Original Article

Evaluation of the anti-arthritic activity of Rhuflex-F – A proprietary Ayurvedic herbomineral formulation in albino rats

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Abstract

Background: Rhuflex-F is a proprietary Ayurvedic herbo-mineral formulation clinically used to combat and relieve stiffness in joints and muscles, reduce edema, restore mobility, and also effective in relieving the symptoms of other autoimmune illnesses that lead to rheumatism. **Aims:** The aim and objective of the research study is to evaluate the efficacy of Rhuflex-F against *in vitro* protein denaturation and *in vivo* Freund's adjuvant-induced arthritis in albino rats. **Materials and methods:** *In vitro* inhibition of protein denaturation activity was carried out using bovine serum albumin. For *in vivo* activity, arthritis was induced by complete Freund's adjuvant in albino rats. Rhuflex-F (135–270 mg/kg, po) was administered for 30th days in arthritic rats, and effects were assessed on primary and secondary paw edema, on pain response, hematological, serum biochemical parameters (serum transaminases, alkaline phosphatase, urea, uric acid, and orosomucoid), and serum anti-oxidant parameters and adrenal ascorbic acid. **Results:** Aqueous extract of Rhuflex-F showed *in vitro* protein denaturation inhibitory activity in a dose-dependent manner. Rhuflex-F showed nonsignificant decrease in primary and secondary paw edema with reduced pain response, some reversal effects on hematological parameters such as white blood cell and red blood cell related parameters and serum orosomucoid and adrenal ascorbic acid in comparison to Fruend's adjuvant control group. Further, Rhuflex-F reversed Freund's adjuvant-induced adverse effects on oxidant status in the serum of albino rats. **Conclusion:** Result of the present study suggested that Rhuflex-F formulation has anti-inflammatory activity, may be due to the inhibition of protein denaturation *in vitro* and *in vivo* anti-arthritic activity against complete Freund's adjuvant-induced arthritis in albino rats.

Keywords: Anti-arthritic activity, anti-inflammatory, complete Freund's adjuvant, dexamethasone, Rhuflex-F

Introduction

In modern, rheumatoid arthritis (RA) is an autoimmune and chronic inflammatory disease of joints characterized by inflammatory cell infiltration, proliferation of synovial tissue, and bone destruction.[1] The production of auto-antigens in certain arthritis diseases may be due to *in vivo* denaturation of proteins. Denaturation of tissue protein is one of the known causes of inflammation and arthritic diseases. Hence, by controlling the production of auto-antigen, inhibiting denaturation of protein and membrane lysis prevent the progression of rheumatic disease and associated inflammatory conditions.[2]

The common side effects of marketed and anti-inflammatory and anti-arthritic drugs are stomach ulcers, gastrointestinal tract bleeding, kidney damage, liver damage, hypertension, etc. Non-steroidal anti-inflammatory drugs (NSAIDs) are consumed massively worldwide and, along with antimicrobial agents, are the most frequent causes of drug-induced liver injury.[3] Hence, the uses of Ayurvedic medicine may play an important therapeutic role in eradicate or minimize the symptoms of RA. Rhuflex-F capsule is a proprietary Ayurvedic herbo-mineral formulation clinically used to get relief in the stiffness in joints and muscles, edema, restore mobility, and also in relieving the symptoms of other autoimmune illnesses that lead to rheumatism. The individual components of the Rhuflex-F capsule are well known for their anti-inflammatory and anti-arthritic activity and reported in the classics; however, they are not used in combination used in the Rhuflex-F.

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Therefore, to prove the scientific claim, the present study was planned with an aim to evaluate the efficacy of Rhuflex-F against *in vitro* protein denaturation and *in vivo* Freund's adjuvant-induced arthritis in albino rats.

Materials and methods

Wistar strain albino rats of either sex weighing between 200 ± 20 g were used in the research study. The animals were reared under standard experimental conditions of temperature (22 ± 03°C), relative humidity (50%–70%), and 12 h light and hour-dark cycles in the animal house attached to the Institute of Teaching and Research in Ayurveda, Jamnagar. The selected animals were kept under acclimatization for 1 week before the administration of Rhuflex-F and the standard drug, dexamethasone. The animals were exposed to the same environmental conditions throughout the experimental period. VRK brand standard rat pellet feed was supplied by Keval Sales Corporation, Vadodara, and drinking water was given *ad libitum*. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC/24/2018/19) in accordance with the guidelines formulated by CPCSEA, India.

The trial sample, Rhuflex-F, is herbomineral proprietary Ayurvedic compound formulation supplied by Zoetic Ayurvedics Pvt., Ltd., Ahmedabad. Name of ingredients, specific names, parts used, and quantity of the drug are given in Table 1. Rhuflex-F powder form was used in the experimental study. Fruend's complete adjuvant (FCA) was purchased from Sigma-Aldrich (product no. F5881). Dexamethasone (batch no. CBU1216) was obtained from Cadila Healthcare Limited, Ahmedabad. Other chemicals of analytical grade were purchased from standard reputed firms to obtain accurate results.

