Research Article

Evaluation of *in vitro* rheumatoid arthritis activity of polyherbal ethanolic extract containing formulations for selected potential Indian herbs

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ABSTRACT

Objective: Arthritis has emerged to be a very common disorder affecting statistically one-third of the population. The present study aims to investigate the effectiveness of a polyherbal formulation *Polygonum glabrum*, *Canthium dicoccum*, *Ochna obtusata*, and *Argyreia nervosa* in treating the disorder arthritis. **Materials and Methods:** Ethanolic extract of different portions are used to investigate by *in vitro* inhibition of protein denaturation method using bovine serum, inhibition of protein denaturation method using egg albumin, and human red blood cell (HRBC) membrane stabilization method. **Results:** The result revealed that the formulations F2 and F4 possessed significant anti-arthritic activity by protein denaturation inhibition and HRBC membrane stabilization comparing with diclofenac sodium, the standard drug used. **Conclusion:** The study concluded that the above two formulations are an effective inhibitor of protein denaturation and HRBC membrane stabilization which can be used potent anti-arthritic activity.

KEY WORDS: *Argyreia nervosa*, *Canthium dicoccum*, Ethanolic extract, *In vitro* rheumatoid, *Ochna obtusata*, *Polygonum glabrum*, Polyherbal

INTRODUCTION

Inflammation is a normal protective response to tissue injury which involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown, and repair.[1] It is characterized by redness, swelling, pain, stiffness of joint, and loss of joint function. Inflammation is associated with membrane alterations, increase in vascular permeability, and protein denaturation.[2] Arthritis is a chronic, inflammatory, systemic autoimmune disorder. It is an inflammation of synovial joint due to immune-mediated response.[3] One-fifth of the world's elderly suffer with arthritis.[4] The current treatment of arthritis includes minimization of this associated pain and inflammation using non-steroidal anti-inflammatory drugs (NSAIDs) as well as deceleration of disease progression using anti-rheumatic drugs.[5,6] Due to adverse reactions of the NSAIDs and disease-modifying antirheumatic drugs, the arthritic patients tend to search for other treatments that are effective and less toxic. Therefore, complementary and alternative medicines are commonly preferred by such patients.[7]

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Polygonum glabrum: The tribes of Chhattisgarh use the root paste as a medicine for snake bite.[8] In some areas, the rootstock is used for the treatment of jaundice and piles.[9] The leaves are used as an antimalarial agent in Sudan.[10] In South India, *P. glabrum* leaves are used for the treatment of dysentery.[11] A decoction of the leaves and seeds are used as cardiotonic, astringent, and anthelmintic.[12] The whole plant decoction is used as a remedy for colic pain, pneumonia, and the boiled paste is applied in cuts and wounds.[13] Apart from medicinal use, the whole plant is powdered and used as bait for fishing. Peels from the stem are used for treating rheumatism.[14]

Ochna obtusata DC. (Family: Ochnaceae) is a small tree up to 8 m tall. The family is characterized by the presence of secondary metabolites such as flavonoids and terpenoids.[15] Moreover, it is extensively used

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in Indian traditional medicine for the treatment of epilepsy, menstrual complaints, lumbago, asthma, ulcers, and as an antidote to snake bites.[16] Several studies conducted on *Ochna* species revealed the presence of glycosides, saponins, steroids, flavones, and fatty acids.[17] The leaves and roots of *O. obtusata* are used for ulcer, asthma, and bronchitis and also possess antiulcerogenic activity.[18]

Canthium dicoccum ethanolic extract of the whole plant of *C. diococcum* for anti-inflammatory activity in Wister albino rats in various models of anti-inflammatory activity, namely, Carrageenan-induced paw edema, formalin-induced paw edema, fresh egg white-induced paw edema, and cotton pellet-induced granuloma model. Results showed the extract with anti-inflammatory activity and suggested a potential alternative to NSAIDs like diclofenac.[19] Ethanolic extract of *C. diococcum* for antidiabetic in an alloxan-induced diabetic rat model. Results showed a significant drop in fasting blood sugar in a dose-dependent manner, with an effect on the beta-cell population in the pancreas. The extract showed almost equipotent antidiabetic activity compared to standard drug glibenclamide.[20] Ethanolic extract for anti-arthritic activity in albino rats. Results showed significant anti-arthritic activity against egg-albumin-induced arthritis model.[8]

Argyreia nervosa seeds are found to possess hypotensive and spasmolytic activity which were due to the mixture of ergot alkaloids, isolated and analyzed by ultraviolet (UV). Due to instability, only one constituent was identified as ergometrine. Other constituents such as caffeic acid and ethyl caffeate were identified[21,22] apart from ergoline alkaloids, N-formylloline alkaloids, flavonoidal sulfates steroids, and triterpenoids were isolated from other parts of *A. nervosa*.[23,24] Para-hydroxycinnamate, scopeltin, and argyroside[25,26] isolated oil from the seed of *A. nervosa* and evaluated the antibacterial effect.[27]

In the recent studies of the author ethanolic extract of the above plants and the polyherbal formulations with different fractions of ethanolic extract showed good antioxidant activity. The present study is designed to evaluate the anti-arthritic activity for the different polyherbal formulations.

