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Anti-inflammatory, antiarthritic and analgesic activity of a herbal formulation (DRF/AY/4012)

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Anti-inflammatory, antiarthritic and analgesic effect of a herbal product (DRF/AY/4012) was evaluated in animal models. Herbal product treatment induced a dose dependent anti-inflammatory activity in acute inflammatory models (carrageenin and egg–albumin induced rat hind paw edema). It also elicited promising anti-inflammatory activity in chronic inflammatory models (cotton pellet granuloma and Freund's adjuvant induced polyarthritis in rats). Further, the product inhibited the increased level of serum lysosomal enzyme activity *viz*. serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, alkaline phosphatase and the lipid peroxidation in liver. In Freund's adjuvant induced polyarthritis, herbal product reduced the increased level of hydroxy proline, hexosamine and total protein content in edematous tissue .The product also exhibited mild to moderate analgesic activity in acetic acid induced writhing in mice. The LD_{50} value of the herbal product was more than 16gm/kg by oral route in mice. The product has distinct advantages over the existing agents and deserves further developmental studies.

Keywords: Analgesic effect, Antiarthritic, Anti-inflammatory, Biochemical parameters

As the result of the inherent problems associated with the current non-steroidal as well as steroidal anti-inflammatory agents, there is continuous search especially from natural sources for alternative agents[1]. Large numbers of herbal extract as well as products being employed in the treatment of inflammatory disorders by natural healers. Herbal extracts of Nirgundi (*Vitex nigundo*), Rasana Patti (*Alpinia galanga*), Prasarini (*Merremmia tridentate*), *Commiphora mukul* and *Boswellia serrata*, the constituents of the herbal product used in the present study are used in folk medicine for joint pain, sprain, inflammatory condition and arthritis including analgesic activities. Anti-inflammatory activity of the some of the plants extracts used in the product have also been used in many other disorders such as asthma, hypolipidemic, chronic laryngitis, cough and many others[2,3].With this background of information the effect of the herbal product has been evaluated on various animal models of inflammation such as carrageenin and egg–albumin induced rat hind paw edema, cotton pellet granuloma and Freund's adjuvant polyarthritis including the analgesic activity.

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Materials and Methods

Animals—Albino mice of Swiss strain weighing 20-30g and Wistar strain rats weighing 110-150g were obtained from Haffkine Institute, Mumbai and Bharat Sera and Vaccines, Thane, respectively. The animals were housed in the groups of 6-8 in plastic cages. The animal house conditions maintained were—25° ± 1°C : 65 ± 10% RH and 10:14 hr L:D cycle. Animals were fed with Amrut brand pelleted standard diet manufactured by Nav Maharashtra Chakan Oil Mils, Ltd., and drinking water *ad libitum*. The animals were allowed to adapt to college's animal house conditions by keeping them for 8-10 days period prior to the experiments.

In all undertaken experiments, 5 groups of animals were taken *viz*. control, standard and three test groups, at different dose levels and 6 animals were used in each group.

The experimental design and research plan along with animals handling and disposal procedure were placed before the animal ethics committee. The committee granted approval after carefully evaluating research project.

Chemicals—Carrageenin and egg albumin were from Sigma Chemical Co., St.Louis, MO. USA, and complete Freund's adjuvant from Diclo Lab., Detroit, MI, USA. SGOT, SGPT, ALP kits were from Span Diagnostics, Surat India. Hydroxyproline (Spectrochem Pvt.Ltd. Mumbai, India). glucosamine (Sisco Labs. Mumbai India) and diclofenac sodium (Themes pharmaceuticals. Mumbai India) were used. The herbal product (DRF/AY/4012) was from Dabur Research Foundation, Ghaziabad, U.P. India.

Anti-inflammatory anti-arthritic activity evaluation

Effect on carrageenin induced hind paw edema in rat[4]—In this test 0.1 ml of 1 % (w/v) carrageenin was injected, s.c., into the planter region of the hind paws of rat.

Effect on egg-albumin induced hind paw edema in rat [5]—Albumin edema was induced by injecting, sc, 0.1 ml of 2% (w/v) bovine albumin prepared in normal saline, into the planter region of the hind paws of rats.

