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ANTIARTHRITIC ACTIVITY OF *BALIOSPERMUM MONTANUM* ROOTS IN COMPLETE FREUND'S ADJUVANT INDUCED ARTHRITIS IN RAT

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ABSTRACT

The present investigation was designed to evaluate the antiarthritic potential of *Baliospermum montanum* roots. *Baliospermum montanum* were defatted by petroleum ether then successive extraction was done with chloroform, ethanol and water. Arthritis was induced by complete freund's adjuvant arthritis assessment was done by measuring paw volume, bodyweight, locomotor activity, haematological parameter, haemoglobin and erthrocyte sedimentation rate and *In vivo* antioxidant parameter (lipid peroxidation, superoxide dimutase, glutathione), histopathological assessment of joints, Radiological studies. Ethanolic extract of *Baliospermum montanum* roots significantly inhibition of paw volume, increased locomotor activity, increased haemoglobin and decreased erythrocyte sedimentation rate and decreased lipid peroxidation and increased superoxide dimustase and gluthathine Histopathological studies showed less influx of inflammatory cells, no pannus formation and absence of necrosis, radiographic study showed reducing the soft tissue swelling and narrowing of joint spaces. The result of the investigation concluded ethanolic extract of *Baliospermum montanum* possesses a significant antiarthritic activity. The observed antiarthritic activity may be due to the presence of phytoconstituent such as flavanoid. However further studies are needed to carry out the isolation of active constituents of ethanolic extract responsible for the activity.

KEYWORDS: Complete freund's adjuvant, Rheumatoid arthritis, *Baliospermum montanum*, Diclofenac, Paw volume.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, progressive and systemic autoimmune disorder characterized by inflammation of the joints, with proliferation of the synovium and progressive erosion of cartilage and bone.[1] Rheumatoid arthritis (RA) is chronic polyarticular inflammatory arthritis that involves notonly small joints of the hands and feet but also medium and large joints. It is associated with excessive disability, mortality and morbidity.[2] Currently available treatments have improved efficiency, the use of non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin; disease-modifying antirheumatoid drugs (DMARDs), such as methotrexate, sulfasalazine, leflunomide and hydroxychloroquine and corticosteroids, such as prednisolone and methylprednisolone is associated with several adverse reactions. Hence, patients with musculoskeletal disorders have sought alternative methods for symptomatic relief. The published literature reveals that patients prefer complementary and alternative medicine. *Baliospermum montanum* commonly known as red physic nut, wild castor, wild sultan seed. The genus Baliospermum is a member of family Euphorbiaceace. Leaves contain β-stiosterol, β-D glucoside, hexacosanol. Roots contain phorbol esters, viz. montanin, baliospermin, β-stiosterol, β-D glucoside, hexacosanol12-deoxyphorbol-13-palmitate, 12-deoxy-16-hydroxyphorbol-13-palmitate, 12-deoxy-5β-hydroxyphorbol. Pharmacological activity reported immunomodulatory activity[3], anthelmintic activity[4], hepatoprotective activity[5], analgesic activity[5], antioxidant activity[6], wound healing activity[7], anti-inflammatory activity[8], anticancer activity[9], antimicrobial activity[10], antidiabetic activity.[11]

MATERIAL AND METHODS

Plant material

Roots were collected from Rachna Devi Hospital and Research Centre, Bhopal and authenticated by Dr. Saba Naaz, HOD, Department of Botany, Safia Science College Bhopal (M.P). The accession number for the specimen is 144/bot/saifia/Sci/College.

Animals

The study was initiated after obtaining approval from the Institutional Animal Ethics Committee (PH/IAEC/VNS/2K17/07). Healthy wistar rats were obtained from the animal house of Faculty of Pharmacy, VNS Group of institutions Bhopal (M.P). Rats were used according to the guidelines given by Committee for the

Purpose of Control and Supervision of Experiments on Animals (CPCSEA) in India. All animals were kept in standard cages and maintained at 25^0C under12 hrs light/dark cycles. The animals were fed with standard feed and water was given after specific interval prior to experiment animal were kept for 12 hrs fasting.

METHODS

Extraction

The Roots of *Baliospermum montanum* was coarsely powdered with the help of electrical grinder and was stored in air tight container for further use and were subjected to successive soxhlet extraction using various solvent (Petroleum ether, Chloroform, Ethanol, Water).

Successive soxhlet extraction method

The dried coarsely powdered drug (25gm) was packed in soxhlet apparatus and defatted with 250 ml of petroleum ether (40-60^{0}C) till complete defeating (21 hours). The defatted material was extracted with different solvents in increasing order of polarity from nonpolar to polar.[12] The percentage yield of extract expressed on dry weight basis of *Baliospermum montanum* roots extract was calculated from the following formula.

$$\% \text{ yield} = \frac{W_1}{W_2} \times 100$$

Where W_1 weight of the completely dried extract residue obtained after solvent removal and W_2 of the weight of dried roots before extraction.

Preliminary phytochemical screening

The petroleum ether (PEBM), chloroform (CLBM), ethanolic (EBM) and aqueous extracts (AQBM) of *Baliospermum montanum* was subjected to qualitative analysis for the various phytoconstituents alkaloid, carbohydrates, glycosides, flavonoid, steroids, tannins and phenolic compounds, fFat and oils.[13]

Evaluation of *in vitro* antiarthritic activity

The *in vitro* antiarthritic activity was studied using Egg albumin denaturation method.[14]

Procedure

The reaction mixture (5ml) was incubated in a water bath at 37^{o}C for 15 min and then heated at 70^{o}C for 5 min. After cooling, absorbance was measured at 660 nm in UV visible spectrophotometer. Percentage inhibition of protein denaturation was determined on percentage basis with respect to control using the following formula.

$$\% \text{ inhibition} = \frac{Ac - At}{Ac} \times 100$$

Ac- Absorbance of control solution, At- Absorbance of test solution.

Dose selection

Acute toxicity studies were not conducted as its safe up to 2000 mg/kg has been reported in an earlier study. The two doses 200 and 400 mg/kg were selected.[11]

Evaluation of *in vivo* antiarthritic activity

Total 30 female Wister rats weighing 190-250gm were selected and divided into 5 Groups of 6 rats in each (n=6). Control, Negative control, Standard, Treatment group1st 200 mg/kg and Treatment group 2nd400mg/kg. Arthritis was induced by subplanter injection of complete freund's adjuvant (2mg/ml) into fore paw and hind paw of animals of all groups on day 0 (except normal control group). Complete freund's adjuvant contains heat killed dead *Mycobacterium tuberculosis* bacterium in liquid paraffin oil in the concentration 2 mg/ml. Group 1 served as Control group treated vehicle only. Group 2 served as Negative control group received of complete freund's adjuvant only. Group 3 served as standard group received Diclofenac sodium (10mg/kg) for 14 days from the 14 thday of induction of arthritis (14th to 28th day treatment) and Group 4 and group 5 served as treatment group 1st and treatment group 2nd received ethanolic extract of *Baliospermum montanum* root (EBM) 200mg/kg and 400 mg/kg for 14 days from the 14th day of induction of arthritis (14th to 28th day treatment).[15]

Table 1: Groups of animals and treatment strategy.

