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Invitro and *Invivo* Anti–Arthritic Activity of Combined Ethanolic Extracts of *Calotropis gigantea and Cardiospermum halicacabum* in Wistar Rats

Narayani P. C[1], Anbu J.[2], Vasuki R.[3], Rajeswari Hari[1*]

[1]Department of Biotechnology, Dr. MGR Educational & Research Institute, University, Maduravoyal, Chennai, India

[2]Department of Pharmacology, Vel's college of Pharmacy, Chennai, India

[3]Department of Biomedical Engineering, Bharath University, Selaiyur, Chennai, India

Abstract

The combined Bi–Herbal Ethanolic (BHE) extract made up of equal quantities of leaves of *Calotropi sgigantea* and *Cardiospermum halicacabum* was evaluated for its synergistic *invitro* and *invivo* anti–arthritic effects and was compared with its individual ethanolic extracts of C*alotropis gigantea* (ECG) and *Cardiospermum halicacabum* (ECH). The *invitro* anti–arthritic potential of these extracts were evaluated in terms of its inhibition of protein denaturation, membrane stabilization and proteinase enzyme inhibition. Freund's Adjuvant Arthritis Model in Wistar rats was used to study the *invivo* anti–arthritic activity. The three extracts (BHE, ECG, and ECH) at the dose level of 300 mg/kg b.w. were ingested orally in the suspension in 2% CMC to the CFA treated rats once daily for 40 days and the percentage inhibition of the paw volume due to the drug treatment was calculated. All the three plant extracts exhibited dose dependent inhibition of protein denaturation and protease inhibition. They also protected the RBC cells by stabilizing the membranes. There was a marked ($p<0.01$) reduction in the paw volume and oedema observed in the drug treated animals when compared to the intoxicant treated animals. The BHE has got a significant reduction of these paw volume and oedema when compared to its individual extract ECG and ECH. The anti–arthritic effects of these plant extracts may attribute either due to its inhibition of protein denaturation by inhibiting the proteinase enzymes or stabilizing the membranes from the free radical attack which are generated due to the immunological and inflammatory reactions observed in most of the arthritic conditions.

Keywords: Anti–arthritic, Freund's adjuvant arthritis, protein–denaturation, synergistic

1. Introduction

Rheumatoid Arthritis (RA) is an autoimmune disease that results in a chronic, systemic inflammatory disorder that causes pain, swelling, stiffness and loss of function in joints [1]. It occurs more frequently in women than in men and its prevalence depends upon age. In humans, RA is the most common inflammatory joint disease where skeletal complications start with focal erosion of cartilage initially followed by marginal and sub–chondral bone loss. Extended joint destruction with ankylosis and generalized bone loss are characteristic for late complications [2]. The steroidal and non–steroidal anti–inflammatory drugs are used in the treatment of the disease, but they offer only temporary relief and produce severe side effects including gastrointestinal bleeding and cardiovascular toxicity. Consequently, there is a need to develop new long acting anti–inflammatory agents with minimum side effects. Herbal medicine is a form of alternative treatment for several ailments and plant derived drugs are gaining popularity both in developing and developed countries due to their natural origin and less side effects in the last few years. The World Health Organization (WHO) has listed 21,000 plants which are

*Author for correspondence
Email: rajihar@gmail.com

used for medicinal purposes around the world and India is known as the "Emporium of Medicinal plants" due to availability of several thousands of medicinal plants in the different bioclimatic zones.

Plant derived secondary metabolites in the plant extracts are the important source of drugs with desired pharmacological activity. Although herbal drugs are effective in treatment of various ailments, very often these drugs are unscientifically exploited and/or improperly used. Therefore, a detailed pharmacological evaluation and documentation of plants used in local health tradition is needed. *Calotropis gigantea Linn*. (Asclepiadaceae family) commonly known as Milkweed or Swallow–wort and *Cardiospermum halicacabum* (Sapindaceae family) known as the Balloon Plant or Love in a puff, are widely distributed in Asia and Africa. Traditionally, the milky juice of *C. gigantea* has been used as a violent purgative, gastrointestinal irritant and abortion inducer [3–5]. It has also been used in the treatment of earache, toothache, headache, sprain and stiff joints [6]. The whole plant of *Cardiospermum halicacabum* is diaphoretic, diuretic, emetic, emmenagogue, laxative, refrigerant, rubefacient and stomachic [7]. It is used in the treatment of rheumatism, nervous diseases, stiffness of the limbs and snakebite [8]. A single drug cannot be effective against all types of severe diseases. Therefore, effective formulations have to be developed using indigenous medicinal plants with proper pharmacological experiments and clinical trials. Since the above mentioned two plants have traditional claim for the treatment of Rheumatic Arthritis and they were scientifically evaluated for their potency individually, the present investigation is undertaken to evaluate the synergistic *invitro* and *invivo*anti arthritic activity of the Bi–Herbal Ethanolic (BHE) extract which is prepared using equal quantities of leaves of *Calotropis gigantea* and *Cardiospermum halicacabum*. The anti arthritic activity of BHE is compared with the individual ethanolic extract of *Calotropis gigantea* (ECG) and individual ethanolic extract of *Cardiospermum halicacabum* (ECH).

