ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

Vol 6, Suppl 4, 2013

ISSN - 0974-2441

Research Article

PHARMACOLOGICAL EVALUATION OF MARKETED POLYHERBAL FORMULATION

VIKAS P PATIL*, AMARJITSING PREMSINH RAJPUT, PANKAJ M CHAUDHARI, SWAPNIL P CHAUDHARI, DHEERAJ T BAVISKAR

KVPS's Sanstha's Institute of Pharmaceutical Education, Boradi- Tal-Shirpur, Maharashtra, India. Email: vp_57@rediffmail.com

Received: 26 June 2013, Revised and Accepted: 19 July 2013

ABSTRACT

Introduction: - Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder that may affect many tissues and organs – skin, blood vessels, heart, lungs and muscles. But principally attacks the joints, producing a non-supportive proliferative sinusitis that often progresses to destruction of the articular cartilage and ankylosis of the joints. Rhumapar tablet is a marketed polyherbal formulation believed to have the potential for providing relief to rheumatoid arthritis (RA) patients.

Objective: - Investigations have been carried out using rats as experimental models, to assess the anti inflammatory and anti arthritic potential of Rhumapar tablet. In-vitro anti-oxidant study has been carried out using different in-vitro models.

Result:- The results obtained demonstrate that Rhumapar tablet can significantly and dose-dependently inhibit carrageenan-induced rat paw oedema (the inhibition at 3 hour was greater than at 1 hour after induction of oedema).Tablet can also significantly inhibit granuloma formation in cotton pellet induced granuloma model. It also showed significant anti-arthritic activity in Freund's complete adjuvant (FCA) induced arthritis. The responses were statistically significant when they were compared with the control.

Conclusion: - It has been indicated that formulation possesses anti- inflammatory and anti-arthritic activities that are probably mediated through inhibition of prostanoid synthesis and free radical scavenging effect.

Keywords: Rhumapar tablet, Rheumatoid arthritis, Anti-inflammatory activity, Anti-arthritic activity, Anti-oxidant activity.

INTRODUCTION

Finding healing power in plants is an ancient idea. It is estimated that there are 2, 50,000 to 5, 00,000 species of plants on earth a relatively small percentage (1-10 %) of these is used as food by humans and other animal species. It is possible that even more are used for medical purpose. [1]

Nitric oxide (NO), which is biosynthesized at the cellular level from L-arginine catalyzed by nitric oxide synthase (NOS), is a very important signalling molecule from the ground of pathophysiologic condition of living entities. The omnipresence of NO in the living body suggests that NO plays an important role in the maintenance of health [2]

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder that may affect many tissues and organs – skin, blood vessels, heart, lungs and muscles. But principally attacks the joints, producing a non-supportive proliferative sinusitis that often progresses to destruction of the articular cartilage and ankylosis of the joints. 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. Arthritis is likely to become severely disabled. The joint changes associated with it probably represent an auto immune reaction that comprises of inflammation, proliferation of the synovium, erosion of cartilage and bone. Arthritis may also develop as the result of increased urate concentration in plasma, resulting in deposition of sodium urate crystals in synovial tissues. The underlying cause being overproduction or impaired excretion of uric acid. Analgesia (painkillers) and anti-inflammatory drugs, including steroids are used to suppress the symptoms, while disease-modifying anti-rheumatic drugs (DMARDs), newer therapies such as anti-tumor necrosis factor (TNF)-α therapy (etanercept, infliximab and adalimumab), anti-CD20 therapy (rituximab) and abatacept 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 towards 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 the adjuvant arthritis is the best available model of rheumatoid arthritis. Management of arthritis is still a challenge to the modern medicine. The modern medicines have many severe side effects. The most modern medicines offer only symptomatic relief from arthritis. Although scientific studies have been done on a large number of Indian botanicals, a considerably smaller number of marketable drugs or phytochemical entities have entered the evidence-based therapeutics. Preclinical biological screening is important not only for establishing the therapeutic efficacy of the medicinal plants but also to validate their historical utilization by traditional healers and herbalists. It's mainly preventive and therapeutic adjuvant arthritis have expanded wide usage for evaluating a drug's potential anti-arthritic activity. [3], [9]

