0.43
HDCR	3.05 ± 0.05$^{*\#}$	36.01 ± 1.51$^{\#}$	7.8 ± 0.25$^{\#}$

Each value represents the mean ± SEM for ANOVA, n=6, *p<0.05, **p<0.001 when compared to healthy control, $^{\#}$p<0.001 when compared to negative control

Table 9: Protein Denaturation Inhibition Study

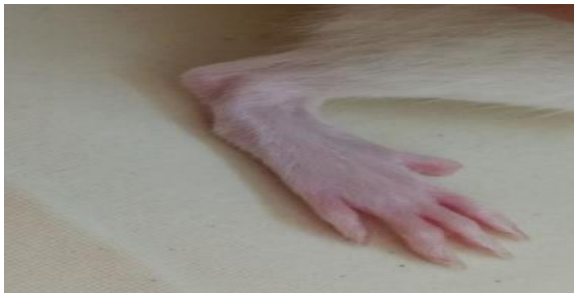
Concentration (mg/ml)	Inhibitory activity of ECR	Inhibitory effect of Acetyl Salicylic Acid (%)
100	24.46 ± 1.12	24.45 ± 1.70
200	33.62 ± 3.08	32.14 ± 2.21
400	45.76 ± 1.98	41.77 ± 1.52
500	54.35 ± 2.37	45.33 ± 2.18
800	76.48 ± 1.92	69.71 ± 2.43*
1000	87.65 ± 3.01	85.17 ± 2.13*

Values are expressed in mean ± SD(n=6), *p<0.05. Statistical significant test for comparison was done by ANOVA followed by Dunnett's t-test. Comparison between acetyl salicylic acid vs *ECR*

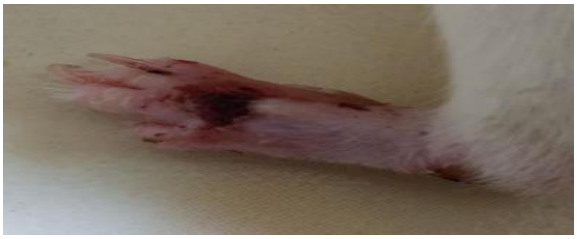


Graph I: % Inhibition of ECR on protein denaturation

Arthritic score



Group 1



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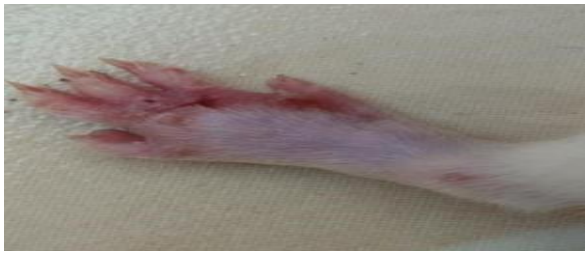

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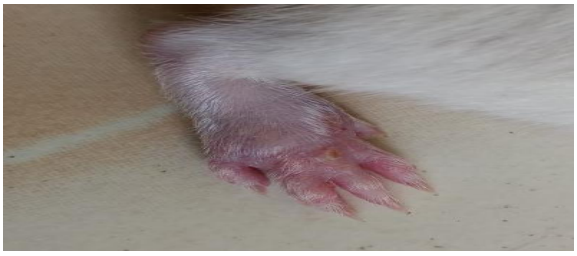