Diclofenac sodium was used as standard anti-inflammatory drug for comparison. The herbal product was administered at different doses viz., 200, 400.800 mg/kg, orally, and diclofenac sodium (15 mg/kg, po) one hr prior to carrageenin and egg-albumin injection.

The paw edema volumes were measured using plethysmometer at various time intervals like 1, 3, 6, 12 and 24 hr after carrageenin and egg-albumin injection. Results were expressed as the percentage inhibition of edema by comparing with the control group.

The anti-inflammatory activity of herbal product was studied at different doses along with various relevant biochemical changes.

Cotton pellet granuloma[6]—Four sterilized cotton pellets each weighing 10 mg were implanted 2 on either side of the ventral region of rats .Cotton pellet inserted rats were randomly divided into 5 groups of 6 rats /group. Different group of rats were treated with herbal product at various doses (200, 400, 800 mg/kg) and diclofenac sodium (15mg/kg) orally, daily for 8 days. The control group received only vehicle, carboxymethylcellulose (CMC; 1 ml/100 gm). On the 9th day, cotton pellets were removed and dried at 60°C for 6 hr. The dry weight was calculated after deducting cotton pellet weight and taken as a measure of granuloma tissue formation.

Freund's adjuvant induced poly arthritis[7]—Rats were injected, sc, 0.1 ml of complete Freund's adjuvant into the planter region of the left hind paw. The changes in the paw volume (left and right) were measured on various days up to 21 days following Freund's adjuvant injection. The herbal product at (200, 400, 800 mg/kg/day) and diclofenac sodium at (15 mg/kg/alternate day) doses were administered orally for 14 days from the day of Freund's adjuvant injection. On the 21st day rats were anaesthetized with diethyl ether and edematous tissue were isolated from the injected hind paw. Blood was withdrawn by cardiac puncture of all the groups. Various biochemical and hematological parameters were estimated in tissue and blood, respectively.

Biochemical estimation—In the preliminary experiments especially carrageenin and egg albumin edema, the biochemical changes observed were maximum at 6 hr as compared to 12 and 24 hr. Hence, biochemical changes were estimated in carragenin and egg albumin hind paw edema at 6 hr only. The biochemical changes in groups of rats of cotton pellet granuloma and Freund's adjuvant polyarthritis experiments were estimated on 9th and 21st day respectively. The rats were anaesthetized under light ether anesthesia and blood was collected by the cardiac puncture for biochemical estimation. Serum was separated from the blood samples and SGOT, SGPT, ALP were determined by the colorimetric method[8, 9] using kits.

Liver was removed and liver per oxidation was measured in liver homogenates by the method of Ohkawa[14]. The percentage inhibition of lipid per oxidation by the test compound or standard anti-inflammatory drug (diclofenac sodium) was calculated by using the formula

$[(A-B) / B] \times 100$; where A represents the control and B represent the diclofenac sodium or herbal product.

The hind paw edematous tissue was separated and hydroxy proline [9,] hexosamine[10, 11] and total protein content[12] were also assayed.

Analgesic activity evaluation

Acetic acid induces writhing in mice[15]—A group of mice were administered 0. 1 ml /10 g of 0.3 %(v/v) acetic acid *ip*. The mice exhibiting the writhing episodes (stretching of hind limbs and bending of trunk) were selected for the study. These mice were randomly divided into various group (6/group). These mice were administered herbal product at different dose (200, 400, 800 mg/kg) and diclofenac sodium

(15 mg/kg) orally 1hr prior the acetic acid injection. The numbers of writhing episodes were counted for 30 min following acetic acid administration.

Toxicity testing—Acute toxicity studies were carried out in mice and LD_{50} was calculated as per Wilcoxon and Litchfield[29]. No mortality was observed upto the dose of 16 gm/kg in mice.

Statistical analysis—All experimental data are expressed as mean ± SE. Statistical analysis were carried out by using one way ANOVA. The level of significance was calculated using Student's '*t*' test. The values at $P < 0.05$ were considered as significant.

