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Ayurvedic Balarista ameliorate anti-arthritic activity in adjuvant induced arthritic rats by inhibiting pro-inflammatory cytokines and oxidative stress☆

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ABSTRACT

Background and aim: Balarista is a fermented ayurvedic liquid preparation recommended as a good therapy for the treatment of rheumatoid arthritis. In the present investigation, the anti-arthritic activity of in-house Balarista formulation and marketed M1, M2, M3 and M4 Balarista formulations at the dose of 2.31 ml/kg were studied on Complete Freund's adjuvant-induced arthritic rat model.

Experimental procedure: Measurement of paw diameter, arthritic index, arthritic score, and body weight were made to assess the anti-arthritic activity. Alterations in hematological and biochemical parameters were carried out to ascertain the disease progression. The inflammatory mediators (TNF-α, IL-1β, and IL-6) were measured by the ELISA method. The oxidative stress parameters were evaluated in tissues of joint, liver, spleen and kidney. The histological and radiological changes in the ankle joint of rats were also studied.

Results and conclusion: Administration of in-house and marketed formulations exhibited significant anti-arthritic activity by reducing all the arthritic parameters. The anomalous alterations in hematological and biochemical parameters were remarkably restored. The expression level of serum pro-inflammatory cytokines was significantly suppressed in treated animals. The oxidative stress, indicated by an increase in lipid peroxidation, decreased in antioxidant enzyme i.e. superoxide dismutase and catalase along with non-enzymatic reduced glutathione in tissues, were strongly counteracted by the formulation. Abnormal changes in arthritic ankle joints shown by X-ray and histological examination were significantly protected by the formulation. The present study suggests that the administration of in-house and marketed Balarista formulations have produced a significant anti-arthritic effect by inhibiting free radicals and inflammatory cytokines.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by the synovial hyperplasia, pannus formation, recruitment of inflammatory cells with continuous impairment of cartilage and bone erosion.[1] Recent studies have revealed that the pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) produced by T helper type-1 (Th1) cells have key role in pathogenesis of RA.[2] It is also evident that the oxidative stress due to amelioration of reactive oxygen and nitrogen species (ROS and RNS) have significant role in the development and progression of RA and also in the age-

Abbreviations: API, Ayurvedic Pharmacopoeia of India; CFA, Complete Freund's adjuvant; IBF, In-house Balarista formulation; IkB, Inhibitor of kappa B; M (1, 2, 3 and 4), Marketed formulations; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; Th1, T helper type-1.

☆ Assessment of anti-arthritic potential of ayurvedic Balarista formulation, use of Complete Freund's adjuvant, study of hematological and biochemical parameters, histological and radiological studies.

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dependent pathogenic processes of cancers, arteriosclerosis, neurodegenerative disorders and other diseased conditions.[3] Free radicals generated from macrophages, lymphocytes, neutrophils, and endothelial cells play important role in the pathophysiology of synovial inflammation.[4] Thus, the degenerative changes in RA could be prevented by eliminating these oxidizing species along with the use of drugs with immunomodulatory activities, will be of interest. The current treatment therapy of RA such as non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDs), TNF-α antagonist, anti-interleukin (IL-6) receptor antibody, IL-1 receptor antagonist, steroidal agents and immunosuppressant/immunomodulators transiently suppress inflammation and ameliorate symptoms. However, they do not help significantly to treat the disease in the long term and may result in serious side effects including gastrointestinal disorders, immunodeficiency, and humoral disturbances.[5]

So, there comes a strong desire to develop potent anti-inflammatory agents, which could be used as preventive and for curative purposes with minimum side effects and it could be effective in the management of RA by preventing the progression of the disease. In this regard, herbal anti-inflammatory medicines provide a broad-spectrum mechanism of action such as interaction with the inflammatory cascade, cytokine production, inhibition of elastase or hyaluronidase, increased anti-oxidative effectiveness and other still unidentified effects that may contribute to joint protective effects.[6] Ayurveda, an Indian system of medicine, is known to generate many useful leads for chronic diseases.[7] Balarista, an ayurvedic fermented arista liquid preparation is recommended for the treatment of RA and other ailments.[8] However, the formulations available in the market are usually not properly standardized which leads to safety and efficacy issues. Therefore, in the present study, an in-house Balarista formulation was prepared according to the Ayurvedic Pharmacopoeia of India (API). As the preclinical scientific data regarding its molecular mechanism of action are not evaluated, so the present work was undertaken to investigate its anti-arthritic potential using Complete Freund's adjuvant (CFA) induced arthritic rat model and was compared with the marketed formulation.

2. Materials and methods

2.1. Plant materials

The roots of *Sida cordifolia* and *Withania somnifera* were collected from Bargarh district, in western part of Odisha, India and other raw ingredients; *Woodfordia fruticosa* (flower), *Ipomoea digitata* (sub-root), *Ricinus communis* (root), *Alpinia galanga* (root), *Elettaria cardamomum* (fruit), *Paederia foetida* (whole plant), *Eugenia caryophyllus* (flower buds), *Vetiveria zizanioids* (root), *Tribulus terrestris* (fruit) were procured from the local market and were authenticated by Prof. Dr. Arun Kumar Das, Dept of Rasa Sastra Bhaisajya Kalpana, Gopabanhu College of Ayurveda, Puri, Odisha. The voucher specimens (SPS/SOAU-22-33) of all ingredients were deposited in the department of Pharmacognosy for future reference. All the ingredients were washed with water, dried under shade and subjected to a coarse powder; and kept in airtight, light-resistant containers.

2.2. Preparation of Balarista

Balarista formulation was made with the ingredients mentioned in API (Table 1S) and coded as IBF (In-house Balarista formulation).[9] Four marketed formulations of different manufacturers were procured from the local market and coded as M1, M2, M3, and M4. *S. cordifolia* and *W. somnifera* were considered as two major ingredients amongst the other ingredients.

2.3. Animals

Wistar rats weighing 150–175 gm (6 weeks) of both sexes were employed in the present study. All the animals were maintained under controlled temperature (22±2 °C) and relative humidity (55 ± 5%) and supplemented with food and water ad libitum. The experimental protocol (1376/PO/Re/S/10/CPCSEA) was approved by IAEC (The Institutional Animal Ethical Committee).