Table 1: Ingredients of Rhuflex-F capsule

Ingredients	Scientific name	Part used
Rasasindur	Red sulphide of hydrargiry	Mineral
Suddha somala	Purified *Oxidum arsenicum*	Mineral
Nag bhasma	Calcined plumbum	Calcined plumbum
Sankh bhasma	Calcined *Turbinella rapa* shells	Calcined shell
Suddha guggul	Purified *Commiphora mukul*	Purified *Niryas*
Yavani	*Trachyspermum ammi* (L.) Sprague	Fruit powder
Shatahva	*Anthem graveolens* Linn.	Fruit powder
Vidanga	*Embellia ribes* Burn.	Fruit powder
Kababchini	*Piper cubeba* Linn.	Fruit powder
Chopchini	*Smilex china* Linn.	Wood powder
Devdaru	*Cedrus deodara* (Roxb.) Loud.	Wood powder
Pushkarmool	*Inula racemosa* Hook.	Root powder
Vishvabhesaj	*Zingiber officinale* Roscoe.	Rhizome powder
Aswagandha	*Withania somnifera* (L.) Dunal	Root powder
Processed with decoction of		
Abhaya	*Terminalia chebula* Retz.	Fruit
Vrudhadaruk	*Rourea santaloides* (Vahl) Wight and Arn.	Root

The rat therapeutic equivalent dose (TED) of Rhuflex-F was calculated as 135 mg/kg, that was obtained from converting the human therapeutic dose, i.e., 1500 mg/day to rat dose-based body surface area ratio.[4] The research study was carried out at two dose levels 135 and 270 mg/kg body weight of albino rats. The drug was suspended in distilled water as per suitable dose level for oral administration in albino rats with the help of an oral feeding cannula. All rats received constant volume of test drug suspension of 10 mL/kg body weight of albino rats.

Experimental protocols

In vitro anti-inflammatory activity

In vitro anti-inflammatory activity was done by the inhibition of the protein denaturation method.[5] Each 50 µL of aqueous extracts of Rhuflex-F in various concentrations (100, 200, and 400 µg/mL) was taken and mixed with 0.45 mL of bovine serum albumin. Diclofenac sodium was used as standard. The pH of the above solutions was adjusted to 6.3 using small amount of 1N hydrochloric acid. The samples were incubated at 37°C for 20 min and heated at 57°C for 3 min which was cooled and 2.5 mL of phosphate buffer (pH 6.3) was added to it. The percentage inhibition of protein denaturation was calculated as follows. Percentage inhibition = (Ac – As)/Ac × 100 (where Ac = Absorbance of control and As = Absorbance of sample).

In vivo anti-arthritic activity

The anti-arthritic effect of Rhuflex-F was evaluated against Freund's adjuvant-induced arthritis in albino rats.[6] Selected albino rats were divided into five groups, i.e., group (I) severed as normal control received distilled water (10 mL/kg, po), group (II) as complete Freund's adjuvant (CFA) control group, and group (III) and (IV) as Rhuflex-F-treated groups (135 and 270 mg/kg, po) (RTED and RTEDX 2), respectively. Group (IV) received dexamethasone (100 µg/kg, po) as standard drug.

On day 0, initially, hind paw volume was measured with the help of digital plethysmometer (IITC, Mumbai). On 1st day, 1 h after test drugs administration, the complete Fruend's adjuvant was made into fine emulsion with the help of a syringe, and 0.1 mL of it was injected beneath the plantar aponeurosis in the left hind paw, and 0.05 mL subcutaneously into the root of the tail of rats. The volumes of left hind paw measured on the 2nd, 3rd, 5th, 10th, 15th, 20th, 25th, and 30th days while for right hind paw on 15th, 20th, 25th, and 30th days. On 20th day and 30th day, test drugs were evaluated for analgesic activity with the help of digital hot plate (IITC, Mumbai) method. The temperature of incremental hot/cold plate was fixed at 55 ± 0.2°C.[7]

On 30th day, the final body weight of overnight fasted rats was taken and thereafter blood was collected from the retro-orbital plexus by capillary under light ether anesthesia and was used for estimations. Hematological analysis was performed using an automatic hematological analyzer (Swelab, Sweden). The parameters include total red blood cell (RBC), hemoglobin (Hb), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), mean corpuscular volume,

packed cell volume, white blood cell (WBC), neutrophils, lymphocyte, eosinophils, monocyte, and platelet.