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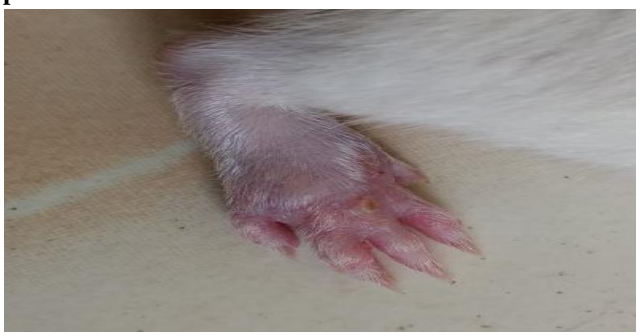
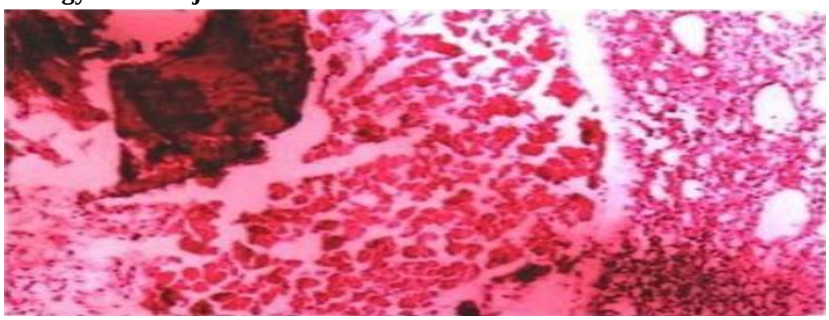
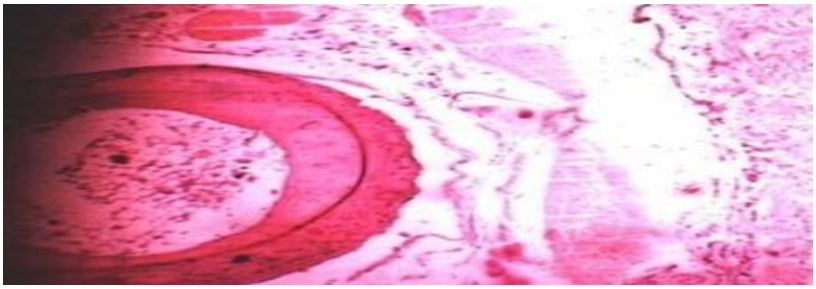


Group 5

Analysis of histopathology of ankle joints



Group 1



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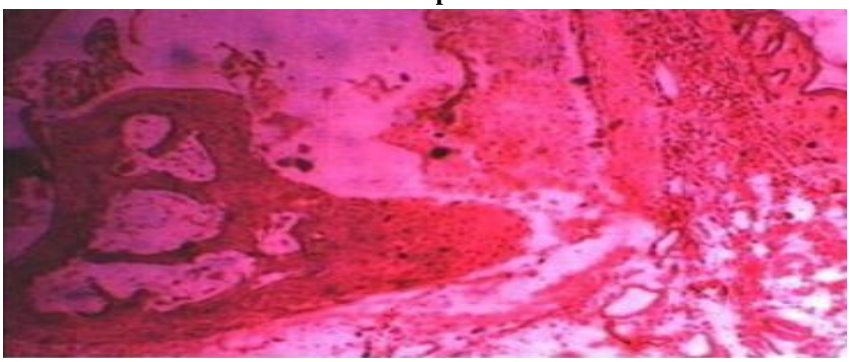

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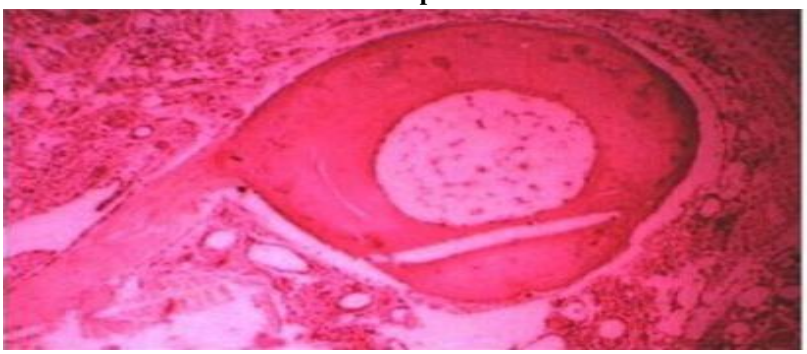