2.4. Experimental design

The dose of Balarista formulation was calculated as per the method described by Reagan-Shaw et al. (2008).[10] According to the API, the approved human dose of Balarista formulation is 15–30 ml. Taking the average value of this it is converted to an animal dose of 2.31 ml/kg. The rats were grouped into eight, each of six animals. Group-1, treated as normal healthy control rats received Tween 80, 2% v/v orally; Group-2, CFA control received Tween 80, 2% v/v, orally and treated as negative control; Group-3, received indomethacin (IND) 1 mg/kg, orally and treated as positive control; Group-4, 5, 6, 7 and 8 received with IBF, M1, M2, M3 and M4 (2.31 ml/kg orally) respectively.

2.5. Induction of arthritis

The rats were immunized with 0.1 ml of CFA (10 mg of heat killed *mycobacterium tuberculosis* per ml of paraffin oil) by injecting into the sub-plantar surface of the right posterior paw. Indication of arthritis was confirmed by inflammatory edema of the injected paw at around 6–8 days of CFA administration.[11] All rats were administered with the indomethacin and formulation from day 15th to 28th day.

2.6. Evaluation of arthritis

2.6.1. Paw diameter

The changes in paws thickness of all experimental rats were examined on days 4, 8, 12, 16, 20, 24 and 28 by using Vernier calipers.[12]

2.6.2. Arthritic index

The arthritic index (AI) was determined as per the methods described by Coelho et al. (2004).[13] It was calculated using the following formula.

$$\mathrm{AI}(\%)=\frac{\mathrm{Hindpawdiameterondayx}-\mathrm{Hindpawdiameterondayo}}{\mathrm{Hindpawdiameterondayo}}\mathrm{X100}$$

2.6.3. Arthritic score

The experimental animals were subjected to macroscopical examination to measure the arthritic score by observing the degree of swelling and redness of joints, edema of periarticular tissues in the injected and non-injected paw.[14]

2.6.4. Bodyweight examination

During treatment, the change in body weight was measured with the help of digital weighing balance from the day of CFA immunization and then subsequently on 4th, 7th, 11th, 14th, 17th, 21st and 28th days.[15]

Table 1
Therapeutic effect of formulation on hematological parameters in adjuvant induced arthritis in rat.

	CNT	CFA	IND	IBF	M1	M2	M3	M4
RBC (X 10^6/mm^3)	6.42 ± 0.19	4.45± 0.11***	6.50± 0.07***	6.85± 0.09***	6.05± 0.11***	7.50± 0.15***	7.42± 0.08***	6.12± 0.12***
WBC (X 10^3/mm^3)	3.57 ± 0.08	9.60± 0.07***	3.07± 0.13***	3.32± 0.06***	3.00± 0.09***	5.85± 0.08**	5.42± 0.06**	3.30± 0.07***
Hb (g/dL)	11.27 ± 0.08	6.60± 0.12***	11.72± 0.08***	12.55± 0.06***	13.52± 0.13***	13.25± 0.06***	13.37± 0.1***	11.45± 0.06***
PCV (%)	34.725± 0.08	29.87± 0.21***	38.52± 0.08***	41.35± 0.1***	42.30± 0.07***	44.87± 0.11***	44.75± 0.11***	36.35± 0.11***
Platelets (X10^3/μl)	5.08 ± 0.01	8.29 ± 0.08***	5.75 ± 0.08***	7.26 ± 0.1**	5.41 ± 0.08***	7.63 ± 0.01**	5.28 ± 0.01***	4.31 ± 0.01***

RBC: Red blood cell; WBC: White blood cell; Hb: Hemoglobin; PCV: Packed cell volume.
Values are expressed as mean ± SEM, n = 6 and was estimated by one-way ANOVA followed by posthoc Tukey HSD test. Comparisons are made between i) Group-1 Vs Group-2, ii) Group-2 Vs Group-3, 4, 5, 6, 7 and 8. ***P< 0.001, **P< 0.01, *P< 0.05 considered as significant.

2.6.5. Measurement of organ weight, hematological and biochemical estimation

The animals were sacrificed by cervical decapitation on 29th day and blood was collected by cardiac puncture and subjected to various studies such as hematological; red blood cell (RBC), white blood cell (WBC), hemoglobin (Hb), packed cell volume (PCV), platelet, erythrocyte sedimentation rate (ESR) and biochemical; aspartate aminotransferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), bilirubin, total cholesterol (TC), total triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), total proteins (TP), albumin (Alb), C-reactive protein (CRP) and rheumatoid factor (RF) estimation. The inflammatory cytokines (TNF-α, IL-1β, and IL-6) were measured in blood serum by enzyme-linked immune sorbent assay (ELISA) method.[16] The liver, kidney, and spleen were anatomized from the body and kept in an ice-cold saline solution. The organ weights were documented and rectified for 100 gms.

2.7. Evaluation of in-vivo antioxidant activity

The *in-vivo* antioxidant activity was studied on joints tissues, liver, spleen and kidney of experimental animals. The tissue homogenates were prepared and subjected to estimation of lipid peroxidation (LPO)[17] and antioxidant enzymes such as superoxide dismutase (SOD),[18] catalase (CAT)[19] along with reduced glutathione (GSH) content.[20]

2.8. X-ray and histopathological examination

The ankle joints were removed from the sacrificed animals on the 29th day and subjected to an X-ray and histological examination.[21]The observation and photography of tissue sections were done under the microscope. The degree of pannus formation, synovial hyperplasia, and erosion of cartilage and bone were blindly investigated by two independent examiners using a semi-quantitative scale (0 = normal), (1 = mild changes), (2 = moderate changes), (3 = severe changes), and (4 = very severe changes) to determine the histological score.[22] Histopathological scores were combined and expressed as the sum of both ankle joints, with a maximum histological score of eight for each histological parameter per rat. The overall score is the mean score of all individual histological parameters per group.

2.9. Statistical analysis

The data of the present study were expressed as mean ± S.E.M (standard error mean) and statistical analysis was performed by one-way ANOVA followed by posthoc Tukey HSD test. P<0.05 was considered significant.

3. Results

3.1. Effect of formulation on paw diameter

All the experimental rats were noticed by the increase in paw diameter on day 4 and reach maximum on day 12 in contrast to normal rats. CFA induced rats were acknowledged with redness, swelling, periarticular erythema, and edema at their joints. Treatment with standard indomethacin had significantly (P<0.001) reduced the paw swelling in contrast to the CFA induced group. Administration of formulations from day 15–28 had significantly decreased the paw diameter in the order of IBF, M3, M1, M2, and M4. However, normal healthy control rats did not show any change in paw diameter (Fig. 1a).