OBJECTIVES

Rhumapar tablet is a polyherbal formulation believed to have the potential for providing relief to rheumatoid arthritis (RA). Rhumapar tablet composed of 14 different plant extract. Many of them are reported to have a potential anti-inflammatory effect. The anti-arthritic properties in this formulation have however not been subjected to any scientifically controlled investigations so far. Investigations have therefore been carried out using rats as experimental models, to assess the anti-inflammatory, anti-arthritic and also for anti-oxidant potential of polyherbal formulation. Although the plant possesses many potential therapeutic activities in traditional system of medicinal practice and possessing rich phytoconstituents, they are not evaluated for their pharmacological activities in detail. [18],[20] Taking these facts into considerations, the present study deals with the evaluation of anti-inflammatory, anti-arthritic activity and its changes in haematological and biochemical parameters of the formulation in Freund's adjuvant induced arthritic rats.

MATERIAL AND METHODS

Phytochemical Screening

The Rhumapar tablet showed positive test for flavonoids, saponins, glycosides, tannins, phenolics compounds, proteins, amino acid and carbohydrate. [21, 22]

Anti-inflammatory Activity Study

Carrageenan induced paw edema

Procedure

The rats were divided into six groups (n=6) and they were fasted for 12 hour and deprived of water only during the experiment. Inflammation of the hind paw was induced by injecting 0.1 ml of 1% carrageenan in normal saline into the sub plantar surface of the right hind paw. The negative control and the positive control group were given normal saline and Diclofenac sodium (10 mg/kg) p.o. respectively. The remaining three groups received the different doses of formulation. The measurement of paw volume was accomplished immediately by displacement technique using the plethysmometer (UGO Basile, Italy) at different time intervals. [12-14]

Cotton pellet induced granuloma

Procedure

The rats were divided into six groups (n=6). Sterile pre weighed cotton pellets soaked in 0.2 ml of distilled water containing penicillin (0.1 mg) and streptomycin (0.13 mg) (6) was implanted subcutaneously bilaterally in groin region under ether anaesthesia. The negative control and the positive control group were given normal saline and Diclofenac sodium (10 mg/kg, p.o.), respectively and the remaining three groups received the different doses of formulation for seven consecutive days from the day of cotton pellet implantation. On the eighth day, the animals were anaesthetized, and the cotton pellets were removed surgically. The pellets were dried at 60°C to constant weight. The weight of the cotton pellet before implantation is subtracted from the weight of the dried granuloma pellets. [2, 3, 16]

Anti -arthritic Activity study

Freund's adjuvant induced arthritis

Procedure

The albino wistar rats of either sex (100-150 gram) were divided into five groups (n = 6). The first group represented control group and receives saline or 5% Tween 80 solution at a dose of 10 mg/kg, p.o. The second group received the standard drug Diclofenac sodium at a dose of 10 mg/kg, p.o. The 3rd, 4th, and 5th groups received the different doses of formulation respectively by oral route. After 30 min, 0.1 ml complete Freund's adjuvant (Sigma, U.S.A) was injected into the sub plantar region of left hind paw on day zero. Saline or extracts were administered orally once daily, from the initial day i.e. from the day of adjuvant injection (0 day) and continued till 21 consecutive days. The anti-arthritic effect of the extracts as well as Diclofenac sodium was evaluate by measuring paw volume of inject paw on 4th, 8th, 14th and 21st day of study by using digital plethysmometer. The mean changes in injected paw volume with respect to initial paw volume are calculated on respective days and % inhibition of paw volume with respect to control group was calculated. On the day 22, blood was withdrawn from retro orbital plexus for estimation hematological parameters.[2,4,10,12]

Anti-oxidant Activity study

Anti-lipid peroxidation effect

Procedure

A 0.5 ml of homogenate was taken and to it 1 ml of 0.15 M KCl and 0.5 ml of test drugs at different concentrations (10, 20, 40, 60, 80 µg/ml) were added. Lipid peroxidation was initiated by adding 100 µl of 1 mM ferric chloride. The reaction was stopped by adding 2 ml of ice cold 0.25 N HCl containing 15% trichloroacetic acid, 0.38% thiobarbituric acid, and 0.2 ml of 0.05% butylated hydroxyl toluene. These reaction mixtures were heated for 60 min at 80ºC then cooled and centrifuged at 6900 rpm for 15 min. The absorbance of supernatant was measured at 532 nm against blank, which contained all reagents except liver homogenate and drug. Same experiments were performed to determine the normal (without drug and $FeCl_3$) and induced (without drug) lipid peroxidation level in the tissue. The percentage of anti-lipid per oxidation effect (% ALP) was calculated by the following formula. [4, 5]