2. Materials and Methods

2.1 Chemicals

Ethanol, Bovine Serum Albumin, Acetyl Salicylic Acid were purchased from SD Fine Chemicals Ltd, India. Perchloric acid and Hydrochloric acid were purchased from Thermo Fisher Scientific India Pvt. Ltd. Enzyme Trypsin was purchased from SISCO Research Laboratories Pvt. Ltd, Mumbai. All other chemicals and reagents used were of analytical grade.

2.2 Collection of Plant Material

The leaves of *Calotropis gigantea* and *Cardiospermum halicacabum* were collected from the IMPCOPS (Indian Medical Practitioners Co–operative Society, Thiruvanmiyur Chennai, India and they were identified and authenticated by Dr. P. Jayaraman, Botanist, Plant Anatomy Research Centre (PARC), Chennai. The voucher specimen is available in the herbarium file of the PARC, Chennai, India.

2.3 Preparation of Plant Extract

The leaves of *Calotropis gigantea* (500 gms) and *Cardiospermum halicacabum* (500 gms) were shade dried and pulverized to a coarse powder. Equal quantities of the powder was passed through 40–mesh sieve and exhaustively extracted with 90% (v/v) ethanol by cold maceration process at room temperature for three days. The extract was filtered and the filtrate was evaporated under pressure until all the solvent had been removed and further removal of water was carried out by freeze drying to give a sample yield of 0.98% (w/w). Similarly, the ECG and ECH were also prepared separately. The ECG extract yield was 0.42% (w/w) and ECH sample yield was 0.38% (w/w). The extracts were stored in refrigerator and used for the present investigations.

2.4 Animals

Adult albino male rats of Wister strain weighing 120–175 g and adult Albino mice were used in the pharmacological and toxicological studies. The inbred animals were taken from animal house in Vel's College of Pharmacy, Chennai, India. The animals were maintained in well ventilated room temperature with natural 12 ± 1 h day–night cycle in the propylene cages. They were fed balanced rodent pellet diet from Poultry Research Station, Nandanam, Chennai, India and tap water *ad libitum* was provided throughout the experimental period. The animals were sheltered for one week and prior to the experiment they were acclimatized to laboratory temperature. The protocol was approved by Animal Ethics Committee constituted for the purpose

as per CPCSEA Guideline. (Reference No:XIII/VELS/PCOL-65/IAEC/CPCSEA/08.08.2012).

2.5 Acute Toxicity Studies

Acute toxicity studies were conducted with BHE, ECG and ECH extracts in adult Albino mice by *Staircase Method* of Ghosh [9]. Albino mice of either sex were selected and segregated into 7 groups of 6 animals each. Single dose of BHE, ECG and ECH extracts dissolved in 0.5% aqueous Tween80, starting from the minimal dose of 50 mg/kg up to 3000 mg/kg were administered orally. The drug treated animals were observed carefully for its toxicity signs and mortality. LD_{50} doses were selected for the evaluation synergistic anti–arthritic activity. From the maximum dose, $1/10^{th}$ of the concentration was considered as therapeutic dose for further studies. All animals were also further observed for 14 days for clinical symptoms and mortality.