3.2. Effect of formulation on arthritic index

The experimental CFA immunized rats were observed with a maximum arthritic index on the 12th day and thereafter it gradually reduced to a minimum on 28th day. This arthritic index was found significantly (P<0.001) decreased in rats treated with indomethacin. However, the supplementation of the formulations also had significantly declined the arthritic index as compared to CFA vehicle-treated rats in the order of M3, IBF, M2, M1 and M4 (Fig. 1b).

3.3. Effect of formulation on the arthritic score

The morphological examination of hind paws of experimental rats was given in Fig. 1c. The arthritic score was found to be maximum (9.5 ± 0.28) on 28th day in vehicle-treated CFA immunized rats in contrast to healthy rats. The animal treated with indomethacin remarkably (P<0.001) declined the arthritic score on the 28th day. However, supplementation of formulations from 15th to 28th day was noticed with a significant reduction in arthritic score in the order of IBF and M1, M4, M2, and M3. The normal healthy control rats did not show any sign of erythema on the hind paw. The macroscopic investigation of hind paws of the experimental rat is given in Fig. 5A.

3.4. Effect of formulations on body weight changes

The vehicle-treated CFA rats were marked with significant (P<0.001) reduction in body weight in between 16th to 28th days in contrast to healthy rats. The normal control rats were noticed with progressive improvement in body weight with respect to time. A continual increase in body weight was also marked in rats treated with indomethacin, IBF, M1, and M4 formulations. The rats supplemented with formulation M2 showed a gradual increase in body weight up to 20th day, and thereafter decreased from 24th to 28th

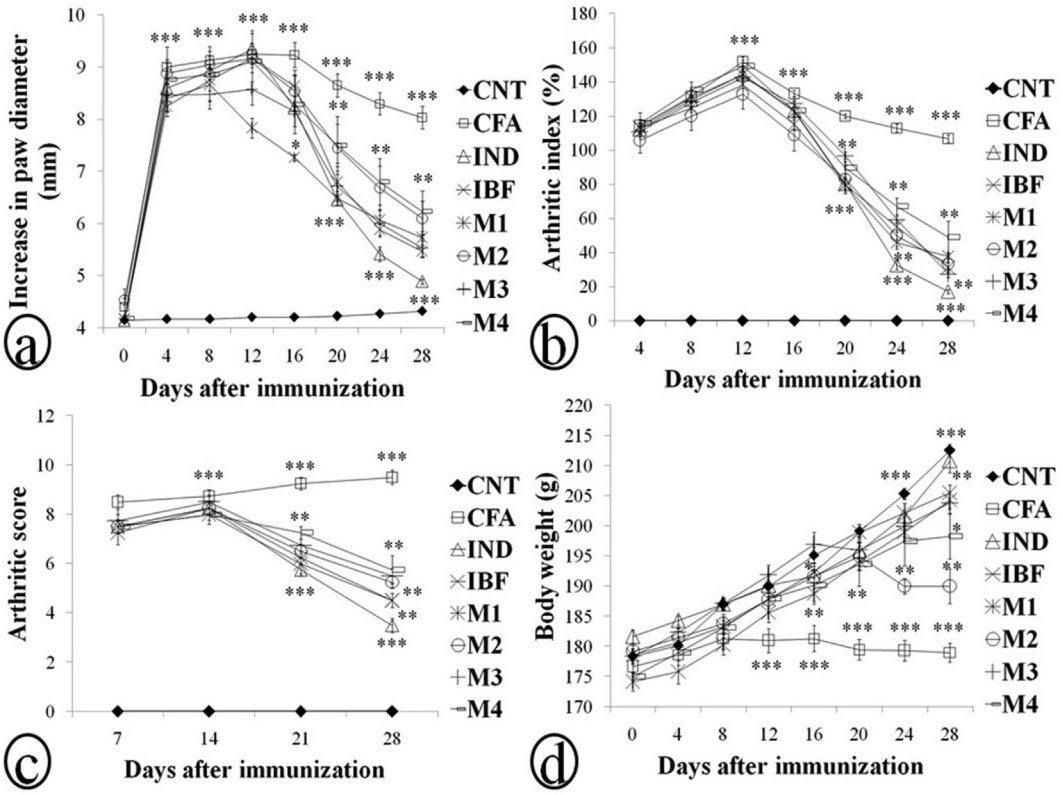

Fig. 1. Therapeutic effect of IBF, M1, M2, M3, M4 and indomethacin on (a) paw diameter, (b) arthritic index, (c) arthritic score and (d) body weight changes of CFA induced experimental rats. Values are expressed as mean ± SEM, n = 6 and was estimated using one-way ANOVA followed by posthoc Tukey HSD test. Comparisons are made between i) Group-1 Vs Group-2, ii) Group-2 Vs Group-3, 4, 5, 6, 7 and 8. ***P< 0.001, **P< 0.01, *P< 0.05 considered as significant.
CNT: Normal control; CFA: Complete Freund's adjuvant; IND: Indomethacin; IBF: In-house Balarista formulation; M1, M2, M3 and M4; Marketed formulation.

day. Similarly, rats administered with formulation M3 showed a continuous increase in body weight up to 16th day, then decreased on the 20th day and thereafter significant regain of body weight took place from 24th to 28th days (Fig. 1d).

3.5. Effect of formulation on organ weight changes

The CFA vehicle-treated rats were noticed with a significant increase in kidney and spleen weight and a decrease in liver weight in contrast to healthy rats. Treatment with indomethacin noticeably (P<0.001) reduced the weight of kidney and spleen and a rise in liver weight in contrast to the CFA control group. The formulation treated rats have also remarkably reduced the weight of kidney and spleen during the treatment and also increased liver weight. The weight of liver in the formulation treated rats was increased in the order of M1, IBF, M2, M4, and M3 whereas the weight of kidney was reduced in the order of IBF, M2, M3, M4, and M1 and of spleen in the order IBF, M1, M2, M3 and M4 (Fig. 2d).

3.6. Effect of formulation on hematological parameters

The experimental rats induced with CFA were distinguished with a significant decrease in RBC count, hemoglobin, packed cell volume and increase in WBC count, ESR and platelet count in contrast to the normal healthy rats. The group treated with indomethacin significantly (P<0.001) retrieved the altered hematological parameter to the normal state. These hematological parameters were also remarkably restored to normal value with the supplementation of in-house and marketed formulations. The data are given in Table 1 and Fig. 2a.