Groups	Group name	Treatment
1.	Control group	Vehicle treated only
2.	Negative control group	0.125ml of complete freund's adjuvant (2mg/ml) Sub planter
3.	Standard group	Diclofenac sodium (10 mg/kg) I.M
4.	Treatment group 1st	*Baliospermum montanum*(EBM) 200mg/kg p.o
5.	Treatment group 2nd	*Baliospermum montanum* (EBM) 400mg/kg p.o

Parameter

Paw volume

Paw volume was measured by plesthysmometer at 0, 7, 14, 21, 28 day.

$$V = \frac{W}{P}$$

Where W is weight and P is gravity of mercury.

Percentage inhibition of paw volume

The mean paw volume of the drug treated rats was compared with arthritic control rats. Percentage inhibition was calculated from this formula.

$$\% \text{ inhibition of Paw volume} = \frac{(Vf - Vi)\text{Negative control} - (Vf - Vi)\text{ Treatment}}{(Vf - Vi)\text{ Negative control}} \times 100$$

Vf=Mean Paw volume at a particular time interval, Vi= Mean Paw volume at a 0 day.

Body weight
Body weight was measured on day 0 and after every 7 days up to 28 days using calibrated electronic balance.

Locomotor activity
Locomotor activity was observed by Actophotometer at 0, 7, 14, 21, 28 day.

Haemoglobin (Hb)
Haemoglobin was measured in rat by Sahli's haemoglobin meter at 0 day, 14th day, 28th day. Matches with the standard colour strips. Observed the reading and reported.[16]

Erythrocyte sedimentation rate (E.S.R)
ESR is measured by Wintrobe tube method at 0th day, 14th day and 28th day.

Estimation of oxidative stress biomarkers
1. The blood was collected from the Female Wister rats was maintained on a standard diet and water.
2. 20µL of 500 IU Heparin was added in 500 µL of blood was collected from retro-orbital plexus under the influence of light ether anaesthesia.
3. Blood was centrifuged at 2500 rpm at 2-8^{0}C for 10 mins.
4. Plasma and buffy coat was removed.
5. Collected erythrocyte was suspended in 4 volumes of ice cold water and Mixed thoroughly.
6. Allow to stand for 5 mins, so that lysis occurs.
7. 800 µL of ice cold chloroform/ethanol (3.75/6.25 v/v) was added in 500 µL of lysate and Shake to 10 sec.
8. Mixture was centrifuged at 2500 rpm at 2-8 ^{0}C for 10 mins.
9. Lysate (upper layer) was used for estimation of SOD, LPO and SOD.

Estimation of lipid peroxidation (LPO)
The solution was kept at 95^0 C in a water bath for 1 hr. The complex formed and gives a pink colour. The samples was centrifuged at 10,000 rpm for 6 mins till the protein precipitated. 0.20 ml of sample (supernatant) was used for the estimation of LPO at 532nm.[17] Calculate based on 1.5×10^5 as the molar extinction coefficient for malondialdehyde. The precipitated protein was used in the samples (after decantation) for the protein measurement and expresses the result as MDA mol/gm of protein.

Estimation of superoxide dimutase (SOD)
1.52 ml was taken of above solution and 0.08 ml of pyrogallol was added. The rate of increased in the absorbance was measured at 420 nm from 30 sec to 5:30 min in UV spectrophotometer. The lag time of 30 sec was allowed for steady state autooxidation of pyrogallol to be attained.[18] Result was expressed in units per gm/mol tissue or per mg of protein for tissue homogenate all experiment was carried out in air conditioned room at 25^{o}C. Protein content was estimated according to the lowry method.

ΔA_{420} nm/min= (A_{420}nm at 5.30 –A_{420}nm at 0.30min/5 min)

Estimation of glutathione (GSH)
Equal volume of 10% TCA and samples (homogenate) was added and allow stand for 10 min and centrifuged at 10000 rpm for 6 min. The supernatant was used for the estimation of GSH. Allow to stand for 10 mins till yellow colour was appeared. Sample was taken and 200µl of solution (with or without sample) in each test. It is required to have corresponding control (without any sample with DTNB and buffer) for GSH assay as assay control. Deduct the detect the absorbance of the assay control from the absorbance of the sample for calculation. Detect the absorbance at 412 nm in spectrophotometer.[19] The results were expressed in µg/mg of protein. All the experiments were carried out in an air conditioned room at 25^{o}C.

GSH (µg/mg of protein) =

$$\frac{\text{Absorbance 412nm} - 0.014 \times \text{DilutionFactor}}{0.0073}$$

Histopathological assessment of joints
For histopathological assessment, the ankle joint was fixed in 10% formalin solution. The fixed tissues of ankle joints were decalcified in formic acid, embedded in paraffin block and trimmed longitudinally into 5mm sections with the help of microtome. A series of transverse sections of the tissue were prepared and stained with hematoxylin and eosin stain. The exposed section was selected for qualitative light microscopic analysis for the cellular infiltration, joint space narrowing, synovial hyperplasia, pannus formation, bone and cartilage erosion of the ankle joint.

Radiological studies
Radiographs were taken for the hind paw of all the experimental animals and examined for soft tissue swelling, bony erosions and narrowing space between joints.[20]

Statistical analysis
The results were evaluated as mean ± SEM and differences among data were determined using one way and two ways ANOVA Followed by Tukey's multicomparison test. P value less than 0.05 were considered to be significant and statistical analysis was performed using Graph pad prism software, San Diego, CA. (Version 8.2.0).

RESULT
Preliminary phytochemical analysis revealed the PBM contain steroids, fat & oils. CLBM contain glycosides, flavonoids, steroids, tannins and phenolic compounds.EBM contain alkaloids, glycosides, flavonoid, steroids, tannins and phenolic compound. AQBM contain glycosides, flavonoids, tannins and phenolic compounds Table 2. Diclofenac sodium at concentration 100µg/ml, 200µg/ml, 300µg/ml,