Serum biochemical parameters were carried out using fully automated biochemical analyzer (BS-200, Lilac Medicare Pvt., Ltd., Mumbai), namely serum glutamic pyruvic transaminase (SGPT), serum glutamic-oxaloacetic transaminase (SGOT), alkaline phosphatase, urea, uric acid, and orosomucoid. The serum was also assessed for antioxidant parameters such as nitric oxide (NO),[8] glutathione (GSH), and GSH peroxidase.[9] At the end, the rats were sacrificed, adrenal glands were carefully dissected out. One adrenal gland was homogenized for the estimation of adrenal ascorbic acid.[10]

The results are expressed as mean ± standard error of the mean. The data generated during the study were analyzed by employing Student's "*t*' test and one-way analysis of variance, followed by Dunnet's multiple "*t*" test for unpaired data to determine significant differences between groups at $P < 0.05$.

Results and Discussion

In vitro anti-inflammatory activity

Denaturation of tissue protein is one of the known causes of inflammatory and arthritic diseases. It is also known that the production of auto-antigens may be due to the denaturation of proteins *in vivo*.[11] Substances which have the ability to prevent protein denaturation could be potential drugs as anti-inflammatory and anti-arthritic activity. Denaturation of a protein involves the disruption of the tertiary and secondary structure of the proteins caused by the application of external stress, leading to the loss of its biological functions.[12] In the present study, part of the investigation on the mechanism of the anti-inflammation activity, the ability of Rhuflex-F to inhibit protein denaturation was studied *in vitro*. Aqueous extract of Rhuflex-F showed *in vitro* protein denaturation inhibitory activity in a dose-dependent manner, [Table 2] whereas diclofenac sodium was used as a standard drug and expressed a pronounced effect as compared to Rhuflex-F.

Table 2: *In vitro* anti-inflammatory activity of water extract of Rhuflex-F

Treatments	Concentration (μg/mL)	% inhibitory activity	IC_{50} (μg/mL)
Diclofenac	100	15.62±0.12	>400
	200	25.00±6.615	
	400	40.62±8.265	
Rhuflex-F	100	3.77±0.88	>400
	200	5.66±2.66	
	400	11.32±6.22	

Data: Mean±SD. SD: Standard deviation, IC: Inhibitory concentration

In vivo anti-arthritic activity

Freund's adjuvant-induced arthritis is thought to occur through cell-mediated autoimmunity structural mimicry between mycobacteria and cartilage proteoglycan in rats. It activates macrophages and lymphocytes by adjuvant inoculation or their products such as monokines, cytokines, and chemokines may be involved in abnormal lipid and protein metabolism.[13] The adjuvant-injected rat provides a model of acute and chronic inflammation where body weight changes can be clearly seen. The involvement of catabolic hormones (glucocorticoids and catecholamine) in weight loss and altered zinc homeostasis in arthritis must also be considered.[14] The present study clarified that the normal control group showed an increase in the body weight of rats while rats of the CFA group showed no weight gain till the 10th day suggesting catabolic effects during arthritic conditions. Rhuflex-F at both dose levels depicted a significant increase in body weight compared to initial body weight recommending, anabolic effects of Rhuflex-F against Freund's adjuvant-induced arthritis in albino rats. [Table 3]

Injection of CFA subcutaneously into hind paw of rats produced localized inflammation and pain. An immediate swelling (primary inflammation) due to the injection of CFA was produced which was followed by secondary inflammation after 8–12 days and the alterations remain detectable for several weeks.[15] CFA produced a significant increase in primary paw edema in the left paw in comparison to the control group from the 2nd day onward in comparison to the control group and secondary edema from the 10th day onward in the right paw in comparison to the control group. The increase in primary paw inflammation suggests the inflammatory response due to Fruend's adjuvant injection and release of chemical mediators and activation of various kinins. The increase in secondary paw inflammation suggests the typical symptoms of arthritis may be due to cell-mediated immune response in albino rats in the present study. The drugs which inhibit primary edema may be due to their anti-inflammatory activity while the drugs which may inhibit secondary edema may be due to their anti-arthritic activity.

Rhuflex-F at the TEDx2-treated group showed a significant decrease in primary paw edema on 2nd day, followed by nonsignificant decrease from the 3rd day up to the 30th day while at TED Rhuflex-F showed a decrease in primary paw edema from the 2nd day to 15th day in comparison to CFA control. The decrease in primary paw edema by Rhuflex-F suggests the anti-inflammatory activity in the test formulation. Symptomatically secondary lesions showed swelling of the non-injected right hind foot from the 10th day onward after injection of CFA in albino rats compared to the control group. Rhuflex-F at TED dose level produced a decrease in secondary paw edema on the 10th, 20th, and 30th days, while a significant decrease on the 15th and 25th day while at TEDx2 dose level produced nonsignificant decrease compared to CFA control group during the course of the experimental period representing significant anti-arthritic activity through cell-mediated immune response in Wistar albino rats. Dexamethasone-treated group showed significant suppression in primary and secondary edema throughout the experimental period in comparison to the CFA control group specifying the presence of significant anti-inflammatory and anti-arthritic activities. [Table 4]