3.7. Effect of formulation on the biochemical investigation

The significant elevation in the level of AST, ALT, ALP, bilirubin, CRP, and RF was noticed in CFA induced groups in comparison to normal control rats. Treatment with indomethacin and formulation noticeably decreased these enzyme levels. There was also a remarkable increase in TC, TG, LDL and VLDL and, a decline of HDL, total protein and albumin in comparison to normal rats. These altered lipid profiles were therapeutically checked in indomethacin and formulation treated rats which are depicted in Table 2 and Fig. 2b and c.

3.8. Effect of formulation on cytokine production

The remarkable elevation of pro-inflammatory cytokines was perceived in CFA induced rats in contrast to normal rats. The experimental groups administered with indomethacin and formulation caused a significant reduction of TNF-α, IL-1β and IL-6 in comparison to CFA induced rats (Fig. 3).

3.9. Evaluation of in-vivo antioxidant activity

The level of LPO, enzymatic SOD and CAT, and nonenzymatic GSH activity were estimated in the tissues of joints, liver, spleen, and kidney of experimental animals. The increase in LPO, decrease in SOD, CAT and GSH activity were observed in vehicle-treated CFA rats in comparison to the normal group. The SOD activity was assessed to determine the endogenous defenses against superoxide radicals. Spleen exhibited the highest SOD activity by 2.73 fold and lowest by 1.71 fold in the kidney of CFA vehicle-treated

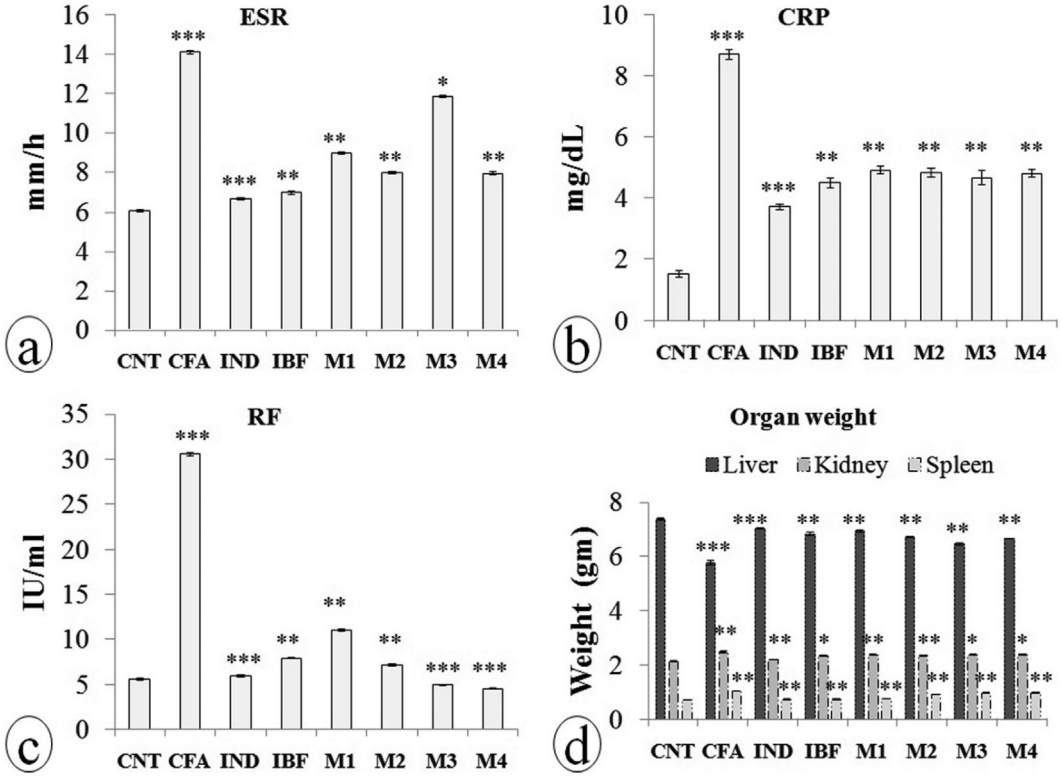

Fig. 2. Therapeutic effect of IBF, M1, M2, M3, M4 and indomethacin on (a) ESR, (b) CRP, (c) RF and (d) Organ weight changes of CFA induced experimental rats. Values are expressed as mean ± SEM, n = 6 and was estimated using one-way ANOVA followed by posthoc Tukey HSD test. Comparisons are made between i) Group-1 Vs Group-2, ii) Group-2 Vs Group-3, 4, 5, 6, 7 and 8. ***P< 0.001, **P< 0.01, *P< 0.05 considered as significant.
CNT: Normal control; CFA: Complete Freund's adjuvant; IND: Indomethacin; IBF: In-house Balarista formulation; M1, M2, M3 and M4; Marketed formulation.

Table 2
Therapeutic effect of formulation on biochemical parameters in adjuvant induced arthritis in rat.

	CNT	CFA	IND	IBF	M1	M2	M3	M4
AST (IU/L)	21.87± 0.08	39.45± 0.06**	22.47± 0.23**	26.40± 0.09**	23.50± 0.14**	28.95± 0.11**	30.40± 0.09**	29.43± 0.01**
ALT (IU/L)	42.50± 0.1	59.90± 0.22**	48.55± 0.13**	53.60± 0.09**	53.42± 0.13**	54.60± 0.12**	51.52± 0.11**	56.50± 0.14**
ALP (IU/L)	234.62± 0.1	317.25± 2.68**	245.00± 0.28**	297.75± 0.15**	207.27± 0.24**	267.25± 0.29**	284.15± 0.27**	249.05± 0.24**
Bilirubin (U/L Blood)	0.37± 0.01	1.27 ± 0.11**	0.347± 0.08**	0.762± 0.01**	0.345± 0.09**	0.552± 0.01**	0.840± 0.02**	0.202± 0.01**
TC (mg/dl)	112.27± 0.25	241.55± 0.13**	119.82± 0.2**	211.90± 0.27**	239.47± 0.35**	240.35± 0.06*	240.37± 0.19*	107.12± 0.31**
TG (mg/dl)	144.02± 0.2	312.45± 0.17**	147.60± 0.15**	172.45± 0.18**	143.27± 0.12**	188.05± 0.15**	192.00± 0.17**	132.27± 0.43**
HDL (mg/dl)	47.57± 0.13	38.52± 0.14**	47.20± 0.21**	43.55± 0.18**	49.17± 0.19**	44.57± 0.13**	47.67± 0.11**	40.52± 0.22**
LDL (mg/dl)	56.47± 0.12	100.72± 0.21**	61.02± 0.41**	65.55± 0.17**	84.52± 0.21**	92.12± 0.16**	93.17± 0.25**	50.12± 0.31**
VLDL(mg/dl)	38.60± 0.1	80.60± 0.09**	44.37± 0.23**	54.62± 0.13**	48.55± 0.14**	69.55± 0.1**	66.52± 0.13**	46.52± 0.12**
TP(mg/ml protein)	18.70± 0.09	13.95± 0.11***	17.50± 0.1***	17.47± 0.04***	16.97± 0.13***	19.82± 0.08***	16.37± 0.13***	18.67± 0.06***
Albumin (g/dl)	4.55 ± 0.13	3.22 ± 0.04***	4.17 ± 0.11**	3.82 ± 0.11*	3.75 ± 0.19*	3.97 ± 0.11**	3.75 ± 0.06*	3.87 ± 0.04**