400µg/ml, and 500µg/ml showed 15.4%, 25.7%, 40.4%, 52.9%, 62.5% inhibition of Egg albumin denaturation. EBM concentration 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml showed 11.7%, 22.7%, 33.8%, 45%, 56.6% inhibition of Egg albumin denaturation. CLBM 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml showed 8.8%, 19.8%, 28.3%, 49.2% inhibition of Egg albumin denaturation. AQBM showed 8.08%, 17.6%, 25.7%, 38.2%, 46.3%inhibition of Egg albumin denaturation. The ethanolic extract of *Baliospermum montanum* showed highest percentage inhibition of protein denaturation Table 3. CFA Induced arthritic rat showed significantly increased of paw volume on 21th and 28th day as compared to control group. Treatment with diclofenac sodium (10 mg/kg) showed significantly decreased paw volume on 21th and 28th day (**P<0.01 and ***P<0.01) as compared to negative control group. Treatment with EBM 200mg/kg showed significantly inhibition of paw volume on 21th day and 28th day (*P<0.01and *P<0.01) as compared to negative control group. EBM 400mg/kg showed significantly inhibition of paw volume on 21th day and 28th day as compared to negative control group (**P<0.01and **P<0.01) Table 4 & Figure 1. Group treated with diclofenac sodium (10mg/kg) showed 35.8% inhibition of paw volume on day 21th and 43.6 % inhibition of paw volume on 28th day. Group treated with EBM 200mg/kg showed 31.6 % inhibition of paw volume on day 21th and 36.5% inhibition of paw volume on 28th day. Group treated with EBM 400mg/kg showed 34.1% inhibition of paw volume on day 21th and 42.06 % inhibition of paw volume on 28th day. Treatment group EBM 400mg/kg showed inhibition of paw volume more as compared to treatment group 200mg/kg Table 5. Bodyweight of CFA Induced arthritic rats were significantly decreased on 28th day as compared to control group. Treatment with EBM 200mg/kg and 400 mg/kg significant increased bodyweight on 21th day (*P<0.01and *P<0.01) and 28th day (*P<0.01and **P<0.01) as compared to negative control group. Treatment group 400 mg/kg was more significant than 200mg/kg Table 6 & Figure 2. Locomotor activity of CFA Induced arthritic rats was significantly decreased on 21th and 28th day as compared to control group. Treatment with EBM 200mg/kg and 400 mg/kg was significantly increased locomotor activity on 21th day (*P<0.01and **P<0.01) and 28th day (**P<0.01 and ***P<0.01) as compared to negative control group. Treatment group 400mg/kg was more significant than 200mg/kg Table 7 & Figure 3. Haemoglobin of CFA Induced arthritic rat was significantly decreased as compared to control group. Treatment with EBM 200mg/kg and 400 mg/kg was significantly increased haemoglobin (*P<0.01 and **P<0.001) as compared to negative control group. Treatment group 400mg/kg was more significant than 200mg/kg Table 8 & Figure 4. ESR of CFA Induced arthritic rat was significantly increased as compared to control group. EBM 200mg/kg and 400 mg/kg significantly decreased ESR (*P<0.01 and **P<0.001) as compared to negative control group. Treatment group 400mg/kg was more significant than 200mg/kg Table 9 & Figure 5.

Lipid Peroxidation of negative control group was significantly increased as compared to control group. EBM 200 and 400 mg/kg significantly decreased LPO levels as compared with the negative control group. (*P < 0.05) and (**P < 0.01). Treatment group 400 mg/kg was more significant than 200mg/kg Table 10 & Figure 6. SOD of negative control group was significantly decreased compared to control group. Treatment with EBM 200 and 400 mg/kg significantly increased SOD levels as compared with thenegative control group. (*P<0.05 and **P<0.01).Treatment group 400 mg/kg was more significant than 200mg/kg Table 11 & Figure 7. The level of GSH of negative control group was significantly decreased as compared to control group. Treatment with EBM 200 and 400 mg/kg was significantly increased GSH levels as compared to negative control group. (**P< 0.01 and *P< 0.05). Treatment group 400 mg/kg was more significant than 200mg/kg Table 12 & Figure 8.

Table 2: Preliminary phytochemical analysis of different extracts of BM roots.

S.NO	TEST	PBM	CLBM	EBM	AQBM
1.	**Alkaloids**				
a.	Dragendorff'sTest	-ve	-ve	+ve	-ve
b.	Hager's Test	-ve	-ve	+ve	-ve
c	Mayer'sTest	-ve	-ve	-ve	-ve
d.	Wagner's Test	-ve	-ve	-ve	-ve
2.	**Carbohydrates**				
a.	Benedict's Test	-ve	-ve	-ve	-ve
b.	Barfoed's Test	-ve	-ve	-ve	-ve
c.	Fehling Test	-ve	-ve	-ve	-ve
3.	**Glycosides**				
a	Legal Test	-ve	+ve	+ve	-ve
b	Baljet Test	-ve	-ve	-ve	-ve
c.	Killer killani Test	-ve	-ve	+ve	+ve
4	**Flavanoids**				
a.	Shinoda Test	-ve	+ve	+ve	+ve

b.	Alkaline reagent Test	-ve	+ve	+ve	-ve
c.	Lead acetate Test	-ve	-ve	-ve	+ve
5.	**Steroids**				
a.	Salkowski Test	+ve	+ve	+ve	-ve
b.	Liebermann Burchard Test	-ve	-ve	+ve	-ve
6.	**Tannins and phenolic compounds**				
a.	Ferric chloride Test	-ve	+ve	+ve	+ve
b.	Potassium dichromate Test	-ve	-ve	+ve	-ve
7.	**Fats and oils**				
a.	Filter paper Test	+ve	-ve	-ve	-ve

Where PBM – Petroleum ether extract of *Baliospermum montanum***, CLBM-** Chloroform extract of *Baliospermum montanum***, EBM-**Ethanolic extract of *Baliospermum montanum*, **AQBM-** Petroleum ether extract of *Baliospermum montanum* **+ve=** Positive**, -ve =**negative.

Table 3: Effect of standard drug and different extracts of BM on Egg albumin denaturation method.

Extract	Concentration (µg/ml)	Absorbance	Percentage inhibition
Diclofenac sodium	100	0.230±0.008	15.4%
	200	0.202±0.009	25.7%
	300	0.162±0.013	40.4%
	400	0.128±0.014	52.9%
	500	0.102±0.001	62.5%
Ethanolic extract of *Baliospermum Montanum (*EBM)	100	0.240±0.004	11.7%
	200	0.210±0.002	22.7%
	300	0.180±0.003	33.8 %
	400	0.148±0.003	45.5%
	500	0.118±0.002	56.6%
Chloroform extract of *Baliospermum montanum (*CBM*)*	100	0.248±0.005	8.8%
	200	0.218±0.002	19.8%
	300	0.195±0.003	28.3%
	400	0.158±0.002	41.9%
	500	0.138±0.002	49.2%
Aqueous extract of *Baliospermum montanum (*ABM)	100	0.250±0.003	8.08%
	200	0.224±0.015	17.6%
	300	0.202±0.002	25.7%
	400	0.168±0.001	38.2%
	500	0.146±0.002	46.3%
Control	NA	0.272±0.051	NA

Table 4: Effect of EBM on Paw volume (ml) in CFA induced arthritic rats.