Table 3: Effect of test drugs on body weight of rats in Freund's adjuvant-induced arthritis

Days	Body weight (g)				
	NC	CFA	DEX	TED	TEDx2
Initial	208.33±7.41	201.33±7.94	222.16±6.71	213.33±5.2	216.50±23.72
10th day	227.5±14.27	207.50±9.64	202.16±8.22##	219.83±6.39	220.16±12.87
20th day	246.0±15.01#	228.33±14.16#	179.5±9.43*.###.@	239.33±7.3##	235.83±15.26#
30th day	233.5±12.56	235.83±20.85	183.66±6.78*.##	247.3±9.17##	244.5±17.08#

*P<0.01, compared to CFA group (unpaired t-test), #P<0.05, ##P<0.01, ###P<0.001, compared with initial body weight (paired t-test), @P<0.05, compared to CFA group (ANOVA followed by Dunnett's multiple t-test). Data: Mean±SEM. SEM: Standard error of mean, NC: Normal control group, CFA: Complete Freund's adjuvant, DEX: Dexamethasone (reference standard) group, TED: Therapeutic equivalent dose of Rhuflex-F capsule (Rhuflex-F capsule lower dose), TEDx2: Therapeutic equivalent dose of Rhuflex-F capsule (Rhuflex-F capsule higher dose)

Table 4: Effect of test drugs on primary and secondary edema (%) in paw of rats in Freund's adjuvant-induced arthritis

Days	NC	CFA	DEX	TED	TEDx2
Increase in primary paw edema (%)					
2nd day	1.76±0.74	81.30±5.09$	41.96±8.86@@	65.70±8.80	51.83±8.29@
3rd day	5.25±2.11	83.68±12.07$	28.52±10.19@@	68.40±20.34	64.91±11.75
5th day	9.14±3.09	80.97±11.93$	35.99±7.99@@	70.57±5.09	72.66±15.34
10th day	7.38±2.57	61.16±11.96$	37.92±11.32@@	58.58±10.75	49.45±7.29
15th day	13.70±4.12	74.35±7.31$	34.19±6.74@@	50.21±9.16	58.34±8.62
20th day	11.85±3.48	62.78±9.86$	25.05±6.34@@	74.09±8.22	64.97±14.21
25th day	4.63±2.79	63.79±12.71$	20.28±7.46@@	67.83±7.32	47.68±12.62
30th day	4.89±2.82	66.86±12.83$	22.08±6.94@@	72.12±8.96	36.28±16.40
Increase in secondary paw edema (%)					
10th day	0.93±0.45	39.55±5.47$	18.21±1.80@@	38.84±8.23	27.85±7.33
15th day	3.77±0.72	47.81±4.04$	28.92±2.42@@	30.19±3.54@@	35.46±5.58
20th day	6.35±1.29	49.51±3.52$	31.60±4.95@@	38.51±2.35	41.24±3.06
25th day	6.72±1.42	51.03±4.03$	30.48±4.79@@	37.59±4.06@	43.95±2.42
30th day	8.79±2.48	47.47±3.30$	25.69±5.04@@	34.49±7.02	42.07±6.68

$$P$<0.01, compared to normal control group, @P<0.05, @@P<0.01, compared to CFA control group (ANOVA followed by Dunnett's multiple t-test). Data: Mean±SEM. NC: Normal control group, CFA: Complete Freund's adjuvant, DEX: Dexamethasone (reference standard) group, TED: Therapeutic equivalent dose of Rhuflex-F capsule (Rhuflex-F capsule lower dose), TEDx2: Therapeutic equivalent dose of Rhuflex-F capsule (Rhuflex-F capsule higher dose), SEM: Standard error of mean

The total WBC count was increased in adjuvant-induced arthritic rats.[16] Previous study advocates that, Fruend's adjuvant increases WBC count, erythrocyte sedimentation rate along with an enhancement of Hb levels and RBC count.[17] Here, the CFA control group showed nonsignificant increase in total WBC count, lymphocytes, monocyte, and Hb level, significant increase in MHC and MCHC while decrease in neutrophil, RBC, and platelet count when compared to the normal control group. Rhuflex-F at both dose levels produced nonsignificant decrease in total WBC count, lymphocytes, monocytes, RBC, MHC, and MCHC in comparison to the CFA control group. [Table 5] The results suggest that test drugs have reversal effects on hematological parameters to Freund's adjuvant-induced adverse effects on hematological parameters.