AST: Aspartate aminotransferase; ALT: Alanine amino transferage; ALP: Alkaline phosphatase; TC: Total cholesterol; TG: Total triglyceride; HDL: High density lipoproteins; LDL: Low density lipoprotein; VLDL: Very low density lipoprotein; TP: Total proteins, Alb: Albumin.
Values are expressed as mean ± SEM, n = 6 and was estimated by one-way ANOVA followed by posthoc Tukey HSD test. Comparisons are made between i) Group-1 Vs Group-2, ii) Group-2 Vs Group-3, 4, 5, 6, 7 and 8. ***P< 0.001, **P< 0.01, *P< 0.05 considered as significant.

rats (Fig. 4a). The negative control group was marked with a decrease in catalase activity by 3.41-fold in the joints followed by spleen (2.87 fold), liver (1.97 fold) and kidney (1.63 fold) (Fig. 4b). The LPO was measured by determining the amount of thiobarbituric acid reactive substances (TBARS) in tissues of experimental rats (Fig. 4c). The elevated level of LPO was perceived in the spleen (7.32 fold) followed by kidneys (6.37 fold), joint (3.82 fold) and liver (1.93 fold) of arthritic rats in contrast to

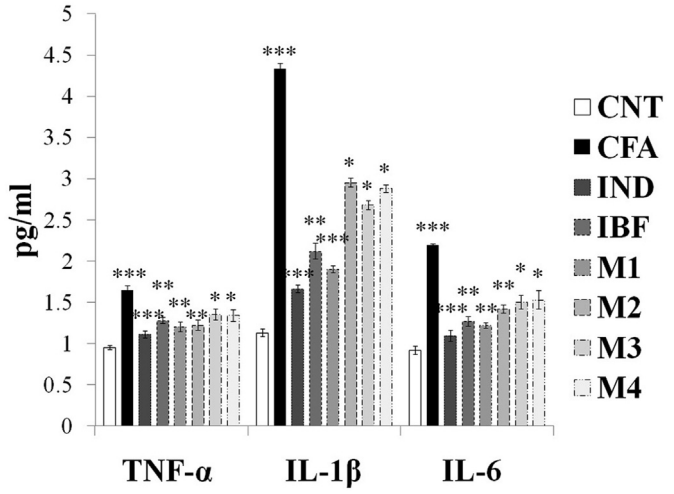

Fig. 3. Therapeutic effect of IBF, M1, M2, M3, M4 and indomethacin on TNF-α, IL-β and IL-6 of adjuvant arthritic rats. Values are expressed as mean ± SEM, n = 6 and was estimated by using one-way ANOVA followed by posthoc Tukey HSD test. Comparisons are made between i) Group-1 Vs Group-2, ii) Group-2 Vs Group-3, 4, 5, 6, 7 and 8. ***P< 0.001, **P< 0.01, *P< 0.05 considered as significant.
CNT: Normal control; CFA: Complete Freund's adjuvant; IND: Indomethacin; IBF: In-house Balarista formulation; M1, M2, M3 and M4; Marketed formulation.

normal rats. This altered level of LPO was remarkably brought to the normal by supplementing with the formulations. The group treated with indomethacin was also noticed with a significant reduction in the LPO level. The GSH level was calculated as acid-soluble sulfhydryl group (-SH) in tissues of normal and CFA induced rats. Fig. 4d showed a significant decline of GSH quantity in negative control rats in contrast to normal control rats. The onset of CFA caused a remarkable depletion in the levels of GSH by 5.01, 3.77, 2.20 and 1.81 fold in the joints, liver, spleen, and kidney respectively. These altered enzymatic and non-enzymatic levels were significantly checked by the indomethacin and formulation treated rats as compared to the negative control rats.

3.10. Effect of formulation on histopathology

The histological study of hematoxylin and eosin-stained ankle joints of normal and CFA treated rats were depicted in Fig. 5B-D. The joints of CFA vehicle-treated rats were noticed with distinct bone and cartilage erosion indicating the successful induction of arthritis by killed *Mycobacterium tuberculosis* followed by the development of synovial hyperplasia and pannus formation. The ankle joints of healthy rats revealed normal histology of joints. The histological section of joints of indomethacin treated rats observed increased in smooth articular surface and articular cartilage layer, normal joint space, and reduction in synovial hyperplasia and pannus formation. The therapeutic administration of formulations from 15th to 28th day had remarkably reduced the histological