Groups	0 day	7 day	14 day	21 day	28 day
Control	1.08±0.10	1.18±0.13	1.11±0.10	1.18±0.13	1.13±0.10
Negative Control	1.20±0.07	1.39±0.07	1.87±0.07	2.40±0.06[a]	2.46±0.04[a]
Standard	1.19±0.08	1.42±0.08	2.04±0.03	1.94±0.01**, [a]	1.88±0.02***, [a]
Treatment 1st	1.18±0.08	1.67±0.09	2.13±0.12	2.0±0.068*, [a]	1.98±0.13*, [a]
Treatment 2nd	1.16±0.07	1.56±0.05	2.12±0.06	1.95±0.01**, [a]	1.89±0.007***, [a]

Value are expressed as Mean ± SEM, n= 6 animal in each group. Data was analyzed using two way ANOVA followed by Tukey's multicomparison test group ***P < 0.001, **P < 0.01, *P < 0.05 compared with negative control groupand [a]P< 0.0001, [b]P< 0.001, [c]P< 0.01, [d]P< 0.05 compared with control group.

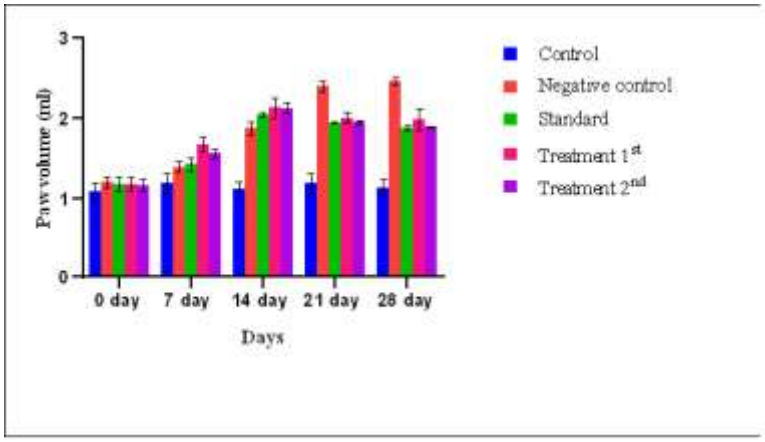


Fig. 1: Effect of EBM on paw volume.

Table 5: Percentage inhibition of paw volume.

Groups	21th day	28th day
Standard	35.8	43.6
Treatment 1st	31.6	36.5
Treatment 2nd	34.1	42.06

Table 6: Effect of EBM on bodyweight in CFA induced arthritic rats.

Groups	0 day	7 day	14 day	21 day	28 day
Control	214±4.49	214.7±4.40	215±4.60	216.5±4.73	218.7±4.27
Negative control	222.2±3.56	214.5±3.20	207.7±3.22	195.7±2.01	193.2±2.21[a]
Standard	220±6.32	211.2±5.02	202.5±4.78	219.2±5.79*	221.7±6.03**
Treatment 1st	227±4.65	218.5±4.27	209.5±3.52	221.2±3.35*	220.2±4.21*
Treatment 2nd	222±7.52	213.5±7.41	201.7±6.25	219.5±7.24*	222 ±6.84**

Value are expressed as Mean ± SEM, n= 6 animal in each group. Data was analyzed using Two way ANOVA followed by Tukey's multicomparison test group ***P < 0.001, **P < 0.01, *P < 0.05 compared with negative control groupand [a]P< 0.0001, [b]P< 0.001, [c]P< 0.01, [d]P< 0.05 compared with control group.

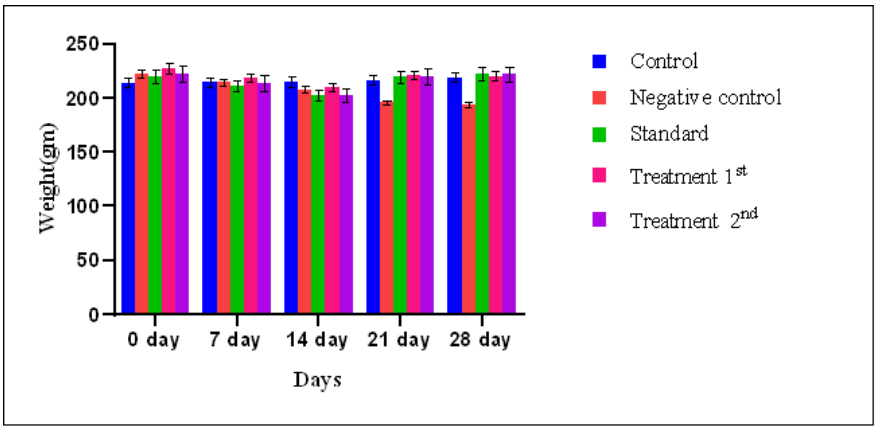


Fig. 2: Effect of EBM on bodyweight.

Table 7: Effect of EBM on locomotor activity in CFA induced arthritic rats.

Groups	0 day	7 day	14 day	21 day	28 day
Control	171.5±0.86	172.7±1.37	174.2±0.25	175.2±1.60	177.7±1.03
Negative control	177±1.29	169±0.70	160.2±3.06	152.5±1.55[a]	147.7±3.70[a]
Standard	183±2.88	172.7±3.09	160.2±3.09	167.2±3.17***	168±2.04***
Treatment 1st	185.5±2.98	172.2±3.56	159.7±2.86	161.5±2.59*,[c]	162.7±2.28**,[c]
Treatment 2nd	181±3.24	169.2±3.03	160±2.85	164.5±1.55**,[c]	166.2±1.54***,[d]

Value are expressed as Mean ± SEM, n= 6 animal in each group, Data was analyzed using Two way ANOVA followed by Tukey's multicomparison test group ***P < 0.001, **P < 0.01, *P < 0.05 compared with negative control groupand [a]P< 0.0001, [b]P< 0.001, [c]P< 0.01, [d]P< 0.05 compared with control group.

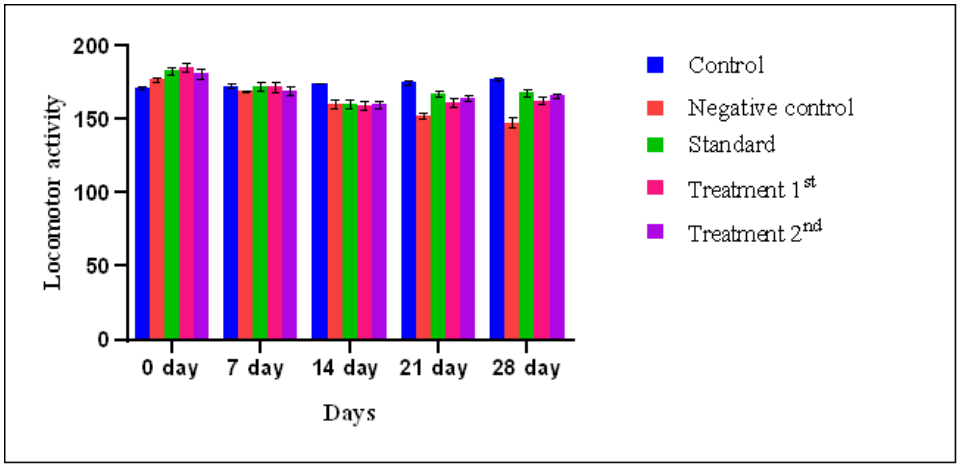


Fig. 3: Effect of EBM on locomotor activity.