Orosomucoid belongs to a group of proteins known as acute-phase proteins. They are a group of plasma proteins which consistently rise as physiological response to different inflammatory states.[18] Orosomucoid also known as α-1 acid glycoprotein is one among the acute-phase reactants, whose serum level elevation indicates underlying inflammation,[19] further antagonized by disease-modifying anti-rheumatic drugs (DMARD), while unaffected by NSAIDs. Thereby, orosomucoid measurement acts as a marker parameter to assess whether the test drug possesses DMARD effect or not. The present study shows a significant increase in serum orosomucoid level in the CFA control group compared to the normal control group which was significantly reverted by Rhuflex-F at both dose levels, while dexamethasone produced nonsignificant decrease in serum orosomucoid level in comparison to CFA control group. [Table 6] Results of the test drug show suppression of induction and formation of acute-phase proteins and, therefore, it can be considered an index of decreased inflammatory reaction. The attenuation effect is probable mediated by modulating the secretion and functioning of the cytokines involved in the expression of acute-phase reaction mentioned above. Hence, we can assume that the test drug possesses DMARD like effect.

Elevated blood urea level was reported in arthritic rats and further hypothesized that substantial fraction of blood urea comes from kidneys synthesizing arginine. Serum uric acid level marks kidney function or catabolic activity.[20] In the present study, blood urea and uric acid levels were not affected

Table 5: Effect of test drugs on hematological parameters in albino rats

Parameters	NC	CFA	DEX	TED	TEDx2
WBC (10^3/mm^3)	10,483.3±785.4	11,016.6±1405.1	8200.0±1946	9750±726.1	10,700±709.9
Neutrophils (%)	24.16±3.58	12.33±2.72$^{\&}$	23.00±8.74	18.66±4.29	21.83±5.98
Lymphocytes (%)	72.33±3.72	83.66±2.94$^{\&}$	73.00±8.88	77.66±4.63	74.00±6.30
Eosinophil (%)	1.83±0.30	1.83±0.16	2.20±0.37	1.83±0.30	2.33±0.33
Monocytes (%)	1.66±0.21	2.05±0.54	1.80±0.20	1.83±0.30	1.66±0.21
RBC (10^3/µL)	8.70±0.09	8.38±0.10$^{\&}$	7.86±0.56	8.24±0.43	8.25±0.33
Hb (g%)	15.13±0.21	15.51±0.08	14.46±0.78	14.90±0.69	14.85±0.36
PCV (%)	48.50±0.70	47.18±0.54	44.32±2.80	46.70±2.38	45.76±1.40
MCH (pg)	17.38±0.16	18.51±0.20$^{\&\&}$	18.50±0.42	18.11±0.19	18.05±0.40
MCHC (g/dL)	31.21±0.22	32.91±0.32$^{\&\&}$	32.72±0.41	31.95±0.2*	32.50±0.28
MCV	55.68±0.63	56.28±0.49	56.50±0.60	56.68±0.61	55.60±0.78
Platelet (10^3/µL)	1274.5±34.2	1132.0±33.2$^{\&}$	1071.4±116.5	977.8±39.9*	1144.8±60.3

*P<0.05, compared with CFA control group (unpaired t-test), $^{\&}P$<0.05, compared with normal control group (unpaired t-test), $^{\&\&}P$<0.05, compared with normal control group. Data: Mean±SEM. NC: Normal control group, CFA: Complete Freund's adjuvant, DEX: Dexamethasone (reference standard) group, TED: Therapeutic equivalent dose of Rhuflex-F capsule (Rhuflex-F capsule lower dose), TEDx2: Therapeutic equivalent dose of Rhuflex-F capsule (Rhuflex-F capsule higher dose), SEM: Standard error of mean, WBC: White blood cell, RBC: Red blood cell, Hb: Hemoglobin, PCV: Packed cell volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, MCV: Mean corpuscular volume

Table 6: Effect of test drugs on serum biochemical parameters in albino rats

Parameters	NC	CFA	DEX	TED	TEDx2
Orosomucoid (mg/dL)	40.93±8.67	185.3±24.26$^{\$}$	144.3±19.44	100.6±11.03$^{@@}$	105.86±18.9$^{@@}$
SGOT (IU/L)	191.5±17.84	115.83±8.16$^{\$}$	148.4±14.08	114.50±7.79	100.50±6.65
SGPT (IU/L)	82.50±5.71	62.33±6.33$^{\&}$	78.00±7.45	64.66±6.17	55.83±5.19
ALP (IU/L)	151.66±12.5	168.66±14.6	204.2±29.0	162.00±21.6	183.66±22.3
Urea (mg/dL)	48.50±2.66	44.33±4.24	34.0±2.75	39.33±2.56	35.33±1.11
Uric acid (mg/dL)	0.71±0.03	0.76±0.15	0.94±0.16	0.48±0.06	0.61±0.09