Free radical scavenging activity by DPPH method

Procedure

Different concentrations of test drug solution and standards were prepared. To this 5 ml of methanolic solution of DPPH was added, shaken well and mixture was incubated at 37°C for 30 min. A blank was prepared in the similar way and the absorbance was measured at 517 nm. Scavenging activity was expressed as the percentage inhibition calculated using the following formula. [5, 17, 19]

Reducing power assay

Procedure

Reducing power of formulation was determined on the ability of antioxidants to form colored complex with potassium ferricyanide. Different concentration of the formulation (10, 20, 40, 60, 80 µg/ml) were mixed with 2.5 ml phosphate buffer (pH 6.6) and 2.5ml potassium ferricyanide (1%). The mixture was incubated at 50ºC for 20 min. 2.5 ml TCA (10%) was added to it and centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was mixed with 2.5 ml of water and 0.5ml of $FeCl_3$ (0.1%) were added to it and absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. [7,8]

Nitric oxide radical scavenging activity

Procedure

Different concentrations of sample solutions were prepared in 100 ml volumetric flasks. To this 0.1489 g of sodium nitroprusside (5 mM) was added and kept for incubation. At different time intervals 5.6 ml was taken, 0.2 ml of Griess reagent A was added, and kept for incubation at 30° C for 10 min. After incubation 0.2 ml of Griess reagent B was added and kept for incubation at 30° C for 20 min. After incubation, absorbance was measured at 542 nm against blank. Concentration of $NO^{\bullet}$ was calculated from standard calibration curve. [5,6,11,15]

RESULT AND DISCUSSION

In phytochemical screening, the formulation showed positive test for saponins, flavanoids, glycosides, tannins, phenolics, proteins, amino acid and carbohydrate. The results are as shown in [Table 1].

Table 1: Phytochemical Screening of Marketed Formulation

Sr. No.	Plant Constituent	Present/Absent
1.	Steroids	Absent
2.	Saponins	Present
3.	Flavonoids	Present
4.	Tannins	Present
5.	Glycosides	Present
6.	Proteins	Present
7.	Carbohydrate	Present
8.	Alkaloids	Absent

In carrageenan induced paw edema method, formulation showed inhibition of paw edema in dose dependant manner. Significant anti inflammatory activity was obtained at 3 hour. There was dose dependant reduction in granular tissue formation in formulation and Diclofenac sodium treated rats after seven days. The activity was found to be statistically significant for the dose ranges used. The carrageenan induced paw edema model in rats is known to be sensitive to cyclooxygenase inhibitors. Based on this reports we can conclude that the inhibitory effect of formulation on carrageenan induced inflammation in rats could be due to inhibition of the

enzyme cyclooxygenase leading to inhibition of prostaglandin synthesis. The results are shown in [Table 2], [Table 3].

Table 2: Effect of Different Doses of Formulation and Diclofenac Sodium on Carrageenan Induced Rat Paw Edema

Group	Dose (mg/kg)	Mean Increase In Paw Volume (Ml)		
		1 HOUR	2 HOUR	3 HOUR
Negative Control	----	0.38 ± 0.060	0.70 ± 0.120	1.00 ± 0.025
Diclofenac sodium	10	0.18 ± 0.024 **	0.22 ± 0.024 **	0.31 ± 0.023 **
Formulation	200	0.38 ± 0.047	0.52 ± 0.050	0.76 ± 0.021 **
Formulation	400	0.30 ± 0.041	0.42 ± 0.039 **	0.63 ± 0.022 **
Formulation	600	0.29 ± 0.014	0.41 ±0.017 **	0.61 ±0.024 **

Table 3: Percentage Inhibition of Carrageenan Induced Paw Edema Treated with Diclofenac sodium and Different Doses of Formulation