Results

The herbal product treatment at 400 and 800 mg/kg, po**,** reduced carrageenin edema formation both at 12 and 24 hr significantly. The greater degree of inhibition in edema formation was observed at 24 hr as compared to 12 hr interval. Whereas diclofenac sodium treatment induced inhibition in edema formation at 3 and 6 hr significantly. The edema inhibition observed was greater at 6 hr interval. (Table 1)

Herbal product treatment at 400 and 800 mg/kg orally inhibited egg albumin edema formation both at 12 and 24hr significantly. The edema inhibition was greater at 24 hr at both the doses. (Table 2). However, diclofenac sodium treatment reduced edema both 3 and 6 hr significantly. The edema inhibition was greater at 6 hr as compared to 3 hr interval (Table 2)

In cotton pellet granuloma, the herbal product treatment orally inhibited both exudatory as well as granulatory phases of inflammation. The degree of anti-inflammatory activity observed was almost same at 400 & 800 mg/kg doses. Diclofenac sodium also elicited inhibitory effect on both exudatory and granulatory phases of inflammation (Table 3).

Table 1—Effect of herbal formulation on carrageenin induced hind paw edema in rats
[Values are mean ± SE from 6 animals in each group]

Treatment and dose (mg/kg, po)	Edema volume (manometer reading) Time interval (hr)					
	0	1	3	6	12	24
Control	0.12 ± 0.002	0.18 ± 0.002	0.2 ± 0.003	0.18 ± 0.003	0.16 ± 0.003	0.15 ± 0.003
DRF/AY/4012 (200)	0.12 ± 0.001	0.17 ± 0.002	0.19 ± 0.002	0.17 ± 0.002	0.15 ± 0.002 *	0.14 ± 0.002
DRF/AY/4012 (400)	0.12 ± 0.004	0.17 ± 0.004	0.18 ± 0.004	0.17 ± 0.004	0.15 ± 0.003*	0.13 ± 0.004 *
DRF/AY/4012 (800)	0.12 ± 0.002	0.16 ± 0.003*	0.17 ± 0.004**	0.16 ± 0.004**	0.14 ± 0.002**	0.13 ± 0.004*
Diclofenac Sodium (15)	0.12± 0.002	0.16 ± 0.003*	0.16 ± 0.002**	0.15 ± 0.004**	0.15 ± 0.002*	0.14 ± 0.003

One way ANOVA is applied for statistical analysis
P values: * < 0.05; ** < 0.001

Table 2—Effect of herbal formulation on egg albumin induced hind paw edema in rats
[Values are mean ± SE from 6 animals in each group]

Treatment and dose (mg/kg po)	Time interval (hr.)					
	0	1	3	6	12	24
control	0.13 ± 0.01	0.21 ± 0.003	0.19 ± 0.003	0.17 ± 0.003	0.16 ± 0.002	0.15 ± 0.002
DRF/AY/4012 (200)	0.13 ± 0.01	0.2 ± 0.003	0.18 ± 0.003	0.16 ± 0.004	0.15 ± 0.002	0.14 ± 0.002 *
DRF/AY/4012 (400)	0.128 ± 0.01	0.2 ± 0.004*	0.17 ± 0.005**	0.15 ± 0.004**	0.14 ± 0.002**	0.14± 0.002*
DRF/AY/4012 (800)	0.128 ± 0.01	0.19 ± 0.004**	0.17 ± 0.002**	0.15 ± 0.004**	0.14 ± 0.002**	0.13 ± 0.003**
Diclofenac Sodium (15)	0.134 ± 0.01	0.2± 0.01 *	0.18 ± 0.003	0.16 ± 0.003	0.16 ± 0.004	0.15 ± 0.005

One way ANOVA is applied for statistical analysis
P values: * < 0.05; ** < 0.001

In adjuvant arthritis, the herbal product treatment prophylactically decreased hind paw edema formation significantly in injected paw. However, the effect on uninjected paw was less significant (Fig. 1)

Biochemical parameters, SGOT, SGPT, ALP in serum and lipid peroxidation in liver were elevated significantly in carrageenin and egg albumin induced hind paw edema. Pretreatment with herbal product or diclofenac sodium prevented significantly the increased level of these biochemical parameters (Tables 4 and 5)

In cotton pellet granuloma experiment too, the increased SGOT, SGPT, ALP and lipid per oxidation were significantly prevented with the concomitant treatment of herbal product as well as diclofenac sodium. (Table 6)