2.6 *Invitro* Anti–Arthritic Activity

2.6.1 Protein Denaturation Inhibition Study

The method of protein denaturation was studied as described by Mizushima and Kobayashi [10]. The reaction mixture contained 0.05ml of crude ethanolic extracts of BHE, ECG and ECH at variable concentrations ranging from 100 to 1000 µg in 10% v/v of polyethylene glycol and 0.45 ml of bovine serum albumin (5% w/w aqueous solution). The pH was adjusted to 6.3 by adding 0.1N HCl and the samples were incubated at 37˚C for 20 min and then heated at 57˚C for 3min. After cooling 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. The resulting turbidity was measured spectrophotometrically at 660 nm. For control tests, 0.05 ml distilled water was used instead of extracts while product control tests lacked bovine serum albumin. The percentage inhibition of protein denaturation was calculated. The control represents 100% protein denaturation. The results were compared with acetyl salicylic acid which was used as a positive control in the present investigation.

2.6.2 Membrane Stabilization Study

The method of RBC membrane stabilization was studied as described by Saket et al. [11]. The total volume (4.5 ml) of reaction mixture contained 2 ml of hypotonic saline (0.36% NaCl), 1 ml of 0.15 M phosphate buffer (pH 7.4) and 1 ml crude extracts of BHE, ECG and ECH at variable concentrations ranging from 100 to 1000 µg in normal saline. To all the test tubes 0.5 ml of 10% v/v rat RBC in normal saline was added. The mixtures were incubated at 56ºC for 30 minutes. The tubes were cooled under running tap water for 20 minutes. The mixtures were centrifuged and hemoglobin content in the suspension was estimated using spectrophotometer at 560 nm. For negative control, 1.0 ml of isotonic saline was used instead of extracts. Acetyl salicylic acid was used as positive control under the same assay conditions. Membrane stabilizing potential of the extracts was calculated accordingly from the decrease in absorbance at 560 nm in comparison with the negative control. Percentage membrane stabilization was calculated.

2.6.3 Proteinase Inhibition Study

The proteinase enzyme inhibitory assay was studied by the method described by Oyedapo et al. [12]. The reaction mixtures (2.0 ml) contained 0.06 mg trypsin, 1.0 ml of 25 mMtris–HCl buffer (pH 7.4) and 1.0 ml of crude extracts of BHE, ECG and ECH at variable concentrations ranging from 100 to 1000 µg in polyethylene glycol. The above mixtures were incubated at 37ºC for 5 minutes and then 1.0 ml of 0.8% (w/v) casein was added. The mixtures were subjected to an additional 20 minutes of incubation at 37ºC and 2.0 ml of 70% (v/v) perchloric acid was added to terminate the reaction. The cloudy suspension was centrifuged. Absorbance of the supernatant was read at 280 nm against buffer as blank. The percentage of inhibition was calculated.

2.7 Assay of Anti–Arthritic Activity

The anti–arthritic activity was performed according to Jubie et al. [13] method. Animals were divided into 6 groups of 6 animals each and each group was given a dose schedule as follows: Group I: Animals received a suspension of 2 ml/Kg CMC (2% v/v of CMC) *p.o.*, once daily. This group served as control.

Group II, III, IV, V & VI: Animals received a suspension of 2 ml/Kg CMC *p.o,* and were injected with 0.1 ml (0.5% w/v) of Freund's Adjuvant in the right hind foot under the plantar region after 30 mins of CMC administration.

Group III, IV and V: Freund's Adjuvant treated animals received 300 mg/Kg body weight of BHE, ECG and ECH respectively in 2 ml/Kg of 2% CMC *p.o,* once daily for 40 days.

Group VI: Freund's Adjuvant treated animals received 45 mg/Kg body weight of Indomethacin in 2ml of 2% CMC *p.o,* once daily for 40 days.

The drug treatment was continued with the respective groups for 40 days. Every day animals were carefully and thoroughly inspected by examining the affected paw and animal's general status. The width and height of the paw and the width of the joints were measured with a caliper ruler before the start of the experiment and on 4^{th}, 13^{th}, 25^{th} and 40^{th} day after injection of Freund's adjuvant.

The percentage inhibition of the paw volume was calculated by the formula:

% Inhibition = (1 – (drug treated/negative control))*100

2.8 Statistical Analysis

The values reported are Mean±SE. The statistical analysis was carried out using ANalysis Of VAriance (ANOVA) followed by Dunnet's 't' test. The p values <0.05 were considered as significant.

3. Results

3.1 Acute Toxicity Studies

No toxic symptoms were observed after administration of different dose levels of extract up to maximum of 3000 mg/kg *p.o.* according to OECD guideline 423; and in addition the higher dose of 5000 mg/kg dose was administered to a group of animals. But symptoms like dyspnea were identified. Hence, one tenth of safe tolerable dose was used as therapeutic dose for further pharmacological study. From this experiment, the maximum therapeutic dose level of BHE, ECG and ECH extracts were studied as 300 mg/kg.