$^{\&}P$<0.001 Compared with FAC of respective group (Unpaired 't' test); $^{\$}P$<0.01, compared to normal control group, $^{@}P$<0.05, $^{@@}P$<0.01, compared to CFA control group (ANOVA followed by Dunnett's multiple t-test). Data: Mean±SEM. SGOT: Serum glutamic-oxaloacetic transaminase, SGPT: Serum glutamate pyruvate transaminase, ALP: Alkaline phosphatase, NC: Normal control group, CFA: Complete Freund's adjuvant, DEX: Dexamethasone (reference standard) group, TED: Therapeutic equivalent dose of Rhuflex-F capsule (Rhuflex-F capsule lower dose), TEDx2: Therapeutic equivalent dose of Rhuflex-F capsule (Rhuflex-F capsule higher dose), SEM: Standard error of mean

in Freund's adjuvant control group and also in drug-treated groups.

Increase in serum transaminases and alkaline phosphatase levels was reported in arthritic rats.[21] Elevation of serum SGPT and SGOT may be due to damaged cells. Mild increase indicates liver damage of determine origin. In the present study, serum transaminases were significantly decrease in the CFA control group, while alkaline phosphatase level was non-significantly increased compared to the normal control group. Rhuflex-F at both dose levels did not affect SGOT, SGPT, and alkaline phosphatase levels in comparison to the CFA control group, [Table 6] although these parameters are not important in determining the anti-arthritic effects of test drugs.

GSH scavenges superoxide anion and protects the protein thiol group from oxidation as being an important constituent of intracellular protective mechanisms against oxidative stress.[22] Previous reports suggest that FCA-treated rats showed an increase in serum GSH peroxidase in arthritic conditions.[23] In the present study, a significant increase in total GSH and non-significant increase in GSH peroxidase were observed in Freund's adjuvant control group may due to the combat of increased oxidative stress in arthritic conditions. Rhuflex-F at both dose levels and dexamethasone produced non-significant reversal of both parameters toward values observed in the normal control group. [Table 7]

NO represents itself as a key molecule in the inflammation and degraded cascade of arthritis contribute.[24] NO can be either protective or damaging to tissues, NO derived from inductive NO synthase, an important arthritic mediator together with other free radicals, significantly adds to the arthritic reactions. In the present study, a significant increase in serum NO was observed in the CFA control group in comparison to the control group while not affected by test drugs when compared to the CFA control group. [Table 7]

Adrenal weight and adrenal ascorbic acid content are used as biomarkers for assessing the involvement of adrenal gland in anti-arthritic activity. Adrenal weight and adrenal ascorbic acid content decreased are considered to indicate an adrenal activity increase and the opposite to indicate decrease in adrenal activity.[25] In the present study, a significant increase in adrenal ascorbic acid content was observed in the CFA control group due to decreased adrenal activity to respond to inflammation. Rhuflex-F at both dose levels and dexamethasone revealed non-significant decrease in adrenal ascorbic acid content in

comparison to the CFA control group which may suggest increase activity of adrenal in albino rats.

Arthritis pain-related electrophysiological changes were measured in primary afferent nerve fibers (peripheral sensitization) and in central nervous system neurons (central sensitization), including neurons in the spinal dorsal horn, therefore, test drugs were evaluated for analgesic activity (20th–30th day) with the help of hot plate method using same rats.[26] Study protocol was selected based on the assessment of arthritic pain in rats by central- and peripheral-mediated effects. The capability of the drug to prolong reaction latency to thermally induced pain by hot plate in rats suggests central analgesic activity. In the present study, dexamethasone and Rhuflex-F at TEDx2-treated groups showed a significant increase in reaction latency compared to initial values as well as CFA control group on 20th–30th days suggest the decline of inflammation and allied pain in arthritic rats. Rhuflex-F at TED shows an increase in latency of response to radiant heat-induced pain in comparison to initial values as well as the CFA control group in the late phase in comparison to the CFA control group. [Table 8] The result of the present study suggests that Rhuflex-F has an anti-arthritic reversal effect and hence also decreases the pain response in albino rats in a dose-dependent manner.

Glucocorticoids were discovered for the treatment of RA in 1949 and today their synthetic analogs, corticosteroids, remain a mainstay in the treatment of the disease. Dexamethasone is a synthetic adrenal corticosteroid with potent anti-inflammatory properties, immunosuppressant properties with 20–30 times the binding affinity for glucocorticoid receptors of endogenous cortisol. Dexamethasone causes reduction in interleukin-1 and tumor necrosis factor-α through inhibitory effect on nuclear factor kB may improve respiration by reducing pulmonary and circulating levels of pro-inflammatory cytokines.[27] In the present study, dexamethasone produced anti-inflammatory, anti-arthritic, and analgesic activity in albino rats may be due to decrease in inflammation response to Freund's adjuvant-induced arthritis.