In Freund's adjuvant arthritis hematological and biochemical changes were investigated in blood and edematous tissue. The elevated levels viz. hydroxy proline, hexosamine and protein observed in edematous tissue during Freund's adjuvant arthritis were significantly inhibited by the herbal product and diclofenac sodium treatment. The herbal product showed dose related inhibition of these biochemical parameters. Diclofenac sodium (15 mg/kg) treatment prevented both hematological as well as biochemical changes in blood and edematous tissue to a greater extent than herbal product. (Table 7) Herbal product (400 & 800 mg/kg) exhibited mild to moderate

Table 3—Effect of herbal formulation on cotton-pellet granuloma in rats
[Values are mean ± SE from 6 animals in each group]

Treatment and dose (mg/kg po.)	Granuloma wet wt. (gm)	Granuloma dry wt. (gm)
Control	0.14 ± 0.003	0.04 ± 0.001
DRF/AY/4012 (200)	0.1 ± 0 .002**	0.03 ± 0.003 **
DRF/AY/4012 (400)	0.1 ± 0 .002**	0.03 ± 0.002**
DRF/AY/4012 (800)	0.1 ± 0.002**	0.02 ± 0.001 **
Diclofenac Sodium (15)	0.07 ± 0 .001**	0.02 ± 0.001**

One way ANOVA is applied for statistical analysis
P values: * < 0.05; ** < 0.001

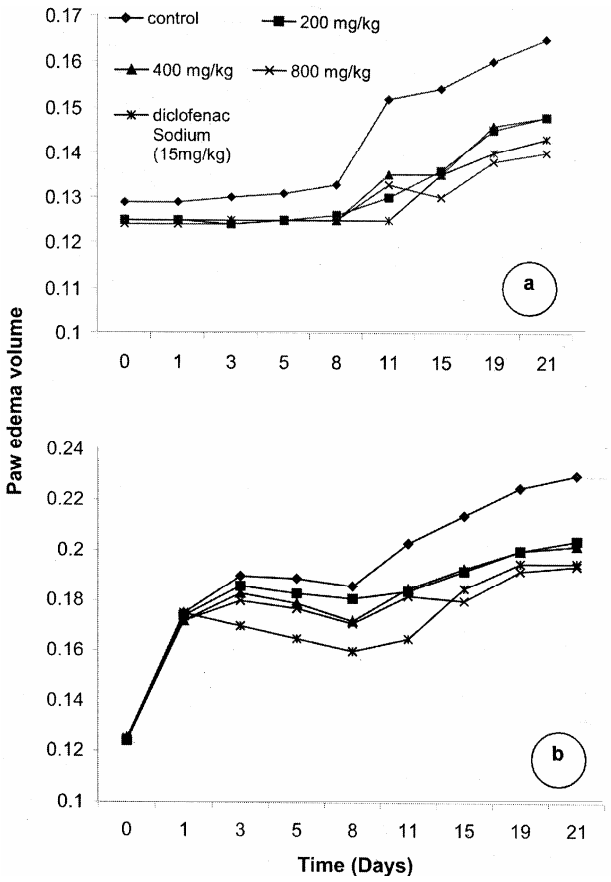

Fig. 1—Effect of herbal product and diclofenac sodium (mg/kg) alternative days for 14 days on adjuvant arthritis induced by complete Freund's Adjuvant in uninjected (a) and injected (b). paw

Table 4—Effect of Herbal formulation on various biochemical changes in carrageenin induced hind paw edema in rats.
[Values are mean ± SE from 6 animals in each group]

Treatment and dose(mg/kg,po.)	SGOT (U/ ml)	SGPT (U/ ml)	Lipid peroxidation (%)	Alkaline phosphatase (U/ml)
Control	112 ± 3.22	78 ± 2.15	100	75 ± 1.39
DRF/AY/4012(200)	82 ± 2.48**	65 ± 1.39**	83 ± 2.27**	70 ± 1.69
DRF/AY/4012(400)	76 ± 2.15**	55 ± 1.29**	70 ± 1.7**	65 ± 1.24**
DRF/AY/4012(800)	70 ± 2.1**	45 ± 0.93**	58 ± 1.53**	63 ± 1.88**
Diclofenac Sodium (15)	65 ± 1.7**	40 ± 1.12**	63 ± 1.88**	60 ± 0.97**

One way ANOVA is applied for statistical analysis
P values: * < 0.05; ** < 0.001

analgesic activity. Diclofenac sodium showed marked analgesic activity in acetic acid induced \writhing mice model. (Table 8).