MATERIALS AND METHODS

Plant Source and Authentication

P. glabrum, O. btusata DC, *C. dicoccum* and *A. nervosa* were collected from Tirumala Hills, Tirupati, and Chittoor district of Andhra Pradesh, near Seshachalam and Tirumala Hills (Rayalaseema region, Andhra Pradesh, India), areas that are geographically located in the Southeastern Ghats, are recognized for their rich flora and fauna. The plant specimen was verified to be of the correct species by Dr. Madhava Setty, a botanist from the Department of Botany, S.V. University, Tirupati, Specimen Voucher No:1972,1220,1012,2162 Preserved for further reference at our laboratory.

Chemicals and Reagents

All the chemical are used analytical grade and the egg albumin, bovine serum, and diclofenac sodium obtained from Sigma-Aldrich.

Preparation of Polyherbal Extract

Aerial parts of *P. glabrum*, *C. dicoccum*, *O. btusata*, and *A. nervosa* were collected and dried. Then, the material was blended to form a fine powder and extracted ethanol using Soxhlet apparatus for 6 h at 50°C and water by maceration. The solvent was completely removed by rotary evaporator (Rotavapor® R-210, BUCHI Corporation) and respective extracts preserved for various investigations.

Preparation of Ethanol Extract of Four Selected Polyherbal Formulations using Different Portions

The above extract used for the pre paration of five different polyherbal formulations with varying proportions and working formula given in Table 1.

Preliminary Phytochemical Studies[28-30]

Previously various preliminary phytochemical tests were performed using standard procedures and the above formulations showed the presence of mainly carbohydrates, alkaloids, glycosides, phenols, tannins, flavonoids, and saponins which majorly responsible for the desired activity.

Evaluation of *In vitro* Anti-arthritis of Polyherbal Formulations

Inhibition of protein denaturation method using bovine serum[31-34]

Preparation of the standard solution: The standard solutions (0.5 ml) were prepared using 0.45 ml of bovine serum albumin (5 % w/v aqueous solution) and 0.05 ml of diclofenac sodium solution in various concentrations (10, 50, 100, 200, 400, 800, and 1000 µg/ml).

Preparation of the test solution: The test solutions (0.5 ml) were prepared using 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of

Table 1: Different ratios of ethanol extract of four plants

Formulations	PGEE	CDEE	OOEE	ANEE
Formulation 1	1	1	1	1
Formulation 2	2	1	1	1
Formulation 3	1	2	1	1
Formulation 4	1	1	2	1
Formulation 5	1	1	1	2

test solution in various concentrations (10, 50, 100, 200, 400, 800, and 1000 µg/ml).

Preparation of the test control solution: This solution (0.5 ml) was prepared using of 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of distilled water.

Experimental Procedure

All the above solutions were adjusted to pH 6.3 using 1N HCl. The samples were incubated at 37°C for 20 min, and the temperature was increased to keep the samples at 57°C for 3 min. After cooling, 2.5 ml of phosphate buffer was added to the above solutions. The absorbance was measured using UV-visible spectrophotometer at 416 nm. The percentage inhibition of protein denaturation was calculated using the formula:

$$\text{Percentage inhibition} = \frac{\text{100 – optical density of test solution – optical density of product control}}{\text{optical density of test control}} \times 100$$

Inhibition of Protein Denaturation Method Using Egg Albumin[2,3,35,36]

Preparation of the standard solution: The standard solutions 5 ml were prepared using 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate buffered saline (pH 6.4), and 2 mL of diclofenac sodium solution in various concentrations (10, 50, 100, 200, 400, 800, and 1000 µg/ml).

Preparation of the test solution: The test solutions 5 ml were prepared using 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate buffered saline (pH 6.4), and 2 mL of different concentrations (10, 50, 100, 200, 400, 800, and 1000 µg/ml).

Preparation of the test control solution: This solution prepared using 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate buffered saline (pH 6.4) and 2 mL of distilled water.