Rhuflex-F formulation contains a total of 16 ingredients, [Table 1] out of which 9 drugs are already proven for strong anti-inflammatory activity and 4 are proven for anti-arthritic activity in experimental research work and clinical studies. *Sankha Bhasma* containing calcium plus Vitamin D supplementation reversed the incidence of RA,[28] *Commiphora mukul* (Hook ex Stocks) has anti-inflammatory effect,[29] *Anethum graveolens* Kurz. fruits, seeds, and oil have anti-inflammatory and pain-reducing effects in rat and mice,[30] *Piper cubeba* Linn. has anti-inflammatory activity and anti-nociceptive activity,[31] *Cedrus deodara* (Roxb.) Loud. wood oil inhibited the Arthus reaction and has

Table 7: Effects of test drugs on serum anti-oxidant parameters and adrenal ascorbic acid in albino rats

Parameters	NC	CFA	DEX	TED	TEDx2
GSH (µmoles/dL)	81.90±6.04	177.29±25.1$^{\$}$	174.54±34.5	119.14±7.84	129.18±10.13
GPx (ng of GSH utilized/mg protein/min)	13.89±0.73	16.93±6.34	9.04±2.31	6.05±0.99	4.11±0.92$^{@@}$
NO (µmoles/dL)	11.28±0.20	17.06±1.97$^{\$}$	18.72±0.52	19.33±0.85	18.11±1.21
Adrenal ascorbic acid (mg/g)	27.18±5.15	71.99±9.88$^{\$}$	51.99±14.09	52.88±11.35	47.02±5.38

$^{\$}P$<0.01, compared to normal control group, $^{@@}P$<0.01, compared to CFA control group (ANOVA followed by Dunnett's multiple *t*-test). Data: Mean±SEM. NC: Normal control group, CFA: Complete Freund's adjuvant, DEX: Dexamethasone (reference standard) group, TED: Therapeutic equivalent dose of Rhuflex-F capsule (Rhuflex-F capsule lower dose), TEDx2: Therapeutic equivalent dose of Rhuflex-F capsule (Rhuflex-F capsule higher dose), SEM: Standard error of mean, GSH: Glutathione, GPx: Glutathione peroxidase, NO: Nitric oxide

Table 8: Effect of Rhuflex-F on radiant heat-induced pain in arthritic rats on 20th day and 30th day at different time intervals

Intervals	Latency of response to radiant heat-induced pain (section)				
	NC	CFA	DEX	TED	TEDx2
			20th day		
Initial	3.41±0.08	3.89±0.22	3.79±0.34	3.88±0.35	4.15±0.12
30 (min)	3.88±0.17$^{\#}$	3.61±0.22	6.89±0.44$^{\#\#,@@}$	4.21±0.45	5.77±0.74$^{@@}$
60 (min)	3.81±0.21	4.21±0.55	6.11±0.50$^{\#\#,@@}$	4.75±0.28	6.63±0.56$^{\#\#,@}$
			30th day		
Initial	4.03±0.10	4.09±0.14	5.48±0.22	4.23±0.11	4.33±0.37
30 (min)	3.16±0.25$^{\#}$	4.25±0.21	5.53±0.37$^{@}$	3.68±0.21$^{\#}$	4.87±0.45
60 (min)	3.90±0.26	4.34±0.25	5.69±0.32$^{@}$	4.25±0.32	5.64±0.47$^{@}$
120 (min)	3.90±0.13	4.03±0.41	6.02±0.60$^{@@}$	4.87±0.26$^{\#}$	5.80±0.41$^{\#,@}$
180 (min)	4.45±0.31	3.81±0.22	7.32±0.23$^{\#\#\#,@@}$	5.35±0.32$^{\#,@}$	6.55±0.59$^{\#\#,@@}$
240 (min)	4.32±0.20	3.89±0.23	7.41±0.23$^{\#\#\#,@@}$	5.64±0.22$^{\#\#,@}$	7.40±0.76$^{\#\#,@@}$

$^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001, compared with initial reading (paired *t*-test), $^{@}P$<0.05, $^{@@}P$<0.01, compared with CFA control group (ANOVA followed by Dunnett's multiple *t*-test). Data: Mean±SEM. NC: Normal control group, CFA: Complete Freund's adjuvant, DEX: Dexamethasone (reference standard) group, TED: Therapeutic equivalent dose of Rhuflex-F capsule (Rhuflex-F capsule lower dose), TEDx2: Therapeutic equivalent dose of Rhuflex-F capsule (Rhuflex-F capsule higher dose), SEM: Standard error of mean

anti-inflammatory and analgesic activity,[32] *Withania somnifera* Linn. Dunal root powder has anti-arthritic activity against adjuvant-induced arthritic rats,[33] and *Terminalia chebula* Retz. was reported for anti-inflammatory activity and anti-arthritic activity.[34,35]

The observed efficacy of Rhuflex capsule in the present study may be due to multiple herbomineral ingredients and their active chemical constituents such as steroid from *Guggulu*, Smilogenine from *Chopachini*, sesquiterpene lactones, mainly alantolactone and isoalantolactone from *Pushkaramoola*, gingirenon from *Curcuma longa* Linn, Withaferin-A form *Ashwagandha,* quercetin, carvacrol, and thymol which have reported for anti-oxidant, anti-inflammatory, anti-arthritic, and analgesic activity may be due to inhibition of inflammatory mediators.