3.2 Inhibition of Protein Denaturation

Anti–arthritic effect of BHE, ECH and ECG were studied significantly by testing various *in–vitro* parameters. Table 1 depicts the inhibition of protein denaturation of different extracts. In the present investigation, all the three extracts inhibited the protein denaturation in a dose dependent manner.

However, the BHE has got a higher inhibitory percentage of protein denaturation when compared ($p>0.05$) with their individual extracts, i.e., ECH and ECG. At the concentrations of 100 and 1000 µg/ml, the inhibitory percentage of BHE was significantly comparable to the positive control Acetyl Salicylic Acid used in this present investigation.

3.3 Membrane Stabilization Study

The stabilization of RBC membranes by plant extracts against heat and hypotonic saline–induced damage in the present investigation further establishes its anti–inflammatory action. The percentage inhibition of the heat induced hemolysis by ECH, ECG, and BHE were shown in the (Fig. 1). In the present investigation, there was a dose dependent protection of RBC by all the three extracts.

Table 1: Effect of BHE, ECH and ECG extracts on inhibition of protein denaturation

Concentration (µg/Ml)	Inhibitory activity of ECH (%)	Inhibitory activity of ECG (%)	Inhibitory activity of BHE (%)	Inhibitory activity of Acetylsalicylic acid (%)
100	17.54 ± 1.55a*	19.90 ± 1.34b*	23.54 ± 1.55c^{ns}	24.31 ± 1.70
200	22.28 ± 2.43a*	21.76 ± 1.95b*	27.28 ± 2.43c^{ns}	30.52 ± 2.53
400	35.61 ± 1.74a*	37.08 ± 1.65b*	39.61 ± 1.74c^{ns}	42.79 ± 1.87
500	42.25 ± 2.21a*	42.50 ± 1.20b*	49.61 ± 1.74c^{ns}	49.36 ± 3.62
800	64.89 ± 1.61a*	66.78 ± 2.13b*	75.67 ± 3.21c^{ns}	77.14 ± 3.45
1000	75.39 ± 1.65a*	77.45 ± 1.32b*	83.99 ± 1.65c^{ns}	86.52 ± 2.41

Values are expressed in mean ± SD (n = 3), statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test Comparison between: a: Acetylsalicylic acid vs ECH; b: Acetylsalicylic acid vs ECG and c: Acetylsalicylic acid vs BHE. *p<0.05, **p<0.1 and NS: Non–Significant.

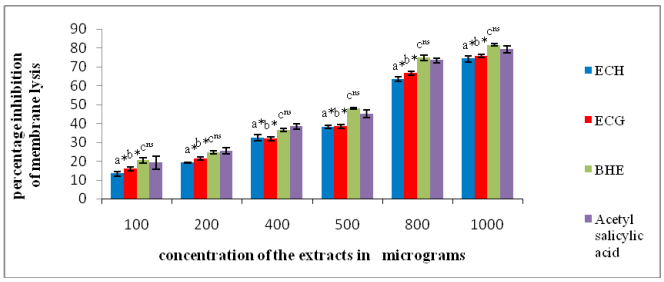

Fig. 1. Membrane stabilization activity of ECG, ECH, BHE extracts and acetyl salicylic acid. Each value represents the mean ± SD (n = 3). Comparison between: a: Acetylsalicylic acid vs ECH, b: Acetylsalicylic acid vs ECG and c: Acetylsalicylic acid vs BHE.*p<0.05, and NS: Non–Significant.

However BHE showed a maximum protection of 81.59% against lysis at the concentration of 1000 µg/ml against its individual extracts ECH and ECG which showed 74.30% and 75.81% protection respectively at the same concentration. It is observed in the study that the BHE exhibited higher protection to the RBC membranes when compared with the positive control (Acetyl Salicylic Acid) which showed only 79.31% inhibition of lysis at the same concentration.

3.4 Proteinase Inhibition Study

The proteinase inhibitory activity of the ECH, ECG, and BHE extracts were shown in Table 2. All the three extracts and the positive control acetyl salicylic acid exhibited a dose dependent anti–proteinase activity.