Table 8: Effect of EBM on Haemoglobin (g/dl) in CFA induced arthritic rats.

Groups	0 day	14th day	28th day
Control	12.8± 0.12	12.8 ± 0.07	13.05± 0.08
Negative control	13.4±0.17	11.8±0.12	10.02±0.23[b]
Standard	13.4±0.27	11.6±0.53	12.1±0.41**
Treatment 1st	13.3±0.44	11.2±0.54	11.9±0.43*
Treatment 2nd	13.8±0.37	11.2±0.40	12.05±0.45**

Value are expressed as Mean ± SEM, n= 6 animal in each group, Data was analyzed using Two way ANOVA followed by Tukey's multicomparison test group ***P < 0.001, **P < 0.01, *P < 0.05 compared with negative control groupand [a]P< 0.0001, [b]P< 0.001, [c]P< 0.01, [d]P< 0.05 compared with control group.

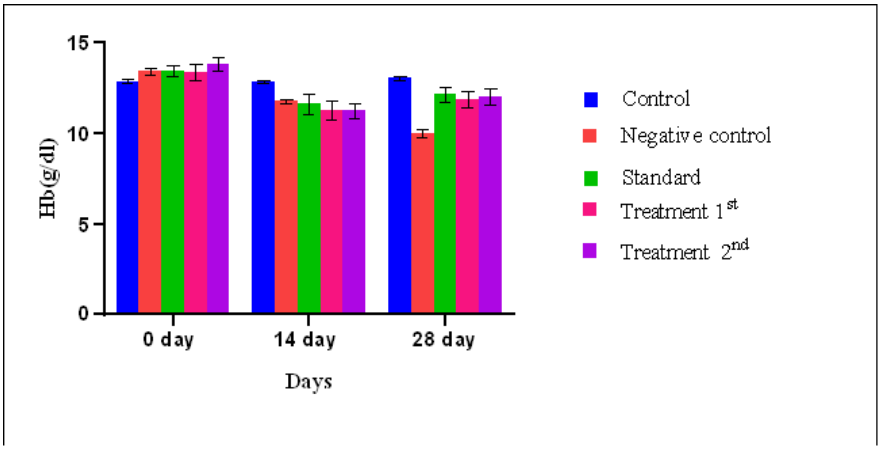


Fig. 4: Effect of EBM on Haemoglobin (g/dl).

Table 9: Effect of EBM on Erythrocyte sedimentation rate (ESR) in CFA induced arthritic rats.

Group	0 day	14th day	28th day
Control	3.5±0.43	3.5±0.39	3.5±0.44
Negative control	3.2±0.32	7.3±0.56	10.2±0.35[a]
Standard	3.2±0.37	9.02±0.80	7.3±0.22**, [b]
Treatment 1st	3.5±0.33	7.8±0.56	7.7±0.63*, [a]
Treatment 2nd	3.7±0.18	7.9±0.51	7.4±0.49**, [b]

Value are expressed as Mean ± SEM, n= 6 animal in each group, Data was analyzed using Two way ANOVA followed by Tukey's multicomparison test group ***P < 0.001, **P < 0.01, *P < 0.05 compared with negative control groupand [a]P< 0.0001, [b]P< 0.001, [c]P< 0.01, [d]P< 0.05 compared with control group.

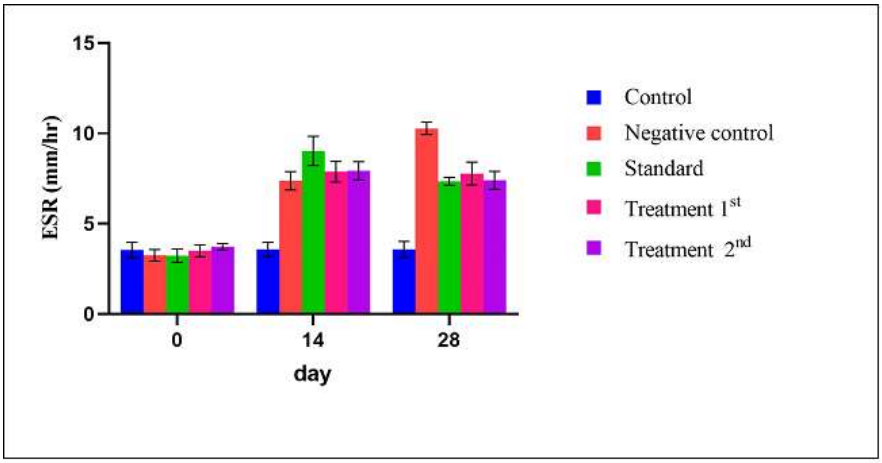


Fig. 5: Effect of EBM on Erythrocyte sedimentation rate (mm/hr).

Table 10: Effect of EBM on lipid peroxidation in CFA induced arthritic rats.

Groups	LPO (µM/mg of TP)
Control	8.8±0.36
Negative control	13.58±0.46[a]
Standard	11.08±0.28**, [d]
Treatment 1st	11.6±0.07*, [c]
Treatment 2nd	10.94±0.60**, [d]

Value are expressed as Mean ± SEM, n= 6 animal in each group, Data was analyzed using One way ANOVA followed by Tukey's multicomparison test group ***P < 0.001, **P < 0.01, *P < 0.05 compared with negative control groupand [a]P< 0.0001, [b]P< 0.001, [c]P< 0.01, [d]P< 0.05 compared with control group.

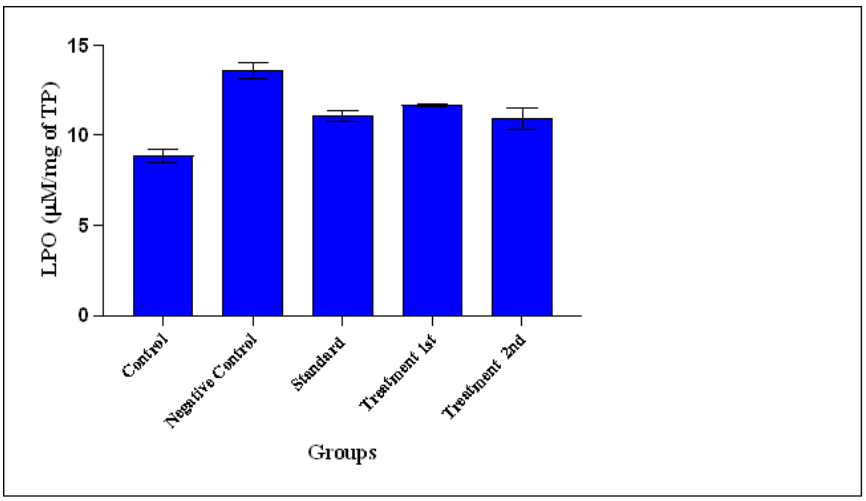


Fig. 6: Effect of EBM on lipid peroxidation.

Table 11: Effect of EBM on superoxide dismutase in CFA induced arthritic rats.