Conclusion

Results of the present study indicated that Rhuflex-F formulation has anti-inflammatory action may be due to inhibition of protein denaturation *in vitro* and *in vivo,* it has anti-arthritic activity against CFA-induced arthritis in albino rats. The activity may be due to multiple herbo-mineral ingredients and their active chemical constituents reported to possess anti-oxidant, anti-inflammatory, anti-arthritic, and analgesic activity.

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Conflicts of interest

There are no conflicts of interest.

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हिन्दी सारांश

रूफ्लेक्स-एफ हर्बोमिनिरल आयुर्वेदिक औषधि का एल्बिनो चूहों में गठिया-विरोधी गतिविधि का मूल्यांकन

वीरेंद्र गुप्ता, बालाजी पाणिग्रही, सुब्रत डे, मुकेशकुमार बी नारिया

पृष्ठभूमि: रुफ़्लेक्स-एफ एक आयुर्वेदिक हर्बोमिनिरल आयुर्वेदिक उत्पादन है जिसका उपयोग चिकित्सीय रूप में जोड़ों और मांसपेशियों में जकड़ाहट से राहत देने, शोथ को कम करने, गतिशीलता को बढ़ाने और गठिया की ओर ले जाने वाली अन्य ऑटोइम्यून बीमारियों के लक्षणों से राहत देने में भी प्रभावी है। **उद्देश्य:** शोध अध्ययन का उद्देश्य इन-विट्रो प्रोटीन विकृतीकरण और अल्बिनो चूहों में इन-विवो फ्रायंड के सहायक-प्रेरित गठिया के खिलाफ रूफ्लेक्स-एफ की प्रभावकारिता का मूल्यांकन करना है। **सामग्री एवं विधि:** गोजातीय सीरम एल्ब्यूमिन का उपयोग करके प्रोटीन विकृतीकरण गतिविधि का इन-विट्रो निषेध किया गया। इन-विवो गतिविधि के लिए, अल्बिनो चूहों में गठिया पूर्ण फ्रायंड के सहायक द्वारा उत्पन्न किया गया था। रुफ़्लेक्स-एफ (135 और 270 मिलीग्राम/किग्रा, पीओ) को गठिया से पीड़ित चूहों में 30वें दिन तक दिया गया था और प्राथमिक और माध्यमिक पॉ-एडिमा, वेदना प्रतिक्रिया, हेमेटोलॉजिकल मापदंडों, सीरम और ऊतक जैव रासायनिक मापदंडों पर प्रभाव का आकलन किया गया था। **परिणाम:** रूफ्लेक्स-एफ के जलीय अर्क ने मात्रा पर निर्भर तरीके से इन-विट्रो प्रोटीन विकृतीकरण निरोधात्मक गतिविधि दिखाई। रूफ्लेक्स-एफ ने वेदना की प्रतिक्रिया में कमी के साथ प्राथमिक और माध्यमिक पॉ-एडिमा में असार्थक कमी दिखाई, हेमेटोलॉजिकल मापदंडों पर कुछ विपरीत प्रभाव, फ्रूएंड के सहायक नियंत्रण समूह की तुलना में सीरम ओरोसोम्यूकोइड और एड्रेनल एस्कॉर्बिक एसिड में कमी देखी गई। इसके अलावा रूफ्लेक्स-एफ ने अल्बिनो चूहों के सीरम में ऑक्सीडेंट स्थिति पर फ्रुएंड के सहायक-प्रेरित प्रतिकूल प्रभावों को उलट दिया। **निष्कर्ष:** वर्तमान अध्ययन के परिणाम से पता चलता है कि, रूफ्लेक्स-एफ फॉर्मूलेशन एंटी-इंफ्लेमेटरी है, जो कि इन-विट्रो में प्रोटीन विकृतीकरण के निषेध और अल्बिनो चूहों में पूर्ण फ्रुएंड के सहायक-प्रेरित गठिया के खिलाफ इन-विवो आर्थेराइटिस विरोधी गतिविधि के कारण हो सकता है।

मुख्य शब्द: गठिया विरोधी गतिविधि, सूजन रोधी, डेक्सामेथासोन, कम्प्लीट फ्रायंड एडजुवेंट, रूफ्लेक्स-एफ।