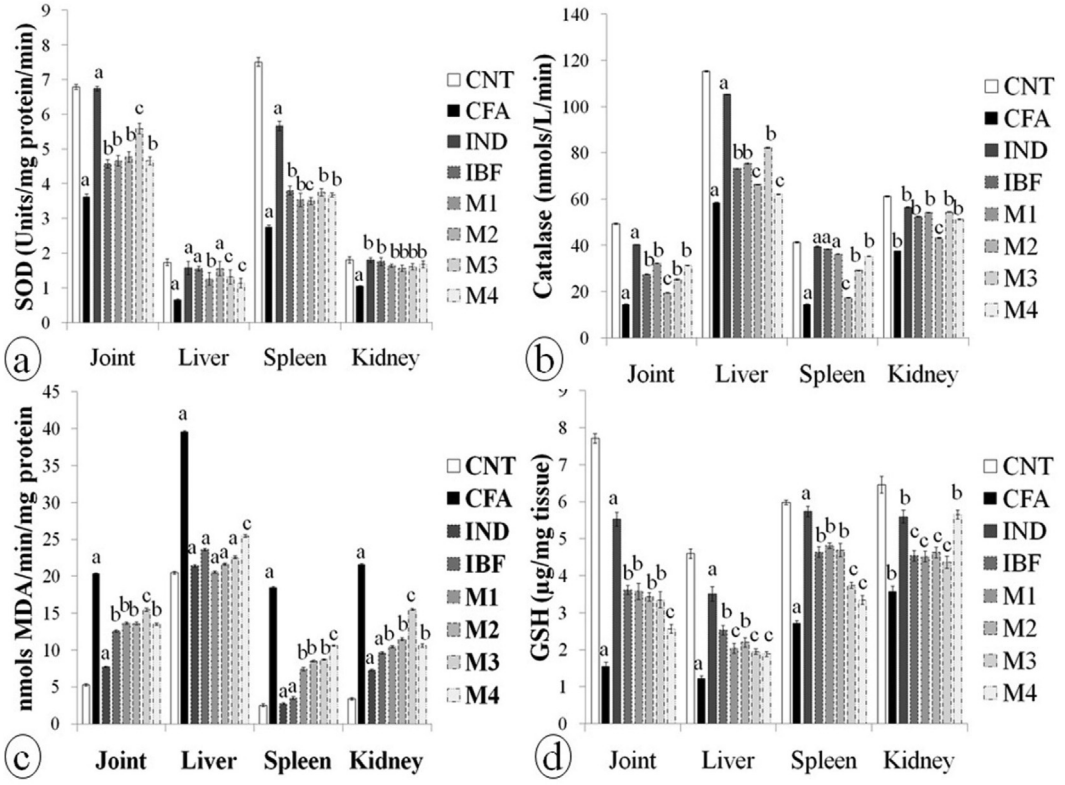

Fig. 4. Therapeutic effect of IBF, M1, M2, M3, M4 and indomethacin on (a) SOD, (b) Catalase, (c) LPO and (d) GSH of CFA induced experimental rats. Values are expressed as mean ± SEM, n = 6 and was estimated using one-way ANOVA followed by posthoc Tukey HSD test. Comparisons are made between i) Group-1 Vs Group-2, ii) Group-2 Vs Group-3, 4, 5, 6, 7 and 8. [a] P< 0.001, [b] P< 0.01, [c] P< 0.05 considered as significant.
CNT: Normal control; CFA: Complete Freund's adjuvant; IND: Indomethacin; IBF: In-house Balarista formulation; M1, M2, M3 and M4; Marketed formulation.

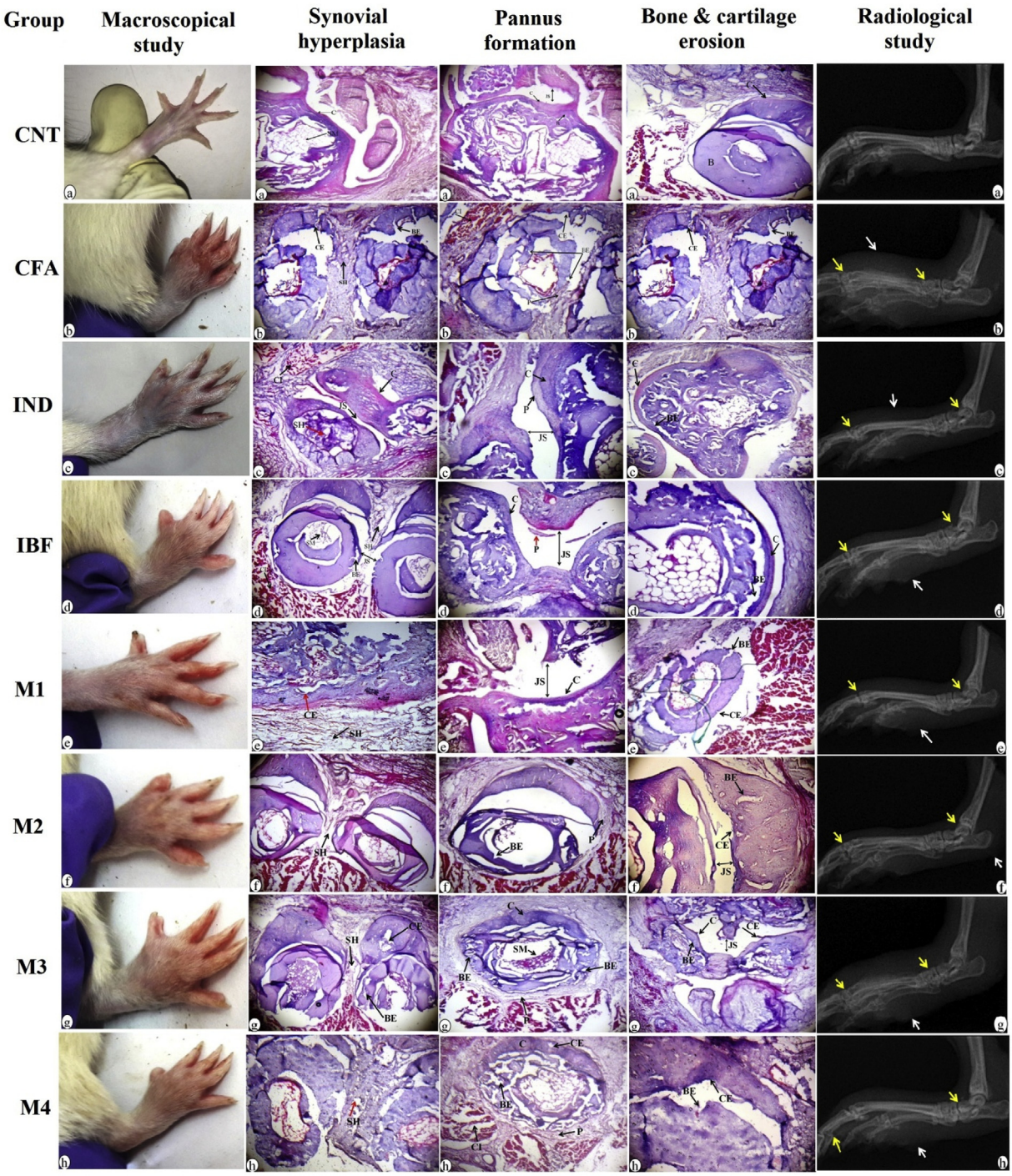

Fig. 5. Therapeutic effect of IBF, M1, M2, M3, M4 and indomethacin on (A) Macroscopical study of hind paw; (B) Synovial hyperplasia; (C) Pannus formation; (D) Bone and cartilage erosion and (E) Radiological study: soft tissue swelling (white arrow), degenerative changes (yellow arrow) of proximal interphalangeal joints (stained with hematoxylin and eosin) of experimental rats. C: Cartilage; CE: Cartilage erosion; CI: Cellular infiltration; B: Bone; BE: Bone erosion; JS: Joint space; SM: Synovial membrane; SH: Synovial hyperplasia; P: Pannus.
CNT: Normal control; CFA: Complete Freund's adjuvant; IND: Indomethacin; IBF: In-house Balarista formulation; M1, M2, M3 and M4; Marketed formulation.

changes in experimental rats.