Group	Dose (mg/kg)	Mean Increase In Paw Volume (Ml)		
		1 HOUR	2 HOUR	3 HOUR
Diclofenac sodium	10	52.63	68.57	69.00
Formulation	200	00.00	25.71	24.00
Formulation	400	21.05	40.00	37.00
Formulation	600	23.68	41.42	39.00

Table 5: Effect of different doses of formulation and Diclofenac sodium on adjuvant induced rat paw edema

Treatment Group	Mean increase in paw volume (ml)							
	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21
Control	1.43± 0.024	2.23± 0.019	2.02± 0.013	1.90 ±0.017	2.03 ±0.016	2.11 ±0.020	2.12 ±0.040	2.25 ±0.026
Diclofenac sodium	1.40± 0.043	1.75** ±0.022	1.81** ±0.036	1.62** ±0.024	1.56** ±0.030	1.55** ±0.025	1.46** ±0.028	1.45** ±0.023
Formulation 200	1.25** ±0.034	1.96** ±0.034	1.90** ±0.022	1.78** ±0.021	1.80** ±0.014	1.82** ±0.018	1.70** ±0.012	1.67** ±0.023
Formulation 400	1.20** ±0.019	1.90** ±0.010	1.84** ±0.024	1.81** ±0.017	1.78** ±0.016	1.82** ±0.019	1.68** ±0.012	1.63** ±0.017
Formulation 600	1.11** ±0.014	1.80** ±0.012	1.81** ±0.024	1.60** ±0.013	1.64** ±0.019	1.58** ±0.017	1.53** ±0.011	1.49** ±0.017

Table 6: Reduction in body weight in adjuvant induced arthritis of rats

Treatment Group	Mean body weight before injection (gm)	Mean body weight on 21 day (gm)	Reduction in body weight (gm)
Control	191.1	175	-16.1±0.99
Diclofenac Na	209.4	199.8	-9.60±0.47**
Formulation 200	192.2	188.1	-4.10±0.58**
Formulation 400	214.3	209.5	-4.80±0.57**
Formulation 600	210.5	195.4	-16.8±0.67

Table 7: Results of hematological parameters in Freund's adjuvant induced arthritis treated with various doses of tablet and Diclofenac sodium.

Parameters	Normal	Control	STD.	Form. 200	Form.400	From. 600
WBC (*10^3/mm^3)	8.45 ±0.89	12.1 ±0.49	9.23 ±0.75	10.9 ±1.09	10.7 ±0.29	9.12 ±0.44
Hemoglobin (gm/dl)	12.6 ±0.59	8.86 ±0.41	11.7 ±0.77	9.01 ±0.34	9.12 ±0.90	11.9 ±0.86
Platelets (10^5/mm^3)	6.48 ±0.49	10.2 ±0.30	8.35 ±0.40	9.07 ±1.05	9.05 ±0.38	8.88 ±0.44
ESR (mm/hr)	3.22 ±0.44	5.03 ±0.86	4.31 ±0.19	4.88 ±0.16	4.86 ±0.29	4.10 ±0.10

The cotton pellet granuloma method has been widely employed to assess the transudative, exudative and proliferative components of chronic inflammation. The results showed good anti transudative and anti proliferative activity. However formulation was less effective than Diclofenac sodium. Percentage inhibition of adjuvant induced paw edema treated with Diclofenac sodium and different doses of tablet is shown in [Figure 1].The results are shown in [Table 5], [Table 6], [Table 7].

The observation made on different days of treatment period in Freund's Complete Adjuvant induced arthritis showed that there was a less increased in paw swelling in Diclofenac sodium and formulation treated animals as compared to control group. In investigation of hematological parameters, formulation showed significant improvement in all parameters measured. Formulation showed significant reduction in total WBC count. It also showed good improvement in hemoglobin level. It showed decrease in platelets count and ESR. In formulation treated group, there was a negative test for CRP and RF level.

In anti-lipid peroxidation assay, the formulation showed a decrease in the absorbance which was comparable with the standard BHA. The results are shown in [Table 8], [Table 9].