Experimental Procedure

All above solutions were incubated at 37 ± 2°C in incubator for 15 min and then heated at 70°C for 5 min. After cooling their absorbance were measured at 660 nm using vehicle as a blank. The percentage inhibition of protein denaturation was calculated using the following formula:

$$\text{Percentage inhibition} = \frac{\text{100 – optical density of test solution – optical density of product control}}{\text{optical density of test control}} \times 100$$

Human Red Blood Cell (HRBC) Membrane Stabilization Method[37-43]

The blood was collected from healthy human volunteer who had not taken any NSAIDs for 2 weeks before the experiment and was mixed with equal volume of sterilized Alsever's solution. The blood solution was centrifuged in a centrifugation machine at 3000 rpm for 15 min and the upper layer was carefully removed with a syringe or sterile pipette. The packed cells remained at the bottom were separated and washed with isosaline solution and a 10% v/v suspension was made with isosaline. HRBCs suspension was used for the study.

Preparation of the standard solution: The standard solution prepared using 1 mL of phosphate buffersolution, 2 mL of hypotonic saline, and 0.5 mL of 10% w/v HRBCs in isotonic saline 0.5 mL of Diclofenac sodium solution in various concentrations (10, 50, 100, 200, 400, 800 and 1000 µg/ml) and 2 ml of distilled water.

Preparation of the test solution: The test solution comprising of 1 mL of phosphate buffer, 2 mL of hypotonic saline, and 0.5 mL of 10%w/v HRBCs in isotonic saline 0.5 mL of extract solution in various concentrations (10, 50, 100, 200, 400, 800, and 1000 µg/ml) and 2 ml of distilled water.

Preparation of test control: The test control solution comprising of 1 mL of phosphate buffer, 2 mL of hypotonic saline, and 0.5 mL of 10%w/v HRBCs in isotonic saline, 2.5 mL of distilled water.

Experimental Procedure

All the assay mixtures were incubated at 37°C for 30 min and centrifuged at the rate of 3000 rpm. The supernatant liquid was poured out, and the hemoglobin content was estimated by UV spectrophotometer at 560 nm. The percentage of HRBC membrane stabilization or protection against hypotonicity induced hemolysis was calculated using the following formula.

$$\text{Percentage protection} = \frac{\text{100 – optical density sample}}{\text{optical density control}} \times 100$$

RESULTS [Tables 2-4 and Figures 1-3]

The present study summarizes the *in vitro* bioassay of anti-rheumatoid arthritis effect against protein denaturation method using egg albumin, HRBC membrane stabilization method and protein denaturation method using bovine serum , the results as fallows.

DISCUSSION

The ethanolic extracts of *P. glabrum*, *C. dicoccum*, *O. obtusata*, *and A. nervosa* are formulated into

Table 2: Inhibition of protein denaturation method using bovine serum

% Inhibition of protein denaturation using bovine serum						
Concentrations µg/ml	**Standard solution**	**F1**	**F2**	**F3**	**F4**	**F5**
10	51.92±0.4	37.12±0.4	44.82±0.24	34.22±0.12	39.36±0.34	32.29±0.2
50	57.81±0.5	43.24±0.6	51.91±0.3	42.91±0.24	46.18±0.4	41.92±0.4
100	63.14±0.9	49.42±0.6	54.64±0.6	45.82±0.19	69.26±0.8	43.19±0.9
200	79.12±1.5	57.12±2.5	71.24±1.3	53.64±0.9	61.92±1.4	52.28±1.5
400	85.46±0.8	65.46±0.4	78.36±0.6	67.74±0.25	64.82±0.6	61.42±0.5
800	92.16±0.9	74.24±0.2	84.29±0.9	72.92±0.16	76.19±0.8	68.26±0.3
1000	94.28±1.0	81.26±0.5	89.16±0.6	79.29±0.24	82.28±0.28	78.18±1.5

Table 3: Inhibition of protein denaturation method using egg albumin

% Inhibition of protein denaturation using egg albumin						
Concentrations µg/ml	**Standard solution**	**F1**	**F2**	**F3**	**F4**	**F5**
10	52.32±0.8	46.12±0.25	49.42±0.24	42.12±0.5	46.89±0.5	43.42±0.46
50	58.24±0.6	48.24±0.62	53.91±0.3	44.24±0.2	49.12±0.12	45.36±0.42
100	65.04±1.2	51.42±0.24	57.64±0.6	48.42±0.4	52.22±0.4	48.32±0.15
200	73.24±1.4	56.12±2.5	69.24±1.3	51.12±1.5	58.25±2.15	52.22±0.4
400	76.08±1.18	63.46±0.4	74.36±0.6	59.46±1.4	64.14±0.41	62.36±0.6
800	79.16±1.5	72.24±0.2	76.29±0.9	68.24±1.2	73.43±0.12	69.34±0.25
1000	86.14±1.2	79.26±0.5	83.16±0.6	74.26±0.5	81.12±0.15	72.84±0.6