At concentrations of 400 and 500 µg/ml the BHE exhibited higher anti–proteinase activity of 49.25% and 75.45% respectively when comparable (p>0.05) to the positive control which showed 46.72% and 73.69% of anti–proteinase activity at the same concentration. But at the maximum concentration of 1000 µg/ml BHE showed 81.39% proteinase inhibition against their individual extracts ECH and ECG which exhibited 71.39% and 73.21% proteinase inhibition against the positive control acetyl salicylic acid which showed the inhibition of 85.88% at the same concentration.

3.5 Evaluation of *invivo* Anti–Arthritic Activity

Table 3 shows the anti–arthritic activity of BHE, ECG, ECH and the Indomethacin on Complete Freund's Adjuvant (CFA) induced arthritis. In the present study, at the dose level 300 mg/kg b.w, all the three ethanol extracts reduced the Complete Freund's Adjuvant (CFA) induced chronic inflammation in the knee joint of rats as compared with that of the standard drug Indomethacin.

There was a significant reduction in the paw volume (122.30 mm on 40th day) of the affected Group III rats which received the BHE when comparable (P<0.001) to the Group IV and Group V rats which received the individual extracts such as ECG and ECH and exhibited the increased paw volume of 151.23 and 150.22 mm on the same day. The cordial signs of the chronic inflammatory reactions like redness, swelling, arthralgia and immobility of affected joints were significantly less in the drug treated Group III, IV and V animals than those of the Group II CFA treated animals.

4. Discussion

Rheumatoid Arthritis (RA) is an autoimmune disease characterized by synovial proliferation, inflammation followed by destruction and deformity of joints or destruction of cartilage and bone, capable of producing severe functional disabilities [14]. Being an inflammatory arthritis, the disease exhibits three basic inter–related processes like inflammation, synovial proliferation and joint tissue destruction. The immune mediated inflammation of the synovial joints by pro inflammatory cytokines, mainly Tumor Necrosis Factor–α (TNF–α), InterLeukin–1β (IL–1β), and IL–6 produced by monocytes, macrophages, and synovial fibroblasts are suggested to play an important role in the pathogenesis and disease progression of RA. All anti–inflammatory drugs are not anti–arthritic because it does not suppress T–cell and B–cell mediated response. Being a chronic disease there is no cure for either rheumatoid or osteoarthritis. Treatment options vary depending on the type of arthritis which includes physical therapy, lifestyle changes (including exercise and weight control) and medication. All the steroidal and non–steroidal anti–inflammatory drugs currently available are probably poly component, in that they are able to modulate more than one mediator or cellular event concerned with the inflammatory response associated with RA [15]. But gastrointestinal bleeding and ulceration is found to be the common side effect for most of the drugs. In recent

Table 2: Effect of BHE, ECG and ECH extracts on invitro model of protease inhibition

Concentration (µg/Ml)	Inhibitory activity of ECH(%)	Inhibitory activity of ECG(%)	Inhibitory activity of BHE(%)	Inhibitory activity of Acetylsalicylic acid(%)
100	15.75 ± 2.41a*	17.63 ± 2.49b*	21.36 ± 2.00c^{ns}	21.87 ± 1.92
200	17.37 ± 2.59a*	21.75 ± 1.30b*	23.28 ± 2.87c^{ns}	27.21 ± 2.36
400	26.19 ± 1.63a*	32.82 ± 1.11b*	37.61 ± 1.03c^{ns}	41.74 ± 1.60
500	37.53 ± 2.99a*	40.00 ± 0.87b*	49.25 ± 2.94c*	46.72 ± 2.55
800	63.45 ± 1.19a*	67.16 ± 2.23b*	75.45 ± 1.68c*	73.69 ± 1.26
1000	71.39 ± 1.79a*	73.21 ± 0.56b*	81.39 ± 1.79c^{ns}	85.88 ± 1.08

Values are expressed in mean ± SD (n = 3), statistical significant test for comparison was done by ANOVA followed by Dunnet's 't' test. Comparison between a: Acetylsalicylic acid vs ECH; b: Acetylsalicylic acid vs ECG and c: Acetylsalicylic acid vs BHE. *p<0.05, **p<0.1 and ns – Non-Significant.