3.11. Effect of formulation on X-ray analysis

The radiographic examinations of ankle joints of normal and treated animals were shown in Fig. 5E. The CFA induced rats were marked by swelling of soft tissue, narrowing of joint space and resorption of bone matrix, whereas no such evidence was noticed in healthy control rats. These radiological changes were significantly protected by indomethacin and formulation supplemented rats in the order of IBF, M1, M2, M4, and M3.

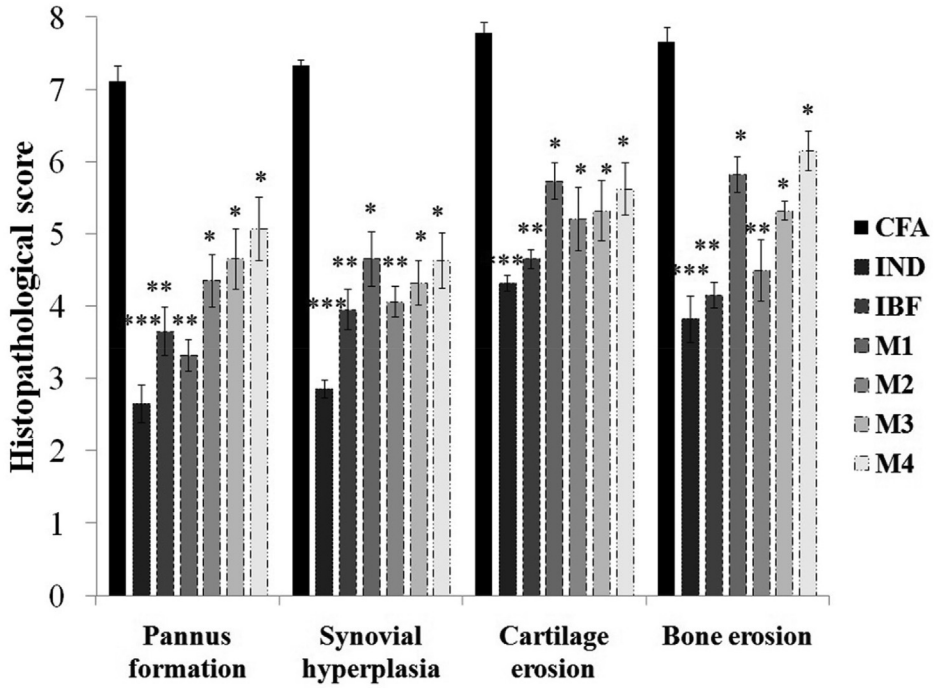

Fig. 6. Prophylactic effect of IBF, M1, M2, M3, M4 and indomethacin on histological score of adjuvant arthritic rats. Values are expressed as mean ± SEM, n = 6 and was estimated by using one-way ANOVA followed by posthoc Tukey HSD test. Comparisons are made between Group-2 Vs Group-3, 4, 5, 6, 7 and 8. ***P< 0.001, **P< 0.01, *P< 0.05 considered as significant.
CNT: Normal control; CFA: Complete Freund's adjuvant; IND: Indomethacin; IBF: In-house Balarista formulation; M1, M2, M3 and M4; Marketed formulation.

3.12. Histological score

The histological changes in CFA treated rats, due to inflammatory mediator, were observed and was scored according to the degree of pannus formation, synovial hyperplasia, and cartilage and bone erosion. The increase in mean pannus formation score and synovial hyperplasia score, in the CFA vehicle-treated group, was significantly reduced by indomethacin. The supplementation of formulations noticeably decreased the mean pannus formation score in the order of M1, IBF, M2, M3 and M4; and the synovial score in the order of IBF, M2, M3, M4, and M1. The increase in mean cartilage and bone erosion score in CFA induced rats was remarkably counteracted by indomethacin, IBF, M2, and M3 as compared to M1and M4 formulations (Fig. 6).

4. Discussion

The result of the present study revealed that the use of both in-house and marketed Balarista formulations successively manages the disease progression in RA by inhibiting the production of inflammatory cytokines and oxidative stress. The rats induced with CFA were noticed with remarkable inflammation on hind paws. In the acute phase of inflammation, there is the production of immune cells that are drifted to the injected area and causes the vascular-exudative phenomenon. In the chronic phase, there is the liberation of pro-inflammatory mediators, which causes synovial hyperplasia, pannus formation, and bone and cartilage destruction.[23] In the present investigation, the significant reduction in the paw edema, arthritic score, arthritic index, and histological score could be due to the interaction of active principles of Balarista formulation with these immune cells and thereby inhibiting the release of inflammatory cytokines. Among the contents of Balarista formulation, *S. cordifolia* and *W. somnifera* are considered to be efficacious. *S.cordifolia* as an important ingredient of an herbal formulation "OA-F2" was found to be significantly protected cartilage degeneration against the collagen-induced arthritic rat model.[24] *S. cordifolia* in "Kerabala", a novel ayurvedic formulation, has been proven as strong antioxidant, anti-inflammatory and anti-arthritic agent.[25] Presence of bioactive compounds such as asparagine, ephedrine, hypaphorine, vasicinone, vasicine, vasicinol, and phytosterols may attribute to the pharmacological importance of *S. cordifolia*.[26] The isolated compounds such as vasicine and vasicinone of *S. cordifolia* have been reported as potent anti-inflammatory in carrageenan and CFA arthritic rat model.[27] The anti-arthritic efficacy of formulations are supported by the anti-oxidant, anti-inflammatory, and anti-arthritic potential of sitoindosides VII-X and withaferin-A of *W. somnifera*.[28–31] *W. somnifera* was also clinically proved in the effective management of RA as reported by Kumar et al. (2015).[32] However, the successful management of arthritis by Balarista formulation may also be attributed to the anti-arthritic activity of *A. galanga*, *E. cardamomum*, *E. caryophyllus*, *V. zizanioids*, and anti-inflammatory activity of *W. fruticosa, I. digitata*, *R. communis*, *E. cardamomum,* and *T. terrestris*.[9]

Rheumatoid cachexia, recognized by a decrease in body weight due to the poor absorption of nutrients through intestine during inflammation,[33] which was noticed in CFA induced rats. The data of the present study revealed significant protection against loss of body mass in formulation treated rats. It is reported that the anti-inflammatory agents can retrieve the impended absorption potentiality of the intestine.[34] Thus, the anti-inflammatory property of ingredients present in Balarista formulation provides significant protection against weight loss.