Table 4: HRBC membrane stabilization method

% HRBC membrane stabilization						
Concentrations µg/ml	**Standard solution**	**F1**	**F2**	**F3**	**F4**	**F5**
10	56.92±0.4	41.56±0.46	44.56±1.2	39.56±0.4	39.96±0.14	38.56±0.2
50	62.81±0.5	49.21±0.42	51.21±1.5	45.21±0.6	50.21±1.3	43.21±0.6
100	89.14±0.9	82.13±1.3	84.13±0.9	82.13±0.25	81.13±1.6	81.13±0.4
200	91.59±1.5	85.14±0.2	89.14±1.4	78.14±1.2	84.14±1.2	72.14±1.2
400	94.26±0.8	89.46±1.4	92.46±0.4	84.46±0.6	86.46±0.9	81.46±1.6
800	96.59±0.9	90.49±1.2	94.49±0.5	89.49±1.3	92.49±0.5	89.49±1.3
1000	98.98±1.0	92.98±0.25	96.98±0.26	93.98±0.4	93.98±0.6	91.98±1.6

HRBC: Human red blood cell

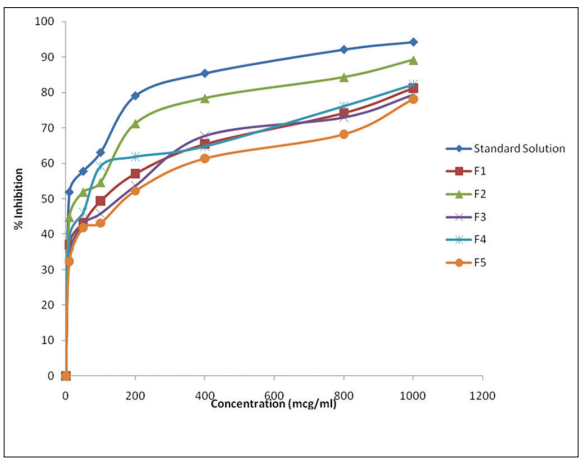

Figure 1: % Inhibition of protein denaturation of bovine serum

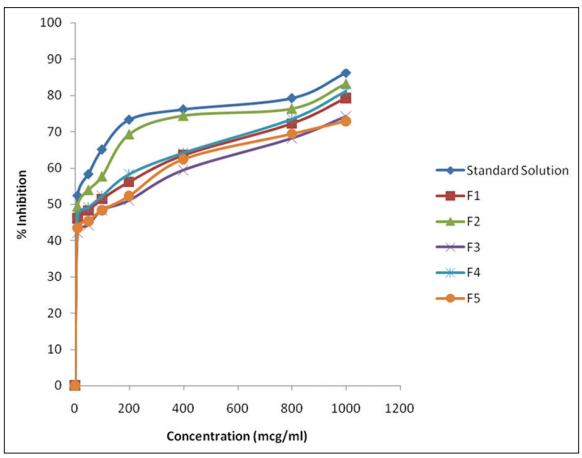

Figure 2: % Inhibition of protein denaturation of egg albumin

five formulations with different portion as given in the above formula and evaluated for *in vitro* anti-arthritis activity with concentrations varying from 10 µg/ml to 800 µg/ml by the *in vitro* inhibition of protein denaturation method using bovine serum, inhibition of protein denaturation method using egg albumin, and HRBC membrane stabilization method comparing with the standard drug as diclofenac sodium in the five formulations F2 and F4 having the significantly more inhibition of protein denature and membrane stabilization which are concentration dependent.

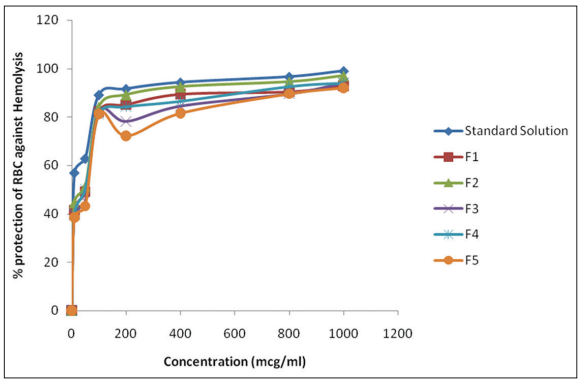

Figure 3: % protection of red blood cell against hemolysis

CONCLUSION

The above result gives a conclusion that the polyherbal formulations with the different concentration have the anti-arthritic activity and the F2 and F4 having significantly more potential. Further investigation is required to use the two formulations in the treatment of rheumatoid arthritis.

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