In DPPH free radical scavenging assay, there was a decreased in the absorbance with increase in concentration, which indicate the antioxidant activity of formulation. The results are shown in [Table 10], [Table 11]. The Percentage inhibition vs. Concentration plot for Formulation, Ascorbic acid and BHA by DPPH free radical method is shown [Figure 3].

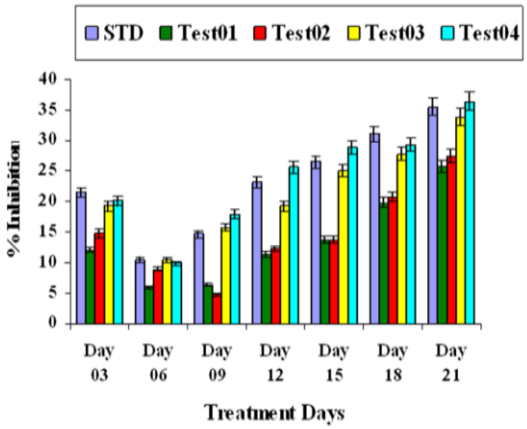

Fig.1: Percentage inhibition of adjuvant induced paw edema treated with Diclofenac sodium and different doses of formulations.

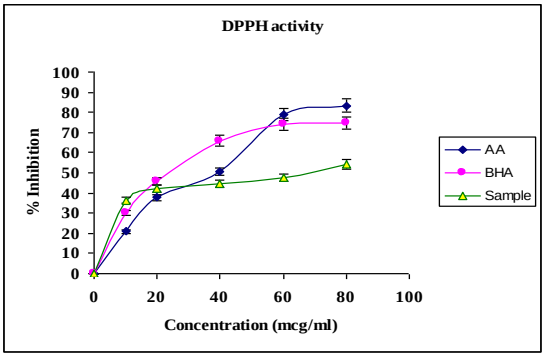

Fig. 3: Percentage inhibition vs. Concentration plot for Formulation, Ascorbic acid and BHA by DPPH free radical method.

In reducing power assay, there was an increased in the absorbance with an increase in the concentration of formulation, which indicates the reducing capacity of Formulation. The results are shown in [Table 11]. The Absorbance vs. Concentration plot for Formulation, Ascorbic acid and BHA by reducing power assay is shown in [Figure 7].

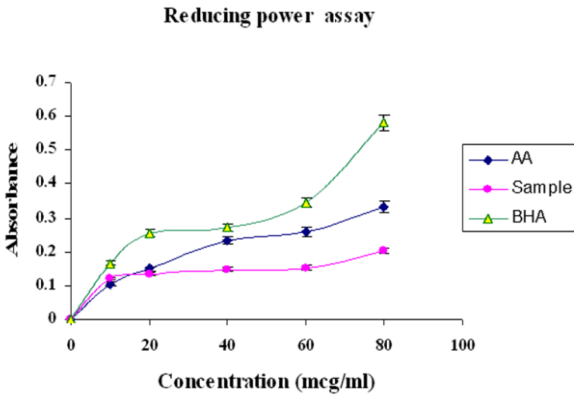

Fig.4: Absorbance vs. Concentration plot for Formulation, Ascorbic acid and BHA by reducing power assay

In nitric oxide free radical scavenging assay, there was a decreased in the absorbance with increase in concentration, which was comparable with the standard ascorbic acid and BHA.

The Percentage inhibition vs. Concentration plot for Formulation, Ascorbic acid and BHA by Nitric oxide free radical scavenging method is shown in [Figure 8].

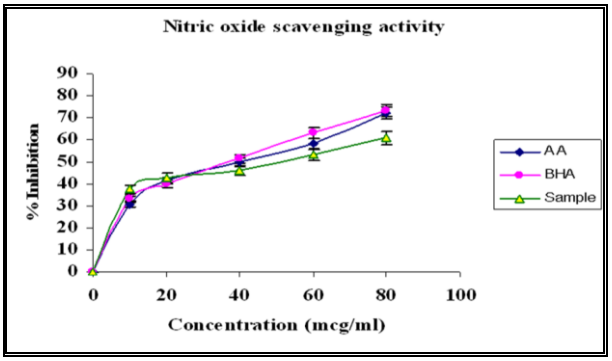

Fig. 8: Percentage inhibition vs. Concentration plot for Formulation, Ascorbic acid and BHA by Nitric oxide free radical scavenging method