Discussion

Carrageenin edema is simple, rapid and gives reliable result with most of clinically active anti-rheumatic drugs[16]. The polyherbal preparation elicited greater anti-inflammatory activity in egg- albumin induced paw edema as compared to the carrageenin induced paw edema (Tables 1 and 2). The delayed effect of herbal product on carrageenin and albumin edema at 12 and 24 hr may be due to slow absorption of the herbal products constituents or due to their metabolites.

There is an increasing evidence that lysosomal enzymes play an important role in the development of acute and chronic inflammation[17-20]. Most of the anti-inflammatory drugs exert their beneficial effect by inhibiting either release of lysosomal enzymes or by

Table 5—Effect of herbal formulation on various biochemical changes in egg albumin induced hind paw edema in rats.
[Values are mean ± SE from 6 animals in each group]

Treatment and dose (mg/kg, po)	SGOT (U/ml)	SGPT (U/ml)	Lipid peroxidation (%)	Alkaline phosphatase (U/ml)
control	99.5 ± 1.98	60 ± 1.81	100	109± 1.23
DRF/AY/4012 (200)	78 ± 2.15**	33 ± 0.96**	81 ± 2.65**	98 ± 1.27**
DRF/AY/4012 (400)	69 ± 0.98**	27 ± 0.82**	65 ± 1.54**	86 ± 1.86**
DRF/AY/4012 (800)	66 ± 1.65**	24 ± 0.77**	50 ± 1.6**	80 ± 1.2**
Diclofenac Sodium (15)	62 ± 1.6**	23 ± 0.93**	59 ± 1.93**	70 ± 1.17**

One way ANOVA is applied for statistical analysis
P values: * < 0.05; ** < 0.001

Table 6—Effect of herbal formulation on various biochemical changes in cotton-pellet granulomma in rats.
[Values are mean ± SE from 6 animals in each group]

Treatment and dose (mg/kg po.)	SGOT (U/ ml)	SGPT (U/ ml)	Lipid peroxidation (%)	Alkaline phosphatase (U/ ml)
Control	104 ± 3.23	47 ± 1.23	100	72 ± 2.51
DRF/AY/4012 (200)	98 ± 3.02	29 ± 1.26**	83 ± 2.52**	63 ± 2.51**
DRF/AY/4012 (400)	91 ± 1.28**	26 ± 1.26**	68 ± 2.21**	60 ± 2.02**
DRF/AY/4012 (800)	88 ± 1.39**	24 ± 1.8**	66 ± 2.29**	59 ± 2.38**
Diclofenac Sodium (15)	75 ± 0.76**	25 ± 1.15**	75 ± 2.71**	57 ± 1.18**

One way ANOVA is applied for statistical analysis
P values: * < 0.05; ** < 0.001

Table 7—Effect of herbal formulation on various hematological & biochemical parameters during freund's adjuvant induce polyarthritis.
[Values are mean ± SE from 6 animals in each group]

	Herbal formulation (mg/kg, po)				Diclofenac sodium (15 mg/kg PO)
Parameter	Control	DRF/AY/4012 (200)	DRF/AY/4012 (400)	DRF/AY/4012 (800)	
ESR (mm) (after 1 hr)	7 ± 1.06	5 ± .94	4.5 ± .91*	3 ± .83**	2.1 ± 0.02**
Total WBC count (cu.mm)	11000 ± 526	8400 ± 460**	8100 ± 490**	8000± 440**	8300 ± 435**
Lymphocyte (%)	52 ± 4.56	35 ± 4.2 **	31 ± 3.34 **	28 ± 3.56 **	44 ± 3.75 **
Neutrophil (%)	55 ± 3.4	60 ± 4.2	70 ± 5.1	75 ± 4.3	60 ± 6.1
Haemoglobin (%)	75 ± 4.4	80 ± 4.1	84 ± 5.8	90 ± 5.5 *	80 ± 4.55%
RBC (million/c.mm)	3.4 ± 0.3	4 ± 0.43	4.2± 0.4	4.3 ± 0.45	3.9 ± 0.5
Total protein (gm%)	8.1 ± 0.045	7.5 ± 0.09	7 ± 0.05 *	6.8 `± 0.08*	5.5 ± 0.04 *
Hydroxy proline (μg/gm)	340±23	290±25	270±20*	245±26*	210±15**
Hexosamine (μg/gm)	1300 ± 25.6	925±27	895 ± 22.5*	850± 20. *	725 ± 15.6**