Table 3: Effect of BHE, ECG and ECH extracts on CFA induced chronic immunological arthiritic model

Treatment	Mean increase in joint diameter in (mm) and Inhibition (%) on			
	4th day	13th day	25th day	40th day
Group I CMC treated (control)	109.12 ± 8.16	107.67 ± 4.52	107.45 ± 9.01	108.23 ± 7.86
Group-II CFA treated	125.16 ± 10.16	188 ± 7.95	224.33 ± 9.52	225.83 ± 8.99
Group-III BHE + CFA treated	111.00 ± 7.55a* (12%)	144.00 ± 6.22a*** (23.40%)	154.00 ± 5.27a*** (31.55%)	122.30 ± 7.00a*** (45.84%)
Group-IV ECG + CFA treated	118.00 ± 8.87b* (5.6%)	177.66 ± 4.56b* (5.5%)	193.13 ± 8.22b*** (13.83%)	151.23 ± 7.93b** (32.88%)
Group-V ECH + CFA treated	121.00 ± 5.66c* (3.2%)	184.03 ± 5.98c* (2.11%)	195.45 ± 6.62c** (12.94%)	150.22 ± 4.63c** (31. 90%)
Group VI Indomethacin + CFA treated	116.83 ± 8.37d** (7.2%)	175.83 ± 7.41d*** (6.47%)	140.66 ± 6.68d*** (37.29%)	132±8.87d*** (41.33%)

Values are mean ± SEM from 6 animals in each group. Statistical significant test for comparison was done by ANOVA, followed by Dunnet's `t' test. Comparison between: a: Group II vs Group III, b: Group II vs Group IV, c: Group II vs Group V, d: Group II vs Group VIP Values: *<0.05, ** <0.01, ***<0.001, NS: Non–Significant.

years, herbal medicine is a form of alternative treatment which includes the search for new phytochemical bioactive components from medicinal plants possessing several pharmacological activities has been on the rise due to their potential use in the treatment and prevention of various chronic and infectious diseases [16, 17].

In the present investigation, all three plant extracts BHE, ECG and ECH have significant *invitro* and *invivo* anti–arthritic activity. The *invitro* anti–arthritic effect of ethanolic extracts of BHE, ECG and ECH were investigated using inhibition of protein denaturation, membrane stabilization and proteinase inhibition. Most of the investigators have reported that denaturation of the protein is one of the cause of rheumatoid arthritis [18]. According to Brown and Mackey [19], production of auto–antigens in certain rheumatic diseases may be due to *in vivo* denaturation of proteins. This denaturation probably is associated with the alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding [20]. In the present investigation, all the three extracts inhibited the protein denaturation in a dose dependent manner. However, the BHE which is made up *Calotropis gigantean* and *Cardiospermum halicacabum* has got a higher inhibitory percentage of protein denaturation when compared with their individual extracts. The preliminary phytochemical screening assay for evaluating the presence of various chemical components in the plants *Calotopis gigantea*, *Cardiospermum halicacabum* and their combination indicated the presence of alkaloid, carbohydrates, phytosterol, tannins, phenol, flavonoids, glycosides, cardiac glycosides, terpenes, and lignin. It is generally accepted that a cumulative synergistic

relationship among the phytochemicals are responsible for the overall beneficial medicinal value of plants. These bioactive substances may either control the production of auto antigens or protect the proteins from denaturation observed in the rheumatic diseases.

The principle involved in membrane stabilization is stabilization of human red blood cell membrane by heat and hypo tonicity induced membrane lysis. As stated by Oyedapo and Famurewa [12], the membrane of RBC is structurally similar to the lysosomal membrane, the effect of any substance on stabilization of RBC membrane may be extrapolated to the stabilization of lysosomal membrane. Protective effect on heat and hypotonic saline–induced erythrocyte lysis is known to be a very good index of anti–inflammatory activity of any agent [1]. In the present investigation our plant extracts were able to inhibit the heat induced hemolysis effectively thereby stabilizing the RBC membranes. The similar effect may be expected in stabilizing the lysosomal membranes and inhibiting the release of lysosomal content of neutrophils at the site of inflammation in the *invivo* condition. The lysosomal constituents present in the neutrophils include bactericidal enzymes and protein degrading enzymes which, upon extracellular release cause further tissue inflammation and damage [21].