Groups	SOD(U/mg protein)
Control	7.3± 1.41
Negative control	1.1± 0.10[b]
Standard	6.7± 0.86**
Treatment 1st	5.8 ± 0.81*
Treatment 2nd	6.2±0.35**

Value are expressed as Mean ± SEM, n= 6 animal in each group, One way ANOVA Tukey multi comparison test group ***P < 0.001, **P < 0.01, *P < 0.05 compared with negative control group and [a]P< 0.0001, [b]P< 0.001, [c]P< 0.01, [d]P< 0.05 compared with control group.

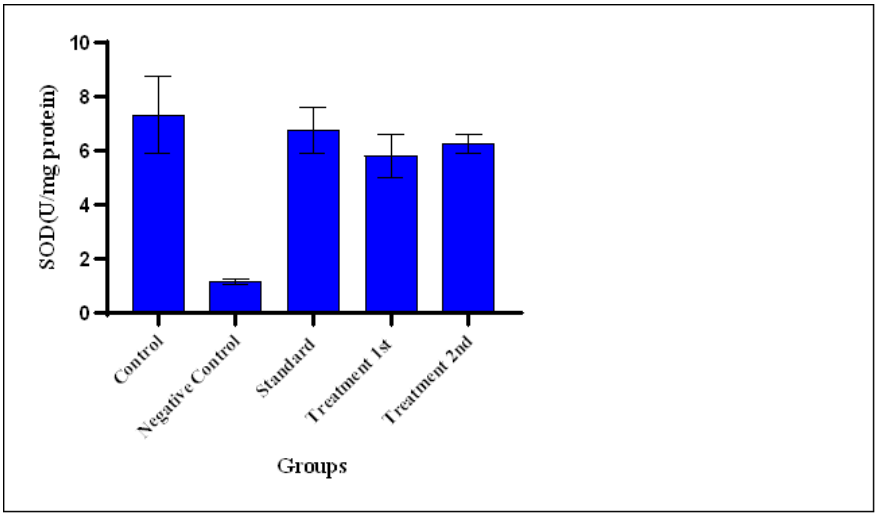


Fig. 7: Effect of EBM on superoxide dismutase (U/mg).

Table 12: Effect of EBM on glutathione in CFA induced arthritic rats.

Groups	GSH(µg/mg TP)
Control	278.8 ± 6.79
Negative control	207.6± 8.03[a]
Standard	249.02 ±6.93**[,d]
Treatment 1st	239.2± 5.79*[, c]
Treatment 2nd	246.89± 4.48**[, d]

Value are expressed as Mean ± SEM, n= 6 animal in each group, One way ANOVA Tukey multicomparison test group ***P < 0.001, **P < 0.01, *P < 0.05 compared with negative control groupand [a]P< 0.0001, [b]P< 0.001, [c]P< 0.01, [d]P< 0.05 compared with control group.

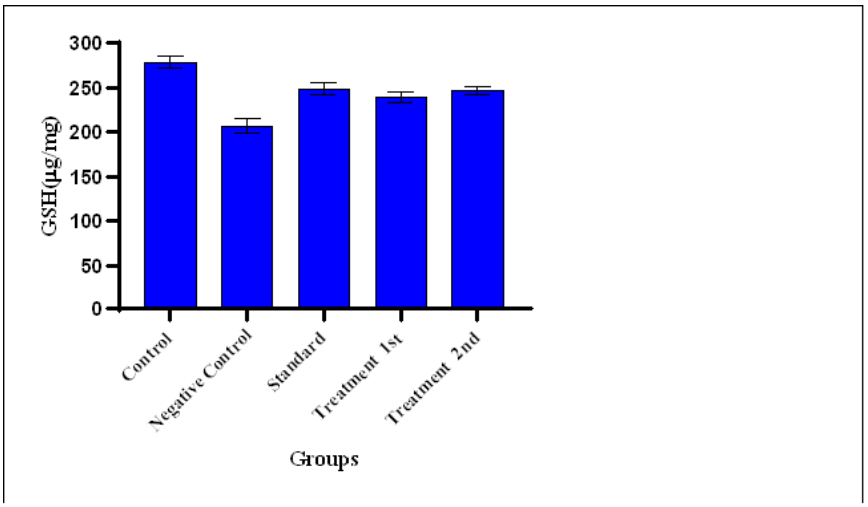
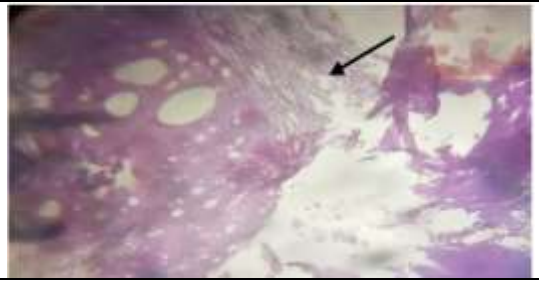
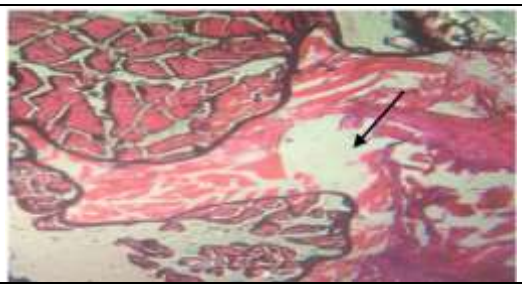


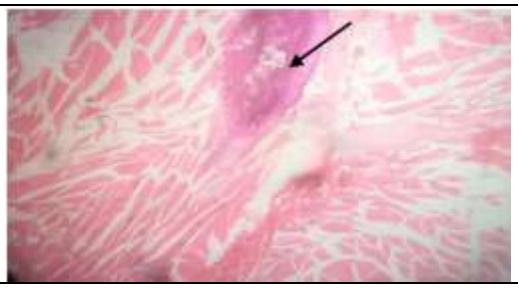
Fig. 8: Effect of EBM on glutathione.

Fig. 9: Effect of EBM ON CFA induced inflammation.

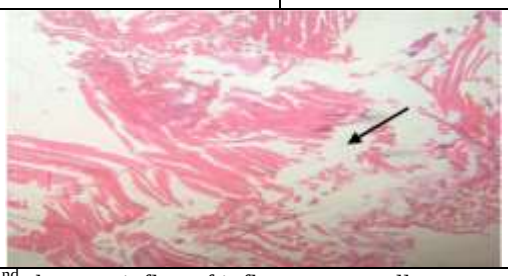
	
Control group showed normal connective tissue structure.	Negative control group showed massive influx of inflammatory cells, synovial hyperplasia with mono and poly-morphonuclear cell accumulation in the joint and edema associated with granuloma formation.



Standard group showed showed reduced synovial hyperplasia, oedematasis in the epidermis weremarkedly less, no infiltration of mononuclear cells and inflammatory cells.