It has been seen that a patient with RA was associated with several hematological and biochemical alterations, which are considered as crucial markers in an arthritic diseased condition indicating a change in the immune system.[35] The variation in hematological parameters such as rise in WBC count, platelet, ESR and

decrease in RBC, Hb and packed cell volume were noticed in experimental rats. The significant increase in serum enzyme level; AST, ALT, and ALP indicates functional impairment of liver and kidney of the body.[36] Pro-inflammatory mediators, which are liberated from macrophages and T-cells, play a pivotal role in the commencement and continuation of RA. The proliferation of TNF-α and IL-1β in the arthritic joint indicates its active participation in the pathogenesis of RA.[37] It is reported that ROS plays a significant role in the pathogenesis of RA.[3] However, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-Kb) has the potentiality to influence the activity of ROS, thus, contributing to the enhancement of pro-inflammatory cytokines.[38] In the present study, the CFA induced rats were marked with an increase in pro-inflammatory cytokines. These elevated cytokines cause an increase in cholesterol and serum triglyceride level by stimulating LDL levels.[39] Furthermore, the release of inflammatory mediators; histamine, bradykinin, and prostaglandins raise the absorption of vascular tissues, which bring down the albumin level in serum.[40]

The significant inhibitory actions of formulations on pro-inflammatory cytokines were noticed by a decrease in cholesterol, triglyceride, and increase in albumin levels. This effect of the formulation could be due to the interaction of bioactive compounds of formulation with inducible TNF-α, IL-1β receptors or by inhibiting the effect of NF-kB phosphorylation. This could be justified by the fact that withaferin-A reduced the activation of NF-kB and thereby decreased the synthesis of TNF-α which leads to a reduction of the cell damage, cartilage and bone erosion in arthritic rats.[41] The inhibition of prostaglandin synthesis by *S. cordifolia* by arresting cyclo-oxygenase was reported by Anil Kumar (2010).[42] *W. somnifera* as a component of UNIM-301 (Unani system of medicine) has proved significant reduction of the pro-inflammatory cytokines in adjuvant-induced arthritic rats.[43] Thus, the reduction in cytokines in the formulation treated rats could be due to the presence of *S. cordifolia* and *W. somnifera*.

The C-reactive protein, a prototype biomarker of systemic inflammation for the acute phase of the reactant, elevates rapidly during the phase of inflammation and is used as a useful serum biomarker for evaluating the active inflammation.[44] The serum RF, a non-self immunoglobulin molecule, is capable of triggering a reaction of the body's immune system. The abnormal change of RF in serum could be acknowledged as a strong indicator of RA.[45] In this study, the significant increase in CRP and RF levels was evident of the inflammatory process in the vehicle supplemented CFA rats. The significant decline in the degree of CRP and RF in rats, treated with formulations, justified its protective effect against RA. In the present work, treatment with formulation remarkably corrected all the hematological and biochemical alterations. These results advocate that the formulation might interact with stimulation and activation of immune cells possibly by blocking the liberation of various inflammatory cytokines and ROS. In arthritic conditions, the *inflamed* cells release free radicals and cause tissue oxidative stress, which was overcame, in this study, by the Balarista formulations by decreasing the inflammation as well as oxidative stress of arthritic rats.

In the present investigation the elevated malondialdehyde (MDA) content was noticed in the tissue of liver, kidney, spleen and joint of CFA induced arthritic rats in contrast to normal rats indicates increased in LPO. This might be due to the compromised intracellular antioxidant defense system with insufficiently scavenge free radicals resulting in increased oxidative stress in RA.[46] Decrease in SOD and CAT activity was observed in CFA induced rats in contrast to the normal healthy group. This may be due to the excessive production of superoxide radicals and H_2O_2 that obstruct the enzymatic activity.[47] It was noticed in the study that excessive lipid peroxidation caused an increase in GSH consumption and thereby depletion of GSH in CFA induced rats as compared to normal rats.[48] The oral administration of formulation in CFA induced rats has improved the SOD and CAT activity followed by GHS and the decreased level of MDA suggesting the antioxidant activity of the formulation. This may be due to the presence of bioactive principles such as withanolides, withaferin-A, flavonoids, and alkaloids in *W. somnifera* root, which have been proven to scavenge the free radicals in the stress-induced animals.[49] Besides, the presence of compounds such as sitoindosides VII–X, polyphenols, flavonoids and vitamin C in *W. somnifera* root were proved to possess antioxidant activity.[30,32,50] It is also reported that the phenolic content of many plants has exhibited antioxidant activity. *S. cordifolia* is an important medicinal plant of the ayurvedic system of medicine, which is rich in poly-phenolic compounds. Thus, the presence of active principles of *S. cordifolia*, *W. somnifera*, and other ingredients could potentiate the antioxidant efficacy of the Balarista formulation.

The experimental rats, subjected to histological study, were marked with degenerative and inflammatory changes in the ankle joint. The decreased histopathological score has indicated a significant protective role of the formulation against arthritic conditions. The reduction in tissue injuries and inflammatory changes in ankle joint might be due to the antioxidant activity of formulation by scavenging the free radicals. Thus, the immunomodulatory effects of formulation against arthritic rats indicate its potential as an anti-arthritic agent. In RA, the destruction of bone and cartilage causes a decrease in joint space which leads to bone fusion.[51] X-ray analyses of experimental rats were marked with the loss of joint space owing to the fusion of ankle joint. Radiographs of the ankle joints of CFA induced rats were noticed with a significant reduction in fusion and bone destruction after treatment with the formulation.

5. Conclusion

This study has demonstrated that the in-house and marketed Balarista formulation at a dose of 2.31 ml/kg has a significant therapeutic effect on CFA induced arthritic rats, justifying its traditional use. The presence of various ingredients in Balarista formulation, which have reported to contain active principles such as flavonoids, phenols, alkaloids, steroids, and terpenoids, etc., with their synergistic effect, might have imparted the potent antioxidant and anti-arthritic potential to this classical formulation. Further, more studies are required to investigate the quantitative presence of different phytoconstituents which are responsible to maintain the therapeutic efficacy in all these formulations.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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