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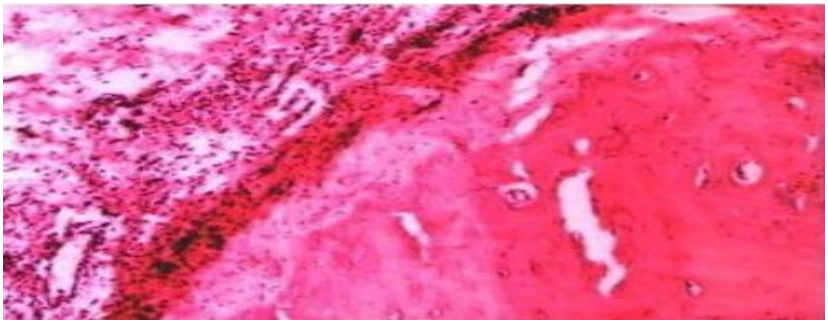
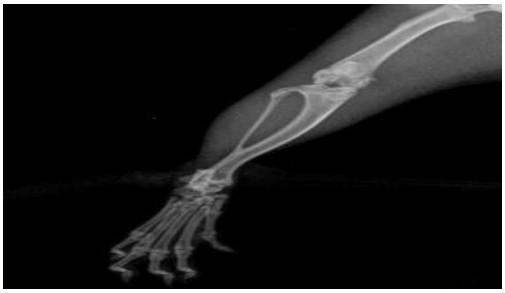
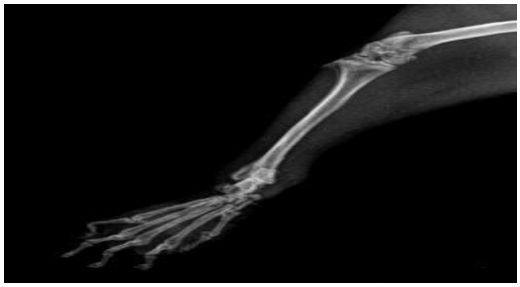


Group 5

Radiography of ankle joints



Group 1



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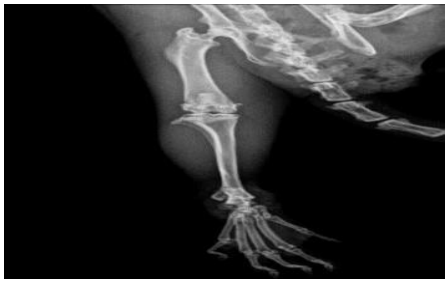

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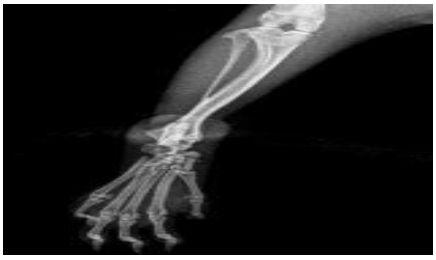

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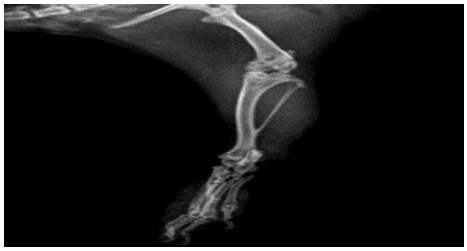

Group 5

Discussion

Rheumatoid arthritis is an inflammatory, autoimmune disorder which destroys it's own immune system. The immunologically mediated Complete Freund's adjuvant induced arthritic model of chronic inflammation is considered as the best available experimental model of rheumatoid arthritis [10]. Complete Freund's adjuvant-induced arthritis is a model of chronic polyarthritis with features that resemble rheumatoid arthritis.

In Complete Freund's 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 Complete Freund"s adjuvant administered rats showed soft tissue swelling around the ankle joints during the development of arthritis, which was considered as edema of the particular tissues.