Several types of proteinases are involved in arthritic reactions. Neutrophils are said to be the storage house of proteinase since they carry many serine proteinases in their lysosomal granules. Neutrophils release elastase and proteases which degrade proteoglycan in the superficial layer of cartilage [22, 23]. The depletion of the proteoglycan allows the immune complexes to precipitate in the superficial layer of collagen and exposes the chondrocytes [24]. Chondrocytes and synovial fibroblasts release Matrix MetalloProteinase (MMPs) when stimulated by the immune molecules such as IL–1, TNF– α, or activated CD4+ and T cells. MMPs, in particular stromelysin and collagenases, are enzymes that degrade connective–tissue matrix and are thought to be the main mediators of joint damage in RA. Apart from this, it was previously reported that leukocytes proteinase also play important role in the development of tissue damage during the inflammatory reactions. So any agent which is acting as a proteinase inhibitor can provide a significant level of protection against the arthritic reactions [25]. Our plant extracts considerably inhibited the proteinase enzyme. Recent studies have shown that many flavonoids and related polyphenols contributed significantly to the anti–inflammatory activities of many plants [26, 27]. The presence of cardiac glycosides, flavonoids and saponins in *Cardiospermum halicacabum* and α– and β–calotropeols and β–amyrin in *Calotorpis gigantea* extract have the potential to reduce pain and swelling (inflammation) as α– and β–amyrin are anti–inflammatory and analgesic agents [28].

The synergistic *invivo* anti–arthritic activity of the Bi–Herbal Ethanolic (BHE) extract which is made up of the leaves of *Cardiospermum halicacabum and Calotorpis gigantea* was further evaluated in the Complete Freund's Adjuvant (CFA) induced arthritis model in rats. The Complete Freund's Adjuvant (CFA) induced arthritis model is considered as a chronic, immunological, cellular and proliferative arthritis similar to clinical RA. In adjuvant induced arthritis model, rats develop a chronic swelling in multiple joints with influence of inflammatory cells, erosion of joint cartilage and bone destruction and remodeling. These inflammatory changes ultimately result in the complete destruction of joint integrity and function in affected animals. Complete Freund's Adjuvant (CFA) induced is considered as cell mediated autoimmunity due to the structural mimicry between mycobacteria and cartilage proteoglycans in rats. This is a most frequently used model for screening the anti–arthritic agents, especially NSAIDs, as the inflammation associated with CFA is highly dependent on Prostaglandin E2 (PGE2) generated by Cycloygenase (COX) [29]. In the present investigation, reduction of paw swelling in the drug treated rats from the third week onwards may be due to immunological protection rendered by the plant extract. Adjuvant inoculation triggers the production of activated macrophages and lymphocytes or their products like monokines, cytokines and chemokines. These in turn produce lipid peroxides due to abnormal lipid peroxidation leading to increased inflammation. In the current study, a significant reduction in the inflammation was shown in Group III BHE treated animals when compared to that of Group II toxin intoxicated animals indicating the antiperoxidative nature of the plant extract. Complex network of cytokines and growth factors with overlapping biological effects are observed in the perpetuation of RA. As a consequence of the inflammatory processes, several immunological

mediators such as TNF–α, IL–1 and IL–6 have been implicated in the pathological mechanism of synovial tissue proliferation, joint destruction and programmed cell death in rheumatoid joint. It was reported that the expression of inflammatory cytokines such as TNF–α and IL–1 β and the tissue enzymes such as metalloproteinases were observed to be increased in the sub–chondral bone region of the knee joint samples from human osteoarthritis or rheumatoid arthritic patients. Biological agents that specifically inhibit the effects of TNF–α or IL–1 or leukocyte migration and accumulation in arthritis may have beneficial effects for joint preservation. Thus, in this study, observing the invitro experiments such as inhibition of protein denaturation, membrane stabilization and proteinase inhibition by the plant extracts and *invivo* anti–arthritic effect analysis indicates proof towards the above theory of joint preservation. Plant phenolic compounds have been found to possess potent anti–inflammatory activity [11, 30]. It has also been reported that the flavonoids significantly inhibit the leukocyte migration in a dose dependant manner [31]. The high presence of flavonoids in *Cardiospermum halicacabum* extract indicates the presence of Luteolin which is a common flavonoid with potent anti–inflammatory activity [32].

5. Conclusion

On the basis of the above results, it could be concluded that BHE, a combination of two herbal plants exert a significant anti–arthritic and anti–inflammatory effects due to different types of active principles each with a single or a diverse range of biological activities, which serves as a good adjuvant in the present armamentarium of these pathological conditions. The results also furnish evidence that the beneficial effects of this extract may either be due to its free radical scavenging activity or its inhibition of protein denaturation by inhibiting the proteinase enzymes thereby stabilizing the membranes from the immunological and inflammatory reactions. Further, clinical studies are required to establish its safety and usefulness medically.

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