One way ANOVA is applied for statistical analysis
P values: * < 0.05; ** < 0.001

Table 8—Effect of herbal formulation on acetic acid induced writhing in mice
[Values are mean ± SE from 6 animals in each group]

Time (min)	Control	DRF/AY/4012 (200 mg/kg, po)	DRF/AY/4012 (400 mg/kg, po)	DRF/AY/4012 (800 mg/kg,po)	Diclofenac Na (15 mg/kg, po)
5-10	14.83 ± 0.83	13.66 ± 0.61	12.83 ± 0.31*	11.83 ± 0.31**	7.6 ± 0.48**
10-20	18 ± 0.73	16 ± 0.85	13.83 ± 0.42**	13 ± 0.68**	9 ± 0.51**
20-25	5.67 ± 0.67	5 ± 0.58**	4.67 ± 0.42**	4.5 ± 0.42**	2.17 ± 0.31**
Total	38.5 ± 2.13	34.33 ± 1.76	31.33 ± 1.62**	30.33 ± 0.9**	18.33 ± 0.82**

One way ANOVA is applied for statistical analysis
P values: * < 0.05; ** < 0.001

stabilizing lysosomal membrane which is one of the major event responsible for the inflammatory process.[21] In carrageenin and egg-albumin induced hind paw edema and cotton pellet granuloma animal models, herbal product treatment significantly decreased the increased level of lysosomal enzyme activity *viz*., transaminase (namely SGOT, SGPT) and alkaline phosphatase.

The changes in connective tissue metabolism are one of the major biochemical events during the process of inflammation. These changes are effected in alteration of relative composition of various constituents of connective tissue such as mucopolysaccharides, glycoprotein, hexosamine and sialic acid.[22-24]. The results clearly showed that following the treatment of herbal product and the standard drug inhibited increased content of hydroxy proline and hexosamine in edematous tissue of adjuvant arthritis.

Lipid peroxidation process was increased during inflammatory conditions [25]. Treatment with herbal product inhibited lipid peroxidation process significantly in carrageenin and egg –albumin induced rat hind paw edema and cotton pellet granuloma animal model. This effect of the herbal product may be correlated to its antioxidant activity.

In order to assess its efficacy against proliferative phase of inflammation we have selected cotton pellet granuloma animal model in which tissue degeneration and fibrosis occurs. During the repair process of inflammation, there is proliferation of macrophages, neutrophils, fibroblasts and multiplication of small blood vessels, which are the basic sources of forming a highly vascularised reddish mass, termed granulation tissue.[27,28] The herbal product treatment (400 and 800mg/kg, *po*) moderately inhibited the granuloma formation. The effect of herbal product was lower as compared to diclofenac sodium (Table 3).

The herbal formulation elicited mild to moderate analgesic activity in acetic acid induced writhing in mice (Table 8).

In Freund's adjuvant arthritic rat model, treatment with herbal product showed significant inhibitory effect on injected hind paw edema and maximum inhibition was observed on the 21st day (Fig. 1). In the present study the increased lymphocyte count and migration of leucocytes into the inflamed area of arthritic rats were significantly prevented with the treatment of the herbal product and the standard drug as reflected from the significant decrease in total WBC count[26]. The erythrocyte sedimentation rate (ESR) level which was markedly elevated in arthritic control group of rats was decreased significantly with herbal product at higher dose (800 mg/kg) and the effect was comparable to standard drug (Table 7).

The present experimental findings of both the pharmacological and biochemical parameters suggest that herbal product is a promising anti-inflammatory agent of plant origin in the treatment of inflammatory disorders and conditions. Hence, it is necessary to evaluate its anti-inflammatory activity on humans in clinical conditions.

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