Table 8: Data for antioxidant activity by Anti- lipid peroxidation method

Test compound	Conc. (µg/ml)	Absorbance A1	A2	A3	Mean ± SEM
Formulation	10	0.237	0.243	0.245	0.241±0.002
	20	0.232	0.235	0.229	0.232±0.001
	40	0.217	0.220	0.221	0.219±0.001
	60	0.189	0.185	0.183	0.185±0.001
	80	0.166	0.169	0.165	0.166±0.001
Ascorbic acid	10	0.409	0.408	0.405	0.407±0.001
	20	0.361	0.364	0.366	0.364±0.001
	40	0.273	0.274	0.273	0.274±0.000
	60	0.202	0.204	0.208	0.205±0.001
	80	0.184	0.181	0.180	0.181±0.001
Butylated hydroxyl anisole	10	0.163	0.162	0.161	0.162±0.000
	20	0.137	0.136	0.134	0.136±0.000
	40	0.133	0.134	0.132	0.133±0.000
	60	0.125	0.127	0.124	0.125±0.000
	80	0.123	0.122	0.126	0.124±0.001

Table 9: Percentage Inhibition and IC_{50} values for Formulation and standard drugs by Anti- lipid peroxidation method

Sr. No	Test compound	Conc. (µg/ml)	% Inhibition	IC_{50}
1.	Formulation	10	49.54	
		20	51.56	
		40	54.21	58.73
		60	61.23	
		80	65.20	
2.	Ascorbic acid	10	15.03	
		20	24.00	
		40	43.00	30.28
		60	57.00	
		80	62.21	
3.	Butylated hydroxyl anisole	10	66.18	
		20	71.60	
		40	72.23	57.33
		60	73.90	
		80	74.11	

Table 10: Data for antioxidant activity by DPPH free radical method

Test compound	Conc. (µg/ml)	Absorbance A1	A2	A3	Mean ± SEM
Formulation	10	0.400	0.411	0.408	0.406±0.003
	20	0.370	0.368	0.370	0.369±0.000
	40	0.350	0.355	0.358	0.354±0.002
	60	0.330	0.334	0.337	0.333±0.002
	80	0.290	0.296	0.291	0.292±0.001
Ascorbic acid	10	0.504	0.508	0.501	0.504±0.002
	20	0.398	0.392	0.397	0.396±0.001
	40	0.312	0.317	0.315	0.315±0.001
	60	0.13	0.134	0.138	0.134±0.002
	80	0.107	0.104	0.106	0.106±0.000
Butylated hydroxyl anisole	10	0.443	0.445	0.449	0.446±0.001
	20	0.342	0.347	0.345	0.345±0.001
	40	0.217	0.218	0.216	0.217±0.000
	60	0.162	0.161	0.165	0.163±0.001
	80	0.161	0.165	0.160	0.162±0.001

Table 11: Data for antioxidant activity by Reducing Power Assay

Test compound	Conc. (µg/ml)	Absorbance A1	A2	A3	Mean ± SEM
Formulation	10	0.118	0.120	0.122	0.120±0.001
	20	0.132	0.138	0.133	0.134±0.001
	40	0.144	0.149	0.151	0.148±0.002
	60	0.155	0.150	0.156	0.153±0.001
	80	0.208	0.201	0.204	0.204±0.002
Ascorbic acid	10	0.102	0.105	0.109	0.105±0.002
	20	0.151	0.152	0.149	0.150±0.000
	40	0.231	0.235	0.239	0.235±0.002
	60	0.263	0.260	0.255	0.259±0.002
	80	0.331	0.329	0.339	0.333±0.003
Butylated hydroxyl anisole	10	0.165	0.162	0.168	0.165±0.001
	20	0.259	0.251	0.258	0.256±0.002
	40	0.275	0.270	0.272	0.272±0.001
	60	0.344	0.344	0.342	0.345±0.000
	80	0.582	0.578	0.586	0.582±0.002

CONCLUSION

From the results & discussion we can conclude that Rhumapar tablet formulation shows significant anti-inflammatory effect in carrageenan induced paw edema and cotton pellet induced granuloma models in rat in dose dependant manner. It also shows significant anti arthritic effect against FCA induced arthritis in rat. It also shows significant anti-oxidant effect in different models of anti oxidant activity.