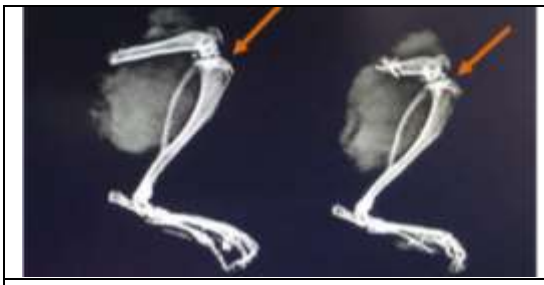


Treatment group 1st showed less influx of inflammatory cells and no pannus formation.

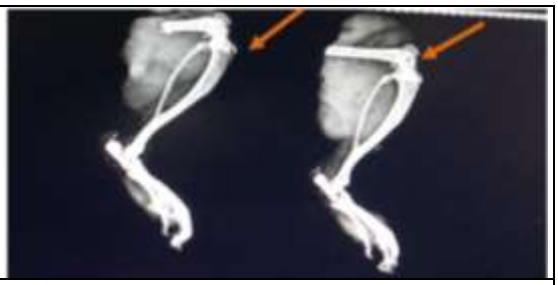


Treatment group 2nd show no influx of inflammatory cells, pannus formation absent.

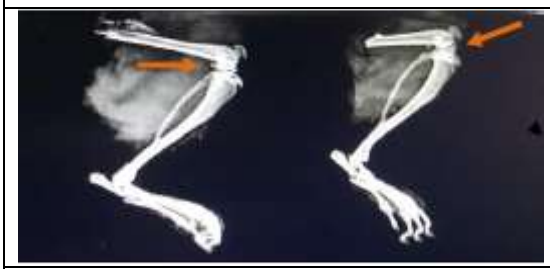
Fig. 10: Histopathology of joint.



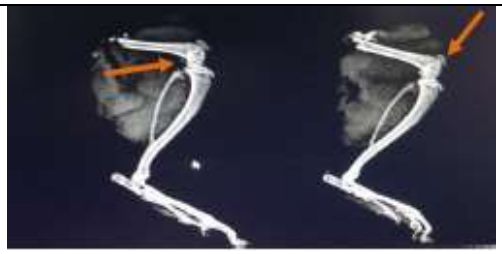
Control group showed the radiology of normal ankle joint.



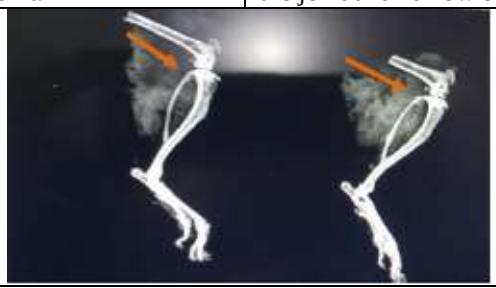
Arthritic control rats showed soft tissue swelling along with the narrowing of the joint spaces and bone destruction.



Standard group treated by diclofenac sodium (10mg/kg) had prevented bony destruction and there is no swelling of the joint.



Treatment group 1st treated with EBM200mg/kg shown no bony destruction and little swelling of the joint and narrow of joint space.



Treatment group treated with EBM 400mg/kg showed significant prevention against bony destruction by showing less tissue swelling and narrow of the joint space.

Fig.11: Radiology of hind leg induced arthritis rats.

DISCUSSION

Rheumatoid arthritis is a chronic inflammatory autoimmune disorder cauterized by persistent inflammatory synovitis involving peripheral joints leading to progressive destruction of cartilages and bones formation resulting in functional impairment. The exact cause of rheumaroid arthritis is not known but the mediators reported to be involved in pathogenesis include proinflammatory cytokines TNF-α, IL-6, IL-1 and inflammatory mediator cyclooxygenase, lipooxgenase. Several classes of drugs such as Non-steroidal anti-inflammatory drugs (NSAIDs), Disease modifying antirheumatic drugs (DMARDs), biological agent and glucocortoid are used for the treatment of rheumatoid arthritis have all been associated with adverse effect. Herbal medicines are being accepted and increasingly use because of the ethnic acceptability and compatibility have fewer side effect. *Baliospermum montanum* commonly known as Danti or Dantimool.it is found throughout India. *Baliospermum montanum* is pharmacologically reported for anti-inflammatory, analgesics, reducing oxidative stress, anthelmintic, hepatoprotective, wound healing, Acetylcholinesterase inhibition and anticancer Activities.[4] The collected roots of *Baliospermum montanum* was confirmed by macroscopic, microscopic and organoleptic character. Extraction was done by successive soxhlet extraction. Soxhlet extraction is continuous solid /liquid extraction with high extraction efficiency that requires less time and large amount of drugs can be extracted with a much smaller quantity of solvent. The selection of solvent was on the basis of polarity from nonpolar to polar. Petroleum ether, chloroform, ethanol and water. The yield value of Petroleum ether extract, chloroform extract, ethanolic extract and aqueous extract of BM rootswas found 11.6%, 12.8%, 15.2% and 16.8%. Preliminary phytochemical analysis revealed the PBM contain steroids, fat & oils. CLBM contain glycosides, flavonoids, steroids, tannins and phenolic compounds.EBM contain alkaloids, glycosides, flavonoid, steroids, tannins and phenolic compound. AQBM contain glycosides, flavonoids, tannins and phenolic compounds it has formerly reported flavonoids, steroid, glycosides reported anti-inflammatory, antioxidant and antiarthritic activities. *Invitro* antiarthritic activity of *Baliospermum momentum* was investigated by Egg albumin denaturation method. Denaturation of protein involves the disruption of secondary, tertiary and quatery structure of the molecules and finally leads to cell death. The mechanism of denaturation probably involves alteration in electrostatic, hydrogen,hydrophobic and disulphide bonding.Mostof the investigator have reported that denaturationof protein is the one of the causes of rheumatoid arthritis due to the production of autoantigen incertain rheumatic diseases maybe due to *in vivo* denaturation of protein. Diclofenac sodium at concentration 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml showed 15.4 %, 25.7%, 40.4%, 52.9%, 62.5% inhibition of Egg albumin denaturation and ethanolic extract of *Baliospermum montanum* concentration 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml showed 11.7%, 22.7%, 33.8%, 45.%, 56.6% inhibition of Egg albumin denaturation chloroform extract 100µg/ml, 200µg/ml, 300µg/ml, 400µg/ml, 500µg/ml showed 8.8%, 19.8%, 28.3%, 49.2% and Aqueous extract showed 8.08%, 17.6%, 25.7%, 38.2%, 46.3%inhibition of Egg albumin denaturation. The ethanolic extract of *Baliospermum montanum* showed highest percentage inhibition of protein denaturation. Complete freund's adjuvant induced arthritis model is the most widely used experimental model of Rheumatoid arthritis as it closely resembles the features of human rheumatoid arthritis. Arthritis is induced by CFA in rats developed a chronic swelling in multiple joints with the influence of inflammmatory cells, erosion of joint and cartilage and bone destruction and remodelling same as human rheumatoid arthritis. Antiarthritic activity of *Baliospermum montanum* was evaluated using complete fruend's adjuvant induced arthritis model. Diclofenac, a nonsteroidal antianflammatory drug was used as a standard for comparison because it is commonly precribed for the treatment of arthritis and its action is mainly through inhibition of cyclooxygenase and prostaglandin production. Diclofenac sodium 10mg/kg dose was selected on basis of previous study. Acute toxicity study was not conducted with the extract in this study as it was safeupto 2000mg/kg which has been reported in earlier study. The two doses were selected 200mg/kg and 400mg/kg. Paw volume, body weight, locomotor activity, haematological parameter (Hb,ESR), antioxidant parameter (LPO,SOD,GSH), histopathological and radiological studies parameter were evaluated. The determination of paw swelling is apparently simple, sensitive and quick procedure for evaluating the degree of inflammation and assessing of therapeutic effects of drugs. Chronic inflammation involves the release of number of mediators like cytokines (IL-1B and TNF-alpha), interferon's and PGDF. These mediators are responsible for the pain, destruction of bone and cartilage that can lead to severe disability. CFA administered rats showed soft tissue swelling around the ankle joints during the development of arthritis, which was considered as edema of the particular tissues.[21] In the present study, we showed that ethanolic extract of *Baliospermum montanum* significantly inhibit the progression of the rheumatoid arthritis in treated animals. However, standard drug and EBM significantly suppressed the swelling of the paws in both acute and chronic phase which may be due to the suppression of inflammatory mediator released due to induction of complete freund's adjuvant. Though the actual mechanism of suppressing inflammation is not known but it can be correlated with the presence of flavonoids, steriods, glycoside, tannin and pheolic compounds. In the present study, EBM significantly suppressed the paw volume induced by the complete Freund's adjuvant (CFA), around tibiotarsal joint and paws. This indicates the anti-inflammatory activity of EBM in rheumatoid arthritis. Weight loss is a powerful