Paw swelling is an index of measuring the anti-arthritic activity of *Catharanthus roseous* Linn. at the dose level 200&400 mg/kg, p.o. *Catharanthus roseous* administered groups showed marked reduction in paw volume when compared with the Negative control group (Group II). It was also found that there was significant weight loss when compared to standard [11]. The result of the present study also indicates that there is a close relationship between the extent of inflammation, loss of body weight and arthritic index. The arthritic scoring was done on the basis of visual observation where it can be seen that there is a marked reduction in the swelling and joint damage of the drug treated groups [12]. It was also noted that the high dose *Catharanthus roseous* Linn. Extract proved its efficacy to reduce

the inflammation of the paws. The locomotor activity of the animals was improved in Group 5 animals (HDMP) when compared to the standard animals.

Histopathology provides a noticeable morphological distinctiveness as a practical and unambiguous pathognomonic sign of Rheumatoid arthritis. The histopathological analysis identified the ability of the bones to re-form upon treatment with *Catharanthus roseous*. Bone structures re-calcified upon treatment with the *Catharanthus roseous* dose dependently. The high dose of the plant extract exhibited good therapeutic potential from the study results and is therefore consistent with earlier findings that the ability of a drug to suppress inflammation, synovitis and protect a joint is desired in rheumatoid arthritis therapy.

Radiographic changes in Rheumatoid arthritis conditions are useful diagnostic measures which indicate the severity of the disease. Soft tissue swelling is the earlier radiographic sign, whereas prominent radiographic changes like bony erosions and narrowing of joint spaces can be observed only in the developed stages (final stages) of arthritis [13]. In Freund‟s adjuvant induced arthritic rat (group II), soft tissue swelling along with narrowing of the joint spaces was severe which implies the bony destruction in arthritic condition. The standard drug Methotrexate (0.75 mg/kg) treated groups have prevented this bony destruction and also there is moderate swelling of the joint. Similarly, according to histopathological studies, extracts of *Catharanthus roseous* have shown significant prevention against bony destruction by showing less soft tissue swelling and narrowing of joint spaces in the 40 days of treatment when compared with Complete Freund's adjuvant (Negative control group).

Summary

Indian sub-continent is a rich source of plant & animal wealth which is due to its varied geographical and agro climate regions. It is a well known fact that traditional system of medicines always played important role in meeting the global health care needs. Arthritis is one of the most common auto-immune inflammatory disorders, foremost cause of disability in western and developing countries. The presently available synthetic drugs in the market are not only economical exploitation but also associated with adverse effects. The synthetic drugs includes NSAIDS and DMARDS like Cyclophosphamide, intramuscular gold, sulfasalazine had the side effects of stomach ulcers, GIT bleeding, kidney, liver damage and hypertension. The given plant *Catharanthus roseous* provides essential compounds with active principles, having no or minimum side effects holds prospect in future rheumatoid arthritis treatment. From the above review it should be manifest that there are many medicinal plants which exert anti-arthritic activity at a particular dose.

The preliminary phytochemical studies discovered the presence of various phytoconstituents.

In vivo study was performed with parameters such as paw edema volume, physical and behavioural changes and arthritic index and the extract possessed a significant effect on the inflammation and joint destruction.

The biochemical analysis were assessed by estimating the serum values which provided favourable effects.

Invitro study showed the effect of the plant extract on the percentage inhibition of protein denaturation and protease enzymes which gave marked responses.

Other methods such as histopathology and the radiographic X-ray analysis of the groups showed good results.

Conclusion

In conclusion, this study has verified that constituents of the plant suppressed the joint inflammation and destruction in adjuvant arthritic rats. We are confident that our data provide mechanistic evidence for anti-arthritic appliance of the plant as a promising candidate for novel therapeutic agent of Rheumatoid arthritis.

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