ROI produced by activated phagocytes in the inflamed joints have been implicated along with prostanoids, leukotrienes and proteases, as mediators of inflammation and the pathogenesis of tissue destruction. Many drugs commonly used in the day to day treatment of rheumatoid arthritis are believed to mediate their therapeutic actions by multiple mechanisms, one of them being suggested is a reduction of oxidant damage at sites of inflammation by drugs either acting as ROI scavengers or inhibitors of ROI production by phagocytes. [1],[7]

As the number of herbal preparations that increasing for the treatment of RA ,further research is needed to examine not only the efficacy of these treatments but also the safety and potential drug interactions of the herbal preparations. Outcome measures should include disability, joint pain and swelling, pain, and both patient and physician global assessment.

REFERENCES

1. Borris P. J. Ethno pharmacol. 1996; 51:29.
2. Achike FI, Kwan CY. Nitric oxide, human diseases and the herbal products that affect the nitric oxide signalling pathway. Clin Exp Pharmacol Physiol 2003; 30: 605-15.
3. Aruoma OI. Use of DNA damage as a measure of pro-oxidant actions of antioxidant food additives and nutrient components.1993: 315–327.
4. Barbier A, Novarro JC, Brelliere RR. Biochemical and clinical changes in rats with the developing arthritis. 1984; 15:103-105.
5. Chattopadhyay P, Besra SE, Gomes A. *Life Sciences* 2004; 74:1839-1849.
6. Chaudhari SC, Chaudhari SR, Chavan MJ. Asian Pacific Journal of Trophical Biomedicine. 2012:S181-S186.
7. Feldman DL, Mogelesky TC, Sharif R, Sawyerw JM. The in vitro and ex vivo antioxidant properties and hypolipidemic activity of CGP 2881.1999; 144: 343–355.
8. Ilavarasan R, Mallika M. *Afr. J. Trad. Cam.* 2005; 2: 70 – 85.
9. Krane SM, Conca W, Stephenson MI, Amento EP, Goldring. *Annals of the New York Academy of Sciences.* 1990; 580: 340–354.
10. Kumar RS, Sivakumar T, Sunderam RS, Gupta M, Mazumdar UK. *Brazilian Journal of Medical and Biological Research*.2005; 38:1015-1024.
11. Mondal SK, Muzumder UK. *Indian J Experimental Biology.* 2006; 44: 39-44.
12. Oyaizu M. *Jpn. J. Nutr.* 1986; 31: 307-315.
13. Patwardhan B, Gautam M. *Drug Discov Today*.2005; 7: 495–502.
14. Prakash BN, Pandikumar P, Ignacimuthu S. *J Ethno pharmaco.* 2009; 125: 356-360.
15. Rajlakshmi D, Banerjee SK. *Journal of Pharmacy and Pharmacology.* 2003; 55:1681-1686.
16. Shinde UA, Phadke AS, Nair AM, Mungantiwar AA, Dikshit VJ, Saraf MN. *J. Ethano Pharmaco.* 1999; 65:21-27.
17. Singh S, Majumdar DK, Rehan HMS. *J. Ethno Pharmaco.* 1998; 54: 19-26.
18. Speroni E, Cervellati R, Innocenti G, Costa S, Guerra MC, Govoni, P. *J. Ethano pharmacol.* 2005; 98:117-125.
19. Sreejayan, Rao MN. *Journal of Pharmacy and Pharmacology*.1997; 49: 105-107.
20. Thangam C, Dhananjayan R. *Indian J. Pharmacol.* 2003; 35: 388-391.
21. Yen.V. *J. Agri. Food Chem.* 1997; 45:30-34.
22. Yin W, Wang TS, Yin FZ, Cai BC. *J Ethno pharmacol.* 2003; 88:205-214.
23. Khandelwal K.R. *Practical Pharmacognosy techniques and experiments*. 6th ed. Nirali Prakashan, Nasik, 1999: 23-28.
24. Kokate, C.K. *Experimental Pharmacognosy* 1st ed. Nirali Prakashan, New Dehli, 1985:24-32.