predictor of health especially in pathological states. RA is associated with weight loss and loss of lean body mass, known as rheumatoid cachexia which leads to the decreased physical activity, muscle strength and decreased daily performance. Some researchers have reported the altered metabolic activities in the arthritic rats. Due to inflammatory condition, intestinal absorption of ^{14}C–glucose and ^{14}C–leucine was reduced and treatment with anti-inflammatory drugs has improved the decreased absorption capacity.[22] CFA induced arthritic rats treated EBM significantly increased bodyweight indicates that restoration of the absorption capacity of the intestine that shows effective management of rheumatoid cachexia.

The measurement of locomotor function appears to be an especially sensitive indicator of functional disability in arthritis. CFA induced arthritic exhibited reduced locomotor activity compared with normal rats. Treated with EBM significantly increased locomotor activity. In CFA-induced arthritis model, arthritic control rats showed reduced Hb count, and increased erythrocyte sedimentation rate (ESR). It is proposed that the reduction in the Hb count during arthritis results from reduced erythropoietin levels, a decreased response of the bone marrow erythropoietin, and premature destruction of red blood cells.[23] ESR is an important haematological tool to index diagnostic or prognostic assessment of inflammatory diseases. ESR of CFA induced arthritic rats was increased. Increase level of ESR is attributed to the accelerated formation of endogenous proteins such as fibrinogen and α/β globulin due to erthrocytes moves closer stack up in a group, become denser and settle faster. With the increases in erthrocyte setting rate, Erythrocyte sedimentation rate also increases.[24] The significant low level of ESR in the EBM and diclofenac treated arthritis rats indicates their anti-inflammatory potential. Oxidative stress contributes to the pathogenesis of Rheumatoid arthritis. Excessive generation of ROS and RNS Produced by activated neutrophils and macrophages inflicts damage to joints mainly through regulation of matrix metalloproteinases and activation of osteoclast activity. Treated with EBM increased SOD and GSH and significantly decreased indicates their antioxidant potential. Lipid peroxidation (LPO) is an important marker of oxidative stress. Prime targets of ROS attack are the polyunsaturated fatty acids in the membrane lipids causing lipid peroxidation (LPO) which may lead to disorganization of cell structure and function.[25] In the present study, EBM significantly decreased the LPO level in CFA-induced arthritis rats probably indicating the prevention of the cell damage by reducing oxidative stress. SOD is the first line of defense antioxidant enzyme against reactive oxygen species. it catalyzes the dismutation of the superoxide anion into hydrogen peroxide.[26] Decreased level of SOD was noted in CFA arthritis rats that indicated participation of superoxide radical which known to produce highly toxic hydroxyl radical through it reaction with hydrogen peroxide. Treatment with EBM elevated the activity of SOD. The level of non-enzymatic antioxidant GSH is also decreased in CFA induced rats. GSH is the main non-enzymatic antioxidant in defending against oxygen free radicals. A reduction in the level of GSH may impair H_2O_2 clearance and promote formation of hydroxyl radical (•OH), the most toxic molecule of the cell, leading to oxidative stress. This decrease contributes to increased cellular damage by favouring attack by free radicals.[27] Treatment with EBM significantly reversed the depleted level of GSH and SOD, probably by competing with scavenging of free radicals and as a result helped to maintain the integrity of cellular membranes in the injured cartilage. Histopathological of negative control group rat showed massive influx of inflammatory cells, synovial hyperplasia with mono and poly-morphonuclear cells accumulation in the joint and edema associated with granuloma formation. It also shows the high degrees of necrosis. Treatment with ethanolic extracts of *Baliospermum montanum* shows less influx of inflammatory cells, no pannus formation and absence of necrosis. Radiographic changes in RA conditions are useful diagnostic measures which indicate the severity of the disease. Soft tissue swelling is the earlier radiographic sign, whereas prominent radiographic changes like bony erosions and narrowing of joint spaces can be observed only in the developed stages of rheumatoid arthritis.[28] In the present study, EBM was found to be effective in reducing the soft tissue swelling and narrowing of joint spaces, especially in developing phase of arthritis. The radiographic report confirms the effective antiarthritic activity of EBM.

CONCLUSION

From the results of the study it may be concluded that ethanolic extract of *Baliospermum montanum* roots posse's significant *in vitro* and *in vivo* antiarthritic activity. This activity was evaluated by *invitro* study using Egg denaturation method and *in vivo* study using a model of CFA induced arthritis on rats. *Baliospermum montanum* were evaluated by complete freund's adjuvant induced arthritis on rat model. This finding justifies the preclinical efficacy and safety data ethanolic extract of *Baliospermum montanum* could be considered as safe and effective intervention for arthritis. The antiarthritic activity of the ethanolic extract may be due to the presence of phytoconstituents such as flavanoid, steroid, glycosides, tannins and phenolic compounds. However further studies are necessary to identify and isolate the active phytoconstituent responsible for antiarthritic activity. The molecular mechanism involved in the arthritic ethanolic extract of *Baliospermum montanum* can be studied in future to develop it as alternate treatment for rheumatoid arthritis.

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